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Breaking the biofilm barrier: harnessing PVA-Nisin technology to safeguard dairy products from persistent pathogens

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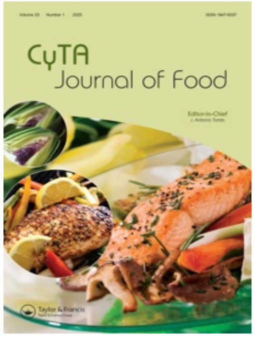

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Breaking the biofilm barrier: harnessing PVA-Nisin technology to safeguard dairy products from persistent pathogens

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ABSTRACT

This study explores the fabrication, characterization, and antimicrobial properties of polyvinyl alcohol (PVA)-based films incorporating nisin. Films prepared via casting display smooth, transparent surfaces, with Scanning Electron Microscopy (SEM) showing increased surface roughness upon nisin addition. Contact angle analysis confirmed enhanced hydrophilicity ($p \le .05$), while Fourier Transform Infrared Spectroscopy (FT-IR) revealed that the casting method preserved the chemical integrity of PVA and nisin. Mechanical testing demonstrated reduced Young's Modulus, suggesting improved elasticity due to nisin's plasticizing effect. Biofilm assays with *Staphylococcus aureus* and *Listeria monocytogenes* showed significant reductions in biofilm populations ($p \le .05$) within 24–48 hours. PVA-nisin films achieved up to 99.98% (3.8 log) and 99.84% (2.8 log) reductions for *Staphylococcus aureus* and *Listeria monocytogenes*, respectively. These findings highlight the potential of PVA-nisin films as antimicrobial materials, providing sustained nisin release to inhibit biofilm formation in dairy food processing environments.

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Antibiofilm material; PVA/nisin film; *Listeria monocytogenes*; *Staphylococcus aureus*; biofilm

Introduction

Biofilm is the main cause of microbial contamination in dairy products since it is found at any point in the production chain and transcends a public health order (Cancino-Padilla et al., 2017). Its presence determines the quality and safety of the product and affects the economic viability of production and the sustainability of the industry (Cancino-Padilla et al., 2017; Flint et al., 2019; Ibarra-Sánchez et al., 2020).

In the dairy industry, *Listeria monocytogenes* and *Staphylococcus aureus* are the most common food pathogens among the 31 identified. Which originate in primary production and is maintained throughout the production process, turning it into a source of pollution. These microorganisms are the cause of the recalls that the dairy industry receives year after year. Process, material, and design failures, in combination with non-compliance with good manufacturing practices, provide favorable conditions for their growth, development, and permanence through the formation of altruistic communities known as biofilm. In addition, handlers act as bridges between food products and microorganisms in any link of the food chain (Paula et al., 2017).

Biofilm is complex, and structured and confers protection on the cells within itself. In this sense, these sessile cells are more resistant to disinfectants and stresses produced by dynamic or hostile factors compared to their planktonic state (Clayton et al., 2021; Merino et al., 2024). Furthermore, the prevalence and resistance of microorganisms with the ability to form altruist communities within a biofilm have many advantages compared to their planktonic stage (Bhosale et al., 2020). Wirtanen and Salo (2016) indicate that this phenomenon is generated because the bacteria within the altruistic communities formed manage to increase their resistance to cleaning agents through defense and regulation mechanisms. While the internal cellular communication system alerts about external stimuli that endanger its survival, the EPS matrix is simultaneously strengthened (Ripolles Avila et al., 2020; Srey et al., 2013; Wirtanen & Salo, 2016).

On the other hand, the prevalence of *Listeria monocytogenes* and *Staphylococcus aureus* in the dairy industry is due to the ability of both microorganisms to adhere to various materials that are part of the machinery and accessories of the food industry (Ripolles Avila et al., 2020). Colagiorgi et al. (2017) show in their study the survival of *Listeria monocytogenes* over time on surfaces such as stainless steel, polystyrene, and glass, materials widely used in the food industry. Similarly, Papadopoulos et al. (2019) isolated strains of *Staphylococcus aureus* from surfaces such as stainless steel, polytetrafluoroethylene (PTFE), polyurethane, and silicone from four plants in north-central and northeastern Greece. In this way, several authors agree on the presence of said microorganisms on surfaces that are in direct and indirect contact with the food being transformed (Antoci et al., 2019; Ferreira et al., 2014; Melero et al., 2019; Oxaran et al., 2017).

Listeria monocytogenes and *Staphylococcus aureus* are particularly difficult to remove from processing surfaces in the

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dairy industry, making them part of the environmental microbiota (Iñiguez-Moreno et al., 2018; Ribeiro et al., 2023). An innovative strategy to prevent the adhesion of microorganisms to the surface is to use antimicrobial peptides (AMP) (Hage et al., 2021). In polymeric matrices, PVA can incorporate a high variety of antimicrobial peptides, such as nisin, chitosan, ε-polylysine, and enterocin-A, into its matrix to develop active polymer matrices (Aymerich et al., 2022; Marvdashti & Koocheki, 2019; Sharma et al., 2023; Yurong & Dapeng, 2020). Due to the high degree of hydroxylation of its molecular chain, PVA-based materials are highly permeable to water vapor, have a high degree of solubility in water, and good physical and chemical properties, biocompatibility and biodegradability, which makes them suitable for the food industry (Andrade et al., 2020; Zanela et al., 2018).

Nisin, an example of these peptides, can be incorporated into materials (Hage et al., 2021; Mauriello et al., 2005; Sebti et al., 2003). It is a peptide that is attributed to antimicrobial properties due to the formation of pores in the cell membrane of microorganisms. Likewise, it is the only bacteriocin approved by the FDA for use in the food industry and has been shown to maintain its activity when incorporated into different types of polymer matrices (Hage et al., 2021; Wu et al., 2023).

Therefore, the main objective of the study is to develop a PVA polymer matrix incorporating nisin to prevent the formation of biofilm generated by *Listeria monocytogenes* and *Staphylococcus aureus*.

Materials

For manufacturing polymeric films, polyvinyl alcohol (PVA, 98–99% hydrolysis degree, Sigma Aldrich), glycerol (Winkler, Santiago-Chile), and nisin ($C_{143}H_{230}N_{42}O_{37}S_7$, N°CAS1414-45-5, Chr. Hansen holding A/S, Dinamarca) were used. Trypticase soy agar and 0.6% yeast extract from Becton (Dickinson and Company) were used as a culture medium. Strains of *Listeria monocytogenes* (ATCC 13,932) and *Staphylococcus aureus* (ATCC6538) were supplied by the Public Health Institute of Chile (ISP-Chile).

Preparation of PVA-based film

Polyvinyl alcohol films were prepared using the solvent casting method according to (Marvdashti & Koocheki, 2019) with modifications. A mixture of PVA (3.6% w/w) and glycerol (0.4% w/w) was hydrated for 24 h in deionized water, after that, this mixture was heated to 90°C and stirred at 300 rpm for 15 min on a hot plate (Nuova II Stirring Hot Plate type 18,400). Subsequently, the mixture was stirred at room temperature for 15 min and 75 mg of powdered nisin was incorporated. The mixture was poured onto 9 cm diameter polystyrene Petri dishes, which were allowed to dry at 40°C for 24 h. The final concentration of nisin in the film matrix was 5,000 IU mL^{-1}, determined based on previous experiments using the Kirby-Bauer disk diffusion method. In these experiments, 3,000, 5,000, and 10,000 IU mL^{-1} of nisin were tested to establish the minimum inhibitory concentration (MIC) required for effective bacterial inhibition.

PVA-based films characterizations

The surface morphology of the PVA films was assessed using a scanning electron microscope (SEM, AURIGA®, Zeiss). The samples were cut into small pieces and placed on a 12.7 mm diameter sample holder (specimen mounts, 12.7 mm, Ted Pella) using carbon tape (EMS). The materials were visualized using different magnifications (100, 500, 2,000, 6,000, and 10,000×) and with an accelerating voltage of 1.00 kV (Romero-Araya et al., 2021). The wettability of PVA films was characterized using a contact angle meter (Contact Angle Meter, Holmarc HO-IAD-CAM-01), equipped with a high-speed camera, a light source, and a 100 µL microsyringe. The materials were cut into pieces of approximately 2·2 cm^2 and stored in a desiccator for 24 h. Then, the samples were mounted on the equipment, and a drop of water was deposited on the surface of the film. The evolution of the drop of water on the materials was recorded in a video program at 7 frames per second (fps) and with a resolution of 2592·1944 bits. The frames were subsequently analyzed to capture the exact moment in which the drop landed on the material, using the ImageJ software (v.1.53k), which allowed the calculation of the angle generated between the drop tangent and the material (Romero-Araya et al., 2021). Both the reagents used for the manufacturing of PVA-based films and PVA films were characterized through Fourier transform infrared spectroscopy (Jasco FT/IR-4600) equipped with an attenuated total reflectance accessory (Jasco ATR PRO 450-S). A scan was performed from 4000 to 500 cm^{-1} with 35 accumulations, at a scanning speed of 2 mm s^{-1}. The assignment of the absorption bands of the infrared spectra for the PVA-based films was made by comparing the spectra of the films with those of the pure substances. The spectra were processed using the Spectra Analysis program (Jasco, version 2.15.15). The mechanical behavior of PVA-based films was studied using stress-strain tests with a universal test texturometer (CT3–1000 Texture analyzer, Brookfield). To measure the mechanical properties of the materials, a tensile test was used. Films were cut with a standard design (ASTM D638, type V, dog-bone), a shape that promotes the material cut in the central section. Films were strained up to 100% deformation with an activation load of 1.0 g and a deformation speed of 0.50 mm s^{-1}. Young's modulus was calculated using the slope of the line segment of the stress-strain graph (Romero-Araya et al., 2021).

Staphylococcus aureus *and* Listeria monocytogenes *biofilm production*

Biofilm production of *Staphylococcus aureus* and *Listeria monocytogenes* on the surface of PVA-based films was assessed following the method proposed by Prieto-Borja et al. (2017) with some modifications. For this, isolated colonies from a trypticase soy agar plate with yeast extract were suspended in 9 mL of BHI broth and incubated for 24 h at 37°C. Subsequently, PVA-based films dimensions were according to the area needed to inhibit microorganisms established in Table 1, which were suspended in 10 mL of BHI broth with inoculum standardized at 10^6 CFU mL^{-1} for 24 h at 37°C. After incubation, the films were removed and washed 3 times with phosphate buffer to remove adherent planktonic cells. Then, the films were sonicated in test tubes with 9 mL of phosphate buffer for 5 min. The sonicated fluid

Table 1. Inhibition Area (cm^2) of Nisin-loaded PVA films against *Listeria monocytogenes* and *Staphylococcus aureus*.

Microorganism	Polymer	IU nisin mL^{-1}	Area needed to inhibit microorganisms (cm^2)
Staphylococcus aureus	PVA	5000	12.7
Listeria monocytogenes	PVA	5000	25.4

was serially diluted. Cycles of 24, 48, and 72 h of culture were carried out. The experiments were performed in triplicate for each strain. The quantification of recovered bacteria was carried out through the microdrop method on the agar plate.

Statistical analysis

All measurements in this study were conducted in triplicate. Additionally, the resulting data were statistically analyzed using an analysis of variance (ANOVA) with LSD (Least Significant Difference) multiple comparison tests to determine significant differences at a 95% confidence level. Statgraphics Centurion XVI software was used for statistical analysis, and Origin Pro 8 was employed for graphing the results.

Results and discussion

PVA-based film manufacturing and characterization

The casting method was used to manufacture PVA films shown in Figure 1, taking into consideration the easiness of this method. At first sight, these are smooth, homogeneous, and transparent. It is noteworthy to highlight that the presence of nisin did not affect the appearance of PVA films. However, through the images obtained by SEM, it is possible to observe that the surface of these films changes when nisin is included. SEM images for the control film (prepared without nisin, Figure 1) show a smooth and homogeneous surface in contrast to those PVA films prepared in the presence of nisin (Figure 2(b.1)) which shows a rough surface at 500× magnification. Then, at 20,000× (Figure 2(b.2)), a significant influence of the addition of nisin is observed. Nostro et al. (2010) present SEM micrographs that resemble those observed in our current study. A high level of nisin results in the presence of rough and uneven surfaces.

Contact angle

The hydrophilic or hydrophobic nature of a material is determined by the intermolecular interactions occurring between a liquid and a solid surface. When water is used as the liquid, the wettability of a solid material can be assessed by evaluating water-surface interactions. Figure 2 presents the contact angle measurements of water droplets deposited on PVA-based films, both in the absence and presence of nisin. The incorporation of nisin into the PVA film formulation resulted in a significant reduction ($p \leq .05$) in the contact angle compared to the control PVA film. This decrease in contact angle indicates that films containing nisin exhibit greater hydrophilicity than pristine PVA films. These findings confirm that nisin itself is a hydrophilic compound, contributing to the enhanced wettability of the modified PVA films. Similarly, Dong and Yang (2015) show a similar effect of the contact angle on surfaces of multilayer nanotubes incorporating nisin (MWCN TS-nisin) indicating that nisin, a macromolecule contains 34 amino acid residues, hydrophobic at the N-terminal and hydrophilic at the C-terminal, allows affinity between the polymer and the nisin. Instead, Ollé Resa et al. (2014) showed an increase in the contact angle in starch and nisin matrices, highlighting that the decrease in water sorption is related to a more hydrophobic surface.

Infrared spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) is a very useful spectroscopic technique to characterize organic and

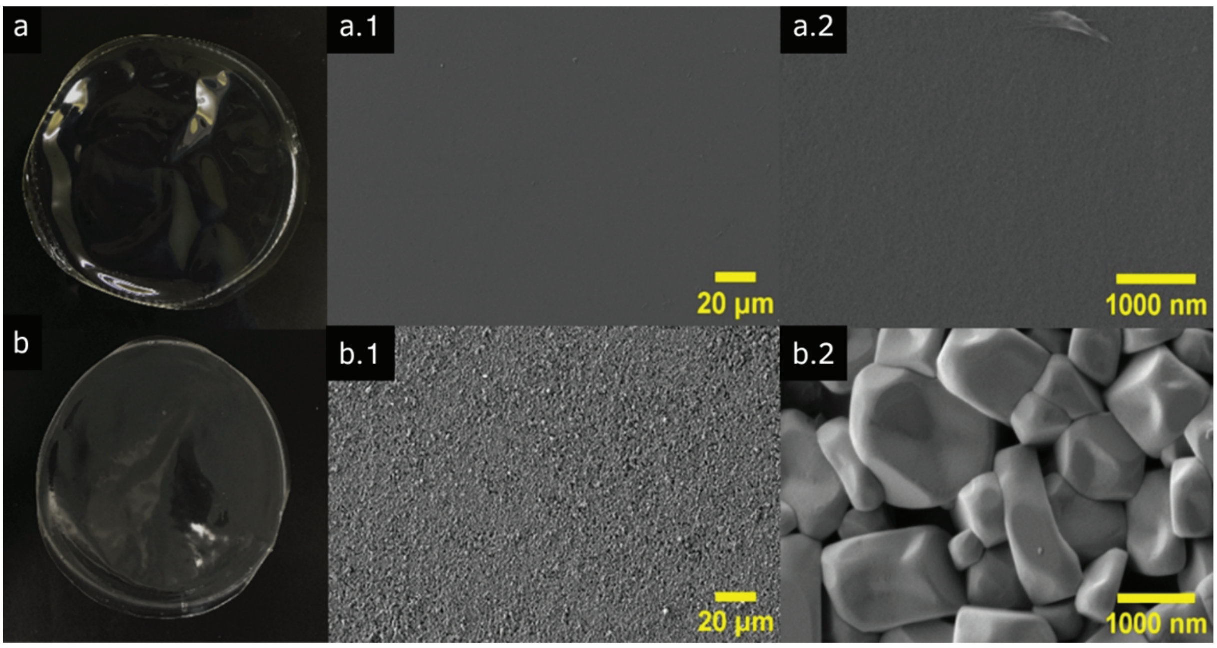


Figure 1. Appearance of PVA-based film manufactured using the casting method. (a) pristine PVA film; (a.1) PVAC-500x; (a.2) PVAC-20,000x; (b) PVA NISIN 5,000 IU mL^{-1}; (b.1) PVA NISIN-500x; (b.2) PVA NISIN-20,000x.

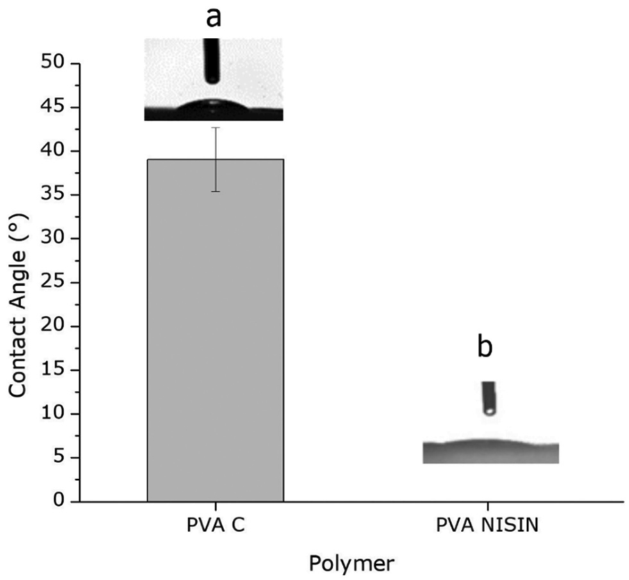


Figure 2. Contact angle: control PVA surface (PVA C); PVA Nisin surface (5,000 IU mL^{-1}). Data were statistically analyzed with the multiple range test using the Fisher least significant difference (LSD) method. Different letters in a column show significant differences ($p \leq .05$).

inorganic compounds since each chemical compound can have a characteristic FT-IR fingerprint, associated with the molecular structure and type of groups functional in the molecule or compound. Figure 3 shows the FT-IR spectra of the raw materials used to prepare the films (PVA polymer and nisin), in addition to the PVA-based films prepared by solvent casting. Considering that the films are prepared using different components, the FT-IR spectrum allows the identification of the different and characteristic vibration signals of the compounds added in the film formulation. Although films are made by dissolving a polymer and then evaporating the solvent, the spectrum of PVA films shares the same absorption bands as pure or unprocessed polymers, indicating that solvent solvent-casting method did not affect the chemical structure of PVA and nisin. The characteristic functional groups of PVA detected by FT-IR are mainly aliphatic (C-H) and ether (C-O) groups, which present vibrational bands in the IR spectrum located at the wavelengths of 2978 and 1074 cm^{-1}, respectively. The presence of primary amide (3400 cm^{-1}), a characteristic functional group of nisin, is attenuated by the characteristic absorption bands of alcohols (stretching of the -OH group).

Mechanical properties

The mechanical properties of a material are quantified by measuring the response of the material after applying deforming forces, for example, a uniaxial deformation, also called the tensile test, which allows evaluation of the hardness of a material through Young's modulus. In Table 2 are listed the Young Modulus values obtained after the tensile test for pristine PVA films and those prepared with nisin. From these results, we observed that the incorporation of nisin into the PVA-based films led to a significant decrease in the film's Young's Modulus (YM) resulting in improved elasticity compared to the one prepared in the absence of nisin. The mechanical properties of a film are generally influenced by various factors, including interactions between the functional groups of the polymers, moisture content, microstructure, mobility of polymer chains, and the ratio of crystalline/amorphous and hydrophilic/hydrophobic groups (Imran et al., 2010; Zhang et al., 2020; Zimet et al., 2019). The addition of nisin likely alters the film network by reducing the intramolecular interactions between polymer chains and diminishing the amount of interchain hydrogen bonds. Thus, these results suggest that the combination of nisin and glycerol has a plasticizing effect on the PVA matrix. Similar results were reported by Sebti et al. (2003) in their study on hydroxypropyl methylcellulose films.

Biofilm formation on the surface of PVA-based film

To assess the interaction of polyvinyl alcohol (PVA)-based films with pathogenic microorganisms, such as *Listeria*

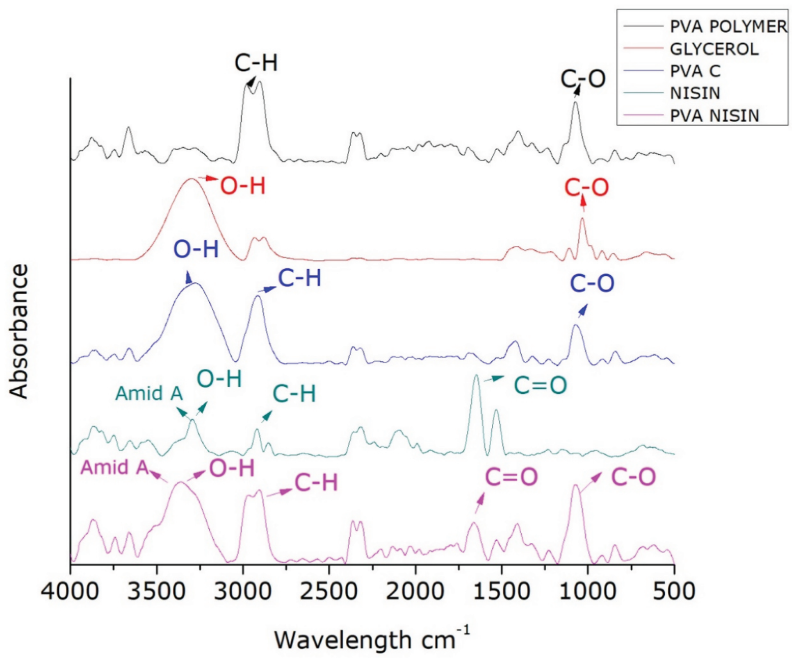


Figure 3. FT-IR spectroscopy of individual and aggregate compounds in polymeric matrices.

Table 2. Young modulus (kPa) and elongation (%) of PVA-based films measured by tensile test.

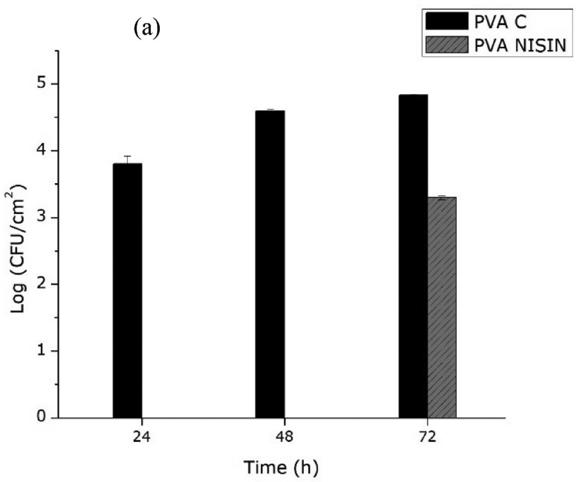
Polymer	Young's modulus (kPa)	Elongation (%)
PVAC	32.61 ± 1.93^{a}	121.00 ± 3.8^{a}
PVA NISIN	27.07 ± 0.21^{b}	124.00 ± 2.3^{a}

Each value represents the average of 3 replicates with its corresponding standard deviation. Data were statistically analyzed with the multiple range test using the Fisher Least Significant Difference (LSD) method. Different letters in a column show significant differences ($p \leq .05$).

PVAC: control film PVA; PVA NISIN: film with active agent (nisin).

monocytogenes and *Staphylococcus aureus*, which are of significant concern in food industries due to their ability to colonize and persist within processing environments (Colagiorgi et al., 2017; Dittmann et al., 2017; Fagerlund et al., 2021), biofilm formation assays were conducted. As a preventive measure during the early stages of biofilm formation by these pathogens, one potential approach could be the inclusion of nisin in polymeric matrices (Hage et al., 2021; Shiroodi et al., 2016; Yu et al., 2020). To form biofilm on the PVA polymer matrix, the samples were submerged in inoculums of *Staphylococcus aureus* and *Listeria monocytogenes* standardized at $1{\cdot}10^{6}$ CFU mL^{-1} for 24, 48, and 72 h. The results were obtained through plate counting of viable cells, expressed as CFU·cm^{-2}. Both *Staphylococcus aureus* and *Listeria monocytogenes* biofilm populations were significantly reduced ($p \leq .05$) during the first 24 and 48 hours in the presence of PVA films containing nisin. The PVA film incorporating nisin demonstrated a 99.98% (3.8 log) reduction in CFU counts (Figure 4) for *Staphylococcus aureus* biofilm, compared to the PVA film without nisin or the control ($p \leq .05$), at 24 hours. Furthermore, the PVA-nisin film achieved a 99.84% (2.8 log) reduction in *Listeria monocytogenes* biofilm CFU at 48 hours. At 72 hours, reductions in CFUs for both *Staphylococcus aureus* and *Listeria monocytogenes* were less than 1 log, in contrast to the control, where no inhibition was observed. These findings suggest that the incorporation of nisin into the polymeric matrix effectively prevents the initial adhesion of the microorganisms under study, providing a sustained antimicrobial action. Several authors prove the effectiveness of biocidal agents incorporated into polymeric matrices that allow a progressive release over time (Jin & Liu, 2020; Jin et al., 2009). In this context, Jin et al. (2010) incorporated potassium sorbate in PLA containers showing an effective release over time against bacterial populations in strawberry pulp in contrast to the direct addition of the preservative. Correia et al. (2015) reported a progressive release of nisin for up to two weeks in PLGA-nisin matrices. Hrabalikova et al. (2016) indicated a cumulative release of nisin in PVA-glutaric acid films after 4 hours, indicating that the trapped nisin molecules require time to be released from the inner core of the polymeric matrix, obtaining the best results at 24 h.

In addition, this analysis showed that the *Staphylococcus aureus* biofilm registered a greater number of viable cells over time ($p \leq .05$) compared to the *Listeria monocytogenes* biofilm, both for the matrix with nisin and for the control film, suggesting that *Listeria monocytogenes* present non-biofilm-forming capacity on its own. Several authors have shown that serotype 4b of *Listeria monocytogenes* is not a good biofilm maker, unlike type 1/2c strains (Borucki et al., 2003; Huang et al., 2018). In the same way, in the environment of the food industry, *Listeria monocytogenes* seeks to make itself compatible with other microorganisms to remain in said environment. These microbial communities provide a protective environment, allowing *Listeria monocytogenes* to persist in food processing facilities and other industrial settings (Fagerlund et al., 2021) pointed out that the affinity of *Listeria monocytogenes* and *Pseudomonas spp*. leads to the formation of a robust biofilm that protects against desiccation and disinfection agents.



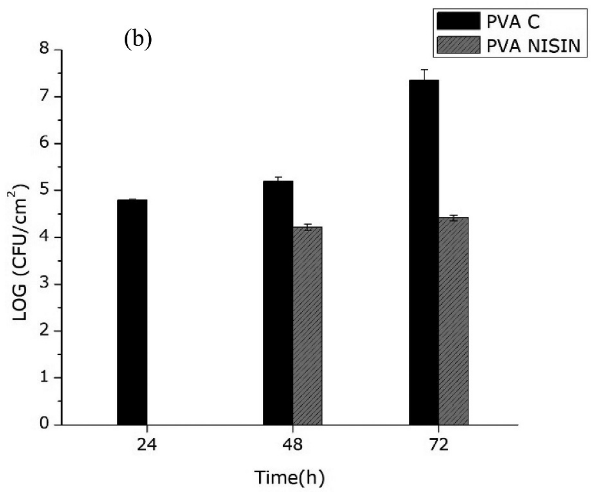


Figure 4. Biofilm of *Staphylococcus aureus* and *Listeria monocytogenes* on PVA films. (a) Biofilm of *Listeria monocytogenes* on PVA films, (b) biofilm of *Staphylococcus aureus* on PVA films. PVAC: control PVA; PVA NISIN: PVA 5,000 IU Nisin mL^{-1}.

Conclusion

This study highlights the potential of incorporating nisin into polyvinyl alcohol (PVA)-based films to enhance their antimicrobial properties. The addition of nisin modified the surface morphology of the PVA films, as observed through SEM, and imparted significant hydrophilic characteristics, as indicated by a decrease in the contact angle. FT-IR analysis confirmed that the chemical integrity of PVA and nisin was preserved during film preparation, ensuring their functional compatibility.

The incorporation of nisin reduced the Young's Modulus of the films from 33 kPa to 27 kPa, improving their elasticity and suggesting a plasticizing effect on the polymer matrix. Importantly, the antimicrobial efficacy of nisin-enriched films was demonstrated through significant reductions in *Staphylococcus aureus* and *Listeria monocytogenes* biofilm populations during the initial 24 to 48 hours of exposure with reductions greater than 99.84%. These results support the effectiveness of nisin as an antimicrobial agent, which acts by progressively releasing biocidal molecules from the PVA matrix over time.

Although *Staphylococcus aureus* exhibited higher biofilm formation than *Listeria monocytogenes*, the inclusion of nisin in the polymeric films was effective in suppressing the biofilm development of both pathogens. These findings underline the potential of PVA-nisin films as a viable strategy to mitigate microbial contamination in food processing environments, offering both immediate and sustained antimicrobial action. Further studies could explore the application of such films in real-world industrial settings and evaluate their performance against multi-species biofilms commonly found in the dairy food industry.

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Author contributions

CRediT: **Adriana Artunduaga-Rojas:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft; **Mario E. Flores:** Data curation, Formal analysis, Investigation, Methodology; **Bernardo Carrillo:** Supervision, Validation; **Wladimir Silva-Vera:** Methodology, Writing – review & editing; **Einar Vargas-Bello-Pérez:** Validation, Visualization, Writing – review & editing; **Romina L. Abarca:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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