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Growth conditions affect biofilms of *Staphylococcus aureus* producing mastitis: Contribution of MALDI-TOF-MS to strain characterization

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ABSTRACT

Bovine mastitis is a disease of dairy cattle prevalent throughout the world that causes alterations in the quality and composition of milk, compromising technological performance. *Staphylococcus aureus* is one of the most important pathogens that produce clinical, subclinical, and chronic mastitis. Biofilms are considered a virulence factor necessary for the survival of *S. aureus* in the mammary gland. Its zoonotic potential is important not only for the dairy industry sector but also for public health. This study aimed to evaluate the effect of different growing culture conditions on the biofilm formation of *S. aureus* isolated from mastitis and to test the MALDI-TOF-MS's ability to discriminate among different biofilm formation levels. Fluids commonly found in the dairy environment were incorporated to approach the pathogen's behavior in natural surroundings. PIA production was also evaluated. All strains were able to form high biofilms in TSB, TSBg, and milk. Milk changed the behavior of some strains which formed more biofilms in this medium than in TSBg. The free iron medium CTSBg and milk whey inhibited the biofilm formation of the most strains. MALDI-TOF-MS performance was an excellent tool to discriminate between high, moderate, and low biofilm producers strains of *S. aureus* in each media, confirming the results of crystal violet assay. PIA production was variable among the strains and showed a media-dependent behavior. Our data highlights the importance of considering the growing conditions that mimic the natural ones to the study of biofilm formation in vitro.

Introduction

Bovine mastitis is a disease of dairy cattle of worldwide distribution that causes alterations in milk quality and composition, compromising technological performance. It causes significant economic losses due to extensive veterinary treatments and the culling of animals with chronic disease (Gussmann et al., 2019). *Staphylococcus aureus* (*S. aureus*) is one of the most important pathogens that produce clinical, subclinical, and chronic mastitis (Rocha et al., 2019). Furthermore, this pathogen is a potentially zoonotic problem for the dairy industry sector and public health. *S. aureus* can be transmitted throughout the production chain to the consumer causing food poisoning and other diseases (Kümmel et al., 2016). *S. aureus* has numerous virulence factors, such as toxins and adhesins that allow it to invade the mammary gland and develop infection (Grunert et al., 2018). The ability to form biofilms is considered a virulence factor necessary for the survival of this pathogen in the mammary gland. Biofilms allow the bacterium to become less susceptible to the action of antibiotic therapies and the host's immune system than the planktonic counterpart (Gomes et al., 2016). In this way, *S. aureus* can persist in the mammary gland and become a reinfection source for all herds (Gomes et al., 2016).

The extracellular matrix that encloses the bacterial community is constituted mainly by the polysaccharide intercellular adhesion (PIA) encoded by the *ica*ADBC operon. Other components of this matrix are

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the secreted or surface bacterial proteins (Eap, Bap, FnBPs, Spa) and extracellular DNA (eDNA) released by autolysis (Moormeier and Bayles, 2017). The mechanism of biofilm formation could be classified as PIA-dependent or PIA-independent according to whether PIA has a primary role in the extracellular matrix constitution (Furukawa et al., 2006; Moormeier and Bayles, 2017).

Biofilm formation is usually analyzed through indirect and colorimetric methods such as the crystal violet staining technique (Stepanović et al., 2007). Nowadays, other techniques are being included in the bacterial and fungal identification and characterization in daily routines of clinical and veterinary laboratories for diagnosis of presumed infections, such as MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight, mass spectrometry) (Clark et al., 2013; Caputo et al., 2018). This high throughput technique would provide a rapid and accurate identification necessary to choose a correct antibiotic therapy for the management of *Staphylococci* infections often related to biofilm formation (Caputo et al., 2018).

Within the dairy industry, the milking routine, handling and hygiene of dairy farms play a critical role in transmitting *S. aureus* among the herd (Dego, 2020). Milk, a fluid with an excellent concentration of nutrients, favors the multiplication of this pathogen within the mammary gland and throughout the production chain (Kümmel et al., 2016). Cheese is one of the largest milk derived-products manufactured worldwide (FAO, 2021). During its production process, the milk whey is obtained. Milk whey constitutes the aqueous phase containing many nutrients such as lactose (66–72% w/w), numerous types of globular proteins (8–15% w/w), and mineral salts (7–15% w/w) (Fernández-Gutiérrez et al., 2017) that make it in a major source of environmental contaminant when it is wasted on soil or waterways without sustainable treatments (Parra Huerta and Campos Montiel, 2014).

Knowledge about the capability of *S. aureus* to form biofilms in conditions related to the surrounding environment, such as in fluids commonly found in the dairy milieu, will be helpful in the development of new strategies to prevent and control this pathogen in dairy farms. Therefore, this study aimed to evaluate the effect of different growing culture conditions on biofilm formation and PIA production of *S. aureus* isolated from bovine mastitis and to test MALDI-TOF-MS as an alternate tool to discriminate among different biofilm formation levels.

Materials and methods

Bacteria and culture fluids

Thirty strains of *S. aureus* producing mastitis duly conserved at -80 °C were randomly selected from our laboratory strain collection (Sordelli et al., 2000). Two reference strains -V329 and RF122- were incorporated (Cucarella et al., 2001; Tormo et al., 2007). Strains were cultured on Trypticase soy agar (TSA) and grown in 5 ml of Trypticase soy broth (TSB) supplemented with 0.25 % glucose (Britania, Buenos Aires, Argentina) (TSBg) overnight at 37 °C before each assay. Bacterial cultures made for MALDI-TOF-MS experiments are detailed in the MALDI-TOF-MS section. The milk used in this study was obtained from a cheese food-processing plant of the Province of Buenos Aires, Argentina, and was previously sterilized by heat treatment. Milk whey was obtained by adding 300 μl HCl (Biopack, Buenos Aires, Argentina) (every 100 ml of milk) and heating in a water-bath at 80 °C for 5 min. After a short centrifugation at 12,000 rpm for 8 min at room temperature, the aqueous phase of the milk (whey) was separated and kept in a refrigerator until later use. Milk-agar and milk whey-agar plates were obtained by adding 1.5 % of melted agar-agar to 120 ml of milk or milk whey, respectively.

Biofilm formation

The biofilm formation ability was evaluated in TSB, TSBg, iron-restricted TSBg by treatment with 3 % de Chelex-100 (BioRad, Hercules, CA, USA) (CTSBg), milk and milk whey through the crystal violet technique according to the protocol described by Dotto et al. (2017) with some modifications. Briefly, bacterial cultures were diluted 1:100 in each medium to reach a bacterial concentration of approximately 10^8 CFU/ml. Then, 200 μl from the diluted suspension was aliquot into 96-well polystyrene microtiter plate and incubated at 37 °C under static aerobic conditions. Six consecutive wells were used per each strain and wells with sterile non-inoculated medium were included as control blanks. After 24 h, the wells were washed with PBS and fixed with 150 μl of methanol. Finally, biofilms formed were stained with 150 μl of an aqueous solution of crystal violet 0.5% (Britania, Buenos Aires, Argentina) for 20 min, and the dye bound to the cells was eluted with 95% ethanol. Optical density was measured at 595 nm (OD_{595}) using a microplate reader (Multiskan EX, Thermo Electron Corporation, Waltham, MA, USA). The division of OD_{595} values of each isolates into quartiles permitted to group the strains according to the ability to form biofilm in each growing condition. The quartile over 75% percentile was classified as high-biofilm former (OD_{595}: 1.35; 1.33; 0.151; 1.72; 0.021 for TSB, TSBg, CTSBg, milk, and milk whey, respectively). Strains, which OD_{595} values were within the quartile below the 25% percentile, were grouped as low-biofilm former (OD_{595}: 0.18; 0.427; 0.026; 0.159; 0.0008 for TSB, TSBg, CTSBg, milk, and milk whey, respectively). Those in between were classified as moderate-biofilm former (OD_{595}: 0.548; 0.928; 0.048; 1.49; 0.01 for TSB, TSBg, CTSBg, milk, and milk whey, respectively). Non-biofilm former strains were categorized when the OD_{595} values were below the control values (Sherry et al., 2014).

MALDI-TOF-MS analysis

Ten *S. aureus* strains were analyzed by MALDI-TOF-MS on-spot extraction approach (Dotto et al., 2021). All strains were processed by biological quadruplicates and technical triplicates on the same day. *S*trains were cultured on TSA, TSAg, milk-agar, and milk whey-agar overnight at 37 °C. Then, a bacterial colony was spread directly on the target plate using a sterile spike, after which 1 μl of formic acid was added to each spot. After the spot was dry, 1 μl of the HCCA matrix (Sigma-Aldrich, St. Louis, MO, USA) was loaded and allowed to dry. Continuous mass spectra were obtained with a Microflex LT mass spectrometer (Bruker Daltonics, Inc., Billerica, MA, USA) (ionization mode: LD+; laserShots: 240; laser repetition: 60) within a mass range of 2,000-20,000 Da. Identification with mass spectra (MS) were internally calibrated and controlled every day using *Escherichia coli* ribosomal proteins (Bacterial Test Standard, Bruker Daltonik GmbH). MS were read as fid/aqus files with MALDIquantForeign (v0.10) (MALDIquantForeign, 2019) and processed using MALDIquant (v1.16.2) R package (Gibb and Strimmer, 2012). Spectra have been square root-transformed and smoothed, employing the Savitzky-Golay algorithm. After, they have been baseline-corrected, applying the Statistics-sensitive Non-linear Iterative Peak-clipping algorithm (SNIP) process across 100 iterations (Ryan et al., 1988). The peak detection was carried out by a function that estimates the noise of mass spectrometry data by computing the median absolute deviation (MAD). The *s*ignal-to-*n*oise-*r*atio (SNR) was set up in 3, with a half window size of 50 and a tolerance of 0.5. Peaks that occur in less than 33% of spectra were discarded. Finally, spectra were averaged by adding technical replicates, resulting in four replicates for each strain. The dataset containing raw mass spectra from each *S. aureus* strains grown in all conditions, including technical and biological replicates, have been deposited in the https://github.com/MarManLed/Biofilms_MALDI. MALDI-TOF-MS data were categorized with the Binda R package (Gibb and Strimmer, 2015). Programmed peak selection was performed in the entire dataset seeking biomarkers of each species by the Binary Discriminant Analysis (BDA) algorithm (MALDIquantForeign, 2019). The best-extracted peaks were then used to run a hierarchical k-means clustering-Principal Component Analysis (PCA) with the Factoextra R package (Factoextra, 2019). The binary distance was used to measure dissimilarity between observations,

ward.D2 was the agglomeration method used, and k was set to 3 or 4, taking into account biofilms levels displayed in each media.

PIA production

PIA production in biofilms formed in TSBg or milk was quantified by ELISA. Briefly, the final culture density from biofilms during 24 h at 37 °C was measured by reading the OD_{595} to normalize PIA quantification. Then, the fixed biofilms were washed and 100 µl of blocking solution (1%, w/v, BSA (Sigma-Aldrich, St. Louis, MO, USA) in PBST (PBS + 0.05 % Tween 20) (Honeywell Fluka, Thermo Fisher Scientific, UK) was added to each well. After incubation for 1 h at 37 °C, the blocking solution was removed and 100 µl per well of 75 ng/ml wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) (Sigma-Aldrich, St. Louis, MO, USA) was added. The level of PIA was measured at OD_{492} and normalized to the OD_{595} of growth as OD_{492}/OD_{595}. The OD_{595} of growth of TSB was used to normalize the OD_{492} measuring of biofilms formed in milk.

Statistical analysis

A Monte-Carlo simulated Chi-squared test and a posterior analysis of Pearson residuals were performed to assess the association between variables and variable factors, respectively using the R packages. The association between factors was considered significant if Pearson residuals were either above 2 or below -2. Nonparametric data were analyzed with the Mann-Whitney test using the Graphpad Software (version 5.0; GraphPad Prism). *P*-values < 0.05 were considered significant.

Results

Biofilm formation

Thirty strains of *S. aureus* isolated from bovine mastitis and two reference strains (RF122 and V329) were grown in TSBg and milk to evaluate their capability to form biofilms. All strains formed biofilms in TSBg with values that ranged from a minimum OD = 0.077 to a maximum OD = 2.339, it being the median value OD = 0.306. The 53.1 % of strains were classified as LB and the rest were considered MB and HB formers (21.9 % and 25.0 %, respectively) after 24 h of growth in TSBg medium. In milk, the quantity of biomass increased significantly ($p < 0.0001$) with a median value bigger than that obtained in TSBg (OD = 1.490) (Fig. 1). A small number (2/32) of strains were considered

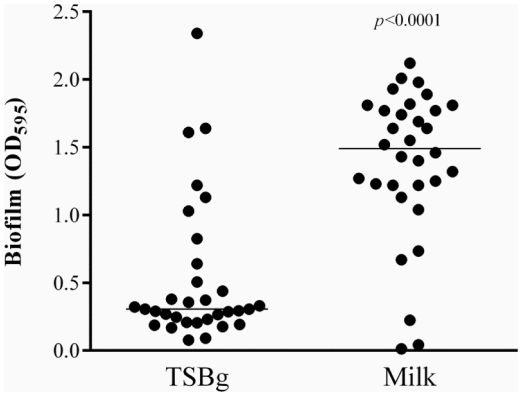

Fig. 1. Biofilm formation of *S. aureus* grown in TSBg and milk. Each point represents the average of three independent assays with six wells of thirty *S. aureus* bovine isolates and two reference bovine strains (V329 and RF122). Lines indicate the optical density (OD) median values, 0.306 and 1.49 for TSBg (TSB + 0.25% of glucose) and milk, respectively ($p < 0.0001$, Mann-Whitney test).

NB (6.3 %) and 43.8 % (14/32) was classified as LB. The rest 50 % of strains were MB and HB.

From the total of thirty *S. aureus* strains analyzed before, eight isolates and the two reference strains were screened for their ability to produce biofilm in six different media. A waste fluid commonly found in the dairy industry such as milk whey and the free iron medium CTSBg were incorporated into this assay. All strains could form high biofilms in TSB, TSBg and milk, with averages OD of 0.813, 0.964 and 1.101, respectively. The strain MB326 was the best biofilm-forming strain and RA24 strain the lowest biofilm forming in TSB and TSBg ($p < 0.0001$) (Fig. 2). Among the ten *S. aureus* isolates tested in this part of the study, only RA18, MB308 and MBb30, did not increase biofilm in milk and RA18 and MB308 strains were the only NB in this medium (Fig. 2). The biofilm formation decreased markedly when strains were grown in CTSBg (OD = 0.208) and it was almost undetectable in milk whey (OD = 0.009) ($p < 0.0001$). It should be noted that only one strain, MB326, was capable of forming a high biomass of biofilm in CTSBg ($p < 0.0001$) (Fig. 2).

Associations between biofilm levels and culture media

The biofilm-levels and culture media relation analysis revealed that these variables were associated (Monte-Carlo simulated Chi-squared test, $p = 0.00049$) (Fig. 3). Notably, a higher frequency of NB and LB biofilm formers were detected on milk whey (Pearson residual > 2.0). Instead, an elevated frequency of HB formers was found on TSB (Pearson residual > 2.0). Significant and negative associations were observed between NB and LB formers and TSBg and milk respectively (Pearson residual < -2.0). This means that both culture media would favor the *in vitro* biofilm formation in *S. aureus*. In fact, MB level of biofilm formation and milk presented a significant and positive association (Pearson residual > 2.0).

MALDI-TOF-MS analysis

A statistical analysis pipeline was implemented to test if MALDI-TOF-MS data could discriminate the biofilm level formation groups in the different culture media. Biofilm data obtained in TSBg and milk was taken to this purpose. First, a supervised classification by the Binary Discriminant Analysis (BDA) algorithm was performed searching for peaks that best differentiated the NB (absent in TSBg), LB, MB, and HB levels (Fig. 4). Of the 20 best peaks selected by the BDA algorithm in each culture media, the ten most discriminated peaks were used for conducting a hierarchical k-means clustering-PCA (Fig. 5). Clusters appeared as three non-superimposed groups on TSBg and the first two principal components explained 80.8% of the variation with good intracluster homogeneity (Fig. 5 A). Specifically, cluster 1 (HB) and 3 (LB) achieved 100% of homogeneity; while cluster 2 (MB) accomplished a 79% homogeneity (Fig. 5 A). In the case of milk, clusters appeared as partially superimposed groups and the first two principal components explained 77.2 % of the variation with group dependent intracluster homogeneity (Fig. 5 B). Cluster 1 (LB) and 4 (NB) reached 100% of homogeneity, cluster 2 (HB) obtained 53% homogeneity, and cluster 3 (MB) 83% homogeneity (Fig. 5 B).

PIA production

Three strains with different abilities to form biofilms in TSBg and milk were chosen to evaluate PIA production. One of them (RA18) was MB in TSBg but unable to form biofilm in milk; the second strain (MB326) was HB in TSBg and MB in milk; and the third strain (RA24) was LB in TSBg but MB in milk (Fig. 2). RA18 produced more PIA than the rest in TSBg but did not produce PIA in milk ($p = 0.0003$) (Fig. 6), which matches with the fact that RA18 did not form biofilm in that medium (Fig. 2). RA18 formed a lower biofilm than MB326 in TSBg. However, RA18 produced more PIA than MB326 in that medium. On the

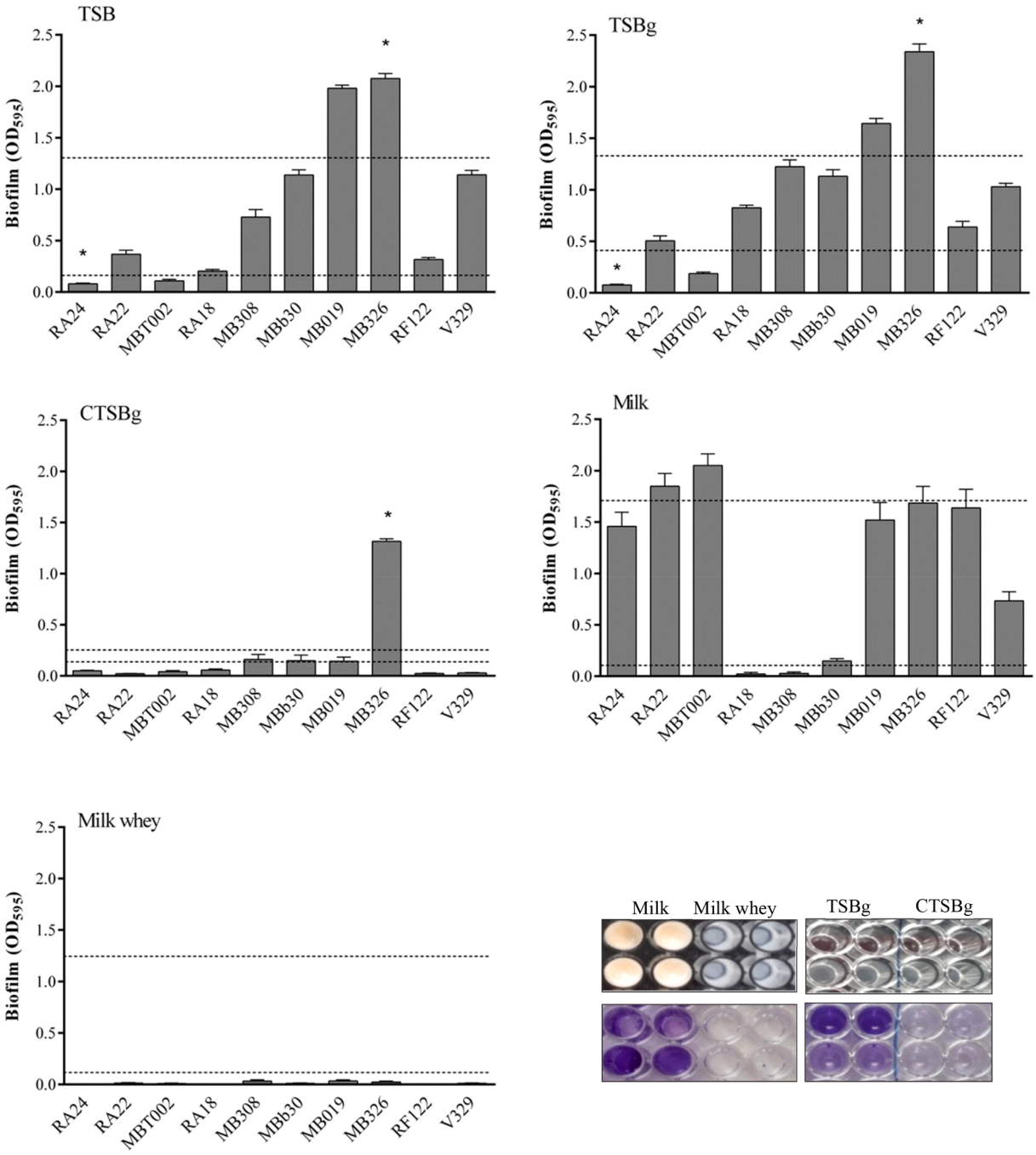

Fig. 2. Effect of different compositions of culture media in the biofilm formation of S. aureus strains. Each bar represents the arithmetic mean ± SEM (standard error of mean) of six wells per strain from three independent assays. The horizontal dotted lines correspond to the Q_{25} and Q_{75} percentile values. Asterisk on the bars represent those strains with significant statistics differences in their biofilm formation –e.g. the highest biofilm-former *vs*. the lowest- ($p < 0.0001$, Mann-Whitney test). Images in the lower right panel are representative and correspond to the biomass formed under different conditions. Trypticase soy broth (TSB); TSB + 0.25% glucose (TSBg); TSBg treated with 3% of Chelex-100 (CTSBg).

other hand, MB326 formed more biofilm in TSBg than in milk but produced more PIA in milk than in TSBg ($p = 0.008$). These data suggest that biofilm formation of RA18 seems to be PIA-dependent in TSBg and milk inhibited this mechanism and consequently the biofilm formation. Conversely, PIA seems to have contributed to biofilm formation of MB326 in milk, but it could also form a biofilm by other mechanisms beyond PIA in TSBg. RA24 produced more PIA in TSBg than in milk ($p = 0.0225$) (Fig. 6). It was noticeable that RA24 strain produced low levels of PIA in milk, although it was MB in this medium (Fig. 2), which suggests that this strain could be forming biofilms by PIA-independent mechanisms in milk.

Discussion

Bovine mastitis is responsible for significant economic losses on dairy farms worldwide and *S. aureus* is a common cause of this disease (Abdi et al., 2021). The veterinary field's concern is that the presence of biofilms in mammary glands results in a decreased effectiveness of antibiotherapy (Pedersen et al., 2021). It is well known that environmental factors such as pH, nutrient availability, among others, can

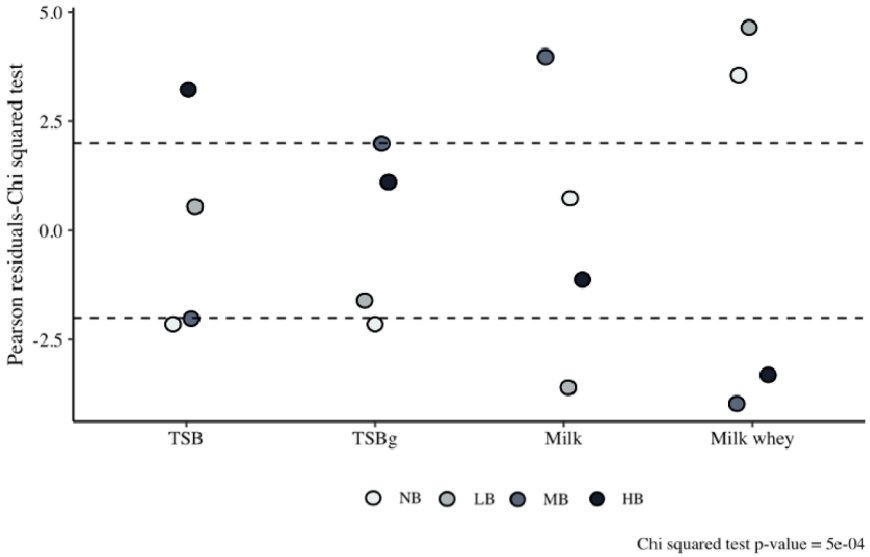

Fig. 3. Biofilms levels according the culture media used. Monte-Carlo simulated Chi-squared test with a posterior analysis of Pearson residuals between biofilm levels and culture media. The association between variables was considered significant if the Pearson residuals were either above 2 or below -2. The dashed lines in the plot point out both cut off values. Trypticase soy broth (TSB); TSB + 0.25% glucose (TSBg). Non-biofilm former (NB); low-biofilm former (LB); moderate-biofilm former (MB); high-biofilm former (HB).

influence bacterial biofilm formation (Atulya et al., 2014). In this study, *S. aureus* strains isolated from subclinical mastitis were cultured in TSBg and milk to evaluate the effects of both media on biofilm formation *in vitro*. In agreement with Fabres-Klein et al. (2015), we found that biofilm formation in milk was significantly higher than in TSBg. Previous reports have demonstrated that milk significantly influenced *Staphylococci* biofilm formation (Melchior et al., 2009; Fabres-Klein et al., 2015; Seixas et al., 2015) and several components of this fluid, such as lactose and caseins, could be a key to stimulate capsule production and biofilm formation (Varhimo et al., 2011; Xue et al., 2014).

A subset of eight strains was randomly selected and their abilities to form biofilms under artificial media and dairy fluids were compared. TSB, TSBg and milk were propitious to form biofilms. All strains produced biofilm in TSB and TSBg, and this is in agreement with other studies that showed the positive effects of adding glucose to medium on biofilm production (Waldrop et al., 2014; Diot et al., 2020). Milk changed the behavior of several strains which become from low-biofilm formers in laboratory media to high-biofilm forming (e.g. RA24, RA22 and MBT002). Nevertheless, the ability of other strains (RA18, MBb30 and MB308) decreased drastically in this medium. As mentioned above, it has been demonstrated that milk and its components could favor the increasing biofilms; however, it is evident that biofilm production is still being strain-dependent, so the strategies taken to prevent and control this pathogen in dairy farms should take it into account (Gomes et al., 2016).

The reduction of free iron in the medium has previously been shown to promote biofilm production by PIA-dependent mechanisms (Dotto et al., 2017). However, in this study, it is shown that only one of these strains produced a robust biofilm in the depleted-iron medium. In concordance with other studies, specific components of milk such as soluble proteins and milk whey, had negative effects on the ability to form biofilms of *S. aureus* (Chaneton et al., 2011; Fijałkowski et al., 2017). Specifically, it was observed that the milk whey caused a high inhibition of the growth of the bacteria and lactoferrin caused a slight decrease in the growth of *S. aureus* (Fijałkowski et al., 2017). Whereby, the presence of soluble proteins with potential antimicrobial action and a deficiency of free iron could explain the results obtained in this study regarding the inhibition of *S. aureus* biofilms in CTSBg and milk whey. Another important factor is the high chemical and biological demand of milk whey which, due the prominent oxidant power of all solid compounds dissolved, may have affected the survival of *S. aureus* and the development of biofilms. Although, all strains were capable of growth when the supernatants were collected from microtiter wells and incubated on TSA for 24 h at 37 °C, suggesting that bacteria could remain viable but could not form biofilms in milk whey (data not shown).

Statistical analysis using the Monte-Carlo simulated Chi-squared test and posterior analysis of Pearson residuals allowed the association between biofilm levels and culture media. The association between culture media and biofilm levels factors imply that these variables were non-randomly distributed. Pearson residuals analysis provides an insight on which factors on one variable were more characteristic or unexpected in the other variable. In concordance with our results of biofilm formation in microtiter plates the analysis with the Chi-squared test revealed that non-biofilm formers presented a significantly lower frequency on TSA and TSAg, which could be interpreted as both media favoring biofilm formation. While, non- and low biofilm formers strains appear in a significantly higher frequency on milk whey, which confirm the negative influence of this media on the *S. aureus* biofilm formation.

In this study, the MALDI-TOF-MS performance with whole colonies and standard experimental settings for microbiological bio-typing was excellent to discriminate between high, moderate and low biofilm producers strains of *S. aureus* in each media, confirming the results of crystal violet assay. An ensemble between supervised and unsupervised algorithms allowed comprehensive discrimination of biofilm level formation status in TSBg, but not in milk. These results showed that each biofilm level on TSBg comprises a distinctive MALDI-TOF-MS pattern which turns this approach into a promising tool to determine different biofilm formation levels. As other studies have investigated, the direct identification after isolation of a bacterial strain as a biofilm producer would allow the development of a valid and effective therapeutic plan (Gaudreau et al., 2017; Caputo et al., 2018; Noumi et al., 2020).

Extracellular polymeric substance which is composed mainly by PIA constitutes the matrix of biofilms (Krukowski et al., 2008). Certainly, the presence of PIA in *S. aureus* infected udders was demonstrated (Schönborn and Krömker, 2016). In this study, PIA production of three strains with different abilities to form biofilm was evaluated in the media which promoted more biofilm formation (TSBg and milk). All of them produced PIA in TSBg, although one strain (RA24) was LB in TSBg, respectively. The production of PIA was more affected by milk and was correlated with the biofilm formation capability, since the non-biofilm forming (RA18) did not produce PIA, while the moderate-biofilm formers (RA24 and MB326) produced a very low amount of PIA in this medium. These results agree with those found by Fabres-Klein et al. (2015) who detected a good correlation between exopolysaccharide production -PIA- and biofilm formation in milk. It has been shown that

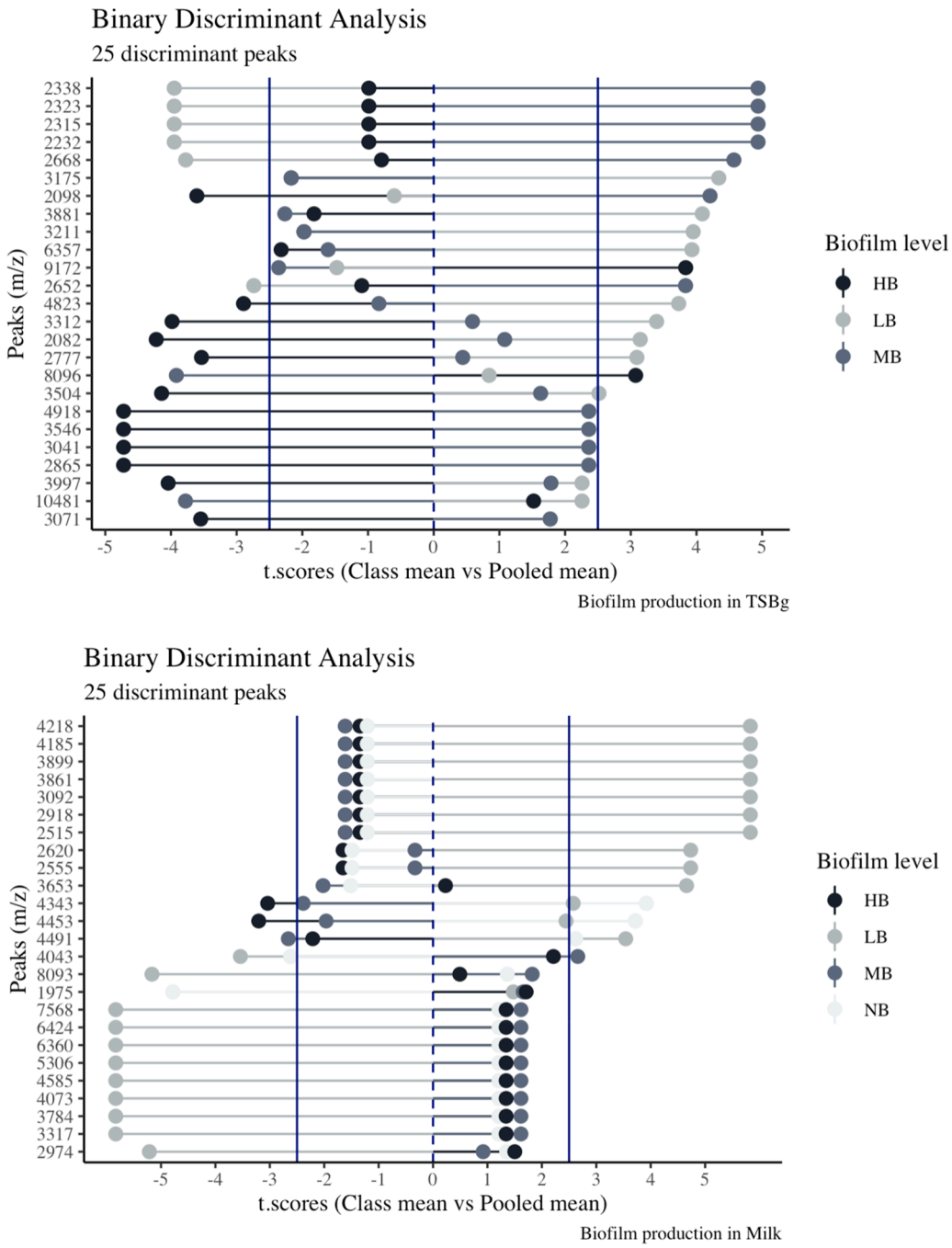

Fig. 4. Binary discriminant analysis (BDA). These plots show the Binary discriminant analysis (BDA) peak selection algorithm result. The algorithm outputs the t. score (x-axis = Class means *vs*. Pooled mean) of each peak (*y*-axis). The sign of the t.score pointed out the presence (positive t.score) or absence (negative t.score) of that peak in each group. A significance level of 95% was achieved when the t.score was equal or higher than 2.5 (up) and equal or less than -2.5 (down).

lactose increased biofilm formation predominantly by inducing PIA production, whereas milk increased biofilm formation through PIA as well as by other biofilm-associated proteins (Xue et al., 2014). That could explain the differences observed between these strains and suggest that RA24 could have produced biofilm by PIA-independent mechanisms in milk.

Conclusion

The present study demonstrated that milk medium changed the performance of biofilm formation by bovine strains of *S. aureus* regarding the common nutritive media used in laboratory conditions. This highlights the importance of considering the growing conditions that mimic the natural ones when analyzing the ability of a strain to form biofilm *in vitro*. In all strains, milk whey led to a significant biofilm biomass decrease. The study of the components and mechanisms underlying this behavior could provide new natural strategies against mastitis. This could help to prevent the indiscriminate use of antibiotics and the reuse of dairy industry waste.

MALDI-TOF MS was an effective tool to confirm the different abilities of biofilm-forming strains by obtaining different protein patterns in each media. Statistical analyses helped to corroborate TSBg and milk as the best culture media which favored the *S. aureus*'s biofilm formation *in vitro*. The incorporation of new high throughput techniques such as

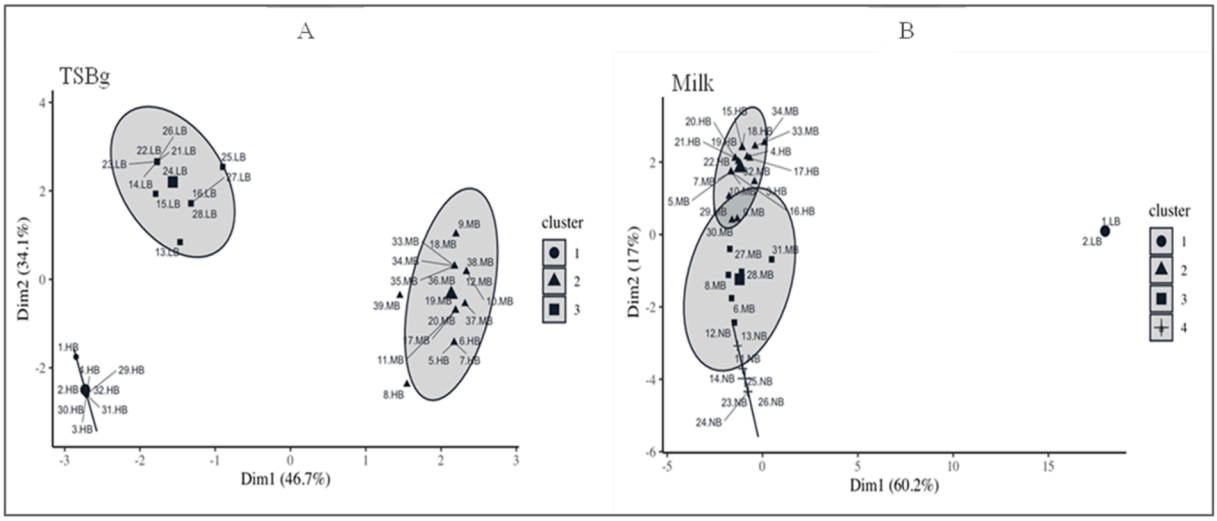

Fig. 5. Unsupervised statistical analysis on TSBg (A) and milk (B). Hierarchical k-means clustering-Principal Component Analysis (HKmC-PCA) cluster plot was made using the top ten peaks selected by the Binary Discriminant Analysis (BDA) algorithm. Labels contain strains replicates ID and biofilm formation level status. Spectra patterns were clustered into three (TSBg, A) and four (Milk, B) groups using the Hierarchical k-means clustering algorithm, represented with different geometric symbols. 95% confidence ellipses were added around cluster means, assuming a multivariate normal distribution. TSB + 0.25% glucose (TSBg). Non-biofilm former (NB); low-biofilm former (LB); moderate-biofilm former (MB); high-biofilm former (HB).

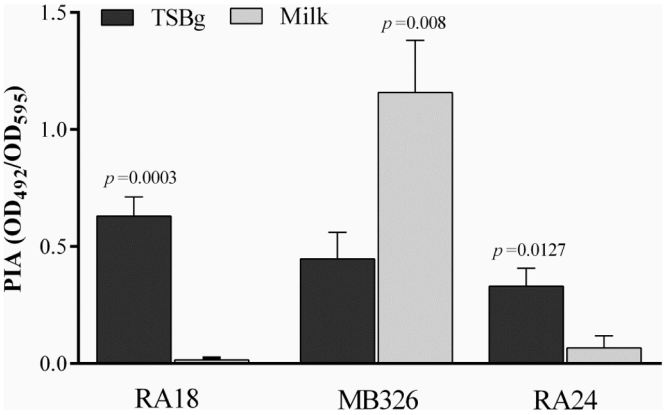

Fig. 6. PIA production by strains with different biofilm formation abilities in TSBg and milk. Each bar represents the arithmetic mean ± SEM (standard error of mean) of 4 to 6 wells per strain from two independent assays. Comparison between PIA (polysaccharide intercellular adhesion) amounts in TSBg vs. milk was calculated statistically (Mann-Whitney test). *P*-values < 0.05 were considered significant. TSB + 0.25% glucose (TSBg).

MALDI-TOF MS in this field could be useful to accelerate and improve the knowledge of the pathogenic potential of *S. aureus* strains that circulate among herds and improve the treatment of mastitis in dairy farms.

CRediT authorship contribution statement

María Emilia Cáceres: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Martín Manuel Ledesma:** Methodology, Software, Formal analysis. **Andrea Lombarte Serrat:** Investigation. **Carlos Vay:** Resources, Validation, Funding acquisition. **Daniel Oscar Sordelli:** Supervision, Project administration, Funding acquisition. **Mónica Nancy Giacomodonato:** Writing – review & editing, Supervision, Funding acquisition. **Fernanda Roxana Buzzola:** Formal analysis, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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