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Design of bioactive biopolymer coating based on *Latilactobacillus curvatus* 54M16 producer of bacteriocins to preserve the safety of minimally processed fennel

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ABSTRACT

In this study *Latilactobacillus curvatus* 54M16 (LAB) producing bacteriocins has been incorporated into a sodium caseinate (SC)/guar gum (GG)/beeswax (BW) blend to develop a bioactive film/coating. Moreover, the coating capacity of preserving the safety and quality of minimally processed fennel has been investigated. Results showed significant antimicrobial activity of the bioactive film against *L. innocua* C6 during 28 days of storage at 4 °C, 10 °C, 20 °C and 30 °C. The presence of LAB did not affect the moisture content, thickness, color, and solubility of the SC/GG/BW films, whereas caused a reduction of the film's stiffness and water vapor permeability.

Counts of *L. innocua* on fennels processed with the active coating showed a significant reduction of about 2 log cycles at the end of storage with respect to the control samples for which *L. innocua* ranged from 3.42 to 4.13 log CFU/cm^2. Moreover, microbial diversity dramatically decreased in samples coated with antimicrobial film, that were dominated by *Lactobacillus* sp. In conclusion, the developed bioactive coating can be used as an antimicrobial coating to improve minimally processed fennel safety.

1. Introduction

The shelf life of minimally processed vegetables can be properly extended by using edible coatings. By forming a thin layer of film on the food, edible coating will regulate the mass transfer of moisture, aroma, or gases. Moreover, it can be used as a carrier of functional compounds i.e., antioxidants or antimicrobials (Volpe et al., 2019; Yousuf, Qadri, & Srivastava, 2018). Active edible coatings increase the safety of food products by inhibiting the growth of undesirable microorganisms during their storage (Khan et al., 2021a; Valdés et al., 2017). All the additives included in an edible coating must be considered generally recognized as safe (GRAS). They must be authorized by food regulatory agencies (Food and Drug Administration and European Food Safety Authority). Moreover, the increasing consumer demand for healthy foods free of chemical additives led scientists to concentrate on bio-preservation by the selection of antimicrobials from biological sources. The growth of pathogenic microorganisms and lipid oxidation have been inhibited by edible active films with bioactive peptides and protein hydrolysates (Tkaczewska, 2020). In addition, the antimicrobial activity of viable LAB cells incorporated into polymeric matrices has also been investigated (El-Sayed et al., 2021; La Storia et al., 2020; Sánchez-González et al., 2013; Sánchez-González et al., 2014).

In the last years, the inclusion of protective cultures into edible films is considered an emerging technology to improve or maintain constant food safety during the shelf-life of the products.

In this study, *Listeria innocua* C6 was used as an indicator strain to perform in vitro antimicrobial activity and challenge test on fennels, for its similarity to *L. monocytogenes* species on environmental growth conditions. *L. monocytogenes* is a ubiquitous food-borne pathogen that causes listeriosis. According to the annual EU One Health zoonosis report, in 2020 listeriosis was the fifth most reported zoonosis (1876 cases), mainly affect the elderly and immune-compromised population. Moreover, was found that the ready-to-eat (RTE) fruit and vegetables occurrence showed an increasing rate over the 2017–2020 (EFSA, 2021). The pathogen was predominantly associated with RTE food and can contaminate them in every phase of production, packaging, and distribution (Silva et al., 2008). To properly design active edible film based on protective cultures, the LAB cell's viability in the edible coating

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should be verified. Moreover, the film composition can affect the antimicrobial activity of LAB. All at once, LAB's presence can affect the film's structure (Leonard et al., 2015; La Storia et al., 2020). Settier-Ramírez et al., 2019 studied the effect of *Lactococcus lactis.sub.lactis* on the morphology and optical properties of polyvinyl alcohol (PVOH) matrices and reported that the moisture content of the film were affected by the functionalization of the film due to the presence of nutrient required for the vitality of the *L. lactis*. Moreover, the same authors showed that the antimicrobial activity of the film could be improved by incorporating phytic acid in a separate film to avoid a negative impact of the additive on the vitaly of the *L.lactic* (Settier-Ramírez et al., 2021).

As reported by Yousuf et al. (2018), protein-based coatings have shown promising results in extending the shelf life of minimally processed F&V related to their excellent gas barrier characteristics and good mechanical properties. Among naturally occurring proteins, casein and its derivatives were found effective due to their availability, low cost, complete biodegradability, and excellent thermoplastic properties. Bioactive coatings based on casein were found successful for the casein's ability to hold small molecules (Khan et al., 2021b).

In this study, cells of *Latilactobacillus curvatus* 54M16 were added to a sodium caseinate matrix to develop a bioactive edible film and coating. The matrix composition has been previously optimized to obtain the required physical properties and it was highlighted the protective effect of the optimized coating on the physiological properties of strawberry (Miele et al., 2022). No preview works focused on the inclusion of *Latilactobacillus curvatus* 54M16 on coating based on protein, polysaccharides and beeswax. Thus, the specific objectives of the work were to study: i) the viability of *L. curvatus* 54M16 cells when added into a sodium caseinate-based edible film-forming solution and in turn on the obtained film; (ii) the effect of the *L. curvatus* 54M16 cells on the physio-chemical properties of the obtained film; (iii) the effect of the bioactive coating against *L. innocua* C6, inoculated on laboratory media and on minimally processed fennels.

2. Materials and methods

2.1. Materials

Sodium caseinate (SC), guar gum (GG), tween 80 (T), span 80 (S), and glycerol (GLY) were purchased from Sigma-Aldrich & Co. (Milano, Italia). Beeswax was purchased from Agraria Ughetto Apicoltura (Giaveno, Torino, Italia). De Man Rogosa and Sharpe (MRS) agar, modified MRS broth (mMRS broth), phosphate buffer (PBS), Trypticase Soy Broth supplemented with 0.5 % yeast extract (TSBY), Trypticase Soy agar supplemented with 0.5 % yeast extract (TSA), Plate count agar (PCA), violet red bile glucose agar (VRBGA), Pseudomonas agar base added with cetrimide-fucidin-cephalosporin (PSA), quarter-strength Ringer's solution and AnaeroGen for generation of anaerobiosis, were purchased from Oxoid (Rodano, Italy). Agar Listeria acc. to Ottaviani & Agosti (ALOA) was purchased from Biolife (Milano, Italy). Fennels were provided by Commerciale Export s.r.l. (Pagani, SA, Italy).

2.2. Culture condition of bacterial strains

The sakacins-producing strain of *L. curvatus* 54M16 (Casaburi et al., 2016) was grown in mMRS broth at 30 °C as described in previous work (La Storia et al., 2020). mMRS broth was used as a nutrient medium for *L. curvatus* 54M16 and was composed only of food-grade ingredients.

The culture was propagated twice in mMRS broth for 16 h at 30 °C before being centrifuged at 6500 g for 20 min at 4 °C. The resulting cell pellet was rinsed twice with PBS and suspended in mMRS broth (about 10 log CFU/ml), before inclusion in films.

The strain of *L. innocua* C6 grown in Trypticase Soy Broth supplemented with 0.5 % yeast extract (TSBY, Oxoid) at 30 °C was used as an indicator of antimicrobial activity (La Storia et al., 2020).

2.3. Coating and film making process

The film-forming solution (FFS) was made as reported by (Miele et al., 2022) with some modifications. SC (8 % (w/v)) was dissolved in deionized water at 90 °C for 1.30 h. Then GG (0.2 % (w/v)) was included and allowed to stir for another 30 min at the same temperature. Finally, BW (2 % (w/v)) was added to the FFS followed by the addition of T/S (0.25 %/0.25 % (v/v)). Gly was included to achieve a glycerol/biopolymer ratio of 0.1. To emulsify the FFS, an Ultra-Turrax T25 system (IKA-Werke, Staufen im Breisgau, Germany) was employed at 15.500 rpm for 5 min. Then the FFS was chilled down at 30 °C and *L. curvatus* 54M16 (4 % (v/v)) suspended in m-MRS broth, was added under stirring for another 15 min to obtain the bioactive film (FL). The control film (FC) was prepared without the addition of *L. curvatus* 54M16. 5 ml of FFS were poured into dishes plates (area 56.7 cm^2). Film were obtained by casting at 50 % RH at 30 °C in a climatic chamber for 24 h (MMM Group, Planegg, Germany).

2.4. Rheological properties of FFS

A strain-controlled rheometer (HAAKE MARS 40 Rheometer, Thermo Fisher Scientific, USA) has been used to measure the viscosity of the FFS, as reported by La Storia et al. (2020). The rheometer was equipped with coaxial cylinders (30 mm outer diameter and 26 mm internal diameter) and all the measurements were performed at 30 °C by increasing the shear rate from 0.1 to 100 s^{-1}.

2.5. Viability of Lb. curvatus 54M16

Viable counts of *L. curvatus* 54M16 were determined on coating solution before the casting process, as previously described (La Storia et al., 2020). The results were expressed as log CFU/ml. Moreover, the LAB viability in the films at different storage time (1, 7, 15, and 28 days) at 4 °C was carried out. The films with and without *L. curvatus* 54M16 (FL and FC, respectively) were dissolved in a Stomacher bag with 56.7 ml of Ringer's solution to obtain an equal correspondence between the area of the film casted (56.7 cm^2) and the volume of the diluent used to dissolve it. Serial decimal dilutions were made in Ringer's solution and each dilution was plated in MRS agar. The plates were incubated in anaerobiosis at 30 °C for 48 h and the results were given as log CFU/cm^2.

2.6. Film characterization

2.6.1. Physiochemical properties

The film surface density (ρ) has been estimated as reported by Volpe et al. (2017) and expressed as mg/cm^2.

Films thickness was assessed by means of a micrometer model H062 with a sensitivity of ± 2 μm (Metrocontrol Srl, Casoria, NA, Italy). The mean film thickness (μm) was determined by averaging five measurements at different locations. Three replications for each film were carried out.

The moisture content (MC) of the films was assessed by gravimetric method and expressed as relative humidity percentage (Torrieri et al., 2015).

Film's colour was assessed by using a colorimeter (Minolta Chroma Meter, CR 300, Japan). The Hunter parameters L* , a* , and b* were calculated and averaged from random positions of each film (Di Giuseppe et al., 2022).

The solubility, expressed as percentage of total soluble matter, was measured as reported by Giancone et al. (2011) with some modifications, to simulate the behavior of the films in contact with food simulants. Film samples (about 20 mg) were weighed and dried at 105 °C for 24 h. Then, the films were incubated at 25 °C for 24 h in a falcon tubes containing 10 ml of 3 % acetic acid (simulant A) and 10 % ethanol (simulant B) by European Standard EN 13130–2005 and European Commission Regulation 10/2011.

2.6.2. Mechanical analysis

The tensile properties of the films were evaluated by using a DMTA V (Rheometrics, Inc., Piscataway, USA) as reported by La Storia et al. (2020). To determine the viscoelastic region, each sample was submitted to a strain sweep test at a given frequency (ω) of 1 rad sec^{-1}. Afterward, a frequency sweep test was carried out by applying a strain amplitude (ε) of 0.005 % (within the linear viscoelastic region) and increasing the frequency from 10^0 rad s^{-1} to 10^2 rad s^{-1} to monitor the storage modulus (E′), and the tangent delta (tanδ). The results were reported as an average of three replicates.

The mechanical properties of the film were determined by using an universal testing machine (Instron, 5900R-4467, USA) according to the ASTM-D882–00 standard (ASTM, 2002; Khan et al., 2022) with some modifications. The speed of the mobile crosshead was set to 10 mm min^{-1}. Then, from the stress-strain curves, the tensile strength (TS, MPa), elongation at break (ε %), and elastic modulus (EM) were calculated.

2.6.3. Water vapor permeability

The water vapor permeability (WVP) of the films was evaluated according to ASTM (1993), as reported by Di Giuseppe et al. (2022). Briefly, 9.89 cm^2 of the film surface was exposed to vapor transmission by being placed on the top of the Payne permeability cup (Carlo Erba, Italy) containing 8 g of silica gel. All the cups were placed into a desiccator at an aw of 0.85, stored in a thermostatic incubator KBF240 (Binder, Italy) at 20 °C, and weighed at scheduled times. The water vapor transmission rate (WVTR) was calculated as reported by Perone et al. (2014).

2.7. Antimicrobial activity of film

The antibacterial activity of the bioactive films during storage at 4 °C was carried out as reported by La Storia et al. (2020), with slight adjustments.

During the storage at 4 °C, pieces of about 2 × 2 cm^2 were placed on plates of TSA soft agar (0.75 % agar) inoculated with *L. innocua* C6. Plates were incubated at 30 °C, 20 °C, 10 °C and 4 °C for 24 h, 72 h, 4 days, and 7 days, respectively. After incubation, the inhibition zones around the films were measured and expressed in cm^2.

In a second method (Sanchez-Gonzalez et al., 2014) with minor modifications, *L. innocua* C6 was spread (about 4 log CFU/cm^2) on the surface of TSA plates, which were then coated with films of the same size as the Petri dishes. The counts of *L. curvatus* 54M16 and of the indicator strain was established after 0, 1, 7, 15, and 28 days of storage at 10 °C. TSA covered with the films (FL or FC) was washed with 56.7 ml of Ringer's solution and the resulting solution was used to make sequential decimal dilutions and each dilution was plated. To count *L. curvatus* 54M16, MRS plates were incubated in anaerobiosis at 30 °C for 48 h whereas to count *L. innocua* C6, ALOA plates were incubated at 30 °C for 48 h. The results were expressed as means of log CFU/cm^2.

2.8. Antimicrobial effect of coating on fennels

Whole fennels deprived of trunk and roots, were washed with tap water. After drying, fennels were inoculated on the surface with an overnight culture of *L. innocua* C6 to get a final concentration of about 3 Log CFU/cm^2 and dried at room temperature under ventilation. Contaminated fennels were then sprayed with the sodium caseinate based solution containing *L. curvatus* 54M16 (samples FL) or with the strain-free sodium caseinate solution (samples FC). Fennels were left to dry at room temperature under ventilation until completely dried. Both samples (FL and FC) were double-covered with respective coatings, left to dry, and then stored at 10 °C for 21 days. Bacterial counts were performed in duplicate at 0, 1, 3, 7, 14, and 21 days of storage. At each sampling time, fennels were dipped in 100 ml of sterile Ringer's solution and rubbed to allow the dispersion of the microbial cells from the surface to the washing solution. The washing liquid was then serially diluted in Ringer's solution to count the following microbial populations: total mesophilic aerobic bacteria (TMA) incubated at 30 °C for 48 h and total psychotropic aerobic bacteria (TPA) at 10 °C for 7 days counted on PCA; *Listeria* spp. counted on selective ALOA incubated for 72 h at 37 °C; LAB counted on MRS agar incubated for 48 h at 30 °C; *Enterobacteriaceae* counted on VRBGA incubated at 30 °C for 24–48 h; *Pseudomonas* spp., incubated at 20 °C for 48 h on PSA. The results were expressed as means of log CFU/cm^2 of the fennel surface.

2.9. Fennel microbiota analysis by high-throughput sequencing

Fennel microbiota was analysed by high-throughput amplicon sequencing for FL (fennels inoculated with *L. innocua* C6 and covered with sodium caseinate-based coating with *L. curvatus* 54M16) and FC (fennels inoculated with *L. innocua* C6 and covered with sodium caseinate-based coating without *L. curvatus* 54M16) samples, to evaluate the effect of the antimicrobial coating in changing microbial dynamics during storage. At each sampling point (baseline, 1, 3, 7, 14, 21 days of storage at 10 °C), two fennels were washed in 200 ml of Ringer's buffer each, shaking the samples in a sterile bag to detach microbial cells from the surface, as previously described (Sequino et al., 2022). One-hundred ml were centrifuged at 13,000 g for 2 min and DNA extraction was carried out starting from cell pellets by using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). The microbiota composition was studied by 16 S rRNA gene sequencing of V3-V4 variable regions (about 460 bp). Primer and PCR conditions were previously described (Berni Canani et al., 2017). Barcoded amplicons were pooled at equimolar concentration and library preparation and sequencing was carried out according to the Illumina metagenomic sequencing library preparation protocol on a MiSeq platform (leading to 2×250bp reads).

Reads were imported into QIIME 2 (q2cli version 2020.11.1; Bolyen et al., 2019), and Amplicon Sequence Variant (ASV) table was obtained following the pipeline recently reported (Sequino et al., 2022).

2.10. Statistical analysis

All data are expressed as means ± standard deviation (SD). ANOVA was performed to evaluate the effect of LAB or time on film properties and microbiological results. Comparisons of mean results were carried out using posthoc Tukey's test at $P \leq 0.05$ significance level. Statistical analyses were performed using the Stata software package version 13 (Stata, 2013). Statistical analyses and plotting of high-throughput sequencing data were carried out in R environment (http://www.r-project.org).

3. Results and discussions

3.1. Rheological properties of FFS

The viscosity of the FFS is an significant physical parameter that impacts the final thickness of the film and of the coating on the food product.

Fig. 1 shows the changes in steady shear viscosity as a function of shear rate. Both the FFSs showed pseudoplastic behavior. The viscosity for FC assumed an average value from 0.17 ± 0.2 (Pa s) to 0.0610 ± 0.0004 (Pa s). The presence of Lab led to a reduction of viscosity; indeed, FL started from an average value of 0.108 ± 0.009 (Pa s) to 0.039 ± 0.001 (Pa s). Similar results were obtained by La Storia et al. (2020).

3.2. Viability of L. curvatus 54M16

One of the requirements that antimicrobial biofilm should have to perform its active properties consist of guaranteeing an adequate viability of bacteria incorporated into the film during the storage period.

The viable count of *L. curvatus* 54M16, immediately after its addition in the FFS and before the casting, was 8.23 log CFU/ml. Counts of the

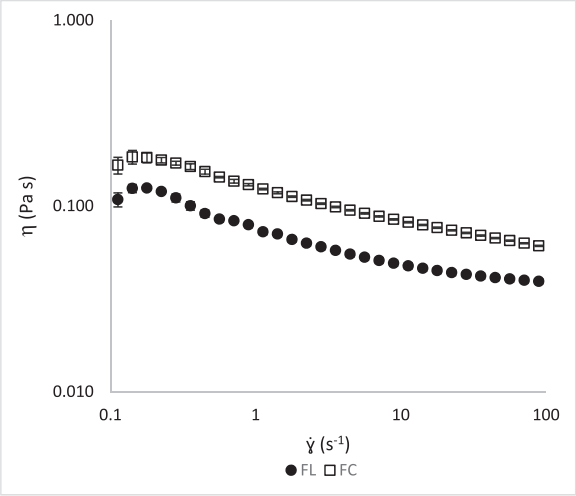

Fig. 1. Viscosity curves of the film forming solutions for FC (•) without *Lb. curvatus* 54M16 and FL (□) with *Lb. curvatus* 54M16.

bacteriocin-producer strain in sodium caseinate-based film were monitored after the casting process and during 28 days of storage at 4 °C. After the casting process at 30 °C for 24 h, the count of *L. curvatus* 54M16 in the film was of about 7.25 log CFU/cm^2. Moreover, high viability of *L. curvatus* 54M16 was detected by assuring a concentration of 7.31, 7.29, and 7.03 log CFU/cm^2 after 7, 14, and 28 days of film's storage, respectively. Therefore, the mean value of the viability of the strain during the storage of the film of 7.22 log CFU/cm^2. An analogous trend was observed in the previous study by La Storia et al. (2020), who investigated the viability of *L. curvatus* 54M16 in whey protein-based films. Therefore, the present study confirms that *L. curvatus* 54M16 effectively adapts and survives to different biopolymer matrices. Moreover, other authors have previously documented that the sodium caseinate matrix is a good carrier of functional lactic acid bacteria (Abdollahzadeh et al., 2018) also when compared to other biopolymers (Ghayoomi et al., 2017; Ghayoomi et al., 2018; Pérez-Chabela et al., 2018; Sanchez-Gonzalez et al., 2014).

3.3. Film physiochemical properties

Film surface density, thickness, moisture content, color parameters, and solubility of the films are reported in Table 1. The surface density for FC and FL films was 10.4 mg cm^{-2} and 13.9 mg cm^{-2}, respectively. The thickness of FC assumed a value of 0.09 ± 0.01 mm, whereas FL showed a slightly higher value ($p > 0.05$) equal to 0.10 ± 0.02 mm. The increase in thickness could be due to the higher solid surface density as also reported by La Storia et al. (2020), even if the authors did not show a significant increase in the thickness due to the addition of LAB cell into the matrix. The addition of LAB did not affect the moisture content (Table 1) which was equal to 14 ± 1 % and 13 ± 1 % for FC and FL, respectively. Regarding the optical properties, the presence of LAB cells did not affect the lightness (L*) (Table 1; both films appeared transparent, homogeneous, and clear). In agreement with La Storia et al. (2020), the colorimetric parameter a* significantly ($p < 0.05$) decreased from 2.0 ± 0.3 to − 0.1 ± 1, whereas b*significantly ($p < 0.05$) increased from − 6 ± 1–3 ± 1 when *Lb. curvatus* 54M16 is added to the matrix. Nevertheless, upon examining the aggregate colour variation (ΔE), no statistical differences were observed between FC and FL samples. Sánchez-González et al. (2014) studied the incorporation of microbial cells of LAB in sodium caseinate and methylcellulose films, and showed that the very small color differences were negligible from a practical point of view.

Solubility, which measures the water resistance and the integrity of the film, has been calculated in two different food simulants. The results are shown in Table 1. Both films were highly soluble in simulant A, with an average of 83 %. In the food simulant B, the solubility is lower for FC and FL with an average value of 70 %. In both simulants, no statistical differences ($p > 0.05$) were observed between the samples, highlighting that the addition of LAB cells did not affect the solubility of the film. However, the high solubility is due to the hydrophilic nature of the used biopolymer, sodium caseinate, and guar gum.

3.4. Mechanical properties and water vapor permeability

In Fig. 2 the dependence of the storage modulus (E′) and loss tangent (tanδ) of FC and FL films are shown. E′ and tanδ are constant for both FC and FL samples by increasing the frequency from 10^0 to 10^2 rad s^{-1}. The parameter E′ and tanδ are related to the elastic properties of the material and on how the material loses energy to molecular rearrangements and internal frictions (Menard, 1999). The results showed that the incorporation of LAB did not affect the structure of the film, which remains brittle. By looking at the tensile properties, reported in Table 2, also the percentage of elongation at break (ε%) is not affected by the addition of the bacteria, assuming for both FC and FL a value of 4 ± 1 %. On the other hand, EM and TS were significantly reduced by the incorporation of LAB in the matrix, from a value of 222 ± 30 MPa to 184 ± 31 MPa, and from 5 ± 1 to 3.7 ± 0.8 MPa for FC and FL, respectively. EM is a measurement of the material's stiffness or its resistance to deformation. The higher modulus, the higher stiffness of the material. La Storia et al. (2020) found that the addition of bacteria led to an increase in EM, TS and ε % of 40 %, 30 %, and 29 %, respectively compared to the control film, probably due to an interaction between the inulin and the bacteria.

The permeability to water vapor (WVP) is reported in Table 2. The WVP of FC and FL is equal to 4.8 ± 0.5 × 10^{-11} g m^{-1} s^{-1} Pa^{-1} and 5.8 ± 0.7 × 10^{-11} g m^{-1} s^{-1} Pa^{-1}, respectively. The WVP were in the same range as those found by Bekhit et al. (2018), but one magnitude order lower than those reported by Abdollahzadeh et al. (2018), Gialamas et al. (2010), La Storia et al. (2020). As can be seen from Table 2, the addition of Lab significantly affects the permeability to water vapor ($p < 0.05$). Sánchez-González et al. (2014) observed significant differences among the barrier properties of sodium caseinate films incorporated with two bacteriocin-producing lactic acid bacteria. The increase in WVP could be attributed to the presence of LAB, which can create discontinuities in the matrix and therefore a higher mass transfer. However, the differences found in our study are very moderate. On the other hand, our results are not in agreement with those found by Abdollahzadeh et al. (2018), Gialamas et al. (2010), La Storia et al. (2020) and Bekhit et al. (2018) who did not find significant differences among the water barrier properties when LAB were incorporated into the matrix. Thus, the impact of the LAB addition on the barrier properties of the film depends on the film composition and structure.

Table 1
Solid surface density (ρ), thickness (Δx), moisture content (MC), colorimetric parameters (L*, a*, b*, ΔE), and solubility in food simulant A (10 % ethanol) and food simulant B (3 % acetic acid) of the control (FC) and bioactive films (FL).

Sample	ρ (mg cm^{-2})	Δx (mm)	MC (%)	Solubility A	Solubility B	L	a*	b*	ΔE
FC	10.4	0.09 ± 0.01^a	14 ± 1^a	81 ± 14^a	69 ± 8^b	97 ± 2^a	2 ± 0.3^b	-6 ± 1^a	8 ± 1^a
FL	13.9	0.10 ± 0.02^b	13 ± 1^a	85 ± 5^a	70 ± 6^a	96 ± 1^a	-0.1 ± 1^a	3 ± 1^b	4 ± 3^a

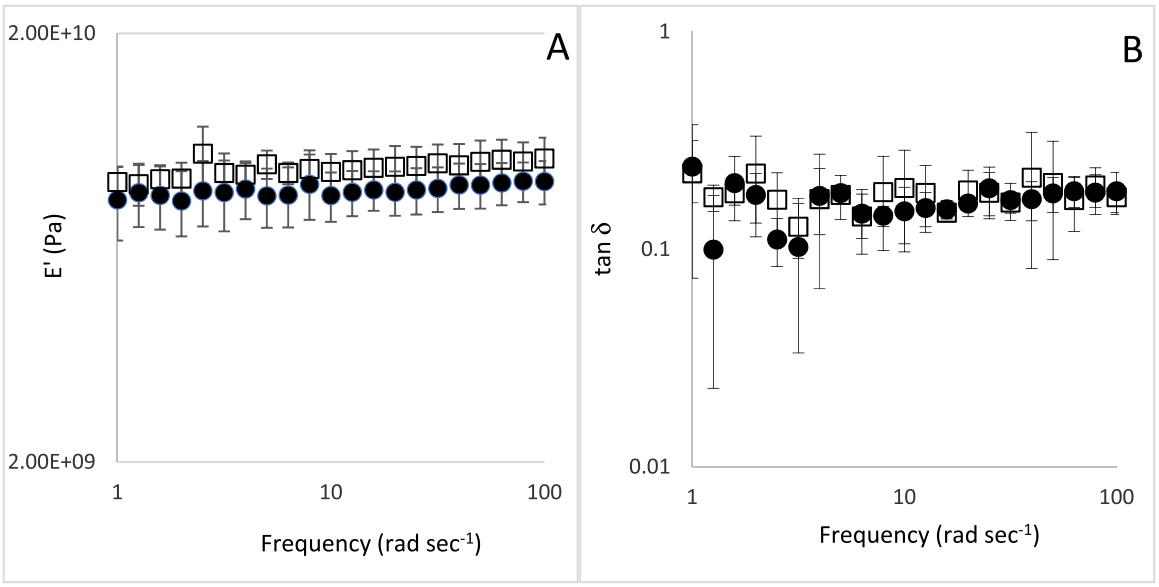

Fig. 2. Elastic modulus (E′) (A) and loss tangent (tan δ) (B) versus the angular frequency (ω) of the sodium caseinate based edible films: based films: FC (●) without *Lb. curvatus* 54M16 and FL (□) with *Lb. curvatus* 54M16.

Table 2
Tensile and water vapor barrier properties of FC and FL films.

Sample	Tensile properties			WVP x 10^{-11}
	EM (MPa)	TS (MPa)	ε%	(g m^{-1} s^{-1} Pa^{-1})
FC	222 ± 30^{b}	5.0 ± 1.0^{b}	4 ± 1^{a}	4.8 ± 0.5^{a}
FL	184 ± 31^{a}	3.7 ± 0.8^{a}	4 ± 1^{a}	5.8 ± 0.7^{b}

3.5. Antimicrobial activity

The antimicrobial activity against *L. innocua* C6 of bioactive films was evaluated on TSA medium at 30 °C, 20 °C, 10 °C and 4 °C and films without *L. curvatus* 54M16 was used as a control. The antilisterial effect was evaluated after testing the high viability of the bacteriocin producer in sodium caseinate-based film throughout the storage period. No significant decrease in activity was detected during the storage period of bioactive film for all temperatures tested (supplementary material). Moreover, control films without bacterial cells did not show antilisterial activity. At each time of storing (0, 7, 15, and 28 days) the inhibition halos were always more extensive when films were stored at lower temperatures (10 °C and 4 °C). These results confirm the previously published by La Storia et al. (2020) on the antimicrobial effect of *L. curvatus* 54M16 in whey protein-based films. Confirming the hypothesis that at low temperature the rate of diffusion of the antimicrobial compound and the growth rate of *L. innocua* C6 were comparable, condition which allow the maximum efficient of the antimicrobial film.

The antilisterial effect was also evaluated on TSA plates spread with the indicator strain then completely covered with the film and incubated at 10 °C for 28 days. The temperature of 10 °C was chosen for the next experiment because it is a suitable temperature used to store fruit and vegetables.

The results significantly varied among the bioactive film and the control one regarding their effects on the growth inhibition of *L. innocua* C6. Fig. 3 showed that in presence of the film with *L. curvatus* 54M16, the indicator strain reduced its concentration by about 2 logs in 24 h and reached values lower than 1 log after 7 days. After 15 days, *L. innocua* C6 reached values below the detection limits. Furthermore, in the activated film, the count of the bacteriocin producer strain was stable until the end of storage.

The overall results confirm the antimicrobial activity of films with *L. curvatus* 54M16 as shown previously (La Storia et al., 2020),

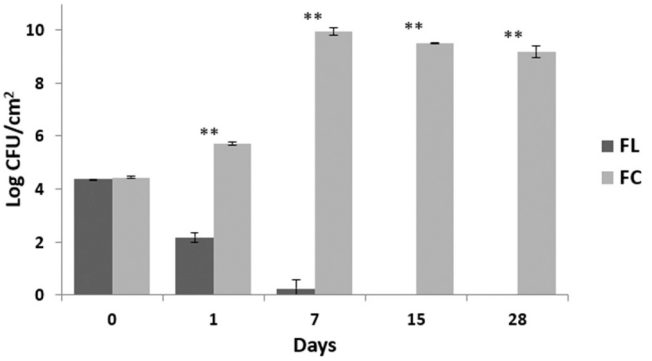

Fig. 3. - Antimicrobial activity of sodium caseinate-based films against *L. innocua* C6 on TSA during storage at 10 °C. FL: sodium casinate-based film with *L. curvatus* 54M16; FC: sodium caseinate-based film without *L. curvatus* 54M16. P value: P ≤ 0.01; * , P ≤ 0.05.

suggesting the ability of *L. curvatus* 54M16 to produce bacteriocins when entrapped in a coating made with biopolymers of different nature.

3.6. Antilisterial activity of coating on fennel

The antilisterial activity of bioactive coating sprayed on fennels contaminated with *L. innocua* C6 is shown in Fig. 4. Counts of fennels coated with an active coating (FL) showed a significant reduction of *L. innocua* C6 about 2 log cycles, whereas for control samples (FC) *L. innocua* increased from 3.42 to 4.13 log CFU/cm^2 during storage. The count of *L. innocua* C6 was significantly different between FL and FC at 1, 3, 7, 14, and 21 days of storing ($p < 0.05$). The mean values of other microbial groups during the storage of fennels are shown in Table 3. In general, no significant differences were found between fennels coated with an active coating (FL) and the control samples (FC) during the storage period, except for LAB. The concentration of LAB was significantly higher in FL at each sampling time compared to the samples FC covered with the coating without *L. curvatus* 54M16. Particularly, in FL the counts of LAB ranged from 6.37 Log CFU/cm^2 at time 0–5.10 Log CFU/cm^2 after 21 days, whereas in FC the concentration of LAB was from 1.30 Log CFU/cm^2 to 0.5 Log CFU/cm^2. The challenge test confirmed in vitro antilisterial activity of the bioactive film previously

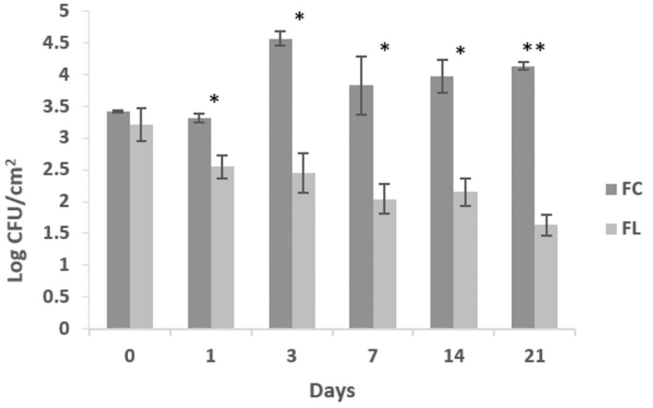

Fig. 4. – Antilisterial activity of bioactive caseinate-based coating sprayed on fennels during storage at 10 °C. FL: fennel inoculated with *L. innocua* C6 and covered with sodium caseinate-based coating with *L. curvatus* 54M16; FC: fennel inoculated with *L. innocua* C6 and covered with sodium caseinate-based coating without *L. curvatus* 54M16. P value: P ≤ 0.01; * , P ≤ 0.05.

Table 3-
Microbial population monitored during the storage of fennels inoculated with *L. innocua* C6 and covered with FC or FL.

Microbial groups[1] at days[3]	[2]Samples	
	FC	FL
APT		
0	$4.74^{a} \pm 0.8$	$4.70^{a} \pm 0.07$
1	$4.90^{a} \pm 0.09$	$5.01^{b} \pm 0.11$
3	$5.30^{b} \pm 0.13$	$5.30^{b} \pm 0.15$
7	$6.21^{c} \pm 0.09$	$6.45^{d} \pm 0.09$
14	$6.24^{c} \pm 0.09$	$6.33^{d} \pm 0.05$
21	$5.99^{c} \pm 0.18$	$6.04^{c} \pm 0.15$
AMT		
0	$4.70^{b} \pm 0.10$	$5.01^{b} \pm 0.19$
1	$4.48^{a} \pm 0.15$	$5.04^{b} \pm 0.20$
3	$4.25^{a} \pm 0.09$	$4.60^{a} \pm 0.09$
7	$6.56^{d} \pm 0.14$	$6.34^{d} \pm 0.14$
14	$6.77^{d} \pm 0.08$	$6.68^{d} \pm 0.08$
21	$6.27^{c} \pm 0.10$	$6.04^{c} \pm 0.13$
Enterobacteriaceae		
0	$3.00^{a} \pm 0.14$	$2.48^{a} \pm 0.14$
1	$3.11^{a} \pm 0.12$	$3.48^{b} \pm 0.11$
3	$4.48^{b} \pm 0.08$	$4.25^{c} \pm 0.17$
7	$4.76^{b} \pm 0.10$	$4.25^{c} \pm 0.10$
14	$5.41^{c} \pm 0.07$	$5.55^{d} \pm 0.08$
21	$5.22^{c} \pm 0.10$	$5.30^{d} \pm 0.15$
Pseudomonas spp		
0	$3.78^{a} \pm 0.11$	$3.54^{a} \pm 0.12$
1	$4.70^{b} \pm 0.10$	$4.78^{a} \pm 0.09$
3	$5.34^{d} \pm 0.12$	$5.30^{a} \pm 0.13$
7	$5.45^{d} \pm 0.10$	$5.25^{a} \pm 0.10$
14	$5.41^{d} \pm 0.09$	$5.24^{a} \pm 0.15$
21	$5.23^{c} \pm 0.06$	$5.32^{a} \pm 0.15$
LAB		
0	$1.30^{Ab} \pm 0.06$	$6.37^{Bd} \pm 0.09$
1	$1.58^{Ac} \pm 0.14$	$6.45^{Bd} \pm 0.10$
3	$1.65^{Ac} \pm 0.11$	$6.41^{Bd} \pm 0.09$
7	$1.37^{Ab} \pm 0.07$	$5.95^{Bc} \pm 0.05$
14	$1.30^{Ab} \pm 0.07$	$5.54^{Bb} \pm 0.13$
21	$0.50^{Aa} \pm 0.14$	$5.10^{Ba} \pm 0.21$

1: AMT, mesophilic bacteria; APT, psychotropic bacteria; LAB, lactic acid bacteria. 2: FL: fennel inoculated with L. innocua C6 and covered with sodium caseinate-based coating with L. curvatus 54M16; FC: fennel inoculated with L. innocua C6 and covered with sodium caseinate-based coating without L. curvatus 54M16. 3: The data are the mean values ± standard deviation of two replicate samples at 0, 1, 3, 7, 14, and 21 days of storage. Statistical Significance: different letters in the same row indicate significant differences among times (P ≤ 0.05). Different capital letters in the columns indicate differences among the samples FC and FL at each time (P ≤ 0.05).

tested in this work. The inclusion of bacteriocin-producing LAB in edible coatings and films has been recently used as a strategy to prevent the growth of pathogenic bacteria in minimally processed fruit and vegetables as reported in the paper by Agriopoulou et al. (2020).

3.7. Effect of L. curvatus 54M16 on microbiota composition

The microbiota composition of fennels during refrigerated storage was monitored by 16 S rRNA gene sequencing. In the FC sample, higher microbial diversity was observed, with *Aeromonadaceae*, *Enterobacteriaceae*, *Pseudomonas,* and *Acinetobacter* as dominant taxa, as recently reported in commercial fennels without any coating (Sequino et al., 2022). In particular, *Pseudomonas* increased from 0 to 7 days, while *Erwinia* took over in the last time points (14 and 21 days) (Fig. 5 A). The addition of *L. curvatus* 54M16 strongly changed the overall microbiota composition (Fig. 5B). Microbial diversity dramatically decreased in samples with antimicrobial coating, that was dominated by *Lactobacillus* sp. In this case, *Pseudomonas* increased from 7 to 21 days of storage (Fig. 5A). Although bacteriocins produced by lactic acid bacteria may have a limited effect against Gram-negative species, several studies reported an antimicrobial effect of lactobacilli strains against *Enterobacteriaceae* (Chen et al., 2019) or *Pseudomonas* (Duan et al., 2020; Sun et al., 2022), possibly due to the production of different compounds (e.g., lactic acid).

In addition, the *Listeriaceae* family showed an increase during storage only in FC, although the difference with FL at the same storage time was not significant, probably for the low number of samples ($p > 0.05$; Fig. 5c).

4. Conclusions

In this study, cells of the bacteriocins-producing strain *Latilactobacillus curvatus* 54M16 were added to a sodium caseinate based matrix to develop a bioactive edible film and coating.

L. curvatus did not affect the structure of the film but the higher solid surface density of the active films slightly affected the physical-chemical properties of the films. Mainly, the active film showed a reduced elastic modulus, indicating a reduction in stiffness, and increased water vapor permeability with respect to the control film. Sodium caseinate-based film ensured the viability and functionality of the bacteriocin-producing strain incorporated. Moreover, the active film showed antilisterial activity in the food model system also confirmed by the study of microbiota composition by rRNA gene sequencing. Thus, sodium caseinate, guar gum, and beeswax can be used as carriers of LAB to produce antimicrobial coating to improve food safety.

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CRediT authorship contribution statement

Marina Giello: Investigation, Dara curation, Writing- Original draft preparation **Stefania Volpe** Investigation, Data curation, writing-Original draft preparation, visualization **Giuseppina Sequino** Investigation, Data curation **Francesca De Filippis**: Methodology-writing-Reviewing. ***Francesco Villani:*** Supervision, Conceptualization, reviewing **Elena Torrieri**: Supervision, Conceptualization, reviewing and editing, funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Elena Torrieri, Francesca De Filippis, Francesco Villani reports financial

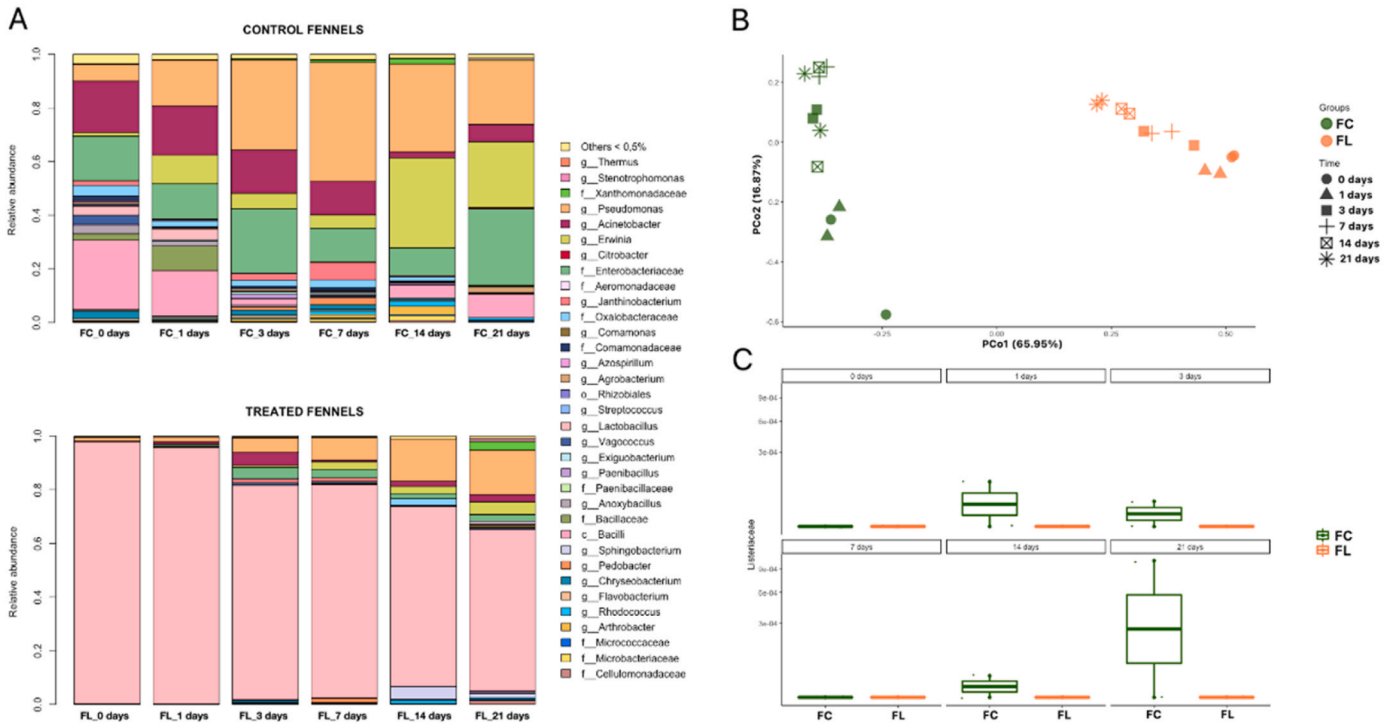

Fig. 5. Stacked bar plot of the microbiota composition during storage of fennels for 21 days. Data are averaged for each time-point (A). Principal Coordinates Analysis (PCoA) based on Bray–Curtis distances matrix obtained from microbiota composition. Each point is colored according to the group (FC or FL) and shaped according to the time of storage (B). Box plots showing the abundance of *Listeriaceae* family (%) in fennels during the storage. Boxes represent the inter-quartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.5 IQR from the first and third quartiles, respectively. (C). FL: fennel inoculated with *L. innocua* C6 and covered with sodium caseinate-based coating with *L. curvatus* 54M16; FC: fennel inoculated with *L. innocua* C6 and covered with sodium caseinate-based coating without *L. curvatus* 54M16.

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Data Availability

I have share the link.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fpsl.2023.101111.

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