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Isolamento e perfil de resistência antimicrobiana de *Enterococcus* spp em linhas de processamento de leite de cabra

Isolation and antimicrobial resistance profile for *Enterococcus* spp in goat milk processing plants

Perfil de aislamento y Resistencia antimicrobiana de *Enterococcus* spp in lineas de procesamiento de leche de cabra

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Resumo

A resistência antimicrobiana de *Enterococcus* spp na indústria de laticínios tem sido relatado ao longo dos anos, tornando se uma potencial fonte de contaminação para o produto acabado pois este micro-organismo é resistente ao processo de pasteurização. Dessa forma o presente estudo teve como objetivo avaliar o perfil de resistência antimicrobiana de *Enterococcu*s spp isoladas de plantas de processamento de leite de cabra. As amostras foram coletadas e semeadas em ágar com 5% de sangue de cabra desfribrinado. Os isolados foram confirmados pela técnica do MALDI TOF. A maior resistência foi observada para norfloxacina, seguindo de tetraciclina e cloranfenicol e vancomicina. Nitrofurantoína e linezolida apresentaram as maiores taxas de sensibilidade para as cepas estudadas. Esta pesquisa demonstra a necessidade de políticas sanitárias para o controle de qualidade do leite de cabra com presença de patógenos resistente aos processos de pasteurização com resistência antimicrobiana tornando se um risco potencial para a saúde da população.

Palavras-chave: Antimicrobianos; Laticínios; Contaminação.

Abstract
The antimicrobial resistance of Enterococcus spp in the dairy industry has been reported over the years, making it a potential source of contamination for the finished product as this microorganism is resistant to the pasteurization process. Thus, the present study aimed to evaluate the antimicrobial resistance profile of Enterococcus spp isolated from goat milk processing plants. Samples were collected and seeded on agar with 5% defibrinated goat blood. The isolates were confirmed by the MALDI TOF technique. The greatest resistance was observed for norfloxacin, followed by tetracycline and chloramphenicol and vancomycin. Nitrofurantoin and linezolid showed the highest sensitivity rates for the strains studied. This research demonstrates the need for sanitary policies for the quality control of goat's milk with the presence of pathogens resistant to pasteurization processes with antimicrobial resistance, making it a potential risk to the health of the population.
Keywords: Antimicrobials; Dairy; Contamination.

Resumen
La Resistencia antimicrobiana de Enterococcus spp en la industria lacteal ha sido reportada a lo largo de los años conviertiendose en una fuente potencial de contaminación para el product terminado. Por lo tanto, el presente studio tuvo como objetivo evaluar el perfil de Resistencia antimicrobiana de Enterococcus spp aislado de plantas processadoras de leche de cabra. Las muestras se recolectaron y sembraron en agar con sangre de cabra desfibrinada al 5%. Los aislamientos fueron confirmados por la técnica Maldi Tof . La mayor Resistencia so observe para norfloxacina, seguida de tetraciclina y cloranfenicol y vancomicina. La nitrofurantoína y el linezolid mostraron las tasas de sensibilidad más altas par alas cepas estudiadas. Por lo tanto, existe contaminación por patógenes del género Enterococcus spp con Resistencia antimicrobiana en las plantas processadoras de leche de cabra investigadas, lo que se traduce en un riesgo potencial para la población.
Palabras clave: Antimicrobianos; Lácteos; Contaminacion.

1. Introduction

Dairy goat farming has a significant role in the socioeconomic and nutritional demands in Brazil. In the North-eats region, most of the production is organized at a local level, represented by cooperatives and associations which are responsible for processing and sale to government programs (Embrapa, 2018).

The state of Paraíba stands out as the largest producer of goat milk in the country, producing over 5.5 million liters of annually (Ibge, 2018). With optimization of goat farming and increase in milk production, there has been a growing concern with milk quality. Despite an improvement in technological level, represented by an increase productivity, production still maintains artisanal characteristics (Embrapa, 2018).

Quality parameters for goat milk in Brazil are regulated by Normative Instruction 37 from October 2000, from the Ministry of Agriculture, Livestock, and Food Supply (Brasil, 2000), which contains conditions for production and identification and minimal quality requirements. Goat milk, like the milk from other mammals, has a high nutritional value, thus being an ideal environment for microorganism growth and multiplication. Aside from primary contamination during animal farming, there are critical points along the production chain for dairy products, such as processing, transportation, and storage of the final products (Weschenfelder, et al., 2016; Agrimonti, et al., 2017), which may compromise quality. Various microorganisms have been isolated from goat milk, such as *Staphylococcus aureus*, *Escherichia coli* (Pádua, et al., 2019), *Acinetobacter* spp. (Ramos & Nascimentos, 2019) and *Enterococcus* (Mcauley, et al., 2015; Hammad, et al., 2015).

Enterococcus spp species are morphologically classified as gram-positive non-spore-forming cocci. Main species are *E. faecalis* and *E. faecium*, who can colonize and infect humans (Souza, et al., 2012). These agents have a wide range of ecological niches, including the gastrointestinal system of humans and animals, thus a concern for public health, considering their frequent association with various clinical manifestation of infection (Cruz, 2019).

The presence of *Enterococcus* in food has been of concern to public health institutions due to its ambiguous nature (Moraes, et al., 2012). Despite showing potential for preservation of cheese due to production of bacteriocins that act against

pathogens (Santos, et al., 2014), they may harbor several genes encoding virulence factors (Moraes, et al., 2012), with isolates being intrinsically resistant to various antimicrobials (Nachtigall, et al., 2013).

Several studies were found in the literature on goat milk quality (Dutra, et al., 2014; Madureira, et al., 2017; Coelho, et al., 2018; Santos, et al., 2019;), but little is known about the presence of *Enterococcus* spp. in the processing environment for goat milk, as well as regarding its antibiotic sensitivity profile. Therefore, the objective of this study was to, identify, and obtain the resistance profile for *Enterococcus* spp. isolates from goat milk processing plants in the Cariri region of Paraíba.

2. Material and Methods

Study location

Four goat milk processing plants from the Cariri region of Paraíba that supply milk to the Food Acquisition Program (PAC), identified as A, B, C, and D, were used. Each mini mill processes an average of 1500 to 2000 liters of goat's milk per day. This program involves government purchase of milk to benefit low-income families in the region. From these locations, samples were collected as follows: 4 samples from the cooling tank, 4 from the pasteurization tank, 4 from the packaging equipment, 4 from packages, 4 from the handler's hand, and 4 from the wall. To evaluate the quality of the milk, in natura milk was selected from the cooling tank and from the pasteurized milk, with 8 samples obtained per plant, totaling 32 samples.

Sample collection and processing

Samples were collected from the equipment after cleaning procedures accordingly The cleaning of the establishment's equipment is carried out in accordance with the Standard Operating Hygiene Procedures implemented by the company with alkaline detergent, acid detergent and sanitizer. The collection was carried out after using the sanitizer and a final rinse. Used a sterile swab with a 12-cm handle, moistened with a 0.1% peptone water solution. Using a sterile mold to restrict the dimensions of the evaluated surface to 100cm^2, the swab was rubbed with a constant pressure, in a rotatory motion and approximate inclination of 30°, initially moving from the left to the right, then from the right to the left. The diluent was then evaluated by plating aliquots in appropriate culture media (Andrade, 2008).

For the handler's hands, a sterile swab with a 12-cm handle, moistened with a 0.1% peptone water solution, was used. The cotton swab was streaked/rubbed three times from the wrist towards each finger. The swab was then rubbed between the fingers before returning to the wrist. The microorganisms collected were transferred to a tube containing 10 ml of the peptone water solution (Andrade, 2008).

Milk *in natura* was collected from the cooling tank after homogenization and placed into a sterile container; pasteurized milk was collected from a packaged product. Both were placed in a cooler with recyclable ice. Samples were taken to the Milk and Derivatives Technology laboratory at UFCG – Patos/PB for analyses in specific media.

Isolation of *Enterococcus* spp.

The samples were seeded in agar with 5% defibrinated goat blood to obtain the isolated colonies. These colonies underwent Gram coloring and a catalase test. Gram-positive cocci arranged in pairs or chains, with a negative catalase test, were submitted to a bile esculin test (BE) and salt tolerance test (BHI with a final concentration of 6.5% NaCl) (Anvisa, 2004).

MALDI TOF MS analysis

To confirm microbial identification, a total of 32 isolates were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), at Qualileite in Pirassununga-Usp/SP, following the protocol described by (Barcelos, et al., 2019).

Antimicrobial sensitivity test

Antimicrobial sensitivity tests were performed using the disc-diffusion method The following antimicrobials were tested: Ampicillin (10 µg), rifamycin (5 µg), erythromycin (15 mcg), chloramphenicol (30 µg), nitrofurantoin (300 µg), linezolid (30 µg), fosfomycin (200 mcg), norfloxacin (10 µg), tetracycline (30 µg), and vancomycin (30 µg) (Clsi, 2020).

3. Results

Of the 32 samples analyzed, only 6 (18.75%) were identified as *Enterococcus spp*. Table 1 shows the prevalence for *Enterococcus* spp, found only at plants C and D. At plant C, *Enterococcus faecium* was found in milk *in natura* and pasteurized milk. At plant D, the agent was isolated from the reception tank, pasteurization tank, packaging equipment, and packages, with a total percentage of 18.75%. *Enterococcus faecium* was present in the reception tank and packaging equipment, while *Enterococus faecalis* was found in the pasteurization tank and packaging equipment.

Table 1 - *Enterococcus* spp species isolated from goat milk plants between January and February, 2019.

Niche	Isolates	
	Enterococcus faecium	*Enterococcus faecalis*
Handler's hand		
Reception tank	D	
Pasteurization tank		D
Packaging equipment	D	
Package		D
Milk *in natura*	C	
Pasteurized milk	C	

Legend: Plants A, B, C, and D. Source: Authors.

Table 2 shows the sensitivity profile for *Enterococcus* spp. and the tested antimicrobials. The highest resistance rate was observed for norfloxacin with four (66.66%) isolates, followed by chloramphenicol, tetracycline, and vancomycin with three (50.00%) each. Nitrofurantoin and rifamycin had only one sample (16.66%) resistant to each. Regarding its multiresistance profile, *E. faecalis* isolated from the reception tank was resistant to seven of ten antimicrobials tested. At plant C, *E. faecalis* was identified in milk *in natura* and pasteurized milk, but with a different resistance profile.

Table 2 - Resistance profile for *Enterococcus* spp. isolated from goat milk plants between January and February, 2019.

Isolates	Niche	Antimicrobial resistance profile
E. faecium	Reception tank	AMP, RIM, ERI, CLO, LNZ, NOR, FOS
E. faecalis	Pasteurization tank	CLO, NOR, TET, VAN
E. faecium	Packaging equipment	AMP, CLO, NOR TET, VAN
E. faecalis	Package	TET, VAN
E. faecium	Milk *in natura*	NOR,
E. faecium	Pasteurized milk	ERI, NIT, FOF

Legend: AMP: ampicillin, RIM: rifamycin, ERI: erythromycin, CLO: chloramphenicol, NIT: nitrofurantoin, LNZ: linezolid, NOR: norfloxacin, FOS: fosfomycin, TET: tetracycline, VAN: vancomycin. Source: Authors.

4. Discussion

Antimicrobial resistance is a worldwide emergency and health surveillance programs focused on this issue are necessary, especially in developing countries such as Brazil. The emergence of antibiotic-resistant *Enterococcus* spp. strains is a concerning issue when considering simultaneous resistance to various drugs.

In the present study, the percentage found for *Enterococcus* spp. was 18.75%. This is a concerning result for this controversial genus among lactic acid bacteria because of the pathogenicity of various species to humans (Cruz, 2019), and their growth in thermally treated milk and refrigerated milk (Girafa, 2002). The genus *Enterococcus* spp. includes 55 species and two subspecies, among which *Enterococcus faecalis* and *faecium* are the most common species found in milk and its dairy products. They persist in milking instruments and milk reservoirs, contaminating the production line because they can survive sanitizing products. This characteristic is probably associated with the presence of biofilms (Gelsomino, et al., 2002).

The presence of *Enterococcus* spp in the processing line for goat milk in various equipments reveals flaws in the cleaning process, and a need to revalidate sanitation standard operating procedures (SSOP). In the literature, *Enterococcus spp* has been identified in dairy products, as well as in the production line for karish cheese, (Hammad, et al., 2015), Minas frescal cheese (Castro, et al., 2017), Turkish white cheese (Ispirli, et al., 2017), and fresh and pasteurized milk (Mcauley, et al., 2015), which makes this microorganism a risk for products processed in the dairy industry.

Resistance of the *Enterococcus* spp. isolated from goat milk plants in the state of Paraíba varied between the different classes of antimicrobials, where norfloxacin had the highest rate with 4 (66.66%) of the samples showing resistance. Norfloxacin is a quinolone which, according to studies by (Lebreton, et al., 2014), inhibit bacterial growth by interfering with DNA replication. Tetracycline, vancomycin, and chloramphenicol were the antimicrobials with the second highest resistance rate in this study. Studies performed by (Nascimento, 2017 & Triveldi, et al., 2011) reported a high resistance rate to vancomycin and erythromycin for *E. faecium* obtained from buffalo cheese. However, there is a discrepancy between findings reported by (Silvetti, et al., 2019, Frazzon, et al., 2010 & Rivas, et al., 2012), who found no resistance to vancomycin in isolates from food, including milk. Vancomycin-resistant *Enterococcus* (VRE) are related with a significant increase in mortality, as well as a tenfold increase in hospital costs (Furtado, et al., 2005; Oliveira & Bettcher, 2010).

For *E. faecalis* and *E. faecium*, species of greater clinical importance, there is an intrinsic resistance to cephalosporins, a resistance to low concentrations of aminoglycosides, lincosamides, and monobactams (Hammerum, 2012), as well as a resistance to sulfonamides, ertapenem, perfloxacin, penicillins, and ampicillin (Lebreton, et al., 2014). Studies have shown that

food is a dissemination route for antibiotic-resistant *Enterococcus* spp. that have the potential to transmit resistance genes (Frazzon, et al., 2010).

5. Conclusion

Enterococcus spp, especially *E. faecium* and *faecalis*, have gained importance due to their pathogenic potential. In this work, it was possible to identify the microorganism even after the thermal processing, demonstrating failures in the quality control of the product. In addition to its pathogenic character, this, combined with the alarming increase in antimicrobial multiresistance, reinforces the need for studies within the milk industry to determine the main causes of antimicrobial multiresistance and implement improvements into the primary production chain and processing plants to ensure the safety of the goat milk in the Cariri region of Paraíba.

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