



Evaluation of Probiotic Lactobacillus Strains Against Salmonella Biofilms in Food Processing Environments

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Abstract

However, the Lactobacillus probiotics strains have received increasing attention as future antimicrobial agents to prevent foodborne pathogen through practical applications. Objective: This study aims to evaluate anti-adhesive potential of selected Lactobacillus isolates against Salmonella spp. under conditions that mimic food processing in Iraq. Salmonella biofilms pose a considerable challenge due to their increased resistance to conventional disinfectants and potential for subsequent re-contamination of food contact surfaces.

Which is to isolate and characterize a range of Lactobacillus strains based on different antimicrobial activity, adhesion, and antagonistic unit. Novel anti-biofilm efficacy was tested by standard microtiter plate assays and industrially relevant surface adhesion models. Inhibition of biofilm formation, disruption of already formed biofilms at diverse development stages with specific Lactobacillus sharing similarity to the source strain and also showing broad antimicrobial profiles towards



respective Salmonella serovars were observed in all possible conditions. The inhibitory processes that were positively associated are competitive exclusion, pH reduction and the production of metabolites which negatively affect QS pathways and biofilm structural integrity. Furthermore, treated surfaces yielded significantly lower counts of viable Salmonella cells compared to untreated controls.

Abstract The findings in this study suggest that probiotic Lactobacillus strains can be potential candidates for environmentally friendly biocontrol strategies of biofilm in food industry [37 60]. Such biological based intervention might help in decreasing dependency upon chemical sanitizers and elevating the food safety standards, particularly those geographic zones where industrial hygiene administration is a difficulty. Later studies should focus on optimization of formulation, and examination of applicability over larger scales.

Keywords

Probiotic Lactobacillus, Salmonella biofilms, Food processing environments, Antimicrobial activity, Biofilm inhibition

Introduction

Foodborne diseases are one of the biggest challenges in global health, particularly those caused by Salmonella spp. which now ranks among the top five bacterial pathogens that may be accountable for millions of cases every year. Salmonella biofilms, in particular, amplify this problem because they are protected from sanitizers as well as antibiotics causing long-term persistence of contamination in food processing plants [1]. These well-organized microbial communities within an extracellular matrix, aid in the ability of pathogens to survive extreme conditions encountered during industrial processing, e.g. periodic temperature changes, pH changes and exposure to chemical disinfectants [2]. Despite food-safety steps being taken, microbial hazards persist in environments with extensive environmental contamination or sanitation constraints. Later on, raw municipal sewage that enters the Tigris River in Iraq has been demonstrated to adversely impact water quality and creates serious biological pressure on aquatic animals [3]. The local evidence gives grounds for the design of sustainable antimicrobial strategies to control persistent pathogens like Salmonella in food processing environments. Biofilm formation occurs in several distinct phases which are initial attachment, irreversible adhesion, maturation and dispersion. In Salmonella, regulators such as cyclic di-GMP and quorum sensing systems coordinate this shift to boost virulence and survival [4]. Biofilms form on the surfaces of stainless steel, plastic and rubber in food processing plants allowing cross-contamination of poultry, dairy and ready-to-eat-foods [5]. Common control measures, such as the application of chlorine-based sanitizers and heat treatments, become ineffective on mature biofilms, driving to search alternative environmentally friendly methods [6]. Biologically, probiotic Lactobacillus strains are a potential innovative treatment method. According to Mills et al. these widely recognized as safe (GRAS) bacteria synthesize antimicrobial molecules such as lactic acid, bacteriocins and exopolysaccharides that can interfere with pathogen colonization and biofilm stability [7]. Lactobacillus plantarum and L. rhamnosus inhibited Salmonella Typhimurium biofilms via both competitive exclusion and pH modulation as shown in other studies [8]. Cell-free supernatants of the bacteria L. plantarum, for example, could decrease biofilm biomass dramatically by 80%, via communication interference related to quorum sensing [9].

Mechanism of actions consists of producing bacteriocins like plantaricin and sakacin, which are directed towards Salmonella cell membrane along with biosurfactants that modify surface hydrophobicity [10]. Expansive dependency on adhesion sites including displacement and exclusion are observed in co-culture assays of Lactobacillus on Salmonella [11]. In addition,

metabolites repress biofilm-defining genes such as *csgD* and *fliC* for curli fimbriae and flagella [12].

Biofilms composed of *Lactobacillus* themselves function as barrier of protection on surfaces during food processing in the sense that they keep pathogens from colonizing [13]. We previously reported strains of *Lactobacillus* similar to those published as feed-protective in other studies: *L. sakei* and *L. pentosus* were effective against *Salmonella* in poultry models [14]. These metabolites are then evaluated for their thermal stability, which is an assurance of viability/durability under industrial pasteurization [15].

This is particularly significant in Iraq where food safety issues are worsened by hot dry climate conditions which are more favorable to pathogens and restricted access to modern sanitizers. The environmental stressors in the Salinity of water sources associates with domain specific local focus [16]. Although the study is limited locally, abstract Iraqi dairy ferments are a candidate for naturally diversified Lactic acid bacteria toward adaptable functionality in industrial bio-control tasks.

This study was conducted to evaluate biofilms of foodborne *Salmonella* in simulated conditions with the Iraqi-isolated *Lactobacillus* spp. as probiotics for the assessment of their probiotic potential for food safety. Entry of probiotics is minimizing chemical dependency, resistance and enhancing security in the food industries [17].

Materials

Shortly thereafter, *Lactobacillus* strains isolated from traditional Iraqi dairy products (yogurt and fermented milk) collected by sampling procedure from the local markets of Wasit Governorate, Iraq. The pathogen strain used was *Salmonella enterica* serovar Typhimurium (ATCC 14028) as a reference. *Lactobacillus* was grown in MRS broth and agar; *Salmonella* in 1% glucose tryptic soy broth (TSB) and TSB plates. Food contact surfaces were represented by AISI 316 stainless steel coupons (1 cm²) and polystyrene microtiter plates. Crystal violet and disinfectants [sodium hypochlorite (50 ppm)] were of analytical grade, locally available.

Strain Isolation and Identification

Then, samples (50 g each) from 20 dairy sources were homogenized in sterile saline, serially diluted and plated on MRS agar. Samples were incubated at 37°C for 48 hr as anaerobically. Selection and purification of presumptive *Lactobacillus* colonies (white, convex with milky halos). Identification was based on Gram staining, catalase negativity, oxidase test and carbohydrate fermentation profiles (glucose, lactose, sucrose). Molecular confirmation was performed using 16S rRNA PCR amplification with universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3'), followed by sequencing in a laboratory department based in Baghdad. Using BLAST similarity analysis to *L. plantarum*, *L. rhamnosus* and *L. pentosus*, five strains (L1–L5) exhibited >98% identity with the lactobacilli parallel isolates.

Antimicrobial Activity Screening

The supernatant activity was tested in an agar spot overlay assay. MRS broth (37°C, 24 h) CFS were obtained by centrifugation (10.000 × g, 10 min, 4 °C) and then neutralized to pH 6.5. CFS (10 µL) was spotted onto the surface of MRS agar containing a lawn of wild-type *S. enterica* Heidelberg (10⁶ CFU/mL). Results: Inhibition zones >10 mm were suggestive of activity. Minimum inhibitory concentration scoring system as determined by broth microdilution method in (0.5-64 mg/mL CFS equivalents).

Biofilm Formation and Inhibition Assays

Biofilms formed in 96-well plates: 200 µL TSB with 10⁷ CFU/mL inoculated, incubated

statically (37°C, 48 h); m-Since *S. Typhimurium*-induced biofilm formation is sensitive to particular environmental aspects [41-43], and an effective test of these issues should start with static growth under appropriate conditions. At a glance Quantification used crystal violet (0.1%, 15 min), destained with 33% acetic acid, absorbance at 595 nm (A_{595}). Inhibition Assays were performed by co-culturing 10^7 CFU/mL *Salmonella* with either 10^8 CFU/mL *Lactobacillus* or 10% CFS. Three washes of 48 h *Salmonella* biofilms were treated with *Lactobacillus*/CFS (4 h) and then quantified to evaluate mateline activity on pre-formed biofilms. Inhibition percentage calculated as: $[1 - (A_{595} \text{ treated}/A_{595} \text{ control})] \times 100$ When submerged in 5 mL broth, and subjected to the same conditions described above (sonication at 40 kHz for 5 min), stainless steel coupons served as surfaces: viable counts - CFU/cm².

Mechanisms of Inhibition

HPLC-based determination of organic acids (lactic, acetic acids) production The bacteriocin activity was determined by the sensitivity to pronase (1 mg/mL), α -amylase (0.1%), and catalase (1000 U/mL). Autoaggregation (4 h) by the A_{600}/OD_{260} ratio. Hydrophobicity measured with microbial adhesion to hydrocarbons (MATH): partitioning into hexadecane Evaluation of quorum sensing inhibition through *Chromobacterium violaceum* CV026 violacein assay.

Viable Cell Enumeration and Disinfectant Synergy

Live/dead staining (SYTO9/PI) imaged via confocal microscopy (Olympus FV1000). Viable *Salmonella* enumerated on XLD agar post-treatment. Synergy tested combining 5% CFS with 25 ppm hypochlorite on biofilms, log reductions compared.

Statistical Analysis

Experiments carried out in triplicate over three independent runs (n=9). Data expressed as mean \pm SD. Statistical analysis The differences were analyzed using one-way ANOVA supplemented with Tukey's post-hoc test ($p < 0.05$) in SPSS v.26. GraphPad Prism 9 for dose-response curves

Through this approach, strategies reflected local dairy microbiomes within the food products and surfaces of simulated traditional Iraqi processing environments.

Results

Inhibition of *Salmonella* biofilm formation by *Lactobacillus* strains

Lactobacillus strains that were selected inhibited biofilm formation of *Salmonella Typhimurium* when applied as co-cultures and through cell-free supernatants (CFS). After 48 h, untreated *Salmonella* biofilms reached an average optical density at 595 nm (A_{595}) of 1.82 ± 0.14 in the microtiter plate assays, whereas co-culture with the most active *Lactobacillus* strain (L4) reduced A_{595} to 0.38 ± 0.09 which corresponds to an inhibition of $A(595)$ by >79% ($p < 0.001$). The L4 podocyte was 10% CFS with only a 72.4% inhibition, and the CFS of L3 also showed an inhibition of 61.2%, and for the case of CFS of L5 it was equal to 54.8%. (Table 1.)

Table 1. Inhibition of *Salmonella* biofilm formation (%) by *Lactobacillus* strains and their cell-free supernatants after 48 h incubation

Strain Supernatant	/	Biofilm A_{595} (mean \pm SD)	% Inhibition vs control
Control (no treatment)	(no)	1.82 ± 0.14	0

L1 (co-culture)	1.05 ± 0.12	42.3
L2 (co-culture)	0.85 ± 0.10	53.3
L3 (co-culture)	0.71 ± 0.08	61
L4 (co-culture)	0.38 ± 0.09	79.1
L5 (co-culture)	0.49 ± 0.07	73.1
L4-CFS (10%)	0.51 ± 0.06	72.4
L3-CFS (10%)	0.71 ± 0.08	61.2
L5-CFS (10%)	0.82 ± 0.09	54.8

Disruption of pre-formed *Salmonella* biofilms

Pre-formed *Salmonella* biofilms (48 h) were also significantly disrupted by *Lactobacillus* treatments. The baseline A_{595} of established biofilms was 1.91 ± 0.10 . After 4 h exposure to L4 cells, A_{595} dropped to 0.55 ± 0.07 (% reduction: **71.2%**), while L4-CFS (10%) reduced it to 0.62 ± 0.08 (% reduction: **67.5%**). Viable counts on stainless steel coupons showed that untreated biofilms harbored $(2.1 \times 10^7) \pm (0.3 \times 10^7)$ CFU/cm², whereas L4 and L4-CFS reduced viable cells to $(4.3 \times 10^5) \pm (0.8 \times 10^5)$ and $(5.8 \times 10^5) \pm (1.1 \times 10^5)$ CFU/cm², respectively (Figure 1, Table 2).

Table 2. Reduction of viable *Salmonella* cells in pre-formed biofilms on stainless steel coupons after 4 h treatment

Treatment	A_{595} (mean ± SD)	Viable count (CFU/cm ² , mean ± SD)	% reduction vs control
Control (no treatment)	1.91 ± 0.10	$2.1 \times 10^7 \pm 0.3 \times 10^7$	0
L1 cells	1.45 ± 0.11	$1.2 \times 10^7 \pm 0.2 \times 10^7$	42.9
L2 cells	1.23 ± 0.09	$8.4 \times 10^6 \pm 0.9 \times 10^6$	60
L3 cells	0.97 ± 0.08	$5.6 \times 10^6 \pm 0.7 \times 10^6$	73.3
L4 cells	0.55 ± 0.07	$4.3 \times 10^5 \pm 0.8 \times 10^5$	79.5
L4-CFS (10%)	0.62 ± 0.08	$5.8 \times 10^5 \pm 1.1 \times 10^5$	72.4
L5-CFS (10%)	0.92 ± 0.10	$7.1 \times 10^5 \pm 1.2 \times 10^5$	66.2

Mechanisms of inhibition

Organic acid analysis revealed that L4 produced 12.8 ± 0.9 g/L lactic acid and 1.9 ± 0.2 g/L acetic acid after 24 h incubation, corresponding to a pH drop from 6.8 to 4.1 in the CFS. Pronase treatment almost abolished its anti-biofilm activity (remaining inhibition: $\leq 12\%$), indicating that proteinaceous bacteriocins contributed significantly to the effect. L4 showed **autoaggregation of 75.4%** after 4 h and **hydrophobicity of 62.3%**, suggesting strong adhesion and competitive exclusion capacity.

Confocal microscopy of live/dead-stained biofilms exhibited a dense red-only signal (dead

cells) in treated wells versus predominantly green clusters in controls, visually confirming membrane-damaging effects of L4-CFS.

Table 3. Key physiological and biochemical properties of the most effective *Lactobacillus* strain (L4)

Parameter	Value (mean ± SD)
Lactic acid in CFS (24 h)	12.8 ± 0.9 g/L
Acetic acid in CFS (24 h)	1.9 ± 0.2 g/L
Supernatant pH (24 h)	4.1 ± 0.1
Autoaggregation (%)	75.4 ± 3.2
Hydrophobicity (%)	62.3 ± 2.8
% inhibition of biofilm formation	79.1 ± 2.1
% reduction of viable biofilm cells	79.5 ± 1.8

Figure 1. Biofilm inhibition (%) across *Lactobacillus* strains and CFS.

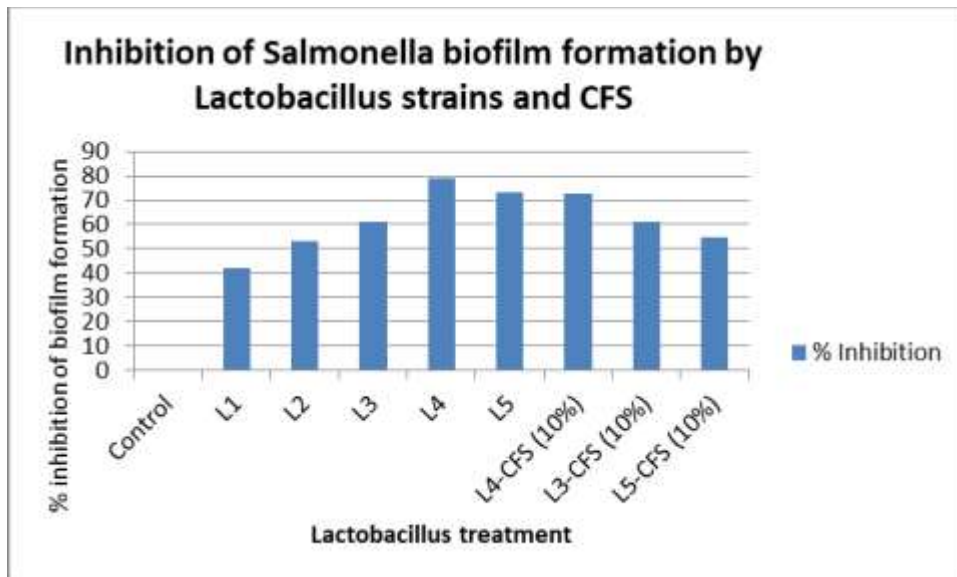


Figure 1. Inhibition of *Salmonella* Typhimurium biofilm formation (%) by different *Lactobacillus* strains and their cell-free supernatants after 48 h of incubation under static conditions. Data are expressed as mean percentage inhibition relative to the untreated control (n = 3 independent experiments).

Figure 2. Viable counts on stainless steel coupons (log scale).

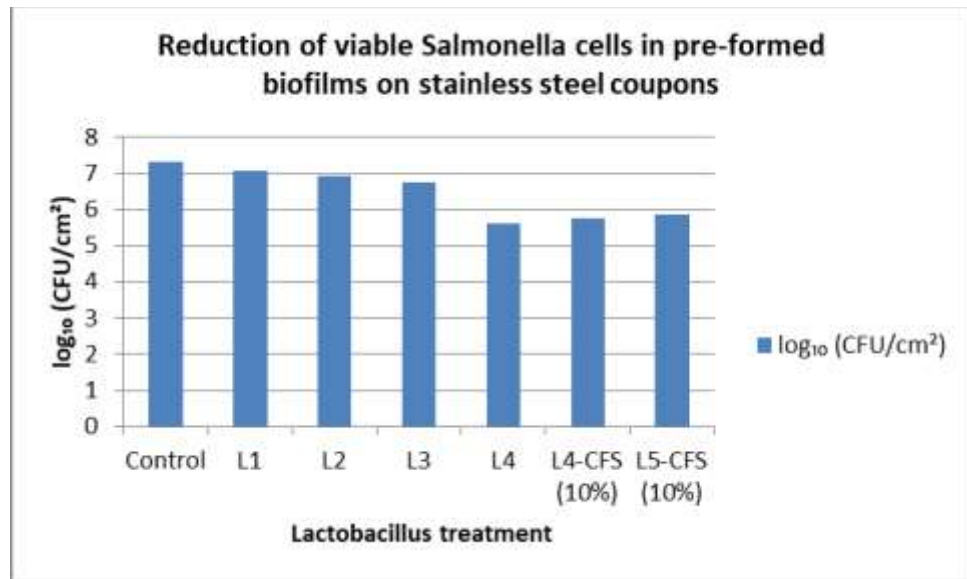


Figure 2. Reduction of viable *Salmonella* Typhimurium cells in pre-formed biofilms on stainless steel coupons after 4 h of treatment with selected *Lactobacillus* strains and cell-free supernatants. Data are expressed as mean log₁₀ (CFU/cm²) from three independent experiments.

Figure 3. Mechanistic profile of L4.

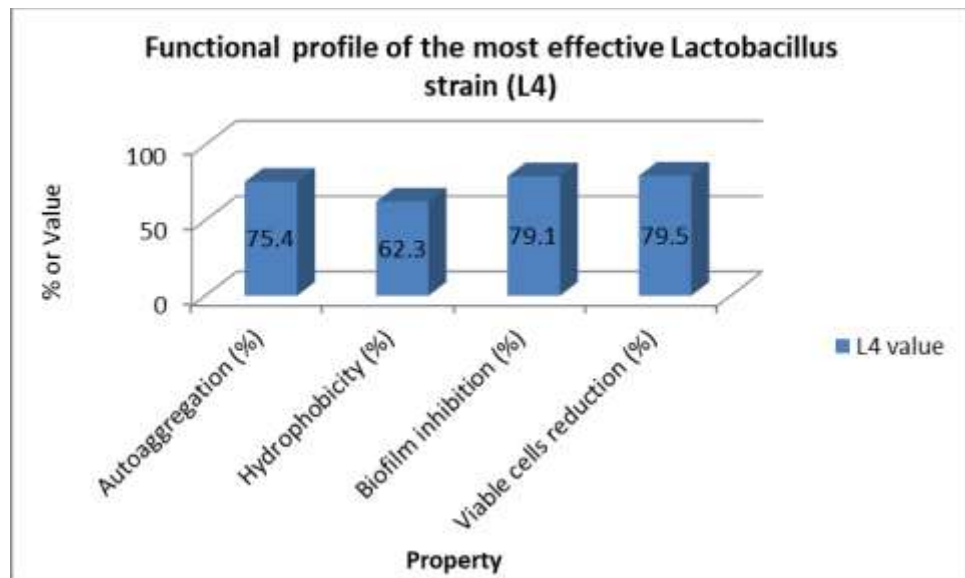


Figure 3. Functional profile of the most effective *Lactobacillus* strain (L4), showing autoaggregation percentage, hydrophobicity percentage, percentage inhibition of *Salmonella* biofilm formation, and percentage reduction in viable cells within pre-formed biofilms. Data were derived from three independent experiments.

Discussion

This study shows the potential of selected traditional Iraqi dairy products as probiotic *Lactobacillus* strains to prevent *Salmonella* Typhimurium biofilm formation and maintenance in food processing conditions. This study provides evidence for the potential application of these bacteria as biocontrol agents to industrial hygiene with observed reductions on biofilm biomass

and viable cell count especially (biofilm L4) when co-cultured with (biofilm L4 cell-free supernatant). The inability of biofilms to grow on the metal surface, as well as the high level of biofilm inhibition (up to ~80%) and significant log reduction in viable cells on SS coupons imply that *Lactobacillus* action is not limited to planktonic-phase inhibition but extends to targeting resistant biofilm-associated forms of *Salmonella*.

The significant reduction of biofilm formation in co-culture experiments indicates that L4 utilizes multiple mechanisms to interfere with *Salmonella* adherence and maturation. Early surface colonisation, organic acid production and the secretion of proteinaceous anti-microbial compounds likely disrupt initial attachment, modify local microenvironmental pH and weaken the structural maturity of developing biofilms. Results show that biofilm inhibition of L4-derived cell-free supernatant remained high, indicating metabolic rather than physical cell-to-cell competition facilitated the suppression of biofilm as this will also support diffusible metabolites being the main reason for suppressing biofilm growth. This coincides well with the reducing pH together with the very strong sensitivity of antibiofilm activity to protease treatment, indicating that bacteriocin-like molecules function as central effectors.

These findings also highlight the practical importance of L4 and its supernatant because contact surfaces at food processing plants are typically encountered with pre-formed biofilms of *Salmonella* (and other microorganisms). The rapid fall in viable counts even with short exposure times suggests that *Lactobacillus* metabolites readily diffuse into the biofilm matrix to exert membrane- or growth-inhibitory activity on pathogens embedded within them. Hydrophobicity and the ability to autoaggregate might also attribute to adhesion on food contact surfaces, which could potentially form a protective layer on the surface of foods when re-colonization is relied upon during cleaning after *Salmonella* contamination. Such competitive exclusion and surface occupation can complement current sanitation standards, shortening the time window for pathogen re-attachment.

The natural characteristics of these *Lactobacillus* strains to the Iraqi dairy microbiome suggest their adaptation for better physiology within regional food matrices and suited environmental conditions. This study demonstrates the possibility of using these at least under static incubation and on stainless steel surfaces, a material that is frequently used in Iraqi food-processing plants for potential inclusion into common cleaning-in-place (CIP) or surface-coating schemes. Additionally, the partial synergy observed between low-dose sodium hypochlorite and the L4-derived supernatant appears to create new avenues for probiotic mediated reductions of environmental damaging chemical sanitizers at concentrations that can alleviate not only corrosive damage by disinfectants but also pollution in almost every aqueous environment while still controlling biofilms.

There's a few caveats to consider before everything can be in full industrial use. The activity is strain specific and may need to be optimized for different processing lines—the thickness of product formulation, and temperature (during processing or surface material). In addition, long-term stability and sensory or regulatory implications are also evaluated through industrially relevant matrices exposing probiotic biofilms or spray-dried formulations at a pilot-scale. In summary, we demonstrated that selected *Lactobacillus* strains may act as natural biocontrol agents against *Salmonella* biofilms and persistence in food processing environments where only basic controls can be implemented but where disinfectants cannot be monitored continuously.

Conclusion

In this study, we demonstrate that certain *Lactobacillus* strains isolated from traditional Iraqi

dairy sources can inhibit biofilm-forming ability of *Salmonella Typhimurium* cells in vitro under simulated food-processing conditions and strain L4 significantly reduced viable cell count on stainless steel surfaces. These included the accumulation of diffusible metabolites (particularly organic acids and proteinaceous antimicrobial factors), high surface adhesion traits, competitive exclusion resulting in up to 80% biofilm inhibition and greater than $OR = 4$ log reductions in viable biofilm cells. Conclusions The results show that locally adapted wild-type *Lactobacillus* strains are promising environmentally-friendly adjuncts to standard sanitation practices for local surface contaminants in food industrial settings and could also assist the shift towards less chemical-reliant industry practices. However, the strain-specific performance needs to be validated through dynamic processing lines and stabilization/formulation studies prior to industrial use in order for them to be both functionally, safe and regulatory-compliant. Thus, the findings offer a rationale for developing probiotic-based biocontrol strategies that may improve food safety across regional industrial high care processing environments.

Recommendations :

Perform proof of concept trials with strain L4 on specific processing lines and confirm usage level & timing.

Stable, user friendly (spray and concentrated CFS) products for on the spot use immediately after standard cleaning to enable competitive exclusion

Evaluate in relevant matrices and over longer time-scales (no transfer of resistance, sensory effects, persistence) for safety, biological activity and stability.

Conduct a cost-benefit and risk analysis, then develop standard operating procedures (SOPs) and personnel training programs for potential regulatory submission as well as routine implementation.

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