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Reducing Dairy Spoilage: The Role of *Limosilactobacillus reuteri* in Controlling *Pseudomonas aeruginosa* and Preserving Starter Culture Functionality

Mohamed F. El-Ssayad[1], Hoda S. El-Sayed[1], Sahar H.S. Mohamed[2]*, Mohammed Aladhadh[2] and Asma E. Al Ghosen[2]

[1]Dairy Science Department, Food Industries and Nutrition Research Institute, National Research Centre, Dokki, Giza, Egypt.
[2]Department of Food Science and Human Nutrition, College of Agriculture and Food, Qassim University, Buraydah 51452, Saudi Arabia.

Abstract

The contamination of food products by psychrotrophic microorganisms, particularly *Pseudomonas aeruginosa* (*P. aeruginosa*), presents a significant challenge to food quality and safety in the dairy industry. Although not a foodborne pathogen, *P. aeruginosa* is highly destructive to food stability, leading to spoilage and reduced shelf life. This study investigates the interactions between *P. aeruginosa*, several foodborne pathogens, yogurt starter cultures, and spoilage fungi using dual-culture assays. *P. aeruginosa* exhibited strong antagonistic effects on various microorganisms and demonstrated inhibition of pathogens through metabolic compounds such as hydroxyurea, pentane-1, 5-diol, ethylmethylsilane, and N-benzylidene-dimethylammonium chloride, identified via GC-MS analysis. To counteract *P. aeruginosa*, the protective lactic acid bacterium *Limosilactobacillus reuteri* (*L. reuteri*) was employed. The bacterium enhanced the sensitivity of *P. aeruginosa* to several antibiotic classes, including aminoglycosides, beta-lactams, cephalosporins, and chloramphenicol. GC-MS profiling of *L. reuteri* extracts revealed potent bioactive compounds, such as derivatives of D-lactic acid, phenyllactic acid, and hydroxyisocaproic acid, which possess robust antimicrobial properties. When applied in fermented milk, *L. reuteri* extracts (2 mg/mL) completely inhibited the growth of *P. aeruginosa* while preserving the functionality of yogurt starter cultures during storage. These findings highlight the potential of *L. reuteri* as a bio-protective agent to mitigate spoilage in dairy products.

Keywords: *Limosilactobacillus reuteri*, *In Vitro* Study, Dual Culture, Antibacterial Activity, Modified Antibiotic Susceptibility, *Pseudomonas* Spoilage of Dairy Products

***Correspondence:** sh.ahmed@qu.edu.sa

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INTRODUCTION

Food contamination by psychrotrophic bacteria, particularly *Pseudomonas aeruginosa*, represents a significant challenge in the dairy industry, undermining both food quality and safety. Although not classified as a foodborne pathogen, *P. aeruginosa* is a destructive microorganism capable of causing spoilage that affects the structural stability, color, flavor, and overall acceptability of dairy products during storage.[1,2] The economic and operational implications of such spoilage are considerable, leading to increased product waste, reduced shelf life, and diminished consumer trust. Furthermore, the growing prevalence of multidrug-resistant strains of *P. aeruginosa* exacerbates the threat, particularly as contamination is often introduced at multiple points in the dairy production chain. From water sources and milking equipment to cooling tanks and raw milk, the dairy farm environment provides numerous opportunities for the proliferation and spread of this microorganism.[3,4]

Contamination by *Pseudomonas aeruginosa* is of particular concern in dairy processing due to its ability to thrive in cold temperatures and its resistance to various sanitization methods.[3,5] Once present, the organism can disrupt microbial ecosystems, especially in products reliant on fermentation. This spoilage mechanism occurs not only because of *P. aeruginosa*'s rapid growth but also due to the production of metabolites that interfere with the activity of starter cultures such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. These starter cultures play an essential role in the production of yogurt and other fermented dairy products, and any disruption can lead to reduced product quality and compromised fermentation efficiency.[1]

Interestingly, *P. aeruginosa* exhibits antagonistic behavior toward other microorganisms, including both prokaryotes and eukaryotes. It produces various bioactive compounds, such as pyocyanin, hydroxyurea, and rhamnolipids, which have antimicrobial properties that inhibit the growth of both Gram-positive and Gram-negative bacteria, as well as fungi.[6,7] While this antagonistic capacity has been extensively studied in clinical and environmental microbiology, its role within the dairy microbiota remains underexplored. Understanding the dynamics of *P. aeruginosa* in polymicrobial environments is crucial for managing its impact, particularly as it is often co-isolated with other pathogens and spoilage organisms.[8] However, not all strains of *P. aeruginosa* exhibit consistent antagonistic behavior, indicating the need for further characterization of its interactions with other microorganisms.[9,10]

Current research has largely focused on the prevalence of *P. aeruginosa* in dairy farms, its incidence in milk and dairy products, and its resistance to antibiotic treatments.[4] However, less attention has been paid to its interactions with beneficial microorganisms, particularly starter cultures that are critical for fermentation. Such polymicrobial interactions can influence the stability and quality of fermented dairy products, making it essential to explore methods for controlling *P. aeruginosa* without disrupting the activity of desirable bacteria.[5]

To address this challenge, the use of protective microorganisms, such as lactic acid bacteria (LAB), has emerged as a promising biocontrol strategy. Among these, *Limosilactobacillus reuteri* has shown considerable potential due to its ability to produce a range of antimicrobial metabolites, including lactic acid derivatives, phenyllactic acid, and other volatile organic compounds.[11] These bioactive compounds can inhibit the growth of spoilage microorganisms like *P. aeruginosa* while preserving the functionality of starter cultures. Additionally, *L. reuteri* has been shown to enhance the sensitivity of *P. aeruginosa* to various classes of antibiotics, making it a viable candidate for mitigating the organism's multidrug-resistance.[12]

Therefore, this study explores the intricate interactions between *Pseudomonas aeruginosa*, dairy starter cultures, and foodborne pathogens within a polymicrobial environment. It further evaluates the potential of *Limosilactobacillus reuteri* as a bioprotective agent to inhibit *P. aeruginosa* and mitigate its adverse effects on fermentation. Using GC-MS profiling, the bioactive metabolites produced by *L. reuteri* are analyzed to uncover their role in its antimicrobial activity.

MATERIALS AND METHODS

Pathogenic and spoilage strains

Escherichia coli (*E. coli*) strain E11 (accession number KY780346.1), *Salmonella enterica* (*S. enterica*) strain SA19992307 (accession number CP030207.1), *Pseudomonas aeruginosa* (*P. aeruginosa*) strain Kasamber5 (accession number KY549641.1), *Bacillus cereus* (*B. cereus*) strain 151007-R3-K09-40-27F (accession number KY820914.1) were isolated and identified by Al-Gamal et al.[13] *Listeria monocytogenes* (*L. monocytogenes*); *Staphylococcus aureus* (*S. aureus*) ATCC 6538 strains were supplemented from the collection of Dairy Microbiological Lab., NRC, Egypt, while *Aspergillus flavus* (*Asp. flavus*) 3357 and *Candida albicans* ATCC 10231 were provided by colleagues of the marine toxin lab., NRC, Egypt.

Bio-protective lactic acid bacterium and Starter

Limosilactobacillus reuteri NRRL B-14171 as well as yogurt starter (*Streptococcus thermophilus* Chr-1 and *Lactobacillus bulgaricus* Lb-12) were obtained from collection of Dairy Microbiological Lab., NRC, Egypt.

Investigating the antagonism of *Pseudomonas aeruginosa*

Interaction via dual culture assay

The antagonistic interaction was inducted between *Pseudomonas aeruginosa* and several indicators including pathogenic and food spoilage strains, as well as dairy starters. The interaction was performed via neighboring dual plate assay that developed the method described by Baptista et al.[14] Agar plates were swabbed from 0.5 McFarland of bacterial suspension. Then, the central well (1 cm) was filled with nutrient agar seeded with *Pseudomonas aeruginosa* (10^8 CFU).

Extraction of bioactive compounds

Heavily-streaked *Pseudomonas aeruginosa* was grown on Tryptic Soy Agar (TSA) at 32 °C for 24 hours. Bioactive compounds were extracted as described by Muller and Merrett,[15] with slight modifications. Equal units (w/v) of the harvested TSA and Chloroform were mixed and agitated for an hour. The filtrate was concentrated in a fuming hood and stored in -20 °C till use.

Evaluation of the extract antimicrobial activity via disc diffusion

The antimicrobial activity of the crude extract is evaluated through disc diffusion assay as recommended in the British Society for Antimicrobial Chemotherapy guidelines.[16] Respectively, Ciprofloxacin (5 µg) and Clotrimazole (1 mg/mL) was used as antibacterial and antifungal positive controls.

GC-MS profiling of both *Limosilactobacillus reuteri* and *Pseudomonas* extract

The analysis was performed in central laboratory, Food Industries and Nutrition Research Institute, National Research Centre, Dokki, Egypt using a GC-MS system (7890A-5975C, Agilent Technologies Inc., Santa Rosa, CA, USA) equipped with an HP-5 MS capillary column (30 m × 0.25 mm, 0.25 mm, Agilent Technologies Inc., Santa Rosa, CA, USA). The injection volume of the sample was 1 µL. Helium (99.999%) was used as the carrier gas at a flow-rate of 1 mL/min. The temperature of the injection port was 280 °C, and the column temperature pro Gram was as follows: 40 °C for 5 min, followed by an increase to 150 °C at a rate of 5 °C/min, and an increase to 210 °C at the rate of 10 °C/min. The MS conditions Capillary column and 5975B Inert XL MS system under electron ionization at 70 eV and Quadrupole mass analyzer. The MS source and Quadrupole were held at 230 °C and 150 °C, respectively. Helium was used as carrier gas at a flow rate of 1 mL/min. The promising Cell-free Supernatant was analyzed on the Headspace-GC–MS instrumentation described with a headspace (HS) oven temperature of 80 °C. The HS loop and transfer line temperatures were set at 90 °C and 100 °C, respectively. Vial equilibration was set at 15 min. The vial pressurization was set at 15 psi for 0.15 min. Injection time was set at 0.50 min.

Evaluation of *Limosilactobacillus reuteri* role in controlling *Pseudomonas aeruginosa*

Interference with antibiotic susceptibility

Antibiotic sensitivity assay on m-Mueller Hinton agar was performed as described in in British Society for Antimicrobial Chemotherapy (BSAC) guidelines.[16] The m-Meuller Hinton agar plates were prepared by pouring a 1st MRS seeded with *Limosilactobacillus reuteri*.

Extraction of bioactive compounds

MRS broth (500 mL) was inoculated with *Limosilactobacillus reuteri* NRRL B-14171 and incubated at 30 °C. After 72 hours, MRS was mixed with Ethyl acetate: Diethyl ether (1:1) and vigorously shacked for an hour. The organic layer was separated and evaporated in fuming hood till dry film was obtained.[17]

Evaluation of the extract against both *Pseudomonas aeruginosa* and yogurt starter

In a dual culture (MRS broth) both of *Pseudomonas aeruginosa* (10^3 CFU/ml) and yogurt starter (10^6 CFU/mL) were inoculated. Different concentrations (0-2000 µg/mL) of *L. reuteri* extract were applied and microbial numbers were recorded after 24 hours incubation in refrigerator.

Evaluation of the preservative potential in a real dairy model

Manufacturing of set yogurt

A whole buffalo's milk was subjected to heat treatment (85 °C/15 min) and inoculated with the commercial yogurt starter (2% v/v). The inoculated milk was divided into four batches; (1) Control (a batch that just contains yogurt starter), (2) Ps. (a batch that contains yogurt starter + 3 log CFU/mL of *Pseudomonas aeruginosa*), (3) re. (Batch No. 2 + 1% v/v of *Limosilactobacillus reuteri*), and (4) ext. (Batch No. 2 + 2 mg/mL of *L. reuteri* extract) (Table 1). After complete coagulation at 42 °C, the four batches were prepared for cold storage. Microbiological analyses investigated the total lactic acid bacteria (log CFU/g), *Pseudomonas aeruginosa* (CFU/g), and Mold and yeast (CFU/g) for 0-15 days with 3 days interval.[18]

Microbiological analyses

Enumeration of *Pseudomonas aeruginosa*

Enumeration was done on the Pseudomonas CN (Oxoid, England) agar.[18] Inoculated plates were incubated at 25 °C for 48 hours.

Enumeration of total LAB on MRS

Enumeration of total lactic acid bacteria, including SLAB and NSLAB on MRS agar (Oxoid) for 48-72 hours at 37 °C.

Enumeration of molds and yeasts

Enumeration of *Asp. flavus* and *Candida albicans* were done on Potato dextrose agar (PDA), the inoculated plates were incubated at 20-25 °C for 5 days.

Statistical analysis

Statistical significance was determined using Minitab 18. The means were determined by analysis of variance test (ANOVA, two-way analysis) ($p < 0.05$).

RESULTS AND DISCUSSION

Estimating the antagonistic potential of *Pseudomonas aeruginosa*

Dual cultures on agar plates

In dual cultures (multispecies interaction), the behavior of *Pseudomonas aeruginosa* (competition/co-operation) with different foodborne pathogens, yogurt starter; *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, as well as some food spoilage and toxin-producing fungi has been assessed (Figure 1). Results were represented as inhibition

Table 1. Different processes of set yogurt manufacturing

Batch	Additives			
	Yogurt starter (2%)	*L. reuteri* (1% v/v)	*Ps. aeruginosa* (10^3 CFU/ml)	*L. reuteri* extract (2 mg/ml)
Ps	√	NA	√	NA
Control	√	NA	NA	NA
St/Ps/re	√	√	√	NA
St/Ps/ex	√	NA	√	√

diameter (mm) in Table 2. Presented zones of inhibition showed a general antagonistic behavior of *Pseudomonas aeruginosa* that began from 15.67 mm in case of *Escherichia coli* and reached 24.67 mm with *Aspergillus niger*. Except the unaffected *Staphylococcus aureus*, there is no significance response among the tested pathogenic bacteria ($p < 0.05$). Both of *Lactobacillus bulgaricus* and *Aspergillus flavus* seems to be the same and all of *Streptococcus thermophilus*, *Aspergillus niger*, and *Candida albicans* appear no significant differences ($p < 0.05$).

The same conclusion was attained by Toppo and Tiwari,[19] when they subjected *Pseudomonas* spp. and *Rhizoctonia soloni*. Also, Rakh et al.[20] reported a good inhibitory potential of a *Pseudomonas aeruginosa* strain against *Sclerotium rolfsii* (reduction by 94% of dry weight).

Disc diffusion of Chloroform extract

This test was performed to measure the contribution degree of *Pseudomonas* antimicrobial metabolism toward the same groups of microorganisms. Chloroform extract was prepared and tested via disc diffusion assay (Figure 2). Results were presented in comparison with positive controls (Table 3). Inhibition zone was the least with *Staphylococcus aureus* (8.67 mm) and gradually increased as 15 mm in case of *Lactobacillus bulgaricus*. All of *Bacillus cereus* (17.07 mm), *Aspergillus niger* (17 mm), and *Streptococcus thermophilus* (16.33 mm) were affected by the same degree without significance ($p > 0.05$). Inhibition reached the greatest with *Candida albicans* (40.67 mm). There is no detected inhibition in case of *Listeria monocytogenes*, *Salmonella enterica*, and *Escherichia coli*.

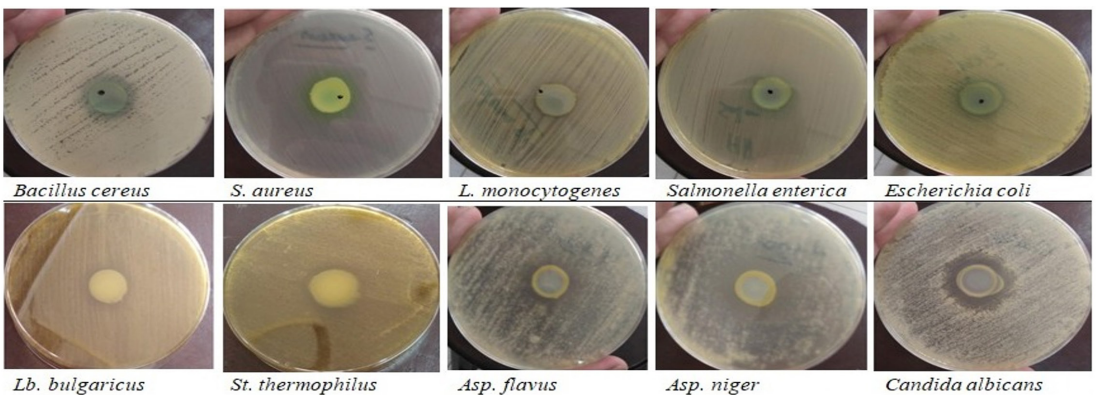

Figure 1. Interaction between *Ps. aeruginosa* and indicator organisms (Dual culture technique)

Table 2. The estimated antagonism of *Ps. aeruginosa* in dual cultures on agar plates

Indicator microorganism		Zone of inhibition (mm)
Pathogenic bacteria	*Bacillus cereus*	16.17 ± 0.17^{C}
	Staphylococcus aureus	ND
	Listeria monocytogenes	17.00 ± 0.59^{C}
	Salmonella enterica	16.00 ± 0.29^{C}
	Escherichia coli	15.67 ± 0.34^{C}
Yogurt starter	*Lactobacillus bulgaricus*	19.17 ± 0.45^{B}
	Streptococcus thermophilus	24.33 ± 0.90^{A}
Spoilage fungi	*Aspergillus flavus*	20.67 ± 0.34^{B}
	Aspergillus niger	24.67 ± 0.90^{A}
	Candida albicans	23.67 ± 0.90^{A}

Means ± SE; Values that share the same letter have no significance

Metabolic profiling of *P. aeruginosa* extract (chloroform) by GC-MS

In a way to understand how different microorganisms were inhibited due to *Pseudomonas aeruginosa*, the later extract was scanned by GC-MS spectrometer as shown by Figure 3 and compounds were listed in Table 4. The most predominant; N-benzylidene-dimethylammonium chloride (area 12.82%) which has a reported activity as surfactant and antimicrobial, followed by the antimicrobial alcohol; Pentane-1,5-diol (area 4.5%), the antibacterial; Ethylmethylsilane (area 3.1%), and the antimicrobial; Hydroxyurea.

Our current results came in agreement with previous studies like Amankwah et al.[25] who reported the potential of *Pseudomonas aeruginosa* isolate to produce antimicrobial alcohols (Hexadecanol and Eicosanol), other like Zhou et al.[26] and Deshmukh and Kathwate,[27] to produce surfactants (Rhamnolipids).

Depending on GC-MS results, the wide spectrum antimicrobial effect of *Pseudomonas aeruginosa* and its extract can be attributed minimally to Hydroxyurea which reported to kill *Escherichia coli* through inhibition of class I ribonucleotide reductase (RNR), depleting dNTP pools and leading to replication fork arrest.[28] Maximum antimicrobial effect is attributed to all of Ethylmethylsilane which enhances the antimicrobial effect,[23] Pentane-1,5-diol,[22] and N-benzylidene-dimethylammonium chloride.[29]

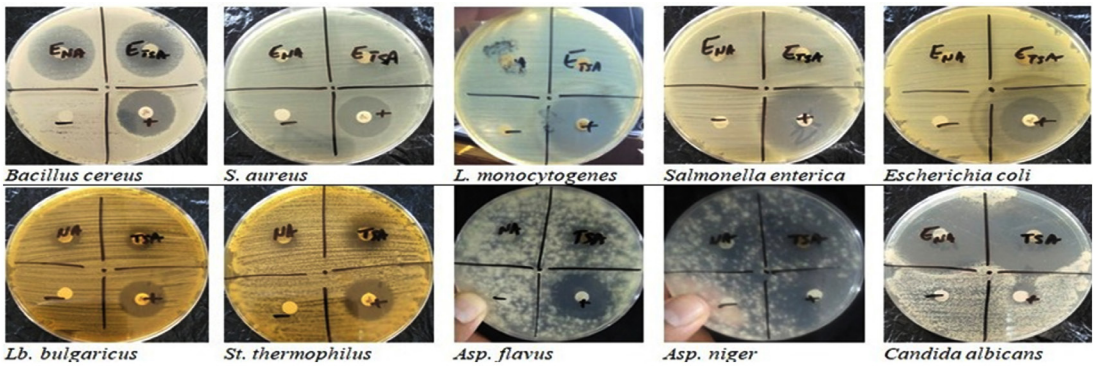

Figure 2. Estimated antimicrobial of *Ps. aeruginosa* extract against indicator organisms (disc diffusion technique)

Table 3. Estimated antimicrobial activity of *Pseudomonas* extract by disc diffusion assay

Indicator microorganism		Zone of inhibition (mm)	
		Pseudomonas extract	Positive control (CIP 5/Clotrimazole)
Pathogenic bacteria	*Bacillus cereus*	17.07 ± 0.07^{Bb}	23.33 ± 0.34^{BCa}
	Staphylococcus aureus	8.67 ± 0.34^{Eb}	22.5 ± 0.29^{Ca}
	Listeria monocytogenes	ND	32.5 ± 0.29^{A}
	Salmonella enterica	ND	33.33 ± 0.34^{A}
	Escherichia coli	ND	23.33 ± 0.34^{BC}
Yogurt starter	*Lactobacillus bulgaricus*	15 ± 0.59^{Ca}	16.67 ± 0.68^{Ea}
	Streptococcus thermophilus	16.33 ± 0.34^{Bb}	18.67 ± 0.34^{Da}
Spoilage fungi	*Aspergillus flavus*	12.33 ± 0.34^{Db}	24.33 ± 0.34^{Ba}
	Aspergillus niger	17 ± 1.02^{Ba}	16.33 ± 0.90^{Ea}
	Candida albicans	40.67 ± 0.34^{Aa}	14.67 ± 0.34^{Fb}

Means ± SE; The same capital letters within the same column means no significance, while the same small letters within a raw indicates no significance; CIP 5 ciprofloxacin antibacterial; Clotrimazole antifungal agent

Bio-protective role of *Limosilactobacillus reuteri* in the control of *Pseudomonas aeruginosa*

Interference with antibiotic susceptibility

This point evaluates the effect of *Limosilactobacillus reuteri* on Pseudomonas response toward different antibiotics. Five classes; aminoglycosides, beta-lactams, mono beta-lactams, chloramphenicol, and cephalosporins were represented as shown in Table 5. *Pseudomonas aeruginosa* was inhibited by all of Amikacin 30 µg (23.33 mm diameter), Cefepime 30 µg (28 mm), and Azetronam 10 µg (27.67 mm), while appeared not affected by Chloramphenicol 30 µg (7.67 mm) and Ampicillin 10 µg (6.67 mm). This response was reported by researches,[30] which attributed this resistance to the MexXY multidrug efflux system that developed by *Pseudomonas aeruginosa*. Pre-hosting with *Limosilactobacillus reuteri* cells increased the zone of inhibition from 23.33 to 25.67 mm (Amikacin 30), 28 to 35.33 mm (Cefepime 30), 27.67 to 31.67 mm (Azetronam 10), 6.67 to 25.67 mm (Chloramphenicol 30), and 6.67 to 10.33 mm (Ampicillin 10). The effect of *Limosilactobacillus reuteri* seemed to inhibit the Pseudomonal-derived stress response systems by suppressing the system effluxes different classes of antibiotics; the MexXY system[30] whose existence is necessary for the development of natural resistance to several classes of antibiotics.[31]

The impact of *L. reuteri* extract on growth of both *Pseudomonas aeruginosa* and yogurt starter

The active extract of *L. reuteri* was studied for its negative impact on both *P. aeruginosa* and yogurt starter and results were presented in figure 4. There is a strong inhibition caused by *Pseudomonas aeruginosa* to yogurt starter whose count was reduced by at least one log cycle (90%). After the addition of *L. reuteri* extract, *Pseudomonas aeruginosa* was reduced from 6.79 to 6.21 log CFU/mL at 0.04 mg extract/mL (0.24 log cycle), 6.02 log CFU/mL at 0.08 mg extract/mL (0.77 log cycle), 4.58 log CFU/mL at 0.4 mg extract/mL (2.21 log cycles), and 4.09 log CFU/ml at 2 mg extract/mL (2.7 log cycles). On the other hand, yogurt starter increased gradually

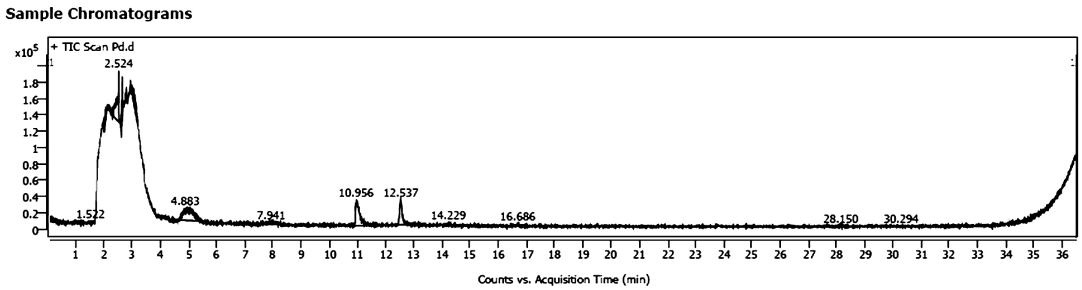

Figure 3. GC/MS chromatogram of *Ps. aeruginosa* extract (chloroform)

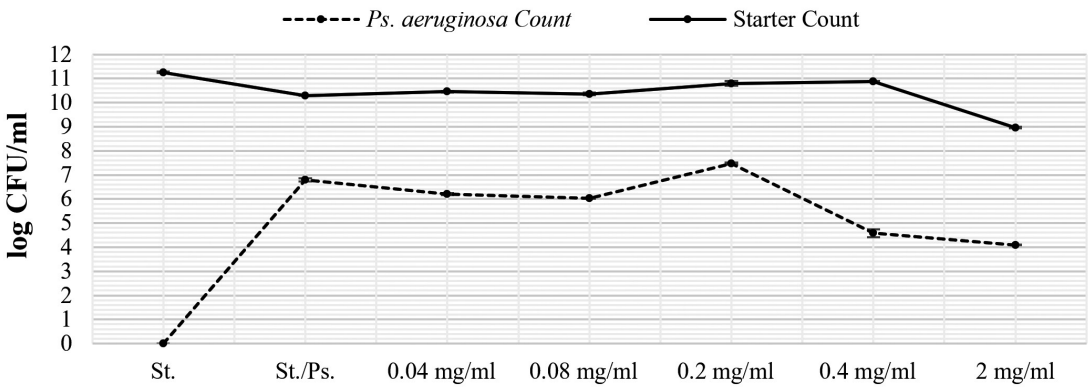

Figure 4. Impact of *L. reuteri* extract on the growth of both *Ps. aeruginosa* and yogurt starter in dual culture

with the increasing in added extract concentration. Yogurt starter count increased from 10.29 log CFU/ml to 10.46, 10.36, 10.80, 10.88 log CFU/ml at extract concentrations 0.04, 0.08, 0.20, and 0.4 mg/mL. A relatively great drop was noted (from 10.29 to ~9 log CFU/ml) at 2 mg/ml of extract concentration.

Metabolic profiling of the protective; *L. reuteri* bacterium using GC-MS analysis

Profiling of *Limosilactobacillus reuteri* extract by GC-MS (Figure 5) revealed predominance of D-Lactic acid derivative with area 35.87%, 1,3-Dioxolane-2-d, 2-methyl (Dioxolane derivative) with area 17.35%, Butanedioic acid, 2TMS derivative (area 4.87%), 2-Hydroxyisocaproic acid, deriva-tive (area 4%), 3-Phenyllactic acid derivative (area 1.39%), Butylated hydroxy toluene (0.93%) as presented in Table 6. After estimation of *L. reuteri*-based metabolic content, the observed inhibition of *Pseudomonas* growth can be understood. The inhibitory contents that produced by *L. reuteri* were majorly Lactic acid derivatives that widely reviewed as strong antimicrobial agents[32] with broad-spectrum activity that was proven against *Pseudomonas aeruginosa*.[11] Considerable contribution to the reduction of spoilage populations was attributed to 1,3-dioxolane derivatives which perfectly antagonized *Pseudomonas aeruginosa* as reported by both.[33] Both of amino acid metabolites; 2-Hydroxyisocaproic acid and Phenyllactic acid

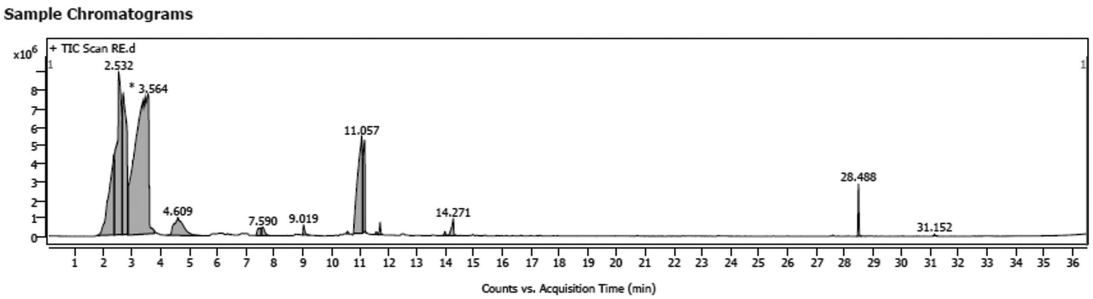

Figure 5. GC-MS profile of *L. reuteri* extract

Table 4. GC-MS profile of the most important bioactive compounds in *Pseudomonas* extract

RT	Compound name	Area%	Biological activity
2.312	Hydroxyurea	0.28	Antimicrobial[21]
2.646	Pentane-1,5-diol	4.5	Antibacterial, antifungal, anti-viral[22]
2.799	Ethylmethylsilane (silane derivatives)	3.1	Antibacterial[23]
12.537	N-benzylidene-dimethyl-ammonium chloride	12.82	Surfactant, antimicrobial[24]

Table 5. Influence of *L. reuteri* on the antibiotic susceptibility of *Pseudomonas aeruginosa*

Treatment	Diameter of inhibition (mm)				
	Amikacin 30 µg	Cefepime 30 µg	Chloramphenicol 30 µg	Ampicillin 10 µg	Azetronam 10 µg
Class	aminoglycoside	beta-lactam	Chloramphenicol	Cephalosporin	Mono beta-lactam
Control	23.33 ± 0.34^{B}	28 ± 0.00^{B}	7.67 ± 0.16^{B}	6.67 ± 0.17^{B}	27.67 ± 0.34^{B}
Response	Sensitive	Sensitive	Resistant	Resistant	Sensitive
L. reuteri	25.67 ± 0.34^{A}	35.33 ± 0.34^{A}	25.67 ± 0.34^{A}	10.33 ± 0.34^{A}	31.67 ± 0.34^{A}
Response	Sensitive	Sensitive	Sensitive	Resistant	Sensitive

Means ± SE; Values that share the same letter have no significance

were produced by *Lactobacillus* spp. and showed a reproducible antagonism against both pathogenic and spoilage bacteria, as well as spoilage and toxigenic fungi.[34,35]

Applying the bio-preservation potential of *Limosilactobacillus reuteri* in Fermented milk

A whole buffalo's milk was subjected to heat treatment (85 °C/15 min) and inoculated with the commercial yogurt starter (2% v/v). The inoculated milk was divided into four batches; (1) Control (untreated), (2) Ps. (contaminated with 3 log CFU/ml of *Pseudomonas aeruginosa*), (3) Re. (Ps. that further supplemented with 1% v/v of *Limosilactobacillus reuteri*), and (4) Ext. (Ps. that further supplemented with 2 mg/mL of *L. reuteri* extract). After complete coagulation at 42 °C, the four batches were prepared for cold storage.

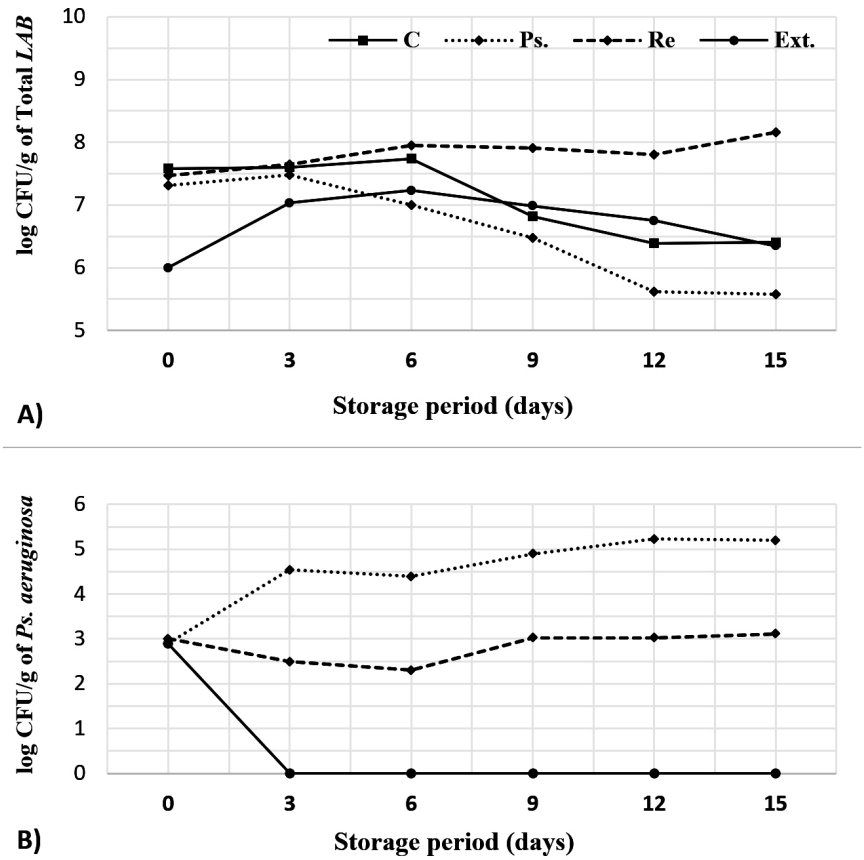

Figure 6. Effect of *L. reuteri* (cells and extract) on microbial growth in fermented milk. The total count of LAB (including *L. reuteri*) was estimated on MRS agar medium, while *Ps. aeruginosa* counts were estimated on CN agar medium

Table 6. GC-MS profile of the most important bioactive compounds in *L. reuteri* extract

RT	Compound name	Area%	Biological activity
2.532	1,3-Dioxolane-2-d, 2-methyl	17.35	Antibacterial and antifungal[33]
3.564	D-Lactic acid derivatives	35.87	Antimicrobial[11]
13.155	2-Hydroxyisocaproic acid, derivative	4	Bactericidal and fungicidal[34]
14.448	Butanedioic acid, 2TMS derivative	4.87	Flavoring agent[36]
18.654	3-Phenyllactic acid derivative	1.39	Broad antimicrobial[37]
28.488	Butylated hydroxy toluene	0.93	Antioxidant[38]

Microbiological analyses investigated the total lactic acid bacteria (log CFU/g), *Pseudomonas aeruginosa* (CFU/g), and Mold and yeast (CFU/g) for 0-15 days with 3 days interval. Results were presented in Figure 6 A and B.

The presented data (Figure 6A) showed general increasing in the total lactic acid populations till the end of 6th storage day. Then numbers gradually decreased to the least (6.4 log CFU/g) by the end of 15 days storage period as widely reported by many researchers.[39] This decline in LAB populations is normal and can be attributed to the accumulation of lactic acid.[40] Inclusion of *Pseudomonas aeruginosa* (2.9 log CFU/ml) contributed to decrease the number of total lactic bacteria by 0.27 log CFU at the beginning of cold storage, and 0.12 log CFU after 3 days. After that, numbers sharply declined from 7.48 log CFU/mL (at the 3rd day) to 7 log CFU/mL (6th day), 6.48 log CFU/mL (9th day), 5.62 log CFU/mL (12th day), reaching to 5.58 Log CFU/mL (15th day) as predicted due to the antagonistic metabolism of *Pseudomonas aeruginosa* that interferes with membrane permeability of the host bacteria as introduced in Table 4 and confirmed by Bharali et al.[41] Addition of *L. reuteri* cells contributed to LAB increasing in greater way than the control. Addition of *L. reuteri* extract firstly caused inhibition in the growth of lactic acid bacteria, but after 6 days enhanced the number of viable lactic acid bacteria which was greater than Ps. - treated batch by 0.23 log CFU/mL (6th day), 0.51 log CFU/mL (9th day), 1.13 log CFU/- (12th day), and 0.77 log CFU/mL (15th day).

Figure 6 B showed that the numbers of *Pseudomonas aeruginosa* increased from 2.9 log CFU/mL at the beginning of storage to 4.55 log CFU/mL (3rd day), 4.40 log CFU/mL (6th day), 4.90 log CFU/mL (9th day), 5.23 log CFU/mL (12th day), 5.19 log CFU/mL (15th day). After the addition of *L. reuteri*, numbers of *Pseudomonas aeruginosa* were declined from 4.55 to 2.49 log CFU/mL (3rd day), from 4.40 to 2.30 log CFU/mL (6th day), from 4.90 to 3.03 log CFU/mL (9th day), from 5.23 to 3.03 log CFU/mL (12th day), and from 5.19 to 3.11 log CFU/mL by the end of 15th day. Similar results were obtained by Tan et al.[42] who concluded that some protective LAB can inhibit *Pseudomonas aeruginosa* in a polymicrobial mixed growth. In case of *L. reuteri* extract, the growth of *Pseudomonas aeruginosa* was no longer detected by the end of 3rd storage day because of containing various compounds that cause pathogen and spoilage inhibition. Similar results were reported by Lima et al.[12] All batches were confirmed as free of mold and yeast along the period of cold storage.

CONCLUSION

The findings of this study highlight the detrimental impact of *Pseudomonas aeruginosa* on yogurt starter cultures, ultimately compromising the quality of fermented dairy products. However, the results also demonstrate the potential of *Limosilactobacillus reuteri* as an effective biocontrol agent. The incorporation of viable *L. reuteri* cells was shown to enhance the sensitivity of *P. aeruginosa* to multiple antibiotic classes, while the application of *L. reuteri* extracts successfully eliminated *P. aeruginosa* populations within three days of cold storage. These promising outcomes suggest that *L. reuteri* could play a vital role in improving the quality and stability of dairy products by mitigating spoilage caused by *P. aeruginosa*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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