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Analysis of virulence traits of *Staphylococcus aureus* isolated from bovine mastitis in semi-intensive and family dairy farms

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ABSTRACT

Background: *Staphylococcus aureus* is one of the main microorganisms that causes bovine mastitis, and its well-known virulence characteristics and interactions with the environment are used to aid the design of more efficient therapies.
Objectives: To determine whether the virulence traits, such as antibiotic resistance and biofilm-forming and internalization abilities, of *S. aureus* isolated from bovine mastitis are related to dairy production system types.
Methods: The study was performed in the Mexican states of Guanajuato and Michoacan. Semi-intensive dairy farms (SIDFs) and family dairy farms (FDFs) (454 and 363 cows, respectively) were included. The 194 milk samples from mastitis affected quarters were collected and 92 strains of *S. aureus* were isolated and identified by biochemical and molecular tests. Antibiotic resistance, biofilm and internalization assays were performed on 30 randomly selected isolated strains to determine virulence traits, and these strains were equally allocated to the 2 dairy production systems.
Results: All 30 selected strains displayed a high degree of resistance (50%–91.7%) to the antibiotics tested, but no significant difference was found between SIDF and FDF isolates. *S. aureus* strains from SIDFs had an average biofilm forming capacity of up to 36% (18.9%–53.1%), while *S. aureus* strains from FDFs registered an average of up to 53% (31.5%–77.8%) ($p > 0.05$). Internalization assays revealed a higher frequency of internalization capacity for strains isolated from FDFs (33.3%) than for those isolated from SIDFs (6.7%) ($p > 0.05$). *fnbpA* gen was detected in 46.6% of FDF strains and 33.3% of SIDF strains, and this difference was significant ($p < 0.05$).
Conclusions: Our findings show that the virulence traits of *S. aureus* isolates analyzed in this study, depend significantly on several factors, such as phenotype, genotype, and environmental conditions, which are significantly related to dairy production system type and daily management practices.

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Guzmán-Rodríguez JJ, Gutiérrez-Chavez AJ; Data curation: Guzmán-Rodríguez JJ, Gutiérrez-Chavez AJ, Valencia-Posadas M; Formal analysis: Guzmán-Rodríguez JJ, Gutiérrez-Chavez AJ; Funding acquisition: Gutiérrez-Chavez AJ, López-Meza JE, Ochoa-Zarzosa A; Investigation: Guzmán-Rodríguez JJ, Gutiérrez-Chavez AJ; Methodology: Guzmán-Rodríguez JJ, Gutiérrez-Chavez AJ, Loeza-Lara PD, Sánchez-Ceja M; Project administration: León-Galván MF, Barboza-Corona JE; Writing - original draft: Guzmán-Rodríguez JJ, Gutiérrez-Chavez AJ; Writing - review & editing: Guzmán-Rodríguez JJ, Gutiérrez-Chavez AJ.

Keywords: Dairy system; biofilm; multi-resistance; internalization; *Staphylococcus aureus*

INTRODUCTION

Bovine mastitis is the most costly pathology that affects the dairy industry, because it decreases milk yield and quality, results in premature culling, and increases veterinarian costs. Numerous pathogens are responsible for mastitis, but most infections are caused by *staphylococci*, *streptococci*, or *enterobacteria*. Mastitis may be clinical or subclinical and depending on the primary reservoir and mode of transmission, this pathology can be contagious or environmental [1]. *Staphylococcus aureus* is one of the main causative agents of subclinical bovine mastitis and produces various virulence factors such as endotoxins and other toxic proteins, in addition to its ability to develop resistance *S. aureus* leads to persistent and recurrent infections. *S. aureus* is particularly resistant to β-lactams, and resistance rates have increased dramatically over recent years [2]. Bacteria can develop resistance to antibiotics in different ways such as by employing antibiotic-resistant genes and producing biofilms. Biofilm formation is a survival strategy for most bacteria because they increase the likelihood of transferring genetic material and antibiotic resistance and provide stability and protection from adverse environmental conditions, which contribute to the successful colonization of hosts. Biofilms are aggregates of microbial cells surrounded by a matrix of exopolymers and are considered an important virulence factor of *S. aureus*. Furthermore, biofilm formation can lead to persistent infection by increasing cellular resistance to sanitary procedures, host immune systems, and antimicrobial agents [3].

Diverse studies have demonstrated *S. aureus* binds to cells and extracellular matrix components and internalizes into bovine mammary epithelial cells (bMECs) and other cells in mammary tissues [4,5]. These abilities enable it to evade host innate immune systems, survive inside host cells, and cause persistent and recurrent infections [6]. *S. aureus* internalization into bMECs occurs mainly by a zipper-like mechanism involving fibronectin-binding protein (FnBP) from *S. aureus* and fibronectin and α5β1 integrin host cell receptors [5,6]. Furthermore, the establishment and development of mastitis depend on environmental factors, such as geographical location, climate, production systems, and good management practices as well as virulence and bacterial pathogenicity [7].

Small family farms are a dominant and widely distributed source of dairy products in Mexico and have been estimated to be responsible for between 35% to 40% of milk production in Central Mexico [8]. The family dairy farm (FDF) system relies on confined or semiconfined herds of predominantly Holstein/Creole crosses [9]. Semi-intensive dairy farms (SIDFs) represent another important production system and are estimated to contribute 21% to total Mexican milk production. These farms use mechanized milking and feeding processes and management and preventive veterinary programs [10]. To generate knowledge that helps improve protocols aimed at diagnosing and controlling bovine mastitis, we isolated, characterized, and analyzed the virulence traits of *S. aureus* isolates in samples obtained from cattle with subclinical bovine mastitis at FDFs and SIDFs in Mexico, and investigated differences between the expressions of virulence traits by isolates with respect to these 2 milk production systems.

MATERIALS AND METHODS

Study area and farms

The study was conducted in the Mexican states of Guanajuato and Michoacan. These regions are located in the center of the country, which has a sub-humid, semi-warm climate with temperatures ranging from 6°C to 30°C. Guanajuato is located at a longitude 101°15′46″ W and a latitude 21°01′08″ N, whereas Michoacan is located at a longitude 101°53′59″ W and a latitude 19°10′07″ N. Four hundred and fifty-four lactating Holstein-Friesian animals at 3 SIDF farms located in Guanajuato state and 363 lactating Holstein/Creole crosses at 30 FDFs in Michoacan state were included in the study. These farms were selected for convenience the availabilities of productive and reproductive data at sampling, and readiness to participate.

Milk sample collection

Subclinical mastitis (SCM) was detected by reactive application (Masti test; BIVE, Mexico) to California Mastitis Test (CMT) in all animals. Results were interpreted according to the procedures issued by the National Mastitis Council [11]. Udder quarters affected by SCM (CMT 2 and 3) were identified and teats were disinfected with swabs soaked in 70% ethyl alcohol. After discarding the first few streams, 10–15 mL milk samples were collected in sterile capped tubes and numbered, as required by the above-mentioned procedures, and described by Hogan et al. [11]. Samples were cooled and immediately transported to the Laboratory of Proteomic and Genic Expression of the Life Science Division at the University of Guanajuato, Irapuato, Guanajuato, Mexico.

Isolation and molecular identification of *S. aureus*

A total of 194 milk samples were collected from udder quarters affected by mastitis and sent for microbiological analysis. Initially, isolates were classified using preliminary bacterial identification protocols, that is, colony morphology, color, and Gram staining.

Subsequently, catalase, coagulase, and mannitol fermentation tests were performed. For molecular identification, genomic DNA was isolated by picking one colony from a fresh culture plate. Isolates were analyzed by amplifying the *nuc* gene encoding staphylococcal thermostable nuclease (**Table 1**) and performing agarose gel analysis [12]. In addition, isolates were subcultured in Luria-Bertani broth (DIBICO CDMX, Mexico) at 37°C for 72 h. After adding 20% (v/v) sterile glycerol, isolates were stored at −80°C. A total of 92 isolates of *S. aureus* were isolated; 30 were randomly selected and allocated equally to SIDFs or FDFs. Isolates were identified with the letters SA and consecutive numbers.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *S. aureus* isolates was performed using the Kirby-Bauer disk diffusion method in Muller-Hinton agar. The antimicrobials used were: penicillin (PE) 6 µg, dicloxacillin (DC) 30 µg, pefloxacin (PEF) 5 µg, cefuroxime (CXM) 30 µg, gentamicin (GE) 120 µg, cefotaxime (CTX) 30 µg, sulfamethoxazole + trimethoprim (SXT) 1.25 and 23.75 µg, tetracycline (TE) 30 µg, ampicillin (AM) 10 µg, erythromycin (E) 15 µg, ceftazidime (CAZ) 30 µg and cephalothin (CF) 30 µg (BioRad, USA).

Biofilm forming

S. aureus was inoculated into LB broth, cultivated overnight at 37°C, and adjusted to an optical density of 0.2 optical density at 600 nm (up to 1×10^8 colony-forming unit [CFU]/mL). The 100 µL of each culture was transferred to a round-bottomed 96-well microtiter polystyrene

Table 1. Oligonucleotides used in the study

Oligonucleotide	Sequence	Alignment temperature (°C)	Reference
blaZ	5′-TAAGAGATTTGCCTATGCTT-3′ 5′-TTAAAGTCTTACCGAAAGCAG-3′	49	18
mecA	5′-GTAGAAATGACTGAACGTCCGATGA-3′ 5′-CCAATTCCACATTGTTTCGGTCTAA-3′	62	20
tetK	5′-GTAGCGACAATAGGTAATAGT-3′ 5′-GTAGTGACAATAAACCTCCTA-3′	49	18
tetM	5′-AGTGGAGCGATTACAGAA-3′ 5′-CATATGTCCTGGCGTGTCTA-3′	49	18
gyrA	5′-AATGAACAAGGTATGACACC-3′ 5′-ACGCGCTTCAGTATAACGC-3′	49	38
gyrB	5′-CAGCGTTAGATGTAGCAAGC-3′ 5′-CCGATTCCTGTACCAAATGC-3′	49	38
icaA	5′-CCTAACTAACGAAAGGTAG-3′ 5′-AAGATATAGCGATAAGTGC-3′	50	39
icaD	5′-AAACGTAAGAGAGGTGG-3′ 5′-GGCAATATGATCAAGATAC-3′	50	39
fndpA	5′-CGACACACCTCAAGACAATAGCGG-3′ 5′-TGTGGCTTACTTTCTGCTGCCGTTC-3′	59	39
clfB	5′-TGAAAGTGCAGATTCCGAAAAAAAC-3′ 5′-CCGTCGGTTGAGGTGTTTCATTTG-3′	59	39
spa	5′-ATATCTGGTGGCGTAACACCTGCTG-3′ 5′-CGCATCAGCTTTTGGAGCTTGAGAG-3′	59	39

plate (Sigma Aldrich, USA) and incubated for 48 h at 37°C. Supernatants were then removed by washing wells twice using 100 µL of phosphate-buffered saline (PBS), and then 100 µL of 0.5% crystal violet (CV) solution was added to wells containing completely dry biofilms. After 15 min of dyeing, the excess CV was removed by washing twice with sterile water. Eventually, the fixed CV was released by 95% ethanol washing. The absorbance was detected at 540 nm in an enzyme-linked immunosorbent assay plate reader (Turner Biosystems, USA).

One well containing sterile medium was used as a blank control. All steps were carried out at room temperature, and 3 independent experiments were performed in triplicate.

Internalization assays of *S. aureus* isolates

Internalization assays were carried out as follows. The bMEC monolayers (approximately 50,000 cells/well) were challenged with *S. aureus* at a multiplicity of infection 30:1. Briefly, bMECs were inoculated with bacterial suspensions and incubated for 2 h in a 5% CO_2 atmosphere at 37°C. The bMEC monolayers were then washed 3 times with PBS (pH 7.4) and incubated in serum-free growth media supplemented with 40 µg/mL GE for 30 min at 37°C to eliminate extracellular bacteria. The bMEC monolayers were detached with trypsin-EDTA (Sigma Aldrich, USA) and lysed with 200 µL of sterile distilled water. Lysates were diluted 100-fold with PBS sterile, plated on LB agar in triplicate and incubated overnight at 37°C. Numbers of CFUs were determined by colony counting, and results are presented as percentage internalizations versus *S. aureus* ATCC 27543 (the positive control).

Polymerase chain reaction (PCR) amplification of resistance, biofilm, and internalization genes

Genomic DNA was obtained by picking one colony from a fresh culture plate. The oligonucleotides used are shown in **Table 1**. The reaction was performed in 25 µL of a reaction mixture containing 0.4 µM of each primer, 200 µM of deoxynucleoside triphosphates

(Invitrogen, USA), 2 mM of magnesium chloride (Invitrogen, PC) and 1 U of Taq DNA polymerase (Invitrogen) using the following conditions: initial denaturation at 95°C for 10 min, followed by 30 amplification cycles of denaturation for 10 min at 94°C, annealing for 1 min at the oligonucleotide specific temperature (**Table 1**), and polymerization for 30 sec at 72°C, and this was followed by a final extension for 7 min at 72°C. PCR products (5 μL) were analyzed by electrophoresis on 1% agarose gels and stained with ethidium bromide.

Data and statistical analyses

Animal data were entered into a spreadsheet using SPSS Smart Viewer ver. 15.0 (IBM, USA) and edited to ensure quality of the analysis. Study variables were analyzed by descriptive analysis. Normality was evaluated for dependent variables to determine the type of statistical analysis used. The analysis was performed using analysis of variance in Statgraphics Centurion ver. 15.2 (Statgraphics Technologies, USA).

Compliance with ethical standards

Animal studies were carried out humanely according to national and international Animal Care and Use Committee protocols.

RESULTS

Frequencies of subclinical and clinical bovine mastitis

Mastitis results showed a frequency of SCM of 43.25% among the 54 lactating cows at SIDFs and of 57.6% among cows from FDFs. SCM frequencies ranged from 31.1% to 53.5% and 14.8% to 85.0% for SIDFs and FDFs, respectively. Concerning CMT quarter reaction extent, reactions of the milk samples of SIDF cows were less severe than those of FDF animals. At least one case of clinical mastitis was detected in all dairy farms studied. CLMs percentages of farms ranged from 1.0% to 25.2% (**Table 2**).

S. aureus isolates

The 194 milk samples were obtained from SCM cases and 92 isolates of *S. aureus* were cultured and confirmed by biochemical and molecular testing by amplifying the *nuc* gene (**Supplementary Fig. 1**). The other 102 cultured milk samples were documented as 'no-growth' or 'another type of staphylococcic agent.' Thirty of the 92 isolates were randomly selected according to characteristics of selected farms (**Table 2**) and allocated equally to SIDFs or FDFs.

Table 2. Frequencies of mastitis and details of main management practices and routines employed by dairy farmers

Variable	Semi-intensive	Family (small-scale)
Mastitis cases (%)		
Subclinical	31.1–53.5	14.8–85.0
Clinical	1.0–7.1	5.0–25.2
Management practice		
Milking technique	Milking machine	Manual milking
Milking frequency (time/d)	2	1
Mastitis screening	Yes	No
Veterinary services	Yes	No
Mastitis control	Yes	No
Milking place	Milking from stanchion/tie stalls	Open space (beside a standing cow)
Housing system	Open (free-stall)	Confinement (tie-stall)
Calf suckling practice	No	Frequently
Hygiene practice	Yes (pre-dipping, disposable paper towels and stripping)	Rarely (use only of water)

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Antimicrobial resistance profile

All 30 selected *S. aureus* isolates exhibited a high degree of resistance to antibiotics (50%–91.7%) (**Fig. 1A**) as compared with the antibiotics tested number. Resistances to antibiotics by *S. aureus* isolates from cattle at SIDFs and FDFs were 75.0% and 70.6%, respectively. However, although this difference was not significant ($p > 0.05$), *S. aureus* isolates from SIDFs presented a 100% resistance pattern to PE, DC, CF, CAZ and AM, and 93.3% resistance to CXM, CTX, and TE. The resistance patterns of *S. aureus* isolated from FDFs were 100% for PE, DC, and AM; 93.3% for CF and TE, and 86.7% for CXM, CTX, and CAZ (**Table 3**).

To characterize the genetic nature of antimicrobial resistance, we investigated the expressions of *blaZ* and *mecA* (β-lactam resistance genes), *tetK* and *tetM* (TE resistance genes), and *gyrA* and *gyrB* (quinolones resistance genes) genes (**Fig. 2**). All 30 isolates were positive for the *blaZ* gene, and 46.6% and 53.3% of the strains from SIDFs and FDFs, respectively, were positive for the *mecA* gene. Interestingly, 26% of the isolates resistant to oxacillin did not express the *mecA* gene. With respect to the *tetK* gene, 86.6% and 66.6% of SIDF and FDF strains, respectively, were positive, whereas for the *tetM* gene positivity's were 73.3% for

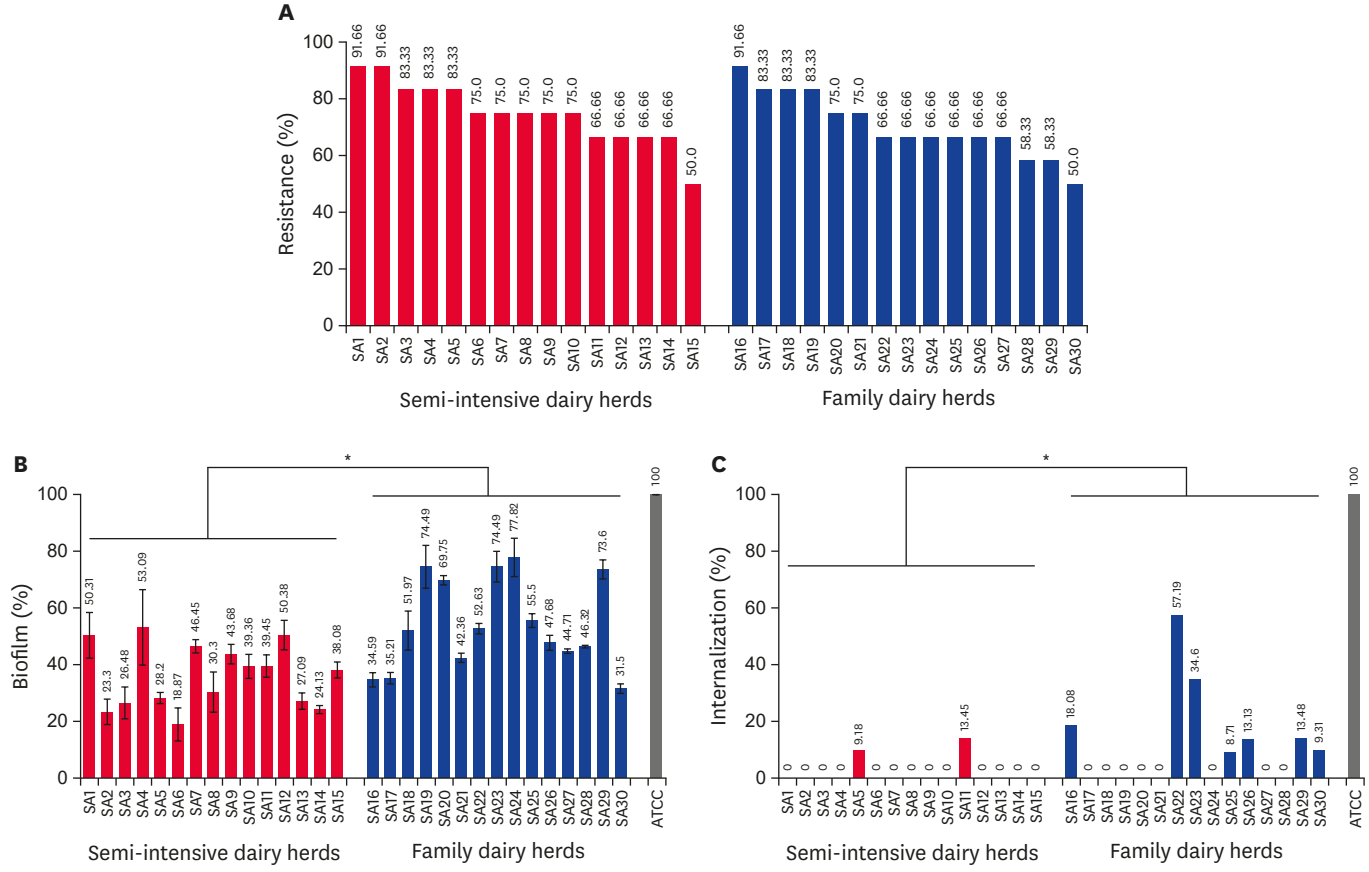

Fig. 1. Virulence traits of *Staphylococcus aureus* isolates. (A) Analysis of antimicrobial resistance to 12 antibiotics were analyzed using the antibiogram test; the graphic represents the number antibiotics isolates were resistant to and are expressed as percentages. (B) Biofilm formation was assessed by crystal violet staining. Biofilm formation percentages were calculated by expressing absorbances as percentages of those of the control strain (*S. aureus* ATCC 27543). Plotted results are the averages of 3 independent experiments performed in triplicate. (C) *S. aureus* invasion percentage values were calculated by expressing percentage colony-forming unit recoveries after bovine mammary epithelial cell lysis as percentages of control (*S. aureus* ATCC 27543). Bars represent the means ± SEs of 3 independent experiments performed in triplicate.
*Statistically significant difference ($p < 0.001$) between the SADF and FDF isolate groups; †Statistically significant difference ($p < 0.001$) between the SADF and FDF isolate groups.

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Table 3. Virulence traits of *Staphylococcus aureus* isolates evaluated

Production system	Isolate	Antibiotic resistance profile
Semi-intensive farms	SA1	PE, DC, AM, TE, CAZ, CXM, PEF, CTX, STX, E, CF
	SA2	PE, DC, AM, TE, CAZ, CXM, PEF, CTX, STX, E, CF
	SA3	PE, DC, AM, TE, CAZ, CXM, CTX, E, CF, PEF
	SA4	PE, DC, AM, TE, CAZ, CXM, PEF, SXT, E, CF
	SA5	PE, DC, AM, TE, CAZ, CXM, CTX, STX, E, CF
	SA6	PE, DC, AM, TE, CAZ, CXM, CTX, SXT, CF
	SA7	PE, DC, AM, TE, CAZ, CXM, CTX, SXT, CF
	SA8	PE, DC, AM, TE, CAZ, CXM, CTX, E, CF
	SA9	PE, DC, AM, TE, CAZ, CXM, CTX, E, CF
	SA10	PE, DC, AM, TE, CAZ, CXM, CTX, E, CF
	SA11	PE, DC, AM, TE, CAZ, CXM, CTX, CF
	SA12	PE, DC, AM, TE, CAZ, CXM, CTX, CF
	SA13	PE, DC, AM, TE, CAZ, CXM, CTX, CF
	SA14	PE, DC, AM, TE, CAZ, CXM, CTX, CF
	SA15	PE, DC, AM, CAZ, CTX, CF
Family farms	SA16	PE, DC, AM, TE, CAZ, CXM, PEF, CTX, STX, E, CF
	SA17	PE, DC, AM, TE, CAZ, CXM, PEF, CTX, SXT, CF
	SA18	PE, DC, AM, TE, CAZ, CXM, CTX, STX, E, CF
	SA19	PE, DC, AM, TE, CAZ, CXM, PEF, SXT, E, CF
	SA20	PE, DC, AM, TE, CXM, PEF, CTX, SXT, CF
	SA21	PE, DC, AM, TE, CAZ, CXM, CTX, E, CF
	SA22	PE, DC, AM, TE, CAZ, CXM, CTX, CF
	SA23	PE, DC, AM, TE, CAZ, CXM, CTX, CF
	SA24	PE, DC, AM, TE, CAZ, CXM, CTX, CF
	SA25	PE, DC, AM, TE, CXM, PEF, CTX, CF
	SA26	PE, DC, AM, TE, CAZ, PEF, CTX, CF
	SA27	PE, DC, AM, TE, CAZ, CTX, E, CF
	SA28	PE, DC, AM, TE, CAZ, CTX, SXT
	SA29	PE, DC, AM, TE, CXM, PEF, CF
	SA30	PE, DC, AM, CAZ, CTX, CF
ATCC 27543		PE, AM

strains from SIDFs and FDFs. The 93% of the isolates were resistant to TE, of which 64.2% have both genes. For the quinolone resistant genes *gyrA* and *gyrB*, 20% and 26.6% of isolates, respectively, from SIDFs were positive and 33.3% and 46.6% of isolates from FDFs were positive (**Fig. 2**).

Biofilm formation capacities

All isolates of *S. aureus* exhibited biofilm forming ability (average 45.06%; range 18.9%–77.8%) with respect to the positive control (*S. aureus* ATCC 27543). *S. aureus* isolated from SIDFs had an average biofilm-forming capacity of up to 36% (18.9%–53.1%), whereas *S. aureus* from FDFs had an average of 53.2% (31.5%–77.8%) (**Fig. 1B**). Interestingly, the biofilm capacities of *S. aureus* from FDFs was 1.5 times higher than that from SIDFs ($p < 0.001$) (**Fig. 3**). We found 66% of SIDF isolates were positive for *icaA* and *icaD* genes, whereas 80% and 73.3% of the FDF isolates were positive for *icaA* and *icaD*, respectively (**Fig. 2**).

Internalization by mammalian epithelial cells

The internalization abilities of isolates were compared with that of *S. aureus* ATCC 27543 (100%). Internalization assays showed that 30% (9/30) of all *S. aureus* isolates internalized into bMECs; mean internalization was 19.68% (range 8.71% [SA25]–57.19% [SA20]) (**Fig. 1C**). The internalization capacity of *S. aureus* isolated from FDFs (46.66%) was greater than of SIDFs (13.33%) ($p > 0.05$) (**Fig. 3**).

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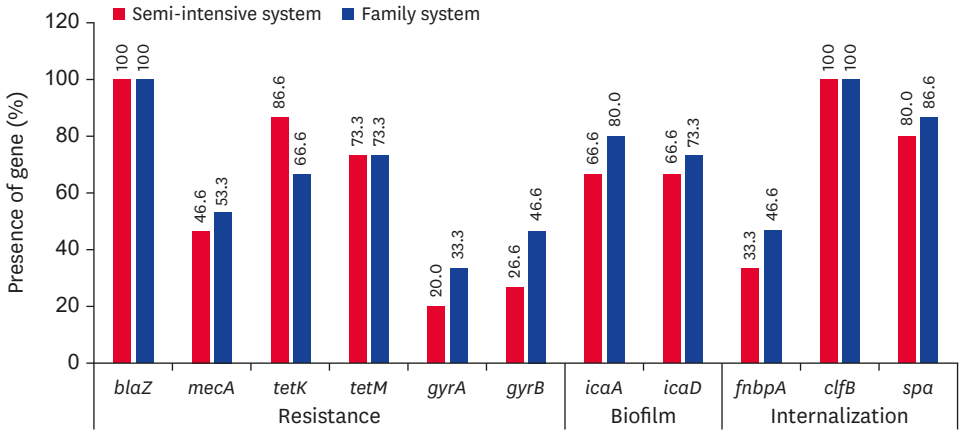

Fig. 2. Genotype profile of *Staphylococcus aureus* isolates. The graphic shows a comparative analysis of percentages of isolates in milk samples from semi-intensive dairy farms and family dairy farms presenting virulence-related genes.

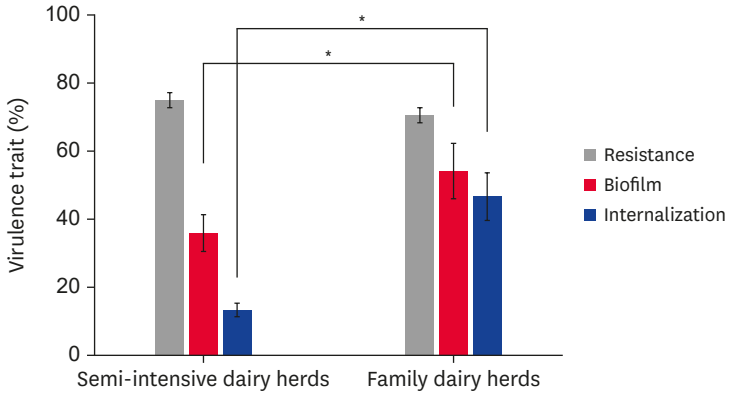

Fig. 3. Comparative analysis of virulence factors in *Staphylococcus aureus* isolates. The graph shows resistance profiles, biofilm formation abilities, and internalization capacities, as determined by antibiograms, crystal violet staining, and gentamicin protection assays tests, of semi-intensive dairy farm and family dairy farm *S. aureus* isolates. Results are presented as median values of 3 independent experiments performed in triplicate.
*Statistically significant difference ($p < 0.001$).

We also examined the expressions of the *S. aureus* internalization-related genes *fnbpA*, *clfb*, and *spa* (**Fig. 2**). The *fnbpA* expression was present in 33.3% of SIDF isolates and 46.66% of FDF isolates. The *clfB* gene expression was observed in all SIDF and FDF isolates. On the other hand, *spa* expression was observed in 80.0% of SIDF isolates and in 86.6% of FDF isolates, which suggested that this gene was a virulence factor (**Fig. 2**).

DISCUSSION

The results of this study showed a high mastitis frequency (subclinical or clinical) among dairy cows in SIDFs and FDFs in the Mexican states of Guanajuato and Michoacan. The majority of subclinical cases were caused by *S. aureus*, a microorganism that exhibits different virulence traits in terms of its antimicrobial multi-resistance pattern, biofilm formation, and internalization ability, regardless of dairy management or production systems. Nonetheless, differences between SIDFs and FDFs especially in terms of dairy facility and milking managements as regards cleaning and hygiene practices, housing conditions, medicinal

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preventive programs, and use and abuse of antibiotics to treat mastitis might be important. All these factors are relevant to dairy farms and might trigger the emergence of bacteria strains with antibiotic multi-resistance, biofilm-forming, or intracellular internalizing ability. For example, in Mexican FDFs, daily management activities are similar to those described by House and Anderson [13], that is, cows are regularly maintained in a tie-stall housing area, manually milked in place, and fed on an individual basis. This type of totally confined animal housing without or with wet straw as bedding material, poor dairy facilities, and incorrect milking hygiene practices influences animal exposure to contagious and environmental bacteria and increase the risk of mastitis caused by opportunistic pathogens [14]. Furthermore, producers with smaller herds believe that milking using a pipeline in a stall is a lower-cost option than a milking parlor capable of milking many more cows. On the other hand, in SIDFs, milking hygiene and health management are often much better than those found in FDFs. However, though this suggests the risk of developing intramammary infections is reduced, this is not always the case [13]. **Table 2** shows mastitis frequencies were higher in FDFs than in SIDFs, and the values obtained were similar to those described by Abebe et al. [15], who reported *S. aureus* is one the most prevalent etiological agents isolated in bovine SCM cases at small dairy farms in Ethiopia. *S. aureus* is a frequent cause of subclinical and clinical mastitis, and chronically infected cows represent an important reservoir of this pathogen. However, it is important to remember that the prevalence of contagious mastitis is markedly influenced by milking procedures. Thus, correct milking procedures such as milking mastitic cows last, and properly sanitizing utensils, milker's hands, and udders before milking might improve the situation [16]. Some risk factors like hygiene practices, registered by the mean multivariable logistic regression model, showed that the herd-level factors significantly and independently associated with the presence of mastitis [15].

Reportedly, approximately 70% of *S. aureus* isolates are resistant to at least 6 antibiotics, and these resistance levels continue to increase [12,16]. Notably, we found 100% of isolates were resistant to at least 6 antibiotics, which represents an important increase. Furthermore, levels of multi-resistance significantly higher than values reported over recent years in Mexico. Ochoa-Zarzosa et al. [12] reported 100% resistance to PE class antibiotics, but low levels of resistance to other antibiotics such as TE and PEF. In 2018, a study carried out in Guanajuato state reported high rates of resistance to β-lactam antibiotics and to PEF, SXT, and E (48%–58%) [17]. Importantly, Mexican farmers commonly treat animals without any previous knowledge of antimicrobial susceptibility, and such practices undoubtedly influence pathogen transmission and resistance and have epidemiological impacts quite different from those of large-scale dairy farms [17].

The presence of the *blaZ* gene in all 30 isolates is consistent with the observation that all *S. aureus* isolates exhibited resistance to PE and express high levels of this gene [18]. The high observed prevalence's of the *mecA* gene in SIDF and FDF isolates (46.6%–53.3%) were similar to those reported by Shrivastava et al. [19]. However, Elhassan et al. [20] found 26% of isolates resistant to oxacillin did not express the *mecA* gene, which suggested other mechanisms were responsible for the hyperproductions of β-lactamases and the reported high prevalence of TE resistance genes *tetK* and *tetM* in isolates. The coexistence of *tetK* and *tetM* and the presence of one of the 2 in microorganisms resistant to TE has been observed in several countries [21]. In addition, Yang et al. [21] found a correlation between the quinolone resistance phenotype of isolates and the presence of the *gyrA* or *gyrB* genes, which was in-line with other studies that showed mutations in genes encoding DNA gyrase were directly

related to levels of *S. aureus* fluoroquinolone resistance [22]. Our results show that Mexican dairy farms management conditions increase the risk of multi-resistant strain development, therefore, the presence of *S. aureus* isolates with a high level of resistance to antibiotics is a key consideration and new strategies should be devised to control bovine mastitis.

Biofilm production by microorganisms is considered an important virulence trait, and *S. aureus* isolates that produce biofilms have been associated with chronic mastitis in dairy animals [23]. All isolates of *S. aureus* analyzed in this study produced biofilms, but our results differ from those of some studies that reported fewer than 50% of isolates produced biofilms [3,4]. These differences may be due to phenotypic and genotypic differences between isolates from different geographical origins [2]. In the present study, the biofilm-forming capacity of *S. aureus* in the FDF isolate group was 1.5 times greater than that in the SIDF isolate group, we suggest this was caused by environmental factors such as the availabilities of nutrients and compounds such as glucose, oxygen, iron and many others that are necessary for bacterial metabolism [24]. For example, nutritional deficiency enhances biofilm formation due to the negative regulations of CcpA, CcpE, RpiRc, and CodY, which control the virulence of gram-positive pathogens and promote survival strategies including biofilm formation [25]. Our analysis of the prevalence's of genes related to biofilm formation (*icaA, icaD*) showed that at least 70% of the isolates presented both genes, which concurs with values reported elsewhere [26]. Percentage prevalence's in samples from FDFs were slightly higher than those in samples from SIDFs, which suggested an association between the genes present and biofilm formation phenotype. However, Notcovich et al. [27] demonstrated that although most *S. aureus* isolates did not possess either *icaA* or *icaB*, they were able to produce biofilms, which indicates biofilm-forming ability also involves entities other than *icaA* and *icaB*, such as biofilm-associated protein, bone-sialoprotein-binding protein, clumping factors A and B, collagen-binding protein, and elastin-binding protein [28], to reinforce the genetic knowledge about the differences in biofilm formation between the *S. aureus* strains of this study and those reported in other work.

Because the internalization of *S. aureus* into bMECs enables the bacterium to evade the host immune system and cause chronic and recurrent infections, we analyzed the internalization abilities of *S. aureus* isolates. In 2008, Ochoa-Zarzosa et al. [12] analyzed the internalization of *S. aureus* isolates from bovine SCM cases in Morelia Michoacán (Mexico) and reported a maximum internalization of 80%, which is substantially higher than values we obtained. In this regard, it has been reported that the *agr* allelic group in *S. aureus* strains is significantly associated with adherence and persistence mechanisms [29]. In addition to classifying *S. aureus* strains in *agr* groups, a recent study analyzed a series of lineages presenting similar virulence regulators and effectors but differing in their abilities to form biofilms, produce toxins, and elicitate bMEC immune response, which is a characteristic feature of the internalization of bacteria [30]. Thus, it appears differences between reported frequencies and magnitudes of *S. aureus* internalization may be due to their belonging to different *agr* groups or lineages. However, further molecular characterization is required to confirm this hypothesis.

S. aureus internalization into bMECs can occur by a zipper-like mechanism, which depends on FnBP, fibronectin, and α5β1 integrin [5,6]. However, other bacterial proteins such as autolysin, iron-regulated surface determinant-B, clumping factor (ClfA and ClfB), bacterial serine aspartate repeat-containing protein C and D, serine-rich adhesin for platelets, and protein A, also are involved in the invasion process [6]. In this study, we investigated relations between the internalization of *S. aureus* and some related genes (*fnbpA*, *clfb,* and *spa*). The

prevalence rates of *fnbpA* among the 30 isolates ranged from 33.3% to 46.6% and were significantly lower than that reported by Pereyra et al. [5], who found the *fnbpA* gene in all isolates analyzed. However, in the same study, the *clfB* gene was also observed in all isolates, which agrees with our findings. Finally, the high prevalence of the *spa* gene in isolates, as determined by our study (> 80%), is consistent with that found in other countries, where the prevalence of this gene in *S. aureus* isolates from bovine mastitis milk samples was reported to be 80.2% [31].

To our knowledge, this is the first report that shows important differences in virulence traits such as antimicrobial resistance, biofilm production, and internalization ability plays an important role in the efforts toward understanding this pathogen to improve the establishment and development of preventive and treatment protocols for dairy cattle. Alternatively, the virulence traits of *S. aureus* as pathogenicity mechanisms in bovine mastitis, it is important to consider other factors such as physiological animal condition and the cow's environment in which the mastitis disease is developed, since it has been previously described that these elements could directly influence the phenotypic characteristics of bacteria present in that environment [32]. We also observed different management conditions, particularly at time of milking and noted that pre-dipping and/or dipping treatments were not used to clean and disinfect milking teats in FDFs. Firth et al. [33] mentioned farm management and hygiene are essential for ensuring udder health and that hygienic milking routines are particularly important. Soiled udders at milking time are another important preventive factor, and we noted frequencies of soiled udders in tie stalls (FDFs) and free stalls (SIDFs) were 23.6% and 13.8%, respectively. In a study conducted in the United States, it was observed that cows kept under tie-stall conditions had a 1.5 times greater risk of intramammary infection than those kept under free-stall conditions [34]. These findings indicate that poor hygiene in milking areas increases animal exposure to organic matter and the risk of intramammary infections, as was observed in the present study at FDFs.

Our study also shows that *S. aureus* drug resistance in dairy farms, which is one aspect of a global sanitary problem. Nevertheless, it reflects the general overuse of antimicrobial drugs in dairy farms, regardless of the production model used.

Biofilm formation is a virulence trait and FDF management conditions would appear to promote *S. aureus* biofilm formation, which would help the bacterium adapt to diverse host environments [24]. Chaiyabutr et al. [35] reported that biofilm formation might be associated with low milk yields and raised concerns in this respect because of the intramammary characteristics of crossbred dairy cows. Interestingly, biofilm formation and internalization were observed at greater levels among isolates from FDFs (**Fig. 1**), possibly because adhesion molecules like clumping factor B and fibronectin are associated directly with these 2 virulence traits during the initial stage of adhesion [5].

Lack of technical assistance leading to failures in animal management and of control measures that prevent udder infections and improve milk quality are problems faced by small dairy farms worldwide. The ability of small farms to invest in farm improvement is much more limited than that of larger farms, and this results in different management practices, equipment, and feeding procedures [36]. The antimicrobial resistance of *S. aureus* is of animal and human health concern because multi-resistant strain infections are often difficult to treat effectively. Furthermore, the environmental conditions in which dairy ruminants live varies considerably and could limit the utility of the One Health approach [37].

The findings of this type of study can be used to promote multi-sectoral responses to food safety hazards and expose risks from zoonoses and other public health threats at the human-animal ecosystem interface and provide guidance on how to reduce these risks in dairy farms.

In conclusion, the presence of isolates of *S. aureus* that exhibit multi-resistance to antibiotics, biofilm formation, and high internalizing ability are relevant to a wide variety of human and animal health issues. This work shows that virulence traits of *S. aureus* isolates from Guanajuato and Michoacán in México are related to a combination of environmental, phenotypic, and genetic characteristics. These factors are part of an evolution complex and the result of multifactorial processes that are significantly related to dairy production system types and their environmental and daily management practices.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

Molecular analysis and *Staphylococcus aureus* identification. The analysis was performed using 1.5% agarose gels, corresponding to the result of the polymerase chain reaction of the *nuc* gene, the figure shows a representative image of this analysis.

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