EVALUATION OF THE ADHESION POTENTIAL OF PSYCHROTROPHIC BACTERIA ISOLATED FROM REFRIGERATED RAW BUFFALO MILK: SIMULATING STORAGE CONDITIONS

Marciéle Bogo, Géssica Aracéli Costa, Ana Paula Guedes Frazzon and Amanda de Souza da Motta*

ABSTRACT

The aim of this study was to evaluate the *in vitro* adhesion potential of psychrotrophic bacteria isolated from buffalo milk. The biofilm formation was evaluated on stainless steel and on a polypropylene surface at temperatures of 7°C, 23°C and 30°C for 24, 48 and 72 h. They were also tested for adhesion to stainless steel at 7°C, in sterilized milk buffalo. Other tests on stainless steel were conducted with the adhesive strains, in co-cultivation with the *Staphylococcus* coagulase positive A710^{-2} strain. The co-culture was tested in sterilized buffalo whole milk on a stainless steel surface at 7°C. Isolates were identified by 16S rRNA gene sequencing in order to consider *Pseudomonas fluorescens* PL5.4 e *Pseudomonas fluorescens* PL7.1. These psychrotrophic bacteria were able to adhere under the conditions tested, with biofilm cell counts exceeding 5.65 and 6.16 log CFU/cm^2 on stainless steel and 6.3 and 6.37 log CFU/cm^2 on polypropylene, for *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 respectively. Both strains showed higher adhesion to a polypropylene. Regarding the test for stainless steel adhesion, at 7°C in sterilized buffalo milk, the tested strains showed counts of 4.85 and 5.09 log CFU/cm^2 for *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 respectively. Both psychrotrophic strains were able adhere in co-culture with the *Staphylococcus* coagulase positive A710^{-2} strain. This study shows that psychrotrophic bacteria, originally isolated from refrigerated raw buffalo milk, can adhere to stainless steel and polypropylene surfaces at different temperatures, indicating that once present in milk, they may become a source of recontamination.

Keywords: *Bubalus bubalis*, buffaloes, bacteria, adhesion, biofilm, contamination, *Pseudomonas*

INTRODUCTION

Biofilm represents a negative impact on all of the dairy industry, as it may be present in all developmental stages of dairy products (Tan *et al*., 2014). Biofilm is defined as microbial cell aggregates embedded in a polymeric matrix, composed of exopolysaccharides (EPS) connected to a biotic or abiotic surface. This bacterial aggregation is a phenomenon in which microorganisms interact with each other and may form a multi-species group (Abee *et al*., 2011). Thus, the microorganisms can create a microenvironment which enhances their survival on the most diverse classes of surfaces

Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil, *E-mail: amanda.motta@ufrgs.br

(Teh *et al*., 2012). This type of organization has the advantage of promoting a high concentration of nutrients and ease of genetic exchange (Nguyen and Yuk, 2013).

Biofilm initiates with the depositing of surface bacterial cells in the presence of organic and inorganic molecules (Bridier *et al*., 2015). According to the study by Teh *et al*. (2012), the number of bacteria normally found in biofilms varies between 10^4 to 10^8 CFU/cm^2. Adhesion of microorganisms initially occurs on the surface. This can also involve accessory bacterial structures, such as fimbriae, which form a bridge between the bacterial cells and available substrate, thus promoting a consistent association (Van Houdt and Michiels, 2010). After the initial adhesion between microcolonies is formed, it develops into a mature biofilm which, in turn, is supported by the bacterial production of exopolysaccharides. The consolidation of biofilm architecture is believed to be attributed to signaling mechanisms among bacteria, which is defined in literature as quorum-sensing (Soheili *et al*., 2015).

In the dairy industry there is a problem of milk residue on surfaces. This residue can supply nutrients to bacteria which are able to survive and proliferate (Teh *et al*., 2011). Microbial adhesion to surfaces of materials and equipment, and subsequent biofilm formation, is a major issue in the industry, since it constitutes a potential source of contamination. The spread of bacterial cells within the production environment threatens the safety and quality of dairy products. This in turn results in diseases being transmitted by food, leading to financial losses and a decrease in food identity and quality standards (Burgess *et al*., 2010; Carpentier and Cerf, 2011).

An important group of biofilm forming microorganisms in the dairy industry are psychrotrophic bacteria because they have the ability to grow at refrigerator temperatures, between 4°C and 10°C, a condition under which products are subjected (Neubeck *et al*., 2015). Although psychrotrophic bacteria account for less than 10% of the total number of microorganisms in fresh milk stored under satisfactory sanitary conditions, this percentage can reach 75% under inadequate conditions of obtaining and processing (Nielsen, 2002). Besides their importance in the formation of biofilm, they have the potential to produce extracellular enzymes, both inside and outside of the biofilm, favoring certain developments in structure, such as protease reservoirs (Marchand *et al*., 2012). The production of proteolytic enzymes in raw milk can cause problems like coagulation, gelation and the deterioration of organoleptic characteristics in milk derivatives (Teh *et al*., 2011). The objective of this study was to evaluate the potential of adhesion to stainless steel and polypropylene surfaces by psychrotrophic bacteria isolated from refrigerated raw buffalo milk, under simulated storage conditions.

MATERIALS AND METHODS

Bacterial cultures and cultivation conditions

This study investigates two cultures of proteolytic psychrotrophic bacteria, previously coded as PL5.4 and PL7.1 (Bogo *et al*., 2017). Both bacteria were had been isolated from samples of refrigerated raw buffalo milk. The cultures were initially kept frozen at -20°C in Tryptone Soy Broth (TSB Himedia, India) with 20% glycerol. For the reactivation of the isolates, TSA (Tryptone Soy Agar, - Himedia, India) was used and were incubated at 30°C for 48 h, to observe the purity of the isolates.

Molecular identification of psychrotrophic bacteria isolated from refrigerated raw buffalo milk

Total bacterial DNA was extracted as described by Donato (2007), with modifications. Cells were collected by centrifugation at 14.000 rpm for 4 to 5 minutes and resuspended in 40 µl of a lysis solution containing 1 M NaOH, sodium dodecyl sulfate (SDS) at 10% and TE 1X buffer (Tris-HCl acid ethylenediaminetetraacetic (EDTA), pH 8.0). This solution was boiled at 100°C for 15 minutes. Thereafter, the sample was diluted with 460 µl of TE 1X buffer and homogenized and centrifuged at 14.000 rpm for 4 to 5 minutes, followed by the removal of the supernatant.

The 16S rRNA gene was amplified from genomic DNA by Polymerase Chain Reaction (PCR) using primers FC27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R530 (5'-CCGCGGCTGCTGGCACGTA-3') (Gontang *et al*., 2007). The PCR reaction system consisted of 2 µl of DNA, 2.5 µl 1X buffer, 2.0 mM $MgCl_2$, 300 µM deoxynucleotide triphosphates (dNTPs), 0.1 pM each primer, 1 U Taq DNA polymerase (Promega, Madison, WI, USA) and sterile distilled water to a final volume of 25 µl.

PCR amplification was performed using a Techne TC-5000 PCR Thermal Cycler under the following conditions: initial phase of denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing temperature of 58°C for 1 minute, 72°C primer extension for 1 minute and finally a primer extension step at 72°C for 5 minutes. Aliquots of 30 to 60 ng of a PCR product of 503 bp and 4.5 pmol of each primer were subjected to automated sequencing (ABI PRISM 3500 Genetic Analyser-) by ACTGene Molecular Analysis Laboratory (Porto Alegre, Brazil). The sequences were identified using standard nucleotide BLAST sequence, in accordance with the 16S rRNA (bacteria and archaea) database, which was optimized for highly similar sequences (Megablast).

Adherence of psychrotrophic bacteria isolated from refrigerated raw buffalo milk to stainless steel AISI 304 and polypropylene coupons

The methodology described by Nörnberg *et al*. (2011); Bayoumi *et al*. (2012), with some modifications, was used. For the experiment, the colonies of bacteria at 24 h growth were transferred to a TSB culture medium and incubated at 30°C for 24 h. An aliquot of 1 ml was transferred to 9 ml of TSB and incubated at 30°C for 24 h with a subsequent adjustment of the optical density (OD) using a spectrophotometer wavelength of 600 nm to $1.5x10^8$ CFU/ml.

Stainless steel (AISI 304) coupons of 1 cm^2 were prepared by soaking in acetone for 30 minutes to remove any fat or fingerprints, with a subsequent washing in sterile distilled water and then autoclave sterilization. Polypropylene coupons were cleaned and sterilized by immersion in 70% ethanol, then rinsed twice in sterile distilled water. For each isolate already prepared at a concentration of $1.5x10^8$ CFU/ml, three coupons of each material were immersed in separate tubes inoculated with a TSB medium. The tubes were then incubated without agitation at 7°C, 23°C and 30°C for 24, 48, and 72 h. Every 24 h, one coupon of each tube was aseptically removed using sterile tweezers and rinsed three times in sterile distilled water to remove non-adherent cells. The coupons containing adherent cells were re-immersed in 10 ml of 0.85% saline bath and subjected to an ultrasonic machine (ultrasonic, USC700, Unique) at a frequency of 40 kHz, and treated for two periods of 10 minutes each in order to release adherent cells. Later, dilutions

were made of treatments which were subjected to plating on TSA and incubated at 30 °C for 24 hours to determine the number of adherent cells, expressed as log CFU/cm^2. Also, the concentration of planktonic cells was determined during 72 h of incubation. Every 24 h, an aliquot was withdrawn and decimal dilutions were made and plated under the same conditions. All counts were made in duplicate and each experiment was repeated twice.

Adherence of psychrotrophic bacteria isolated from refrigerated raw buffalo milk to stainless steel AISI 304 in sterile whole buffalo milk

For this experiment, new colonies of isolates were transferred to the TSB broth culture medium and incubated at 30^{o}C for 24 h. Then, an aliquot of 1 ml was transferred into 9 ml of sterilized whole buffalo milk (at 121^{o}C for 10 minutes) and incubated at 30^{o}C for 24 h, taking up an inoculum of approximately 1.5x10^8 CFU/ml.

The stainless steel coupons (AISI 304) 1 cm^2 were prepared as previously described. Three stainless steel AISI 304 coupons were immersed in tubes with buffalo milk, which had been inoculated with the isolates separately. The tubes were incubated without agitation at a temperature of 7^{o}C for 72 h. Every 24 h, a coupon of each tube was aseptically removed using sterile forceps and then washed and treated as previously described. Counts were made on each of the coupons removed to evaluate the amount of sessile and planktonic cells. All counts were performed in duplicate and each experiment was repeated twice.

Adherence of psychrotrophic bacteria in co-culture with *Staphylococcus* coagulase positive A710^{-2} isolated from refrigerated raw buffalo milk to stainless steel AISI 304 in sterile whole buffalo milk

In this test, we evaluated the ability of the strains PL5.4 and PL7.1 to adhere to stainless steel coupons with another microorganism. Initially the cultures had their standardized inoculum of 1.5x10^8 CFU/ml in sterilized buffalo milk, separately. Then, these were prepared by adding the culture of *Staphylococcus* coagulase positive A710^{-2} (a strain known to be biofilm forming) (1.5x10^8 CFU/ml) and mixed in sterile whole buffalo milk. A group of three stainless steel AISI 304 coupons (1 cm^2) were immersed in each tube containing sterile whole buffalo milk and the mix of cultures.

The tubes were incubated at 7^{o}C and aliquots were collected for counting at 24, 48 and 72 h. As a control, cultures were evaluated separately. The cell counts on the coupon and planktonic cells were performed according to the method previously described above, using the differential selective culture medium Mannitol Salt Agar (Himedia, India) for a *Staphylococcus* coagulase positive A710^{-2} count and MacConkey Agar (Himedia, India) for counting the *Pseudomonas* isolates. In addition, counts were made of recovered cells and planktonic cells. All counts were performed in duplicate and each experiment was repeated twice.

Statistical analysis

Three replicates were performed for each experiment, on two separate occasions. Statistic 12.5 Software was used for analysis of variance, using the ANOVA test with a critical probability of $P \leq 0.05$ followed by Tukey's test.

RESULTS AND DISCUSSION

Molecular identification of psychrotrophic bacteria isolated from refrigerated raw buffalo milk

The isolates previously encoded as PL5.4 and PL7.1, were identified as *Pseudomonas fluorescens* PL5.4 with the code gb/KM579624.1 and *Pseudomonas fluorescens* PL7.1 with the code gb/JF327445.1 in the Standard Nucleotide BLAST (available from http://www.ncbi.nlm.nih.gov). Both isolates demonstrated a match of at least 98% to the sequences in the database.

The microbiota of raw milk is diverse and complex, due to its high nutritional content (Quigley *et al*., 2011). The species of the genus *Pseudomonas* are described as the main bacteria in refrigerated raw milk associated with biofilm formation (Abdallah *et al*., 2015) as well as the deterioration of milk and milk derivatives (Decimo *et al*., 2014; Scatamburlo *et al*., 2015).

Adherence of psychrotrophic bacteria isolated from refrigerated raw buffalo milk to stainless steel AISI 304 and polypropylene coupons

Microorganism adhesion and biofilm formation are of great importance and can occur at any moment or environment of the production chain, from the milking at the processing site to the making of dairy products (Vlková *et al*., 2008; Hamadi *et al*., 2014). The planktonic cell count of *P. fluorescens* PL5.4 at temperatures of 7°C, 23°C and 30°C ranged from 8.12 to 8.39 log CFU/ml, 8.18 to 8.58 log CFU/ml and 7.86 to 8.64 log CFU/ml, respectively. For *P. fluorescens* PL7.1, the planktonic cell count ranged from 7.98 to 8.19 log CFU/ml, 7.74 to 8.48 log CFU/ml and 8.01 to 8.55 log CFU/ml, at temperatures of 7°C, 23°C and 30°C respectively. Numerous previous studies have already shown that this bacterium is able to form biofilm on various surfaces, both abiotic and biotic ones (Song and Leff, 2006; Borges *et al*., 2014; Pozo *et al*., 2014; Lemos *et al*., 2015; Malegori *et al*., 2016).

In the evaluation of sessile cells, it was observed that both cultures were able to adhere to both types of surfaces. The count of bacterial cells adherent to the polypropylene surface were higher than those adherent to stainless steel AISI 304 for both psychrotrophic isolates. The number of adherent cells of *P. fluorescens* PL5.4 ranged from 5.31 to 6.37 log CFU/cm^2 on the surface of stainless steel AISI 304 and 6.3 to 7.44 log CFU/cm^2 for the polypropylene surface. On the stainless steel AISI 304 surface, no significant difference was observed between the times and the temperatures tested. However, the polypropylene surface showed differences depending on the treatment. The highest score was recorded at a temperature of 30°C after 72 h of incubation (Figure 1A).

The bacterial cell count of *P. fluorescens* PL7.1, which adhered to the surface of stainless steel AISI 304, ranged from 6.16 to 6.97 log CFU/cm^2, and these counts showed significant differences in relation to the different times and temperatures tested. The bacterial cell count which adhered to the polypropylene surface ranged from 6.16 a 7.52 log CFU/cm^2 (Figure 1B).

This study sought to simulate long-term milk storage at higher temperatures in relation to two contact surfaces: stainless steel AISI 304 and polypropylene. According to the results above, the strains of *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 can adhere to both stainless steel AISI 304 and polypropylene surfaces, and a larger number of adhered cells could be observed on polypropylene surfaces for both isolates. The fact that more cells adhered to polypropylene surfaces can be due to the greater roughness that characterizes this type of surface. According to Dias *et al.* (2010) macroscopic and microscopic characteristics of

surfaces in particular, are key to greater or lesser microbial adhesion. The microtopography of the surface can make cleaning procedures more difficult. Likewise, these microgrooves or other imperfections create conditions for planktonic cells to adhere and form biofilm.

Di Ciccio *et al.* (2015) also showed increased adhesion to polypropylene surfaces in comparison with stainless steel surfaces. Additionally, several authors have reported the ability of bacteria to form biofilm on many different materials that are usually employed in food processing environments, such as stainless steel, glass, rubber, polycarbonate, polyurethane, polystyrene, polypropylene, aluminum, titanium, and ceramics (Simões *et al*., 2010; Vázquez-Sánchez *et al*., 2013; Hamadi *et al*., 2014). It was also observed that the two bacteria tested have the ability to adhere at different temperatures, indicating that, once present in milk, they may become a source of recontamination.

Adherence of psychrotrophic strains to stainless steel AISI 304 coupons in sterile whole buffalo milk

In the adhesion assay with stainless steel AISI 304, in sterilized buffalo milk at 7°C, *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 showed adherence, with an increasing number of cells constituting this structure throughout the experimental time of 72 h. The count of bacterial cells of *P. fluorescens* PL5.4 which adhered was 4.85 log CFU/cm^2 at 24 h, 5.07 log CFU/cm^2 at 48 h and 5.68 log CFU/cm^2 at 72 h. For *P. fluorescens* PL7.1, bacterial cell counts were 5.09 log CFU/cm^2 at 24 h, 5.41 log CFU/cm^2 at 48 h and 5.36 log CFU/cm^2 at 72 h (Figure 2).

A higher cell count of *P. fluorescens* PL5.4 adhered to the surface of stainless steel AISI 304 at 72 h of incubation, showing a significant difference from other counts. The count of planktonic cells from strains of *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 ranged from 8.87 to 9.2 log CFU/ml and 8.87 to 9.54 log CFU/ml, respectively.

These results show that the presence of these bacteria in milk may represent a problem for the dairy industry, since the biofilm provides a protective character for the cells present, thus creating a recontamination point. As such, cleaning and disinfecting processes may become less effective.

Adherence of psychrotrophic bacteria in co-culture with Staphylococcus coagulase positive A710^{-2} to stainless steel AISI 304 coupons in sterile whole buffalo milk

Undoubtedly, bacterial strains, even the ones belonging to the same species, differ in many phenotypic responses, including biofilm formation and/or kinetic growth parameters (Blana *et al*., 2015). This variability has been taken into account in the present study. The evaluation of multi-species adherence to surfaces was also studied because this is considered a situation most commonly found in processing environments.

All employed cultures demonstrated growth and biofilm formation under the conditions tested. The number of recovered bacterial cells of *P. fluorescens* PL5.4 after cell adhesion was 4.68 log CFU/cm^2 at 24 h, 5.12 log CFU/cm^2 at 48 h and 5.32 log CFU/cm^2 at 72 h (Figure 3A). For *Staphylococcus* coagulase A710^{-2} bacterial cell counts were 5.22 log CFU/cm^2 at 24 h, of 5.26 log CFU/cm^2 at 48 h and 5.48 CFU/cm^2 log at 72 h (Figure 3A). The number of bacterial cells recovered from *P. fluorescens* PL5.4 was lower than in single culture. However, *Staphylococcus* coagulase positive A710^{-2} showed higher counts of

adhered bacterial cells on the surface of stainless steel AISI 304 than *P. fluorescens* PL5.4. Neither showed significant adherence at 72 h of incubation (Figure 3A).

Moreover, the reduction of the bacterial cell count of *Staphylococcus* coagulase positive A710^{-2} and *P. fluorescens* PL5.4 in co-culture was also observed in the planktonic cell count. The values range from 6.63 to 7.54 log CFU/ml for *P. fluorescens* PL5.4 and from 6.16 to 7.31 log CFU/ml for *Staphylococcus* coagulase positive A710^{-2}.

The recovered *P. fluorescens* PL7.1 bacterial cell counts on the stainless steel AISI 304 surface was 5.87 log CFU/cm^2 at 24 h, 6.03 log CFU/cm^2 at 48 h and 5.76 log CFU/m^2 at 72 h of culture (Figure 3B). In assessing the co-cultivation of *P. fluorescens* PL7.1 and *Staphylococcus* coagulase positive A710^{-2}, lower cell adherence was observed than in isolated cultivation. Conversely, when in individual cultivation, the culture of *Staphylococcus* coagulase positive A710^{-2} showed high counts of bacterial cell adherence to the stainless steel AISI 304 surface. For *Staphylococcus* coagulase positive A710^{-2} the number of recovered cells was 6.19 log CFU/cm^2 at 24 h, 6.41 log CFU/cm^2 at 48 h and 6.36 log CFU/cm^2 at 72 h of culture. However, the association between cultures the *P. fluorescens* PL7.1 and *Staphylococcus* coagulase positive A710^{-2} showed a reduction in the number of bacterial cells adhered at 72 h of evaluation over other times (Figure 3B).

It was also observed that co-culture *P. fluorescens* PL7.1 and *Staphylococcus* coagulase positive A710^{-2} stimulated the adhesion with increasing numbers of *Staphylococcus* coagulase positive A710^{-2}cell adherence, while co-cultivation of *P. fluorescens* PL5.4 and *Staphylococcus* coagulase positive A710^{-2} showed a lower adherence of *Staphylococcus* coagulase positive A710^{-2} cells. The count of planktonic cells in co-culture of *Staphylococcus* coagulase positive A710^{-2} and *P. fluorescens* PL7.1 also showed decline when compared with the simple culture of each isolate. Their scores ranged from 7.09 to 7.51 log CFU/ml for *P. fluorescens* PL7.1 and from 7.32 to 8.17 log CFU/ml for *Staphylococcus* coagulase positive A710^{-2}. The co-culture *P. fluorescens* PL7.1 with *Staphylococcus* coagulase positive A710^{-2} favored the accession of Gram-positive bacteria tested. Multiple factors may underlie the observed dominance of strains in each mixed-culture biofilm community. These may include higher ability for initial attachment, higher growth rate in the biofilm, higher propensity for cellular aggregation, better adaptation in less favorable conditions, better entrapment in the developing biofilm structure (and thus reduced dispersal), as well as increased resistance to chemical disinfectants (Giaouris *et al*., 2005). According to Lindsay *et al.* (2002); Teh *et al.* (2012), exopolysaccharide production by Gram-negative bacteria favors the setting of Gram-positive bacteria on stainless steel surfaces. Thus, cells from several different species may coexist in a pre-formed biofilm. An example is a combined culture of *Listeria monocytogenes* and *Pseudomonas* biofilm (Marchand *et al*., 2012). However, in real food processing environments, the presence of many other microbial species clearly adds additional complexity to the behavior of multi-species biofilms, since all incorporated microorganisms are able to compete, cooperate, and communicate with each other (Gkana *et al*., 2017).

Biofilm formation is multifactorial and growth media containing different carbon and nitrogen sources at different temperatures are important for adherence and biofilm maturation. In food processing environments, biofilm is a

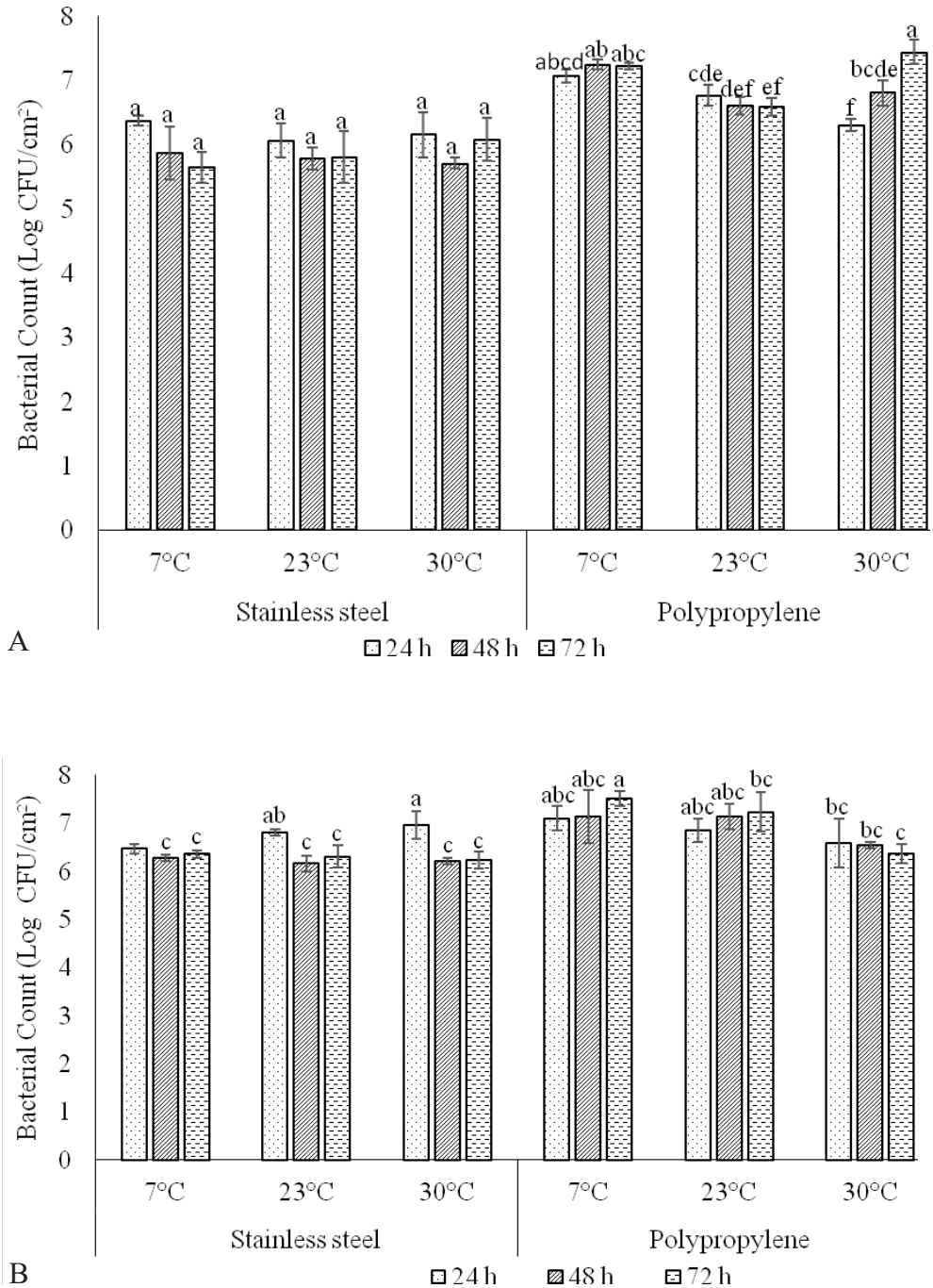


Figure 1. Biofilm formation on stainless steel AISI 304 and polypropylene in temperature of the 7°C, 23°C and 30°C for 24, 48 and 72 h. (A) Strain *P. fluorescens* PL5.4. (B) Strain *P. fluorescens* PL7.1.

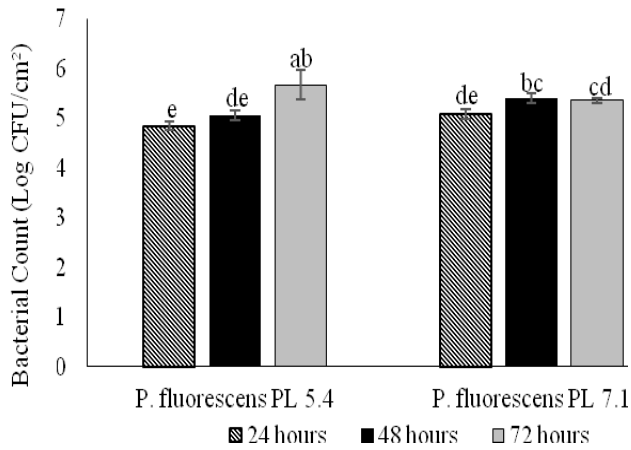
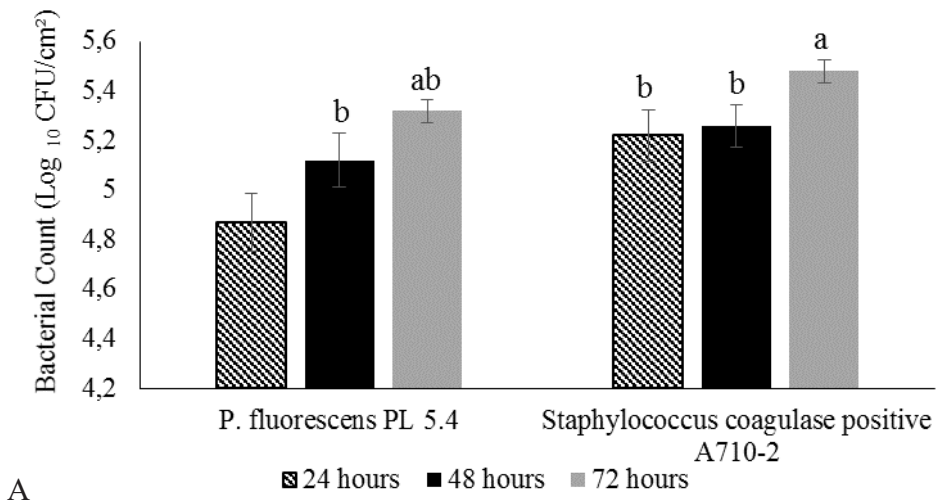
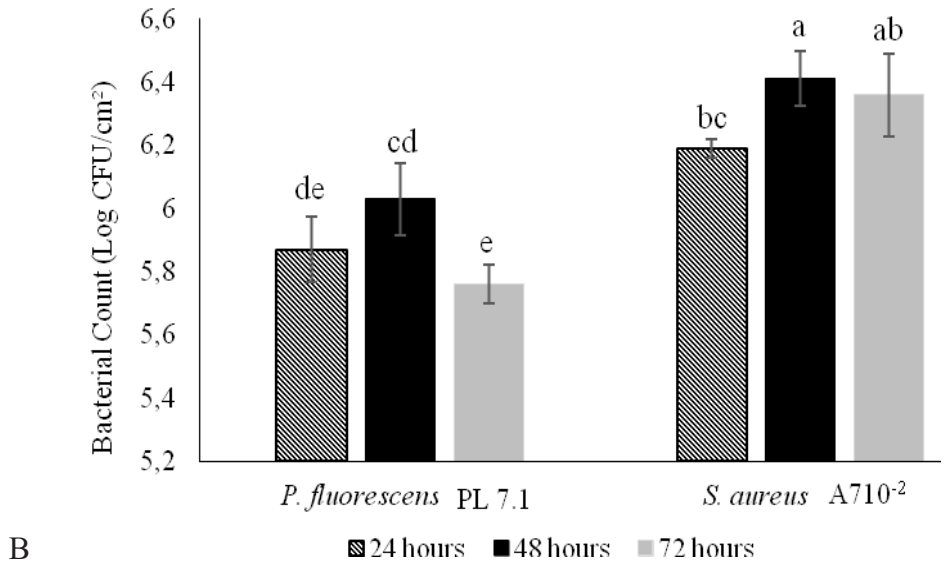


Figure 2. Biofilm formation of the strains *P. fluorescens* PL5.4 and *P. fluorescens* PL7.1 in stainless steel AISI 304, with milk culture medium at a temperature of 7°C for 24, 48 and 72 h.



A

Figure 3. (A) Biofilm formation of *P. fluorescens* PL5.4 strain and co-cultivation with *Staphylococcus* coagulase positive A710^{-2}.



B

Figure 3. (B) Biofilm formation of *P. fluorescens* PL7.1 strain and co-cultivation with *Staphylococcus* coagulase positive $A710^{-2}$. Both co-cultivation in stainless steel AISI 304 in sterile buffalo milk medium at a temperature of 7°C for 24, 48 and 72 h.

constant concern and understanding the effect of nutrients and storage conditions on biofilm formation is essential to develop effective biofilm prevention and control strategies (Aswathanarayan and Vittal, 2014).

CONCLUSION

In conclusion, these microorganisms, originally obtained from refrigerated buffalo milk, have the potential to adhere to stainless steel AISI 304 surfaces and demonstrate even greater adhesion to polypropylene surfaces. Moreover, these psychrotrophic bacteria can grow and adhere under conditions similar to those of refrigerated raw milk storage, as well as in co-culture with other bacteria. These results reinforce the need for adequate milk storage temperatures, along with the requisite time and care, in order to obtain good quality milk from the point of origin.

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