An Investigation of Proteolytic, Lipolytic Activity and Biofilm Formation by Psychrotrophic Bacteria Isolated from Buffalo Milk

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Abstract: The aim of this study was to investigate the enzymatic activity of 21 bacteria isolated from refrigerated raw buffalo milk, as well as to evaluate the production of biofilm by these bacteria. Proteolytic, lipolytic and lecithinase activity, as well as the production of exopolysaccharides were evaluated at different temperatures. For all of the psychrotrophic bacteria, biofilm formation on microtiter plates was evaluated at different temperatures and in the presence of residual buffalo and bovine milk. All cultures showed a proteolytic profile while 9 cultures showed lipase activity. Lecithinase production was found in 7 of the evaluated psychrotrophic bacteria. The ability to produce exopolysaccharides was found in 12 bacteria. Of the 21 bacterial isolates, 16 were biofilm producers at 7°C. At 23°C, 20 isolates were found to be biofilm producers. At a temperature of 37°C, biofilm formation by 17 isolates was weak. In the presence of residual buffalo milk, 7 were biofilm producers, while 16 bacteria produced biofilm in residual bovine milk. The results of this study show that many isolates of psychrotrophic bacteria from raw buffalo milk have the potential to produce extracellular enzymes as well as biofilm. This deserves special attention when considering the best practices to recommend during the collection of raw milk in establishments which process milk.

Keywords: Psychrotrophic bacteria, proteolysis, lipolysis, adhesion, milk.

1. INTRODUCTION

The production of buffalo milk in Brazil began to be recognized in the 1990s. This milk has special physical and chemical characteristics when compared to cow milk, including higher protein, fat and lactose contents [1]. Due to these characteristics, the processes of industrialization have generated differentiated products, principal among them buffalo mozzarella cheese [2].

The quality of milk depends on its microbiological characteristics which, in turn, are directly related to the management processes of the dairy herd, including the obtaining and maintenance of this livestock [3]. Regarding the milk microbiota, these may be affected by factors such as temperature, handling of the animals, utensil sanitization and storage time [4].

On farms, the milk-cooling process immediately after milking allows for the multiplication of mesophilic bacteria, a major cause of acidification [5]. This treatment is part of a legal requirement to keep livestock property [6]. However, the storage temperature of the milk often varies between 4°C and 10°C, which allows for the development of psychrotrophic microorganisms [7].

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Buffalo milk production in the southern region of Brazil achieves an annual output of 130 thousand liters of milk per year, corresponding to 8.71% of the total production in the country [8]. Due to these numbers, the buffalo milk sector in this region is concerned about the quality of products made with this raw material since, according to Osman *et al*. [9], buffalo milk also presents microbial contaminants that may compromise the production of dairy products.

Psychrotrophic bacteria are able to grow at a low temperature of around 7°C, but exhibit more growth at a higher optimal temperature, between 20°C to 30°C. These bacteria are the principal agents of deterioration in refrigerated raw milk and dairy products. The spoilage action of psychrotrophic bacteria is mainly due to the production of extracellular enzymes that hydrolyze the main components of milk such as proteins and fats [10].

The action of proteolytic enzymes is associated with a bitter taste in milk, due to the hydrolysis of peptide bonds [11]. As these enzymes are present in low concentrations in milk and dairy products, over time, they may alter the physicochemical properties of these goods, resulting in changes which remain active even after heat treatment (such as changes in color and flavor) [11, 12]. Lipases are enzymes that catalyze the hydrolysis of triglycerides (triacylglycerols), the major lipid components of milk. The products of the reactions

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are non-esterified fatty acids, partial glycerides (mono- and diglycerides) and, in some cases, glycerol. The lipolysis of milk fat contributes to off-flavors, such as rancid, soapy or occasionally bitter tastes [13].

Another feature of psychrotrophic bacteria is their ability to survive in liquid which adheres to contact surfaces, through the formation of cell aggregates called biofilm. The presence of biofilm in the dairy industry represents a risk to consumer health, due to the likelihood of the spread of pathogenic bacteria and their toxins; it can also cause financial losses due to the decrease in product shelf life [14].

According to Abe *et al.* [15], biofilm comprises microbial cell aggregates embedded in a polymeric matrix formed by exopolysaccharides (EPS), and is connected to a biotic or abiotic surface. The bacteria forming the biofilm have the advantages of a higher concentration of nutrients and ease of genetic changes. Additionally, because of the protection provided by the EPS, the biofilm-producing bacteria display an increased ability to withstand both a reduction in nutrients due to pH changes, as well as the highest concentrations of antibiotics [16].

The use of buffalo milk has increased in recent years [17], but the study of these microorganisms in buffalo milk is still not well established. The objective of this study was to evaluate the production of proteolytic and lipolytic enzymes and check the biofilm production ability of psychotropic bacteria isolated from refrigerated, raw buffalo milk.

2. MATERIALS AND METHODS

2.1. Bacterial Cultures and Cultivation Conditions

The 21 strains of psychrotropic bacteria were previously isolated from refrigerated raw buffalo milk samples which were obtained from cooling tanks on a dairy farm. The cultures were initially kept frozen at -20°C in 20% glycerol. For the reactivation of the isolates, the medium Tryptone Soy Broth (TSB, Himedia, India) was used in the incubation of these isolates at a temperature of 30°C for 48 hours. After, they were spread in Tryptone Soy Agar (TSA, Himedia, India) for 48 hours, in order to observe the purity of the cultures.

2.2. Identification of Bacterial Isolates

The identification of the psychrotrophic bacterial isolates was performed with the use of morphological and biochemical tests, in accordance with MacFaddin [18]. The morphological, cultural and physiological assessments were compared with the data described in Bergey's Manual of Determinative Bacteriology [19]. For biochemical evaluation, an API 20E kit was also used, in accordance with the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). The API galleries were examined after 24 and 48 hours respectively, after incubation at 37°C, and using *Escherichia coli* ATCC 10536 as a positive control. Identification was performed manually by sending the seven-digit code to Apiweb ™, an online database.

2.3. Evaluation of Proteolytic, Lipolytic and Lecithinase Activity

The verification of the enzymatic properties was performed in accordance with Ruaro *et al.* [20], with some modifications. To test for proteolytic activity, bacterial cultures were inoculated in milk agar (5 g L^{-1} meat peptone, 3 g L^{-1} yeast extract, 12 g L^{-1} agar, 10 % bovine skim milk) and incubated at 30°C for 48 hours. *Pseudomonas aeruginosa* ATCC 27853 was used as a positive control. For lipolytic activity, the psychrotrophic bacterial isolates were inoculated in a Tributyrin Agar (Sigma, EUA) culture medium and incubated at a temperature of 30°C for 48 hours. *Staphylococcus aureus* ATCC 25923 was used as a positive control. In both tests, the appearance of clear zones around the colony represented enzymatic activity and results were expressed in millimeters (mm).

Verification of lecithinase production was carried out in accordance with the methodology proposed by Marques *et al.* [21], with some modifications. The psychrotrophic bacteria were inoculated on Baird Parker Agar (Himedia, India) supplemented with a 10% egg yolk emulsion (Kasvi, Italy). The inoculated plates were incubated at 30°C for 48 hours. *S. aureus* ATCC 25923 was used as a positive control. The appearance of opaque zones around the colonies was indicative of enzyme production. All tests were performed in duplicate.

2.4. Evaluation of Exopolysaccharide Production by the Congo Red Agar Method

The EPS detection was conducted in accordance with the method of Freeman *et al*. [22], using Congo Red Agar (CRA) prepared as described by this author. Plates of the Congo Red Agar medium were inoculated and incubated at 30°C for 24 hours and at 7°C for 72 hours. For the controls, *Staphylococcus epidermidis* ATCC 35984 (a strong EPS producer) and

Staphylococcus carnosus P-9-4 isolated morcilla (not an EPS producer) were used. EPS positive strains produced black-colored colonies while EPS negative strains were pink colored.

2.5. Evaluation of Biofilm Formation

For biofilm formation, the method described by Stepanovic *et al.* [23], was employed. After bacterial growth on TSA plates, the colonies were resuspended in a 0.85% saline solution and turbidity was standardized according to the McFarland scale of 0.5, which corresponds to 1.5 x 10^8 CFU/ml.

The assay was performed on 96 well polystyrene microtiter plates (NEST, China). The wells were filled with 180 µl of TSB (Himedia, India), plus 0.25% glucose and 20 µl of bacterial suspension. Each isolate was inoculated in octuplicate. For negative control, wells were inoculated under the same conditions but without the presence of the isolates. For positive control, a strong biofilm culture producer, *S. epidermidis* ATCC 35984, was used. Plates were prepared and three replicates were used for incubation under different conditions at 7°C for 72 hours, at 23°C for 24 hours and at 37°C for 24 hours - to evaluate the formation of biofilm. After incubation, the wells were aspirated and samples were washed three times with 200 µl of 0.85% saline solution. The fixing of the bacteria was performed using 200 µl of methanol PA for 20 minutes. After, the methanol was aspirated and the microplates were inverted and allowed to dry overnight at room temperature.

The staining procedure was performed with 200 µl of crystal violet solution (0.5%) for 15 minutes, followed by the washing of the plate with sterile distilled water. After drying the plate, the bacterial cells fixed and stained at the bottom of the wells were resuspended in 200 µl of 95% ethanol for 30 minutes; then, the quantification of the biofilm was carried out. The optical density (OD) of the bacterial biofilm was quantified with the aid of a microplate reader spectrophotometer at a wavelength of 450 nm (Anthos 2010 Type 17 550 S. Nº 17 550 4894).

For easier interpretation of the results, strains may be divided into the following categories: non-biofilm producer, weak biofilm producer, moderate biofilm producer and strong biofilm producer, based upon the previously calculated OD values (for this type of calculation, average OD value of the strain should not be reduced by ODc value): OD ≤ODc = non-biofilm producer; ODc ≤2XODc = weak biofilm producer; 2XODc <OD ≤4xODc = moderate biofilm producer; 4XODc <OD = strong biofilm producer [23].

2.6. Biofilm Formation Assay with Residual Milk

To simulate the presence of residual milk on the surface of sterile microtiter plates, the wells were filled with 200 µl of bovine and buffalo milk (pasteurized whole), before the biofilm formation assay. The incubation was conducted at 7°C for 24 hours to simulate the conditions of a milk storage tank. After incubation, the entire volume of milk was aspirated from the microtiter plate wells. The procedure for biofilm formation, reading and interpretation was carried out in accordance with item 2.5. The biofilm production capacity of psychrotrophic bacteria in the presence of milk residue was evaluated by incubating the cultures at 7°C for 72 hours and 30°C for 24 hours.

RESULTS AND DISCUSSION

3.1. Identification of Psychrotrophic Bacteria

The psychotropic bacteria were characterized and identified by biochemical tests and by using the API 20E kit. According to the tests, the following bacterial genera have been identified: *Pseudomonas, Chryseobacterium, Enterobacter, Burkholderia, Acinetobacter* and *Oligella* (Table **1**). Among the Gram-negative bacteria, *Pseudomonas* was the most isolated bacterial genus. We know the importance of this genus because it contains species that cause deterioration in milk and milk products [24]. These bacteria are present in the environment and can be transmitted to the raw milk from soils, water and vegetation as well as from dairy farm environments [25]. The species belonging to the genera *Chryseobacterium, Burkholderia* and *Acinetobacter* are common food contaminants and were also isolated from the milk. These have a high potential for food spoilage [26].

3.2. Proteolytic, Lipolytic and Lecithinase Production by Psychrotrophic Bacteria

In this study, the 21 bacterial isolates showed proteolytic degradation halos on agar milk with values observed between 4 mm and 17 mm (Table **1**). This profile demonstrates their ability to hydrolyze casein, the principal milk protein component, through the production of proteolytic enzymes. The production capacity of these compounds has been shown by other authors, who have identified the *Pseudomonas* genus as the main one involved in this profile [11, 27, 28].

Table 1: Evaluation of Enzymatic Activity and Production of Exopolysaccharides by Psychotropic Bacteria Isolated from Refrigerated, Raw Buffalo Milk

Strains	PA (mm)	LA (mm)	L	EPS	
				7°C	30°C
B. mallei PL3.2	8	-	-	+	+
P. aeruginosa PL3.4	13	5	-	-	-
P. fluorescens PL3.5	10	5	+	-	-
P. putida PL4.1	17	7	-	-	+
P. putida PL4.2	8	5	-	-	+
Enterobacter sp. PL4.4	6	-	-	-	-
P. fluorescens PL4.5	10	-	-	-	-
P. fluorescens PL5.2	4	-	-	+	+
Pseudomonas sp. PL5.4	7	7	+	-	+
A. johnsonii PL5.6	8	-	-	-	+
P. putida PL6.2	4	-	-	-	-
P. putida PL6.3	6	-	-	-	-
C. indologenes PL6.4	10	8	-	-	+
P. fluorescens PL7.1	7	7	-	-	-
O. urethralis PL7.2	7	5	-	-	+
Enterobacter sp. PL7.3	9	-	-	+	+
A. radioresistens PL7.4	15	-	+	-	+
C. indologenes PL8.1	11	5	+	-	+
A. radioresistens PL8.2	14	7	+	-	-
A. radioresistens PL8.3	13	-	+	-	+
A. radioresistens PL8.5	15	-	+	-	-
P. aeruginosa ATCC 27853	15	NA	-	NA	NA
S. aureus ATCC 25923	NA	11	+	NA	NA
S. epidermidis ATCC 35984	NA	NA	NA	+	+
S. carnosus ATCC 12228	NA	NA	NA	-	-

PA: Proteolytic Activity; AL: Lipolytic Activity; L: Lecithinase; NA: Not applicable.

Technologically, these activities have been linked to the loss of cheese yield, the formation of off-flavors, gelation and the coagulation of UHT milk proteins (at ultra-high temperatures) during storage which, in turn, limit the shelf life of milk and dairy products [27].

Under the lipolytic profile, nine (42.85%) isolates showed lipid degradation halos with values between 5 mm and 8 mm (Table **1**), and *Chryseobacterium* sp. PL6.4 displayed the largest halos. Lipase activity is a problem for the dairy industry because the hydrolysis of lipids present in the layer of fat globules leads to alteration of the flavor and properties of milk and dairy products [29]. This compromises the quality of dairy products and reduces shelf life. A similar effect occurs when bacteria produce the lecithinase enzyme since this also acts on milk fat globules, causing changes in cream, for example [30]. Lecithinase production was found in seven (33%) isolates (Table **1**). Through these tests, it was observed that four (19%) bacteria showed the three evaluated enzymatic properties, which highlights the negative impact of the presence of these isolates on buffalo milk and on the quality of dairy products.

3.3. Evaluation of Exopolysaccharide Production by the Congo Red Agar Method

Of the 21 isolates of psychrotrophic bacteria, it was found that three of these cultures tested positive for the production of this compound at 7°C; twelve were positive for EPS production at 30°C. The production of

EPS was evidenced by the appearance of colonies exhibiting a black color and a dried crystalline consistency.

The ability to form EPS in Congo Red Agar is indicative that these bacteria have the ability to produce biofilms, and the importance of this polysaccharide in the structure of the biofilm is well known. It is also known that the Congo Red can interact directly with certain polysaccharides, forming a colored complex [31]. However, for the formation of biofilm, bacteria are also dependent on other factors such as the presence of substrate surfaces as well as determining types of secondary structures which permit the adhesion process to a specific area [14].

3.4. Biofilm Formation

In the biofilm formation evaluations, it was observed that the bacterial isolates presented different behavior when evaluated at different temperatures as well as in the presence of residual milk (Table **2**).

Of the 21 psychrotrophic bacteria studied, 16 (76.19%) were observed producing biofilm at 7°C. Of these 16 isolates, 2 (9.52%) were classified as moderate biofilm producers and 14 (66.6%) as weak biofilm producers. At 23°C, 20 isolates (95.23%) were able to form biofilm. Of these, 3 (14.28%) were moderate producers and 17 (80.95%) weak producers. At a temperature of 37°C, 17 (80.95%) were classified as weak biofilm producers. None of the isolates was determined to be strong biofilm producers, under the conditions which were employed. Eleven isolates have been shown to be weak biofilm producers under three evaluated conditions. The isolated *Pseudomonas* sp. PL5.4 and *P. fluorescens* PL7.1 presented as weak biofilm producers at 37°C. At the lower temperatures of 7°C and 23°C, both behaved as moderate producers.

Table 2: Biofilm Formation Under Different Conditions by Psychotropic Bacteria Isolated from Refrigerated Raw Buffalo Milk

Strains	Non-residue biofilm			Residual buffalo milk biofilm		Residual cow milk biofilm	
	37°C	23°C	7°C	23°C	7°C	23°C	7°C
B. mallei PL3.2	Weak	Weak	NF	NF	NF	NF	NF
P. aeruginosa PL3.4	Weak	Weak	Weak	NF	NF	NF	Weak
P. fluorescens PL3.5	Weak	Weak	Weak	NF	NF	NF	Weak
P. putida PL4.1	Weak	MD	Weak	NF	NF	NF	NF
P. putida PL4.2	Weak	Weak	NF	NF	NF	Weak	Weak
Enterobacter sp. PL4.4	Weak	Weak	Weak	MD	NF	NF	Weak
P. fluorescens PL4.5	Weak	Weak	Weak	MD	NF	Weak	Weak
P. fluorescens PL5.2	Weak	Weak	NF	MD	NF	NF	Weak
Pseudomonas sp. PL5.4	Weak	MD	MD	NF	NF	NF	Weak
A. johnsonii PL5.6	Weak	Weak	Weak	NF	NF	NF	NF
P. putida PL6.2	NF	Weak	Weak	NF	Weak	Weak	Weak
P. putida PL6.3	Weak	Weak	Weak	NF	NF	NF	NF
C. indologenes PL6.4	NF	NF	Weak	NF	NF	Weak	Weak
P. fluorescens PL7.1	Weak	MD	MD	NF	Weak	Weak	MD
O. urethralis PL7.2	Weak	Weak	Weak	NF	Weak	NF	NF
Enterobacter sp. PL7.3	Weak	Weak	NF	NF	Weak	Weak	Weak
A. radioresistens PL7.4	Weak	Weak	Weak	NF	NF	Weak	Weak
C. indologenes PL8.1	Weak	Weak	Weak	NF	NF	NF	Weak
A. radioresistens PL8.2	NF	Weak	Weak	NF	NF	NF	Weak
A. radioresistens PL8.3	Weak	Weak	Weak	NF	NF	Weak	Weak
A. radioresistens PL8.5	NF	Weak	Weak	NF	NF	NF	Weak
S. epidermidis ATCC 35984	Strong	Strong	NF	NF	NF	NF	NF

NF: Non-Forming Biofilm; MD: Moderate-Forming Biofilm.

When biofilm formation occurred in the presence of residual milk, 4 isolates (19.04%) were weak producers at 7°C and 17 (80.95%) did not produce biofilm. Three (14.28%) were moderate producers at 23°C and 4 did not produce biofilm in residual buffalo milk. In the presence of residual bovine milk, 15 (71.42%) bacteria were weak producers at 7°C, 1 (4.76%) was a moderate producer and 5 (28.57%) did not produce biofilm. At 23°C, it was observed that only 8 (38.09%) isolates were weak biofilm producers and 13 (61.9%) did not produce these structures.

In the evaluations made without residual milk, the isolates of *Pseudomonas* sp. PL5.4 and *P. fluorescens* PL7.1 were moderate producers at temperatures of 7°C and 23°C. Another isolate which showed similar behavior at 23°C was *P. putida* PL4.1.

These isolates represent a problem for the dairy industry because, according to Bogino *et al.* [32], the bacteria these structures organize acquire several advantages including protection against predation, desiccation and exposure to antibacterial substances, as well as a better acquisition of nutrients released into the cooling processor or storage environment.

Improper cleaning can lead to the deposition of organic matter and microorganisms, with the result of possible adherence and biofilm production by bacteria [33]. In the evaluation of the biofilm formation test in the presence of residual milk, it was observed that some isolates showed moderate biofilm production, whereas in the absence of residual milk they were classified as weak producers. These results indicate that variables such as temperature and the presence of residue need to be controlled to minimize deposition of these structures which, in turn, compromise the quality and safety of food.

4. CONCLUSION

The results of this study show that many psychrotrophic bacteria isolated from refrigerated, raw buffalo milk have the potential to produce proteolytic, lipolytic and lecithinase enzymes, bacterial adhesion and biofilm production. Thus, all these combined factors presented by psychrotrophic bacteria represent a source of contamination and deterioration in food, potentially causing problems for the economy and public health.

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