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PRELIMINARY INVESTIGATIONS REGARDING THE ANTIBIOFILM EFFECT OF CHITOSAN-BASED BIOMATERIALS

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Abstract

The purpose of this study was to assess the antibiofilm effect of chitosan-based materials, including chitosan films (Chi), as well as chitosan-nisin films at concentrations of 5 mg/mL (ChiN5) and 10 mg/mL (ChiN10). Chitosan, a natural polymer, finds applications in tissue engineering and regenerative medicine, either alone or in combination with other polymers. Current research seeks to improve chitosan-based materials for wound healing, bone, and cartilage regeneration. Before proceeding with experiments to evaluate the antibiofilm properties of chitosan-based materials, preliminary tests were conducted to measure the antibacterial and antibiofilm effects of nisin on three selected bacterial strains. Monitoring took place at 24 and 48 hours to observe if the antibacterial effect depends on exposure time. The efficacy of nisin was determined through Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) methods. To investigate the antibiofilm potential of chitosan-based materials, a static bacterial biofilm cultivation method was utilized. Bacterial biofilm on chitosan materials was visualized using the TTC assay, and the impact of chitosan films on biofilm formation in wells was assessed using the Crystal Violet staining method. The results revealed that the nisin solution exhibited a pronounced antibiofilm effect against both Gram-positive and Gram-negative bacteria. However, concerning the antibiofilm effect of chitosan-based biomaterials, the findings showed that none of the chitosan-based films completely inhibited bacterial biofilm formation due to slow release of nisin from their structure. These preliminary results serve as a launching point for further research into the antibiofilm properties of chitosan-based materials, contributing to the understanding of the intricate interaction between chitosan-based biomaterials and bacterial biofilm formation, thereby paving the way for the development of innovative therapeutic strategies to combat biofilm-associated infections.

Introduction

Bacterial biofilms are complex aggregates of microorganisms surrounded by an extracellular polymeric substance, providing protection against external factors. The ability of the biofilm to resist various extreme conditions, including radiation, extreme temperatures, and chemicals, is highlighted in studies. Chitosan, a polysaccharide derived from chitin, is presented as a substance with antimicrobial potential. Mechanisms through which chitosan inhibits microbes include interactions with the negatively charged functional groups of microbial cell membrane proteins, causing changes in cell surface and permeability. Several experiments have emphasized the importance of these mechanisms in chitosan-induced membrane damage.

Material and method

The experiments involved evaluating the antibacterial and antibiofilm effects of nisin on three bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*). Nisin's efficiency on planktonic cells was evaluated using the Minimum Inhibitory Concentration (MIC) determination method. A nisin stock solution (5 mg/mL) was tested at eight concentrations. After measuring optical density at 620 nm, samples were transferred to solid medium using a 96-pin replicator. Plates were incubated overnight at 37°C, and MIC and Minimum Bactericidal Concentration (MBC) values were determined based on visual assessment the following day. The antibiofilm effect of nisin was assessed in 96-well microplates using Crystal Violet staining. Successive dilutions of nisin (0.078 mg/mL - 5 mg/mL) were tested against bacterial strains (*E. coli*, *P. aeruginosa*, *S. aureus*). After 48 hours of biofilm formation and washing, the wells were fixed, stained with 0.25% Crystal Violet, and dried. The biofilm was solubilized with 30% acetic acid, and absorbance was measured at 550 nm compared to a control using 30% acetic acid.

Chitosan-based materials, including chitosan films (Chi) and chitosan + nisin at concentrations of 5 mg/mL (ChiN5) and 10 mg/mL (ChiN10), were investigated for their antibiofilm effect on various bacterial strains. The strains included *Bacillus cereus* DSM 4312, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* MRSA U50, *Enterococcus faecium*, *Enterobacter cloacae* DSM 106614, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853.

The bacterial biofilm on chitosan materials was highlighted using triphenyl tetrazolium chloride (TTC). The experiment took place in 48-well microplates, where chitosan fragments were exposed to bacterial strains in TSB medium for 48 hours at 37°C. After incubation, the chitosan films were washed and then treated with a 0.5% TTC solution in TSB for 4 hours. The biofilm on the surface of the films was visualized by turning red, indicating the presence of bacteria attached to the chitosan materials.

To examine the effect of chitosan films on biofilm formation in wells, the Crystal Violet staining method was employed. After removing the chitosan films from the wells, the protocol described earlier, involving washing the microplates and staining the biofilm formed in the wells, was followed.

Conclusions

In preliminary experiments, a nisin solution demonstrated a 100% inhibitory effect on both Gram-positive and Gram-negative bacterial strains at the maximum tested concentration (5 mg/mL). However, chitosan-based films did not completely inhibit bacterial biofilm formation, attributed to the slow release of nisin from the films. These findings suggest avenues for further research to optimize concentrations and usage strategies for chitosan biomaterials in achieving an effective antibiofilm effect.

Acknowledgement:

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Results and discussions

The initial experiments focused on evaluating the antibacterial effects of nisin in solution, determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Monitoring at 24 and 48 hours revealed that MIC was consistently reached at 1.25 mg/mL for all strains. These results (Figures 1, 2, 3) underscored the potent antibacterial impact of nisin, extending even to Gram-negative strains. For MBC determination, samples were transferred using a 96-pin replicator, confirming MBC at 2.5 mg/mL. At 1 mg/mL nisin, bacterial growth inhibition was approximately 90%, reaching 100% at the maximum tested concentration. Regarding the antibiofilm effect (Figure 6), nisin concentrations between 0.078 mg/mL and 0.625 mg/mL exhibited up to 30% inhibition, while higher concentrations (1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL) inhibited biofilm formation by around 80%, falling short of complete inhibition. Analyzing the biofilm on chitosan films, none of the materials completely prevented bacterial biofilm development. Crystal Violet staining (Figure 7) revealed weak biofilm inhibition, indicating a gradual release of nisin at low concentrations from chitosan films, resulting in a reduced antibiofilm effect.

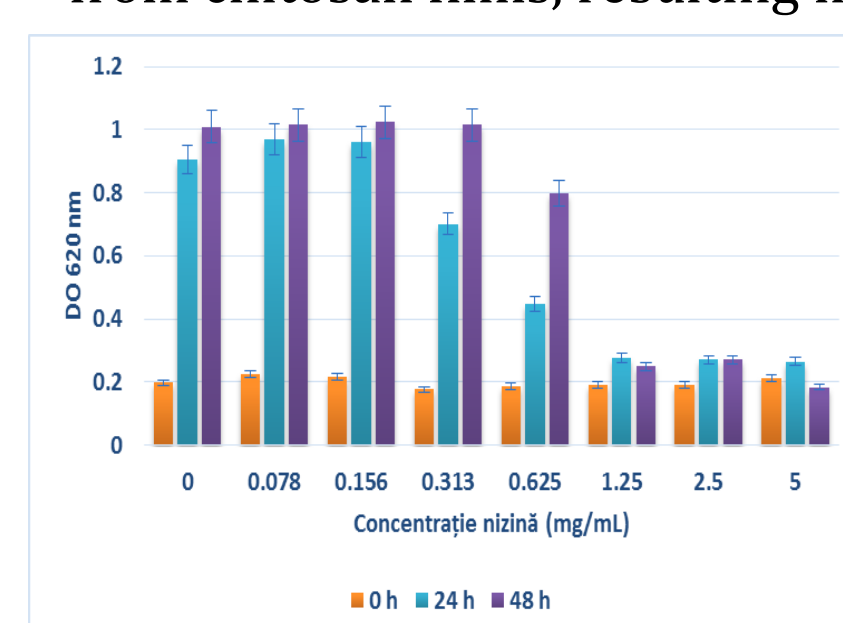


FIG. 1. Minimum Inhibitory Concentration (MIC) of nisin on *E. coli*.

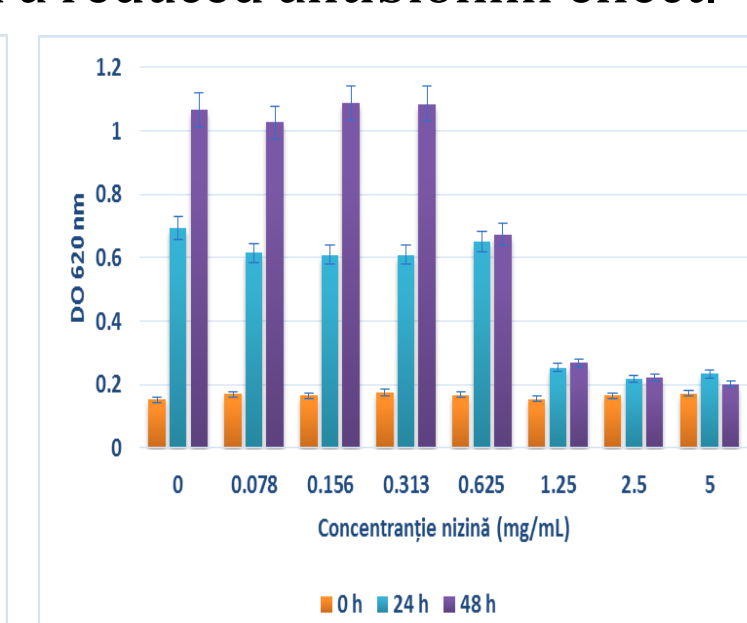


FIG. 2. Minimum Inhibitory Concentration (MIC) of nisin on *Pseudomonas aeruginosa*.

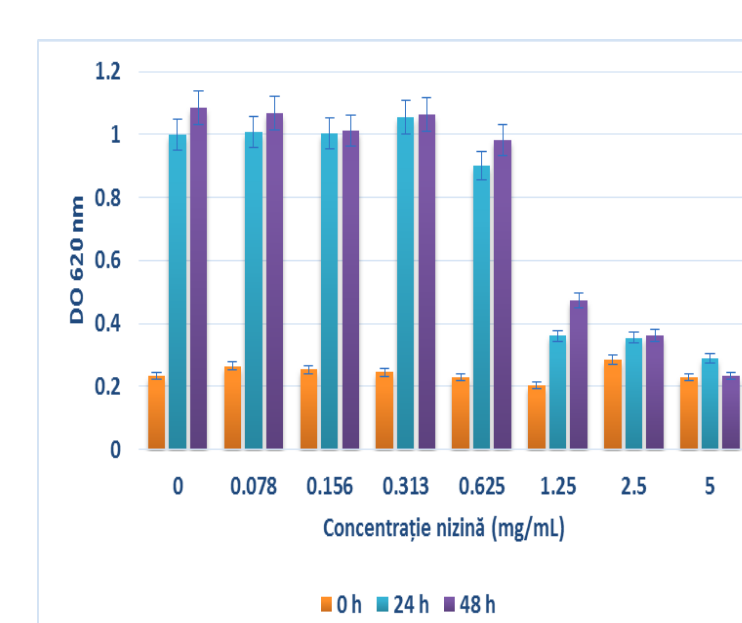


FIG. 3. Minimum Inhibitory Concentration (MIC) of nisin on *S. aureus*.

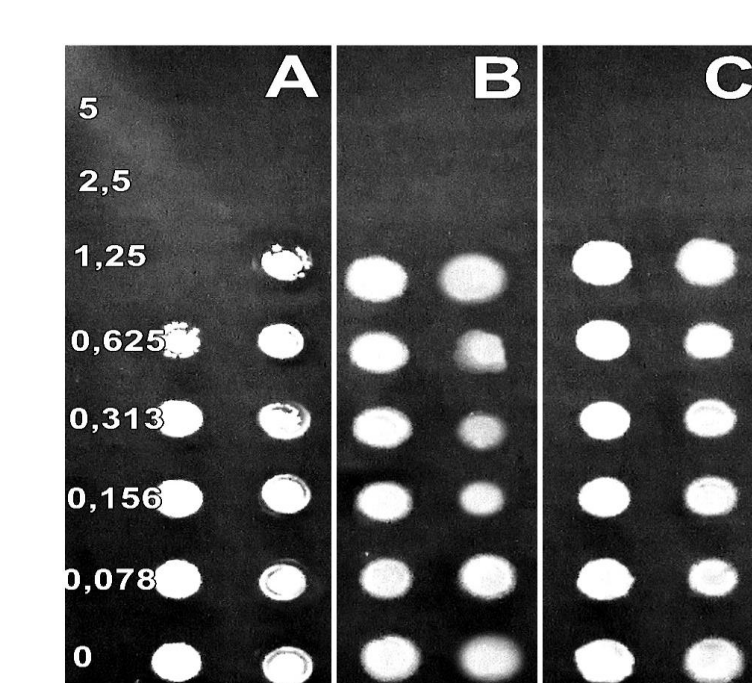


FIG. 4. Assessing the Minimum Bactericidal Concentration (MBC) of nisin on bacteria *E. coli* (A), *P. aeruginosa* (B), and *S. aureus* (C).

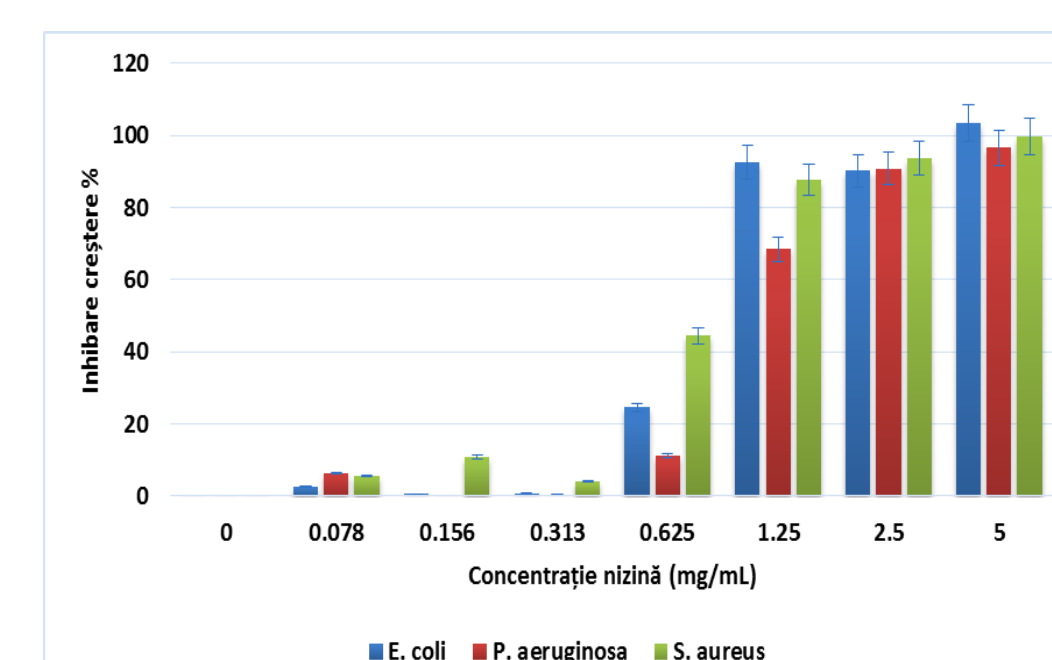


FIG. 5. The Effect of Nisin on Bacterial Growth: Highlighting the Percentage of Bacterial Growth Inhibition for *E. coli*, *P. aeruginosa*, and *S. aureus* Strains

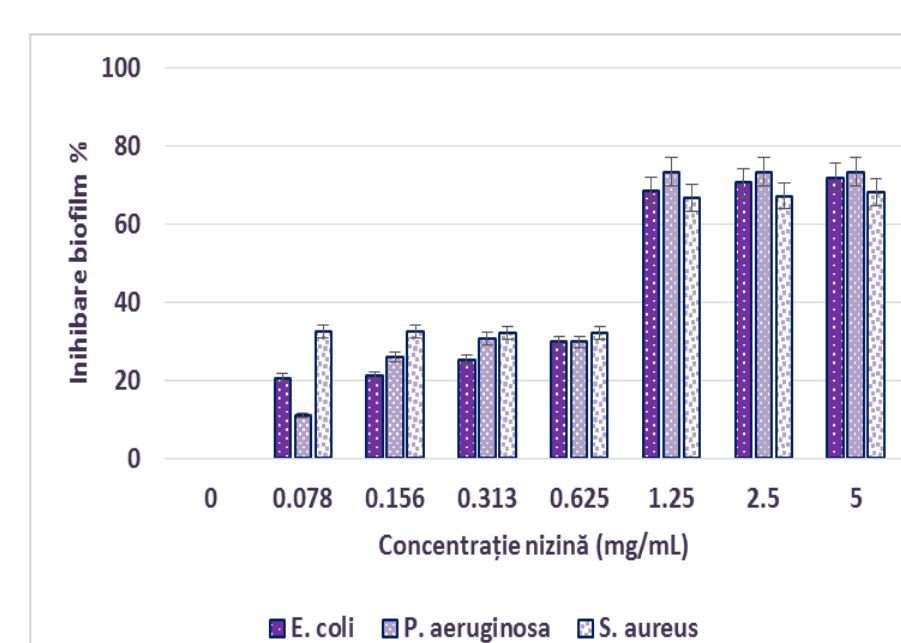


FIG. 6. The Antibiofilm Effect of Nisin: Inhibiting Bacterial Biofilm Formation for *E. coli*, *P. aeruginosa*, and *S. aureus* Strains

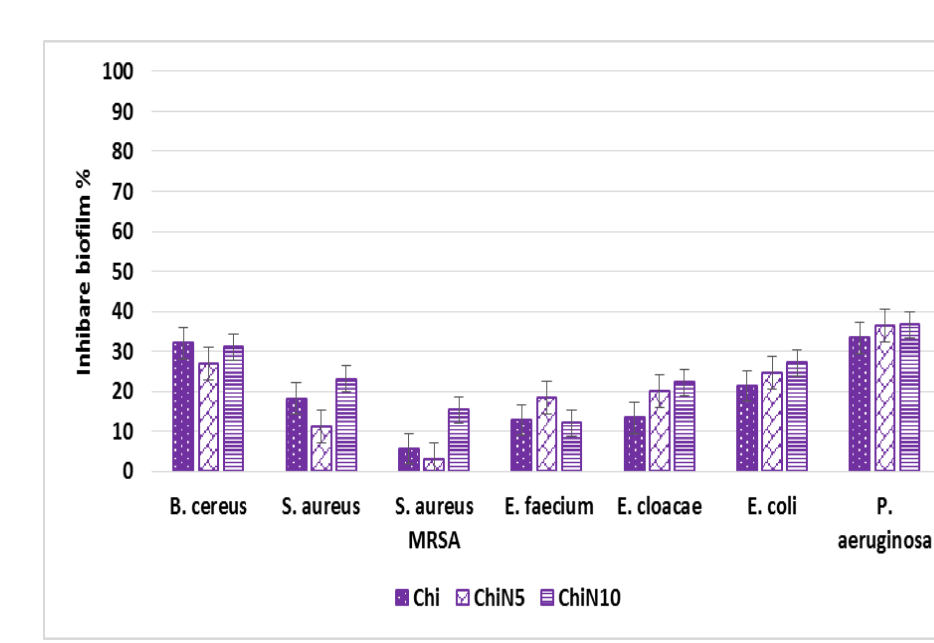


FIG. 7. Antibiofilm effect of Chitosan-Based Films: Highlighting Biofilm Formation in Microplate Wells