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Antimicrobial activity and physical properties of protein films added with cell-free supernatant of *Lactobacillus rhamnosus*

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ABSTRACT

Antimicrobial and physical properties of whey protein isolate (WPI) or calcium caseinate (CC) films added with cell-free supernatant of *Lactobacillus rhamnosus* NRRL B-442 were evaluated. Antimicrobial activity was probed against *Listeria monocytogenes* Scott A, *Staphylococcus aureus* ATCC 29413, *Escherichia coli* ATCC 25922, or *Salmonella enterica* serovar Typhimurium ATCC 14028. Cell-free supernatant was obtained by centrifugation and filtration from cultures of *L. rhamnosus* in MRS broth, freeze-dried, and rehydrated to add 6, 12, or 18 mg/ml to protein film solution. Films' inhibition zones were determined by agar disk diffusion assay against tested microorganisms. Films' thickness, puncture strength, color, moisture content, solubility, and water vapor permeability (WVP) were evaluated. Noticeable antimicrobial activity (about 3 mm) was observed against *E. coli*, *L. monocytogenes*, *S. aureus*, or *S.* Typhimurium when 18 mg/ml of cell-free supernatant were added. Gradual and significant ($p < 0.05$) increments were obtained in thickness, color, and solubility when different increasing concentrations of supernatant were added. Reductions in WVP and puncture strength were observed when adding supernatant. Antimicrobial films were brown colored and with variable moisture contents. WPI films were significantly ($p < 0.05$) better than CC films for most evaluated properties. Protein films added with cell-free supernatant are an interesting alternative to develop natural antimicrobial films that can have a potential food application as wrappings or coatings.

1. Introduction

Current trends in food active packaging include the search for biopolymers and natural antimicrobial compounds in order to obtain friendly packages and reduce human consumption of synthetic preservatives. Milk proteins are common sources to formulate edible films and coatings. Typical composition of milk proteins contains caseins (80%) and whey proteins (20%). Caseins are a blend of 4 fractions of proteins named α_{s1}-, α_{s2}-, β-, and κ-caseins which represents approximately 37, 10, 35, and 12%, respectively (Fox & McSweeney, 1998). Whey protein isolates (WPI) consist of four main types of globular proteins: β-lactoglobulins (50%), α-lactalbumin (20%), inmunoglobulins (10%), and albumin (7%), as well as other minor proteins such as lactoferrin, lysozyme, and lactoperoxidase (Fox & McSweeney, 1998). WPI and calcium caseinate (CC) have been widely used to elaborate films with good barrier properties. WPI and CC films are transparent, odorless, and tasteless, as well as excellent oxygen, lipid, and aroma barriers (Fernández-Pan, Royo, & Maté, 2012; Kristo, Biliaderis, & Zampraka, 2007). Several studies have shown protein films are good vehicles for antimicrobial substances, e.g. essential oils (Fernández-Pan et al., 2012; Matan, 2012; Shakeri, Shahidi, Beiraghi-Toosi, & Bahrami, 2011; Zinoviadou, Koutsoumanis, & Biliaderis, 2009), organic acids salts (Kristo, Koutsoumanis, & Biliaderis, 2008), glucose oxidase (Murillo-Martínez, Tello-Solís, García-Sánchez, & Ponce-Alquicira, 2013), nisin (Cao-Hoang, Grégoire, Chaine, & Waché, 2010; Kristo et al., 2008; Murillo-Martínez et al., 2013), thymol (Li, Yin, Yang, Tang, & Wei, 2012), and viable cells of *Lactobacillus sakei* (Gialamas, Zinoviadou, Biliaderis, & Koutsoumanis, 2010). Antimicrobial packaging is important to enhance the shelf life of meat, poultry, fish, vegetables, and dairy products, among others. *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*, and *Staphylococcus aureus* are some of the microorganisms of interest in these foods. Moreover, some of these microorganisms are indicators of bad manufacturing practices or fecal contamination;

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therefore, they are representative bacterial genera and of interest for the food industry.

Lactic acid bacteria (LAB) typically produce a wide range of antimicrobial substances like organic acids (lactic and acetic), hydrogen peroxide, reuterin, nisin, pediocin, or other bacteriocin-like substances. *Lactobacillus rhamnosus* is a homofermentative LAB that typically produces lactic acid from glucose. Individual and purified metabolites are frequently used for antimicrobial purposes. Nevertheless, more than one antimicrobial substance is necessary to achieve the desirable level of microbial inhibition. Cell-free supernatants (rich in metabolites with antimicrobial activities) could act as appropriate antimicrobial agents since they contain a wide variety of antimicrobials. Potential uses have been suggested for cell-free supernatant of *L. rhamnosus*, such as possible treatment of allergic airway inflammation in neonatal mouse model (Harb et al., 2012), and as a cosmetic antioxidant (Tsai et al., 2013). Based on the variety and amount of metabolites present in its cell-free supernatant, it could be possible to add it as an antimicrobial agent. Additionally, cell-free supernatant could be considered as a natural antimicrobial with good acceptance. Beristain-Bauza (2015) demonstrated that antimicrobial activity of cell-free supernatant of *L. rhamnosus* against *E. coli*, *S.* Typhimurium, *L. monocytogenes*, and *S. aureus* can be attributed to lactic acid and a bacteriocin-like substance when native supernatant was tested, but when the supernatant was neutralized (pH 6.5) antimicrobial activity was assumed to the bacteriocin-like substance. Furthermore, supernatant treated with proteinase entirely lost its antimicrobial activity. Based on these results *L. rhamnosus*' cell-free supernatant could be added to protein films. The aim of this work was to evaluate the antimicrobial activity and physical properties of whey protein isolate and calcium caseinate films added with *L. rhamnosus* cell-free supernatant. Antimicrobial activity was probed against four target bacteria (*L. monocytogenes*, *S. aureus*, *E. coli*, and *S. enterica* serovar Typhimurium).

2. Materials and methods

2.1. Bacterial strains, culture conditions, and materials

L. rhamnosus NRRL B-442 was acquired in lyophilized form from the USDA (Agricultural Research Service, Peoria, Illinois). The strain was activated and routinely sub-cultured in MRS broth (Difco™ BD, Sparks, Maryland) under anaerobic conditions at 37 °C. For cell-free supernatant production, *L. rhamnosus* was cultured in 100 ml of MRS broth at 37 °C for 18 h under anaerobic conditions. Cell-free supernatant was obtained by centrifugation at 8000 × *g* for 10 min at 4 °C (Marathon 21K/R, Fisher Scientific, Germany), and filtered through cellulose nitrate filter of 0.45 μm (Advantec, MFS, Dublin, California) in order to separate the remaining cells. 100 ml of cell-free supernatant was freeze-dried in a stoppering tray dryer (Labconco Corp., Kansas, Missouri) in order to concentrate the supernatant. Freeze-dried supernatant (0.6 g) was rehydrated in 10 ml of water and adjusted the pH to 8.0 with NaOH (10 M) and was stored at 5 °C until it was utilized. Films' antimicrobial activity was probed against target microorganisms: *E. coli* ATCC 25922, *L. monocytogenes* Scott A, *S. aureus* ATCC 29413, and *S. enterica* serovar Typhimurium ATCC 14028. Target cultures were activated and routinely sub-cultured in trypticase soy broth (Bioxon®BD, Edo. de Mexico, Mexico) at 37 °C for 16 h. Whey protein isolate (WPI) Nutrilac HA7510, was acquired from ArlaFoods Ingredients (Union, New Jersey), calcium caseinate C335 (CC) from Cedrosa (Edo. de Mexico, Mexico), sodium alginate from FMC Biopolymers (Rogaland, Norway), and glycerol and calcium chloride were purchased from RBM (Puebla, México). Every solution and dispersion was prepared with distilled water.

2.2. Preparation of films

Film-forming solutions were prepared according to the modified method reported by Quintero-Salazar, Vernon-Carter, Guerrero-Legarreta, and Ponce-Alquicira (2005). Briefly, 50 ml of aqueous dispersions of WPI or CC (6%) pH 8.0 were heated at 90 °C for 30 min and cooled to 50 °C; then 25 ml of sodium alginate solution (1%) pH 8.0, 10 ml of rehydrated cell-free supernatant, 2.2 g of glycerol, and water pH 8.0 were added to complete 100 ml (protein final concentration was 3%). The blend was heated at 60 °C for 20 min under stirring in order to homogenize components. Four ml of film-forming solution were poured in silicon casts (diameter 5 cm and height 2 cm) and dried at 30 °C for 24 h in an oven (Cole–Parmer, Vernon Hills, Illinois). Dried films were peeled off and immersed in calcium chloride solution 10% for 10 s in order to cross-link alginate of the films, and then films were dried at 30 °C for 30 min. Three rehydrated cell-free supernatant concentrations were added to film solutions (6, 12, or 18 mg/ml). In addition, cell-free supernatants were heat treated for 30 min at 92 °C to evaluate antimicrobial compounds' stability. Films without cell-free supernatant were made as controls. Furthermore, films with MRS broth were prepared in order to observe growth medium effect. All films were conditioned at 33% relative humidity for 48 h at 25 °C before testing.

2.3. Antimicrobial activity of films

Agar disk diffusion assay (López-Malo, Palou, Parish, & Davidson, 2005) was used to evaluate films' antimicrobial activity. Target strains were inoculated in trypticase soy broth at 37 °C for 16 h, serial dilutions were made to obtain a density of about 10^7 cells/ml. Antimicrobial activity was carried out by spreading 0.1 ml of the bacterial cells in the agar surface. Three film disks (0.6 cm of diameter) were placed on the surface of the lawn culture and plates were incubated at 37 °C for 24 h. Inhibition zones were measured with a digital caliper (Mitutoyo Corp., Kawasaki, Japan).

2.4. Characterization of films

2.4.1. Thickness

Film thickness was measured with a digital micrometer (Mitutoyo Corp., Kawasaki, Japan). The average of ten measurements was used in calculations.

2.4.2. Puncture strength

Puncture strength was determined using a texture analyzer TA.XT2 (Stable Micro Systems, Godalming, United Kingdom). Each film was fixed on the puncture frame (diameter of 16 mm), and the stainless steel spherical probe (diameter of 14 mm) was placed just above the center of film and moved through the film at a cross-head speed of 1 mm/s. Puncture strength was calculated as the maximum force (N) which was loaded on the film to puncture the sample. Six determinations were performed to each tested film.

2.4.3. Water vapor permeability

Water vapor permeability (WVP) of the films was determined using a modification of the "cup method" from the gravimetric technique of ASTM E96-95. For this study, glass cups with a diameter of 25 mm were used. Granular calcium chloride (8 g) was added within the cups, and each film was placed on the top of the cup tightened by Parafilm®. The entire cup was then placed within a desiccator containing saturated $MgCl_2$ solution (33% relative humidity) at 25 °C. The water transferred through the film was determined from the weight gain of the system (cup plus $CaCl_2$) over 88 h at 8 h intervals and weighted to the nearest 0.1 mg using