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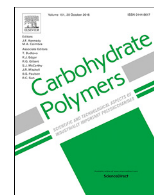
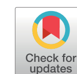


Decontamination of seeds destined for edible sprout production from *Listeria* by using chitosan coating with synergetic lysozyme-nisin mixture



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ABSTRACT

This study aimed at decontamination of seeds destined for edible sprout production from *Listeria* using chitosan (CS) coatings incorporated with synergetic lysozyme-nisin (LYS-NIS) mixtures. Low molecular weight (LMW) CS coating showed the highest potency against *Listeria innocua,* followed by medium molecular weight (MMW) and high molecular weight (HMW) CSs. The LMW CS film with LYS-NIS also caused almost 1.5-fold greater log reduction (~5 log) in initial *L. innocua* load of broth culture than MMW and HMW CS films with LYS-NIS within 6 days. Moreover, LMW CS coating with LYS-NIS reduced the initial *Listeria* loads of inoculated mung beans, lentils, and wheats by 3.3, 3.4 and 4.1 log, respectively. Antimicrobial coating did not affect seed germination rates considerably. The LYS-NIS addition increased yellowness and opacity of films, and caused limited changes in their mechanical and morphological properties. LMW CS coating with LYS-NIS reduces risk of listeriosis from sprouted seeds.

1. Introduction

Raw ready-to-eat sprouted legumes and cereals might bear great microbial risks since they are germinated at high humidity and warm temperature conditions that are highly favorable for the growth of pathogenic contaminants (Iacumin & Comi, 2019; Piernas & Guiraud, 1997; Trząskowska, Dai, Delaquis, & Wang, 2018). This explains why raw sprouts are frequently associated with many recalls and outbreaks originated from critical pathogens such as *Listeria monocytogenes* (CDC, 2014), *Escherichia coli* O104:H4 (EFSA, 2011a), *Salmonella* (CDC, 2016; EFSA, 2011b), *Yersinia enterocolitica* and *Bacillus cereus* (EFSA, 2011b). The *L. monocytogenes* recalls cause particular concerns among consumers since this bacterium may lead to deadly infections in susceptible individuals such as pregnant women, elderly people, and immunosuppressed subjects (Álvarez-Ordóñez, Leong, Hickey, Beaufort, & Jordan, 2015; Vázquez-Boland, Domínguez-Bernal, González-Zorn, Kreft, & Goebel, 2001). Thus, the decontamination of seeds to cause a minimum 3 log reduction in their microbial pathogen load is essentially needed before sprouting (CFIA, 2018). Different studies conducted for decontamination of seeds include application of chemical disinfectants (e.g., organic acids, chlorine, calcium hypochlorite, chlorine dioxide, ozone, etc.), protective cultures, heating, pulsed UV light, irradiation, supercritical CO_2, high hydrostatic pressure and ultrasound (Millan-Sango, Sammut, Van Impe, & Valdramidis, 2017; Studer, Heller, Hummerjohann, & Drissner, 2013; Trząskowska et al., 2018). However, studies related to use of antimicrobial edible coatings for decontamination of seeds destined for sprout production are scarce.

Chitin, the second most abundant polysaccharide on the Earth after cellulose, is a linear polysaccharide that is composed of D-glucosamine and *N*-acetyl-D-glucosamine subunits linked through β (1-4) linkage. Chitosan (CS) is commercially obtained by deacetylation of chitin extracted from shells of crustaceans such as crab, shrimp, lobster, and crawfish (No, Meyers, Prinyawiwatkul, & Xu, 2007; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). The conditions of deacetylation process such as the concentration of the alkaline solution and the time-temperature combinations affect the degree of acetylation and molecular weight of CS that are highly effective on its solubility, viscosity and inherent antimicrobial properties (Synowiecki & Al-Khateeb, 2003; Vargas & González-Martínez, 2010). The broad antimicrobial spectrum of CS is attributed to its ability to bind negatively charged bacterial surfaces with cationic -NH_3^+ of its glucosamine residues (Wang, Qian, & Ding, 2018). It was thought that the binding of chitosan to cell wall polymers triggers secondary cellular effects that cause disruption of membrane functions (e.g., barrier functions, and membrane-bound energy generation pathways) and initiate various stress responses (Dutta, Tripathi, Mehrotra, & Dutta, 2009; Raafat, von Bargen, Haas, & Sahl, 2008; Tantala, Thumanu, & Rachtanapun, 2019).

In the literature, different studies exist to combine inherent

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antimicrobial activity of CS films and coatings with natural and generally recognized as safe (GRAS) antimicrobials such as LYS or NIS (Duan, Park, Daeschel, & Zhao, 2007; Duan, Kim, Daeschel, & Zhao, 2008; Guo, Jin, Wang, Scullen, & Sommers, 2014; Imran, Klouj, Revol-Junelles, & Desobry, 2014; Park, Daeschel, & Zhao, 2004; Pranoto, Rakshit, & Salokhe, 2005). CS coatings with NIS, an antimicrobial peptide produced by certain strains of *Lactococcus lactis* spp. *lactis,* or with LYS, an antimicrobial enzyme obtained from hen egg white, has been applied to inhibit critical food pathogens in fresh or minimally processed fruits, fresh meat, fish products, deli foods, cheeses and whole eggs (Duan et al., 2007, Cé, Noreña, & Brandelli, 2012; Duran et al., 2016; Guo et al., 2014; Mehyar, Al Nabulsi, Saleh, Olaimat, & Holley, 2018; Thumula, 2006; Ye, Neetoo, & Chen, 2008a; Ye, Neetoo, & Chen, 2008b; Yuceer & Caner, 2014). The addition of LYS-NIS mixture into different food has also attracted a particular interest since these biopreservatives can show synergetic antimicrobial action on different Gram-positive bacteria including *L. monocytogenes* (Bhatia & Bharti, 2015; Chung & Hancock, 2000; Gill & Holley, 2000; Mangalassary, Han, Rieck, Acton, & Dawson, 2008; Sozbilen, Korel, & Yemenicioğlu, 2018).

In the current study, the inherent antimicrobial activity of CS was combined with the synergetic mixture of LYS-NIS to develop antimicrobial coatings capable to reduce risk of listeriosis from seeds such as mung bean, lentil, and wheat destined for the production of sprouts. The novelty of this work is that for the first time in the literature, it adopts antimicrobial coating technology to seeds destined for the production of sprouts without interfering seeds' germination capacities.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (≥40,000 U/mg protein) (L6876), nisin (≥1000 IU/mg) from *Lactococcus lactis* (N5764), *Micrococcus lysodeikticus*, low [degree of deacetylation (DDA): 75–85 %, molecular weight (MW): 50 − 190 kDa], medium [DDA: 75–85 %, MW: 190 − 300 kDa], and high [DDA: > 75 %; MW: 310 − 375 kDa] molecular weight chitosans were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, USA). MRS broth was obtained from Merck (Darmstadt, Germany). The surrogate of *L. monocytogenes, L. innocua* NRRL-B 33314 ATCC 1915 was from the culture collection of the microbiology laboratory of the Department of Food Engineering at Izmir Institute of Technology IYTE, Izmir. The strain of *Lactobacillus plantarum* (NRRL-B4496) obtained from ARS Culture Collection (NRRL) was kindly provided by Dr. Burcu Öztürk, from IYTE. Mung bean, lentil, and wheat were purchased from a local market in İzmir.

2.2. Methods

2.2.1. Preparation of films

CS films were prepared by a slight modification of the method given by Ünalan, Ucar, Arcan, Korel and Yemenicioğlu (2011). Briefly, 1.5 % w/w LMW, MMW or HMW CS was dissolved in a 0.5 % acetic acid solution by stirring almost 20 h at 300 rpm. Then, glycerol 100 % of CS by weight was added into the solution with stirring at 300 rpm for 30 min. The film solution was filtered by using cheesecloth to remove insoluble residues, and different concentrations of LYS and/or NIS were added into solution. The film making solution was then homogenized using a Silent Crusher M with a 12 F shearing tool, Heidolph Instruments GmbH, Germany at 10000 rpm for 3 min, and then centrifuged to remove air bubbles. The classical casting method was used to obtain self-standing films needed for characterization studies films used in Sections 2.2.2 to 2.2.4). For this purpose, film making solutions that contain different amounts of antimicrobial(s) (sufficient amounts to reach final concentration of 0.5 mg/cm^2 for NIS, and 0.5–3.5 mg/cm^2 for LYS in dried films) were poured (20 g portions) into disposable Petri dishes (8.5 cm in diameter). The films were then dried for 22 ± 2 h at 45 °C. On the other hand, the film making solution with or without LYS and NIS (LYS-NIS) was used directly when it was employed in seed coating applications (see Sections 2.2.5 and 2.2.6).

2.2.2. Antilisterial activities of films and film constituents in broth media

Before incorporation of LYS-NIS combination into CS films, the synergy between soluble forms of these two natural preservatives was demonstrated within broth media using the classical dynamic flask method. The tests were conducted at 4 °C to prevent the rapid growth of culture, and to observe maximal differences among antimicrobials. In order to adopt *L. innocua* to refrigeration conditions, the inoculum was grown at 4 °C for 24 h following incubation at 37 °C for 24 h. The initial load of culture was adjusted to 10^3 CFU/mL. The solutions of LYS, NIS and LYS-NIS were prepared in 0.05 M Na-phosphate buffer at pH 6.0. The concentrations of antimicrobials (1, 4 and 8 μg/mL) were determined carefully with a preliminary test to avoid instant inactivation of bacteria and identify magnitude of synergy easily. Briefly, 5 mL of culture, 40 mL of nutrient broth at pH 6.0 and 5 mL of antimicrobial solution (with LYS, NIS or LYS-NIS) prepared with buffer were distributed into the sterile capped Erlenmeyer flasks. The flasks were incubated at 4 °C for 9 days under continuous shaking at 80 rpm. The change in *L. innocua* count during incubation was monitored by counting of flask content at different time intervals (0th, 1st, 2nd, 5th, 7th and 9th days) using the spread plate method on nutrient agar. The nutrient agar plates were enumerated after 24 h incubation of at 37 °C. This experiment was conducted in duplicate and the enumeration for each sample group was carried out in triplicate.

For determination of antilisterial activities of films in broth medium, LYS and NIS concentrations in the films were kept minimal (each at 0.5 mg/cm^2) to prevent the rapid inactivation of *L. innocua* by solubilized antimicrobials and to make comparisons among LMW, MMW, and HMW CSs above the detection limit of microbial counting. The tests were also conducted at 4 °C to prevent the rapid growth of culture, and to observe maximal differences among antimicrobial activities of different films. The CS film discs (13 mm in diameter) were prepared with a cork-borer under aseptic conditions. After that, disk-shaped films were put into sterile tubes containing 2.4 mL of nutrient broth (at pH 6.0) and 0.265 μL of inoculum of *L. innocua*. The enumeration was performed on the 0th, 1st, 2nd, 6th, and 12th days of cold-storage at 4 °C. Two tubes were analyzed for each day of sampling. No film added tubes were also prepared to monitor the growth of the bacteria. The enumeration was conducted by the spread plate method on nutrient agar. The colonies were enumerated after 24 h incubation of plates at 37 °C. The analysis was carried out in duplicate and the enumeration was performed in triplicate. The tests were conducted at the initial *L. innocua* load of 10^6 CFU/mL unless otherwise was stated. The tests were also repeated at an initial *L. innocua* load of 10^4 CFU/mL to see the dependency of obtained trends on initial microbial load.

2.2.3. Release profiles of LYS and NIS from films

The release profiles of LYS and NIS were determined for LMW CS films since films of this type of CS showed more potent antilisterial activity than those of MMW and HMW CS. Briefly, the films were cut into 16 cm^2 squares pieces (4 cm × 4 cm), and they were placed into an Erlenmeyer flask containing 15 mL of 0.05 M Na-phosphate buffer at pH 6.0. The flasks were stored at 4 °C in an incubator to prevent loss of LYS and NIS activity, and shaken with an orbital shaker working at 160 rpm. The release test was continued for 12 days to ensure equilibrium reached for the release of LYS or NIS.

LYS activity was measured spectrophotometrically at 660 nm using *Micrococcus lysodeikticus* as a substrate as described by Boyacı and Yemenicioğlu (2018). Aliquots (3 × 0.2 mL) taken at different time intervals (3 h, 6 h, 48 h, 120 h, 192 h, and 288 h) were assayed 3 times. The activities were expressed as Units (0.001 absorbance change in 1 min per 1 mL of enzyme) released per cm^2 of films tested.

NIS released was determined by the classical agar diffusion method (Teerakarn, Hirt, Acton, Rieck, & Dawson, 2002) using *L. plantarum* NRRL-B4496 as test microorganism. Briefly, the bacteria culture was inoculated into MRS broth and incubated for 24 h at 30 °C. Then, the culture of freshly grown cells was adjusted to 0.5 Mac Farland unit with 0.1 % of pepton water, and the diluted culture was seeded into MRS test agar which was prepared by adding 0.75 % of agar and 20 mL/L of 50 % of Tween 20 into MRS broth. Twenty mL of the inoculated agar was then poured into Petri dishes. Three 6 mm-diameter wells were then opened on the surface of solidified agars by using a sterile cork-borer, and 50 μL (3 repeats) aliquots from NIS release medium collected at different time periods (3 h, 6 h, 30 h, 54 h, 144 h, and 288 h) were added into the wells. Serial dilutions of NIS (500 IU/mL) in sterile 0.05 M Na-phosphate buffer (pH 6.0) was used to prepare the calibration curve. The NIS concentration was expressed as International Units (IU) released per cm^2 of films tested.

The LYS and NIS recoveries from the films were calculated by the equation-1.

$$\%\ \text{LYS or NIS recovered} = \frac{\text{Maximum U (LYS) or IU (NIS) released from film}}{\text{Total U (LYS) or IU (NIS) incorporated into film}} \times 100 \quad (1)$$

2.2.4. Colour, opacity, morphology and mechanical properties of films

The color of films was measured using a digital colorimeter (chromometer type, Konica Minolta, CR-410, Tokyo, Japan) standardized with a white board (Y = 93.80, X = 0.3159, y = 0.3322). Results were expressed as CIE (Commission International de l'Eclairage); L* (0, dark; 100, light), a* (-a, greenness; +a, redness; 0, neutral) and b* (-b, blueness; +b, yellowness; 0, neutral). The measurements were done at illuminant condition: C, D65, and observer condition: 2° standard observer. Average of five measurements were used to calculate different parameters.

The film opacity was determined by using absorbance values of films at 600 nm (Abs_{600nm}) with a spectrophotometer (Shimadzu Model 2450, Japan). Average of five absorbance measurements were used to calculate opacity according to Zimet et al. (2019) by Eq. (2).

$$\text{Opacity} = Abs_{600nm}/\text{film thickness (mm)} \quad (2)$$

The cross-sectional morphology of films was determined by using scanning electron microscopy (SEM) (Philips XL 30S FEG, FEI Company, Netherlands) under high vacuum mode at an operating voltage varying between 2 and 3 kV. The films were placed into liquid nitrogen for fast freezing and crashed for SEM examination. After that, the samples were gold coated with a sputter coater (Emitech K550X, Quorum Technologies Inc., UK) under 15 mA for 1 min. The thickness of the films was measured from SEM cross-sectional views of films from 500 × magnified images.

Tensile strength, elongation at break and Young's modulus of the films were determined by using a Texture Analyzer TA-XT2 (Stable Microsystems, Godalming, UK) according to ASTM Standard Method D 882-02 (ASTM, 1999). The conditioning of the films was performed at 25 °C and 50 % RH for 24 h in an environmental chamber. The conditioned films were then cut into 8 mm × 80 mm strips. The initial grip distance and crosshead speed were set to 50 mm and 50 mm/min, respectively. At least seven replicates of each film were tested.

2.2.5. Antimicrobial coating studies with different seeds

For the antimicrobial coating tests, seeds were firstly soaked in 5 % (v/v) sodium hypochlorite solution for 15 min and washed with sterilized distilled water. After that, they were dried in a laminar flow cabin at room temperature. The *L. innocua* was activated by transferring one loop of frozen culture into a tube containing 9 mL of nutrient broth, and incubating the tube at 37 °C for 24 h. The culture was then diluted tenfold with nutrient broth to adjust its final count in this medium at 10^7 CFU/mL. After that, each type of seed (10 g portions) was immersed into the culture and stirred with a sterile glass rod for 15 min to distribute the inoculum evenly. The culture was then drained and the inoculated seeds were placed into sterile Petri dishes and dried for 2 h in a laminar flow cabin.

LMW CS film solutions with or without LYS (at 9.9 mg/g) and NIS (at 1.4 mg/g) were prepared as described in Section 2.2.1. Ten gram portions of each type of inoculated seed were immersed into flasks containing the film solutions, and the contents were stirred with a glass rod to distribute the solution evenly. The excess amount of the film solution was then drained, and the seeds treated with the film solution were then dried in sterile Petri dishes kept at 25 °C for 4 h under aseptic conditions. During drying, seeds were mixed with a sterile glass rod at 1 h intervals. At the end of drying, each sample was diluted 10-fold with 0.1 % peptone water, and it was stirred vigorously in an Erlenmeyer flask for 60 s. The serial decimal dilutions were then spread plated onto Oxford *Listeria* Selective Agar supplemented with Oxford *Listeria* Selective Supplement. The enumeration of small black colonies with a halo on the plate was performed after 48 h incubation at 37 °C. The enumeration was conducted in triplicate plates. Two separate samples from each replicate were used in the microbiological analysis. Uncoated seeds were considered as the control group.

2.2.6. Effect of antimicrobial coating on seed germination rate

The germination rate of seeds was determined by applying a slight modification of the method given by Pierre and Ryser (2006). Briefly, 40 portions of LMW CS coated (see Section 2.2.5) or uncoated seeds were placed onto moistened cotton placed into Petri dishes (3 × 40 seeds per replicate). The Petri dishes were then incubated in an environmental chamber at 22 °C and 50 % RH for 5 days in the dark for the germination of seeds. Seeds were moistened daily with 5 mL of water. Seed germination was monitored daily, and a seed was considered germinated when its radicle was 2 mm long. The germination rate was determined according to equation-3 at 3rd, 4th and 5th days.

$$\text{Germination rate (\%)} = \frac{\text{Number of sprouted seeds per plate}}{\text{Total number of seeds per plate}} \times 100 \quad (3)$$

2.2.7. Statistical analyses

The results presented are averages and standard deviations that were calculated from two replicates (Microsoft Excel, Microsoft Corporation, Redmond, WA). The Analysis of Variances (ANOVA) and Fisher test were applied to determine statistically significant differences (at $P < 0.05$) using the statistical software of Minitab release 16 (Minitab Inc., State College, Pa., USA).

3. Results and discussion

3.1. Synergy of LYS-NIS combination

The synergy between LYS and NIS solubilized in Na-phosphate buffer (pH 6.0) at 1, 4 and 8 μg/mL was demonstrated in broth media (see supplementary file, Table 1S). The LYS alone at 1 μg/mL caused ≤ 0.4 and ~0.6 log reductions in initial *L. innocua* counts within 7 and 9 days, respectively while NIS alone at 1 μg/mL did not show significant antibacterial activity. In contrast, LYS-NIS combined at 1 μg/mL caused 1.3–1.5 log reduction in initial *L. innocua* count within the first 5 days ($P < 0.05$). The *L. innocua* showed rapid inactivation and remained below detection limit (< 0.69 log CFU/mL) between 2nd and 9th days in presence of LYS-NIS combined each at 4 μg/mL. In contrast, counts of cultures with LYS or NIS at 4 μg/mL were 1.9–4.1 log higher than detection limit during 9-days incubation. Therefore, in respect to control, the sum of separate decimal reductions at a given day caused by LYS and NIS at 4 μg/mL is always lower than that caused by LYS-NIS combined each at 4 μg/mL. LYS alone at 4 and 8 μg/mL caused similar antibacterial activity, but both NIS and LYS-NIS with each agent at

Table 1
Effect of different CS films with LYS and/or NIS (each at 0.5 mg/cm^2) on *L. innocua* in broth media incubated at 4 °C (initial microbial load at 6 log CFU/mL) (n = 6; $P < 0.05$).

Type of film/ antimicrobial	*L. innocua counts* (log CFU/mL)				
	Day 0	Day 1	Day 2	Day 6	Day 12
LMW CS					
Control (no film)	6.5 ± 0.2 ^{d,A,A}	6.8 ± 0.2 ^{c,A,A}	6.9 ± 0.1 ^{c,A,A}	7.7 ± 0.3 ^{b,A,A}	8.2 ± 0.2 ^{a,A,A}
Control (with film)	6.5 ± 0.3 ^{a,AB,B}	5.5 ± 0.5 ^{b,B,B}	5.2 ± 0.6 ^{b,B,B}	3.9 ± 0.7 ^{c,B,B}	3.0 ± 0.6 ^{d,B,C}
LYS	6.7 ± 0.1 ^{a,A,A}	5.5 ± 0.3 ^{b,B,A}	4.5 ± 0.1 ^{c,C,A}	2.4 ± 0.4 ^{d,C,B}	2.5 ± 0.1 ^{d,B,B}
NIS	6.3 ± 0.3 ^{a,B,B}	3.9 ± 0.5 ^{b,C,A}	3.2 ± 0.8 ^{bc,D,B}	2.8 ± 0.6 ^{c,C,B}	2.5 ± 0.6 ^{c,B,B}
LYS-NIS	5.7 ± 0.3 ^{a,C,A}	3.1 ± 0.6 ^{b,D,B}	2.5 ± 0.2 ^{c,E,B}	1.4 ± 0.2 ^{d,D,B}	2.6 ± 0.2 ^{bc,B,B}
MMW CS					
Control (no film)	6.5 ± 0.2 ^{d,A,A}	6.8 ± 0.2 ^{c,A,A}	6.9 ± 0.1 ^{c,A,A}	7.7 ± 0.3 ^{b,A,A}	8.2 ± 0.2 ^{a,A,A}
Control (with film)	6.7 ± 0.2 ^{a,A,AB}	6.4 ± 0.1 ^{a,B,A}	6.4 ± 0.2 ^{a,B,A}	4.9 ± 0.6 ^{b,B,A}	4.7 ± 0.2 ^{b,B,B}
LYS	6.7 ± 0.1 ^{a,A,A}	4.3 ± 0.2 ^{b,C,B}	3.2 ± 0.5 ^{c,C,B}	4.0 ± 0.7 ^{b,C,A}	4.0 ± 0.3^{b,CD,A}
NIS	6.7 ± 0.1 ^{a,A,A}	4.3 ± 0.0 ^{b,C,A}	4.1 ± 0.1 ^{b,D,AB}	4.0 ± 0.4 ^{b,C,A}	4.4 ± 1.1 ^{b,BC,A}
LYS-NIS	5.7 ± 0.8 ^{a,B,A}	3.2 ± 0.2 ^{b,D,B}	2.1 ± 0.2 ^{c,E,B}	3.2 ± 0.3 ^{b,D,A}	3.6 ± 0.4 ^{b,D,A}
HMW CS					
Control (no film)	6.5 ± 0.2 ^{d,A,A}	6.8 ± 0.2 ^{c,A,A}	6.9 ± 0.1 ^{c,A,A}	7.7 ± 0.3 ^{b,A,A}	8.2 ± 0.2 ^{a,A,A}
Control (with film)	6.9 ± 0.3 ^{a,A,A}	6.9 ± 0.1 ^{a,A,A}	6.9 ± 0.2 ^{a,A,A}	5.4 ± 0.2 ^{b,B,A}	5.4 ± 0.1 ^{b,B,A}
LYS	6.7 ± 0.4 ^{a,A,A}	4.6 ± 0.5 ^{b,B,B}	3.7 ± 0.5 ^{c,C,B}	3.8 ± 0.5 ^{c,C,A}	3.8 ± 0.5 ^{c,C,A}
NIS	6.6 ± 0.3 ^{a,A,AB}	4.3 ± 0.1 ^{bc,BC,A}	4.6 ± 0.2 ^{b,B,A}	4.0 ± 0.3 ^{cd,C,A}	3.7 ± 0.4 ^{d,CD,AB}
LYS-NIS	6.5 ± 0.1 ^{a,A,A}	4.2 ± 0.1 ^{b,C,A}	3.5 ± 0.4 ^{c,C,A}	3.3 ± 0.2 ^{c,D,A}	3.3 ± 0.3 ^{c,D,AB}

$^{a-d,\ A-C\ and\ \mathit{A-C}}$ Values at each row (lower case letters) and column (capital letters among data of each film type, italic capital letters among data of all film types) followed different letters indicate significant differences.

8 μg/mL showed high potency and dropped bacterial counts below detection limit within 1 day. However, LYS-NIS combined at 8 μg/mL kept bacterial counts below detection limit during 9-days incubation while bacterial regrowth in culture with 8 μg/mL of NIS initiated at 5th day. These results clearly proved the potential benefit of combining LYS-NIS against *Listeria* in CS films.

3.2. Selection of most potent CS type for antimicrobial coating

The antimicrobial effects of LMW, MMW, and HMW CS films with or without LYS, NIS or LYS-NIS mixture (each at 0.5 mg/cm^2) on *L. innocua* were compared in broth medium at pH 6.0 during 12-days incubation at 4 °C (Table 1). The comparison of antimicrobial activities of control CS films at three different molecular weights clearly showed that the inherent antimicrobial potential of LMW CS film on *L. innocua* was significantly higher than those of MMW and HMW CS films (at both 10^4 CFU/mL or 10^6 CFU/mL initial inoculation levels) (Fig. 1). The *L. innocua* counts of cultures containing LMW, MMW, and HMW CS films at the end of 12 days were 5.3, 3.5, and 2.8 log lower than that of control culture incubated for 12 days, respectively. Moreover, the

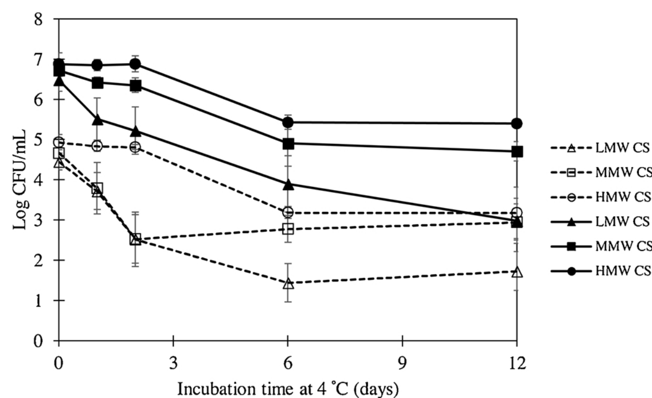

Fig. 1. Inherent antimicrobial activity of LMW, MMW and HMW CS films on *L. innocua* (initial *L. innocua* counts = 10^4 CFU/mL (dashed lines); 10^6 CFU/mL (continuous lines)).

LMW, MMW and HMW CS films caused almost 3.5, 1.8 and 1.1 log reduction in initial *L. innocua* count of broth culture (log CFU/mL = 6.53) within 12 days at 4 °C, respectively. These results showed parallelism with those of Kim, No and Prinyawiwatkul (2007), and Zheng and Zhu (2003) who found a greater antimicrobial activity of LMW CS than HMW CS against *Salmonella* Enteritidis and *Staphylococcus aureus*, respectively. In contrast, Leleu et al. (2011) found that *S. enterica* serovar Enteritidis was more efficiently inhibited by HMW CS than LMW CS. These findings supported different reports that antimicrobial activity of CS is influenced not only by its molecular properties (e.g., molecular weight and degree of deacetylation), but also by type of bacteria tested (Qin et al., 2006; Shin, Yoo, & Jang, 2001; Zheng & Zhu, 2003).

The incorporation of LYS, NIS or LYS-NIS mixture (each at 0.5 mg/cm^2) into LMW, MMW or HMW CS films improved the inherent antimicrobial activity of respective CS films against *L. innocua* significantly. The LMW CS films with LYS, NIS or LYS-NIS mixture showed better antimicrobial performances than MMW and HMW CS films with LYS, NIS or LYS-NIS mixture. However, none of the CS films with LYS and/or NIS showed a considerable antimicrobial effect on *L. innocua* between 6 and 12 days of incubation. LYS and NIS continuously disintegrated the membranes of bacteria and caused leakage of their cytoplasmic fluids. Thus, at the final stages of the test, the media contained high numbers of dead cells, components of hydrolyzed cell wall carbohydrates, and cytoplasmic components such as protein, enzymes (e.g., proteases), organelles, etc. that might interact with NIS and LYS to reduce their antimicrobial performances. Moreover, it was also possible that *Listeria* had developed a resistance against LYS and NIS after 6 days of incubation. The capacity of *Listeria* to develop resistance against NIS was reported (Gallo, Pilosof, & Jagus, 2007; Harris, Fleming, & Klaenhammer, 1991; Schillinger, Becker, Vignolo, & Holzapfel, 2001), but reports about the development of resistance by *Listeria* against LYS are scarce. On the other hand, it is also important to note that all films with LYS-NIS performed better than those with LYS or NIS alone. The reduction in initial *L. innocua* count of broth culture within 6 days was almost 5 log for LMW CS films with LYS-NIS, while it was almost 3.3 log for both MMW and HMW CS films with LYS-NIS. Moreover, on the 6th day, the count of culture containing LMW CS films with LYS-NIS was 6.3, 2.5, 1.9 and 1.8 log lower than those of control culture without CS,

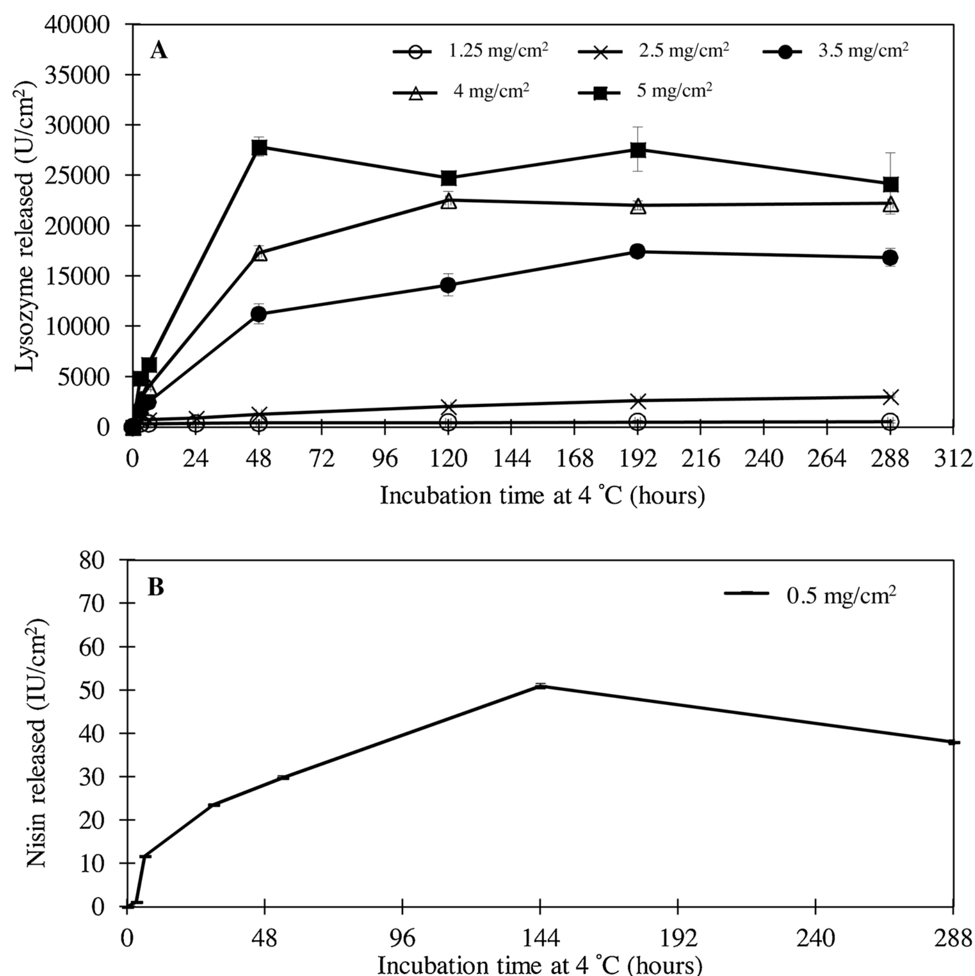

Fig. 2. Release profiles of LYS (A) and NIS (B) from different LMW CS films (The labels indicate concentrations of LYS or NIS incorporated into films).

and cultures with LMW CS film, MMW CS film with LYS-NIS, and HMW CS film with LYS-NIS, respectively. Finally, it is also important to note that the LMW CS film with LYS-NIS tested at initial *L. innocua* inoculation level of 10^4 CFU/mL (instead of 10^6 CFU/ml) was also more effective than LMW CS films with LYS or NIS alone (see supplementary file, Table 2S). Thus, LMW CS film with LYS-NIS combination was selected as the most suitable film for seed coating application.

3.3. Release profiles of LYS and NIS from LMW CS films

The release profiles of LYS and NIS at pH 6.0 were determined for LMW CS that was selected as the most potent antimicrobial CS form on *L. innocua* (Fig. 2). The lack of any enzyme activity release from LMW CS films with LYS at 1.25 mg/cm^2, and only a slight release of the enzyme from films with LYS at 2.5 mg/cm^2 clearly showed that LYS was bind by the CS film matrix (Fig. 2A). The retention of LYS by positively charged CS film should not be due to charge-charge attractions since LYS is also positively charged at pH 6.0. Thus, it seemed that the H-bonding, as proposed by Yao and Li (1994), and/or other factors (e.g., hydrophobic interactions and physical entrapment) could have played roles in the binding of LYS on CS matrix. However, a significant and concentration-dependent LYS release started from LMW CS films as enzyme concentration increased $\geq$ 3.5 mg/cm^2. The recoveries of LYS from films with 3.5, 4.0 and 5 mg LYS/cm^2 were 46, 59 and 74 %, respectively (see supplementary file, Fig. 1S-A). Thus, it appears that the CS matrix failed to bind and retain excessive LYS. These results were in line with the findings of Park et al. (2004) who also observed a concentration-dependent increase in LYS release from CS films. However, further studies are needed to explore exact mechanisms effective on the binding of LYS by the CS matrix.

The NIS at 0.5 mg/cm^2 was also bound by LMW CS matrix effectively (Fig. 2B), but unlike LYS, NIS at this concentration released slowly from the films with a recovery of almost 10 % (see supplementary file, Fig. 1S-B). On the other hand, the incorporation of NIS or LYS alone did not cause a considerable change in the dense structure of LMW CS films observed with SEM, but this resulted in the formation of some limited number of visible disordered structures within the film matrix (Fig. 3A–C). The addition of LYS-NIS mixture increased the disordered heterogeneously distributed structures observed within the films (Fig. 3D). During the drying of films, the concentration of solutes in the film forming solution increased the interactions of film components (CS, NIS, and LYS). Thus, it is possible that the complexation

of LYS and/or NIS molecules caused the aggregation of part of these protein-based agents due to increased concentration effect. It seems that these aggregations caused some local interruption of the ordered CS film network. However, all these observations did not suggest major changes in the overall dense film morphology of CS films following incorporation of LYS-NIS. Similar heterogeneous structures were also reported for NIS incorporated CS films by different workers (Cé et al., 2012, Zimet et al., 2019), and attributed to interruption of polymer-polymer (CS-CS) interactions by NIS (Gharsallaoui, Joly, Oulahal, & Degraeve, 2016; Zimet et al., 2019).

3.4. Colour and opacity of LMW CS films

The control LMW CS films showed the general characteristics of CS

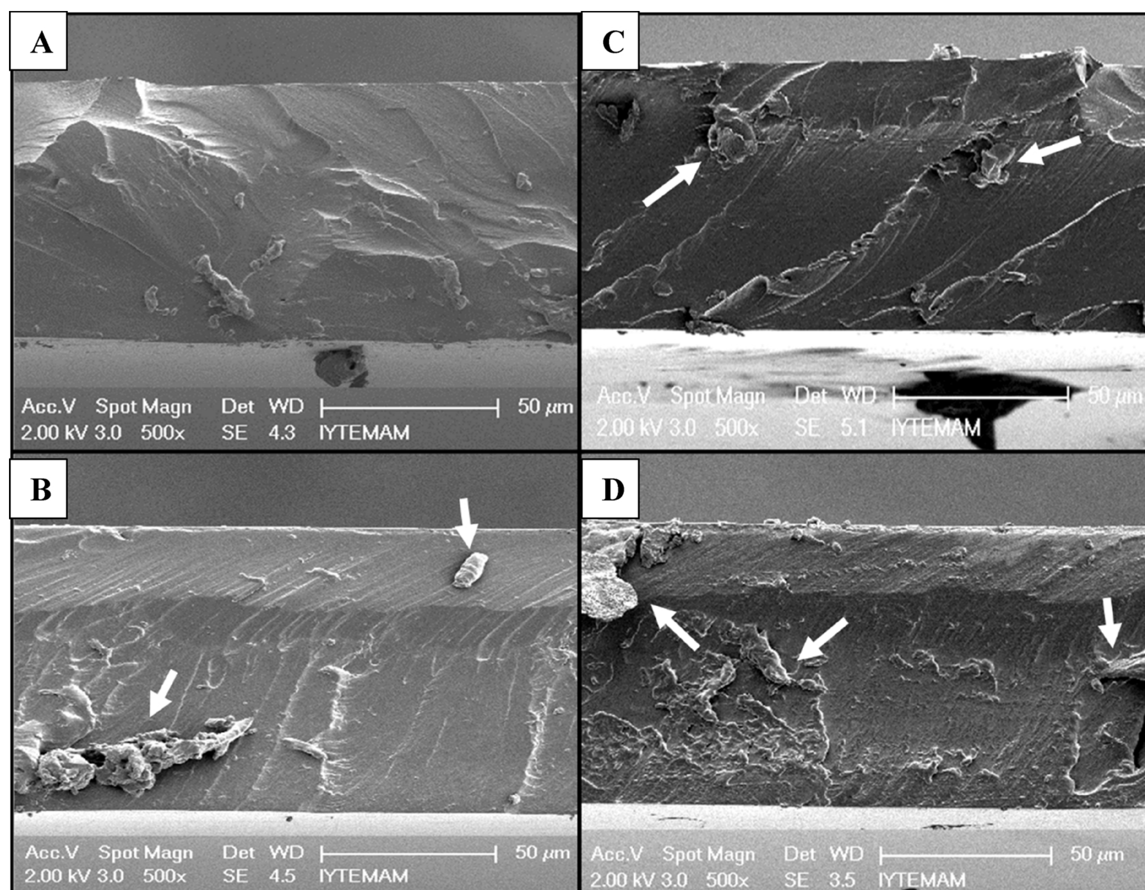

Fig. 3. SEM photographs of LMW CS films (White arrows indicate heterogeneously distributed aggregates in films) (Magnification 500×; A: Control; B: 3.5 mg/cm^2 LYS; C: 0.5 mg/cm^2 NIS; D: 3.5 mg/cm^2 LYS and 0.5 mg/cm^2 NIS).

Table 2
Color and opacity of films incorporated with different antimicrobials.

Type of Film*	L* (Lightness)	a* (Redness)	b* (Yellowness)	Opacity (A_{600}/mm)
Control	84.3 ± 0.6^B	0.1 ± 0.0^A	2.8 ± 0.3^C	0.5 ± 0.1^C
LYS	85.3 ± 0.3^A	−0.2 ± 0.1^B	3.7 ± 0.4^B	0.4 ± 0.0^C
NIS	85.0 ± 0.4^A	0.1 ± 0.0^A	3.8 ± 0.4^B	2.1 ± 0.2^A
LYS-NIS	83.2 ± 0.2^C	0.1 ± 0.0^A	4.7 ± 0.3^A	1.4 ± 0.2^B

$^{A–C}$ Values at each column followed by different letters indicate significant differences (n = 5; $P < 0.05$); *The concentration of LYS and/or NIS incorporated into the CS films was 3.5 and/or 0.5 mg/cm^2, respectively.

films that are colourless and transparent in nature (see supplementary file, Fig. 2S-A). The incorporation of LYS, NIS or LYS-NIS did not cause dramatic changes in the visual appearance of films, but a slight darkening was observed in the film with LYS-NIS (Fig. 2S-B to D) The changes in L* (lightness) values of films by incorporation of LYS and/or NIS were significant, but these values varied at a narrow range (Table 2). No significant changes were observed in a* values (redness) of films by incorporation of NIS and LYS-NIS while a limited decline was observed in the a* of films with the addition of LYS alone. On the other hand, films with antimicrobials, particularly those with LYS-NIS, showed an apparent increase in their b* values (yellowness). The increase in yellowness of pre-cast polysaccharide films loaded with protein based antimicrobials and dried at 45 °C was expected due to the possibility of the Maillard reaction occurred between carbonyl groups of CS with amino groups of LYS and NIS. However, such browning reactions could be controlled during coating applications on seeds that could be dried with dehumidified air at room temperature. On the other hand, the opacity of films did not change significantly by the addition of LYS while the addition of NIS or LYS-NIS caused a significant increase in film opacity.

3.5. Mechanical properties of LMW CS films

Mechanical properties of developed films: tensile strength (TS), elongation at break (E) and Young's modulus (YM), are presented in Table 3. No significant changes occurred in TS and E of CS films by incorporation of NIS or LYS alone. The incorporation of LYS-NIS gave a film with a lower TS than that of control CS films, possibly due to local interruptions in the film matrix caused by formed LYS-NIS aggregates (see SEM micrographs). However, such changes should have limited effects on overall dense structures of films with LYS-NIS since their TS was not significantly different than those of films with LYS or NIS alone. The E of the film with LYS-NIS was also not significantly different than those of control CS films and films with NIS alone, but it is significantly

Table 3
Mechanical properties of LMW CS films after incorporation of LYS and/or NIS.

Conc. (mg/cm^2)		Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)
LYS	NIS			
–	–	2.98 ± 0.86^A	30.67 ± 7.19^{AB}	0.09 ± 0.01^D
3.5	–	2.26 ± 0.46^{AB}	36.99 ± 6.69^A	1.78 ± 0.20^A
–	0.5	2.79 ± 1.04^{AB}	34.50 ± 7.49^{AB}	0.72 ± 0.16^B
3.5	0.5	1.79 ± 0.18^B	28.15 ± 7.42^B	0.43 ± 0.18^C

$^{A-D}$ Values at each column followed by different letters indicate significant differences (n = 7; $P < 0.05$).

Table 4
Effect of LMW CS with or without LYS-NIS coating on *L. innocua* load of inoculated seeds (n = 6; P < 0.05).

Samples	Uncoated	LMW CS coated	LMW CS with LYS-NIS coated*
Mung bean	5.23 ± 0.14 [a,B]	2.72 ± 0.22 [b,A]	1.93 ± 0.33 [c,A]
Wheat	5.75 ± 0.12 [a,A]	2.14 ± 0.52 [b,B]	< 1.69
Lentil	5.65 ± 0.13 [a,A]	2.83 ± 0.04 [b,A]	2.23 ± 0.33 [c,A]

[a-c and A-B]Values at each raw (lower case letters) and column (capital letters) followed by different letters indicate statistically significant differences; *LYS and NIS concentrations of LMW CS film solutions used for coating were 9.9 mg/g and 1.4 mg/g, respectively.

lower than that of the film with LYS alone. On the other hand, the YM of films showed a greater variation than their other parameters. In particular, the significant increase in YM of films by incorporation of LYS alone indicated increased film networking possibly due to the binding of LYS by the LMW CS matrix.

3.6. Antilisterial effects of LMW CS coatings with LYS-NIS on different seeds

The antimicrobial effects of LMW CS coating with or without LYS-NIS mixture on *L. innocua* inoculated onto mung bean, lentil, and wheat are presented in Table 4. The differences between the *Listeria* loads of uncoated control and LMW CS coated mung bean, lentil, and wheat were 2.5, 2.8 and 3.6 log, respectively. These results clearly proved the effectiveness of LMW CS alone as an antimicrobial coating. However, it is important to note that the LMW CS coating of mung beans and lentils alone is still insufficient to reach the 3 log reduction requirement of CFIA for seed disinfection methods targeting food pathogens (CFIA, 2018). On the other hand, the higher antilisterial effect achieved for wheat than mung bean and lentil could be related to the high amount of LMW CS retention and/or high affinity of LMW CS on wheat surface. The wheats have a rough surface that might increase the amount of coating retained at their surface (Cromey, Wright, & Boddington, 1998). In contrast, the lentils had an uneven surface covered with distinctive conical shaped papillae that might impair the coating homogeneity (Hughes & Swanson, 1986) while mung beans have a smooth surface that might limit amount of coating retained at their surfaces (Miano, da Costa Pereira, Castanha, da Matta Júnior, & Augusto, 2016). On the other hand, it is important to note that the LMW CS with LYS-NIS mixture caused 3.3, 3.4 and > 4.1 log reduction in initial *L. innocua* loads of mung beans, lentils, and wheats, respectively. The *L. innocua* counts of mung beans and lentils coated by CS with LYS-NIS were significantly lower than those of mung beans and lentils coated by CS alone. Moreover, *L. innocua* load of wheats coated by LMW CS with LYS-NIS (< 1.69 CFU/g) was minimum 0.46 log lower than that of wheats coated with LMW CS alone. These results clearly showed the possibility of obtaining a minimum 3 log reduction in *Listeria* loads of studied seeds by LMW CS coatings incorporated with LYS-NIS mixture.

3.7. Effect of LMW CS coating on germination rate of seeds

The effects of LMW CS coating on germination rates of mung beans, lentils, and wheats were also determined to evaluate the applicability of developed treatment (Fig. 4). The results clearly showed that the LMW CS coating had no significant effects on germination rates of mung beans and wheats ($P > 0.05$). In contrast, LMW CS coating caused a statistically significant, but limited reduction (almost 5.4 %) in germination rates of lentils at the end of 5 days. The germination rates of LMW CS coated seeds at the end of 5 days were minimum 93 % for lentil, and minimum 98 % for mung bean and wheat. In the literature, similar germination tests were applied to determine the effects of disinfection methods such as hot water or steam heating on sprouted seeds such as alfalfa seeds and mung beans (Studer et al., 2013; Trząskowska et al., 2018). However, the current study is the first study that investigated the effects of antimicrobial coating on germination rates of seeds destined for edible sprout production.

4. Conclusions

This work clearly showed that the LMW CS alone could be applied as a coating to reduce *Listeria* load of seeds destined for sprout production. However, it is also proved that the LMW CS coating alone is not sufficient to obtain a 3 log reduction in *L. innocua* at the surfaces of lentils and mung beans. In contrast, the combination of inherent antimicrobial activity of LMW CS coating with that of incorporated synergetic LYS-NIS mixture gives a potent active coating that could be employed to achieve > 3 log reduction in *Listeria* load at the surfaces of all tested seeds. No considerable negative effect of LMW CS coating on seeds' germination rates was determined, but further tests (e.g., effects on yield) are needed with different types of seeds at different coating conditions. Moreover, the effect of developed antimicrobial coating on other pathogenic bacteria should also be evaluated. This work provided a basis to employ natural antimicrobial coating as a novel decontamination method that eliminates risk of listeriosis from sprouted legumes and cereals.

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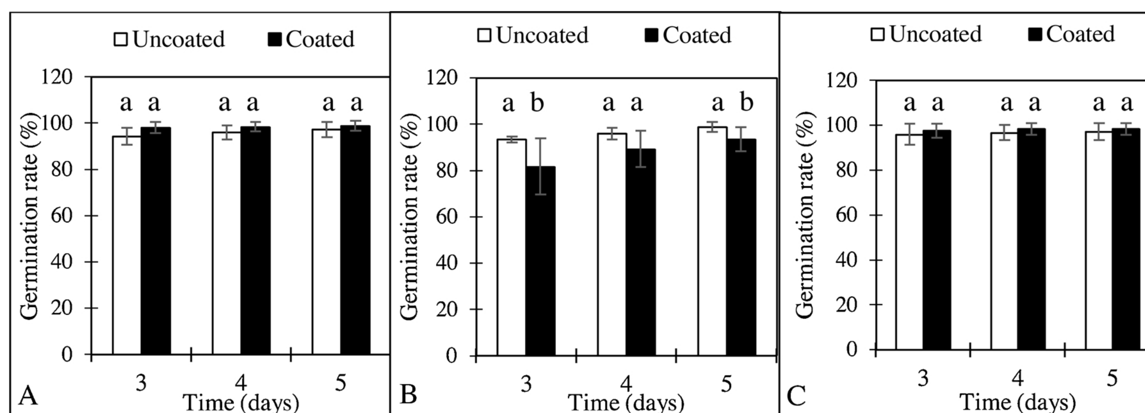

Fig. 4. Germination rates of LMW CS coated and uncoated mung bean (A), lentil (B) and wheat (C) (Different lower case letters indicate significant differences between uncoated and coated seeds at indicated days) (n = 9; $P < 0.05$).

analysis. We thank PhD student Pelin Barış Kavur for kindly conducting color and opacity measurements. All other experiments were conducted by Dr. Gözde Seval Sözbilen as part of her PhD thesis.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2020.115968.

References

Álvarez-Ordóñez, A., Leong, D., Hickey, B., Beaufort, A., & Jordan, K. (2015). The challenge of challenge testing to monitor *Listeria monocytogenes* growth on ready-to-eat foods in Europe by following the European Commission (2014) Technical Guidance document. *Food Research International, 75*, 233–243.

ASTM (1999). *Standard test methods for tensile properties of thin plastic sheeting. D882-97. Annual book of ASTM standards. Philadelphia.* Pa: ASTM Intl. p163–171.

Bhatia, S., & Bharti, A. (2015). Evaluating the antimicrobial activity of Nisin, Lysozyme and Ethylenediaminetetraacetate incorporated in starch based active food packaging film. *Journal of Food Science and Technology, 52*(6), 3504–3512.

Boyacı, D., & Yemenicioğlu, A. (2018). Expanding horizons of active packaging: Design of consumer-controlled release systems helps risk management of susceptible individuals. *Food Hydrocolloids, 79*, 291–300.

CDC (2014). *Center for disease control and prevention. Sprouts and investigation of human Listeriosis cases (Final update)*. Wholesome soy products, Inc.. Accessed June 2019 https://www.cdc.gov/listeria/outbreaks/bean-sprouts-11-14/index.html.

CDC (2016). *Center for disease control and prevention. Multistate outbreak of Salmonella Reading and Salmonella abony infections linked to alfalfa sprouts (Final update)*. Accessed June 2019 https://www.cdc.gov/salmonella/reading-08-16/index.html.

Cé, N., Noreña, C. P., & Brandelli, A. (2012). Antimicrobial activity of chitosan films containing nisin, peptide P34, and natamycin. *CyTA-Journal of Food, 10*(1), 21–26.

CFIA (2018). *Canadian Food Inspection Agency. Preventive controls for the hygienic production of sprouted seeds.* Accessed June 2019 http://www.inspection.gc.ca/food/general-food-requirements-and-guidance/preventive-controls-food-businesses/fresh-fruits-or-vegetables/sprouted-seeds/eng/1524179755850/1524179758065.

Chung, W., & Hancock, R. E. (2000). Action of lysozyme and nisin mixtures against lactic acid bacteria. *International Journal of Food Microbiology, 60*(1), 25–32.

Cromey, M. G., Wright, D. S. C., & Boddington, H. J. (1998). Effects of frost during grain filling on wheat yield and grain structure. *New Zealand Journal of Crop and Horticultural Science, 26*(4), 279–290.

Duan, J., Park, S. I., Daeschel, M. A., & Zhao, Y. (2007). Antimicrobial chitosan-lysozyme (CL) films and coatings for enhancing microbial safety of mozzarella cheese. *Journal of Food Science, 72*(9), M355–M362.

Duan, J., Kim, K., Daeschel, M. A., & Zhao, Y. (2008). Storability of antimicrobial chitosan-lysozyme composite coating and film-forming solutions. *Journal of Food Science, 73*(6), M321–M329.

Duran, M., Aday, M. S., Zorba, N. N. D., Temizkan, R., Büyükcan, M. B., & Caner, C. (2016). Potential of antimicrobial active packaging 'containing natamycin, nisin, pomegranate and grape seed extract in chitosan coating'to extend shelf life of fresh strawberry. *Food and Bioproducts Processing, 98*, 354–363.

Dutta, P. K., Tripathi, S., Mehrotra, G. K., & Dutta, J. (2009). Perspectives for chitosan based antimicrobial films in food applications. *Food Chemistry, 114*(4), 1173–1182.

EFSA (2011a). *European food safety authority.* Available at: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2011.EN-176 Accessed March 2019.

EFSA (2011b). *European food safety authority.* Available at: https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2011.2424 Accessed March 2019.

Gallo, L. I., Pilosof, A. M. R., & Jagus, R. J. (2007). Effective control of *Listeria innocua* by combination of nisin, pH and low temperature in liquid cheese whey. *Food Control, 18*(9), 1086–1092.

Gharsallaoui, A., Joly, C., Oulahal, N., & Degraeve, P. (2016). Nisin as a food preservative: Part 2: Antimicrobial polymer materials containing nisin. *Critical Reviews in Food Science and Nutrition, 56*(8), 1275–1289.

Gill, A. O., & Holley, R. A. (2000). Inhibition of bacterial growth on ham and bologna by lysozyme, nisin and EDTA. *Food Research International, 33*(2), 83–90.

Guo, M., Jin, T. Z., Wang, L., Scullen, O. J., & Sommers, C. H. (2014). Antimicrobial films and coatings for inactivation of *Listeria innocua* on ready-to-eat deli turkey meat. *Food Control, 40*, 64–70.

Harris, L. J., Fleming, H. P., & Klaenhammer, T. R. (1991). Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A, and UAL500 to nisin. *Journal of Food Protection, 54*(11), 836–840.

Hughes, J. S., & Swanson, B. G. (1986). Microstructure of lentil seeds (*Lens culinaris*). *Food Structure, 5*(2), 8.

Iacumin, L., & Comi, G. (2019). Microbial quality of raw and ready-to-eat mung bean sprouts produced in Italy. *Food Microbiology, 82*, 371–377.

Imran, M., Klouj, A., Revol-Junelles, A. M., & Desobry, S. (2014). Controlled release of nisin from HPMC, sodium caseinate, poly-lactic acid and chitosan for active packaging applications. *Journal of Food Engineering, 143*, 178–185.

Kim, S. H., No, H. K., & Prinyawiwatkul, W. (2007). Effect of molecular weight, type of chitosan, and chitosan solution pH on the shelf-life and quality of coated eggs. *Journal of Food Science, 72*(1), S044–S048.

Leleu, S., Herman, L., Heyndrickx, M., De Reu, K., Michiels, C. W., De Baerdemaeker, J., et al. (2011). Effects on *Salmonella* shell contamination and trans-shell penetration of coating hens' eggs with chitosan. *International Journal of Food Microbiology, 145*(1), 43–48.

Mangalassary, S., Han, I., Rieck, J., Acton, J., & Dawson, P. (2008). Effect of combining nisin and/or lysozyme with in-package pasteurization for control of *Listeria monocytogenes* in ready-to-eat turkey bologna during refrigerated storage. *Food Microbiology, 25*(7), 866–870.

Mehyar, G. F., Al Nabulsi, A. A., Saleh, M., Olaimat, A. N., & Holley, R. A. (2018). Effects of chitosan coating containing lysozyme or natamycin on shelf-life, microbial quality, and sensory properties of Halloumi cheese brined in normal and reduced salt solutions. *Journal of Food Processing and Preservation, 42*(1), e13324.

Miano, A. C., da Costa Pereira, J., Castanha, N., da Matta Júnior, M. D., & Augusto, P. E. D. (2016). Enhancing mung bean hydration using the ultrasound technology: Description of mechanisms and impact on its germination and main components. *Scientific Reports, 6*, 38996.

Millan-Sango, D., Sammut, E., Van Impe, J. F., & Valdramidis, V. P. (2017). Decontamination of alfalfa and mung bean sprouts by ultrasound and aqueous chlorine dioxide. *LWT-Food Science and Technology, 78*, 90–96.

No, H. K., Meyers, S. P., Prinyawiwatkul, W., & Xu, Z. (2007). Applications of chitosan for improvement of quality and shelf life of foods: A review. *Journal of Food Science, 72*(5), R87–R100.

Park, S. I., Daeschel, M. A., & Zhao, Y. (2004). Functional properties of antimicrobial lysozyme-chitosan composite films. *Journal of Food Science, 69*(8), M215–M221.

Piernas, V., & Guiraud, J. P. (1997). Microbial hazards related to rice sprouting. *International Journal of Food Science & Technology, 32*(1), 33–39.

Pierre, P. M., & Ryser, E. T. (2006). Inactivation of *Escherichia coli* O157: H7, *Salmonella* typhimurium DT104, and *Listeria monocytogenes* on inoculated alfalfa seeds with a fatty acid–based sanitizer. *Journal of Food Protection, 69*(3), 582–590.

Pranoto, Y., Rakshit, S. K., & Salokhe, V. M. (2005). Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin. *LWT-Food Science and Technology, 38*(8), 859–865.

Qin, C., Li, H., Xiao, Q., Liu, Y., Zhu, J., & Du, Y. (2006). Water-solubility of chitosan and its antimicrobial activity. *Carbohydrate Polymers, 63*(3), 367–374.

Raafat, D., von Bargen, K., Haas, A., & Sahl, H. G. (2008). Chitosan as an antibacterial compound: Insights into its mode of action. *Applied and Environmental Microbiology*.

Rabea, E. I., Badawy, M. E. T., Stevens, C. V., Smagghe, G., & Steurbaut, W. (2003). Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules, 4*(6), 1457–1465.

Schillinger, U., Becker, B., Vignolo, G., & Holzapfel, W. H. (2001). Efficacy of nisin in combination with protective cultures against *Listeria monocytogenes* Scott A in tofu. *International Journal of Food Microbiology, 71*(2-3), 159–168.

Shin, Y., Yoo, D. I., & Jang, J. (2001). Molecular weight effect on antimicrobial activity of chitosan treated cotton fabrics. *Journal of Applied Polymer Science, 80*(13), 2495–2501.

Sozbilen, G. S., Korel, F., & Yemenicioğlu, A. (2018). Control of lactic acid bacteria in fermented beverages using lysozyme and nisin: Test of traditional beverage boza as a model food system. *International Journal of Food Science & Technology, 53*(10), 2357–2368.

Studer, P., Heller, W. E., Hummerjohann, J., & Drissner, D. (2013). Evaluation of aerated steam treatment of alfalfa and mung bean seeds to eliminate high levels of *Escherichia coli* O157: H7 and O178: H12, *Salmonella* enterica, and *Listeria monocytogenes*. *Applied and Environmental Microbiology, 79*(15), 4613–4619.

Synowiecki, J., & Al-Khateeb, N. A. (2003). Production, properties, and some new applications of chitin and its derivatives. *Critical Reviews in Food Science and Nutrition, 43*(2), 145–171.

Tantala, J., Thumanu, K., & Rachtanapun, C. (2019). An assessment of antibacterial mode of action of chitosan on *Listeria innocua* cells using real-time HATR-FTIR spectroscopy. *International Journal of Biological Macromolecules, 135*, 386–393.

Teerakarn, A., Hirt, D. E., Acton, J. C., Rieck, J. R., & Dawson, P. L. (2002). Nisin diffusion in protein films: Effects of film type and temperature. *Journal of Food Science, 67*(8), 3019–3025.

Thumula, P. (2006). *Studies on storage behaviour of tomatoes coated with chitosan-lysozyme films.* Doctoral dissertationMcGill University.

Trząskowska, M., Dai, Y., Delaquis, P., & Wang, S. (2018). Pathogen reduction on mung bean reduction of *Escherichia coli* O157: H7, *Salmonella enterica* and *Listeria monocytogenes* on mung bean using combined thermal and chemical treatments with acetic acid and hydrogen peroxide. *Food Microbiology, 76*, 62–68.

Ünalan, İ. U., Ucar, K. D. A., Arcan, I., Korel, F., & Yemenicioğlu, A. (2011). Antimicrobial potential of polylysine in edible films. *Food Science and Technology Research, 17*(4), 375–380.

Vargas, M., & González-Martínez, C. (2010). Recent patents on food applications of chitosan. *Recent Patents on Food, Nutrition & Agriculture, 2*(2), 121–128.

Vázquez-Boland, J. A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J., & Goebel, W. (2001). Pathogenicity islands and virulence evolution in *Listeria*. *Microbes and Infection, 3*(7), 571–584.

Wang, H., Qian, J., & Ding, F. (2018). Emerging chitosan-based films for food packaging applications. *Journal of Agricultural and Food Chemistry, 66*(2), 395–413.

Yao, Y. J., & Li, S. F. Y. (1994). Capillary zone electrophoresis of basic proteins with chitosan as a capillary modifier. *Journal of Chromatography A, 663*(1), 97–104.

Ye, M., Neetoo, H., & Chen, H. (2008a). Control of *Listeria monocytogenes* on ham steaks by antimicrobials incorporated into chitosan-coated plastic films. *Food Microbiology, 25*(2), 260–268.

Ye, M., Neetoo, H., & Chen, H. (2008b). Effectiveness of chitosan-coated plastic films incorporating antimicrobials in inhibition of *Listeria monocytogenes* on cold-smoked salmon. *International Journal of Food Microbiology, 127*(3), 235–240.

Yuceer, M., & Caner, C. (2014). Antimicrobial lysozyme–chitosan coatings affect functional properties and shelf life of chicken eggs during storage. *Journal of the Science of Food and Agriculture, 94*(1), 153–162.

Zheng, L. Y., & Zhu, J. F. (2003). Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydrate Polymers, 54*(4), 527–530.

Zimet, P., Mombrú, Á. W., Mombrú, D., Castro, A., Villanueva, J. P., Pardo, H., et al. (2019). Physico-chemical and antilisterial properties of nisin-incorporated chitosan/carboxymethyl chitosan films. *Carbohydrate Polymers, 219*, 334–343.