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Adhesion and Production of Degrading Enzymes by Bacteria Isolated from Biofilms in Raw Milk Cooling Tanks

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Abstract

Biofilms in milk cooling tanks compromise product quality even on farms. Due to the lack of studies of this topic, this study evaluated the microbiological conditions of raw milk cooling tanks on farms and characterized the microorganisms isolated from these tanks. Samples were wiped off with sterile swabs from seven milk cooling tanks in three different points in each tank. Mesophiles and psychrotrophic counts were performed in all samples. The isolation of *Pseudomonas* spp., *Bacillus cereus* and atypical colonies formed on selective media were also performed, totalizing 297 isolates. All isolates were tested for protease and lipase production and biofilm formation. Of the total isolates, 62.9% produced protease, 55.9% produced lipase, and 50.2% produced biofilm. The most widespread genus inside the milk cooling tank was *Pseudomonas* since it was not possible to associate this contamination with a single sampling point in the equipment. High counts of microorganisms were found in some cooling tanks, indicating poor cleaning of the equipment and providing strong evidences of microbial biofilm presence. Moreover, it is worth mentioning the milk potential contamination with both microbial cells and their degrading enzymes, which compromises milk quality.

Keywords: psychrotrophic bacteria; cleaning process; lipase; protease.

1 Introduction

Biofilms are ubiquitous, the major form of microbial growth (Costerton, 1987; Costerton et al., 1995). In the food industry, they are considered one of the major sources of cross contamination (Shi & Zhu, 2009). This is due mainly to the fact that the extracellular matrix of biofilms might be responsible for the high resistance of microorganisms to sanitizers, allowing pathogenic and spoilage bacteria to survive the sanitization process (Meyer, 2003).

The current Brazilian legal recommendations for milk cooling in the dairy farm (Brasil, 2011) represents the concretization of many benefits for the maintenance of the microbiological quality of milk and dairy products. However, inadequate cleaning of milk cooling tanks favors biofilm formation on the inner surfaces of these devices (Simões et al., 2010). Therefore, controlling biofilm formation on the farm poses considerable challenge, and there are relatively few published results about microbiological contamination and cleaning of milk cooling tanks. Several studies focus mainly on the microorganisms isolated from raw milk.

In cooling tanks, biofilms can compromise milk quality, preventing it from being used by the food industry. This occurs mainly by the action of psychrotrophic species like *Bacillus cereus* (also an enterotoxin producer) and *Pseudomonas*, which reproduce at the usual refrigeration temperatures and produce thermostable enzymes that in turn are able to alter the food attributes, even after microbial inactivation (Pinto et al., 2006).

This study aimed to evaluate the microbiological conditions of raw milk cooling tanks on farms and to characterize the bacteria remaining after the cleaning procedure.

2 Materials and methods

2.1 Experimental design

The study was conducted on seven dairy farms in Erechim (RS, Brazil). The main criteria used to select the farms was the proximity to downtown Erechim, the interest of the farmers in participating, and the availability of an empty (and clean) milk cooling tank on the farm on the day and time established for the sample collection.

2.2 Sample collection

From each milk cooling tank, three samples were collected from the inside of the tank, the homogenizer, and the milk drain valve. Surface areas (10cm × 10cm) of the tank (inside) and the homogenizer were wiped off with sterile swabs moistened in phosphate buffer (pH 7.2). The drain valve was also wiped off thoroughly using a swab; it was measured and the area was calculated to express counts in CFU/cm².

The samples were transported to the laboratory in a thermal box containing ice and analyzed immediately upon arrival. The time elapsed between cleaning the tank and sample collection (when the equipment remained off at room temperature) was about 6h.

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2.3 Count and microorganism isolation

Swabs containing microbial cells were resuspended in phosphate buffer solution for 1 min. The suspension was serially diluted in peptone water (0.1%) and inoculated into *Pseudomonas* Agar Base and Cetrimide Agar plates (Himedia) for isolation of *Pseudomonas* spp. and were incubated at 30°C for 48 h. The suspension was also inoculated into Cereus Agar (MERCK) for isolation of *Bacillus cereus* (30°C for 48 h) and Plate Count Agar (PCA - Acumedia) for heterotrophic mesophiles (35°C for 48 h) and psychrotrophic (6.5°C for 240 h) total counts.

Plates of PCA agar with 25 to 250 colonies and their consecutive dilutions were selected for counting (Brasil, 2003). In plates of selective media for *Pseudomonas* and *B. cereus*, typical and atypical colonies were isolated in order to obtain isolates with different colonial morphologies, which were representative of the diversity of morphologies observed. At least 21 colonies of each milk cooling tank were isolated. All isolates obtained were maintained frozen (-20°C) in Tryptone Soy Broth (TSB - Himedia) with glycerol (25%).

2.4 *Pseudomonas* spp. and *Bacillus cereus* identification

Typical colonies of *B. cereus* in selective medium were tested for confirmation of species (Brasil, 2003). Isolates that showed typical colonies of the genus *Pseudomonas* in selective media were grouped according to colonial and Gram stain morphology, and they were subjected to biochemical tests (Oxidation / Fermentation of glucose, nitrate reduction, oxidase, and catalase production) to confirm genus (Macfaddin, 2000; Brener, 2005). One or two isolates representing each of the typical colony morphology groups were confirmed as *Pseudomonas* by amplification and sequencing of a fragment from the 16S rRNA gene.

The DNA was extracted according to Misbah et al. (2005), with modifications. After growth in Tryptone Soy Agar (TSA - Himedia), two or three colonies were transferred to a microtube with 100 µL of TE buffer (Tris-HCl / EDTA; pH 8.0) and boiled for 10 min. Then, 100 µL of chloroform-isoamyl alcohol (24:1) was added. The microtube was centrifuged at 12.000 x g (10 min), and 1 µL of this supernatant was used as a template in the PCR reaction, performed using a Mastercycler Personal thermocycler (Eppendorf AG 22331 - Hamburg). The primers used and the amplification conditions were set according to Spilker et al. (2004). Aliquots of 30–60 ng of a 618-bp PCR product and 4.5 pmol of primers were subjected to automated sequencing (ABI-PRISM 3500 Genetic Analyser) at the ACTgene Molecular Analysis Laboratory (Porto Alegre, RS). Both strands were sequenced with the same primers used for amplification.

The sequences were identified using Standard Nucleotide BLAST (available on <http://www.ncbi.nlm.nih.gov>) using the 16S ribosomal RNA sequences (Bacteria and Archaea) database, optimized for highly similar sequences (megablast). Isolates were considered *Pseudomonas* spp. if their sequences showed the highest degree of similarity (99%) to sequences of *Pseudomonas* spp.

2.5 Enzymes production

All isolates obtained from selective media were tested for production of protease and lipase. To verify the production of protease, they were inoculated into milk agar (skimmed milk 100 g/L; yeast extract 1.5 g/L; agar 15 g/L) and incubated for 48 h at 30°C. The presence of degradation halos around the colonies indicated positive result (Budi et al., 2000). *B. cereus* strain ATCC 11778 was used as a positive control for the test.

For lipase production, the isolates were inoculated into culture medium containing olive oil and rhodamine B and incubated for 72 h at 30°C. The emission of orange fluorescence under U.V. light represented a positive result (Kouker & Jaeger, 1987, with modifications). *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 23229 strains were used as positive and negative controls for the test, respectively.

2.6 Quantification of biofilm production

Biofilm-forming capacity of all isolates with typical and atypical colonies obtained from selective media was quantified according to Stepanovic et al. (2000; 2007), with modifications. The isolates were subcultured on Tryptone Soy Agar (TSA - Himedia) for two consecutive days for cell activation. Thereafter, a cell suspension was performed in Tryptone Soy Broth (TSB- Himedia) with turbidity adjusted according to McFarland standard 0.5 (equivalent to 1.5×10^8 CFU/mL). Subsequently, triplicated aliquots of 200 µL of the cell suspension were placed in a 96-well polystyrene microplate and incubated at 30°C/24 h; 200 µL sterile TSB was used as negative control in each microplate. Each well was washed three times with sterile saline solution (NaCl 0.9%) to remove loosely adhered cells. The adhered cells were fixed with 200 µL of 99% methanol (15 min), and the microplate was air-dried. The cells were stained with 200 µL of Hucker crystal violet solution (2%, 5 min). The dye was drained and rinsed with tap water, with subsequent drying of the microplate. Finally, the dye remaining in the wells was resolubilized in 160 µL of glacial acetic acid (33%).

The reading of the optical density (OD) was performed using a microplate reader (EL800 Biotek Instruments, INC.) at λ of 490 nm. The mean and standard deviation of the negative control were also calculated, and the cutoff (OD_c) was established as three standard deviations above the mean of negative control. The isolates with $OD \leq OD_c$ were considered non-biofilm forming; isolates with $OD_c < OD \leq (OD_c \times 2)$ were considered weak formers; isolates with $(OD_c \times 2) < OD \leq (OD_c \times 4)$ were considered moderate formers; and isolates with $(OD_c \times 4) < OD$ were considered strong formers.

2.7 Statistical analysis

The results of mesophiles and psychrotrophic counts were analyzed statistically using ANOVA (Analysis of Variance) with multiple comparisons of means by the Bonferroni test. The different sampling points were compared using the mean counts of the samples collected from the seven farms investigated. Mesophiles and psychrotrophic counts of each sampling points were added. The farms were compared using the mean counts

of the samples collected from the three sampling points on each farm. All cell count results were natural log-transformed.

To analyze the results of biofilm formation, the Kruskal Wallis test was used. The results of lipase and protease production were analyzed using the X^2 test (SPSS software version 18.0 - Nucleus for Statistical Analysis – NAE/UFRGS).

3 Results

On three dairy farms, the predominance of mesophiles was observed in all sampling points. Dairy farm six was the only one with psychrotrophic predominance in all sampling points (Table 1). The contamination in the milk drain valve differed statistically from the other points ($p < 0.05$). However, it was not possible to associate mesophiles or psychrotrophic counts with a particular sampling point, which indicates homogeneous distribution of these microorganisms inside the tank. There was also no significant difference in the

Table 1. Mesophiles and psychrotrophic counts inside the milk cooling tanks (CFU/cm²) in the seven farms evaluated. Sample collections were performed after routine cleaning of the equipment.

Farm	Collection point	Mesophiles Count (UFC/cm ²)	Psychrotrophic Count (UFC/cm ²)
1	Inside the tank	5.9×10 ¹	1.3×10 ²
	Homogenizer	4.3×10 ²	1.9×10 ²
	Drain Valve*	1.0×10 ⁵	4.0×10 ⁴
2	Inside the tank	2.4×10 ⁴	2.3×10 ⁴
	Homogenizer	2.2×10 ³	2.1×10 ³
	Drain Valve*	3.2×10 ⁷	1.8×10 ⁷
3	Inside the tank	5.6×10 ²	1.1×10 ²
	Homogenizer	1.5×10 ³	1.1×10 ³
	Drain Valve*	1.6×10 ⁵	4.2×10 ⁴
4	Inside the tank	4.0×10 ³	2.2×10 ³
	Homogenizer	6.4×10 ³	5.7×10 ³
	Drain Valve*	3.3×10 ⁵	2.0×10 ⁵
5	Inside the tank	4.1×10 ²	4.9×10 ²
	Homogenizer	2.6×10 ⁴	4.0×10 ³
	Drain Valve*	1.6×10 ⁵	1.1×10 ⁵
6	Inside the tank	9.2×10 ⁴	1.4×10 ⁵
	Homogenizer	1.7×10 ⁵	8.4×10 ⁵
	Drain Valve*	9.3×10 ⁶	1.3×10 ⁷
7	Inside the tank	3.0×10 ⁴	1.8×10 ⁴
	Homogenizer	2.7×10 ³	3.4×10 ³
	Drain Valve*	2.8×10 ⁷	5.7×10 ⁷

*Significant difference ($p < 0.05$).

contamination between the farms, possibly due to the high variability in the results.

From the typical and atypical colonies formed on selective medium, 297 isolates were obtained. Isolates from atypical colonies were not identified, and 66 of the typical colonies were identified as *Pseudomonas*, and five as *Bacillus cereus*. The most widespread genus inside the milk cooling tank was *Pseudomonas* since it was not possible to associate its contamination with a single collection point in the equipment ($p < 0.05$).

All 297 isolates were tested for lipases and proteases production and biofilm formation (Tables 2 and 3). It was observed that 62.9% were able to produce protease, and 55.9% produced lipase. All isolates of *B. cereus* produced protease, but none was a lipase producer. One isolate from *B. cereus* was considered weak biofilm former, while the others were unable to produce biofilm.

There was a positive correlation between lipase production and the milk homogenizer isolates ($p < 0.05$), whereas no production of this enzyme was associated with the isolates from the inside of the homogenizer. There was no association between the sampling points in the cooling tank with protease production.

Half of the isolates (50.2%) showed some ability to form biofilms (Tables 2 and 3). The homogenizer sampling point was statistically different from the milk drain valve sampling point ($p < 0.05$) in terms of biofilm formation. In addition, isolates from the milk drain valve had a tendency to be classified as non-biofilm formers. Oppositely, isolates from the homogenizer tended to be classified as weak or moderate formers.

4 Discussion

The high microorganism counts (up to 10⁶ CFU/cm²) found in some cooling tanks indicate poor cleaning of the equipment and provide strong evidence about the presence of microbial biofilms. Hood & Zottola (1995) reported that bacterial counts of 10³ CFU/cm² do not characterize a microbial biofilm, but even the relatively low contamination levels found in some of the farms have to be considered because of the biotransfer potential of microbial cells and the likely increase in sanitizing resistance (Peng et al., 2001).

The predominance and/or high counts of mesophiles found in the equipment were not expected since low temperature disfavors their growth. Thus, two main hypotheses can be formulated to explain this result: (i) the contamination of the equipment with mesophiles occurred during the cleaning

Table 2. Source of atypical colonies and *Bacillus cereus* isolates, their enzyme production, and biofilm formation.

Collection point	Isolates of non-typical colonies	<i>B. cereus</i> Isolates	Extracellular enzyme		Quantification of biofilm formation*			
			Protease	Lipase	NOT ⁱ	WEA ⁱⁱ	MOD ⁱⁱⁱ	STR ^{iv}
Homogenizer *,#	67	0	36	40*	26	28	11	2
Inside	51	5	33	24	27	20	7	2
Drain Valve*	108	0	58	52	59	36	10	3
Total (Percentage)	231		127 (54.9%)	116 (50.2%)	112 (48.5%)	84 (36.4%)	28 (12.1%)	07 (3.0%)

ⁱ not former; ⁱⁱ weak former; ⁱⁱⁱ moderate former; ^{iv} strong former; *,# Significant ($p < 0.05$).

Table 3. Source of *Pseudomonas* spp. isolates, their enzyme production, and biofilm formation.

Collection point	<i>Pseudomonas</i> isolates	Extracellular enzyme		Quantification of biofilm formation*			
		Protease	Lipase	NOT ⁱ	WEA ⁱⁱ	MOD ⁱⁱⁱ	STR ^{iv}
Homogenizer ^{*,#}	17	16	15 [#]	5	8	4	0
Inside	12	10	7	7	4	1	0
Drain Valve*	37	34	28	24	9	4	0
Total (Percentage)	66	60 (90.9%)	50 (75.6%)	36 (54.5%)	21 (31.9%)	9 (13.6%)	0

ⁱ not former; ⁱⁱ weak former; ⁱⁱⁱ moderate former; ^{iv} strong former; ^{*,#} significant ($p < 0.05$).

procedure (from water, cleaning helpers, or environment); (ii) the cooling process of the cooling tank was not performed appropriately, allowing these microorganisms to remain in the equipment and form a biofilm.

Law and regulations governing the production of raw milk in Brazil (Brasil, 2011) requires the refrigeration of the product on the dairy farm and the cleaning of the cooling tank according to the manufacturer's recommendation. However, a quick evaluation (visual observation or informal talk with the farmers) was enough to reveal that the cleaning of the cooling equipment was performed by workers who often were not adequately trained to do so. Many factors might result in insufficient and inappropriate cleaning, such as choice of sanitizers, inadequate cleaning method and/or tools, and lack of financial resources, and create the conditions for formation of microbial biofilms (Simões et al., 2010).

Therefore, the development of training programs for farmers is recommended in order to improve the quality of the cleaning process on dairy farms. These initiatives should focus on aspects such as: the importance of acquiring chemicals for cleaning, as recommended by the manufacturer and/or technical assistance; the importance of mechanical action in the cleaning method; quality of water; and the need to thoroughly rinse the drain valve and to keep the cooling tank closed after cleaning and during milking intervals.

The predominance of biofilm formers in the homogenizer was not observed in the cooling tank drain valve. One possible reason could be the difference in selective pressure for colonization in this sampling point: the homogenizer is in constant motion during operation, which might have stopped the colonization of non-adherent microorganisms. Figueiredo et al. (2009) showed an inversely proportional relationship between the flow speed of milk over a surface and the number of *Pseudomonas* that remained attached, which became constant at speeds from 1.0 m/s.

The contamination level of the milk drain valve can also be related to the fact that this device is difficult to clean and also to poor hygiene procedures: in some cooling tanks, the swab used for sampling showed signs of milk residues, even after the tanks were cleaned. Moreover, the homogenizer is easily reachable and, besides the selective pressure associated with movement, it would select microorganisms capable of withstanding the mechanical action exerted by the handler at the time of cleaning.

Although the milk drain valve is not in contact with milk constantly, it is an important source of contamination of the raw material when it is transferred to the isothermal transport

vehicle. In addition, the remaining milk waste not only favors the permanence of microorganisms, but also supports microbial growth during scheduled stops required for milk sampling.

In general, biofilms that are formed in milk cooling tanks and in the food industry are multi-species. In the biofilm formation process and in the metabolic activity of a mature biofilm, each microbial species has a quite peculiar role and *Pseudomonas*, besides being important in contamination of refrigerated raw milk (Ercolini et al., 2007), is one of the active genera in the initial rapid colonization of surfaces. After consolidation of adhesion, it causes significant changes in the physicochemical properties of the surface and probably favors the incorporation of several other species with reduced adhesion capacity (Zottola & Sasahara, 1994; Ibusquiza et al., 2012).

Due to ubiquitous distribution of *Pseudomonas*, water, air, food handlers, skin/fur, dairy cattle feed and many others may be considered sources of milk contamination by (Dogan & Boor, 2003). Thus, their presence in cooling tanks might be associated with minor failures in the sanitization of these devices, and even in dairy cattle management. Fagundes et al. (2006) observed significant differences in *Pseudomonas* counts in freshly and refrigerated raw milk when comparing farms with adequate and inadequate management. The origin of water (surface or underground) also played a significant role in the microbial search of this bacterium. Identifying the origin of *Pseudomonas* isolates was not the objective of the present study, but it is possible that many of these bacteria had just been deposited on the surface of the equipment during or after the cleaning process. This may have contributed to the increase in the number of isolates classified as non-biofilm formers. In addition, most *Pseudomonas* isolates were obtained from the drain valve sampling point, which is associated with less selective colonization pressure.

Although the sequencing result of the amplified 16S rRNA gene fragment was sufficient to confirm genus, it was not conclusive for *Pseudomonas* species determination. Though almost the entire 16S rRNA gene was sequenced, intraspecific differentiation was difficult because the *Pseudomonas* genus has quite conserved sequences in this region (Anzai et al., 2000). The species with the highest similarity with the tested isolates were *P. fluorescens*, *P. putida*, *P. aeruginosa*, *P. stutzeri*, *P. poae*, *P. tolaasii*, *P. gessardii*, *P. mucidolens*, and *P. koreensis*. Considering that many of them originate from the farm environment and cause spoilage of the milk and dairy products (Eneroth et al., 2000), this result was expected.

The formation of biofilm by the isolates obtained corroborates the results of the counts and their association

with the presence of biofilms in the equipment used; more than half were considered capable of forming biofilms. It is worth mentioning that the presence of biofilms in milk cooling tanks is not merely a perennial source of milk contamination by microorganisms. In this study, milk contamination with degrading enzymes (lipase and protease) bacteria should also be taken into consideration, given the significant milk spoilage potential exhibited by the isolated microorganisms.

Enzyme production by spoilage microorganisms has been extensively studied. However, only recently were biofilms seen as potential reservoirs not only of undesirable microorganisms but also of enzymes that might compromise the product quality (Teh et al., 2014). The biofilm structure protects the enzyme, creates microenvironments favorable to the production, and changes the cellular physiology of the microorganisms (Wang & Chen, 2009). Teh et al. (2012, 2013), found that proteolytic and lipolytic activities were higher in cells growing in biofilms than in planktonic form. *P. fluorescens*, for example, retained proteolytic activity at 37°C only when present in biofilms. In the present study, of the 297 isolates, 62.9% were able to produce protease and 55.9% produced lipase; therefore, more than half showed potential for milk spoilage. Approximately 90% and 75% of the *Pseudomonas* strains were, respectively, protease and lipase producers, and most of them came from the drain valve. Thus, this site is a potential source of milk spoilage bacteria, whose enzymes may remain active in the milk during all manufacturing steps, reducing shelf life and causing financial loss for the industry.

The microbial diversity found in the biofilms may also contribute to the increased potential for food alterations. Teh et al. (2013) found that the lipolytic activity of *Streptococcus uberis* and *P. fluorescens* only occurred in the biofilms formed by strains in co-culture. Moreover, the importance of biofilms in terms of enzyme production is such that the concepts of cell-associated enzyme and enzyme-free cells were adapted to this form of cell growth: enzymes attached to the biofilm matrix are still considered as cell-associated enzymes; the enzymes released into the environment that surrounds the biofilm are considered as cell-free enzymes (Wang & Chen, 2009).

Proteolytic enzymes are associated with the bitter flavor of milk and its gelatinization after prolonged storage due to the hydrolysis of peptide bonds. Lipolytic enzymes are associated with rancidity and instability of beverages based on milk foam. Due to their heat resistance, both categories of enzymes are associated with reduced shelf life of UHT milk (Sørhaug & Stepaniak, 1997; Teh et al., 2012), among other problems. Of the 66 isolates of *Pseudomonas*, 47 (71.2 %) were able to produce protease and lipase, confirming the prominence of the genus, considered the major deteriorating agent of dairy products (Ercolini et al., 2007; Marchand et al., 2009).

5 Conclusion

The results indicate that the cleaning procedure of milk cooling tanks is not effective on the dairy farms investigated and should be reviewed since these routines contribute to the establishment of biofilms in equipments. Most microorganisms

isolated following the cleaning procedure were able to produce degrading enzymes and to form biofilms, indicating potential to contaminate raw milk not only by microorganisms, but also by their enzymes.

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