Original article

Antimicrobial activity of nisin incorporated in pectin and polylactic acid composite films against Listeria monocytogenes

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Summary
An extruded composite food packaging film containing pectin, polylactic acids (PLAs) and nisin was developed to inhibit Listeria monocytogenes. The mechanical properties and surface structure of the film were also examined. Cells of L. monocytogenes were reduced by 2.1, 4.5 and 3.7 log units mL⁻¹ by the pectin plus PLA (pectin/PLA) film containing nisin (1000 IU mL⁻¹ of tested liquid) in Brain Heart Infusion (BHI) broth, liquid egg white and orange juice, respectively, after 48 h at 24 °C. Pectin played an important roll in embedding nisin into the film. The pectin/PLA film had a similar stiffness but lower tensile strength, elongation and fracture energy than the pure PLA film. These data suggested that nisin incorporated into the pectin/PLA film was an effective approach to reducing L. monocytogenes in a typical growth medium (e.g. BHI broth) as well as in foods (e.g. orange juice and liquid egg).

Keywords
Films, liquid egg, Listeria monocytogenes, nisin, orange juice, packaging, pectin, polylactic acids.

Introduction
In recent years, there has been a growing interest in the use of natural antimicrobials, especially nisin, in food packaging applications. Nisin is a bacteriocin produced by Lactococcus lactis ssp. lactis. It is effective against gram-positive bacteria and ineffective against fungi and gram-negative bacteria (Jay, 1996). Nisin was affirmed GRAS by Food and Drug Administration in 1988 (FDA, 1988), and now used as a biopreservative in 57 countries around the world. Because it is non-toxic, heat stable and does not contribute to off-flavours, nisin is commercially used in a variety of foods including dairy, eggs, vegetables, meat, fish, beverages and cereal-based products to inhibit growths of foodborne pathogens including Listeria monocytogenes (Schillinger et al., 1996).

Listeria are a concern in food with extended shelf life because they tolerate salt, pH changes, inadequate thermal pasteurisation and refrigerated temperatures (Conner et al., 1986; Cole et al., 1990; Harris et al., 1991). They are ubiquitous and can contaminate many foods and beverages (Adams & Moss, 2000). Some strains can grow in the pH range of approximately 4.1–9.6 and in the temperature range of approximately 1–45 °C (Yuste & Fung, 2002). Cells of L. monocytogenes have been implicated in several fatal outbreaks of foodborne illness (Ryser & Marth, 1991; Mead et al., 1999). The presence of L. monocytogenes in ready-to-eat foods is a special concern for at-risk populations. The USDA’s Food Safety Inspection Service has set a zero-tolerance level for L. monocytogenes in ready-to-eat foods (Klima & Montville, 1995). Cells of L. monocytogenes have been isolated from commercially processed liquid whole egg in the United States (Leasor & Foegeding, 1989) and Northern Ireland (Moore & Madden, 1993). Although no outbreaks of listeriosis have been attributed to eggs, the potential exists for survival and growth of L. monocytogenes in egg products. Acidic foods such as fruit juices were not recognised as vehicles for organisms causing foodborne illness until their survival in acidic fruit juices was demonstrated recently (Mazzotta, 2001). No outbreaks involving L. monocytogenes in fruit juices have been reported; however, this pathogen can survive in the presence of

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possible inhibitory compounds in acidic food, such as apple and orange juices, cider and tomato products, even if it does not proliferate during normal refrigerated storage (Beuchat & Brackett, 1991; Buchanan & Golden, 1998; Sado et al., 1998). The fact that this pathogen may survive well beyond the normal shelf-life of non-sterile acidic fruit juices suggests that these products are potential vehicles of infection with *L. monocytogenes*. Therefore, the National Advisory Committee on Microbiological Criteria for Foods recommended that, in the absence of known specific pathogen-product associations, *L. monocytogenes* should be used as one of the target organisms, as appropriate (FDA, 1998).

An approach to controlling the growth of *Listeria* or other gram-positive bacteria in foods is adding nisin directly to foods by formulation. However, the nisin is eventually exhausted due to interactions with the food matrix and bacterial cells (Zhang et al., 2004). Interest in the use of films as nisin delivery systems to reduce undesirable bacteria in foodstuff has increased in recent years. Various approaches have been proposed and demonstrated by using edible and polymer films to deliver nisin (Baldwin et al., 1997; Cuq et al., 1998; Debeaufort et al., 1998).

Polylactic acid (PLA) is a biodegradable and compostable polymer with hydrophobic surface properties, which can be derived from renewable resources. Polymers of PLA are of current interest not only because of the need to ultimately replace many fossil fuel-derived polymers but also due to the growing global problems associated with plastic waste disposal (Plackett et al., 2002; Frederiksen et al., 2003). There have been developments in Europe and in North America that have involved the use of PLA-based packaging for supermarket products, such as Biota™ PLA-bottled water, Noble™ PLA-bottled juices and Dannon™ yogurts.

Pectin is a water-soluble hygroscopic polymer. Pectin has been used as a thickening, coating and encapsulating material. It can be used as a vehicle to carry and deliver nisin (Baldwin et al., 1997; Cuq et al., 1998; Debeaufort et al., 1998).

**Materials and Methods**

**Film Preparation**

PLA resin was obtained from Cargill Dow (Minneapolis, MN, USA) and pectin sodium salt was purchased from Danisco (Danisco Cultor USA, New Century, KS, USA). The pectin had an average molecular weight of 90 000, a degree of esterification of 60% and a water content of 7.8%. Pectin plus PLA (pectin/PLA) composite films and PLA films were prepared according to Liu et al. (2007). Briefly, the films were prepared with a Brabender single-screw extruder with four temperature zones (150°–170°–170°–150 °C). The high shear mixing zone screw had a 3:1 ratio. Ribbons were extruded using a hangar-type die at 150 °C. The thickness of resultant films was measured using a micrometer (Ames, Waltham, MA, USA).

Nisin (Nisaplin, Danisco Cultor USA) was reported to be 2.5% pure (1 000 000 IU g⁻¹), with the remaining components being listed as sodium chloride and milk solids. Nisin was loaded into pectin/PLA and PLA films by a diffusion coating method post-extrusion according to Liu et al. (2007). Briefly, five pieces of film (1.6 cm in diameter each) were placed in a Petri dish (60 × 15 mm) containing 10.0 mL of 1% nisin solution adjusted to pH 2 with 1 N HCl and shaken at room temperature at 80 r.p.m. for 18 h. The film pieces were removed from the nisin solution, washed three times with 10 mL of 1 N NaCl (pH 2) and three times with deionised water by shaking in the solutions for 1 min for each time. The washed films were dried under laminar flow for 30 min and stored at 4–7°C in a refrigerator prior to bacterial inhibition tests.

**Nisin in Films**

To determine the nisin concentration entrapped in the film pieces, a standard curve was prepared using a modified seeded lawn overlay spot method (Siragusa et al., 1999). *Listeria monocytogenes*, BHI agar and stock solution of BHI broth were used in this study as bacterial inhibition testing of films was performed with *Listeria in BHI broth/BHI agar*. Petri plates containing BHI agar were overlaid with 8 mL of semi soft BHI agar (0.5% w/v agar) seeded with 10 μL of an overnight culture of *L. monocytogenes*. The seed density was approximately 1 × 10⁶ CFU mL⁻¹ of overlay. Nisin standard solutions (100–1 000 IU mL⁻¹) were freshly prepared by diluting nisin stock solution in BHI broth. Twenty microlitres of nisin standard solution were spotted on the agar surface. The plates were incubated at 37°C for 24 h and examined for zones of inhibition. The size of the zone was proportional to the amount of nisin applied. The regression line describing the relationship between zone size and known nisin
concentration served as a standard curve. A pectin/PLA with nisin (pectin/PLA + nisin) film sample was placed in a glass tube with 10 mL of BHI broth, heated in a beaker with boiling water (100 °C) for 5 min, cooled and stirred at room temperature for 10 days. The concentration of nisin in the extract was determined using the seeded lawn overlay spot method previously described and the amount of nisin incorporated in the film calculated. The pectin/PLA + nisin films contained approximately 500 IU nisin per mg of film as determined from the standard curve.

Bacterial inhibition test
Pathogenic bacteria L. monocytogenes Scott A 724 used for stock cultures was obtained from the culture collection of the US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center. Stock cultures were maintained at −80 °C in BHI medium (Difco Laboratory, Detroit, MI, USA). Working Cultures of L. monocytogenes were maintained on BHI agar at 4 °C and were sub-cultured bi-weekly and grown aerobically at 37 °C in BHI broth. Prior to inoculation of product, cultures were grown in BHI broth at 37 °C for 16–18 h.

Bacterial inhibition studies were evaluated using agar diffusion and liquid incubation methods (Chung et al., 2003). For liquid incubation tests, five pieces of film composed of either pectin/PLA or PLA were placed in a glass bottle containing 50 mL liquid medium (BHI broth, orange juice or liquid egg white). Preservative-free orange juice and pasteurised liquid egg white were purchased in a local store. The orange juice was autoclaved before use. The pH of liquid egg white was 8.46 and the pH of the orange juice was 3.78. Aliquots containing 1 mL of an overnight culture of L. monocytogenes (approximately 1 × 10⁹ cells) were transferred into the bottles and shaken at 150 r.p.m. at 24 °C. At each sampling time, aliquots containing 1 mL of incubated sample were serially diluted with sterile phosphate buffer (Hardy Diagnostics, Santa Maria, CA, USA), then pour plated onto BHI agar. Plates were incubated at 37 °C for 24 h. Film-free inoculated medium served as controls. Room temperature (24 °C) was selected for the incubation tests after considering the worst case scenario in which food was left at room temperature, at which the pathogens grow much faster, rather than refrigerated.

The agar diffusion test was used to simulate a test for solid food packaging. Each film sample was placed on a surface-inoculated BHI agar plate, on which 10⁶ CFU mL⁻¹ of L. monocytogenes were seeded. The agar plates were incubated at 37 °C for 24 h. The diameters of the growth inhibition zones were measured with a caliper. The ratio of the diameter of inhibition zone to the diameter of the film specimen (16 mm) was used to determine antimicrobial activity.

Mechanical test
Film samples were cut into ASTM D638-99 Type I tensile bars (16.42 cm × 1.91 cm, w × l) for mechanical property tests. Properties were measured at 21 °C and 65% relative humidity with a gauge length of 102 mm. An upgraded Instron mechanical property tester (model 1122) and Testworks 4 data acquisition software (MTS Systems Corp., Minneapolis, MN, USA) were used throughout this investigation. The strain rate (cross-head speed) was set at 50 mm min⁻¹.

Confocal laser microscopy
Film specimens were glued to a 1 cm × 3 cm microscope slide and placed on an optical microscope (model IRBE; Leica Microsystems GmbH, Wetzlar, Germany) with a 10× lens integrated with a model TCS-SP laser scanning confocal microscope (Leica Microsystems, Exton, PA, USA). Images were made at 633 nm for confocal reflection and at 425/475 nm (ex/cm.) for autofluorescence at two channels.

Statistical analysis
Antimicrobial experiments were conducted in triplicate on different days, with two observations per film treatment for each replication (n = 6). For mechanical testing, all measurements were performed on five samples (n = 5). Data points were expressed as the mean ± SD. All data were analysed by analysis of variance using sas version 9.1 software (SAS Institute, Cary, NC, USA). Duncan’s multiple range tests were used to determine the significant difference of mean values at a significance level of 0.05.

Results and discussion
The PLA film and pectin/PLA film thickness (0.54 mm and 0.55 mm, respectively) were not significantly different (Table 1). The addition of nisin to films had no effect on the thickness of films.

Table 1 Composite and thickness of polyactic acid (PLA) film and pectin/PLA film

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thickness (mm)</th>
<th>PLA content (%)</th>
<th>Pectin content (%)</th>
<th>Water content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA</td>
<td>0.54 ± 0.02</td>
<td>100</td>
<td>0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>pectin/PLA</td>
<td>0.55 ± 0.02</td>
<td>75.2 ± 3</td>
<td>19.1 ± 6.2</td>
<td>6.7 ± 1.5</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± sd (n = 5).
on the film thickness or the variation of film thickness compared with the film without nisin (data not shown).

Table 2 presents mechanical properties of PLA and pectin/PLA films. The Young’s moduli of PLA and pectin/PLA films were similar, both around 2500 MPa, indicating that the two materials were similar in stiffness. Decreases in tensile strength of about 19% and fracture energy of about 40% for the composite Pectin/PLA film were observed. These decreases were mainly attributed to the reduction of the PLA phase. The addition of pectin to PLA brought water to the formula, which could have had a plasticising effect. This could have affected the physical properties of the pectin/PLA film. The nisin loading steps and incorporation of nisin did not affect the tensile strength, flexibility and toughness of pectin/PLA films (data not shown).

Figure 1 shows growth curves of *L. monocytogenes* in BHI broth at 24 °C. Control (film-free) sample in BHI showed a typical S-shape growth curve. However, pectin/PLA + nisin film significantly decreased bacterial counts compared with the control due to the rapid antimicrobial action of nisin against *L. monocytogenes* in the first 4 h, and both the growth rate and the final cell density of *L. monocytogenes* were reduced at 24 h and 48 h, respectively, in the liquid incubation method. The maximal nisin concentration migrating/diffusing from the films to the medium was equivalent to 1000 IU mL\(^{-1}\) of BHI broth. PLA film coated with nisin (PLA + nisin) or pectin/PLA film without nisin had little or no effect on the growth of *L. monocytogenes* as shown in Fig. 1, which was further confirmed by the agar diffusion test. The agar diffusion test showed the absence of zones of inhibition around both the pectin/PLA film without nisin and the PLA film with nisin (Fig. 2).

The ratio of the diameter of inhibition zone to the diameter of the film specimen (16 mm) was 1.02 for the PLA film coated with nisin and 1.56 for the pectin/PLA + nisin film (Fig. 2). The small antilisterial effect of PLA films with nisin indicated that there was little release of nisin from PLA + nisin film. The reason may be explained by the surface characteristics of films and the nisin coating method. It was not surprising that PLA films without nisin had no antimicrobial activity. Liu et al. (2007) also observed that PLA films and PLA + nisin films did not show antimicrobial activity against *Lactobacillus plantarum*. Because PLA + nisin films and pectin/PLA films without nisin showed little antilisterial activity, they were not included in the later experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Modulus (MPa)</th>
<th>Tensile strength (MPa)</th>
<th>Elongation (%)</th>
<th>Fracture energy (J cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA</td>
<td>2492 ± 99</td>
<td>53.4 ± 3.5</td>
<td>3.00 ± 0.21</td>
<td>0.63 ± 0.11</td>
</tr>
<tr>
<td>Pectin/PLA</td>
<td>2598 ± 100</td>
<td>40.2 ± 1.1</td>
<td>1.98 ± 0.07</td>
<td>0.35 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 1 Effect of film treatment on growth of *Listeria monocytogenes* in BHI broth at 24 °C. PLA film, PLA + nisin film and pectin/PLA + nisin film containing 50 000 IU nisin were tested in 50 mL of BHI broth (approximately 1000 IU nisin per mL of BHI broth). Error bars represent the standard deviation of the mean from three separate tests.

Figure 2 Antilisterial activity of films determined by agar diffusion method: Pectin/PLA film with nisin (1), Pectin/PLA film without nisin (2) and PLA film with nisin (3).
Figure 3 shows images of reflection and fluorescence in stereo projection for the surface structure of the two components, PLA and pectin. The reflection areas coloured red contained PLA fibres and the green-coloured area represented pectin particles. Confocal reflection microscopy revealed a continuous, smooth surface for pure PLA films (Fig. 3a), and a relatively rough morphology for pectin/PLA films, showing a ragged layer of 20–30 µm in thickness lying on the pectin/PLA film surfaces (Fig. 3b). The hydrophobic characteristics of PLA and the smooth film surface as shown in Fig. 3 limited the capability to incorporate nisin onto the film surface. After films were washed in the nisin coating procedure, they lost almost all of the nisin. On the contrary, the incorporation of pectin in the film created a rough and ragged surface, which was hydrophilic and facilitated the access and adsorption of nisin. These observations suggested that the pectin in the pectin/PLA film played an important role in embedding nisin during the diffusion coating process. Bower et al. (1995) studied the efficacy of nisin adsorption to siliconised surfaces and found that more nisin activity was retained when nisin was adsorbed to less hydrophobic surfaces.

Figure 4 shows the inhibitory effect of pectin/PLA film against \textit{L. monocytogenes} in liquid egg white. The cell population of \textit{L. monocytogenes} in liquid egg white with PLA/pectin + nisin film (approximately 1000 IU nisin per mL of liquid egg white) was reduced from 6.8 logs to 2 logs while the control remained at 6.5 logs after 48 h.

When \textit{L. monocytogenes} was inoculated into orange juice, the cell populations decreased over the 48 h incubation at 24 °C (Fig. 5). Pectin/PLA + nisin film significantly increased the reduction in the bacterial populations by 3–4 log cycles from 8 to 48 h. Orr et al. (1998) reported that refrigerated milk samples inoculated with \textit{L. monocytogenes} had a reduction of approximately 2 logs when exposed for 48 h to cast corn zein films containing nisin.

To examine whether a direct addition of nisin into a liquid medium would achieve a similar inhibitory effect on the growth of \textit{L. monocytogenes}, a comparison study was conducted. Growth curves of \textit{L. monocytogenes} in
BHI broth with nisin solution or Pectin/PLA + nisin film were determined. One piece of PLA/nisin film (20 mg) in 10 mL medium was used. The maximal amount of nisin diffusing from the film to medium was equivalent to 1000 IU mL\(^{-1}\) of BHI broth. The final concentrations of nisin directly added to the BHI broths were 200, 1000 and 5000 IU mL\(^{-1}\) of BHI broth, respectively. These concentrations were the same, or were five times greater or five times smaller than the nisin level released from the pectin/PLA + nisin film.

Figure 6 shows the effects of direct or indirect nisin treatments on the growth curves of L. monocytogenes in growth medium as affected by direct or indirect nisin treatments. The growth curve of the control cells (absence of nisin or films) represented a typical growth behaviour of L. monocytogenes in BHI. When nisin or pectin/PLA + nisin films were added, a 3 to 3.5-log reduction in the Listeria cell counts was observed at 4 h compared to the control. At that time, increasing the nisin concentration in liquid medium resulted in greater reductions in L. monocytogenes counts; and the antilisterial activity of films was equivalent to the direct treatment of 5000 IU mL\(^{-1}\). The cell populations in growth medium at 8 h ranged from log 5.8 in the control to 2.5 logs in the 200 IU mL\(^{-1}\) nisin treatment, 1.9 logs in the 1000 IU mL\(^{-1}\) nisin treatment, and 1.0 log in the 5000 IU mL\(^{-1}\) treatment. At 24 h, the cell populations increased in all the direct nisin treatments but not in the film with nisin treatment which remained unchanged from the first recorded measurement at 8 h. Similarly, at 48 h the cell populations remained unchanged in the film with nisin treatment while the cell populations for the other treatments increased, reaching cell counts close to those of the control. When nisin was rapidly added directly to the medium, Listeria counts dropped initially and then increased after 4 or 8 h. This could suggest that the surviving cells were resistant to nisin and could continue to multiply, or that the excessive nisin in the system did not improve the efficacy of antimicrobial activity. When studying antilisterial activity of instantaneous or slowly added nisin, Zhang et al. (2004) also observed similar results.

These data demonstrated that nisin in pectin/PLA film retained more activity against L. monocytogenes over 48 h than a direct addition of a similar total dose. Direct addition of antimicrobials to food results in an immediate reduction of bacterial populations but may not prevent the recovery of injured cells or the growth of cells that were not destroyed by direct addition. The application of antimicrobial films allows for release of the antimicrobial to the film surface and therefore a continued antimicrobial effect on the food during extended exposure. Our data were similar to previously reported data demonstrating retention of nisin activity by heat-pressed in zein film coating against Lact. plantarum by Hoffman et al. (1998) and against L. monocytogenes by Janes et al. (2002). Cutter & Siragusa (1996a, 1998) also reported that immobilisation of nisin in an edible calcium alginate gel or a meat-binding system may be a more effective delivery system for nisin to the beef carcass surface than direct application.

Nisin had a transitory bactericidal effect on L. monocytogenes for short incubation times. However, inhibition of growth was not observed for long incubation times (Boussouel et al., 1999). Several studies have indicated an immediate bactericidal effect, with reduction of a population by 1–3 log cycles on addition of nisin, and with little or no affect upon further incubation (Harris et al., 1991). Similar phenomena were observed in food systems where nisin effectiveness was reduced by food characteristics such as pH, high fat content, particle size and non-uniform distribution of nisin in food (Bell & DeLacy, 1987; Jung et al., 1992). The use of polymers as nisin carriers not only controlled nisin release, but also prevented dramatic reduction of nisin activity due to its affinity for food particles and its inactivation by proteolytic enzymes in foods (Cutter & Siragusa, 1996b; Wan et al., 1997).

Conclusions

In this study, pectin/PLA films incorporating nisin showed promise for the inhibition of pathogenic L. monocytogenes in orange juice or liquid egg. The use of pectin and PLA in combination with nisin has a great potential in antimicrobial food packaging to reduce post-process growth of food pathogens. The use

![Figure 6](image-url)
of nisin containing films as packaging materials with antimicrobial activity against *L. monocytogenes* for solid foods, such as meat products, will be further investigated.

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**References**


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