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# Antilisterial properties of PVOH-based films embedded with *Lactococcus lactis* subsp. *lactis*

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ABSTRACT

The incorporation of lactic acid bacteria (LAB) in edible films and coatings has recently emerged as an innovative strategy to provide packaging films with new functionalities in order to ensure food quality and safety. In this work, bioactive films were made by incorporating *Lactococcus lactis* subsp. *lactis* in cast polyvinyl alcohol (PVOH) matrices, alone or blended with a small percentage of proteins, protein hydrolysates, or yeast extract, and the effect of incorporating them on the morphology and optical properties of the PVOH films was studied. Moreover, the viability of *L. lactis* in the films developed stored at 20 °C and 43.2% relative humidity for four weeks, and the antimicrobial activity against *L. monocytogenes* were determined.

The thickness, color properties, and morphology of the films incorporating LAB and nutrients did not show significant differences compared with plain PVOH films, but moisture content increased slightly with nutrient incorporation. *L. lactis* remained viable for 4 weeks of storage, but viability depended on the matrix composition, being lower in plain PVOH films and higher in films supplemented with proteins, hydrolysates, or yeast extract. The highest antimicrobial activity was observed in PVOH matrices with hydrolyzed gelatin or casein, and the lowest in plain PVOH films. The growth of *L. lactis* was similar in all the films after incubation in contact with *L. monocytogenes* at 37 °C in liquid medium, regardless of the initial concentration. The films developed could be applied in the design of food packages with the purpose of inhibiting growth of *L. monocytogenes*.

## 1. Introduction

Nowadays, there is an increasing tendency for food consumption to be linked intrinsically with a healthy lifestyle. Consumers now demand less processed foods made with natural ingredients, which means a preference for products free of synthetic additives and preservatives. In order to satisfy the wishes of consumers while maintaining food safety, the use of naturally occurring antimicrobials has been presented as an alternative to synthetic ones, causing great interest in the food industry (Xu et al., 2007). Many studies have shown that incorporating antimicrobials in polymer films or coatings is more effective than adding them directly to the food product. In fact, films and coatings not only immobilize the antimicrobial compound and provide a protective environment for it, but also modulate its release to the packaged food (Aloui & Khwaldia, 2016; Realini & Marcos, 2014).

Bacteriocins are antimicrobial peptides and products of the metabolism of certain bacteria. The use of bacteriocins from lactic acid bacteria (LAB) in the food industry has been an advance for improving the shelf-life of foods while guaranteeing the safety and health of consumers (Reis, Paula, Casarotti, & Penna, 2012). The incorporation of bacteriocins from LAB into packaging films and coatings has been studied, with results that only show good effectiveness of the antimicrobial at the beginning of the food storage period (Coma, Sebti, Pardon, Deschamps, & Pichavant, 2001; Ercolini et al., 2009; Marcos, Aymerich, Monfort, & Garriga, 2008). This is due to the gradual depletion of the bacteriocin from the matrix reservoir. In order to solve this problem, some studies propose the incorporation of LAB directly into the film as a natural tool to extend film antimicrobial activity over time by means of the ability of bacteria to produce bacteriocins (Espitia, Batista, Azeredo, & Otoni, 2016). In this regard, other mechanisms such as competition between bacteria and pathogens for nutrients and the production of organic acids can help bioactivity. *Lactococcus lactis* subsp. *lactis* is used in food preservation because of its ability to produce nisin (Deegan, Cotter, & Ross, 2006). This bacteriocin is a polycyclic peptide with antibacterial properties that has been well characterized and that is classified as GRAS (Generally Recognized as Safe) by the FDA (Food and Drug Administration). *L. lactis* inhibits the growth of certain Gram + bacteria, such as *Listeria monocytogenes*, a pathogen that causes listeriosis, a serious foodborne disease (Benkerroum & Sandine, 1988).

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Currently, there are few studies regarding the development of antimicrobial films incorporating bacteria, and all of them have been done with naturally occurring protein and polysaccharide polymers derived from plants and animals (Concha-Meyer, Schöbitz, Brito, & Fuentes, 2011; Gialamas, Zinoviadou, Biliaderis, & Koutsoumanis, 2010; López De Lacey, López-Caballero, Gómez-Estaca, Gómez-Guillén, & Montero, 2012; Sánchez-González, Quintero Saavedra, & Chiralt, 2013, 2014), because they create a favorable environment for LAB survival. However, studies regarding the use of synthetic polymers as carriers of LAB intended for antimicrobial packaging applications are scarce (Iseppi et al., 2011).

Polyvinyl alcohol (PVOH) is a synthetic polymer that is completely biodegradable and biocompatible. It can be obtained from raw materials that are not oil derivatives, such as natural gas (De Prisco et al., 2002; Dorigato & Pegoretti, 2012). PVOH is soluble in water and has excellent film-forming, emulsifying, and adhesive properties. PVOH films and coatings are easily obtained by casting (Dorigato & Pegoretti, 2012; López-De-Dicastillo, Jordá, Catalá, Gavara, & Hernández-Muñoz, 2011), they have great flexibility and mechanical resistance, and are water soluble, odorless, colorless, and non-toxic (cc. Schoneker, 2003; Goodship, V., & Jacobs, 2009). Furthermore, PVOH is approved by the FDA for use in food contact and as a food additive with INS No. 1203 (Codex Alimentarius) (FAO/WHO (Food and Agriculture Organization/ World Health Organization), 2004). In the EU it is approved by the EFSA as a food additive in food supplements in accordance with Annex II to Regulation (EC) No. 1333/2008.

The viability of *Lactobacillus plantarum* has been shown to be greater in protein films than in polysaccharide films (Sánchez-González, Quintero Saavedra, & Chiralt, 2014). Soukoulis, Singh, Macnaughtan, Parmenter, and Fisk (2016) have recently found that protein films are better matrices than starch films to sustain *Lactobacillus rhamnosus* viability, but a synergistic effect on viability has been observed when blending starch with proteins. Léonard et al. (2013) found higher viability values and higher antilisterial activity of *L. lactis* entrapped in matrices of gelled alginate blended with sodium caseinate than in plain gelled alginate, and greater survival of LAB in the caseinate-rich phase. Gelatin and sodium caseinate are edible proteins of animal origin that are commonly used in the food industry. In this regard, using PVOH matrices, blended with these proteins or their hydrolysates, as carriers of *Lactococcus lactis* could improve bacteria viability. For the same purpose, yeast extract (a cocktail of amino acids and vitamins) could also be used to promote bacterial growth and nisin production when added to PVOH as a coadjuvant agent.

Therefore, the main purpose of this work was to develop antilisterial PVOH-based films incorporating *L. lactis*. For this purpose, a series of films consisting of plain PVOH matrices or matrices blended with gelatin, sodium caseinate, gelatin or casein hydrolysates, and yeast extract was obtained and the bacteria viability in the films over time was studied. Moreover, the antimicrobial activity of the films against *L. monocytogenes*, and the survival of LAB after the films had been in contact with the pathogen in liquid medium during the antimicrobial assay were also evaluated. Furthermore, the effect of incorporating LAB and proteins on the optical and morphological properties of the PVOH films was determined.

## 2. Materials and methods

### 2.1. Bacterial strains

Bacterial strains were supplied by the Spanish Type Culture Collection (CECT).

The *L. lactis* strain (CECT 539, ATCC 11454) was selected for the development of nisin-producing films. The strain was maintained at −80 °C in Man, Rogosa, and Sharpe broth (MRS) with 20% glycerol. The microbial culture was regenerated and maintained by regular subculture at 4 °C on MRS broth. Prior to beginning work, a subculture was made by transferring a loopful of the strain to 10 ml of MRS broth and incubating it at 30 °C for 24 h.

The *Listeria monocytogenes* strain (CECT 934, ATCC 19114) was chosen because of its importance in foodborne illness. The strain was kept frozen at −80 °C in Tryptone Soy Broth (TSB) supplemented with 20% glycerol. For experimental use, the stock culture was maintained by regular subculture at 4 °C on Tryptone Soy Agar (TSA) and transferred monthly. Prior to the experiments, a loopful of the strain was transferred to 10 ml of TSB and incubated overnight at 37 °C. All microbiological products were provided by Scharlau, Barcelona, Spain.

### 2.2. Determination of minimum inoculum of L. lactis active against L. monocytogenes

The minimum initial inoculum of *L. lactis* able to reduce microbial growth of *L. monocytogenes* was determined in liquid medium. *L. lactis* cells were harvested by centrifugation at 2500 RCF for 15 min at 4 °C and washed twice with peptone water; then they were resuspended in TSB with 0.3% of yeast extract (TSB + YE). Appropriate dilutions were made in order to inoculate tubes with 10 ml of TSB + YE with concentrations ranging from 7 log/ml to 0.01 log/ml; then all the tubes were inoculated with 3 log CFU/ml of *L. monocytogenes*. One tube without *L. lactis* and another one without *L. monocytogenes* were used as controls. The tubes were incubated at 37 °C for 24 h. After incubation, serial dilutions with peptone water were made and plated in Petri dishes with 15 ml of Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol agar (PALCAM agar) to study logarithmic reduction of *L. monocytogenes,* and also in MRS to study growth of *L. lactis* in contact with *L. monocytogenes*. MRS agar plates and PALCAM plates were incubated at 30 °C and 37 °C for 4 days and 48 h, respectively. Thus, *L. lactis* colonies were counted in MRS agar and *L. monocytogenes* colonies were counted in PALCAM agar. Tests were carried out in triplicate.

### 2.3. Film formation

Five different film-forming solutions (FFS) were prepared by dissolving 2% of polyvinyl alcohol (PVOH, Gohsenol GH17, Nippon Synthetic Chemical Company, Osaka, Japan) in distilled water as the main polymer matrix, and gelatin (gelatin from porcine skin, type A, Sigma-Aldrich, USA), sodium caseinate (casein sodium salt from bovine milk, Sigma-Aldrich, New Zealand), casein and gelatin hydrolysates (peptone from casein and gelatin, enzymatic digest, Sigma-Aldrich, France), and yeast extract (yeast autolysate, Sigma-Aldrich, France) as nutritional supplements for *L. lactis*. They were prepared in a mass ratio of 1:0.125. *L. lactis* cells were harvested by centrifugation at 2500 RCF for 15 min at 4 °C and washed twice with peptone water. Then they were incorporated into the film-forming solution in order to obtain 7 log CFU/ml of FFS, and 15 g of it was cast in Petri dishes (90 mm) and dried at 37 °C for 24 h. PVOH films without nutritional supplement were used as control films.

### 2.4. Film characterization

#### 2.4.1. Film thickness

Film thickness was measured with a digital micrometer (Mitutoyo Manufacturing Co., Ltd., Tokyo, Japan) with a sensitivity of 1 μm. Five readings were taken randomly for each film sample.

#### 2.4.2. Moisture content

Film samples of approximately 0.5 g were placed on aluminum plates and stored in a desiccator containing a saturated solution of potassium carbonate anhydrous (Acros Organics, France) in order to obtain 43.2% RH at 20 °C. After reaching weight equilibrium, in approximately two weeks, they were weighed and placed in desiccators with phosphorus pentoxide (Fluka, Sigma-Aldrich, France) for