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Genipin cross-linked antimicrobial nanocomposite films and gamma irradiation to prevent the surface growth of bacteria in fresh meats

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ABSTRACT

A 125 μg/mL of nisin and 30 mM of disodium ethylenediaminetetraacetate (EDTA) were immobilized on the surface of the nanocrystal (CNC)/chitosan nanocomposite films by using genipin as a cross-linking agent. The effect of low-dose gamma irradiation on the antimicrobial activity of the films was tested *in vitro* against *Escherichia coli* and *Listeria monocytogenes*. The genipin cross-linked films prepared by irradiating at 1.5 kGy demonstrated the highest antimicrobial activity against both the bacteria at the end of 35 days of storage at 37 °C showing an inhibition zone of 27.1 mm for *E. coli* and 27.7 mm for *L. monocytogenes* as compared to 23.4 mm and 23.8 mm for the same respective bacteria at day 1. The films restricted the growth of psychrotrophs, mesophiles and *Lactobacillus* spp. (LAB) in fresh pork loin meats and increased the microbiological shelf-life of meat sample by more than 5 weeks. The films also reduced the count of *E. coli* and *L. monocytogenes* in meat samples by 4.4 and 5.7 log CFU/g, respectively, after 35 days of storage.

Industrial relevance: Foodborne diseases are responsible for 9.4 million illnesses, 55,961 hospitalization and 1,391 deaths each year in the United States. In the context of a constantly growing population and globalization of markets, and the increase of the demand for ready to eat foods without synthetic additives, the development of new technologies to prevent food contamination and to reduce foodborne illnesses is important.

The development of antimicrobial packaging containing natural antimicrobials has been proposed as a novel technology to assure food safety. This technology is gaining interest from researchers and industries due to its potential to prevent the surface growth of pathogenic bacteria in meat products.

The limit of the use of natural polymers, in order to reduce the packaging wastes, is their high water permeability and low resistance. The use of nanocellulose can permit to reinforce film and can improve their physico-chemical properties. We have developed a novel biopolymeric matrix reinforced with a nanofiber. The nanofiber is non-toxic, natural and obtainable from renewable sources. Chitosan, is also obtained from renewable sources, non-toxic, biodegradable, has biocompatible properties, and found application in several fields including food packaging.

1. Introduction

In the context of a constantly growing population and globalization of markets, prevention of food contamination by microorganisms or pathogens, is becoming increasingly important. Foodborne diseases are responsible for 9.4 million illnesses, 55,961 hospitalization and 1391 deaths each year in the United States (Scallan et al., 2011). Microbial contamination, which is considered to be the main reason for food spoilage, can drastically reduce the shelf-life of foods and increase the risk of foodborne illnesses. Consumption of contaminated fresh meats or ready-to-eat (RTE) products possesses serious health risk and can result in hospitalization or even deaths. Microbial contamination of meat products usually occurs at the surface due to post-processing handling, processing or cutting. Antimicrobial packaging is gaining interest from researchers and industries due to its potential to prevent the surface growth of pathogenic bacteria in meat products (Quintavalla & Vicini, 2002). Direct incorporation of antimicrobials onto meat surface may provide limited efficacy due to the migration of the active substances into the bulk food matrix. Also, drastic loss of antimicrobial activity may occur due to the interaction and/or inactivation of the active substances by food components (Coma, 2008). Antimicrobial packaging provides an innovative alternative to some of the traditional meat preservation methods and can reduce the addition of larger quantities of

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antimicrobials that are usually incorporated directly into the bulk of the food (Cooksey, 2005; Quintavalla & Vicini, 2002).

Bionanocomposites can be defined as a novel class of materials consisting of a biopolymeric matrix, which is reinforced with a nanofiber (Khan, Huq, Khan, Riedl, & Lacroix, 2014a). The nanofiber should be non-toxic, natural and obtainable from renewable sources. Cellulose-based nanofiber reinforced bionanocomposite films have attracted significant attention during recent years, due to their renewable nature and high potential in the field of food packaging (Khan et al., 2014a; Moon, Martini, Nairn, Simonsen, & Youngblood, 2011). Cellulose based nanofiber such as Cellulose NanoCrystal (CNC) can be extracted though selective cellulosic sources by a controlled acid hydrolysis process (Habibi, Lucia, & Rojas, 2010). CNC is made up of highly crystalline, rod shaped, nanoparticles and exhibits an average length of 100–110 nm and width of 5–10 nm width (Dong, Revol, & Gray, 1998). CNC has been found very effective to improve the mechanical properties of biopolymeric films such as, chitosan, alginate, carrageenan, starch etc. (Chang, Jian, Zheng, Yu, & Ma, 2010; Huq et al., 2012; Khan et al., 2012; Pereda, Dufresne, Aranguren, & Marcovich, 2014; Sanchez-Garcia, Hilliou, & Lagaron, 2010). Chitosan is a natural hetero-polysaccharide composed of 2-amino-deoxy-β-D-glucopyranose and 2-acetamido-deoxy-β-D-glucopyranose (chitin) residues. It is a partially deacetylated derivative of chitin, which is the second most abundant natural polysaccharide in nature after cellulose. Chitosan, due to its non-toxic, biodegradable and biocompatible properties, has found application in several fields including food packaging (Prashanth & Tharanathan, 2007).

Lactic acid bacteria (LAB) produce a wide range of antimicrobial agents including bacteriocins such as, nisin. Nisin is produced by the lactic acid bacterium *Lactococcus lactis*, subsp. *lactis* (Hyde, Parisot, McNichol, & Bonev, 2006). It is composed of 34 amino acids and belongs to the class of small peptides (<4 kDa) called lantibiotics (Hyde et al., 2006; O'Sullivan, Ross, & Hill, 2002). Nisin is used as food additive in at least 48 countries and is the only bacteriocin that has GRAS (generally considered as safe) status by Food and Drug Administration (FDA) (Delves-Broughton, 1990). Nisin can inhibit a broad range of gram-positive bacteria including pathogens such as, *Listeria monocytogenes* (Khan, Vu, Riedl, & Lacroix, 2015). However, lack of inhibition against gram-negative bacteria remains a bottleneck for the use of nisin in food products, which requires inhibition of both gram-positive and gram-positive bacteria. Combination of nisin with other antimicrobials such as EDTA has proved to be effective to improve the antimicrobial activity of nisin against gram-negative bacteria (Vaara, 1992).

The activity of nisin may be lost in fresh and RTE meat products due to an interaction with glutathione (GSH). GSH is a strong reducing agent and is found in plants, animals, microorganisms as well as meats such as, beef, chicken and pork (Rose, Sporns, Stiles, & McMullen, 1999; Stergiou, Thomas & Stergiou, Thomas, & Adams, 2006). Nisin can be cross-linked onto the surface of the packaging in order to protect its activity in meat products. Genipin represents a highly potential cross-linking agent for food packaging applications due to its biocompatibility and low toxicity compared to other similar cross-linking agent such as glutaraldehyde (Sung, Huang, Huang, & Tsai, 1999). It is derived from the fruit *Genipa americana* and has the ability to cross-link with amino acids or proteins (Mi & Sung, 2002). In our previous study, the genipin cross-linked bionanocomposite films has demonstrated better antimicrobial activity against *L. monocytogenes* compared to the uncross-linked films, by reducing the growth rate of the bacteria in ready-to-eat (RTE) meat samples and protected the efficiency of nisin during storage (Khan et al., 2014b). However, the antimicrobial activity was limited to only *L. monocytogenes*.

The objective of the present study was to investigate the antimicrobial efficacy of genipin cross-linked bionanocomposite films containing nisin and EDTA. The antimicrobial efficacy of the films was tested *in vitro* and *in situ* against *E. coli* and *L. monocytogenes* on fresh pork loin meat samples during storage at 4 °C. The microbiological quality of the meat samples was also tested during storage.

2. Materials and methods

2.1. Bacterial strains

E. coli 0157:H7 (strain EDL933) was obtained from INRS-Institut Armand Frappier (Laval, Quebec, Canada). The *L. monocytogenes* strains HPB 2569 1/2a, 2558 1/2b, 2371 1/2b, 2812 1/2ba and 1043 1/2a were obtained from Health products and Foods Branch of Health Canada (Ottawa, Ontario, Canada). All the microorganisms were kept frozen at −80 °C in Brain heart infusion (BHI, Alpha Biosciences Inc., Baltimore, MD, USA) broth containing glycerol (10% v/v). Before use, the stock cultures were resuscitated through two consecutive 24-h growths in BHI at 37 °C to obtain the working cultures containing approximately 10^9 CFU/mL. The five *L. monocytogenes* were mixed together (2 mL each) to obtain a mixture containing approximately 10^9 CFU/mL.

2.2. Preparation of nisin–EDTA antimicrobial formulation

The nisin–EDTA mixture was prepared according to the procedure described by Khan et al. (2015). A 125 μg/mL of nisin solution was prepared by dissolving the Niprosin™ powder (2.5% nisin, 77.5% salt and 20% vegetable protein, Profood, IL, USA) in deionized water. The pH of the solution was adjusted to 3.0 with dilute lactic acid (Laboratoire Mat, Beauport, Quebec, Canada), followed by centrifugation for 15 min at 5000 *g*. A 30 mM of disodium ethylenediaminetetraacetate (EDTA, Laboratoire Mat) was mixed with the nisin supernatant and the pH of the formulation was adjusted to 5.5 with 1 M NaOH (Laboratoire Mat). The nisin–EDTA formulation was then filtered through a 0.45-μm filter and stored at 4 °C.

2.3. Preparation of the nanocomposite films

In the current study, the nanocomposite films were prepared from the optimized CNC content, microfluidization pressure and number of cycles; obtained from our previous study (Khan et al., 2014c). At first, 2% w/v of high mol. wt. chitosan (DD: 85–90%, 85/2500 Heppe-medical GmbH, Germany) and 0.5% ethylene glycol (Laboratoire Mat, Beauport, Quebec, Canada) was dissolved into a 2% w/v of aqueous acetic acid (Laboratoire Mat, Beauport, Quebec, Canada) solution. A 2% w/v of aqueous CNC (FP Innovations, Pointe-Claire, QC, Canada) suspension was prepared and incorporated (8% w/w of chitosan) into the chitosan/ethylene glycol solution. The CNC/chitosan nanocomposite suspension was then pre-homogenized for 3 h with IKA RW-20 (IKA® Works Inc., Wilmington, DE, USA), microfluidized at 7000 psi and six microfluidization cycles. The nanocomposite films were made by casting the microfluidized suspension on Petri dishes which was allowed to dry at room temperature and 30–35% RH. Films were then treated with 1 M NaOH (Laboratoire Mat, Beauport, Quebec, Canada) for 2 min, washed several times with deionized water and were allowed to dry.

2.4. Preparation of the antimicrobial nanocomposite films

The antimicrobial films were prepared by casting 15 mL of the nisin–EDTA antimicrobial solution on the surface of the nanocomposite films. The genipin cross-linked films were prepared by mixing 0.05% w/v of genipin (Challenge Bio-products, Yun-Lin Hsien, Taiwan) with the nisin–EDTA formulation at pH 5.5 and the reaction was carried out for 24 h at room temperature. The films were allowed to dry for 2 days. After drying the films were peeled off the Petri dishes and stored were stored at 4 °C in a desiccator filled with deionized water. All the films were γ-irradiated at doses 0.5 and 1.5 kGy at the Canadian Irradiation Centre (CIC, Laval, Quebec, Canada) at room temperature. The uncross-linked films were coded as N-0 kGy, N-0.5 kGy and N-1.5 kGy. The genipin cross-linked films were coded as G-0 kGy, G-0.5 kGy and