Bacteriocin-Mediated Antagonistic Activity of *Lactococcus lactis S93* against Foodborne Pathogens

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Abstract
Lactic acid bacteria (LAB) have long been recognized for their significant role in food preservation, fermentation and human health. They exhibit antimicrobial properties through the production of organic acids, hydrogen peroxide and bacteriocins which can effectively inhibit the growth of spoilage organisms and pathogenic bacteria. Among LAB, Lactococcus lactis is widely studied for its potential as a natural biopreservative due to its ability to produce antimicrobial peptides known as bacteriocins. This study aims to investigate the antagonistic effect of Lactococcus lactis subsp. lactis S93 against several foodborne pathogens, including Escherichia coli, Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes and Enterococcus faecalis. By utilizing the agar overlay technique, inhibition zones were measured to determine the extent of bacterial suppression. The inhibitory compounds were further characterized by evaluating the effects of neutralized pH and enzymatic treatments. The results demonstrated that Lactococcus lactis S93 effectively inhibited the growth of all tested pathogens, primarily through the production of bacteriocins and organic acids.

Furthermore, the observed effect was determined to be bacteriostatic rather than bactericidal, suggesting its potential application as a biopreservative in food products. These findings contribute to the growing body of research supporting the use of Lactococcus lactis strains for improving food safety and shelf-life. Future research should focus on optimizing bacteriocin production and assessing its efficacy in real food matrices.

Keywords: *Lactococcus lactis* S93, lactic acid bacteria, pathogenic bacteria, bacteriocin, lactic acid, inhibition, antagonism, food preservation.

Introduction

Lactic acid bacteria (LAB) are a diverse group of Gram-positive, facultatively anaerobic microorganisms that play a vital role in food and beverage fermentation. Their ability to convert sugars into lactic acid contributes not only to flavor development but also to the inhibition of undesirable microbial contaminants. Beyond their fermentative capabilities, LAB produced various antimicrobial substances, including organic acids, hydrogen peroxide and bacteriocins, which make them valuable agents in food biopreservation[1,4,10,11,21].

Among LAB, *Lactococcus lactis* has received significant attention due to its broad-spectrum antimicrobial activity. This species is commonly used in dairy fermentation and is recognized for its Generally Recognized As Safe (GRAS) status. One of the key mechanisms through which *Lactococcus lactis* exerts its antimicrobial effects is the production of bacteriocins, small ribosomally synthesized peptides with bactericidal or bacteriostatic activity against closely related or even some unrelated bacterial species[3,19,15,22].

The increasing demand for natural food preservation strategies has fueled interest in LAB as bio-protective cultures. Foodborne pathogens such as *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Enterococcus faecalis* pose significant risks to public health, often leading to foodborne illnesses and outbreaks. Conventional preservation methods, including chemical preservatives and thermal processing, may alter food quality and raise consumer concerns regarding chemical additives. Consequently, alternative approaches such as the application of LAB-derived antimicrobial agents are gaining momentum in the food industry[2,5,8,16].

This study aims to assess the antagonistic activity of *Lactococcus lactis* subsp. *lactis* S93 against selected pathogenic bacteria. Specifically, it seeks to determine the extent of inhibition, identify the key antimicrobial compounds responsible and evaluate whether the inhibition is bactericidal or bacteriostatic. The findings of this research could contribute to the development of natural, LAB-based preservation strategies to enhance food safety and to extend product shelf-life.

Material and Methods

Microbial Strains: The bacterial strains used in this study included *Lactococcus lactis* subsp. *lactis* S93 as the test strain, along with five pathogenic and spoilage bacteria: *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes and Enterococcus faecalis*. The test strain was maintained at -20°C in M17 broth supplemented with 15% glycerol until further use. Pathogenic bacterial

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strains were obtained from the laboratory culture collection. Prior to experimentation, all bacterial strains were revived by subculturing on their respective media and incubated at optimal growth conditions.

Antagonism Tests: The antagonistic activity of *Lactococcus lactis* S93 was evaluated using the well-established Fleming agar overlay method. Briefly, the test strain was first cultured in M17 broth at 30°C for 24 hours. Subsequently, 100 µL of the culture was spread onto M17 agar plates and incubated for another 24 hours to allow bacterial growth. Pathogenic bacteria were separately grown in BHI broth at 37°C overnight and then diluted to an optical density (OD600) of 0.1 to standardize the bacterial load. The prepared pathogen suspensions were then mixed with 0.7% soft agar (BHI) and poured as an overlay onto the M17 agar plates containing the *Lactococcus lactis* S93 colonies. The plates were incubated at 37°C for 24 hours and inhibition zones were measured using a digital caliper to assess the antagonistic effect.

Identification of the Inhibitory Factor: To determine the nature of the inhibitory effect, the culture supernatant of *Lactococcus lactis* S93 was collected by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was filtered through a 0.22 µm membrane to remove any residual bacterial cells before further analysis.

- **Effect of Lactic Acid:** The acidity of the supernatant was neutralized to pH 6.7 using 0.1N NaOH followed by incubation at 30°C for 1 hour. The neutralized supernatant was then tested for residual inhibitory activity using the agar overlay method. The loss or reduction of inhibition zones after neutralization indicated that lactic acid contributed to the antimicrobial activity.
- **Effect of Bacteriocins:** To assess the presence of bacteriocins, the supernatant was treated with pepsin (1 mg/mL final concentration) at 37°C for 2 hours. The enzyme-treated supernatant was then tested against the pathogenic bacteria using the same overlay method. If the inhibition zones disappeared or significantly reduced after pepsin treatment, it suggested that the inhibitory compounds were proteinaceous in nature, confirming the presence of bacteriocins.

Results and Discussion

Antibacterial Activity of Strain S93: Figure 1 illustrates the antibacterial activity of strain S93 against various pathogenic bacteria, as indicated by the formation of inhibition zones. Strain S93 exhibits notable antibacterial effects, leading to visible alterations in pathogen growth. The acid produced by strain S93 selectively inhibits *Listeria* and *Enterococcus*, demonstrating specificity in its antimicrobial action. Furthermore, strain S93 synthesizes a bacteriocin that is highly active against *E. coli* and is sensitive to pepsin digestion (Figure 2).

For *S. aureus*, *B. cereus* and *Listeria*, the inhibition zones are reduced in the presence of pepsin compared to the enzyme-free medium. This suggests the presence of an additional bacteriocin of glycoprotein or lipoprotein nature which does not act on *Enterococcus*. Interestingly, for *Enterococcus*, the inhibition zone in the pepsin-containing medium is larger than in the pepsin-free medium, indicating the possible involvement of other inhibitory factors (Figure 3).

Growth Dynamics in Mixed Culture: Figure 4 presents the interaction between strain S93 and pathogenic bacteria in mixed culture within skimmed milk over time and the results indicate that strain S93 exhibits a time-dependent inhibitory effect on pathogenic bacteria, leading to their eventual suppression in mixed culture. In the presence of strain S93, *Bacillus* initially grows rapidly for the first 2 hours, followed by a slow growth phase until 4 hours, after which a rapid decline occurs, leading to total disappearance at 28 hours.

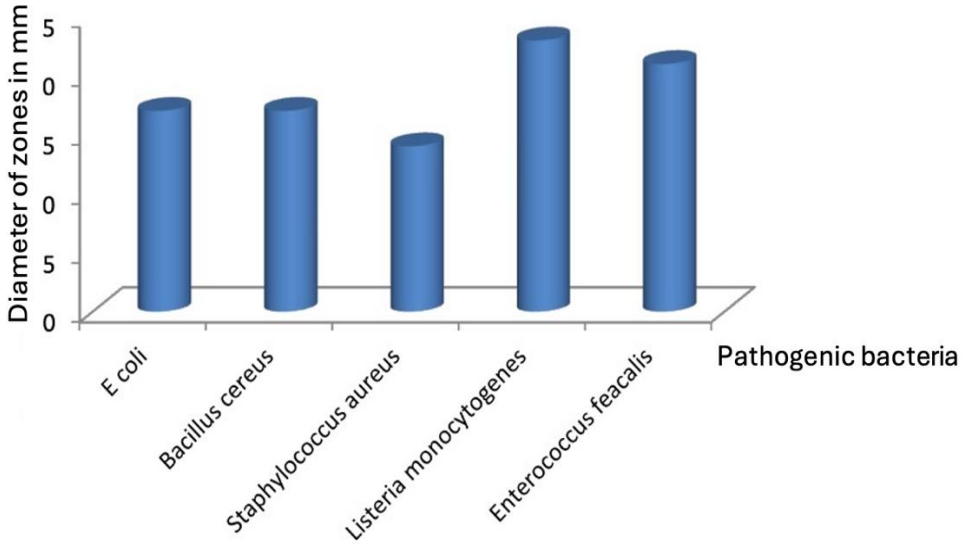

Figure 1: Inhibition diameters formed by strain S93 confronted with harmful bacteria.

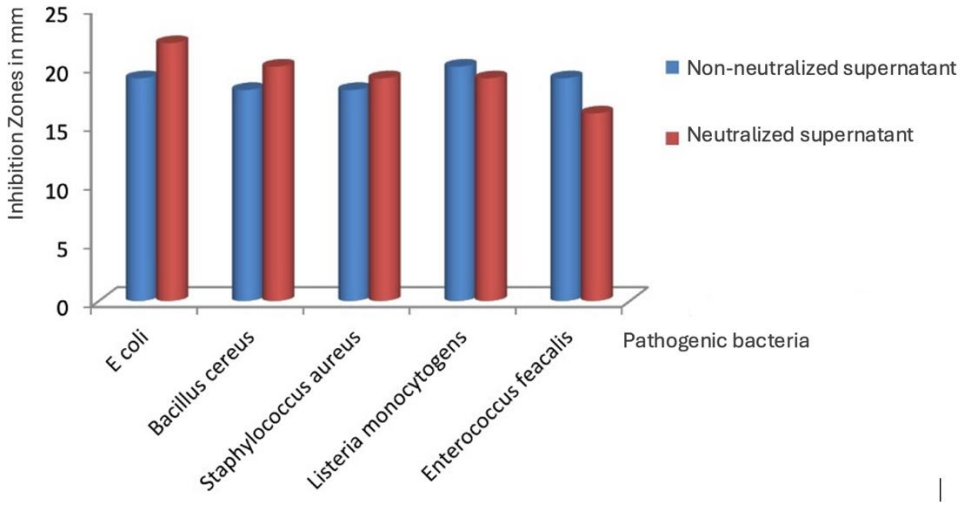

Figure 2: Acid inhibition of pathogenic bacteria.

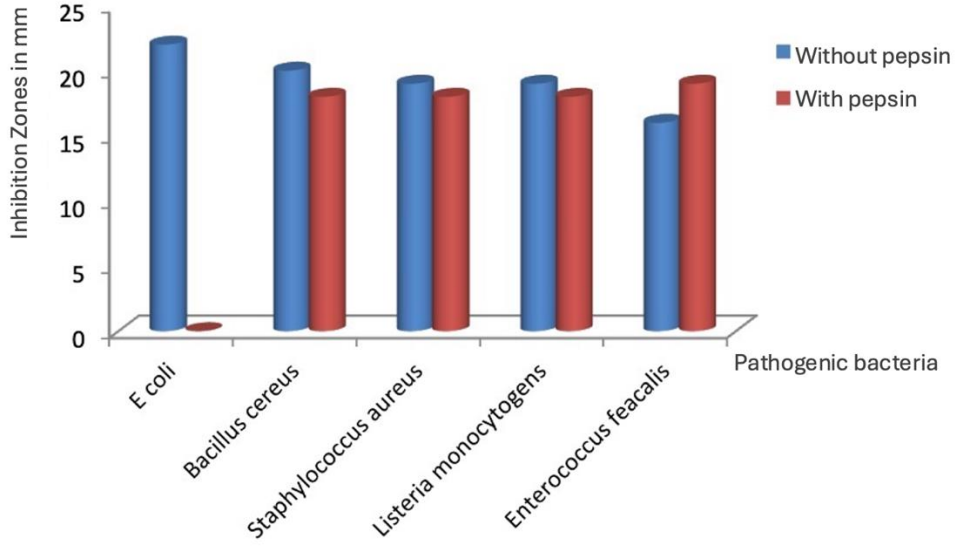

Figure 3: Inhibition of pathogenic bacteria by S93 using pepsin.

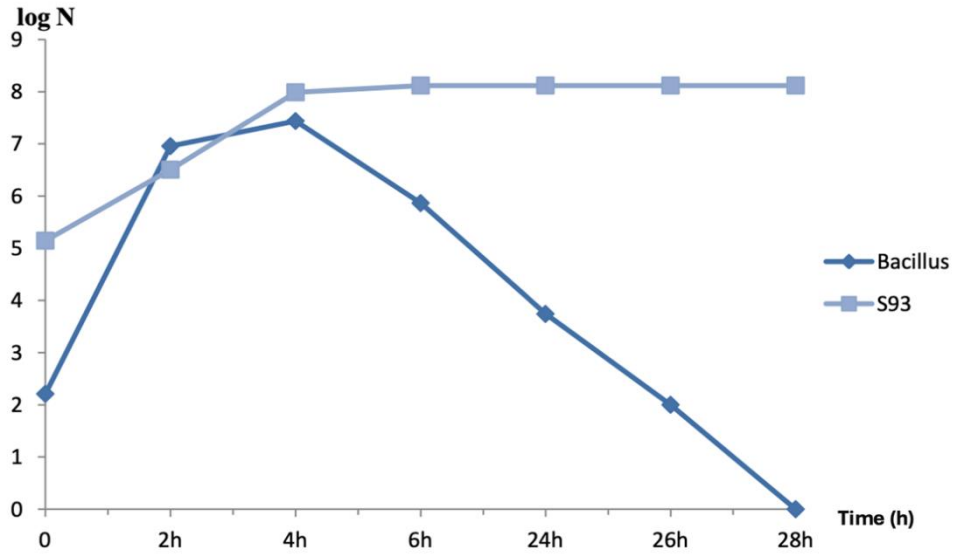

Figure 4: Growth kinetics of strain S93 and Bacillus interacting in skimmed milk

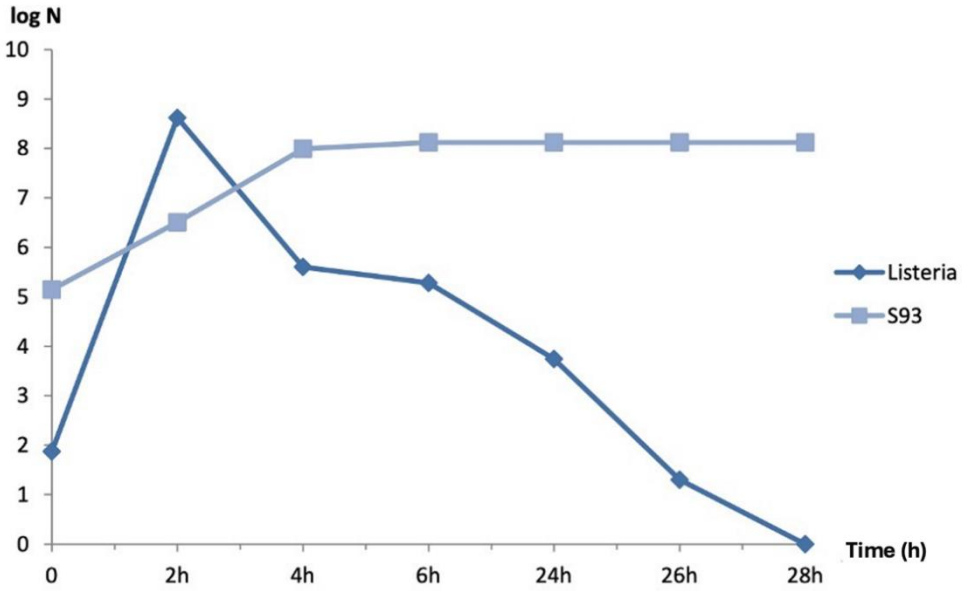

Figure 5: Growth kinetics of strain S93 and Listeria interacting in skimmed milk

For the *Listeria* species, maximum growth is observed within the first 2 hours followed by a steady decline, ultimately resulting in complete elimination at 28 hours. Meanwhile, the lactic strain exhibits continuous growth for 6 hours before reaching a stationary phase (Figure 3). Lactic acid bacteria are known for their ability to inhibit the development of pathogenic bacteria and spoilage in food[7,23], by producing a wide variety of antimicrobial substances.

The results of this study clearly indicate that *Lactococcus lactis* S93 exhibits a strong antagonistic effect against all tested pathogenic bacteria. The inhibition zones measured confirmed the ability of this strain to suppress the growth of *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes and Enterococcus faecalis*. Notably, the largest inhibition zones were observed for *Listeria monocytogenes* (23 mm) and *Enterococcus faecalis* (21 mm), suggesting that these species are particularly susceptible to the antimicrobial activity of *Lactococcus lactis* S93. This aligns with previous studies that highlight the susceptibility of Gram-positive bacteria to bacteriocins produced by lactic acid bacteria[12,14,18].

Further analysis of the inhibitory factors revealed that lactic acid contributes significantly to the suppression of *Listeria monocytogenes* and *Enterococcus faecalis*, as the neutralized supernatant showed reduced inhibition. However, bacteriocin production was confirmed through pepsin treatment, which abolished the inhibitory effect on *Escherichia coli*, indicating that proteinaceous compounds play a role in its suppression. These findings reinforce the importance of bacteriocins in controlling Gram-negative pathogens.

Additionally, the mode of inhibition was determined to be bacteriostatic rather than bactericidal, as regrowth of the pathogens was observed after the removal of inhibition zones. This suggests that while *Lactococcus lactis* S93 can effectively suppress bacterial proliferation, its effect is not lethal. This is consistent with previous research on lactic acid bacteria, where bacteriostatic effects were commonly observed due to competition for nutrients and production of growth-inhibitory compounds[6,13,17,19,20]. The potential applications of *Lactococcus lactis* S93 in food preservation are particularly promising. The significant inhibition observed in milk models indicates that this strain could serve as an effective biopreservative in dairy products, helping to prevent contamination by harmful bacteria. Further research should explore the optimization of its application in food matrices and assess its efficacy in real-world food storage conditions.

Despite the promising antagonistic activity of *Lactococcus lactis* S93 against foodborne pathogens, several limitations must be acknowledged. First, the study primarily relied on *in vitro* agar overlay assays, which may not fully replicate the complex conditions found in real food matrices. The efficacy of *L. lactis* S93 in diverse food systems with varying pH, nutrient composition and microbial interactions remains to be thoroughly evaluated. Secondly, while the inhibition of pathogens was observed, the mode of action was determined to be bacteriostatic rather than bactericidal. This suggests that *L. lactis* S93 may require prolonged exposure or combination with other hurdles (e.g. refrigeration, natural antimicrobials) to ensure effective pathogen control.

Further studies should investigate potential synergistic effects with other food preservation techniques. Another limitation is the partial characterization of the inhibitory compounds. Although the role of lactic acid and bacteriocins was confirmed, the specific identity and structure of the bacteriocins produced by *L. lactis* S93 remain unknown. Advanced molecular techniques such as proteomics and gene sequencing could provide deeper insights into the

nature and biosynthetic pathways of these antimicrobial peptides. Additionally, safety and regulatory aspects must be considered for industrial applications.

Although *L. lactis* is generally recognized as safe (GRAS), strain-specific safety evaluations including potential antimicrobial resistance genes and cytotoxicity tests are crucial before widespread implementation in food products.

Future research should focus on optimizing bacteriocin production by investigating the effects of fermentation parameters such as pH, temperature and nutrient composition to enhance yield and activity. Additionally, *in situ* studies in real food matrices including dairy, meat and plant-based products, are necessary to evaluate the strain's efficacy under practical storage conditions. A deeper mechanistic understanding of *L. lactis* S93's antimicrobial activity through transcriptomic and proteomic analyses could provide valuable insights into its mode of action. Safety and regulatory assessments including whole-genome sequencing to screen for potential virulence or antimicrobial resistance genes, are also crucial for ensuring its suitability for industrial applications. Furthermore, exploring potential synergistic effects with other LAB strains, could enhance its biopreservative potential and broaden its applications in food preservation.

Conclusion

This study highlights the antagonistic potential of *Lactococcus lactis* S93 against several foodborne pathogens. The observed inhibition was primarily due to the production of lactic acid and bacteriocins, which acted in a bacteriostatic manner rather than bactericidal. These findings support the potential application of *Lactococcus lactis* S93 as a natural biopreservative in food products, offering a safer alternative to chemical preservatives. Future research should focus on optimizing bacteriocin production, investigating its mechanism of action at the molecular level and conducting *in situ* trials in food matrices to confirm its efficacy under real-world conditions.

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(Received 10th April 2025, accepted 15th June 2025)