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Microbial persistence in pasteurized milk: Biocontrol and heat treatment optimization

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Abstract

Background and Objective: Increasing interests in healthy nutrition stimulate use of lactic acid bacteria with functional characteristics in production of dairy products. Lactic acid bacteria are included in the composition of starters for cheese making as well as other dairy products. In addition to their general health benefits, they show pronounced antimicrobial activities, partly due to their metabolite complexes. The primary method of ensuring milk safety is heat treatment. Pasteurization at 72°C ±2 is commonly used in cheese production. Nevertheless, the survival of viable *Pseudomonas* (*P.*) *aeruginosa* cells, capable of growing at refrigeration temperatures, may lead to spoilage during storage.

Material and Methods: Antagonistic activity of industrial *Lactobacillus* (*L.*) *helveticus*, against *P. aeruginosa* strains as well as the effectiveness of pasteurization at 72 °C ±2 (common in cheese production) was assessed. *L. helveticus* demonstrated high antagonistic activity against the reference collection strain of *P. aeruginosa* ATCC 25668 and the wild-type isolate *P. aeruginosa* 47, verifying its potential as a biological control agent.

Results and Conclusion: Limitations of standard pasteurization have been identified. During the heat treatment of sterile milk contaminated with the wild-type strain *P. aeruginosa* 47, 1.9×10^4 CFU ml^{-1} of viable cells survived after a 20-s exposure, while only 1.8×10^1 CFU ml^{-1} survived after a 40-60 s exposure. Cell growth was observed at up to 3.5×10^6 CFU ml^{-1} during 7 d of storage. Microscopy revealed morphological changes in the cells (elongation and filamentous structures), indicating adaptive mechanisms. Similar results were detected for the reference collection strain of *P. aeruginosa* ATCC 25668. A 15-min pasteurization time at 72°C ±2 was effective against the two strains. The study suggests an integrated approach to ensuring the safety of dairy products by combining biological control with the optimization of heat treatment regimes.

Keywords: Microbial persistence, Heat treatment, Lactic acid bacteria, *Pseudomonas aeruginosa*

What is "already known" on this topic:	➢ LAB are known for production of functional dairy products. ➢ LAB are usually included with starter culture for cheese making ➢ Antimicrobial activity is observed due to LAB metabolites. ➢ Pasteurization at (72±2) °C is commonly used in cheese production.
What this article adds:	➢ The limitations of standard pasteurization have been identified. ➢ *P. aeruginosa* 47 survived after a 20-second exposure ➢ Survival reduced by enhancing exposure time. ➢ A 15-minute pasteurization at (72±2) °C was effective against both strains. ➢ Combining biological control with the optimization of heat treatment is an integrated approach to ensuring the safety of dairy products ➢ Survival of *P. aeruginosa* may lead to spoilage during refrigerator storage.

1. Introduction

The increasing interest in healthy nutrition stimulates use of lactic acid bacteria (LAB) with functional characteristics in production of dairy products. Metabolites from these cultures show pronounced antimicrobial activity in addition to their general health benefits. The LAB and their metabolic complexes may be promising agents for the study [1, 2]. Throughout their life cycle, LAB can produce a range of antimicrobial compounds, including organic acids (lactic, acetic, propionic and butyric acids), bacteriocins, hydrogen peroxide and bacteriocin-like inhibitory substances [3]. Their use can be considered as a natural strategy of pathogen control, including *P. aeruginosa*, and a basis for the development of innovative technologies in various industries [4, 5]. Moreover, the antagonistic activity of LAB against pathogen growth varies within species and strains. This antimicrobial effect results from the synergistic action of various metabolites [2, 3]. The primary method of ensuring milk safety is heat treatment. Pasteurization at 72 °C ±2 is commonly used in cheese production. Nevertheless, survival of viable *P. aeruginosa* cells, capable of growing at refrigeration temperatures, may lead to spoilage during storage. The LAB with strong antagonistic characteristics against *P. aeruginosa* can serve as additional barriers to ensuring the product quality and safety, when used as starter cultures.

Raw milk delivered to dairy plants is frequently contaminated with psychrotrophic bacteria. Within psychrotrophic microorganisms, *P. aeruginosa* is a Gram-negative pathogen with high spoilage potentials, significant resistance to antimicrobial agents and capacity to form biofilms [6]. Moreover, increase of multidrug-resistant strains due to the improper and excessive use of antibiotics increases morbidity and mortality within immuno-compromised patients. The *P. aeruginosa* is included in the top three causes of opportunistic infections in humans, affecting more than 2 million patients and causing approximately 90,000 deaths annually [7]. Despite being often underestimated as a foodborne pathogen, *P. aeruginosa* plays an increasingly significant role in contaminating industrial equipment and finished dairy products [8]. Moreover, *P. aeruginosa* can quickly become resistant to various stress factors, including antimicrobial agents and conventional disinfectants, which increases concerns about the potential transmission of resistant strains through the food chain [9,10]. The spoilage of products by *P. aeruginosa* poses a serious concern for consumers and food safety regulators. Milk is an excellent medium for the growth of various microorganisms. The psychrotrophic *P. aeruginosa* can continuously increase its population in milk at refrigeration temperatures (4–6 °C). Its short generation time—less than 4 h—enables it to reach cell counts exceeding 10^6 CFU ml^{-1} in milk after 8 d of storage [11]. The count of psychrotrophic bacteria in raw milk delivered to dairy plants reflects the effectiveness of sanitary and hygienic measures on the farm. Contamination of the dairy plant with *P. aeruginosa* lessens adequate sanitation. This is due to the *P. aeruginosa* ability to form biofilms actively and rapidly acquire resistance to disinfectants. Moreover, strains with various susceptibility to antimicrobial agents, including potentially resistant ones, may co-exist within a single facility [12]. Based on the International Dairy Federation's recommendations, milk should be cooled to 4–10 °C within 1.5–3 h. In some countries, milk is delivered to plants only once a day, which may promote bacterial growth due to *P. aeruginosa* ability to multiply under a wide temperature range. Regarding its multidrug resistance and high adaptability, there is an urgent need to develop effective strategies to control *P. aeruginosa* contamination in the food industry.

Currently, the primary most effective method for eliminating pathogenic and spoilage-causing bacteria in milk is heat treatment. It is used in production of almost all dairy products [13]. Milk pasteurization is achieved by heating it to a temperature less than 100 °C for a time sufficient to eliminate pathogenic bacteria that may present. There are various pasteurization regimes and the choice depends on the type of product. For example, pasteurization at 72 °C ±2 is widely used for cheesemaking. Low-temperature pasteurization was previously assumed sufficient to prevent transmission of *P. aeruginosa* through dairy products. This assumption was based on the premise that non-spore-forming pathogenic bacteria should be eliminated at temperatures greater than 60 °C due to the denaturation of their essential cellular proteins [14,15]. However, recent studies have reported presence of heat-resistant bacteria that can survive standard pasteurization regimes [16]. Studies on the heat resistance of pathogens such as *Listeria monocytogenes* have shown that short-time pasteurization of milk at 71.7 °C for 15 s is insufficient [17]. Cells may be able to survive even after exposure to 72 °C for up to 4 min [17]. Frequency of *P. aeruginosa* detection in milk after pasteurization can relatively be high [18]. In the Czech Republic, a study of pasteurized milk samples for a year detected that 4% of the samples included *P. aeruginosa*. The effectiveness of pasteurization depends on the initial microbiological contamination of milk and the strain composition of the microbial contaminants [19]. The heat resistance of microorganisms varies widely and depends on factors that affect them before, during and after heat treatment [20].

The mechanisms enabling microorganisms to adapt to high temperatures are not fully understood. Scientific evidence suggests that the cellular response to heat shock involves synthesis of various proteins known as heat shock proteins [21]. Heat shock disrupts folding of existing and newly synthesized proteins. Intermolecular interactions within misfolded proteins lead to the formation of aggregates and their number increases proportionally with the intensity of heat treatment [22]. Thus, loss of essential cellular proteins compromises cell viability and may ultimately result in cell death. To modify these processes, bacteria produce cellular disaggregases that solubilize protein aggregates, promoting their refolding and recovery; thereby, enhancing heat resistance. A stronger adaptive response at higher temperatures is associated with the accumulation of larger qiantities and/or induction of a distinct subset of heat shock proteins. Other heat protective mechanisms may exist [20].

Most studies have focused on clinical isolates. Analysis of wild-type isolates may reveal previously unknown characteristics of *P. aeruginosa*, including enhanced heat resistance acquired under exposure to external stressors [23, 24]. This study included a reference strain of *P. aeruginosa* and a wild-type strain isolated from a dairy farm. The aim of this study and its novelty included investigation of the ability of *P. aeruginosa* cells to survive under pasteurization regimes used in cheese making, as well as their ability for reactivation during refrigerated storage. The study assessed potential use of industrial LAB strains as protective cultures within starter compositions, serving as additional antimicrobial barriers against *P. aeruginosa*.

2. Materials and Methods

2.1. *Pseudomonas aeruginosa* strains used in the study

The reference strain of *P. aeruginosa* ATCC 25668 was provided by the State Collection of Pathogenic Microorganisms and Cell Cultures "GCPM-Obolensk", Russia. The wild-type strain of *P. aeruginosa* 47 was isolated during microbiological monitoring of equipment surfaces at a private dairy farm. Identification was carried out according to GOST ISO 16266-2018, "Water Quality. Detection and Enumeration of *P. aeruginosa*. Membrane Filtration Method." Nutrient broth and dehydrated media (nutrient broth, dehydrated nutrient agar) from NPC LLC "Biocompas-S," as well as pseudomonas agar from Himedia, India, were used to achieve 24-h cultures and to inoculate. Compared to the reference strain of *P. aeruginosa* ATCC 25668, the isolate of *P. aeruginosa* 47 produced further pigments on nutrient and pseudomonas agars. This trait is generally addressed as an indicator of higher virulence [6]. A bacterial suspension was prepared from a 24-h culture in physiological saline, adjusted to a cell count of approximately 1.5×10^8 CFU ml^{-1}, corresponding to an optical density of 0.5 of the McFarland. Experiments were carried out using sterile milk of the "Standard" brand (Complimilk, Belarus). Strains from the probiotic and lactic acid bacterial collection of the All-Russian Dairy Research Institute (FGANU "VNIIMI") were used to assess antagonistic activity of the industrially promising LAB against *P. aeruginosa* 25668 and *P. aeruginosa* 47.

2.2. Lactic acid bacterial strains used in the study

The LAB strains were stored in lyophilized form at -50 °C ±1. Lyophilized cultures were reconstituted in sterile skim milk by incubation for 16 h. Strains were incubated at 37 °C ±1 (*L. helveticus*, *Lacticaseibacillus rhamnosus* and *Streptococcus thermophilus*) or at 30 °C ±1 (*L. lactis*).

2.3. Study of antagonistic activity using co-cultivation method

In general, *P. aeruginosa* and LAB cultures were prepared as described in Sections 2.1 and 2.2. Antimicrobial activity was assessed by co-cultivation of LAB with *P. aeruginosa* strains. Briefly, 1 ml of inocula from the selected LAB strains and *P. aeruginosa* strains was added to 20 ml of sterile skim milk and incubated at 37 °C ±1 for 48 h. Milk samples inoculated with 1 ml of the *P. aeruginosa* strain served as controls. After incubation, serial 10-fold dilutions were prepared from each sample and *P. aeruginosa* counts were reported by plating on cetrimide agar (Himedia, India), a selective medium for pseudomonads. Plates were incubated at 37 °C ±1 for 24 h; then, colony-forming units (CFU) were counted. The growth inhibition of *P. aeruginosa* was calculated regarding control sample (monoculture), which was reported 100%.

2.4 Study of the effectiveness of heat treatment against *Pseudomonas aeruginosa*

To assess effectiveness of the heat treatment against *P. aeruginosa*, sterile milk in test tubes was inoculated with a bacterial suspension of the *P. aeruginosa* strains to reach a final cell count of approximately $n \times 10^5$ CFU ml^{-1}. The cell count was verified by plating aliquots from serial dilutions of the sample. The contaminated milk was processed using UKT-150 heat resistance control device, which simulated a pasteurizer, providing 99.99% efficiency equivalent to standard pasteurization (Figure 1) [25].

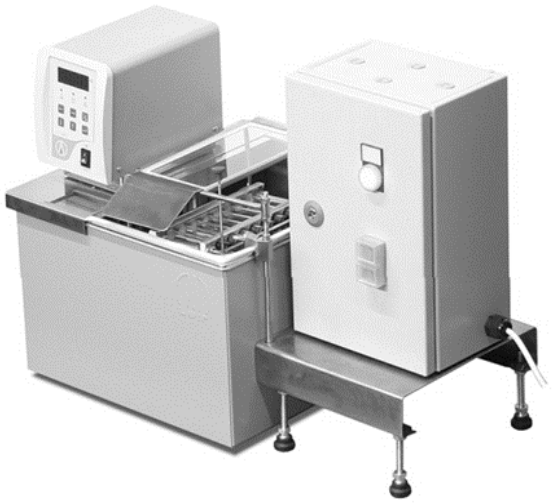


Figure 1. Heat resistance control device UKT-150

After system activation, a rack with sealed, heat-resistant test tubes containing contaminated milk was transferred into a silicone solution. The oscillatory movements of the device contributed to the uniform heating of samples within the test tubes. The pasteurization regime (72 °C ±2), with a typical exposure time of 20 s for cheesemaking, was used with various exposure times of 40 s, 60 s, 5 min, 10 min and 15 min. Temperature control was carried out using control test tube with milk and thermometer to monitor the time needed to reach the target temperature. Upon completion of heat treatment, test tubes were rapidly cooled down using ice bath. The heat-treated and cooled contaminated milk was plated onto solid nutrient media and incubated at 37 °C ±1 for 24-48 h. Results were recored based on colony counts. To simulate shelf-life and storage conditions of the pasteurized milk, the experimental test tubes were stored in a refrigerator at 6 °C ±2 for up to 14 d. To assess the potential recovery of heat-stressed *P. aeruginosa* cells, samples were removed from the experimental test tubes after 7 and 14 d of storage, followed by plating and colony enumeration. Microscopic analysis was carried out to detect potential phenotypic changes in the cells.

2.5. Statistics

The MS Office Excel 2016 was used for data analysis and graph construction. Experiments were carried out in three independent replicates and results were expressed as mean ±SD (standard deviation). Differences were considered statistically significant at $p \leq 0.05$ using two-tailed Student's t-test followed by Tukey's HSD test (for multiple group comparisons). The study was carried out using equipment from the Collaborative Center of the All-Russian Dairy Research Institute (CKP VNIMI).

3. Results and Discussion

3.1. Study of the antagonistic activity of LAB against *P. aeruginosa* using co-cultivation method

Results of the study on the antagonistic activity of industrial LAB strains are present in Table 1. As shown in Table 1, *L. helveticus* Bbn4 and *L. helveticus* NK1 were most effective in growth inhibition of the reference strain of *P. aeruginosa* and the wild-type isolate. After 48 h of co-cultivation, growth inhibition reached approximately 70%. *Streptococcus thermophilus* 16t effectively inhibited growth of the reference strain of *P. aeruginosa* 25668 but showed a limited activity against the wild-type isolate (growth inhibition of 24%). The antagonistic activity of *L. rhamnosus* F was less than that of *L. helveticus* strains, with growth inhibition reaching approximately 50%. In contrast, *Lactococcus* strains showed negligible or no inhibitory effects. These results were similar to those of previous studies reporting strong antagonistic activity of *L. helveticus* strains, including that against *P. aeruginosa* [26, 27]. Inhibitory effect of *L. helveticus* Bbn4 might be attributed to the synthesis of antimicrobial compounds, primarily organic acids, which effectively inhibit growth of pathogenic bacteria [28, 29]. It is known that *L. helveticus* strains are active acid producers [29, 30]. Additionally, *L. helveticus* strains can synthesize bacteriocins that inhibit the growth of pathogens, including *P. aeruginosa* [26]. The potential presence of antimicrobial compound-encoding genes in metagenome of *L. helveticus* Bbn4 warrants further investigations. The strains of *L. helveticus* Bbn4 and *L. helveticus* NK1 show potentials for use in production of fermented dairy products to decrease risks of *P. aeruginosa* contamination. However, further studies into the mechanisms of their antimicrobial action are necessary.

Table 1. Growth inhibition of *Pseudomonas aeruginosa* by lactic acid bacterial strains after 48 h of co-cultivation

Strain	Isolation source	Growth inhibition (%) *P. aeruginosa* 25668	Growth inhibition (%) *P. aeruginosa* 47
1. *Lactobacillus helveticus* Bbn4	NFDP	84±9	73^{b}±7
2. *Streptococcus thermophilus* 16t	Naturally fermented dairy product	68^{a}±6	24±2
3. *Lactobacillus helveticus* NK1	Human feces	58^{a}±6	70^{b}±4
4.*Lacticaseibacillus rhamnosus* F	Human feces	43±4	54±4
5. *Lactococcus lact*is 55	Naturally fermented dairy product	10±1	8±1
6. *Lactococcus lactis* 9078	Naturally fermented dairy product	no inhibition	no inhibition
7. *Lactococcus lactis* 711	Naturally fermented dairy product	no inhibition	no inhibition

[a-b] Statistically significant differences ($p \leq 0.05$).

3.2. Study on the effectiveness of milk pasteurization at 72 °C ±2

Results of the study on the effectiveness of milk pasteurization at 72 °C ±2 with exposure times of 20, 40 and 60 s for samples contaminated with *P. aeruginosa* 47 are present in Figures 2a,b.

As shown in Figure 2a, viable cells forming colonies on nutrient agar were detected in all samples pasteurized at 72 °C ±2, regardless of exposure time. The shortest exposure time (20 s) was the least effective for *P. aeruginosa* 47 elimination, as the cell count decreased by only one order of magnitude from 1.8×10^5 to 1.9×10^4 CFU ml^{-1}. After 7 d of refrigerated storage (Figure 2b), cell counts increased to 3.5×10^6 CFU ml^{-1}, with a slight increase to 5.4×10^6 CFU ml^{-1} on Day 14. The cell count of *P. aeruginosa* 47 decreased by four orders of magnitude, reaching 1.8×10^1 CFU ml^{-1} following exposure at 72 °C ±2 for 40 and 60 s. After 7 d of refrigerated storage, the cell counts increased to 3.0×10^6 and 5.7×10^5 CFU ml^{-1} in samples pasteurized for 40 and 60 s, respectively. Thus, none of the tested regimes resulted in the complete elimination of *P. aeruginosa* 47, with the 20-s pasteurization as the least effective. An increase in viable cell counts was observed in all samples after 7 d of refrigerated storage, exceeding the initial values, followed by a gradual stabilization on Day 14. Results of the study on the effectiveness of milk pasteurization at 72 °C ±2 with exposure times of 20, 40 and 60 s for samples contaminated with *P. aeruginosa* 25668 are present in Figures 3a,b.

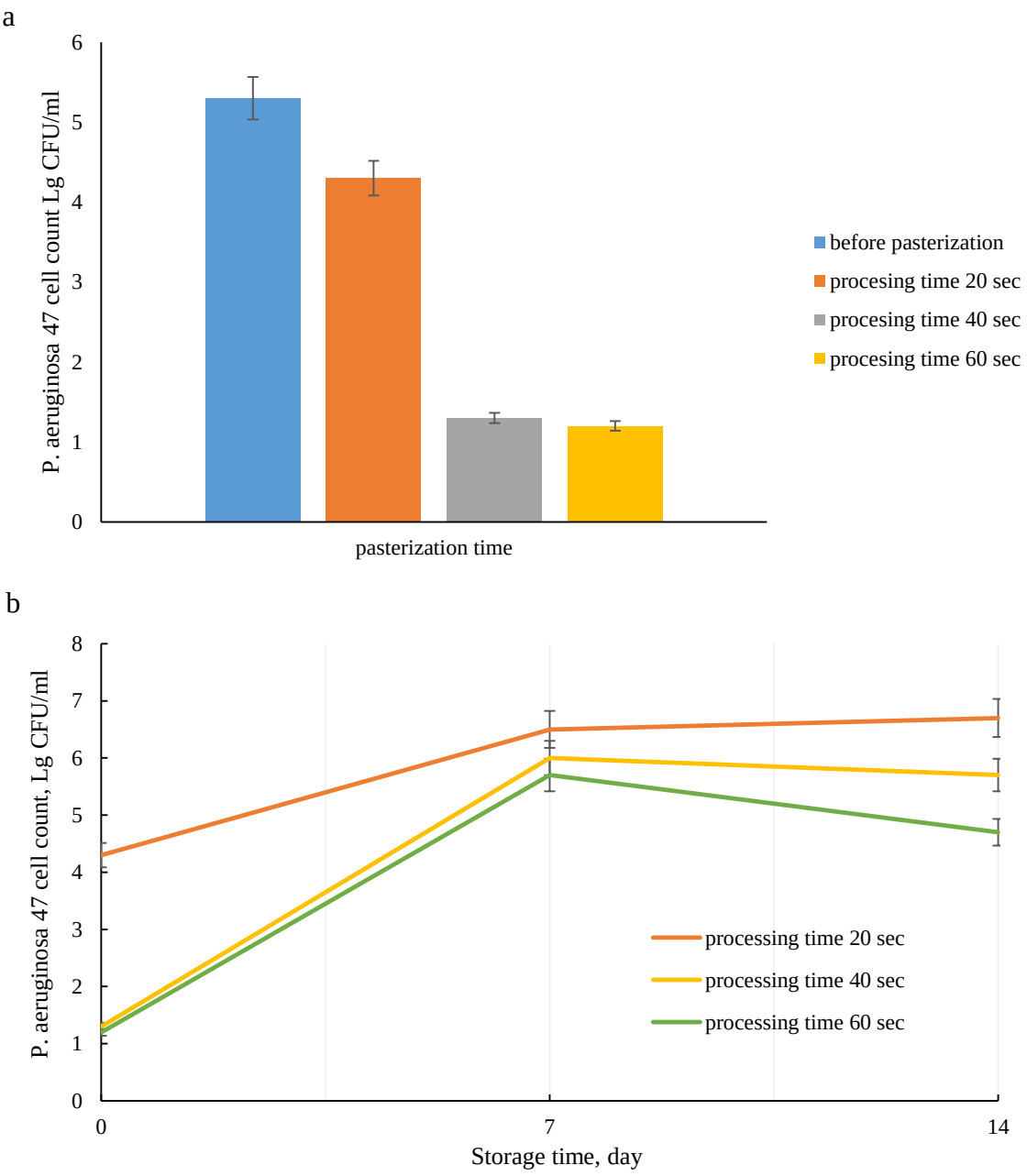


Figure 2. Changes in *P. aeruginosa* 47 cell count after pasteurization at 72 °C ±2 (a) and during storage (b)

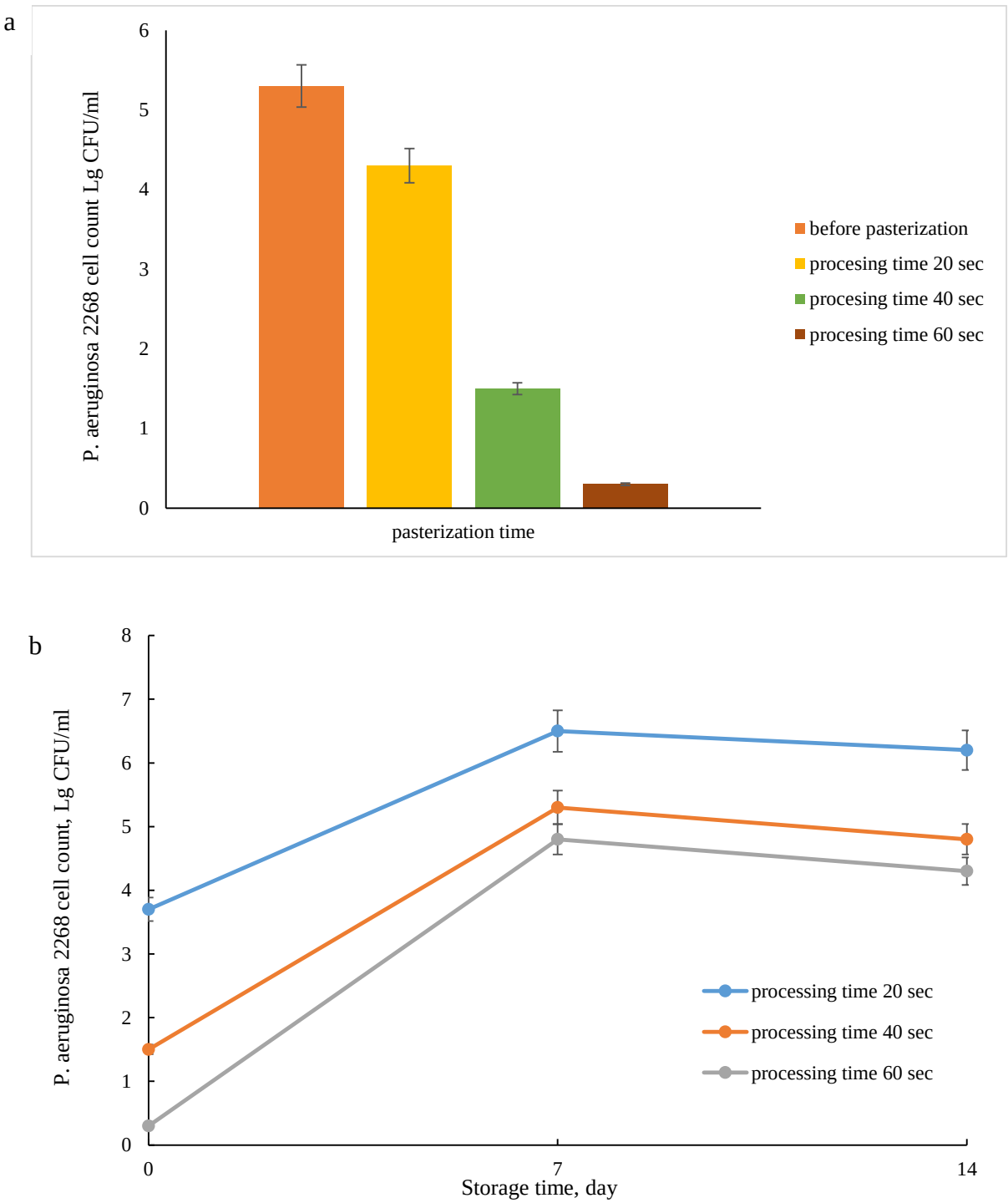
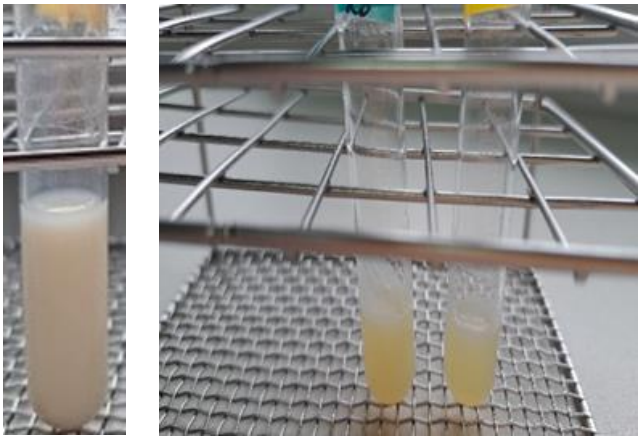


Figure 3. Changes in *Pseudomonas aeruginosa* 25668 cell count after pasteurization at 72 °C ±2 (a) and during storage (b)

For the reference strain of *P. aeruginosa* 25668, viable cells were detected immediately after all the pasteurization regimes were used. After 20 s of exposure, the cell count decreased by two orders of magnitude, from 1.9×10^5 to 5.3×10^3 CFU ml^{-1}. A 40-s pasteurization decreased the cell count by four orders of magnitude to 1.8×10^1 CFU ml^{-1}. After 60 s, only single colonies were observed (Figure 3a). On Day 7 of refrigerated storage (Figure 3b), cell counts increased to 3.3×10^6, 1.8×10^5 and 7.7×10^4 CFU ml^{-1} for 20, 40 and 60-s pasteurization regimes, respectively. A slight decrease was observed on Day 14, with cell counts reaching 1.7×10^6, 3.4×10^5 and 1.6×10^4 CFU ml^{-1} for similar pasteurization regimes. On Day 14, visible spoilage was seen in the test tubes, characterized by color change and formation of a surface film (Figure 4).

To establish the time needed for the effective elimination of *P. aeruginosa* during pasteurization at 72 °C ±2, contaminated milk samples were heat-treated for 5, 10 and

15 min. A 15-min pasteurization at 72 °C ±2 was sufficiently effective against the reference and wild-type strains, with initial titers of 9.5×10^5 and 9.8×10^5CFU ml^{-1}, respectively. No bacterial growth was detected under this pasteurization regime through 14-day refrigerated storage time. Pasteurization regime of 72 °C ±2 with exposure times of 5 and 10 min did not guarantee the absence of forms capable of reactivation, as growth of individual colonies was seen.



A) Control milk and B) spoiled milk

Figure 4. Visible spoilage of milk on Day 14 of refrigerated storage, following pasteurization at 72 °C ±2 for 60 s

This study verified high antagonistic activity of *L. helveticus* Bbn4 and *L. helveticus* NK1 against the reference strain and the wild-type isolate of *P. aeruginosa* from a dairy farm. Their inhibitory effects might be attributed to various metabolic products that effectively inhibited growth of the pathogen. It is known that *P. aeruginosa* produces siderophores, low-molecular-weight (LMW) compounds that chelate and solubilize iron [6]. Lactobacilli, unlike most microorganisms, are iron-independent, making them unaffected by siderophores produced by *P. aeruginosa*. Moreover, organic acids, the primary metabolites from LAB, possess chelating characteristics and can bind iron from the substrate; thereby, limiting its availability for *P. aeruginosa* growth. Antimicrobial activity of *Lactobacillus* spp. might be associated to the induction of enzymes that degraded peptidoglycan layer of the cell walls of Gram-negative bacteria. Thus, antimicrobial activity of the industrially linked LAB strains of *L. helveticus* NK1 and *L. helveticus* Bbn4 against *P. aeruginosa* verified their functional characteristics. These strains show promise for use in development of functional foods, especially in dairy formulations.

The study demonstrated that short-time pasteurization (20–60 s at 72 °C ±2) was insufficient to eliminate *P. aeruginosa*, as viable cells with an initial titer of approximately 10^5 CFU ml^{-1} were detected immediately after heat treatment. According to Sviridenko et al., the *P. aeruginosa* culture, known for its psychrotrophic characteristics, showed the ability of individual surviving cells to multiply after heat treatment at 72 °C ±2, with an initial contamination level of approximately 10^6 CFU ml^{-1} and to preserve ability to reactivate, similar to the present findings [19].

Pasteurization for 5 and 10 min at the highlighted temperature did not guarantee complete inactivation of *P. aeruginosa*, as cells preserved ability to recover and resume growth during storage. On Day 14 of storage, no growth was observed in the sample that was heat-treated for 15 min. Based on the scientific data on *P. aeruginosa* survival, heating milk for at 63.5 °C for 30 min with an initial contamination level of 5.8×10^5 CFU ml^{-1} resulted in a 4-log dcrease in viable cell count. At the same time, a significant proportion of the cells was metabolically active but unable to form colonies on solid media. Thirty percent of *P. aeruginosa* strains showed signs of metabolic recovery within 24 h, following heat treatment at 72 °C ±2 for 16 s, supporting the present results. Under low and high-temperature pasteurization regimes of 72 °C ±2 and 80 °C ±2 for 10–20 s, individual *P. aeruginosa* cells preserved their ability to resume growth and reactivate following heat treatment at high initial contamination levels (> 10^6CFU ml^{-1}) [21]. The authors' findings regarding the heat resistance of another Gram-negative opportunistic bacterium—*E. coli*—indicated that the reference strain of *E. coli*, as well as wild strains, included heat stability. Moreover, under certain conditions, cells might survive pasteurization and later resume growth and multiplication. The authors suggest that wild-type strains of microorganisms may show increased heat resistance. The current results demonstrated a higher recovery rate for the wild-type strain of *P. aeruginosa* 47, compared to the reference strain. Therefore, these findings verified that certain bacterial strains could survive heat treatment under standard processing conditions.

In general, bacterial responses to stress factors, including heat shock, result in similar adaptive outcomes as transition to a dormant state. Bacteria decrease their metabolic activity, slowing down growth and division while decreasing expression of virulence-linked genes to prioritize survival. Sometimes, *Pseudomonas* spp. are capable of forming cyst-like dormant forms. After heating *P. aeruginosa* cell suspensions at 70 °C for 5 min, small pinpoint microcolonies (≤ 0.5 mm in diameter) were seen. These colonies were difficult to detect and became visible after prolonged incubation (7–14 d and in cases up to 1 m). The efficiency of microcolony reversion to the normal phenotype after subculturing on agar media did not exceed 20% . In this study, heat stress resulted in growth of non-pigmented pinpoint colonies on solid media (Figure 5ab).

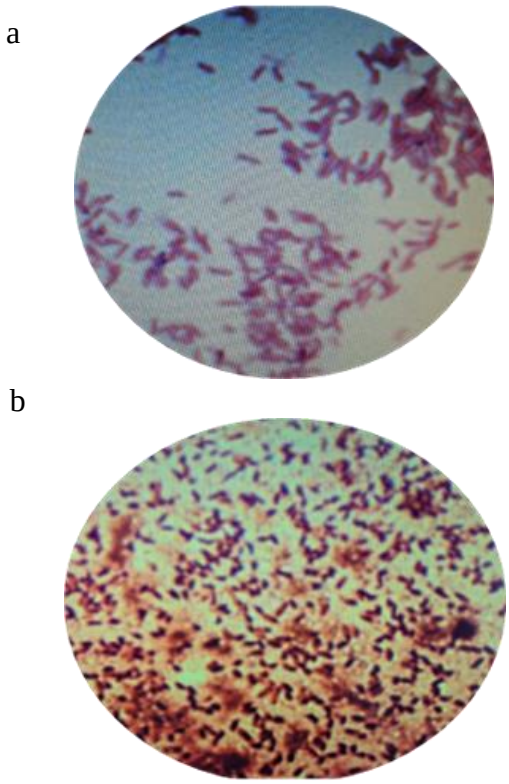


Figure 5. *Pseudomonas aeruginosa* 47 cells after pasteurization at 72 °C ±2 for 60 s (a) and without pasteurization (b)

Exposure to various stress factors has been reported to cause similar morphological changes in microbial cells, which preserve their functionality and serve as universal markers of adaptation mechanisms. For example, antibiotic exposure induces cell variability, characterized by heteromorphic growth, including cell elongation and formation of filamentous structures. On solid media, primarily pinpoint colonies were seen. Microscopy of *P. aeruginosa* cells after heat exposure revealed atypical elongation and occasional chain formation, which might indicate involvement of proteins that enhanced heat resistance. The present study revealed heat-induced morphological changes in *P. aeruginosa* cells, characterized by elongation, compared to untreated samples.

Bacterial response to heat stress includes synthesis of heat shock proteins, cellular disaggregases capable of solubilizing and refolding damaged protein aggregates; thereby, enhancing microbial heat resistance [22]. Within bacterial disaggregases, two proteins are particularly well known for protecting cells from heat-induced damages, including ClpB and ClpG. The ClpG shows stronger disaggregation activity and is associated with increased heat resistance in bacteria. An increase in disaggregase levels reflects the bacterial ability to adapt to extremely high temperatures during sterilization and pasteurization processes in food industry and medicine. Presence of ClpG has been verified in several Gram-negative bacteria, including *P. aeruginosa*. In *P. aeruginosa*, ClpG levels increase during the stationary growth phase, while ClpB activity predominates during the logarithmic phase. These proteins can functionally compensate for each other within specific temperature ranges: ClpG is stable at 70°C whereas ClpB is inactivated at 60–65 °C. Furthermore, ClpG may enable *P. aeruginosa* to survive pasteurization temperature of 72 °C ±2. Acquisition of an additional disaggregase, $ClpG_{GI}$, has contributed to the widespread dissemination of *P. aeruginosa* C-clone populations; in which, it has been identified. Additionally, $ClpG_{GI}$ is likely not a species-specific protein and has been detected in unrelated Gram-negative bacteria, suggesting recent acquisition by *P. aeruginosa* and a possible role in enhancing heat resistance of wild-type strains [22]. Genes encoding ClpG are horizontally transferable, facilitating the rapid spread of heat resistance traits in microbial communities. This may undermine efficacy of the present heat treatment regimes, including those used in the food industry.

4. Conclusion

Carried out studies demonstrated antimicrobial activity of industrially associated LAB strains against *P. aeruginosa*. The most pronounced inhibitory effect was shown by *L. helveticus* Bbn4 and *L. helveticus* NK1 strains. These strains and their metabolic complexes can be considered promising biological agents to control *P. aeruginosa* contamination, a particularly challenging pathogen.

Pasteurization at 72°C ±2 for 20, 40 and 60 s was insufficient to eliminate *P. aeruginosa* in the reference strain and the wild-type isolate. Ability of *P. aeruginosa* to survive under these pasteurization conditions should be addressed for technological process optimization in dairy production, particularly in facilities prone to microbial contamination. Exposure time was identified as a critical efficiency parameter; a 15-min treatment at 72°C resulted in the complete elimination of the pathogen. The available literature provides limited data on the ability of *P. aeruginosa* to survive milk pasteurization and reactivate during refrigerated storage. This characteristic may contribute to product spoilage and pose a potential risk to public health. The current findings highlight practical uses for dairy production. They highlight needs to adjust pasteurization regimes for resistant strains, potential use of LAB as additional barriers against *P. aeruginosa* and importance of monitoring survival and recovery of pathogens during storage. The study suggests an integrated approach to ensuring safety of dairy products by combining biological control with the optimization of heat treatment regimes.

5. Acknowledgements

The authors express their gratitude to Galstyan A.G. for supporting our work.

6. Declaration of competing interest

The authors report no conflicts of interest

7. Authors' Contributions

Conceptualization, I.R., N.P., V.L., A.P.; methodology S.K., E.I.; validation, S.K., E.I.; formal analysis, I.R., V.L., S.K.; investigation, E.I., S.K.; data curation, S.K., E.I.; writing—original draft preparation, S.K.; writing—review and editing, V.L.,I.R.; visualization, S.K., VL; supervision, AP; project administration, IR., AP.

8. Using Artificial Intelligent Chatbots

The authors did not use artificial intelligence

9. Ethical Consideration

This study does not require approval from an ethics committee.

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پایداری میکروبی در شیر پاستوریزه: کنترل زیستی و بهینه‌سازی عملیات حرارتی

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چکیده

سابقه و هدف: علاقه روزافزون به تغذیه سالم، استفاده از باکتری‌های اسید لاکتیک (LAB) با خواص فراسودمند را در تولید محصولات لبنی تقویت می‌کند. LAB در ترکیب با آغازگرهای پنیرسازی و سایر محصولات قرار می‌گیرند. آنها علاوه بر فواید عمومی سلامتی بخش، فعالیت ضد میکروبی قابل توجهی دارند که تا حدودی به دلیل متابولیت های کمپلکس آنها است. روش اصلی تضمین ایمنی شیر، عملیات حرارتی است. معمولاً پاستوریزاسیون در دمای ۷۲±۲° C در تولید پنیر استفاده می‌شود. با این وجود، بقای سلول‌های زنده *سودوموناس آئروژینوزا* (قادر به رشد در دمای یخچال)، ممکن است منجر به فساد در طول نگهداری شود.

مواد و روش ها: این مطالعه به طور کلی فعالیت آنتاگونیستی LAB صنعتی مانند *لاکتوباسیلوس هلویتیکوس*، را در برابر سویه‌های *سودوموناس آئروژینوزا* و همچنین اثربخشی پاستوریزاسیون در دمای ۷۲±۲ درجه سانتیگراد، (دمای معمول تولید پنیر) بررسی می‌کند. *لاکتوباسیلوس هلویتیکوس* فعالیت آنتاگونیستی شدیدی علیه سویه مرجع و وحشی *سودوموناس آئروژینوزا* ATCC 25668 و 47 نشان داد که توانایی بالقوه آن را به عنوان یک عامل کنترل بیولوژیکی تأیید می‌کند.

یافته‌ها و نتیجه‌گیری: در این تحقیق محدودیت‌های پاستوریزاسیون استاندارد مشخص شده است. در طول عملیات حرارتی شیر استریل آلوده به سویه وحشی 47، به مدت ۲۰ ثانیه 1.9×10^4 CFU/ml از سلول‌ها زنده ماندند، در حالی که پس از ۴۰-۶۰ ثانیه این تعداد به 1.8×10^1 CFU/ml رسید. رشد سلولی در طول ۷ روز نگهداری تا 3.5×10^6 CFU/ml مشاهده شد. مطالعات میکروسکوپی تغییرات ریخت‌شناسی در سلول‌ها (کشیدگی، ساختارهای رشته‌ای) را نشان داد که حاکی از سازوکار سازگاری است. نتایج مشابهی برای سویه مرجع P. aeruginosa ATCC 25668 نیز مشاهده شد. پاستوریزاسیون ۱۵ دقیقه‌ای در دمای ۷۲±۲° C برای هر دو سویه مؤثر بود. این مطالعه، رویکردی یکپارچه برای تضمین ایمنی محصولات لبنی با ترکیب کنترل بیولوژیکی با بهینه‌سازی رژیم‌های عملیات حرارتی را پیشنهاد می‌کند.

واژگان کلیدی: پایداری میکروبی، عملیات حرارتی، باکتری‌های اسید لاکتیک، *سودوموناس آئروژینوزا*