

### Proteolytic and Biofilm-forming Capacity of Bacteria Isolated from a UHT Milk Processing Plant

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### ABSTRACT

The destabilization of milk during processing, caused by bacteria, represents a significant challenge for the dairy industry. This study aimed to determine the spoilage potential of 31 psychrotrophic, mesophilic, or thermophilic strains isolated from the UHT-milk production chain. This encompassed the biofilm-forming ability on polystyrene and stainless-steel surfaces, along with the strains' caseinolytic activity and heat resistance. The effect of 5 mM MgCl<sub>2</sub> on the biofilm-forming capacity was also determined. The results indicated that the spoilage potential is highly strain-dependent. On polystyrene, *Pseudomonas brenneri* C10.3.4, *Staphylococcus pasteurii* RE1.9, and *B. licheniformis* SDP.15 were the strongest biofilm-formers of each group, whereas on stainless-steel, *Pseudomonas lactis* D1.11, *Lacticaseibacillus paracasei* P2.20 and *Bacillus sonorensis* C4.13 exhibited the highest capacity. *Staphylococcus pasteurii* RE1.9 and *Staphylococcus epidermidis* E3.6 biofilm cells showed the highest heat resistance.

Moreover, biofilm-born cells of the spore-forming *B. licheniformis* SDP.15 and *B. sonorensis* C4.13 were significantly more heat-resistant than their free-cell equivalents. Furthermore, 22 of the assessed strains showed proteolytic activity, although only the proteases produced by *P. lactis* E6.10 maintained their enzymatic activity after being treated at 92°C. These findings may contribute to developing strategies to enhance the dairy industry's long-term economic sustainability.

**Keywords:** UHT milk; spoilage potential; caseinolytic activity; biofilm-forming ability; thermal resistance

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### INTRODUCTION

Bovine milk is one of the most consumed foods worldwide due to its high nutritional content and energy contribution<sup>1</sup>. Due to these properties, milk constitutes a proper medium for the proliferation of multiple microorganisms. Therefore, microbial contamination throughout milk's industrial processing might lead to the proliferation of potential pathogenic or spoilage microorganisms in the commercial product<sup>2</sup>. The organisms found in raw dairy originate from different sources associated with cattle feed, cowshed environment, microbial contamination on the cow's udder's surface, and inadequate milking equipment cleaning<sup>3</sup>. In addition, the sanitization of containers and the storage of physical-chemical conditions can also affect the microbial composition of raw milk<sup>4</sup>. Two main strategies have been widely applied in industrial milk processing to control or eliminate its microbial population<sup>5</sup>. In the first instance, raw milk is usually stored at refrigeration

temperatures (4 - 7 °C) for a maximum of 48 hours to limit the growth of mesophilic and thermophilic bacteria <sup>6</sup>. Although this practice contributes to the temporary preservation of milk, it may also stimulate the growth of psychrotrophic microorganisms <sup>7</sup>. These are microorganisms adapted to grow at low temperatures (0 – 7 °C), even though their optimal and maximal growth temperatures are higher than 15 and 20 °C, respectively <sup>8</sup>. Subsequently, as a second strategy, raw bovine milk undergoes industrial thermal processes to reduce or eliminate microbial load and to obtain a safe commercial product for human consumption <sup>9</sup>. Pasteurization involves milk exposure to a temperature of 72 °C for 15 s <sup>10</sup>. This aims to reduce the number of pathogenic bacterial cells to levels that do not represent a significant health hazard <sup>11</sup>. Likewise, in the ultra-high temperature (UHT) treatment, the heating of milk, between 135 to 150 °C for 1 to 4s, pursues a 9-log inactivation of its initial microbiota <sup>12</sup>. Combined with aseptic packaging, this process leads to the obtention of a commercially sterile product <sup>13</sup>.

Despite the efficiency of the UHT process, several studies have reported the isolation of multiple bacterial species in UHT milk samples, suggesting the occurrence of post-heat treatment contamination (e.g., container filling) or bacterial survival during thermal processing <sup>14,15</sup>. In this regard, several mechanisms can be associated with microbial resistance to heating steps during milk processing. For instance, bacterial endospores are dormant forms of vegetative cells resistant to adverse environmental conditions like starvation, bactericidal chemicals, and heat, among others <sup>16</sup>. Therefore, an elevated concentration of spore-forming bacteria in raw milk, particularly of highly heat-resistant strains, may increase these microbes' survival <sup>17</sup>. Furthermore, most bacteria can form biofilms by adhering to multiple surfaces, such as stainless steel (SS), commonly used in the food industry <sup>18</sup>. A biofilm is a biologically active microbial community formed by cells of one or more species immersed in a matrix of exopolysaccharides <sup>19</sup>. The structure of this community creates an appropriate environment for microbial cells and is a self-protective mechanism against harsh conditions, including UV radiation, microbicidal compounds, and extreme pressure, pH, and temperature <sup>20</sup>. Therefore, this form of life enhances the survival of bacteria during heat treatments of milk, and its occurrence could constitute a source of cross-contamination in milk processing plants <sup>21</sup>. Moreover, the microorganisms in a biofilm could disperse in the food matrix immediately after contacting the contaminated surface <sup>22</sup>. This may compromise the quality and shelf-life of the product due to uncontrolled coagulation of the milk and the proliferation of bacteria resistant to thermal and chemical treatments <sup>23</sup>. Likewise, biofilms can generate hygiene inconveniences and financial impacts due to microorganisms catalyzing chemical and biological reactions that cause fouling in storage tanks and SS pipelines <sup>24</sup>.

In addition, certain extracellular enzymes produced by these bacteria can maintain their catalytic activity despite exposure to high temperatures <sup>25</sup>. Among them, lipases are lipolytic enzymes that break down lipids, including triglycerides in milk fat, into free fatty acids <sup>26</sup>. Proteases, on the other hand, degrade the major proteins present in milk, especially casein <sup>27</sup>. The destabilization of this protein by the action of proteolytic enzymes leads to changes in the organoleptic characteristics of the finished product, such as coagulation, gelation, and the formation of bitter flavors <sup>28,29</sup>.

Some conventional cleaning and disinfection processes are ineffective for the control of biofilm formation in dairy production and processing facilities. For this reason, various studies have focused on the suitability of potential biofilm inhibitory compounds. Recently, it has been reported that magnesium ions (Mg<sup>2+</sup>) affect the biofilm formation of *Bacillus* spp. through the downregulation of genes involved in the production of

exopolysaccharides<sup>30</sup>. Therefore, utilizing  $Mg^{2+}$  ions may constitute an alternative strategy to improve the efficiency of sanitary practices in milk processing. Based on the background above, this research aimed to determine the spoilage potential of psychrotrophic, mesophilic, and thermophilic bacteria isolated from raw, pasteurized, and ultra-high temperature (UHT) processed milk by *in vitro* evaluation of their biofilm formation capacity, along with their response to 5mM magnesium chloride as an inhibitory agent. In addition, the proteolytic activity of each bacterial isolate was evaluated. The results obtained in this research could contribute to developing strategies focused on controlling bacterial contamination during milk processing and preventing commercial product spoilage.

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## MATERIAL AND METHODS

### Bacterial strains

For this study, 14 psychrotrophic, 13 mesophilic, and 4 thermophilic strains were provided by the Food Microbiology Laboratory of the Microbiology career at PUCE. These strains were previously isolated from a UHT milk processing plant<sup>31</sup>. Accession numbers of the 16S rDNA sequences of the evaluated strains are stated in the supplementary material.

The selected strains were reactivated on nutrient agar under the optimal temperature and growth time conditions for each strain to establish the collection of bacterial cultures used in the subsequent experiments. This was achieved using cultures previously preserved in BHI broth with 20% (v/v) glycerol.

Then, each bacterial isolate was propagated in 5 mL of sterile BHI broth under the same environmental conditions. Upon completion of incubation, an equal volume of glycerol, with a final concentration of 20%, was added, and the resulting suspension was divided into three aliquots of 1 mL, which were cryopreserved at -20 °C.

### Screening of the biofilm-forming capacity in polystyrene microplates

The biofilm-forming capacity of the assessed strains was determined using 96-well flat-bottomed translucent polystyrene plates. Each well was loaded with 200  $\mu$ L of sterile brain heart infusion (BHI) broth plus 20  $\mu$ L of 24-hour liquid culture of each bacterial isolate. This suspension was incubated for 24 hours at 35 °C or 55 °C for mesophilic or thermophilic bacteria. Meanwhile, psychrotrophic strains were incubated at 7 °C for 10 days. The negative control consisted of individual aliquots of 220  $\mu$ L sterile BHI broth dispensed in three independent wells.

After incubation, the content of the wells was discarded, and the plates were rinsed three times with Milli-Q water. Subsequently, the plates were allowed to dry at room temperature, and the biomass, adhered on the polystyrene surface, was fixed for 10 minutes by adding 220  $\mu$ L of pure methanol into each well. After this period, methanol was poured off, and after a drying step at room temperature, a 220  $\mu$ L volume of 0.05% crystal violet solution was added to each well. This step was followed by a 10-minute incubation period at room temperature. Right after this lapse, the dye was discarded, and the wells were rinsed with Milli-Q water. After drying, a 220  $\mu$ L volume of an alcohol-acetone solution (50%-50% v/v) was added to each well, and the

plates were incubated at room temperature for 15 minutes. Finally, this solution was transferred to a new polystyrene plate, and the optical density of each well was spectrophotometrically measured at a wavelength of 492 nm ( $OD_{492nm}$ ). The cut-off (CP) value was calculated through the sum of the mean  $OD_{492nm}$  of three replicates of the negative control plus three standard deviations<sup>18,32</sup>.

### **Assessment of the biofilm-forming ability in the presence of 5 mM magnesium chloride as an inhibitory agent**

Those strains that previously showed the ability to form biofilms ( $OD_{492nm} > CP$ ) were selected to evaluate their response to magnesium chloride as a biofilm inhibitory agent. For this purpose, the above-detailed methodology was followed, but a single variation involving the addition of magnesium chloride into the culture medium (final concentration = 5 mM) was incorporated into the experimental protocol<sup>30</sup>.

### **Biofilm-forming capacity on SS plates submerged in milk**

This assay was performed according to the method described by Sadiq et al.<sup>18</sup>. Briefly, a 24-hour culture of biofilm-forming bacterial strains was individually inoculated into sterile glass flasks containing 100 mL of sterile skimmed milk. These suspensions were incubated for 24 hours at 30 °C for psychrotrophic strains, while mesophilic and thermophilic strains were incubated at 35 °C and 55 °C, respectively. Afterward, three sterile SS coupons (grade 316; 1 cm<sup>2</sup>; 2B finish) were immersed into the content of each of the flasks. In the case of mesophilic and thermophilic isolates, the cultures were further incubated for 24 hours, while psychrotrophic bacteria were incubated at 7 °C for 10 days. After incubation, the SS plates were aseptically removed and washed with sterile Milli-Q water. Subsequently, the SS coupons were placed in 50 ml conical tubes containing 10 ml of phosphate buffer solution (PBS) and 5 g of sterile glass beads to disperse the cells adhered on their surface. This preparation was stirred at 3000 rpm for 2 minutes, and subsequently, the contents of the conical tubes were diluted adequately in PBS. Thereafter, the dilutions were plated in triplicate on the surface of Tryptone Soy Agar (TSA) and incubated according to the optimal growth temperature of each bacterial group for 24 hours. At the end of this period, the formed colonies were quantified to determine the number of log UFC/mL and log UFC/cm<sup>2</sup> developed in skimmed milk and on the surface of the SS plates, respectively.

### **Heat resistance of planktonic or biofilm cultures**

Planktonic cultures in milk and biofilm cell suspensions of each bacterial isolate, obtained in the previous assay, were aliquoted in 1 mL fractions. These aliquots were thermally treated in a thermal block for 10 minutes at 80 °C. Non- and thermally treated suspensions were serially diluted in PBS. Subsequently, each dilution was inoculated in triplicate on the surface of TSA plates and incubated at the optimal growth temperature of each bacterium for 24 hours. Finally, bacterial colonies were counted to determine the number of viable log UFC/ml or log UFC/cm<sup>2</sup> before or after the heat treatment<sup>33</sup>.

## Screening of the proteolytic capacity of bacterial strains

The initially selected bacterial strains were individually, propagated in 5 ml of sterile BHI broth and incubated at their optimal growth temperature for 24 hours. After that, the cells were sedimented by centrifugation at 4000 rpm for 10 minutes<sup>34</sup>. The resulting supernatant was discarded, and under the same centrifugation parameters, the cells were washed with 2.5 ml of PBS. Subsequently, the sediment was suspended in the same volume of PBS, and its optical density, spectrophotometrically determined at a wavelength of 600 nm ( $OD_{600nm}$ ), was adjusted in the range of 0.5 to 0.8 by diluting each suspension in the required amount of PBS. Then, 25  $\mu$ l of each bacterial suspension were inoculated in triplicate in perforated wells on a Plate count medium supplemented with skim milk 10%. The psychrotrophic strains were incubated at 7 °C for 10 days, while the mesophilic and thermophilic isolates were incubated for 48 h at 35 and 55 °C, respectively. After incubation, the formation of clarification halos around the inoculated wells was observed as a result of the proteolytic capacity of the bacterial isolates, and their diameters were measured.

## Heat resistance of microbial extracellular proteases

The strains that showed proteolytic capacity in the previous assay were inoculated into conical tubes containing 10 ml of sterile BHI broth with skimmed milk 10% and incubated for 72 hours under the abovementioned environmental conditions. Thereafter, the cells were centrifuged at 6500 rpm for 30 minutes, and the supernatant was filtered and sterilized using a filter with a pore size of 0.22  $\mu$ m.<sup>35</sup> Each cell-free supernatant (CFS) was evaluated at three pH levels. For this purpose, 200  $\mu$ l aliquots of each CFS were individually mixed in a 1:1 ratio with 0.1 M sodium acetate buffer (pH 5.0), 0.1 M sodium phosphate buffer (pH 6.5), and 0.1 M Tris-HCl buffer (pH 8.0)<sup>36</sup>. In addition, the production of proteases was also evaluated without mixing the CFS with any buffer. Subsequently, to verify the presence of thermostable proteolytic enzymes, the supernatants were heat treated at 92 °C for 8 minutes and inoculated into perforated wells of milk agar. The untreated CFS was used as a positive control. After 24 hours of incubation, the formation of casein hydrolysis halos surrounding the inoculated well was observed, and their diameters were measured<sup>37</sup>.

## Inoculation effect of protease-producing bacteria in milk before sterilization

The bacterial strains that formerly showed proteolytic capacity were inoculated into 5 ml of sterile BHI broth and incubated for 24 hours at the optimal growth temperature for each strain until reaching an  $OD_{600nm}$  corresponding to 0.5 - 0.8. Subsequently, 1 mL of a 1:100 dilution of each culture in PBS was inoculated into 5 ml of sterile skim milk. Afterwards, all bacterial suspensions were incubated at 4 °C for 48 hours. Thereafter, 1 ml of each homogenized sample was dispensed into sterile conical tubes, which were heat-treated at 130 °C for 30 seconds and immediately cooled down on ice. A sterility test was then conducted by seed inoculating 100  $\mu$ l of each sample onto BHI agar. The tubes were incubated at 45 °C for 45 days. During this time, a daily visual inspection of each sample was performed to identify any effects on the milk, such as casein coagulation<sup>34</sup>.

## Statistical analysis

All experiments in this research were carried out in triplicate. The differences between the evaluated strains, corresponding to the mean of the OD<sub>492nm</sub> of biofilms developed on polystyrene, the concentration of planktonic or sessile cells in milk or the surface of SS, respectively, and the diameters of the casein digestion halos were determined through One-Way ANOVA. The post hoc Tukey multiple comparison test was used to analyze all possible strain pair combinations and ascertain whether the observed differences were statistically significant ( $P < 0.05$ ). On the other hand, the Student's t-test was applied to compare the biofilm-forming capacity of each strain without MgCl<sub>2</sub> against the OD<sub>492nm</sub> value obtained when cells of the same isolate were exposed to 5 mM MgCl<sub>2</sub>. Similarly, the same statistical analysis was used to compare the viability of planktonic or biofilm cells before and after thermal treatment. Significance was set at 0.05 for both statistical analyses.

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## RESULTS

### Screening of the biofilm-forming capacity in polystyrene microplates

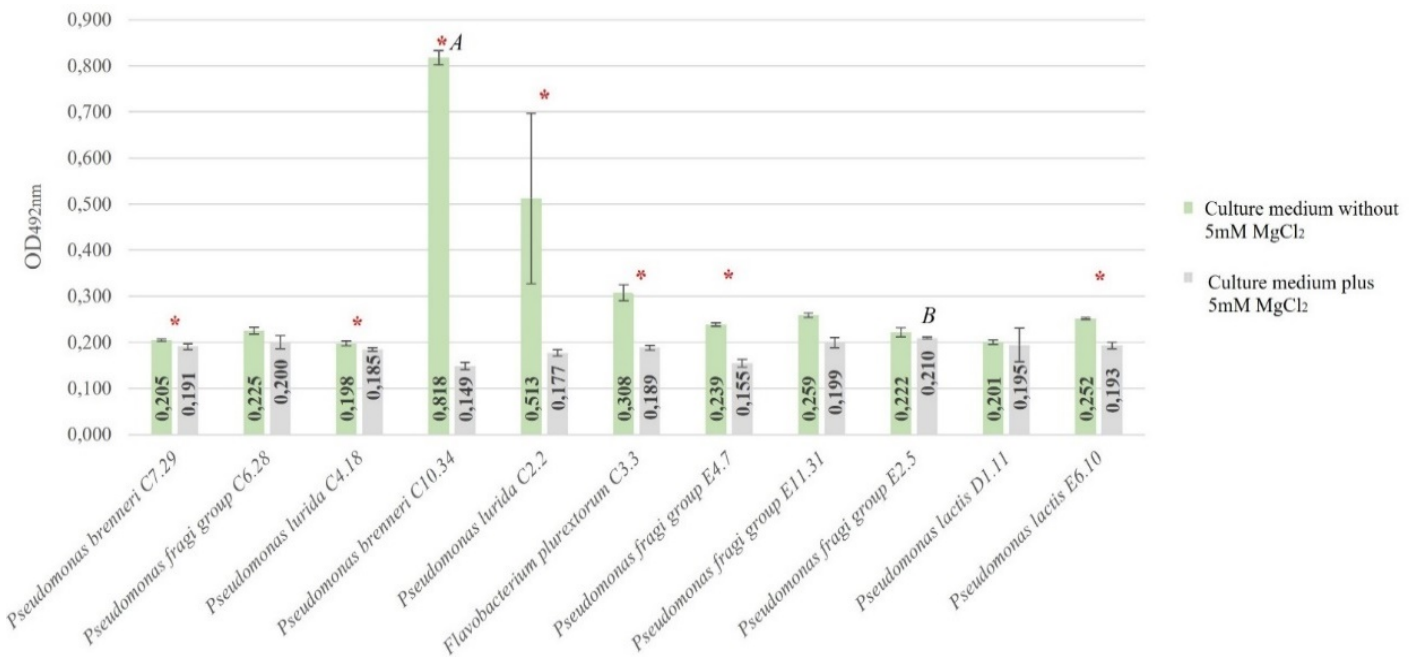
To evaluate the ability of biofilm formation, a cut-off point (CP) of 0.173 was established for the three analyzed bacterial groups. In the case of the psychrotrophic strains, it was observed that the OD<sub>492nm</sub> of 11 strains exceeded the CP value and, therefore, were considered biofilm-forming isolates. *Pseudomonas brenneri* C10.34 (OD<sub>492nm</sub> 0.818 ± 0.01) exhibited the highest biofilm-forming capacity. This was significantly higher ( $P < 0.05$ ) than the OD<sub>492nm</sub> of all psychrotrophic strains.

For mesophilic bacteria, 12 out of 13 strains showed an OD<sub>492nm</sub> higher than that threshold. *Staphylococcus pasteurii* RE1.9 (OD<sub>492nm</sub> 0.337 ± 0.02) was the strain that demonstrated the highest biofilm-forming capacity ( $P < 0.05$ ) compared to the absorbance of all strains. In the case of the thermophilic group, it was observed that three out of four isolates were capable of biofilm formation. This ability was significantly higher ( $P < 0.05$ ) for *Bacillus licheniformis* SDP.15 (OD<sub>492nm</sub> 0.483 ± 0.05) when compared to the other two biofilm-forming strains.

### Biofilm-forming ability in the presence of 5 mM magnesium chloride as an inhibitory agent

To assess the biofilm-forming ability of the isolates in the presence of 5 mM MgCl<sub>2</sub>, a unique CP was defined at 0.162. Of the 11 biofilm-forming psychrotrophic isolates, only *Pseudomonas brenneri* C10.34 and *Pseudomonas fragi* group E4.7 were inhibited since their OD<sub>492nm</sub> values were lower than the established CP. Additionally, it is essential to highlight that the absorbance, related to biofilm biomass, of every strain exposed to 5 mM MgCl<sub>2</sub> was significantly lower than that developed in the absence of this compound, except for *Pseudomonas fragi* group C6.28, *Pseudomonas fragi* group E2.5, and *Pseudomonas lactis* D1.11 (Figure 1).





**Figure. 1** OD<sub>492nm</sub> corresponding to the biofilm formation of psychrotrophic strains in the absence and presence of 5mM MgCl<sub>2</sub>

(\*) Statistically significant reduction after treatment with 5mM MgCl<sub>2</sub>. <sup>A</sup> Strain showing a significantly higher biofilm-forming capacity in the absence of 5mM MgCl<sub>2</sub> compared to all strains. <sup>B</sup> Strain showing a significantly higher biofilm-forming capacity in the presence of 5mM MgCl<sub>2</sub> compared to all strains except C6.28, E11.31, and D1.11.

On the other hand, the biofilm-forming capacity of none of the mesophilic strains was inhibited by the presence of 5mM MgCl<sub>2</sub>, since their OD<sub>492nm</sub> was higher than the CP value. Although the absorbance of all the mesophilic strains, exposed to 5mM MgCl<sub>2</sub>, was lower than the value determined in the absence of this compound, a statistically significant reduction was only observed for *Pantoea ananatis* P.40, *Pantoea conspicua* D.23, *Staphylococcus pasteurii* RE1.9, *Enterobacter hormaechei* E8.16, *Raoultella terrigena* E7.12, and *Lactococcus lactis* subsp. *lactis* D.24 (Figure 2)

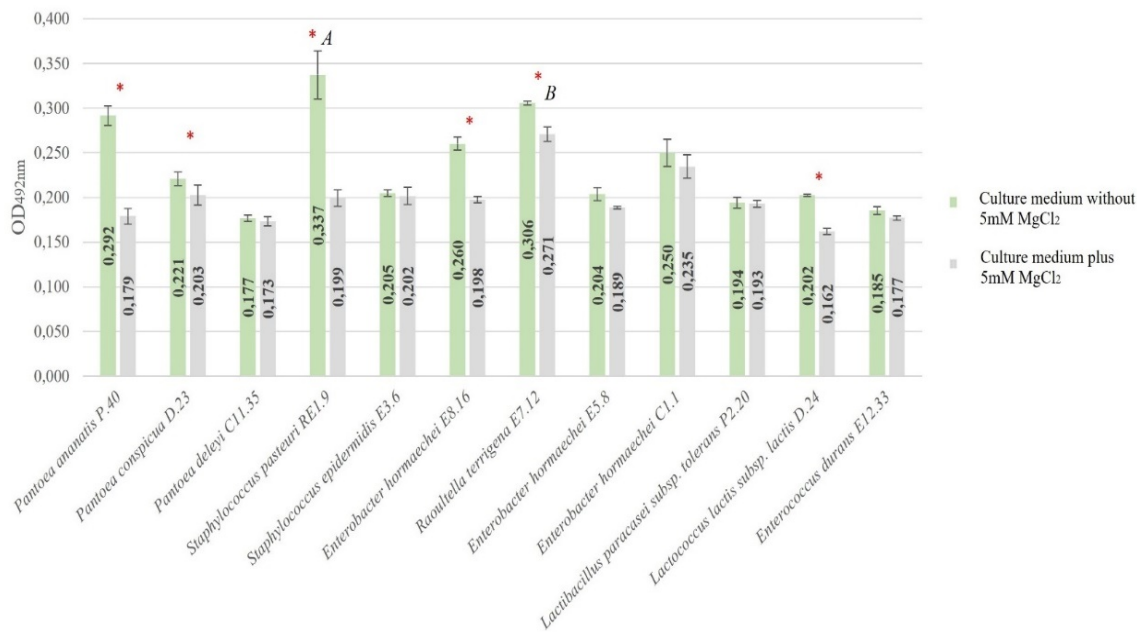


Figure. 2 OD<sub>492nm</sub> corresponding to the biofilm formation of mesophilic strains in the absence and presence of 5mM MgCl<sub>2</sub>

(\*) Statistically significant difference after treatment with 5mM MgCl<sub>2</sub>. <sup>A</sup> Strain showing a significantly higher biofilm-forming capacity in the absence of 5mM MgCl<sub>2</sub> compared to all strains. <sup>B</sup> Strain showing a significantly higher biofilm-forming capacity in the absence of 5mM MgCl<sub>2</sub> compared to all strains.

Finally, only the biofilm formation capacity of *Enterococcus durans* E1.4 was significantly reduced and inhibited by 5mM MgCl<sub>2</sub> since its OD<sub>492nm</sub> value was lower than the established CP. Likewise, this capacity, compared to the previous treatment, was significantly reduced (P < 0.05) for *Bacillus licheniformis* SDP.15 when exposed to this compound (Figure 3).

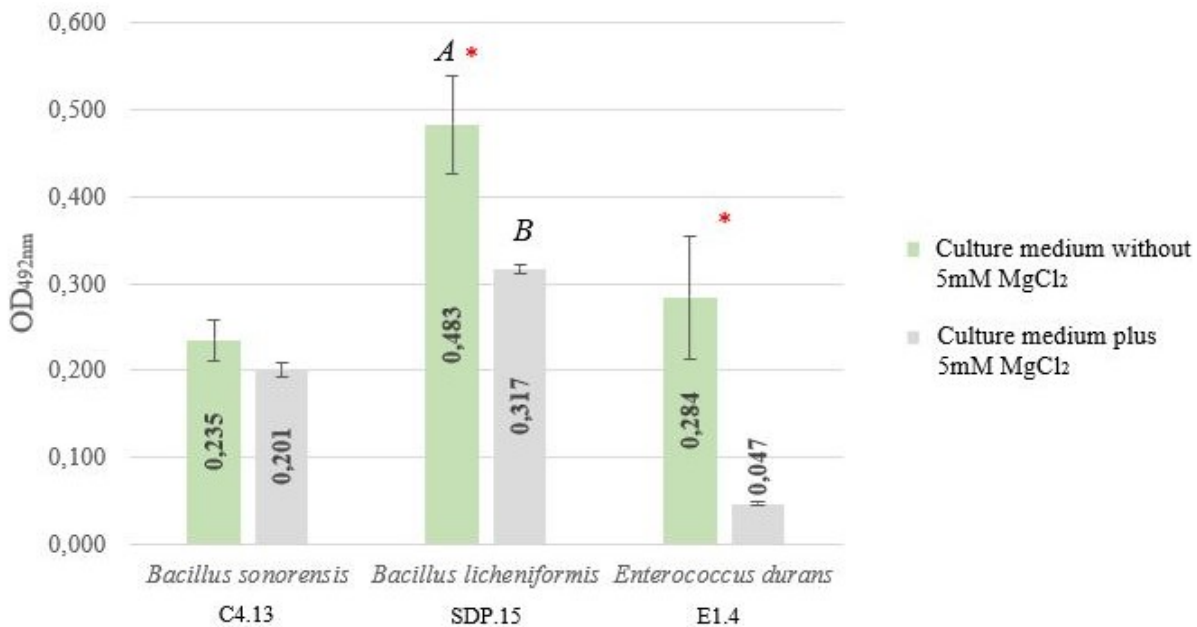


Figure. 3 OD<sub>492nm</sub> corresponding to the biofilm formation of thermophilic strains in the absence and presence of 5mM MgCl<sub>2</sub>

(\*) Statistically significant difference after treatment with 5mM MgCl<sub>2</sub>. <sup>A</sup> Strain showing a significantly higher biofilm-forming capacity in the absence of 5mM MgCl<sub>2</sub> compared to all strains. <sup>B</sup> Strain showing a significantly higher biofilm-forming capacity in the absence of 5mM MgCl<sub>2</sub> than all strains.



## Biofilm-forming capacity on SS plates submerged in milk

In this study, the cell counts of biofilms recovered from SS plates were compared to the number of planktonic cells for each strain. Among the psychrotrophic bacteria group, it was observed that the highest biofilm cell concentration, equivalent to  $8.56 \pm 0.49 \log \text{CFU/cm}^2$ , corresponded to *Pseudomonas lactis* D1.11. In comparison to the rest of the psychrotrophic strains, this was statistically higher than the sessile cell counts of *Pseudomonas fragi* group E11.31, *Pseudomonas lactis* E6.10, *Pseudomonas fragi* group C6.28, *Pseudomonas brenneri* C10.34, *Pseudomonas brenneri* C7.29, *Pseudomonas fragi* group E2.5, *Pseudomonas lurida* C4.18, and *Pseudomonas fragi* group E4.7 (Table 1). In contrast, this latter strain exhibited the lowest biofilm-forming capacity on SS ( $6.86 \pm 0.07 \log \text{CFU/cm}^2$ ).

On the other hand, the planktonic cell concentration of all the psychrotrophic strains propagated in milk, was higher than  $7 \log \text{CFU/mL}$ . In this regard, it was observed that *Pseudomonas fragi* group C6.28 achieved the highest count ( $9.59 \pm 0.03 \log \text{CFU/mL}$ ), which was significantly higher than all strains, except for *Pseudomonas fragi* group E11.31, *Pseudomonas lactis* E6.10, and *Pseudomonas fragi* group E4.7. However, the cells of *Pseudomonas fragi* group C6.28, recovered from the SS plates, exhibited the second lowest concentration ( $7.14 \pm 0.01 \log \text{CFU/cm}^2$ ) in this group. Additionally, *Pseudomonas fragi* group E2.5 was the strain that showed the lowest counts of planktonic cells ( $7.11 \pm 0.07 \log \text{CFU/mL}$ ) compared to the concentration of all strains.

Likewise, *Lactocaseibacillus paracasei* subsp. *tolerans* P2.20 was the mesophilic isolate with the highest concentration ( $8.98 \pm 0.01 \log \text{CFU/cm}^2$ ) of biofilm cells attached to SS. This result was statistically higher than the counts obtained for all strains, except for *Enterococcus durans* E12.33 and *Enterobacter hormaechei* E8.16 (Table 1). Interestingly, *L. paracasei* subsp. *tolerans* P2.20 also showed the highest count of planktonic cells ( $8.96 \pm 0.02 \log \text{CFU/mL}$ ) within this group. On the contrary, the cells of *Dermacoccus nishinomiyaensis* C1.1, adhered to SS plates, reached a concentration of  $7.38 \pm 0.01 \log \text{CFU/cm}^2$ . This count was significantly lower ( $P < 0.05$ ) than all the analyzed strains.

Regarding the thermophilic strains, it was observed that *Bacillus sonorensis* C4.13 exhibited significantly higher counts of both sessile cells ( $8.23 \pm 0.01 \log \text{CFU/cm}^2$ ) and planktonic cells ( $8.83 \pm 0.02 \log \text{CFU/mL}$ ) compared to all the other isolates. On the other hand, *Bacillus licheniformis* SDP.15 was the thermophilic strain that reflected the less efficient biofilm-forming capacity on SS, reaching a concentration equal to  $5.25 \pm 0.08 \log \text{CFU/cm}^2$ . For free cells, the lowest value was  $8.03 \pm 0.10 \log \text{CFU/mL}$  for *Enterococcus durans* E1.4 (Table 1).

Group	Strain	Planktonic cell count (log CFU/mL)	Biofilm cell count (log CFU/cm <sup>2</sup> )
Psychrotrophic	<i>Pseudomonas fragi</i> group E11.31	$9.49 \pm 0.06$	$7.51 \pm 0.06$
	<i>Pseudomonas lactis</i> E6.10	$9.56 \pm 0.09$	$7.26 \pm 0.01$
	<i>Pseudomonas fragi</i> group C6.28	$9.59 \pm 0.03^C$	$7.14 \pm 0.01$
	<i>Pseudomonas fragi</i> group E4.7	$9.49 \pm 0.13$	$6.86 \pm 0.07^B$

	<i>Flavobacterium plurextorum</i> C3.3	8.08 ± 0.03	8.42 ± 0.10
	<i>Pseudomonas brenneri</i> C10.34	8.97 ± 0.16	7.76 ± 0.04
	<i>Pseudomonas lurida</i> C2.2	9.43 ± 0.08	8.39 ± 0.08
	<i>Pseudomonas fragi</i> group E2.5	7.11 ± 0.07 <sup>D</sup>	7.72 ± 0.10
	<i>Pseudomonas brenneri</i> C7.29	7.28 ± 0.02	7.03 ± 0.05
	<i>Pseudomonas lurida</i> C4.18	8.50 ± 0.04	8.20 ± 0.17
	<i>Pseudomonas lactis</i> D1.11	8.29 ± 0.01	8.56 ± 0.49 <sup>A</sup>
<b>Mesophilic</b>	<i>Pantoea deleyi</i> C11.35	8.10 ± 0.01	8.34 ± 0.21
	<i>Pantoea conspicua</i> D.23	8.87 ± 0.02	8.79 ± 0.03
	<i>Dermacoccus nishinomiyaensis</i> C1.1	7.75 ± 0.02	7.38 ± 0.01 <sup>F</sup>
	<i>Pantoea ananatis</i> P.40	7.81 ± 0.02	8.42 ± 0.01
	<i>Enterococcus durans</i> E12.33	8.90 ± 0.06	8.95 ± 0.01
	<i>Staphylococcus pasteurii</i> RE1.9	8.05 ± 0.01	8.06 ± 0.02
	<i>Lactocaseibacillus paracasei</i> subsp. <i>tolerans</i> P2.20	8.96 ± 0.02 <sup>G</sup>	8.98 ± 0.01 <sup>E</sup>
	<i>Lactococcus lactis</i> subsp. <i>lactis</i> D.24	8.80 ± 0.06	7.91 ± 0.16
	<i>Enterobacter hormaechei</i> E8.16	8.78 ± 0.02	8.95 ± 0.02
	<i>Staphylococcus epidermidis</i> E3.6	7.36 ± 0.04 <sup>H</sup>	8.04 ± 0.04
	<i>Enterobacter hormaechei</i> E5.8	8.12 ± 0.01	8.31 ± 0.02
<i>Raoultella terrigena</i> E7.12	7.78 ± 0.02	8.38 ± 0.02	
<b>Thermophilic</b>	<i>Enterococcus durans</i> E1.4	8.03 ± 0.10 <sup>L</sup>	6.88 ± 0.09
	<i>Bacillus licheniformis</i> SDP.15	8.35 ± 0.05	5.25 ± 0.08 <sup>J</sup>
	<i>Bacillus sonorensis</i> C4.13	8.83 ± 0.02 <sup>K</sup>	8.23 ± 0.01 <sup>I</sup>

Planktonic cells were grown in sterile skimmed milk 10%, and biofilms were developed on the surface of stainless-steel plates submerged in the same matrix. The numerical values correspond to the mean count of three replicates ± the standard deviation of planktonic or biofilm cell concentration. <sup>A</sup> Biofilm cell count significantly higher (P < 0.05) than the concentration of all the psychrotrophic evaluated strains, except C3.3 and C2.2. <sup>B</sup> Biofilm cell count significantly lower compared to the count of all psychrotrophic isolates, except C6.28 and C7.29. <sup>C</sup> Planktonic cell count significantly higher than the concentration of C3.3, C10.34, C2.2, E2.5, C7.29, C4.18, and D1.11. <sup>D</sup> Planktonic cell count significantly lower than the concentration of all psychrotrophic strains. <sup>E</sup> Biofilm cell count significantly higher than the mean of all mesophilic isolates, except E12.33 and E8.16. <sup>F</sup> Biofilm cell count significantly lower than the concentration of all mesophilic strains. <sup>G</sup> Planktonic cell count significantly higher than the count of all mesophilic strains. <sup>H</sup> Planktonic cell count significantly lower than all concentrations of mesophilic isolates. <sup>I</sup> Biofilm cell count significantly higher compared to the count of all thermophilic strains. <sup>J</sup> Biofilm cell count significantly lower than the concentration of all thermophilic isolates. <sup>K</sup> Planktonic cell count significantly superior to the count of all thermophilic strains. <sup>L</sup> Planktonic cell count significantly lower than all thermophilic strains' mean.

**Table 1. Concentration of planktonic cells grown in milk and biofilm bacteria developed on SS**

## Heat resistance of planktonic or biofilm bacterial cells

After the exposure of the psychrotrophic biofilm cells to thermal treatment, only *Flavobacterium plurextorum* C3.3, *Pseudomonas lurida* C2.2, and *Pseudomonas lurida* C4.18 showed detectable survival ( $> 3 \log$  CFU/mL). The viability of these three strains suffered a reduction ranging from  $3.57 \pm 0.23 \log$  CFU/mL for *Flavobacterium plurextorum* C3.3 to  $4.10 \pm 0.35 \log$  CFU/mL for *Pseudomonas lurida* C4.18. These values were statistically lower ( $P < 0.05$ ) compared to the reduction observed for the planktonic cells of each strain (Table 2). Therefore, the sessile cells of these strains might exhibit more resistance to high temperatures than their free-cell counterparts. Conversely, it was observed that the planktonic cells of *Pseudomonas lactis* D1.11, *Pseudomonas fragi* group C6.28, *Pseudomonas fragi* group E11.31, and *Pseudomonas lactis* E6.10 suffered a reduction between  $3.19 \pm 0.17 \log$  CFU/mL to  $5.32 \pm 0.18 \log$  CFU/mL. This result suggests that the free cells of these four strains exhibit more excellent resistance at that temperature (Table 2).

On the other hand, after the heat treatment, the biofilm cell viability, of all the evaluated mesophilic isolates, remained above  $4.32 \log$  CFU/mL (Table 2). During this assay, the viability loss oscillated between  $0.03 \pm 0.02$  for *Staphylococcus pasteurii* RE1.9 and  $3.59 \pm 0.43$  for *Lactococcus lactis* subsp. *lactis* D.24. The log reduction exhibited by sessile cells of *Staphylococcus pasteurii* RE1.9 was statistically lower than the viability loss of *Pantoea conspicua* D.23, *Enterococcus durans* E12.33, *Lacticaseibacillus paracasei* subsp. *tolerans* P2.20, *Lactococcus lactis* subsp. *lactis* D.24, and *Enterobacter hormaechei* E8.16. Similarly, planktonic cell survival of all the mesophilic strains, except for *Pantoea deleyi* C11.35, was evidenced throughout the heat treatment. However, the viability loss of every heat-resistant strain was significantly lower for cells in a sessile state than those in planktonic form. This finding suggests that biofilm cells of this bacterial group exhibit more excellent resistance compared to their planktonic counterparts (Table 2).

Moreover, the three thermophilic evaluated strains could survive the thermal treatment, either in a biofilm or planktonic state. Nevertheless, a biofilm cell reduction of  $0.04 \pm 0.01$  and  $1.76 \pm 0.12 \log$  CFU/mL for *Bacillus sonorensis* C4.13 and *Bacillus licheniformis* SDP.15, respectively, were significantly lower than the viability decreases experienced by planktonic cells of these strains (Table 2)

Group	Strain	Initial planktonic cell count (log CFU/mL)	Planktonic cells count after treatment at 80°C (log CFU/mL)	Initial biofilm cell count (log CFU/mL)	Biofilm cells count after treatment at 80°C (log CFU/mL)
Psychrotrophic	<i>Pseudomonas fragi</i> group E11.31	$9.49 \pm 0.06$	$4.20 \pm 0.17^B$	$7.51 \pm 0.06$	0
	<i>Pseudomonas lactis</i> E6.10	$9.56 \pm 0.09$	$4.24 \pm 0.10^B$	$7.26 \pm 0.01$	0
	<i>Pseudomonas fragi</i> group C6.28	$9.59 \pm 0.03$	$4.39 \pm 0.36^B$	$7.14 \pm 0.01$	0
	<i>Pseudomonas fragi</i> group E4.7	$9.49 \pm 0.13$	0	$6.86 \pm 0.07$	0
	<i>Flavobacterium plurextorum</i> C3.3	$8.08 \pm 0.03$	0	$8.42 \pm 0.10$	$4.85 \pm 0.13^{4'}$

	<i>Pseudomonas brenneri</i> C10.34	8.97 ± 0.16	0	7.76 ± 0.04	0
	<i>Pseudomonas lurida</i> C2.2	9.43 ± 0.08	0	8.39 ± 0.08	4.32 ± 0.28 <sup>d</sup>
	<i>Pseudomonas fragi</i> group E2.5	7.11 ± 0.07	0	7.72 ± 0.10	0
	<i>Pseudomonas brenneri</i> C7.29	7.28 ± 0.02	0	7.03 ± 0.05	0
	<i>Pseudomonas lurida</i> C4.18	8.50 ± 0.04	0	8.20 ± 0.17	4.10 ± 0.17 <sup>d</sup>
	<i>Pseudomonas lactis</i> D1.11	8.29 ± 0.01	5.10 ± 0.17 <sup>b</sup>	8.56 ± 0.49	0
<b>Mesophilic</b>	<i>Pantoea deleyi</i> C11.35	8.10 ± 0.01	0	8.34 ± 0.21	8.11 ± 0.02 <sup>d</sup>
	<i>Pantoea conspicua</i> D.23	8.87 ± 0.02	5.09 ± 0.03	8.79 ± 0.03	5.20 ± 0.03 <sup>d</sup>
	<i>Dermacoccus nishinomiyaensis</i> C1.1	7.75 ± 0.02	5.43 ± 0.38	7.38 ± 0.01	7.31 ± 0.01 <sup>d</sup>
	<i>Pantoea ananatis</i> P.40	7.81 ± 0.02	4.81 ± 0.10	8.42 ± 0.01	8.26 ± 0.02 <sup>d</sup>
	<i>Enterococcus durans</i> E12.33	8.90 ± 0.06	5.27 ± 0.01	8.95 ± 0.01	7.77 ± 0.02 <sup>d</sup>
	<i>Staphylococcus pasteurii</i> RE1.9	8.05 ± 0.01	6.38 ± 0.08	8.06 ± 0.02	8.02 ± 0.01 <sup>d*</sup>
	<i>Lactocaseibacillus paracasei</i> subsp. <i>tolerans</i> P2.20	8.96 ± 0.02	4.20 ± 0.17	8.98 ± 0.01	6.13 ± 0.02 <sup>d</sup>
	<i>Lactococcus lactis</i> subsp. <i>lactis</i> D.24	8.80 ± 0.06	4.20 ± 0.10	7.91 ± 0.16	4.32 ± 0.28 <sup>d</sup>
	<i>Enterobacter hormaechei</i> E8.16	8.78 ± 0.02	5.38 ± 0.08	8.95 ± 0.02	6.93 ± 0.03 <sup>d</sup>
	<i>Staphylococcus epidermidis</i> E3.6	7.36 ± 0.04	4.96 ± 0.03	8.04 ± 0.04	7.97 ± 0.01 <sup>d</sup>
	<i>Enterobacter hormaechei</i> E5.8	8.12 ± 0.01	4.43 ± 0.23	8.31 ± 0.02	8.16 ± 0.01 <sup>d</sup>
<i>Raoultella terrigena</i> E7.12	7.78 ± 0.02	4.03 ± 0.01	8.38 ± 0.02	8.30 ± 0.01 <sup>d</sup>	
<b>Thermophilic</b>	<i>Enterococcus durans</i> E1.4	8.03 ± 0.10	5.73 ± 0.05 <sup>b</sup>	6.88 ± 0.09	3.52 ± 0.07
	<i>Bacillus licheniformis</i> SDP.15	8.35 ± 0.05	4.85 ± 0.29	5.25 ± 0.08	3.49 ± 0.20 <sup>d</sup>
	<i>Bacillus sonorensis</i> C4.13	8.83 ± 0.02	5.33 ± 0.35	8.23 ± 0.01	8.19 ± 0.02 <sup>d+</sup>

Each value corresponds to the mean count ± standard deviation of three replicates of planktonic or biofilm cells before and after the thermal treatment at 80°C. <sup>d</sup> Reduction of biofilm cells significantly lower (P < 0.05) than the reduction of planktonic cells after the thermal treatment. <sup>b</sup> Reduction of planktonic cells significantly lower than the reduction of biofilm cells after the thermal treatment. (!) Strain that showed the lowest reduction of biofilm cells compared to all psychrotrophic isolates. (\*) Mesophilic strain that showed the lowest reduction of sessile cells compared to D.23, E12.33, P2.20, D.24, and E8.16. (+) Strain that showed the lowest reduction of sessile cells compared to all thermophilic isolates.

**Table 2. Heat resistance of planktonic or biofilm cells of psychrotrophic, mesophilic, or thermophilic isolates**

## Screening of protease-producing bacterial strains

As a result of their proteolytic activity, the growth of 22 out of 31 evaluated strains led to the formation of casein degradation halos around each inoculum (Table 3). Among psychrotrophic bacteria, eleven isolates exhibited halos whose diameters ranged from  $1.1 \pm 0.1$  for *Pseudomonas brenneri* C10.34 to  $3.2 \pm 0.2$  cm for *Pseudomonas lurida* C2.2. The latter strain showed a significantly higher ( $P < 0.05$ ) caseinolytic capacity in comparison to the rest of the psychrotrophic strains. Furthermore, eight mesophilic strains showed halos whose diameters oscillated from  $1.1 \pm 0.1$  to  $2.9 \pm 0.1$  cm for *Lacticaseibacillus paracasei* subsp. *tolerans* P2.20 and *Staphylococcus epidermidis* E3.6, respectively. This latest isolate exhibited the highest proteolytic activity compared to the other strains within this group (Table 3).

On the other hand, three thermophilic strains, out of four, exhibited protease-producing capacity. Their digestion halos showed diameters ranging between  $2.8 \pm 0.4$  cm for *Bacillus sonorensis* C4.13 and  $3.3 \pm 0.6$  cm for *Bacillus licheniformis* SDP.15. On average, these three strains exhibited higher proteolytic activity compared to those belonging to the other two bacterial groups (Table 3).

Group	Strain	Diameter of casein degradation halo (cm)
Psychrotrophic	<i>Pseudomonas brenneri</i> C7.29	$1.5 \pm 0.1$
	<i>Janthinobacterium lividum</i> C5.27	$1.4 \pm 0.1$
	<i>Pseudomonas fragi</i> group C6.28	$1.2 \pm 0.1$
	<i>Pseudomonas brenneri</i> C10.34	$1.1 \pm 0.1$
	<i>Pseudomonas lurida</i> C2.2	$3.2 \pm 0.2^A$
	<i>Pseudomonas fragi</i> group E4.7	$1.4 \pm 0.1$
	<i>Pseudomonas lurida</i> C4.18	$1.6 \pm 0.1$
	<i>Pseudomonas lactis</i> D1.11	$1.2 \pm 0.2$
	<i>Pantoea ananatis</i> D.22	$2.3 \pm 0.3$
	<i>Pseudomonas fragi</i> group E11.31	$1.8 \pm 0.3$
	<i>Pseudomonas fragi</i> group C9.32	NP
	<i>Flavobacterium plurextorum</i> C3.3	NP
	<i>Pseudomonas fragi</i> group E2.5	NP
	<i>Pseudomonas lactis</i> E6.10	$1.6 \pm 0.1$
Mesophilic	<i>Staphylococcus pasteurii</i> RE1.9	$1.6 \pm 0.1$
	<i>Raoultella terrigena</i> E7.12	$2.1 \pm 0.2$
	<i>Enterococcus durans</i> E10.19	$1.7 \pm 0.2$
	<i>Enterobacter hormaechei</i> E5.8	$2.3 \pm 0.3$
	<i>Lacticaseibacillus paracasei</i> subsp. <i>tolerans</i> P2.20	$1.1 \pm 0.1$
	<i>Staphylococcus epidermidis</i> E3.6	$2.9 \pm 0.2^B$
	<i>Lactococcus lactis</i> D.24	$1.4 \pm 0.1$
	<i>Enterococcus durans</i> E12.33	$1.2 \pm 0.1$

	<i>Pantoea deleyi</i> C11.35	NP
	<i>Pantoea ananatis</i> P.40	NP
	<i>Pantoea conspicua</i> D.23	NP
	<i>Dermacoccus nishinomiyaensis</i> C1.1	NP
	<i>Enterobacter hormaechei</i> E8.16	NP
<b>Thermophilic</b>	<i>Bacillus sonorensis</i> C4.13	2.8 ± 0.4
	<i>Bacillus licheniformis</i> SDP.15	3.3 ± 0.6
	<i>Bacillus licheniformis</i> E9.17	3.1 ± 0.1
	<i>Enterococcus durans</i> E1.4	NP

The numerical values correspond to the average of three replicates ± standard deviation of the diameter of casein digestion halos. <sup>A</sup> Strain that exhibited the highest proteolytic capacity compared to all psychrotrophic strains ( $P < 0.05$ ). <sup>B</sup> Strain that showed the highest caseinolytic activity compared to all mesophilic strains. "NP" Indicates that it did not exhibit proteolytic capacity.

**Table 3. Screening of the proteolytic capacity of psychrotrophic, mesophilic, and thermophilic bacterial isolates**

### Heat resistance of bacterial extracellular proteases

After thermally treating the CFS, individually obtained from the culture of protease-producing bacterial strains, it was evidenced that, uniquely, the inoculation of the supernatant extracted from the cultivation of *Pseudomonas lactis* E6.10, led to the development of casein degradation halos. Like the control without heat treatment, the diameter of clearing zones, around the inoculated wells, was  $1.6 \pm 0.1$  cm. This was evident in the case of unbuffered CFS and when pH was adjusted to 5, 6.5, and 8.

### Inoculation effect of protease-producing bacteria in milk before sterilization

Except for milk inoculated with *Pseudomonas brenneri* C7.29, *Pseudomonas fragi* group C6.28, *Pseudomonas brenneri* C10.34, *Pseudomonas lactis* D1.11, or *Pseudomonas fragi* group E11.31, most of the samples, previously contaminated with individual protease-producing strains and subsequently sterilized, suffered syneresis during storage at 45 °C. This phenomenon was evidenced by the casein coagulation and its subsequent separation from the whey. This effect was noticeable during the initial seven days for samples containing remaining metabolites of psychrotrophic and thermophilic bacteria. On the other hand, changes were observed in milk containing metabolic products of mesophilic bacteria only after 30 days of incubation (Table 4)

Group	Strain	Effect	Days of incubation when changes were observed for the first time
<b>Psy- chrotrophic</b>	<i>Pseudomonas brenneri</i> C7.29	NP	-
	<i>Janthinobacterium lividum</i> C5.27	Casein coagulation	7
	<i>Pseudomonas fragi</i> group C6.28	NP	-
	<i>Pseudomonas brenneri</i> C10.34	NP	-
	<i>Pseudomonas lurida</i> C2.2	Casein coagulation	7



	<i>Pseudomonas fragi</i> group E4.7	Casein coagulation	7
	<i>Pseudomonas lurida</i> C4.18	Casein coagulation	7
	<i>Pseudomonas lactis</i> D1.11	NP	-
	<i>Pantoea ananatis</i> D.22	Casein coagulation	7
	<i>Pseudomonas fragi</i> group E11.31	NP	-
	<i>Pseudomonas lactis</i> E6.10	Casein coagulation	7
<b>Mesophilic</b>	<i>Staphylococcus pasteurii</i> RE1.9	Casein coagulation	30
	<i>Raoultella terrigena</i> E7.12	Casein coagulation	32
	<i>Enterococcus durans</i> E10.19	Casein coagulation	30
	<i>Enterobacter hormaechei</i> E5.8	Casein coagulation	30
	<i>Lacticaseibacillus paracasei</i> subsp. <i>tolerans</i> P2.20	Casein coagulation	30
	<i>Staphylococcus epidermidis</i> E3.6	Casein coagulation	30
	<i>Lactococcus lactis</i> D.24	Casein coagulation	31
	<i>Enterococcus durans</i> E12.33	Casein coagulation	30
<b>Thermophilic</b>	<i>Bacillus sonorensis</i> C4.13	Casein coagulation	7
	<i>Bacillus licheniformis</i> SDP.15	Casein coagulation	7
	<i>Bacillus licheniformis</i> E9.17	Casein coagulation	7

NP: No effect on sterile milk after 45 days of incubation at 45°C. (-) No changes were observed on any day.

**Table 4. Effect of inoculation of psychrotrophic, mesophilic, and thermophilic protease-producing bacteria in milk before sterilization**

## DISCUSSION

This study revealed that Gram-negative psychrotrophic *Pseudomonas brenneri* C10.34 exhibited the highest biofilm-forming capacity on polystyrene wells. In this connection, it has previously been described that multiple milk-originated strains belonging to *Pseudomonas* spp., including *P. fluorescens* and *P. brenneri*, displayed an efficient biofilm-forming capacity, on polystyrene plates<sup>38–40</sup>. Additionally, consistent with our outcomes, Rossi et al.<sup>39</sup> have documented that an extended incubation time ( $\geq 48$ h) at a low temperature (10 °C), stimulates biofilms development of *P. fluorescens* and *P. brenneri* strains. This finding also suggests that this temperature enhances motility and, consequently, the initial adhesion of bacteria cells to the substrate and the occurrence of homogeneous properties of their exopolysaccharides. Similarly, Gram-positive thermophilic *Bacillus licheniformis* SDP15 and mesophilic *Staphylococcus pasteurii* RE1.9, along with Gram-negative mesophilic *Raoultella terrigena* E7.12 showed a relatively high biofilm-forming ability on the same substratum. This capacity of the mentioned milk-contaminant bacteria has been well described in other

investigations. For example, several strains of *B. licheniformis* have been reported as robust biofilm-formers, particularly when isolated from dairy production environments<sup>41</sup>. In the same context, milk isolates of coagulase-negative staphylococci, which include *S. pasteurii*, possess key factors, mainly polysaccharide intercellular adhesin (poly-N-acetylglucosamine), that allow this genus to form robust biofilms on multiple surfaces<sup>42-44</sup>. Moreover, in the present investigation, it was observed that biofilm-forming ability was highly variant, even among strains of the same species. For instance, the OD<sub>492nm</sub> of *P. brenneri* C10.34 was approx. 4 times higher than that of *P. brenneri* C7.29. In like manner, *Pseudomonas lurida* C2.2 displayed a more robust biofilm-forming capacity (OD<sub>492nm</sub> 0.513 ± 0.1) than that of *Pseudomonas lurida* C4.18 (OD<sub>492nm</sub> 0.198 ± 0.005). This phenomenon has also been observed in different studies, suggesting that the ability to form biofilms is strain-dependent and closely tied to the adaptive potential to new environmental conditions<sup>39,45</sup>. This intra-species variability relies on multiple aspects like the adhesion ability, related to cell surface hydrophobicity and the development of appendages (pili, curli, fimbriae, flagella)<sup>46</sup>. Factors determining biofilm maturation (e.g., type and concentration of produced EPS plus transport mechanisms through the cell membrane) are also associated with strain-dependent biofilm-forming capacity<sup>47</sup>.

On the other hand, various strategies have been explored in the food industry to control biofilm formation, encompassing different bacterial species or strains. Among these strategies, using MgCl<sub>2</sub> as an inhibiting agent stands out as a potential alternative. Despite the crucial role those divalent cations like Mg<sup>2+</sup> and Ca<sup>2+</sup> play in the physiology of bacterial cells, an excessive amount is detrimental<sup>48</sup>. It has been demonstrated that these ions can influence the electrostatic interactions and surface adhesion processes, dependent on bacterial physiology, by acting as cellular cations and enzymatic cofactors<sup>49</sup>. Furthermore, it has also been demonstrated that Mg<sup>2+</sup> ions affect biofilm formation by negatively regulating the expression of extracellular matrix genes<sup>50</sup>. Thus, in the present study, we assessed the effect of 5 mM MgCl<sub>2</sub> on the bacterial isolate's biofilm-forming capacity on 96-well polystyrene plates. This treatment repressed the biofilm formation (OD<sub>492nm</sub> < CP) of *Pseudomonas brenneri* C10.34, *Pseudomonas fragi* group E4.7, and *Enterococcus durans* E1.4. Furthermore, in response to 5mM MgCl<sub>2</sub> exposure, the OD<sub>492nm</sub> associated with the adhered biomass of 13 out of 23 remaining strains was significantly reduced (P < 0.05), although their biofilm-forming ability was not inhibited. Between the abovementioned isolates, the OD<sub>492nm</sub> of *Bacillus licheniformis* SDP.15, *Pantoea ananatis* P.40, *Staphylococcus pasteurii* RE1.9, *Pseudomonas lurida* C2.2, *Pseudomonas brenneri* C10.34, and *Enterococcus durans* E1.4 (ascendingly ordered), was reduced by approx.1.5 to 6 times when exposed to the inhibiting compound. Consistent with our outcomes, Mulcahy and Lewenza<sup>51</sup> have reported that Mg<sup>2+</sup> limitation triggers the biofilm formation of *Pseudomonas aeruginosa* strains, which is closely related to the *Pseudomonas* species included in our investigation. Similarly, it has been reported that the biofilm biomass of *Enterobacter cloacae* BF-17 was significantly reduced as a response to 0.5 – 256 mM MgCl<sub>2</sub>.6H<sub>2</sub>O exposure<sup>52</sup>. Moreover, concerning the effect of this compound on *Bacillus* spp. biofilm formation, variable results have been described. For instance, Ben-Ishay et. al.<sup>30</sup> observed that MgCl<sub>2</sub>, in concentrations equal to or higher than 5 mM, is detrimental to biofilm formation of *Bacillus* spp. strains, while Oknin, Steinberg and Shemesh<sup>50</sup> suggest that this effect only occurs on *Bacillus subtilis* when exposed to at least 25 mM MgCl<sub>2</sub>. This may also explain that in contrast to *Bacillus licheniformis* SDP.15, biofilms of *Bacillus sonorensis* C4.13 were not significantly altered during this treatment. Along with the latter, in this study biofilm formation ability was not changed for the other nine strains, including *Pseudomonas fragi* group C6.28 and E2.5, *Pseudomonas lactis* D1.11, *Enterobacter hormaechei* E5.8 and C1.1, *Enterococcus durans* E12.33, and *Staphylococcus epidermidis* E3.6. This suggests that 5 mM MgCl<sub>2</sub> might not be effective regarding biofilm inhibition for the latter strains. In this

connection, it has been proposed that 16 mM  $Mg^{2+}$  stimulates biofilm formation on plastic surfaces instead of inhibiting<sup>53</sup>. In addition, based on the results as mentioned earlier, it was noted that the strains evaluated in the present investigation, even belonging to the same species, responded variably to 5 mM  $MgCl_2$  exposure. Consistent with this, other studies suggest that the biofilm-inhibiting effect of  $Mg^{2+}$  may also be a concentration, species, or strain-dependent property<sup>54</sup>. Based on this, in further studies, the response of our isolates to  $MgCl_2$  in a concentration range from 5 to 50 mM shall be evaluated to determine the most efficient value in terms of the biofilm inhibitory capacity of this compound.

Moreover, it was also observed that all bacterial isolates could grow in milk, reaching concentrations exceeding 7 log CFU/mL in a planktonic state. Nevertheless, robust growth in this matrix did not necessarily lead to a high concentration of cells adhered to SS plates. For example, *Pseudomonas fragi* group E4.7, *Enterococcus durans* E1.4, and *Bacillus licheniformis* SDP. 15 reached a concentration lower than 7 log CFU/cm<sup>2</sup> adhered to SS, whereas *Pseudomonas lactis* D1.11, *Lacticaseibacillus paracasei* subsp. *tolerans* P2.20 and *Bacillus sonorensis* C4.13 were the strains that exhibited the highest concentrations (> 8 logs CFU/cm<sup>2</sup>) of attached cells on the same surface. In this connection, it has been settled that biofilm formation, in the presence of milk, results from the interaction of several abiotic and biotic factors. These involve the proportion of metal ions in surface composition (Ni, Fe, Mn, among others), its hydrophobicity, a solution pH close to the isoelectric point of bacteria, and electrostatic attraction between cells and the metal surfaces<sup>55,56</sup>. Additionally, raw milk proteins may reduce the bacterial ability to form biofilms on SS<sup>57</sup>, whereas the occurrence of milk surfactant compounds and heat-denatured protein deposits stimulates this capacity<sup>59</sup>. At the same time, bacterial adhesion on SS is also a strain-dependent property and compromises the formation of S-layer, EPS production, and the presence of filamentous appendages<sup>56</sup>. In addition, it has been outlined that butyric acid in milk is a critical factor that triggers *B. licheniformis* biofilm formation<sup>58</sup> and that SS provides better support than polystyrene for biofilm development of this species<sup>18,60</sup>. Nevertheless, the present research results do not directly support that claim since *B. licheniformis* SDP. 15 exhibited a high biofilm-forming ability on polystyrene but was the less efficient biofilm-former strain on SS in contact with milk. This finding aligns with the outcomes of Parkar et al.<sup>61</sup> who observed that the adhesion of both spores and vegetative cells of *B. licheniformis* was reduced when SS plates were immersed in 1% and 10% skimmed milk. Furthermore, it has been demonstrated that the nature of this effect appears to vary depending on the organism, substrate, and milk proteins<sup>62</sup>

On the other hand, *Lacticaseibacillus paracasei* subsp. *tolerans* P2.20 was one of the weakest biofilm formers strains on polystyrene but the strongest on SS. Consistently, Dat et al.<sup>63</sup> have suggested that *Lacticaseibacillus paracasei* is a strong biofilm former on SS in the presence of milk. The precipitation of milk proteins might also stimulate this phenomenon due to bacterial fermentation and a pH decrease to 5.5<sup>63</sup>. In contrast, it has been demonstrated that UHT milk (whole, semi-skimmed, and skimmed) significantly alters the hydrophobicity of steel, regardless of contact time<sup>64,65</sup>. This alteration promotes the pre-adsorption of  $\alpha$ ,  $\beta$ , and  $\kappa$ -casein and results in a 20% reduction in *Staphylococcus aureus* adhesion compared to clean steel. It is presumed that this effect could be similar in other species of this genus<sup>62</sup>. Our results support those findings since *S. pasteurii* RE1.9 showed the fourth lowest count of biofilm cells on SS plates immersed in skimmed milk, while on polystyrene, it proved the most efficient.

As a proof-of-concept, biofilm-originated cells of all the mesophilic strains along with psychrotrophic *Flavobacterium plurextorum* C3.3, *Pseudomonas lurida* C2.2, and C4.18; when exposed to heat treatment (80 °C for 10 min), exhibited a significantly lower viability loss compared to their planktonic counterparts. As a function of several mechanisms, the heat-stress response of biofilms is generally more effective than that of free planktonic cells<sup>66,67</sup>. In the same context, the log reduction suffered by the sessile cells of the thermophilic isolates *Bacillus licheniformis* SDP.15 and *Bacillus sonorensis* C4.13, after heat treatment, was 1.98 and 87.5 times, respectively, lower than the viability loss of the corresponding bacteria in the planktonic state. Our results suggest that the heat resistance might be enhanced when bacteria of those strains are biofilm-originated, although a remarkable survival was expected due to their intrinsic capacity to produce heat-resistant spores. In this connection, Simmonds et al.<sup>68</sup> demonstrated that spores of *B. licheniformis*, *B. cereus*, and *Geobacillus stearothermophilus* adhered to SS were significantly more resistant ( $P < 0.05$ ) to high-temperature exposure (90°C, 95°C, 100°C, and 110°C) than non-biofilm-originated spores. Similarly, Huang, Flint and Palmer<sup>33</sup>, have described that the  $D_{90^\circ\text{C}}$  values of biofilm spores are significantly extended by at least 6 minutes compared to the  $D_{90^\circ\text{C}}$  corresponding to free-floating spores. This might occur due to additional protection against adverse environments provided by the polymeric matrix surrounding the endospores embedded in a biofilm<sup>41,69</sup>.

Surprisingly, an insignificant viability loss of biofilm-originated cells of *Staphylococcus pasteurii* RE1.9 and *S. epidermidis* E3.6 resulted from exposure to high temperature. In line with this, a study conducted by Alma-troudi et al.<sup>70</sup> reported that biofilm cells of *S. aureus* experienced a 2 log reduction when exposed to 121°C for 20 minutes, whereas planktonic cells suffered a viability loss higher than 8 log. This remarkable resistance might be attributed to the elevated proportion of anteiso-C19 fatty acids formerly described as the membrane of staphylococcal biofilm cells<sup>71</sup>. The high melting point of these molecules leads to a reduction in membrane fluidity and consequently enhances resistance to thermal stress<sup>71</sup>. In addition, according to a study conducted by Montanari et al.<sup>72</sup>, this great thermal resistance is present in other species of this genus, and thus, it may support our findings.

On the other hand, the concentration of thermally treated viable planktonic cells of *Pseudomonas fragi* group E11.31, C6.28, along with *Pseudomonas lactis* E6.10 and D1.11, remained close to 4 log CFU/mL. In contrast, biofilm cells suffered a loss of viability below the assay's detection limit after heat treatment. In this connection, most strains within this group exhibited an initial concentration of sessile cells close to 7 log CFU/mL, whereas planktonic cells exceeded 8 log CFU/mL. This observation suggests that the higher initial concentration of planktonic cells influenced the resulting survival above the assay's detection limit despite biofilm cells' commonly associated heat resistance. Moreover, it should be considered that biofilm cells of *Pseudomonas lurida* C4.18 and C2.2, closely related to the above strains, suffered an average cell death of approximately 4 log CFU/mL from an initial concentration of  $8.20 \pm 0.17$  log CFU/mL and  $8.39 \pm 0.08$  log CFU/mL, respectively. In a hypothetical scenario in which sessile cells of *Pseudomonas fragi* group E11.31 and C6.28 plus *Pseudomonas lactis* E6.10, with initial concentrations close to 7 logs CFU/mL, suffered the same log reduction, a non-detectable survival might be observed as it resulted in the present study. This notable observation might be reinforced by the pattern observed for *Pseudomonas fragi* group E2.5 and *Pseudomonas brenneri* C7.29, whose initial populations of both cell types were approx. 7 log CFU/mL and, consistently, no survival was detected. In line with this, it has been observed that high population density might be associated with enhanced cell heat resistance, owing to the accumulation of protective compounds against thermal stress (e.g., trehalose and catalase), in addition to the synthesis of heat shock proteins<sup>73,74</sup>. Therefore, it is logical to expect that cells

with lower concentrations would be more susceptible to adverse conditions, thus supporting the findings of this study.

On the other hand, *Enterococcus durans* E1.4 exhibited a significantly higher initial concentration of planktonic cells ( $8.03 \pm 0.10$  log CFU/mL) than the concentration of its counterparts recovered from biofilms ( $6.88 \pm 0.09$  log CFU/mL). It is noteworthy that, despite biofilm cells' relatively low initial cell density, a survival of at least 3 log CFU/mL was detected after thermal treatment. However, this value was lower when compared to that of free-living cells. Overall, it has been widely described that *Enterococcus* species possess high thermal resistance attributed to genetic adaptations that stabilize cellular structures or may even repair the damage caused by heat<sup>75,76</sup>. The characteristic peptidoglycan-rich cell wall of these Gram-positive bacteria provides greater rigidity and protection<sup>77</sup>. Therefore, it is well known that this type of bacteria may exhibit more excellent resistance compared to Gram-negative species. These advantages enable them to maintain cell viability throughout thermal treatments of up to 80°C, consistent with the outcomes observed in the present investigation.

In contrast, *E. durans* E12.33, classified in this study as a mesophilic growth strain, showed a remarkable heat resistance since they suffered a viability loss corresponding to  $3.63 \pm 0.07$  and  $1.18 \pm 0.03$  log CFU/mL for planktonic and sessile cells, respectively, after an initial concentration close to 9 log CFU/mL of both cell states, was thermally treated. In this connection, our findings align with those reported by McAuley et al.<sup>76</sup>, who previously highlighted the significant variability regarding heat resistance among enterococci strains. This variability could result from a complex interplay of factors, including regulating gene expression in the biosynthetic pathways of spermidine and putrescine<sup>75</sup>. These polyamines play a pivotal role in bacterial survival, as they possess the ability to stabilize DNA structure and uphold cellular membrane integrity under thermal stress conditions<sup>78</sup>.

In addition, 22 out of 31 analyzed strains exhibited proteolytic capacity, as evidenced by the formation of digestion halos around the inoculated wells in milk agar. Within this context, a notable variation in caseinolytic potential was observed among closely related strains. For example, two out of the five strains belonging to the *fragi* group of *Pseudomonas* were not capable of casein degradation. This discrepancy may be attributed to species diversity within this taxonomic group, which includes *P. fragi*, *P. lundensis*, *P. taetrolens*, and *P. psychrophila*<sup>79,80</sup>. Interestingly, this variability was also evident among strains of the same species, as demonstrated in the case of *P. lurida* C2.2, which exhibited significantly larger diameters of casein degradation halos than all other psychrotrophic isolates, including *P. lurida* C4.18. In line with this Caminero et al.<sup>81</sup>, have suggested that the type and proportion of proteases vary significantly among different taxonomic groups and strains. Consistently, Ribeiro Júnior et al.<sup>82</sup> reported the variability, in terms of proteolytic ability, of different *Pseudomonas* spp. isolates in bovine milk might be related to its capacity to produce the AprX metalloprotease, which catalyzes the hydrolysis of  $\alpha$ ,  $\beta$ , and  $\kappa$ -casein<sup>83</sup>. This enzyme is codified by the *aprX* gene, which, in the genome of *Pseudomonas* spp., is part of the *aprX-lipA* operon. In this connection, it has been reported that the operon structure is variable among species or strains of this genus<sup>84,85</sup> since this operon contains genes related to secretion mechanisms (*aprD*, *aprE*, *aprF*, *prtAB*), a protease inhibitor (*aprI*), and a lipase (*lipA*)<sup>84</sup>. Consequently, Maier et al.<sup>84</sup> demonstrated that the strain-specific proteolytic capacity relies on the genetic structure of the *aprX-lipA* operon. Besides these intrinsic traits, environmental parameters like temperature<sup>83</sup>, calcium, and iron content<sup>85-87</sup> influence the casein digestion ability<sup>88</sup>.



Furthermore, significant variability of the caseinolytic potential was observed among the *Enterococcus durans* strains evaluated in this study. At the end of the incubation period, only the two mesophilic growth isolates displayed hydrolysis zones on the culture medium, whereas the thermophilic strains exhibited no proteolytic ability. Within this context, it has been documented that the production of gelatinase (GelE), an extracellular zinc metalloprotease, and serine protease (SprE), which form part of the proteolytic system of enterococci strains isolated from dairy products, are not representative of all species of this genus<sup>89-93</sup>. The respective encoding genes, *gelE*, and *sprE*, are co-transcribed within the same operon<sup>92,94</sup>. Variability of the enzymatic activity may be attributed to the fact that these genes are only expressed when the enterococci population has reached high cell densities and are thus under the control of a regulatory *fsr* quorum sensing (QS) system consisting of three regulatory genes (*fsrA*, *fsrB*, and *fsrC*)<sup>91,94,95</sup>. In this regard, Pessione et al.<sup>92</sup> have suggested that modifying these regulatory genes might impair protease functionality. This could account for the absence of proteolytic activity.

In addition, it was evident that, on average, *Bacillus* spp. strains exhibited notably more giant degradation halos than those in the other two evaluated groups. Previously, it has been reported that the genus *Bacillus* can produce large amounts of enzymes such as alkaline serine proteases and zinc metalloproteases, which represent over 95% of their extracellular proteolytic activity<sup>96,97</sup>. Within this context, Mehta et al.<sup>98</sup> observed that the enzymes produced by this genus can cleave  $\beta$  and  $\kappa$ -casein. Likewise, previous studies have documented that the proteolytic potential of *B. licheniformis* might vary as a result of the incubation temperature<sup>100</sup>. In this connection, and consistent with our results, Sayem, Alam and Hoq<sup>101</sup> reported an optimal temperature range between 37°C and 50°C for the activity of *B. licheniformis* proteases, and consequently, extensive casein hydrolysis zones were observed when cultivated in skim milk agar medium (SMA).

Moreover, it has been outlined that most heat-resistant proteases related to UHT milk spoilage are represented by the AprX metalloprotease produced by species belonging to *Pseudomonas* spp.<sup>25,102</sup>. Nevertheless, in this research, only the CFS, heat-treated at 92°C, of *Pseudomonas lactis* E6.10 could form casein hydrolysis zones when inoculated into plate count agar supplemented with skim milk 10%. Although the *aprX* gene is widespread among *Pseudomonas* spp., its nucleotidic sequence shows variability within different strains of this genus<sup>28</sup>. This affects the flexibility of the protein's three-dimensional structure and, consequently, its ability to accurately refold after exposure to high temperatures<sup>103</sup>. This aspect and the *aprX* gene's expression level could elucidate why heat-resistant proteases were not observed for *P. lactis* D1.11 or the rest of the *Pseudomonas* spp. isolates evaluated in this study. As previously mentioned, this residual enzymatic activity is one of the primary factors contributing to the deterioration of UHT dairy products<sup>104-106</sup>. Therefore, in future research, it would be pertinent to extract and characterize this protease and its thermo-stability at 135°C to determine its potential impact during the storage of the final product, which shall be assessed.

Although the heat resistance of proteases was demonstrated only for those produced by *Pseudomonas lactis* E6.10, when bacterial strains were individually inoculated into milk 48 h before its sterilization, casein coagulation, associated with bacterial metabolism, was observed in 17 out of 22 sterile milk samples throughout 45 days of storage. This outcome might be linked to a pH decrease in milk due to the production of bacterial metabolites, mainly organic acids<sup>107</sup>. Previous investigations have highlighted that casein reaches its isoelectric point at a pH of 4.6 leading to increased compaction of its micellar structure<sup>108,109</sup>. Therefore, our findings suggest that casein coagulation, observed in this assay, and syneresis were likely caused by the bacterial metabolism before the sterilization process rather than protease activity.



Milk-spoilage-related microorganisms represent critical challenges for the food industry. In our study, a remarkable biofilm-forming capacity on both polystyrene and SS surfaces, as well as heat resistance of sessile cells and proteolytic ability, was exhibited by several strains. This includes isolates like *S. pasteurii* RE1.9, *S. epidermidis* E3.6, *E. durans* E12.33, *Lacticaseibacillus paracasei* subsp. *tolerans* P2.20, *B. sonorensis* C4.13, *B. licheniformis* SDP.15, *P. lurida* C2.2, and *Raoultella terrigena* E7.12. In this connection, the presence of bacteria, showing such spoilage potential, in a UHT milk production plant represents a considerable hazard that could affect the quality and safety of dairy products and the operational efficiency of related industries. Likewise, the control of thermostable protease production, such as those secreted by *P. lactis* E6.10, should be further studied since they also challenge the stability of the commercial product during its shelf life.

On the other hand, the properties exhibited by *Lacticaseibacillus paracasei* subsp. *tolerans* P2.20, *Lactococcus lactis* D.24, and *Bacillus licheniformis* SDP.15 shall be considered within the investigation of their putative biotechnological applications. This may involve their probiotic potential, the acidification rate of lactic acid bacteria strains during milk fermentation, and the production of industrial-relevant metabolites, among others.

This research provides a comprehensive insight into the variability of biofilm-forming capacity, caseinolytic activity, and heat resistance among bacterial strains isolated from the production chain of UHT milk. Thus, these findings may contribute to developing strategies to enhance the efficiency of processes like milk collection and transportation, thermal treatment, packaging, and industrial storage. We emphasize the need for pilot studies to confirm the applicability of our findings in practical settings. Additionally, it is essential to conduct further research to elucidate the underlying mechanisms of microbial spoilage. Finally, developing and assessing new control strategies for managing spoilage microorganisms is necessary. These integrated approaches are crucial for enhancing the dairy industry's safety, integrity, and long-term sustainability.

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## CONCLUSION

This research suggests that the milk spoilage potential of bacteria could be strain-dependent, showing significant variations even between different strains of the same species. Among the 31 evaluated isolates, 26 could form biofilms on polystyrene. In this connection, *Pseudomonas brenneri* C10.34 was the strongest psychrotrophic biofilm-former on polystyrene surfaces, whereas, on the same substratum, *Staphylococcus pasteurii* RE1.9 and *Bacillus licheniformis* SDP.15 were the most efficient biofilm-producers among mesophilic and thermophilic strains, respectively. Moreover, it was observed that a concentration of 5 mM MgCl<sub>2</sub> significantly reduced this capacity for 16 strains, suggesting that the utilization of this compound, after further investigation of adequate concentrations, could be efficient in terms of biofilm control. However, the biofilm biomass of isolates like *Lacticaseibacillus paracasei* subsp. *tolerans* P2.20, among others, were not affected when exposed to 5 mM MgCl<sub>2</sub>, although it did not behave as a strong biofilm-former on polystyrene. The latter mesophilic bacterium, the psychrotrophic isolate *P. lactis* D1.11, and the thermophilic culture of *B. sonorensis* C4.13 exhibited the highest capacity of biofilm development on SS in each of the evaluated groups. On the other hand, when exposed to high temperatures, the sessile cells of 17 strains were substantially more resistant than their planktonic counterparts.

Interestingly, among the psychrotrophic isolates, only cells recovered from biofilms of *Flavobacterium pluxextorum* C3.3, *P. lurida* C2.2, and *P. lurida* C4.18 maintained their viability after the heat treatment. Similarly, although the heat-resistance of bacterial endospores has been widely described, biofilm-born cells of the spore-forming strains *B. licheniformis* SDP.15 and *B. sonorensis* C4.13 were significantly more resistant to high temperatures than their free cells equivalents. However, the biofilm cells of *S. pasteurii* RE1.9 and *S. epidermidis* E3.6 proved to be the most heat-resistant of the entire experiment.

Moreover, although 22 of the assessed strains secreted extracellular proteases, only those produced by *P. lactis* E6.10 maintained their enzymatic activity after being treated at 92°C. This research highlights the critical need for optimized strategies involving the control of bacterial contamination during UHT milk production based on the determination of strain-dependent unique characteristics in terms of their spoilage potential. This approach would mitigate the risks associated with biofilm-forming, heat-resistant, and proteolytic bacteria and could ensure that processes in a dairy production plant remain robust and efficient. Moreover, the observed properties of some of the evaluated strains should be considered for further studies related to the potential biotechnological applications of these microorganisms or their metabolites.

**Supplementary Material:** Accession numbers of the 16S rDNA sequences of the evaluated strains are stated in the supplementary material.

**Author Contributions:** Erika Molina: Writing – original draft; Investigation; Writing – review & editing; Formal analysis; Data curation; Methodology. Romina Camacho: Methodology. Daniela Mejía: Methodology. Martín Marcial-Coba: Conceptualization; Funding acquisition; Writing – review & editing; Investigation; Supervision; Methodology; Formal analysis.

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