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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Resistance profile and biofilm production of *Enterococcus* spp., *Staphylococcus* sp., and *Streptococcus* spp. from dairy farms in southern Brazil

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Abstract

Milk is a high nutritional value food that helps in human development and growth. However, it can also harbor microorganisms. Therefore, the objective of this study was to isolate, identify and evaluate the resistance profile and pathogenicity factors of gram-positive cocci isolated from liners in milking rooms in the south of Rio Grande do Sul, Brazil. Biochemical and molecular tests were performed for the identification. The following were isolated: *Enterococcus faecalis* (10), *Enterococcus faecium* (4), *Staphylococcus intermedius* (1), *Streptococcus uberis* (1), and *Streptococcus dysgalactiae* (1). The susceptibility of isolated microorganisms to eight antibiotics was evaluated according to CLSI, and the genus that proved to be resistant to most of those was *Enterococcus*. In addition, all 17 isolates were able to form biofilm, which remained viable after the use of neutral, alkaline and alkaline-chlorinated detergent. The only product that was effective against biofilm of all microorganisms was chlorhexidine 2%. The results obtained highlight the importance of pre- and post-dipping tests on dairy properties, in which chlorhexidine is one of the disinfectants used. As observed, products indicated for cleaning and descaling pipes were not effective on biofilms of the different species tested.

Keywords Dairy production · Food contamination · Mastitis

Introduction

Among the main agricultural activities in Brazil, dairy production is responsible for a major part of the national income and tax collection [1]. The largest production is found in the South region of the country, comprising the states of Rio Grande do Sul, Paraná, and Santa Catarina [2]. Milk is a food of high nutritional value, composed of proteins, carbohydrates, fats, and mineral salts that assist in human development and, thanks to its nutritional composition, it enables the development of undesirable microorganisms [3].

The presence of microorganisms in milk is a constant problem in Brazil. *Pseudomonas* spp., *Bacillus* spp., *Enterococcus* spp., *Micrococcus* spp., *Staphylococcus* spp., and *Streptococcus* spp. are among the main deteriorating microorganisms in milk [4, 5]. The most efficient method to reduce the growth of psychotropic bacteria is to store raw milk at a temperature below 4°C. The dairy production chain is not always easy to measure. Therefore, the reduction of bacterial, psychotropic substances in milk can be a useful control of contamination [6].

Moreover, these bacteria in dairy properties are often related to cases of mastitis, especially the *Staphylococcus aureus* [7, 8]. Mastitis, an inflammatory process of the mammary gland, is the disease that prevails within milk production, negatively affecting the economy of this sector [9].

To reduce infection sources and increase production, the use of antibiotics is still the most used method in milk production to treat bovine mastitis, which ends up accelerating the resistance process caused by mutations in the

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Highlights
- Method of in vitro evaluation of biofilm formation in milking machine.
- Bacteria isolated from the milking parlor produce biofilm.
- Chlorhexidine is effective in disinfecting milking parlor equipment.

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microorganisms [10]. DNA mutations, transformation by incorporation of foreign DNA or phage-mediated transduction or conjugation are resistance factors commonly presented by bacteria [11].

Among the resistance factors, the ability to form biofilms is mentioned as a contributor to the persistent colonization of food processing environments [12]. In vitro studies show that bacteria in biofilms become more resistant to the effects of antimicrobial agents, when compared to free cells of the same bacteria [13, 14]. The permanence of biofilms is influenced by several processing methods found in the food industry, such as sub-ideal temperatures and/or inadequate disinfection [15].

In view of the above, the correct hygiene in the different stages of milk production is essential, as well as to evaluate the efficiency of the products used routinely in these places. Therefore, the objective of the present study is to identify and evaluate the resistance profile and the ability to biofilm formation of gram-positive cocci isolated from the milking parlor.

Material and methods

Bacterial isolates

In this study, 17 gram-positive coccis were isolated from the milking parlor equipment of eight different farms located in four different cities (Pelotas (2), Capão do Leão (4), Piratini (1), and Morro Redondo (1)) in the south of Rio Grande do Sul, and three ATCCs: 25904® (*Staphylococcus aureus* subsp. *aureus* Rosenbach), 12600® (*Staphylococcus aureus* subsp. *aureus* Rosenbach), and 51299® (*Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz).

The samples were isolated from initial teat cups using sterile swabs that were placed in previously identified tubes, packed and sent to the Animal Products Inspection Laboratory of the Federal University of Pelotas. This collection point was chosen because of the contact between the animal and the pipes that take the milk to the refrigerator.

Biochemical identification

All samples were biochemically characterized in the Bacteriology Sector of the Regional Diagnostic Laboratory of the Faculty of Veterinary Medicine. The methodology used followed the Cowan and Steel bacterial identification manual [16]. The samples were subjected to the Catalase test to differentiate *Staphylococcus* spp. (catalase-positive) from *Streptococcus* spp. and *Enterococcus* spp. (both catalase-negative). Catalase-positive samples were submitted to MR-VP (Methyl Red, Voges-Proskauer), coagulase test, and biochemical analyses against ribose, nitrate, galactose, maltose, mannitol, and trealose, in addition to polymyxin resistance. CAMP was performed in catalase-negative samples and esculin, inulin, mannitol, salicin, sorbitol, and trehalose tests.

Molecular identification

A DNA template was prepared by emulsifying 5 colonies in 100 µL of ultra-pure water and adding 20 µL to the PCR reaction mixture prior to thermal cycling, following the study of Ellington et al. [17]. The PCR reaction was performed with the commercial kit GoTaq® Colorless Master Mix (ProMega). Each PCR reaction contained 6.5µL of nuclease (solvent), 12.5µL of Mix (Buffer, magnesium chloride and taq DNA polymerase), 0.5µL of direct primer, 0.5µL of reverse primer, and 5µL of DNA, all listed in Table 2. PCR amplifications were performed at 94°C for 2 min, followed by 35 repetitive cycles of 94°C for 1 min, 50°C for 30s, and 72°C for 1 min, ending with a final extension at 72°C for 5 min in a thermocycler (T100 Thermal Cycler, Bio-Rad, Hercules, CA, EUA). The DNA fragments were analyzed on a 1.5% agarose gel. The fragments were visualized by UV fluorescence using a Kodak L-Pix EX/EDAS photo documentation system stained with Blue Green Loading Dye. The sequence of base pairs used for molecular identification of bacterial are described in Table 1.

Inoculum preparation

The inoculants were seeded on BHI agar and remained in the oven at 37°C for 24 h. Afterward, the colonies were diluted in distilled water with approximately 1.5×10^8. Colony-forming units (CFU)/mL. This preparation was carried out for the antibiogram, biofilm accumulation test and disinfectant action.

Antibiogram

Eight antibiotics were used to assess susceptibility: Rifampicin (RIF) 5µg, clindamycin (CLI) 2µg, imipenem (IPM) 10µg, levofloxacin (LVX) 5µg, ampicillin + sulbactam (APS) 20µg, cefuroxime (CRX) 30µg, oxacillin (OXA) 1µg, and vancomycin 30 µg, according to the CLSI document M100 28th Edition (*Clinical Laboratory Standard Institute*) [18].

Inoculum were placed in petri dishes containing the Agar Mueller Hinton medium. Then, with the aid of tweezers, the antibiotic discs were fixed in the middle, at the concentrations previously described. All plates were incubated overnight in the oven at 37°C. After this period, the diameter of the bacterial growth inhibition halos of each antibiotic was measured, where the largest halo is the most effective. The CLSI (2018) was used to determine whether the bacteria

Table 1 Sequence of base pairs used for molecular identification of bacterial bacteria

Bacteria	Sequence 5′ – 3′	Gene	Number of bases	bp	Reference
Enterococcus faecalis	ATCAAGTACAGTTAGTCTT ACGATTCAAAGCTAACTG	ddl *E. faecalis*	19 18	941	Dutka-Malen et al. [46]
Enterococcus faecium	GCAAGGCTTCTTAGAGA CATCGTGTAAGCTAACTTC	ddl *E. faecium*	17 19	550	Dutka-Malen et al. [46]
Streptococcus dysgalactiae	GAACACGTTAGGGTCGTC AGTATATCTTAACTAGAA AAAACTATTG	16S-23S	18 27	270	Forsman et al. [47]
Streptococcus uberis	TAAGGAACACGTTGGTTA AG TTCCAGTCCTTAGACCTT CT	16S-23S	20 20	330	Forsman et al. [47]
Staphylococcus intermedius	CCGTATTAGCTAGTTGGT GG GAATGATGGCAACTAAGT TC	16S	20 20	901	Wakita, et al. [48]
Staphylococcus aureus	CCTATAAGACTGGGATAA CTTCGGG CTTTGAGTTTCAACCTTG CGGTCG	16S	25 24	791	Mason et al. [49]

Table 2 Biochemical tests for preliminary identification of isolated micro-organisms

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Catalase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
BHI+NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
CAMP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Insulin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+		
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		–
Sorbitol	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+		–
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
Maltose																+	
Galactose																+	
Nitrate																+	
Ribose																+	
MR-VP																–	
Esculin																	–
Inulin																	–
Coagulase																–	

(+) positive; (–) negative (2); (1–10) *Enterococcus faecalis*; (11, 14) *Enterococcus faecium*; (15) *Streptococcus uberis*; (16) *Staphylococcus intermedius*; (17) *Streptococcus dysgalactiae.* The concentration of NaCl used was 6.5%; CAMP (Christie, Atkins, and Munch-Petersen); MR-VP (methyl red-voges proskauer test)

Table 3 Susceptibility profile of gram-positive cocci isolated from milking equipment according to the document M100 28th Edition do CLSI, classified as sensitive (S), intermediate (I), and resistant (R)

Sample/ATB	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
LVX	S	S	S	S	R	S	R	S	I	R	I	I	S	S	S	S	S	R	S	R
IPM	S	S	I	I	S	R	R	R	S	I	S	I	I	I	R	R	R	S	S	S
APS	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
OXA	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S
CLI	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	I
RIF	S	S	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R	R
CRX	S	S	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S
VAN	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Levofloxacin (LVX) 5 µg, cefuroxime (CRX) 30 µg, imipenem (IPM) 10 µg, ampicillin (APS) 20 µg, oxacillin 1 µg (OXA), clindamycin (CLI) 2µ g, rifampicin (RIF) 5µg, vancomycin (VAN) 30 µg; (1) *Staphylococcus aureus* subsp. *aureus* – ATCC25904; (2) *Staphylococcus aureus* subsp. *aureus* – ATCC 12600; (3) *Enterococcus faecalis* – ATCC 51299; (4-13) *Enterococcus faecalis*; (14-17) *Enterococcus faecium*; (18) *Staphylococcus intermedius*; (19) *Streptococcus uberis*; (20) *Streptococcus dysgalactiae*

were susceptible (S), intermediate (I), or resistant (R) to the tested antibiotics.

Biofilm formation

For the biofilm formation test, specimens were made from 1 cm long polyvinyl chloride (PVC), non-toxic and sterile fragments, to leave them suspended in BHI broth, in 24-well plates, following the methodology of Peralta [19]. A 1.8mL of BHI broth was added to each well of the plate, followed by 180µL of the inoculum. The material was incubated at 37°C for 72h to induce the biofilm formation. The specimens were washed every 24 h with 0.9% NaCl solution (saline), with the culture medium being changed, in order to keep only the sessile cells and discard cells that were free in the medium, that is, that did not adhere to the specimens.

The experiment was carried out in triplicate. Each plate had a negative control, with the BHI broth medium and the specimen. As a positive control, to assess biofilm formation, ATCC 25904 (*Staphylococcus aureus* subsp. aureus Rosenbach) was used.

At the end of 72h, the hoses were collected, washed with 0.9% NaCl solution to dispense free cells, transferred to an Eppendorf with 1mL of 0.9% NaCl solution and sonicated for 30s at 30W (Cole-Parmer Ultrasonic Processor ® 60648 USA) to release the entire biofilm in the saline solution, without cell lysis. In the following, serial dilutions of the suspensions were performed until the dilution of inoculum equivalent to 10^{-7} was obtained.

All samples were plated on BHI agar with two 10µL aliquots of each Eppendorf and subsequently incubated at 37°C for 24 h to count the colony-forming units (CFU), following Peralta [19] and applying the following equation:

CFU = (n° CFU / inoculum volume) × dilution

In this study, the concentration of 10^{-4} was used as a standard for all samples, as it was the largest dilution in which it was possible to differentiate and count colonies.

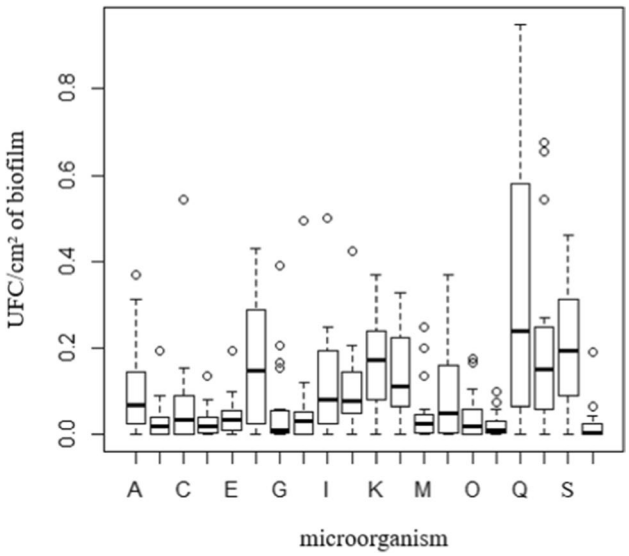


Fig. 1 Box plot with biofilm formation of micro-organismos in UFC/cm^2 of biofilm. *Streptococcus dysgalactiae* (**A**); *Enterococcus faecalis* (**B**, **C**, **D**, **F**, **G**, **H**, **M**, **N**, **O**, **P**); *Streptococcus uberis* (**E**); *Enterococcus faecium* (**I**, **J**, **K**, **L**); *Staphylococcus intermedius* (**Q**); ATCC 12600 (**R**); ATCC 25904 (**S**); ATCC 51299 (**T**)

Action of chemical products on biofilm

All microorganisms were tested for biofilm viability against commercial products used in the milking parlor routine, such as neutral detergent (composition: anionic surfactants, coadjuvant, preservative, neutralizer, thickener, and vehicle; active ingredient: linear sodium alkylbenzene sulfonate), acid detergent, alkaline-chlorinated detergent (composed of sodium hydroxide, sodium hypochlorite, sequestrant, adjuvant, vehicle, and anionic surfactant) and 2% chlorhexidine-based solution (each 100mL contains 1.0 g chlorhexidine

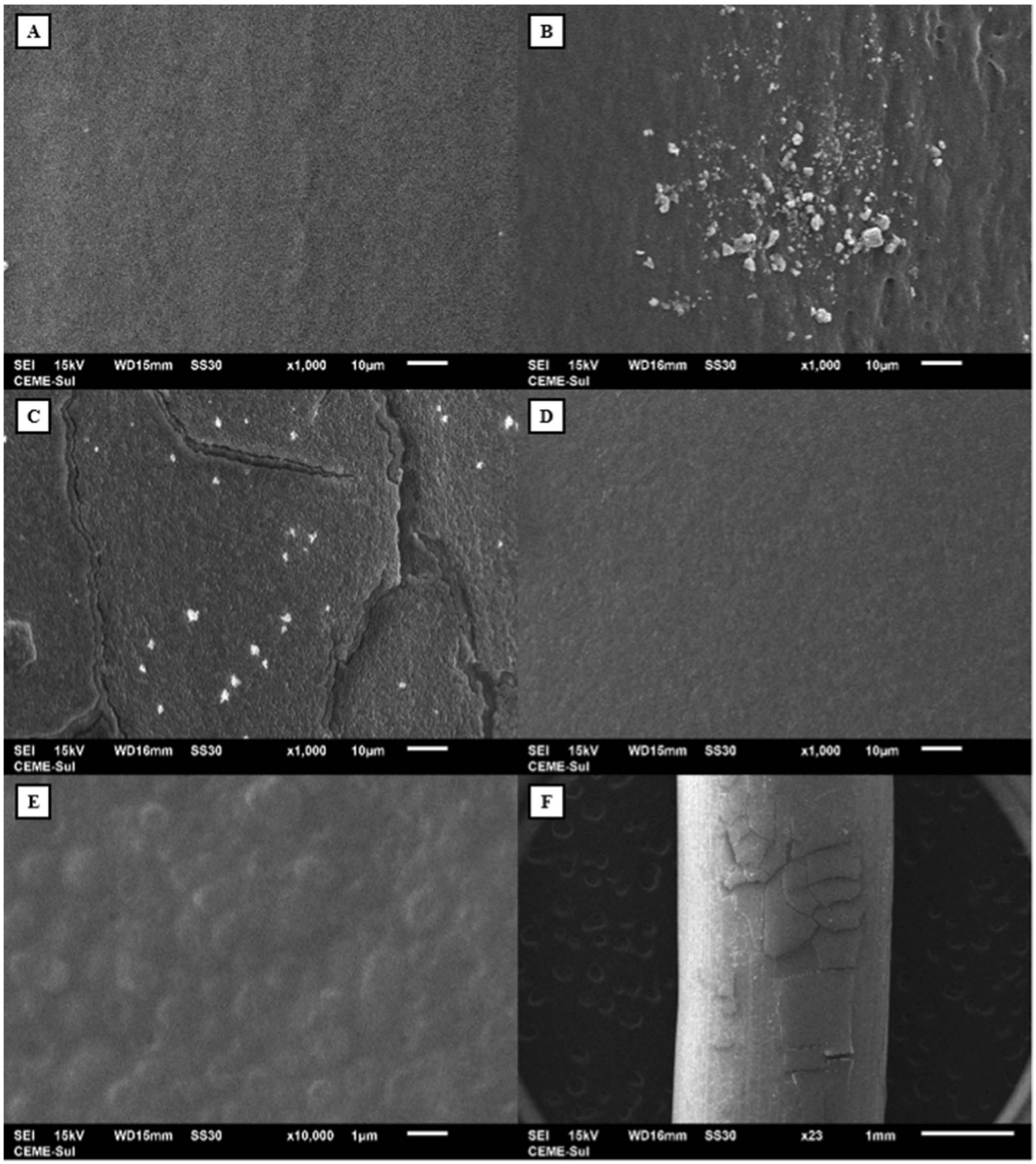


Fig. 2 Biofilm formation in the control group (*Staphylococcus aureus* ATCC 25904) with a 1000× magnification: **A** the biofilm formed in the hoses of the milking parlor at hour 0, where it is possible to observe that adhesion of sessile cells has not yet occurred. The adhesion of microorganisms to PVC begins in **B** in 24 h, where it is possible to observe adhered cells and predominance of EPS; **C** the biofilm at 48 h, well consolidated due to the high multiplication index and greater EPS production. This conclusion is possible due to the greater cellular aggregation and observation of gaps between these clusters that appeared after the preparation and drying of the samples for the realization of the images, characteristic of a place with accumulation of humidity and that is subsequently subjected to drying; **D** the biofilm formed after 72 h, with difficult cell differentiation that is justified by the advanced stage that the biofilm is in, with a thick layer of EPS, which ends up providing protection and making it difficult to visualize the cells that are under this layer. **E** The approximation (5000× magnification), thus allowing the verification of a large number of cells under the EPS matrix, while **F**, less approximate (23× magnification), allows the visualization of the crust formed by the EPS of the biofilm formed on the PVC

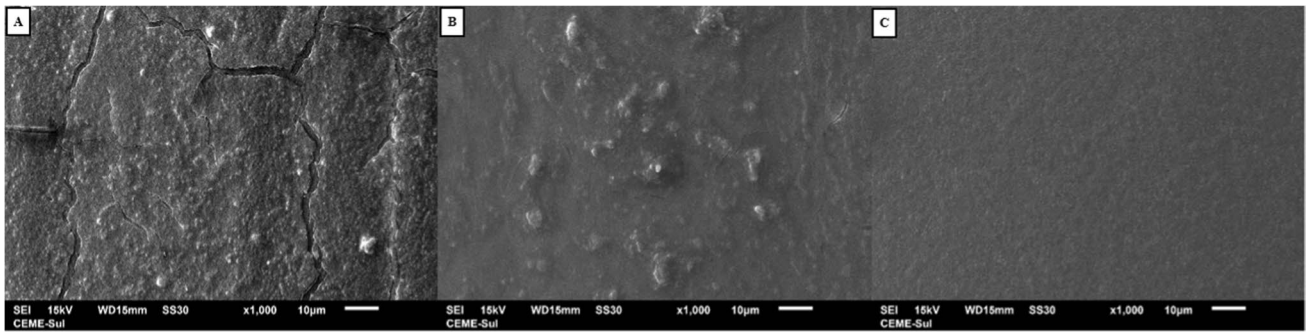


Fig. 3 Biofilm formed after 72 h of contact with PVC, inoculum and culture medium in the three different isolated genera: **A** *Enterococcus* (*E. faecalis*); **B** *Streptococcus* (*Streptococcus uberis*); **C** *Staphylococcus* (*Staphylococcus aureus*). All images it is possible to visualize mature biofilm, where in **A**, it is possible to visualize the cracks that appeared in the drying process of the samples after their loss of H_2O; **B** regions with biofilm growth and elevation in relation to the place where they are adhered; **C** intense formation of EPS on the cells adhered to the surface

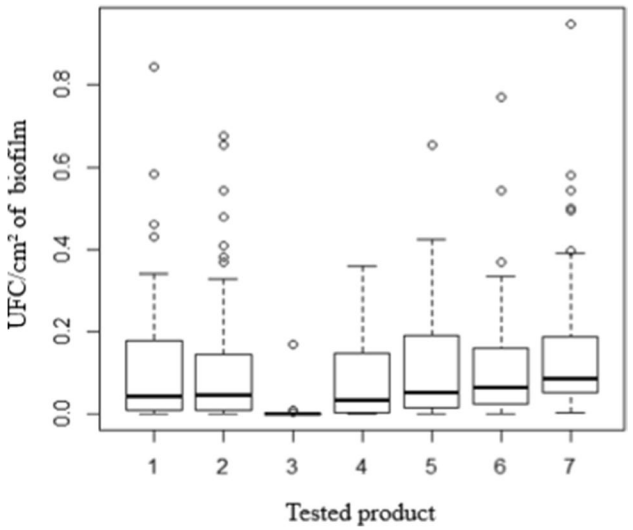


Fig. 4 Box plot of production and biofilm with the treatments tested, where the dark lines (median) represent the growth of microorganisms for the following treatments: control with distilled H_2O (1); neutral detergent (2); clorexidin (3); acid detergent (4); alkaline-chlorinated detergent (5); alkaline-chlorinated detergent at 45°C (6); distilled H_2O at 45°C

digluconate 20%, benzalkonium chloride 1.0 g, and vehicle q.s.p. 100mL).

The alkaline-chlorinated disinfectant was used at room temperature and 45° C, the temperature indicated by the manufacturer. In order to assess whether the action of the disinfectant on the isolates was of the product or the temperature (45°C), a control was also used with distilled water at 45°C, thus totaling seven groups. For this test, the methodology of Peralta et al. [19] with modifications was used. After the first 48 hours of growth of the microorganisms and adhesion of the same in the hoses, they were washed with 0.9% NaCl solution, left for 10 minutes in contact with the product in the concentrations recommended by the manufacturer and washed again with 0.9% NaCl solution.

A control of each tested isolate was used to evaluate the action of each product, without exposure to the products, for later comparison. After the 72h of the experiment, the samples were taken to the sonicate in Eppendorf with 1mL of saline for 30s at 10W (Sonicator de S500, R2D091109 Brazil) and later two 10µL aliquots were plated on BHI agar. Finally, these were taken to the incubator at 37°C for 24 h to count the CFU following the study of Peralta [19].

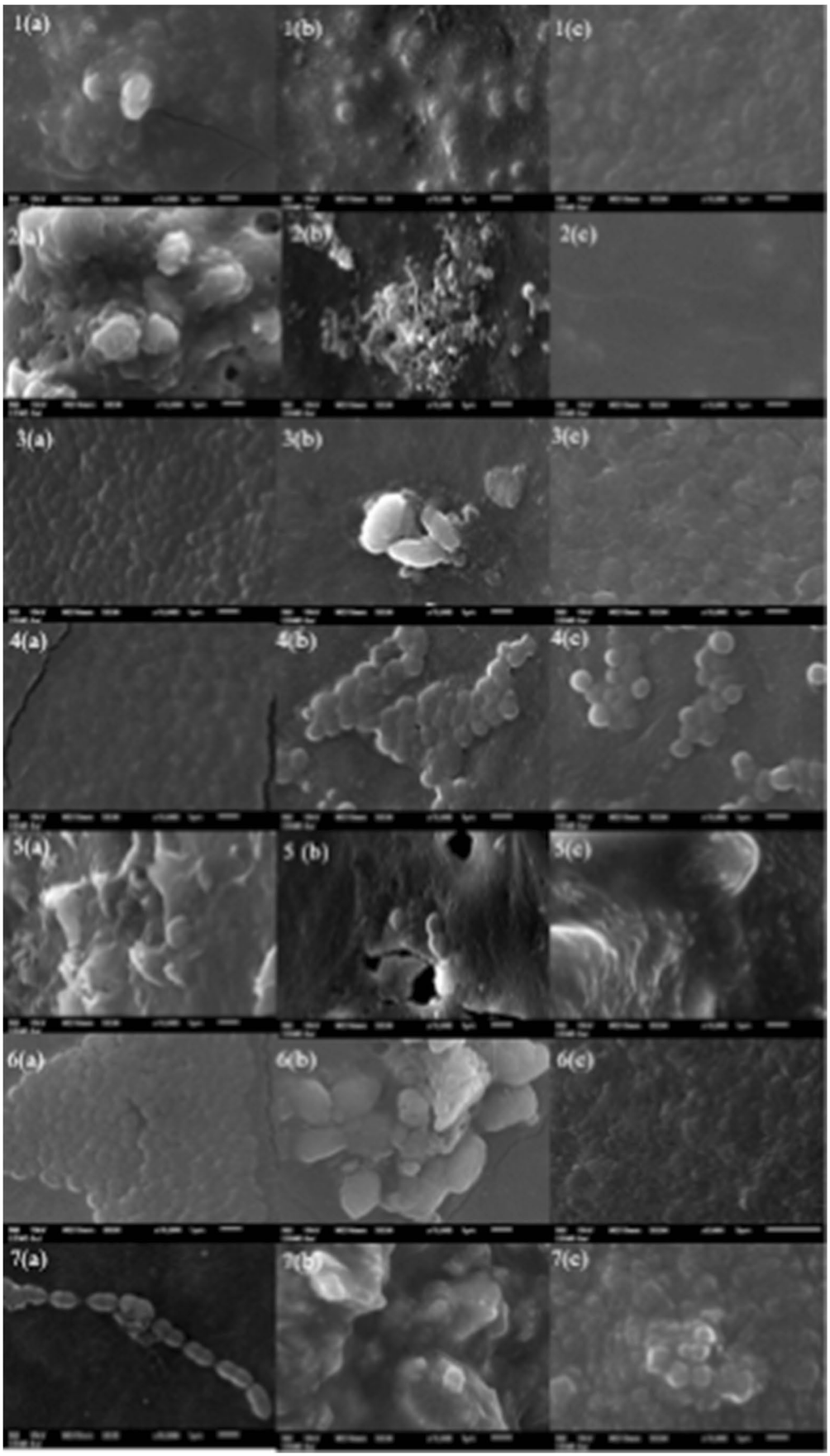
Analysis of samples in scanning electron microscopy

For the SEM analysis, three strains were used, one from each genus to check the different patterns of biofilm formation between genders, which were ATCC 25904 (*Staphylococcus aureus subsp. Aureus*), *Enterococcus faecalis* (1), and *Streptoccocus uberis* (1).

With the methodology used to evaluate disinfectants described by Peralta [19], ATCC 25904 assessed biofilm formation at different times: 0h, 24 h, 48h, and 72h. The action of disinfectants applied after 48 h of biofilm formation was also evaluated. For the *Enterococcus faecalis* and *Streptococcus uberis* samples, the action of the control groups (72h without product) and the action of chlorhexidine and neutral, alkaline-chlorinated and acid detergents were evaluated. A microplate well was used as a negative control to monitor the sterility of the culture medium.

At the end of the period of biofilm formation of the samples, the PVCs were removed from each well. With the aid of tweezers, these were placed in previously identified Eppendorfs, which were taken to the incubator at 40°C for 120h to dry the material.

After this period, the samples were deposited on double-sided tape in metallic stubs, metalized with gold and


1(a)
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◂**Fig. 5** Biofilm formation and its treatments. Different species of bacteria tested are represented by letters, being *Staphylococcus aureus* ATCC 25904 (**a**), *Enterococcus faecalis* (**b**), and *Streptococcus uberis* (**c**). Tested compounds are felt through numbers. Control with distilled H_2o (1), neutral detergent (2), acid detergent (3), alkaline-chlorinated detergent (4), 2% chlorhexidine-based solution (5), alkaline-chlorinated disinfectant at 45 °C (6), the temperature indicated by the manufacturer, and a control of temperature with distilled water at 45°C (7). All this pictures were taken after 72h of biofilm formation and contact with the products within 48 h

observed/photographed in a scanning electron microscope (Jeol, JSM - 6610LV), emphasizing the magnifications of 10,000×, 5000×, and 1000× at the Center for Electronic Microscopy of the South Region (CEME-SUL) of the Federal University of Rio Grande.

Statistical analysis

The data obtained were not parametric fand. Thus, the Kruskal-Wallis test was used, indicated for independent samples, considering $p < 0.05$ in the BioEstat® software version 5.3.

Results

Biochemical Identification

The result of each bacteria against sugars and other biochemical tests is described in Table 2.

Molecular Identification

The genus *Enterococcus* spp. prevailed in the identification, with ten species of *Enterococcus faecalis* and four of *Enterococcus faecium*. The species *Streptococcus uberis* (1), *Streptococcus dysgalactiae* (1), and *Staphylococcus intermedius* (1) were also identified.

Antibiogram

The result of the antibiogram against the isolates with the susceptibility profile is described in Table 3, following the document CLSI M100, 28th Edition [18].

The only antibiotics that showed results in relation to all tested microorganisms were ampicillin (APS) 20µg. All *Enterococcus* isolates and the only *Streptococcus uberis* isolate are multidrug-resistant, showing resistance to three or more classes of antibiotics [20]. The isolated genus that showed the greatest resistance was *Enterococcus*, with 100% of the results detected, with only one of the tested antibiotics being ampicillin (APS) 20µg. The same-gen ATCC (51299) also stood out in relation to the others tested.

The species *S. intermedius* and *S. dysgalactiae* showed resistance to levofloxacin. In addition, *S. intermedius* was also resistant to clindamycin, while *Streptococcus dysgalactiae* was resistant to rifampicin, not being considered multidrug-resistant isolates. An isolate of *Streptococcus uberis* was obtained, which proved to be resistant to the antibiotics Cefuroxime, rifampicin, clindamycin, and oxacillin.

Biofilm formation and disinfectant action

All selected samples and ATCCs were able to form biofilm, being the only ones of S. intermediates and *S. dysgalactiae*, all *E. faecium*, three samples of *E. faecalis*, and one ATCC 12600 formed biofilm statistically equal to ATCC 25904. The means and standard deviations for each isolate were as follows: ATCC 12600 (0.19±0.20), ATCC 25904 (0.20±0.13), ATCC 51299 (0.02±0.04), *S. intermedius* (0.32±0.30), *S. dysgalactiae* (0.10± 0.10); *S. uberis* (0.04± 0.04), *E. faecalis* (0.03±0.04; 0.08±0.15; 0.02±0.03; 0.15±0.14; 0.05± 0.09; 0.05±0.10; 0.04±0.06; 0.08±0.10; 0.03±0.05; 0.02±0.02), and *E. faecium* (0.11±0.12; 0.10±0.09; 0.16±0.11; 0.13±0,10). These results are described in Fig. 1.

Figure 2 shows the biofilm formed by ATCC 25904 in PVCs at different times of contact with the culture medium: 0h, 24 h, 48h, 72h. Figure 2 E presents the approximate image 2 (D), with a 5000× magnification, where a dense exopolysaccharide (EPS) matrix and a large number of cells under it are seen. It is also possible to observe the very thick biofilm showing some cracks because of the methodology of drying the material and loss of H_2O. The increase used for Fig. 2 F was 23×.

Figure 3 presents biofilm formations, after 72 h, in different isolated species, following different forms of formation evolution in the isolated genera in this study.

Figure 4 illustrates a box plot of production and biofilm with the treatments tested in dark lines (medians, standard deviation and outliers).

In Fig. 5, biofilm is shown in different species and submitted to different treatments.

Discussion

The microorganisms isolated and identified were *Enterococcus faecalis* (10), *Enterococcus faecium* (4), *Streptococcus uberis* (1), *Streptococcus dysgalactiae* (1), and *Staphylococcus intermedius* (1). Within the genus *Enterococcus*, a higher prevalence of *E. faecalis* was expected, when compared to *E. faecium*, since the first species is the

most prevalent in the genus and is described as an important environmental pathogen that causes bovine mastitis [21, 22]. Both (*E. faecalis* and *E. faecium*) are related to poor hygienic-sanitary conditions in dairy farms and serve as indicators of fecal contamination [23].

Within the genus *Streptococcus*, the two isolated species, *S. dysgalactiae* and S. *uberis,* are among the four most found species in cattle herds, being frequently related to cases of environmental mastitis [24]. The only isolate of the genus *Staphylococcus*, *Staphylococcus intermedius*, is also related to cases of mastitis, although less frequently when compared to other species [25].

Regarding the antibiogram, *Enterococcus* showed susceptibility to ampicillin and vancomycin (100%) only and resistance to levofloxacin (21.42%), imipenem (42,85%), oxacillin (100%), clindamycin (92.85%), rifampicin (92.85%), and cefuroxime (92.85%). This resistance of *Enterococcus* is described in several studies [26–28].

About 40 years ago, the *Enterococcus* spp. was considered a harmless genus with microorganisms present in the gastrointestinal tract of humans and animals in commensal form. However, the emergence of species causing infections in hospitals has been observed, mainly those found in this study, *Enterococcus faecalis* and *Enterococcus faecium*, which are mainly due to the indiscriminate use of antimicrobial agents [29–31]. In 2002, Shepard and Gilmore described that this resistance is caused by genes intrinsic to the classes of antibiotics, such as sulfonamides, clindamycin and low levels of β-lactamases and aminoglycosides. Also, according to the authors, the genus is able to resist all classes of antimicrobials [32].

The presence of antibiotic-resistant *Enterococcus* spp. in the bovine gastrointestinal tract can promote the spread of antibiotic resistance genes (ARGs) to other bacteria, especially if associated with mobile genetic elements (MGEs) [29, 30]. The bacteria in this study were isolated from liners, where the pipes that take bovine milk to storage tanks begin. This may result in a public health problem since resistance genes can reach humans through the food chain [33]. In addition to the transfer of plasmids, the resistance acquired by *Enterococcus* may be related to the transfer of transposons, chromosomal exchanges, and mutation [34].

In 1992, Noble and colleagues found, in the laboratory, the ability of *Enterococcus faecalis* to transfer vancomycin resistance genes to *Staphylococcus aureus*, the main etiologic agent of mastitis in cattle [35]. Rosvoll et al. evaluated the presence of plasmids in 93 strains of *E. faecium* using the PCR technique. They concluded that 88 strains had one to seven plasmids inserted in their genetic material and that these were related to a large part of the horizontal gene transfer [36].

An article recently published by Amidi-Fazli and Hanifian [37] shows interesting results. They evaluated the biodiversity, antibiotic resistance and virulence traits of Enterococcus species in artisanal dairy products. Surprisingly, of the 426 dairy products evaluated, 262 (62.91%) were Enterococci positive with 48.55% *E. faecalis* (168), and 40.17% *E. faecium* (139). *E. faecalis* (17 isolates) and *E. faecium* (6 isolates) matched all seven virulence genes tested: asa1, esp, gelE, hyl, cylA, efaA, and ace, emphasizing the importance of knowing the biodiversity of this genus in dairy products. The high isolation index is directly related to failures in one or several steps during milk collection on the properties. This is why we highlight the importance of carrying out all steps, as one does not exclude the other.

Regarding the location where the microorganisms in this study were isolated, Terra et al. [38] highlight the danger, because according to a study carried out by their research group and published in 2019, milk is an appropriate environment for the transfer of vancomycin resistance genes from *E. faecium* to *E. faecalis*. Another important point is in relation to biofilm formation and resistance to antibiotics. Pajohesh et al. [39] revealed a significant correlation between biofilm production and resistance to antibiotics in microorganisms isolated from raw milk intended for humans. A large number of multidrug-resistant Staphylococcus aureus strains have been found to carry multiple biofilm-related genes.

ATCC 25904 (*Staphylococcus aureus*) was used as it has been used in other research as a control of biofilm formation [40, 41]. Of the two isolated species of *Enterococcus*, *E. faecalis* has a greater aptitude for biofilm formation when compared to *E. faecium* [42], although the opposite was observed in this study (greater biofilm formation by *E. faecium*). Enterococcal surface proteins, aggregating substances and collagen-binding proteins are adhesion factors found in *Enterococcus* that facilitate the establishment of biofilm [42]. For Verran, these structures are a potential for bio transfer, where microorganisms present on the surface of the equipment, before or after the hygiene procedure, can serve as possible contaminants in food products during processing [43].

In this study, the hoses in the milking parlor can serve as a source of contamination, even after the use of acidic, alkaline-chlorinated and neutral detergents. The only product that affected biofilms of different species was chlorhexidine 2%. The results are in line with those found by Bohrz, when he analyzed the hygienic-sanitary conditions of liners and other equipment inside a milking parlor and confirmed the presence of bacteria, even after using sanitizers [44].

Medeiros et al. obtained good results when they tested 2% chlorhexidine in *Staphylococcus* isolated from subclinical bovine mastitis and found that the longer the product is applied, the better its effectiveness is. The longest time tested for chlorhexidine in contact with the bacterium was 5 min. During this, it was observed that it killed 93.30% of *Staphylococcus aureus* isolates and 81.8% of non-aureus coagulase-positive *Staphylococcus* [45].

Regarding the neutral detergent, the concentration indicated by the manufacturer varies according to the dirtiness of the material to be cleaned. For light cleaning, the manufacturer indicates 50 to 200mL of the product for each 10L

of water, while for heavy cleaning the concentration can vary from 200 to 1000mL of the product for 10L of water. In this study, a concentration of 200mL was used, which can be used for all types of cleaning. However, we did not see any results in the breakdown of biofilms formed at the end of 72h.

It is important to emphasize that the detergents chosen for this study are not aimed directly at the microorganisms, but at the descaling and cleaning of the equipment. That is why it is extremely important to carry out all stages. Failure in the pre-dipping, where the product used in the studied properties is 2% chlorhexidine, can result in the permanence of microorganisms on the ceiling and subsequent contamination of the pipes, where they can multiply and have greater protection against external agents.

Conclusion

All microorganisms were able to form biofilm and adhere to the piping material in the milking parlor. These results show the importance of carrying out pre- and post-dipping tests to control contamination inside the milking parlor, since after the biofilm is established in the pipes, detergents would hardly act on them. The microorganisms studied here showed resistance to most antibiotics, with 100% effectiveness being observed only to ampicillin (APS) 20 µg and vancomycin (VAN) 30 µg. The only disinfectant that proved effective was chlorhexidine. In addition, a meticulous hygiene routine, frequent change of liners and proper handling of animals would certainly reduce the rates found here. Our conclusions lead to alternatives to be used to inhibit microbial contamination, while not offering risks for contamination of the product (milk).

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Declarations

Conflict of interest The research was conducted with the support of the Laboratory of Molecular Biology of Microorganisms (LBMM), Laboratory of Inspection of Products of Animal Origin (LIPOA), and the Bacteriology sector of the Regional Diagnostic Laboratory of the Faculty of Veterinary Medicine, all belonging to the Federal University of Pelotas.

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