JOURNAL OF FOOD QUALITY & HAZARDS CONTROL
SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 7 (2020) 67-74

Antibiotic Resistance and Biofilm Production in Catalase-Positive Gram-Positive Cocci Isolated from Brazilian Pasteurized Milk

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HIGHLIGHTS

- *Kocuria varians*, *Macrococcus caseolyticus*, and *Staphylococcus epidermidis* were detected in pasteurized milk samples.
- Four isolates of *K. varians* exhibited multidrug resistant phenotype.
- Five isolates of *K. varians* and one isolate of *S. epidermidis* were biofilm producers.
- Antibiotic-resistance of the isolates highlights the possible role of milk as a reservoir of resistance genes.

Article type
Original article

Keywords
Gram-Positive Cocci
Drug Resistance, Microbial
Biofilms
Milk
Brazil

Article history
Received: 16 Apr 2020
Revised: 18 May 2020
Accepted: 22 May 2020

Acronyms and abbreviations
CFU=Colony Forming Unit
CRA=Congo Red Agar
MDR=Multidrug Resistant

ABSTRACT

Background: Milk is a reservoir for several groups of microorganisms, which may pose health risks. The aim of this work was to assess the antibiotic resistance and biofilm production in catalase-positive Gram-positive cocci isolated from Brazilian pasteurized milk.

Methods: The bacteria were isolated using Baird-Parker agar and identified by Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight (MALDI-TOF) mass spectrometer. Disk diffusion technique was used to evaluate antimicrobial susceptibility. For qualitative evaluation of biofilm production, the growth technique was used on Congo Red Agar.

Results: Totally, 33 out of 64 isolates were identified, including *Staphylococcus epidermidis* (n=3; 4.7%), *Macrococcus caseolyticus* (n=14; 21.9%), and *Kocuria varians* (n=16; 25%). Twenty-two isolates were resistant to at least one antibiotic. Biofilm production was detected in only 5 isolates of *K. varians* and 1 isolate of *S. epidermidis*. All 14 *M. caseolyticus* isolates were resistant to at least one antibiotic; but, multidrug resistant (MDR) isolates were not detected. Among all *K. varians* isolates, 4 were resistant to at least one antibiotic from three different classes and were considered to be MDR.

Conclusion: The presence of antibiotic-resistant *M. caseolyticus*, *S. epidermidis*, and *K. varians* isolates, especially MDRs, in milk samples highlights the possible role of milk as a reservoir of resistance genes.

Introduction

Milk and dairy products are suitable sources of several essential nutrients, such as proteins, carbohydrates, lipids, and vitamins (Alegbeleye et al., 2018). However, because of its nutritional richness, milk is also a habitat

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To cite: Machado M.A.A., Ribeiro W.A., Toledo V.S., Ramos G.L.P.A., Vigoder H.C., Nascimento J.S. (2020). Antibiotic resistance and biofilm production in catalase-positive Gram-positive cocci isolated from Brazilian pasteurized milk. *Journal of Food Quality and Hazards Control*. 7: 67-74.

DOI: 10.18502/jfqhc.7.2.2886 Journal website: http://www.jfqhc.com

for a wide variety of microorganisms originating from different sources, such as the milker or utensils and equipment used for milking and, transporting or storing milk (Ali et al., 2017).

Mastitis is a common disease in dairy animals that can be treated using several antibiotics; however, excessive or inappropriate use of these drugs can lead to the acquisition of resistance by bacteria. *Staphylococcus* sp. is one of the main microorganisms associated with mastitis and is often found in milk (Ruegg, 2017). Species belonging to this genus, particularly *S. aureus*, are also associated with several diseases in humans, including food poisoning. In addition, these bacteria often show resistance to antibiotics used in both the clinical and veterinary fields. In some cases, genes carrying resistance can even be transferred to other bacteria (Vanderhaeghen et al., 2010).

Natural gene transfer usually occurs through the mechanisms of transformation, transduction, and conjugation, involving free exogenous DNA, bacteriophages, and plasmids. This is thought to be the primary way in which antibiotic-resistance genes are transferred (Thomas and Nielsen, 2005). Raw foods, or those that have been contaminated after processing, are potential vehicles for the introduction of antibiotic-resistant bacteria into the human gastrointestinal tract. Resistance genes, in turn, can be transferred to opportunistic or pathogenic bacteria. Some studies have already reported the transfer of antibiotic-resistance genes from food bacteria, including enterococci, staphylococci, and lactobacilli, through *in vitro* conjugation experiments (Nawaz et al., 2011; Vignaroli et al., 2011; Zhang et al., 2008). However, a recent experiment carried out in Hungary showed that raw milk samples sold for human consumption in public markets contained genetic material from many bacterial species, including several antimicrobial resistance genes. The authors suggest that antimicrobial resistance can be acquired not only through residual antibiotics in milk but also through the ingestion of antimicrobial resistance genes in animal products, which can then be transferred to other bacteria (Tóth et al., 2019).

Other genera of Gram-positive and catalase-positive cocci related to *Staphylococcus* spp., such as *Kocuria* and *Macrococcus*, are found in milk and dairy products (Martinez et al., 2017; Ribeiro-Júnior et al., 2018) and can also transfer resistance genes to each other and to other bacteria (MacFadyen et al., 2018). In some cases, this transference can be enhanced by the presence of the biofilm.

Biofilms are highly organized multicellular complexes wrapped in a self-produced matrix of extracellular polymeric substances (Donlan, 2002; Yuan et al., 2019). This structure enables bacteria to adhere to various surface types, such as stainless steel, plastic, glass, and food products (Donlan, 2002; Yuan et al., 2018, 2019). The formation of biofilms in the dairy industry is a persistent contamination problem that has been constantly addressed in the field of food safety, since the biofilm cells are resistant to sanitization processes and antimicrobial agents (Song et al., 2016). Biofilm-forming bacteria may cause serious damage, ranging from economic loss to food spoilage and diseases (Møretrø and Langsrud, 2017; Yuan et al., 2018). Biofilms can increase the corrosion rate of equipment in the production chain, interfering with the heat treatment processes (Mnif et al., 2020; Mogha et al., 2014).

Although, pasteurization is the most common thermal process used to eliminate pathogenic microorganisms in milk, this technique is not able to destroy the entire bacterial population (Knight et al., 2004; Ribeiro-Júnior et al., 2018), in some cases due to the production of biofilm by microbial population. This study aimed to determine antibiotic resistance and biofilm production in catalase-positive Gram-positive cocci isolated from Brazilian pasteurized milk.

Materials and methods

Isolation and identification of catalase-positive Gram-positive cocci

Nine convenience samples (L1-L9) of pasteurized milk sold in the city of Rio de Janeiro, Brazil were selected as the food matrix used for the initial isolation of *Staphylococcus* and to detect other possible catalase-positive Gram-positive cocci. The samples were from the two most commercialized brands in Rio de Janeiro and were collected from October 2019 to February 2020.

According to ISO (1999), 25 ml of milk were diluted in 225 ml of 0.1% peptone water (Biocen, São Paulo, Brazil), homogenized for 60 s, and diluted until the 10^{-3} dilution. Dilution aliquots were inoculated on Baird Parker agar (Kasvi, São Paulo, Brazil), and the plates were incubated at 37 °C for 48 h. After the incubation period, typical (circular, black, surrounded by an opaque zone and/or transparent halo extending beyond the opaque zone) and atypical colonies (morphologically similar to the typical ones, without the presence of halos) were selected. The isolates were stored by freezing in cryotubes with tryptone soy broth with 40% (v/v) glycerol (Merck, São Paulo, Brazil).

The isolates were identified using a Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight (MALDI-TOF) mass spectrometer (Microflex LT, Bruker Daltonics, USA). Each isolate was inoculated on tryptone soy agar (Merck, São Paulo, Brazil) at 37 °C for 24 h, then transferred to a 96-well microplate (Bruker,

Billerica, USA) with the aid of a sterile toothpick. The bacterial cells were lysed with 70% formic acid (Sigma-Aldrich, USA) with subsequent addition of an aliquot of 1 µl of the matrix solution [alpha-cyano-4-hydroxy cinnamic acid diluted in 50% (v/v) acetonitrile and 2.5% (v/v) trifluoroacetic acid (Sigma-Aldrich, USA)]. The spectra of each sample were generated by the equipment and analyzed using the MALDI Biotyper 3.1 software (Bruker Daltonics, Bremen, Germany).

Antibiotic susceptibility profiles

The disk diffusion technique was used to assess the *in vitro* antimicrobial susceptibility profile of the isolates, according to CLSI (2018). The *S. aureus* strain ATCC 25923 was used as a control in the antimicrobial susceptibility tests. The isolates were classified as susceptible, moderately susceptible (intermediate resistance), or resistant to the tested antibiotics. The following antimicrobials were used: ciprofloxacin (5 µg), clindamycin (2 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), norfloxacin (10 µg), penicillin (10 µg), and tetracycline (10 µg), which are representative classes of fluoroquinolones, lincosamines, phenicols, macrolides, aminoglycosides, penicillins, and tetracyclines.

Biofilm production potential

For the qualitative evaluation of biofilm production, the growth technique on Congo Red Agar (CRA) was used including 15 g/L of Brain Heart Infusion agar (Kasvi, São Paulo, Brazil), 50 g/L sucrose (Pro Analysis, São Paulo), and 0.8 g/L of Congo Red (VETEC, Brazil) as described by Freeman et al. (1989) and adapted by Cruzado-Bravo et al. (2019) for use with *Staphylococcus* sp.

The isolates were grown on tryptic soy agar at 37 °C for 24 h. Then, a bacterial suspension was prepared in 0.85% saline until the solution reached a turbidity of 0.5 on the McFarland scale which was approximately 1.5×10^8 Colony Forming Unit (CFU)/ml. The suspensions were inoculated on CRA plates and incubated under aerobic conditions for 48 h at 37 °C. The biofilm-producing isolates presented as dark-gray or black colonies, while the non-producing isolates were colorless or reddish.

Results

Table 1 indicates the counts of typical (black, circular, small colonies with transparent halo) and atypical (black, circular colonies, without the presence of a transparent halo) suggestive colonies of *Staphylococcus* sp. in the nine milk samples varied between $<10^2$ and 1.0×10^5 CFU/ml.

Ten isolates from each sample were selected, which 64 isolates were catalase-positive. After being subjected to mass spectrometry by MALDI-TOF, 31 (48.4%) of them did not present a reliable identification at the gender level and were discarded. The remaining isolates (n=33) were identified as *S. epidermidis* (n=3; 4.7%), *M. caseolyticus* (n=14; 21.9%), and *K. varians* (n=16; 25%), all of which were catalase-positive Gram-positive cocci. All isolates of *M. caseolyticus* were obtained from sample 1, while *K. varians* and *S. epidermidis* isolates were obtained from sample 5.

Susceptibility testing revealed that 22 of the isolates were resistant to at least one antibiotic. Resistance to penicillin was verified in 18 isolates, while resistance to tetracycline was found in 11 isolates. Resistance to clindamycin, chloramphenicol, and norfloxacin was found in five, three, and one isolates, respectively. All 14 *M. caseolyticus* isolates were resistant to at least one antibiotic; however, multidrug resistant (MDR) isolates were not detected. Among all the *K. varians* isolates tested, 4 were resistant to at least one antibiotic from three different classes and were considered to be MDR (Table 2).

Of all the isolates, few showed biofilm production, as evidenced by black color on CRA (Figure 1). This characteristic was detected in only 5 isolates of *K. varians* and 1 isolate of *S. epidermidis*. No isolate of *M. caseolyticus* was found to be biofilm producer.

Table 1: Counts of typical and atypical suggestive colonies of *Staphylococcus* sp. in the milk samples

Sample No.	*Staphylococcus* sp. (CFU/ml)
L1	1.0×10^5
L2	$<10^2$
L3	$<10^2$
L4	6.6×10^4
L5	9.1×10^4
L6	4.2×10^3
L7	$<10^2$
L8	$<10^2$
L9	$<10^2$

Table 2: Resistance profiles and biofilm production activity of Gram-positive cocci isolates

Identification	Isolates	Antibiotic resistance	Qualitative production of biofilm
Micrococcus caseolyticus	2-L1T	PEN	No
	4-L1T	PEN	No
	5-L1T	PEN, TET	No
	6 -L1T	PEN	No
	7-L1T	PEN	No
	8-L1T	PEN	No
	9-L1T	PEN	No
	11-L1T	PEN, TET	No
	12 -L1T	PEN, TET	No
	13-L1T	PEN	No
	14-L1T	PEN, TET	No
	15-L1T	PEN	No
	16-LIT	PEN, TET	No
	27-L1T	PEN, TET	No
Kocuria varians	60-L5T	-	No
	61-L5T	-	No
	62-L5T	TET	No
	63-L5T	-	No
	64-L5T	CLI, PEN, TET, (MDR)	No
	65-L5T	CLI, PEN, TET, (MDR)	No
	66-L5T	CLI, CLO, PEN (MDR)	No
	67-L5T	CLI, CLO	Yes
	68-L5T	CLO, TET	Yes
	69-L5T	CLI, PEN, TET (MDR)	Yes
	70-L5T	-	Yes
	71-L5T	-	No
	72-L5T	-	No
	74-L5A	NOR	No
	75-L5A	-	No
	76-L5A	-	Yes
Staphylococcus epidermidis	77-L5A	-	Yes
	78-L5A	-	No
	79-L5A	-	No

MDR: Multidrug Resistant; CLI: Clindamycin; CLO: Chloramphenicol; NOR: Norfloxacin; PEN: Penicillin; TET: Tetracycline

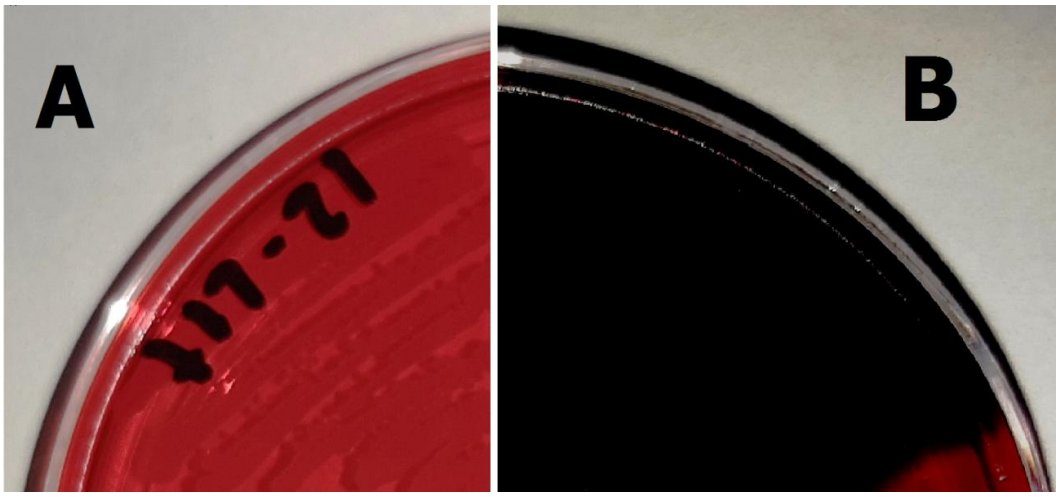

Figure 1: Biofilm production on Congo Red Agar. A: non biofilm producer isolate (*Micrococcus caseolyticus* 12-L1T); B: biofilm producer isolate (*Staphylococcus epidermidis* 77-L5A)

Discussion

Although, *S. aureus* is considered to be one of the main pathogens associated with outbreaks of food-borne diseases worldwide, other staphylococci, including coagulase-negative ones, such as *S. epidermidis*, may be associated with these diseases (Benkerroum, 2018; Osman et al., 2019). As milk is a potential reservoir of these microorganisms, the initial objective of this study was characterization of these microorganisms in pasteurized milk samples marketed in Rio de Janeiro. However, staphylococci were not the most frequently detected microorganisms. Only 3 out of 33 isolates were identified as staphylococci and the other isolates were *M. caseolyticus* and *K. varians*.

Organji et al. (2018) isolated *Macrococcus*, *Kocuria*, and *Staphylococcus* from dairy and meat products produced in Saudi Arabia using selective media culture for *Staphylococcus* sp. including mannitol salt agar and *Staphylococcus* medium 110. El-Ashker et al. (2015) used Baird-Parker agar with the aim of isolating of *S. aureus* from quarter milk samples of dairy cattle and buffaloes with clinical and subclinical mastitis from a farm in Egypt. Most of the isolates were from the genus *Staphylococcus*, but the authors also found *Kocuria* sp., *Macrococcus* sp., and other Gram-positive and Gram-negative bacteria.

It is important to mention that until 1988, *M. caseolyticus* was included in the genus *Staphylococcus* as *S. caseolyticus* (Kloos et al., 1998). *K. varians*, in turn, was known as *Micrococcus varians*, but was included in its new genus in 1995 (Stackebrandt et al., 1995). The isolation of these genera together with *Staphylococcus* sp. can be explained by the evolutionary relationship between the genera and by the sharing of niches, such as milk. Two other studies performed in India (Joishy et al., 2019) and Slovakia (Kačániová et al., 2019) also reported the isolation of these three genera from milk and dairy products.

Bacteria in the *Macrococcus* genus, despite being closely related to the *Staphylococcus* genus, are not pathogenic for humans. However, some species are becoming increasingly recognized as veterinary pathogens and for carrying antibiotic resistance. In this study, *M. caseolyticus* isolates did not present a varied resistance profile, showing resistance only to penicillin or penicillin and tetracycline. Some studies, however, showed the presence of *M. caseolyticus* isolates resistant to a variety of antibiotics, including methicillin, in cases of bovine mastitis (MacFadyen et al., 2018) and in samples of pasteurized milk (Schwendener et al., 2017). It has been stated that strains of *Macrococcus* sp. can encode resistance to various antibiotics, including methicillin, and may be involved in the spread of resistance to staphylococci (MacFadyen et al., 2018).

The *K. varians* isolates detected in this work presented a more varied profile. Like other species of this genus, *K. kristinae* and *K. varians* are not commonly associated with human infections. However, over the past few years, some evidences have highlighted these species as opportunistic pathogens, especially in hosts that have a serious pre-existing disease (Purty et al., 2013). Studies on antibiotic resistance in isolates of *K. varians* from food are scarce. Rodríguez-Alonso et al. (2009) described the detection of MDR isolates of *K. varians* from artisanal cheeses made with raw milk in Spain. Similarly, *K. varians* and other species of the genus were isolated from Brazilian dairy farms and also showed proteolytic and lipolytic activity, indicating a deteriorating potential and offering technological risk to milk (Ribeiro Júnior et al., 2018).

None of the three isolates of *S. epidermidis* detected in this work showed resistance to the tested antibiotics. It is known that *S. epidermidis* is one of the species most related to bovine mastitis, as it is frequently present in raw and pasteurized milk. In these foods, *S. epidermidis* presents high rates of antimicrobial resistance, often higher than other staphylococcal species (Kim et al., 2019). Because it is present in raw milk, *S. epidermidis* can constitute a problem in many dairy products, such as artisanal cheeses. Chajęcka-Wierzchowska et al. (2019) reported that the majority of *S. epidermidis* isolates obtained from artisanal cheeses produced in Poland showed resistance to penicillin, clindamycin, tetracycline, and erythromycin, with 16% of these being classified as MDR. Still, 50% of these isolates showed biofilm producing activity. In a similar research performed in Saudi Arabia with pasteurized milk, 91% of *S. epidermidis* isolates were capable of producing biofilm (Eladli et al., 2019). All of these data are indicative of the virulence of *S. epidermidis* and its potential for transmitting resistance genes to the contaminating milk microbiota. It is important to highlight that in addition to being resistant to antibiotics, *S. epidermidis* and other coagulase-negative staphylococci can also produce enterotoxins and biofilms, thus representing a potential risk to public health.

In this study, one of the three isolates of *S. epidermidis* was able to produce biofilms on CRA. The CRA growth technique has been used by several authors for the qualitative evaluation of biofilm production by strains of *Staphylococcus* sp. isolated from milk and dairy products (Castro et al., 2020; Cruzado-Bravo et al., 2019; Fabres-Klein et al., 2015; Gutierrez et al., 2012) and has been shown to be effective in determining whether an isolate has the potential for biofilm production. Tests for the quantitative evaluation of biofilm production in

polystyrene plates and Polymerase Chain Reaction (PCR) amplification of *ica* genes can also be used for confirmation, especially of the negative results in CRA.

Four isolates of *K. varians* were producers of biofilm in CRA, one of which also presented the MDR phenotype. This is concerning, because this species generally shares the same niche as staphylococci and micrococci, and may even form mixed biofilms (Leriche et al., 2003; Sanchez-Vizuete et al., 2015). In addition to providing greater protection for the microorganisms involved, mixed biofilms increase the chance of transferring resistance genes. The production of biofilm by the isolates of *K. varians* in this study that was associated with resistance to antimicrobials suggests a considerable quality problem in the analyzed milk samples. This could become a health problem if the genes that encode these phenotypes can be transferred to other microorganisms in the accompanying microbiota of the food. Interactions between species, including *Kocuria* spp., have been reported in the literature, resulting in increased biofilm formation in food or associated utensils and equipment. Carpentier and Chassaing (2004) succeeded in growing a variety of bacterial isolate, including *K. varians*, in binary culture biofilms with *Listeria monocytogenes* in stainless steel coupons to mimic the production of biofilms on food industry equipment. Using epifluorescence microscopy verified that the *L. monocytogenes* cells arranged around the *K. varians* microcolonies in the biofilm, proposing some kind of synergistic interaction between them. Based on a recently research, *K. rhizophila* isolate from a food-processing environment in different consortia of multiple bacterial species showed synergistic biofilm formation (Røder et al., 2015).

Conclusion

The presence of antibiotic-resistant isolates of *M. caseolyticus*, *S. epidermidis*, and *K. varians*, especially MDRs, highlights the possible role of milk as a reservoir of resistance genes. The abundance of this reservoir could lead to the dissemination of resistance genes between pathogenic staphylococci and other related groups commonly found in milk, by gene transfer mechanisms. Additionally, the virulence potential of *K. varians* is evidenced by its ability to form biofilms, which should serve as a warning to the dairy industry.

Author contributions

M.A.A.M., W.A.R., and V.S.T. conducted the experimental work; G.L.P.A.R., H.C.V., as well as J.S.N. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that the research was conducted in the absence of any conflict of interest.

Acknowledgements

The authors thank the Laboratório de Investigação Médica, from Instituto de Microbiologia Paulo de Góes (IMPG/UFRJ) for performing the mass spectrometry analysis (MALDI-TOF) identification. This work was finnancially supported by the program Prociência from the Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, Brazil.

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