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Antibiofilm activity of postbiotics derived from *Lacticaseibacillus paracasei*

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Abstract
Biofilm-associated contamination by *Pseudomonas* and *Bacillus* remains a major challenge in dairy processing environments, necessitating natural and effective antimicrobial alternatives. This study evaluated the antimicrobial and antibiofilm efficacy of a postbiotic preparation derived from *Lacticaseibacillus paracasei* UBLP-35 against these predominant dairy-associated spoilage organisms. The postbiotic was produced through heat inactivation, ultrasonic lysis and lyophilization, yielding a cell-free bioactive preparation. Antimicrobial activity assessed by agar disc diffusion demonstrated a strong, concentration-dependent inhibitory effect (P=0.001). *Pseudomonas aeruginosa* inhibition zones increased from 6.60±0.15 mm at 300 mg/mL to a maximum of 13.93±0.28 mm at 900 mg/mL, while *Bacillus* inhibition expanded from 7.23±0.10 mm to 16.15±0.41 mm over the same concentration range. Antibiofilm assays revealed substantial suppression of biofilm formation, with optical density values decreasing significantly at higher postbiotic concentrations (P=0.001). At 900 mg/mL, biofilm biomass was reduced to 0.043±0.002 in *P. aeruginosa* and 0.032±0.001 in *Bacillus subtilis*, corresponding to 78.71 and 72.41 per cent inhibition, respectively. Lower concentrations (300-800 mg/mL) also produced graded reductions, confirming a clear dose-response trend. Overall, the results demonstrate that *L. paracasei*-derived postbiotics exhibited potent, broad-spectrum antimicrobial and antibiofilm activities. The high efficacy at ≥ 800 mg/mL highlights the potential of this postbiotic as a clean-label biopreservative for mitigating biofilm-mediated spoilage in dairy processing systems. These findings support the industrial applicability of LAB postbiotics as stable, natural and safe interventions for enhancing microbial safety and extending product shelf life.

Keywords: Postbiotics, *Lacticaseibacillus paracasei*, *Pseudomonas*, *Bacillus*, Antibiofilm, zone of inhibition

Introduction
Biofilm formation is widely recognized as one of the most persistent, complex and economically important challenges faced by the modern dairy industry. In dairy processing environments, microorganisms proliferate in surfaces that are frequently exposed to residual nutrients, moisture, fluctuating temperatures and turbulent flow conditions. All these factors promote the rapid attachment and colonization of bacteria. Once initial adhesion occurs, microorganisms begin producing extracellular polymeric substances (EPS), resulting in a three-dimensional, highly protective biofilm matrix (Abdalla *et al.*, 2024) [1]. These biofilms shield embedded cells from environmental stress, disinfectants, antibiotics and mechanical cleaning forces, making them extremely difficult to eradicate through routine sanitation procedures. Consequently, biofilms become reservoirs of spoilage and pathogenic organisms, continually releasing cells into dairy products and compromising product quality, safety and shelf life. Economic losses stem not only from contaminated batches but also from equipment damage, increased downtime for cleaning and the need for more aggressive chemical sanitizers. Within dairy facilities, biofilms are commonly detected on stainless steel surfaces, pipelines, gaskets, pasteurizers, milk silos and even packaging machinery. The structural complexity of biofilms enables microorganisms to survive for extended periods, persist through Cleaning in Place (CIP) operations and recolonize surfaces shortly after sanitation.

The resilience of biofilms makes them a long-standing concern for food safety agencies and dairy processors worldwide. According to Khani *et al.* (2023) [10], biofilms contribute significantly to recurrent contamination in dairy environments and represent a major factor in microbial spoilage and equipment corrosion. Because dairy products are highly perishable and rich in nutrients, even minor microbial contamination can accelerate spoilage, resulting in defects such as souring, off-flavours, coagulation, rancidity and undesirable textural changes.

Interest in novel, natural, and safe antibiofilm agents has grown substantially in recent years because traditional disinfectants often fail to penetrate dense biofilm matrices or may leave harmful residues on equipment surfaces. This has prompted the exploration of biological alternatives such as probiotics, bacteriocins, enzymes and postbiotics. Postbiotics defined as preparations of inactivated microbial cells, cell-wall fragments, metabolites or secreted compounds have shown exceptional potential due to their stability and multifunctional benefits. Unlike probiotics, postbiotics do not depend on the presence of live cells for activity, making them more suitable for incorporation into product or sanitation strategies that involve heat, pressure, variable pH or prolonged storage. The resistance to technological stressors allows them to maintain antimicrobial properties through processing.

Postbiotics derived from lactic acid bacteria (LAB) are especially promising because LAB naturally produce metabolites such as organic acids, short-chain fatty acids, antimicrobial peptides, enzymes, exopolysaccharides and biosurfactants. These substances not only inhibit pathogenic and spoilage bacteria but also have demonstrated significant antibiofilm capabilities. As discussed by Wegh *et al.* (2019) [19], the wide range of bioactive components in postbiotics can disrupt quorum-sensing pathways, inhibit EPS synthesis, alter cell membrane integrity, and prevent microbial adhesion at the earliest stages of biofilm formation. Among the LAB species investigated, *Lacticaseibacillus paracasei* stands out due to its ability to produce a diverse spectrum of antimicrobial and antibiofilm metabolites. Postbiotics from *Lacticaseibacillus paracasei* have been shown to suppress biofilm formation in several pathogenic and spoilage organisms by altering their metabolic activity and structural integrity. Aguilar-Toalá *et al.* (2018) [2] highlighted that postbiotics from strains like *L. paracasei* contain peptides and organic acids capable of targeting both Gram-positive and Gram-negative microorganisms. Their broad efficacy makes them valuable for dairy environments where biofilm-forming bacteria such as *Pseudomonas and Bacillus* pose major risks. Because postbiotics do not rely on bacterial viability, they avoid regulatory complications commonly associated with the introduction of live cultures into processing environments.

Pseudomonas spp., are the most troublesome organisms in milk and dairy products. These bacteria proliferate at refrigeration temperatures and secrete heat-stable proteases and lipases that remain active even after pasteurization. Such enzymes cause defects like bitterness, gelation and rancidity and reduced shelf stability issues that undermine consumer acceptance. Furthermore, *Pseudomonas* readily attaches to stainless steel and forms dense biofilms, contributing to recurring contamination in cheese vats, storage tanks and milk lines. Their resistance to sanitizers makes them extremely difficult to eliminate once established. Similarly, *Bacillus spp.,* including *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis*, present significant hygiene and safety challenges. Their endospores survive pasteurization and germinate during storage, leading to spoilage and potential toxin production. Spores and vegetative cells of *Bacillus* can anchor firmly to processing equipment, forming multilayered biofilms that resist CIP routines. These biofilms are associated with defects such as off-flavours, curdling, gas formation, and textural disruptions in dairy products. Because *Bacillus* biofilms can persist for months, they represent a long-term sanitation challenge for dairy processors.

The application of *L. paracasei* postbiotic offers a promising approach to overcoming these biofilm-related issues. Its broad-spectrum activity, natural origin, stability, and strong antibiofilm mechanisms align well with industry goals for cleaner-label preservation systems. By interfering with microbial adhesion, suppressing quorum sensing, and disrupting the structure of mature biofilms, postbiotics have the potential to significantly reduce contamination levels on dairy equipment. Additionally, their compatibility with existing sanitation procedures makes them suitable for integration into routine hygiene management protocols. As global demand for minimally processed, chemical-free dairy products continue to rise, the implementation of natural antibiofilm agents like *L. paracasei* postbiotic could contribute substantially to safer and more sustainable dairy processing systems. Further research and industrial-scale validation will help determine optimal concentrations, application methods, and regulatory pathways for incorporating postbiotics into dairy hygiene programs. Ultimately, these innovations may support improved product safety, extended shelf life, and enhanced consumer confidence in dairy products.

2. Materials and Methods

2.1 Maintenance of bacterial cultures

The freeze-dried culture of *Pseudomonas aeruginosa* and *Bacillus subtulis* were propagated in sterilized Nutrient broth by incubating at 37 °C for 24 h. After sufficient growth, it was sub cultured at weekly intervals on Nutrient agar and stored at 5 °C for further use.

2.1 Propagation and maintenance of *Lacticaseibacillus paracasei*

The *Lacticaseibacillus paracasei* (UBLP-35) culture was aseptically transferred in to skim milk and sub cultured at weekly intervals and were stored at 4 °C (Moradi *et al*., 2019) [12].

2.2 Preparation of Postbiotics from *Lacticaseibacillus paracasei* UBLP-35

The *L. paracasei* strain was cultured in MRS broth and incubated at 37±1 °C for 48 h in a CO_2 incubator to achieve optimal biomass. The culture was subsequently heat-inactivated at 121 °C for 15 min, followed by ultrasonic disruption in an ice bath at 300 W with 10 son /10 s off cycles for 15 min to ensure complete cell disintegration. The absence of intact cells was confirmed microscopically. The suspension was centrifuged at 6000 rpm for 10 min at 4 °C and the resulting supernatant was sequentially filtered through 0.45 µm and 0.22 µm membrane filters. The clarified cell-free supernatant was lyophilized (−40 °C freezing temperature, 100 mTorr pump pressure, −60 °C shelf temperature) and stored as the postbiotic preparation for subsequent assays (Moradi *et al*., 2019) [12].

2.3 Agar Disc Diffusion Assay

The antibacterial activity of the postbiotic was evaluated against *Pseudomonas aeruginosa* and *Bacillus subtulis* using the agar disc diffusion method described by Tejero-Sariena *et al*. (2012) [17]. Standardized bacterial suspensions (0.5 McFarland) were spread onto Mueller-Hinton agar plates. Sterile discs were loaded with three concentrations of the postbiotic solution and placed onto the inoculated plates. Following 24 h incubation at 37 °C, the diameter of the inhibition zone (mm) surrounding each disc was measured to quantify antimicrobial activity (Sevin *et al*., 2021) [15].

2.4 Minimum Inhibitory Concentration (MIC) Determination

The MIC was determined using the Inhibitory Concentration in Diffusion (ICD) method with modifications based on Guerin-Faublee *et al*. (1996) [9]. Mueller-Hinton agar plates were lawned with bacterial suspensions adjusted to 0.5 McFarland. Discs impregnated with graded postbiotic concentrations were placed on the agar surface and incubated at 37 °C for 18-24 h, depending on the bacterial species. The lowest concentration that produced a visible inhibition zone was recorded as the MIC for each strain.

2.5 Antibiofilm potential of postbiotics against *Bacillus.* spp and *Pseudomonas.* spp

The anti-biofilm activity of postbiotics against the test organisms was determined as per the method described by Kim *et al*. (2023) with some modifications. To measure the anti-biofilm activity, 100 µL each of *Bacillus* and *Pseudomonas* cultures at 1×10^8 colony-forming units (CFU/mL) in nutrient broth were dispensed to the 96-well plates. Next, 100 µL of postbiotic solution was added and absorbance was measured at 595 nm after incubation at 37 °C for 24 h. Negative control wells were administrated with 200µL of uninoculated broth only and positive control wells were inoculated with 200 µL of isolates only. Afterwards, the culture was removed and gently washed thrice with Phosphate-buffered saline (PBS). The adherent biofilm layer was stained with 200 µL of 0.1 percent crystal violet at 25 °C for 20 min. After washing the crystal violet thrice with PBS, 200 µL of 99 percent ethanol was added to the well, and the absorbance was measured at 595 nm.

3. Results and Discussion

3.1 Zone of inhibition of *Lacticaseibacillus paracasei* against *P. aeruginosa* and *B. subtulis*

The *Lacticaseibacillus paracasei* postbiotic exhibited a strong and highly significant (P=0.001) concentration dependent antimicrobial activity against both *Pseudomonas* and *Bacillus*, as reflected in the progressive increase in zone of inhibition values across concentrations ranging from 300 to 900 mg/mL. In *Pseudomonas*, the inhibition zone increased from 6.60±0.15 mm at 300 mg/mL to 6.63±0.20 mm and 7.60±0.15 mm at 400 and 500 mg/mL respectively, indicating no significant difference at lower doses. A marked increase in activity was observed at 600 mg/mL (8.85 ± 0.13^b mm) and 700 mg/mL (9.79 ± 0.30^b mm), both forming a distinct statistical cluster (b), demonstrating a significant enhancement in inhibitory potential. The antimicrobial effect continued to intensify at 800 mg/mL (11.73 ± 0.18^c mm) and reached its maximum at 900 mg/mL (13.93 ± 0.28^d mm) with each concentration confirming strong dose responsiveness.

A similar trend was observed against *Bacillus*, where inhibition zones increased from 7.23 ± 0.10^a mm at 300 mg/mL to 7.76±0.08 mm and 8.53±0.17 mm at 400 and 500 mg/mL. Significant increases emerged at 600 mg/mL (9.65±0.22 mm) and 700 mg/mL (10.60±0.07 mm), while 800 mg/mL (13.00±0.27 mm) produced a substantial increase in inhibition zone. The highest activity was observed at 900 mg/mL (16.15±0.41 mm), representing a distinct and significantly elevated antimicrobial response. The consistent elevation in inhibition with ascending concentrations demonstrates a clear and robust dose-response relationship for both organisms.

These findings are in line with previous work showing that *L. paracasei* and other lactic-acid-bacteria-derived postbiotics produced a rich mixture of organic acids, peptides and other metabolites capable of exerting strong, dose-dependent antibacterial and antibiofilm effects. Duan, *et al.* (2020) [8] reported that a natural antimicrobial substance from *L. paracasei* FX-6 markedly inhibited *Pseudomonas putida* via disruption of genomic DNA, demonstrating potent activity against Gram-negative pseudomonads similar to the high zones observed at 800-900 mg/mL in the present study. Alexandre *et al*. (2014) [3] showed that several *L. paracasei* strains possessed anti-elastase and anti-biofilm properties toward *Pseudomonas aeruginosa*, supporting the capacity of this species to interfere with virulence and surface colonization rather than merely planktonic growth. Rahman *et al*. (2025) [14] and Thorakkattu *et al*. (2022) [18] emphasized that lactic-acid-bacteria postbiotics act as promising natural preservatives in foods, with broad-spectrum activity against both Gram-positive and Gram-negative bacteria, reflecting the dual activity you observed against *Pseudomonas* and *Bacillus*. Studies using cell-free supernatants from LAB have also demonstrated strong antibiofilm and antibacterial effects against *P. aeruginosa* and other pathogens in a clearly concentration-dependent manner, which closely parallels the graded increase in inhibition zones seen here. Recent work on *L. paracasei* lysates and postbiotics further confirms their multifunctional activity combining antibacterial, anti-inflammatory and antioxidant effects which underscores their potential as clean-label biopreservatives for high-risk matrices such as dairy products. Taken together, the data strongly support the view that *L. paracasei* postbiotic, particularly at ≥ 600 mg/mL and optimally at 900 mg/mL, functions as a powerful, antimicrobial agent with clear relevance for controlling *Pseudomonas* and *Bacillus*-associated spoilage and biofilm formation in the dairy industry.

Table 1: Zone of inhibition of postbiotics of *L. paracasei* against *P. aeruginosa* and *Bacillus subtulis*

L. Paracasei **Postbiotic concentration (mg/mL)**	**Zone of inhibition (mm)** ***Pseudomonas*** **(Mean ± SE**	**Zone of inhibition (mm)** ***Bacillus*** **(Mean ± SE)**
300	$6.60^a\pm0.15$	$7.23^a\pm0.10$
400	$6.63^a\pm0.20$	$7.76^a\pm0.08$
500	$7.60^a\pm0.15$	$8.53^a\pm0.17$
600	$8.85^b\pm0.13$	$9.65^b\pm0.22$
700	$9.79^b\pm0.30$	$10.60^b\pm0.07$
800	$11.73^c\pm0.18$	$13.00^c\pm0.27$
900	$13.93^d\pm0.28$	$16.15^d\pm0.41$
P value	0.001**	0.001**

Overall, the data strongly validate the efficacy of *L. paracasei* postbiotic as a potent natural antimicrobial agent capable of significantly inhibiting spoilage-associated *Pseudomonas* and *Bacillus*, with maximal performance at 900 mg/mL. These findings support its application as a promising biopreservative candidate for enhancing microbial safety and quality in dairy and other food systems.

3.2 Antibiofilm potential of postbiotics against *P. aeruginosa* and *Bacillus subtulis*

The antibiofilm potential of postbiotics derived from *Lacticaseibacillus paracasei* against *Pseudomonas* and *Bacillus* biofilms. The postbiotic concentrations of 700, 800 and 900 mg/mL based on the mean OD values obtained from the microplate assay are presented in Table 2. The percentage reduction in biofilm formation corresponding to each concentration is depicted in Figure 1. A clear concentration-dependent antibiofilm effect was observed in both test organisms. At 900 mg/mL, the postbiotic exhibited the maximum antibiofilm activity, as reflected by the lowest OD values recorded for *Pseudomonas* (0.043±0.002) and *Bacillus* (0.032±0.001). These values were significantly lower (P=0.001) than those obtained at 700 and 800 mg/mL, indicating enhanced disruption or inhibition of biofilm matrix formation at higher concentrations. In comparison, the positive control showed markedly higher OD values for *Pseudomonas* (0.202±0.00) and *Bacillus* (0.116±0.00), confirming the robustness of the assay. The negative control OD values remained close to the untreated baseline for both organisms.

Overall, the data demonstrate that *L. paracasei* postbiotics possess strong antibiofilm potential against both Gram-negative (*Pseudomonas*) and Gram-positive (*Bacillus*) strains, with statistically significant differences among treatments (P=0.001). The pronounced decrease in OD at 900 mg/mL validates it as the most effective concentration for biofilm inhibition in the present study.

Table 2: Antibiofilm activity against *Pseudomonas and Bacillus* (Mean ± SE)

***L. Paracasei* Postbiotic concentration (mg/mL)**	**Antibiofilm activity (OD value) *Psedomonas* (Mean ± SE)**	**Antibiofilm activity (OD value) *Bacillus* (Mean ± SE)**
300	0.103^{d}±0.001	0.085^{f}±0.002
400	0.098^{d}± 0.003	0.081^{f}±0.001
500	0.081^{c}±0.001	0.072^{e}±0.001
600	0.074^{c}±0.002	0.065^{d}±0.001
700	0.067^{c}±0.001	0.057^{c}±0.001
800	0.052^{b}±0.001	0.048^{b}±0.001
900	0.043^{a}±0.002	0.032^{a}±0.001
Negative control	0.045 ±0.00	0.043±0.00
Positive control	0.202 ±0.00	0.116 ±0.00
P value	0.001**	0.001**

The percentage reduction in biofilm formation by *L. paracasei* postbiotics at different concentrations is presented in Figure 1. A clear dose-dependent antibiofilm effect was observed against both *Pseudomonas* and *Bacillus*. Biofilm inhibition in *Pseudomonas* increased from 49.01 per cent at 300 mg/mL to 78.71 per cent at 900 mg/mL, while in *Bacillus* it rose from 26.72 per cent to 72.41 per cent over the same concentration range, indicating that higher postbiotic doses substantially enhance antibiofilm efficacy.

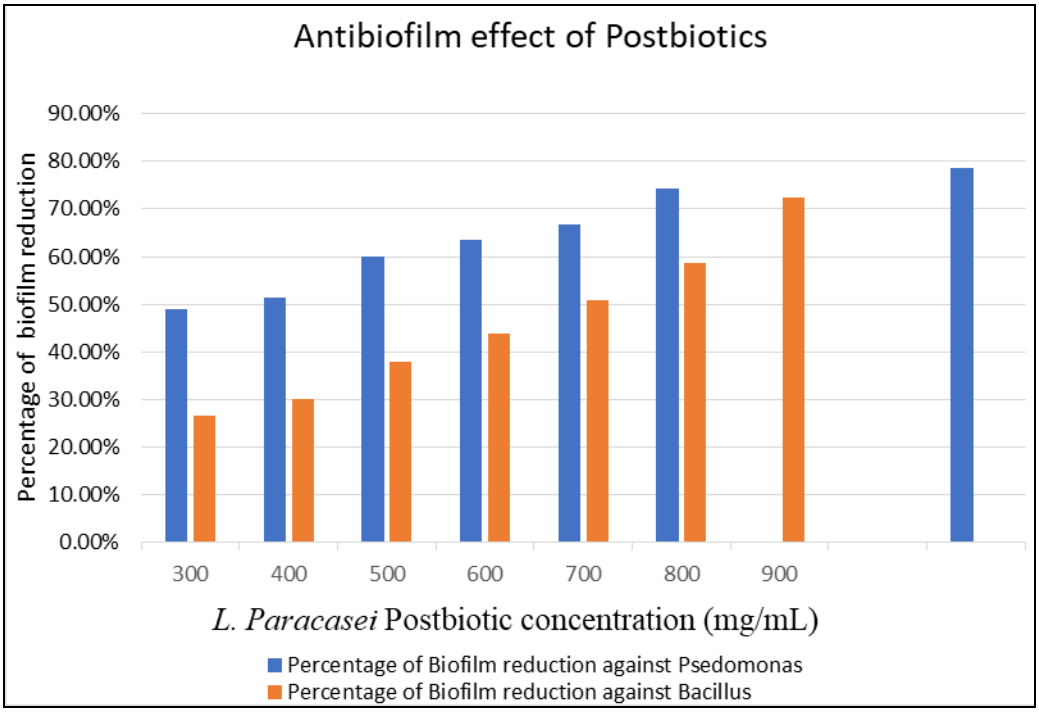

Fig 1: Antibiofilm activity against *Pseudomonas and Bacillus* (Mean ± SE)

This level of inhibition is comparable to or higher than that reported for other *L. paracasei* derived preparations. For instance, Mouafo *et al*. (2023) [13] demonstrated that a biosurfactant from *L. paracasei* N2 significantly reduced biofilm formation by multi-antibiotic-resistant foodborne pathogens. Likewise, Aydın *et al*. (2023) [5] reported that exopolysaccharides (EPS) from *L. paracasei* L1 inhibited *Pseudomonas aeruginosa* biofilms by ~54% at 500 mg/mL, supporting the strong antibiofilm potential of *L. paracasei* metabolites. Similarly, Behbahani *et al*. (2024) [6] found that a strain of *L. paracasei* produced a cell-free supernatant which reduced biofilm formation of other pathogens in a dose-dependent fashion. *L. paracasei* postbiotics further strengthens these findings. Algieri *et al*. (2023) [4] showed that a postbiotic fraction from *L. paracasei* CNCM I-5220 (LP-PBF) disrupted *Salmonella* biofilms in vitro and reduced intestinal colonization in vivo, highlighting the capacity of cell-free metabolites to interfere with biofilm architecture and pathogen persistence. Zhao *et al*. (2023) [20] similarly demonstrated that postbiotics from *L. paracasei* ET-22 inhibited *Streptococcus mutans* biofilms and down-regulated genes involved in EPS production and virulence, indicating that postbiotic action extends beyond simple growth inhibition to modulation of biofilm-related pathways. Studies on *Lacticaseibacillus paracasei* B31-2 also report anti-biofilm activity against *Listeria monocytogenes*, further underscoring the broad-spectrum biofilm-inhibitory properties of this species. More broadly, recent reviews emphasize that LAB-derived postbiotics (including organic acids, bacteriocins, biosurfactants, and EPS) can achieve 40-90% inhibition of pathogen biofilms, depending on strain, matrix composition and assay conditions, and are considered promising biofilm-control tools in food and biomedical settings. In this context, the 72-79% maximum inhibition observed at 900 mg/mL in the present study positions *L. paracasei* postbiotics at the upper end of reported antibiofilm efficacies and confirms a robust, statistically significant (P=0.001) concentration-dependent effect against both Gram-negative (*Pseudomonas*) and Gram-positive (*Bacillus*) biofilms (Drumond *et al*., 2023 and Sornsenee *et al*., 2021) [7, 16].

4. Conclusion

The present study clearly demonstrated that postbiotics derived from *Lacticaseibacillus paracasei* UBLP-35 possess strong and concentration-dependent antimicrobial and antibiofilm activities against two major dairy-associated spoilage organisms, *Pseudomonas* and *Bacillus*. The progressive increase in inhibition zones from 6.60 to 13.93 mm for *Pseudomonas* and 7.23 to 16.15 mm for *Bacillus* together with the substantial reduction in biofilm biomass (up to 78.71 and 72.41 per cent respectively), confirms the robust efficacy of the postbiotic preparation across all tested concentrations. These results highlight the potential of *L. paracasei* postbiotics as natural, stable and technologically compatible alternatives to chemical sanitizers for controlling biofilm-mediated contamination in dairy processing environments. Their broad-spectrum activity, combined with safety and ease of incorporation into existing hygiene workflows, underscores their promise as clean-label biopreservatives for improving product safety, extending shelf life and supporting more sustainable dairy production systems.

Future studies focusing on mechanism of action, synergy with CIP systems, and pilot-scale validation will further enhance their industrial applicability.

Conflict of Interest
Not available

Financial Support
Not available

Reference

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