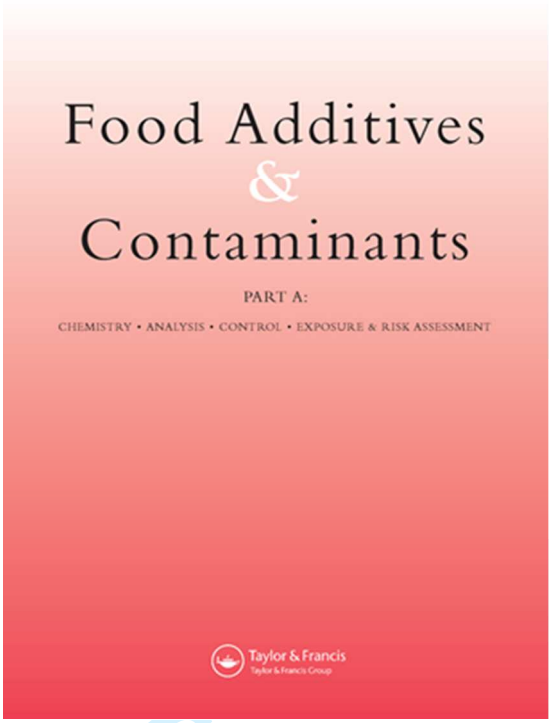

Development of an active wheat gluten film with *L. curvatus* CRL705 bacteriocins, antimicrobial performance study during aging

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Development of an active wheat gluten film with *L. curvatus* CRL705 bacteriocins and a study of its antimicrobial performance during aging

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Abstract

Antimicrobial wheat gluten film was obtained at pilot scale by *Lactobacillus curvatus* CRL705 bacteriocins inclusion in the film forming solution. Bacteriocins minimum inhibitory concentration for the film activation was 2133 AU cm^{-3} (lactocin AL705) and 267 AU cm^{-3} (lactocin 705). Mechanical and barrier properties as well as film aging kinectics were not significantly affected by bacteriocins addition. The antimicrobial film performance during aging was assessed. Film activity against *Listeria innocua* 7 and *Lactobacillus plantarum* CRL691 was observed over 50 days of aging. Even when bacteriocins release from the film upon water contact was observed for both bacteriocins at the beginging of aging period, and anti-*Listeria* activity was delivered to the simulant up to the 15th day of aging, film residual activity for both bacteriocins was

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Keywords: active bio-based polymer, anti-*Listeria*, bacteriocins, antimicrobial film performance during aging

Abbreviations

WG (Wheat gluten)

LAB (Lactic acid bacteria)

LLDPE (Linear low denstity polyethylene)

PHB (Polyhydroxybutyrate)

BCE (bacteriocins crude extract)

MIC (minimum inhibition concentration)

Introduction

Food packaging is designed not only to contain and protect food, but also to keep food safe and secure, to retain food quality and freshness, and to increase its shelf-life (Imam et al 2008). Determining factors in food consumption include price, income, culture, and food safety. Food-related health crises in recent times have decreased consumer confidence (Pllana et al 2012). Meat and meat food products are consumed extensively throughout the world, and among the meat borne pathogens, *L. monocyto*genes has been associated with cooked, ready-to-eat (RTE) meat and poultry products (Williams et al 2011; Hereu et al 2012; Thippareddi, 2012). To solve the problem comprising RTE food contamination, antimicrobial additives have been included in polymers as a preservation

strategy (Vermeiren et al 2002; De Jong et al 2005; Cooksey 2005; Blanco Massani et al 2008; Koontz et al 2010). Furthermore, as an eco-friendly hurdle technology, bio-based polymers have been used keeping safe and extending shelf life of foods (Cagri et al 2004; Cha & Chinnan 2004; Juneja et al 2006; Coma 2008; Iturriaga et al 2012). In the last few years, special attention was given to bacteriocin incorporation into these materials for food applications. Many studies can be found on active packaging with antimicrobial action by the inclusion of nisin and other bacteriocins such as enterocin, pediocin and lacticin 3147 (Scannell et al 2000; Grower et al 2004; Luchansky & Call 2004; Lungu, & Johnson 2005; Guiga et al 2010 ; Ibarguren et al 2010). Regulatory requirements for active packaging technologies in the United States are not very different from the requirements for conventional antimicrobial additives. Packaging materials that has no intented technical effect on the food should be notified to the FDA at least 120 days before its introduction to the market and can be sold unless the FDA objets to the notification. However, the material exerting antimicrobial effect on food throuhg migration or controlled release would constitute a ‘‘direct additive” and would be subject to much stricter FDA regulatory requirements (Cho et al, 2009; Restuccia et al 2010). On the other hand, for Mercosur member states, plastic articles intended to come in contact with foods should comply with the requirements of Mercosur Regulation (GMC/RES 32/07), which is updated acoording to the European Union Regulation (1935/2004/EC). Even when current Mercosur regulation on active packaging materials is not available, general requirements stated in Regulation 1935/2004/EC for the safe use of active and intelligent packaging have been recently integrated by Regulation 450/2009/EC (Restuccia et al 2010). Lactocin 705 and lactocin AL705, are bacteriocins produced by *Lactobacillus curvatus* CRL705, with antagonist effect against Lactic acid bacteria (LAB), *Brochothix thermosphacta*, and *Listeria*

species, respectively (Castellano & Vignolo 2006). Previous studies have demonstrated activity retention when lactocin 705 and AL705 were adsorbed on a synthetic LLDPE-based film (Blanco Massani et al 2008). Either lactocin 705 or AL705 were found to be stable in a range of pH from 3.0 to 6.0, and up to 70ºC (Palacios 2000; Castellano 2005). Among the renewable resources available for biodegradable polymers obtaining, our laboratory has worked with PHB (Botana et al 2010), starch, corn zein, soy and WG proteins. While for PHB, starch and corn zein, film processing temperatures are higher than 70ºC (Zhang & Han 2006; Marcos et al 2007; Ghanbarzadeh & Oromiehi 2009; Botana et al 2010), films from wheat gluten or soy proteins can be obtained at lower temperatures (Irissin-Mangata et al 2001; Denavi et al 2009). Proteins are hetero-polymers comprising amino acids generally classified by groups that could interact via hydrogen bonds (non-ionized polar amino acids), ionic interactions (ionized polar amino acids), non-polar interactions (non-polar amino acids) or covalent bonds (disulfide or dityrosine bonds). This heterogeneous structure provides many reaction sites for potential cross linking or chemical grafting (Guilbert & Cuq 2005). Proteins must be denatured by heat, acid, base, and/or solvent in order to form the more extended structures that are required for film formation. Once extended, protein chains can associate through hydrogen, ionic, hydrophobic and covalent bonding (Bourtoom 2008). Film and film-forming properties are strongly dependent on the pH of the dispersion, and they are normally lower close to the isoelectric point. Thus, soy proteins isolated films cannot be formed close to a pH of 4.5 (Wu & Bates 1972; Sian & Ishak, 1990) and are mostly formed at alkaline conditions (Ou et al 2005; Wan et al 2005; Denavi et al 2009). Many studies have focused on the production of gluten films either by casting a film forming solution obtained in humid conditions, or by thermal processing (Gennadios, Brandenburg et al 1993; Gennadios, Weller et al 1993; Hochstettera et al

2006; Song et al 2008; Marcuzzo et al 2010; Zhang et al 2010). As wheat gluten films can be formulated either at basic or acid conditions (isoelectric point around 7.5) (Herald et al 1995; Olabarrieta, Cho et al 2006), this protein could be an addecuate support for *L. curvatus* CRL705 bacteriocins, since either 705 or AL705 are able to withstand film forming conditions (acid pH and temperatures lower than 70ºC). The use of gluten films has been studied for food applications such as lipid barriers reducing fat uptake in deep-fried products, postharvest shelf life extention for refrigerated strawberries, reducing breakage of egg shell and egg microbial contamination, among others (Albert & Mittal 2002; Xie et al 2002; Tanada-Palmu & Grosso 2005). Moreover, gluten films containing bacteriocins and other antimicrobials has been proposed for foods that can be potentially contaminated with *Listeria monocytogenes*, such as meat foods, fruits and vegetables (Ko et al 2001; McCormick et al 2005; Ibarguren et al 2010). Nevertheless, gluten films are known to suffer from aging due to plasticizer lost (glycerol), protein aggregation or oxidation, and as a consequence material ductility lowers, while stiffness and strength raises, incrementing film brittleness (Morel et al 2000; Micard et al 2000; Olabarrieta, Cho et al 2006). The purpose of our study was to obtain WG-film containing *L. curvatus* CRL705 bacteriocins and to assess the influence of aging on its antimicrobial performance, for the potential use to avoid bacteria proliferation on meat products.

Materials and Methods

Bacterial strains and growth conditions

Lactobacillus curvatus CRL705, lactocin 705 and lactocin AL705 producer, and *Lactobacillus plantarum* CRL691, used as an indicator of lactocin 705, were isolated from dry-fermented sausages (Vignolo et al 1993) and grown in MRS broth (Britania

Laboratories, Buenos 20 Aires, Argentina) at 30º C. *Listeria innocua* 7, used as an indicator of lactocin AL705, was obtained from the Unité de Recherches Laitières et Génétique Appliquée, INRA (France) and grown in trypticase soy broth (TSB, Britania) with 5mg cm^{-3} of added yeast extract (YE, Britania) at 30ºC. All strains were maintained and stored at -20ºC in 0.15 g cm-3 of glycerol until use.

Bacteriocins preparation and antimicrobial assays

An active powder containing lactocin 705 and lactocin AL705 was obtained as earlier reported (Blanco Massani et al 2008). A bacteriocins crude extract (BCE) was prepared by the active powder water re-suspension.

Lactocin 705 and AL705 antimicrobial activity in aqueous solution expressed as AU cm^{-3} was determined by the agar well diffusion method (Pongtharangkul & Demirci 2004).

Antimicrobial activity of the activated and control films (see below) was determined by placing 1.0 cm diameter punched circles directly on the semisolid agar plates seeded with the sensitive organisms. Film activity was evidenced as an inhibition zone of the indicator organisms beneath and around the film and expressed as relative inhibition area (Blanco Massani et al 2008).

Antimicrobial WG-film pilot scale preparation

Wheat gluten (77,9% of protein, 13,3% starch and 1,0% lipids w/w, on dry weight base) was kindly supplied by Molinos Juan Semino S.A. (Carcarañá, Pcia de Santa Fe). To obtain a WG-film at pilot scale, a film forming solution was prepared by stirring wheat gluten, sodium sulfite (Merck, Germany), glycerol (Cicarelli, Argentina) and ethanol 96% (Merck, Germany) with a mechanical stirrer (Heidolph RZR 2041). After a

homogeneous solution was attained, water and the BCE were added, and the pH was adjusted to 5.0 with acetic acid (Sintorgan, Argentina). The film forming solution was spread onto a continuous Teflon® tape and dried in a warm tunnel with forced air at 50ºC for 4 hours.

Concerning the investigation on antimicrobial minimum inhibitory concentration (MIC), BCE different concentrations were added to the film forming solution (0.01; 0.1 y 1% v/v, BCE on formulation base). The obtained films were assayed for antimicrobial activity as previously described and compared to a film without bacteriocins (control).

Film properties as a function of aging time

Active and control (without bacteriocins) films were conditioned for 2 days in an environmental chamber at 50% relative humidity and 23 ºC. The films were aged in the mentioned conditions for 50 days and different properties were determined as a fuction of time.

Water content, mechanical and barrier properties.

Residual water content in the bio-based films (active and control) was determined by drying each sample at 100ºC until constant weight. The water content of the films was determined from the weight difference between films before and after drying.

Control and active films´ tensile strength and elongation at break were evaluated in quintuplicates with an Instron universal testing machine (model 1125), according to ASTM D638-10. Initial grip separation was set at 65 mm and cross-head speed was set at 50 mm/min.

Films water vapor permeability (WVP) was gravimetrically determined as described in ASTM E96/E 96M-12. The tested films were placed on the top of hermetically sealed aluminum cups, containing anhydrous silica desiccant (0.00181 m^2 exchange film area)

and placed in a chamber (75% RH and 23ºC). At least three samples of each type of film were tested. The WVP, determined by the increase in cup weight over time at the mass transfer steady-state, was calculated from the following equation:

$$WVP = \Delta w x / A \Delta t \Delta p \quad (\text{mol m}^{-1}\ \text{s}^{-1}\ \text{Pa}^{-1})$$

Where Δw is the weight gain of the permeation cell in the steady state (mol), x is the film thickness (m), Δt is the time of weight gain (s), A is the area of exposed film (m^2), and Δp (Pa) is the water vapor pressure differential across the film.

The thickness of each sample was taken as the average of three measurements made at random points in the film using a hand-held micrometer.

Lactocin 705 and lactocin AL705 residual antimicrobial activity on the films after water and sunflower oil contact

Active and control wheat gluten films (2.5 cm^2) were contacted with water and sunflower oil (2 cm^3) representing hydrophilic and hydrophobic food simulant media, respectively. After 10 days of contact (5ºC), films were removed. Residual antimicrobial activity of the films was determined by placing them directly on the semisolid agar plates seeded with the sensitive organisms. Bacteriocins activity in water and sunflower oil after the films were removed, was determined by the well agar diffusion assay. Experiment was run in duplicates.

Statistical analysis

Experimental data were subjected to analysis of variance (ANOVA), and the Tukey test was applied with a level of significance of 95%.

All statistical analyses were performed using Minitab Statistic Program, release 12. Results are informed by mean ± Standar Deviation of replications.

Results and discussion

Active film preparation. MIC determination

Different concentrations of de BCE (0.01%, 0.1 and 1%) were added to the wheat gluten formulation. Films doped with BCE 0.1 % (267 AU cm^{-3} lactocin 705, 2133 AU cm^{-3} AL705) and 1% (4267 AU cm^{-3} lactocin 705, AU cm^{-3} 12800 AL705) exerted antimicrobial activity against *L. plantarum* CRL691 and *L. innocua* 7; while for films with BCE 0.01% added (67 AU cm^{-3} lactocin 705, 400 AU cm^{-3} AL705) antagonistic effect was displayed only against *L. innocua* 7 (Figure 1). Thus 0.1% BCE was defined as the MIC, since at this concentration the WG-films were active against both sensitive microorganisms. Even when the activation method as well as the polymer matrix were different from the present work, lactocin 705 and AL705 showed similar MIC for a LLDPE-based film activation by contact (bacteriocins adsorption) with a solution from *L. curvatus* CRL 705 (Blanco Massani et al 2008).

Film properties as a function of aging time

The functional properties of the wheat gluten film formulated with BCE (0.1%) and stored for 50 days (23ºC, RH 50%) were periodically determined and compared to a control film without bacteriocins. During the storage time, both the control and active wheat gluten residual water content was around 10%. Tensile strength and percentage elongation at break of the control and active film as a function of storage time are shown in Figure 2a and 2b. At the beginning of the experiment, the mechanical properties of the control and active films (Figure 2a and b) were significantly different ($P<0.05$) (tensile strength 2.0 ± 0.1 MPa and 2.7 ± 0.3 MPa; elongation at break 251 ± 14% and 190 ± 13%, respectively for the control and active film); nevertheless, the observed difference was negligible (Figure 2 a and b). Mechanical properties values

obtained in our work are in the range of those already reported for WG-films (Olabarrieta, Cho et al 2006, Irissin-Mangata et al 2001). After 5 days of aging, the tensile strength as well as elongation at break of the active film reached the values of those obtained for the control (3.5 ± 0.3 MPa and 3.4 ± 0.4 MPa; 155 ± 21.% and 163 ± 14%, respectively). Until the 20th day of storage, strength marginally increased, while elongation decreased (3.8 ± 0.3 MPa and 3.8 ± 0.3 MPa; 137 ± 29 % and 146 ± 18 %, respectively for the control and active film), (Figure 2 a and b). From the 20th day up to the 50th, mechanical properties remained fairly constant reaching tensile strength values of 3.3 ± 0.3 MPa, 3.6 ± 0.2 MPa, and elongation at break, 177 ± 29 % and 160 ± 22 %, respectively for the control and active films (Figure 2 a and b). WG-films change in mechanical properties as a function of time was already studied and explained by an increase in thiol oxidation during aging, leading to the formation of protein polymers of large molecular size (crosslinking) (Morel et al 2000; Micard et al 2000, Olabarrieta, Cho et al 2006). Water vapor permeability was not significantly ($P \geq 0.05$) different between the control and active film ($8\ 10^{-12} \pm 3\ 10^{-13}$, $8\ 10^{-12} \pm 3\ 10^{-13}$, respectively), maintaining the magnitude order until the end of the storage period ($8\ 10^{-12} \pm 6\ 10^{-13}$, $8\ 10^{-12} \pm 8\ 10^{-13}$, respectively). Water vapor permeability values obtained in our work are in the order of those found for wheat gluten films obtained at acid pH (Irissin-Mangata et al 2001). When activating a packaging polymer to render antimicrobial properties, some physico-mechanical properties as well as processability can be affected. General properties of packaging materials include mechanical properties such as tensile strength, elongation, burst strength, tearing resistance, stiffness, and physical properties such as oxygen (and other gas) permeability, water vapor permeability, among others (Han 2000). From the comparison between active and control WG-films mechanical and

barrier properties it could be observed that addition of bacteriocins had no effect on the kinetics of WG film aging.

To fulfill the objective of protecting and extending food shelf life, active packaging materials should maintain their activity properties over a time period, determined in agreement with shelf life of the food they packed. To study the effect of aging in the WG-film antimicrobial properties, activity of the bacteriocins in the wheat gluten film with elapsed time (50 days at 23ºC) was assayed. A slight decrease in anti-*Listeria* activity (latocin AL705) was observed by the end of the storage period (Figure 3); while for lactocin 705, activity decrease was more pronounced. Nevertheless, antimicrobial activity of the wheat gluten film was not completely lost during storage (Figure 3). The polymer matrix is the internal network with free space in which the antimicrobials are entrapped. The distribution and compactness of the polymer molecules, as well as interactions between antimicrobials and polymer determine the movement of these additives within the matrix (Balasubramanian 2012). Electrostatic interactions between the cationic nisin and the anionic stearic fatty acid, added to a HPMC film was found to decrease bacteriocin desorption from the film leading to a decrease in antimicrobial activity (Sebti & Coma 2002). Nevertheless, when HPMC films were made at acid conditions, nisin was released from the films since protonated species from nisin and stearic acid were favored (Sebti et al 2002). Chemical crosslinking in a HPMC matrix decreased antimicrobial activity of nisin in the polymer (Sebti et al 2003). In our work, even when mechanical properties variations could suggest that WG-film suffered from aging (crosslinking), no correlation between antimicrobial activity behavior (Figure 3) and structural changes (Figure 2) was observed. Contrary, to our results on lactocin 705 and AL705 antimicrobial activity from the WG-film, are similar to that obtained for the bacteriocins adsorbed on a PE-based film; in which case film activity was related to the

bacteriocin stability over the time, rather than to an interaction with the film matrix, and more stability of AL705 than 705 in the film was found (Blanco Massani et al 2012). Antimicrobial stability was earlier reported for bacteriocins immobilized in different bio-based matrices. Activity retention of cellulose based inserts showed an initial decrease in the first week of storage but remained stable for the remaining 3 months of the trial, while a cellulose based coating conserved its activity during 12 weeks both at room temperature and under refrigeration (Scannell et al 2000, Neetoo et al 2007).

Other factor to be considered in active packaging food application is the migration of the antimicrobial substances from the film, as well as its antimicrobial activity performance on the presence of food simulants. The release kinetics of antimicrobial agents has to be designed to maintain the concentration above the critical inhibitory concentration with respect to the microorganisms' growth kinetic. Foods have different chemical and biological characteristics; they provide different environmental conditions to microorganisms and included antimicrobial agents (Han 2000). The influence of hydrophobic (sunflower oil) and hydrophilic (water) simulants media on antimicrobial activity of the wheat gluten film; as well as the residual activity in the media after contact with the film was periodically assayed. Results of these experiments are shown in Figure 4 and Table 1. Wheat gluten films residual activity after water contact was observed in each day of the experiment as shown in Figure 4 b and e (day 50 of storage). Lactocin 705 activity in water after active WG-film contact was observed at the beginning of the experiment, while for lactocin AL705, activity was also detected in water contacted with films that were aged for 15 days (Table 1). Antimicrobial release from polymers is influenced by several factors; (i) active component size, (ii) antimicrobial and polymer matrix compatibility, (iii) polymer matrix characteristics, (iv) processing method, (v) food composition, (vi) storage conditions. Controlled release of

antimicrobials is feasible only if the antimicrobials are physically entrapped within the polymer matrix rather than being chemically bound (Balasubramanian 2012). In our work, even when lactocin 705 and AL705 were delivered from the active WG-film after water contact, bacteriocins activity was still retained inside the film matrix (Figure 4 b and e). Even when WG-films did not age as dramatically as other WG films obtained in acid conditions (Olabarrieta, Cho et al 2006; Olabarrieta, Gällstedt et al 2006), film cross-linking during the elapsed time (Micard et al 2000; Morel et al 2000; Irissin-Mangata et al 2001) could lead to the observed bacteriocins entrapment within the WG-matrix and loss in film bacteriocins release upon water contact after 15 days of film storage. After active film contact with sunflower oil (Figure 4 c and f, Table 1), the film exerted antimicrobial activity of lactocin AL705 only (Figure 4 f) and no activity in the hydrophobic media was detected for each assayed storage time (Table 1). *L. curvatus* CRL705 bacteriocins lack of activity in sunflower oil could be related to their insolubility in that media, while inactivation of lactocin 705 in the WG-film after contact is in coincidence with the results found for this bacteriocin adsorbed in a PE-based film, which is related to its mode of action and interaction with hydrophobic compounds (Castellano et al 2007; Blanco Massani et al 2012; Blanco Massani et al 2013). An active compound which is immobilized into polymeric materials could act directly from the film without being released into the packaged foodstuff (Conte et al 2007). Protein packaging films can act as a reservoir and gradually release antimicrobial agents to maintain a constant microbial inhibitory effect (Dawson et al 2003). In our work, changes in properties observed after WG-film aging favored activity retention inside the film without a significant change in antimicrobial activity, thereby allowing the active agent to act at a food surface level. Furthermore, the efficacy of the bacteriocin activity could be improved by control of migration of the bacteriocin into

the packaged media, enabling its antimicrobial effect to be preserved beyond consumer purchase (Sobrino-López & Martín-Belloso 2008). According to our results, it should be expected that in the time scale of vacuum packaged cooked sausages (aproximately 30-40 days) (Korkeala & Björkroth, 1997), bacteriocins activity in the film and the intimate contact provided by vacuum, give an efficient anti-microbial effect over these products.

The positive impact on antimicrobial activity properties with gluten aging demonstrated in this work, could help to understand the film performance applied in more complex real cases (RTE meat food products), which is part of current studies.

Conclusions

A WG-film obtained at pilot scale demonstrated effectiveness as an antimicrobial support for *L. curvatus* CRL705 bacteriocins. Mechanical properties as well as aging kinectics were not significantly affected by bacteriocins addition. Promising antimicrobial release properties were observed in contact with substances commonly used as food simulants for cooked meat products (sunflower oil and water). Moreover, anti-*Listeria* activity was retained in the film even after contact with simulants. Changes in the properties observed after WG-film aging (50 days) favored activity retention inside the film without a significant change in antimicrobial activity, this time period being cosistent with the shelf life of some vacuum packaged cooked sausages. The results suggest that a WG-film could be used as lactocin 705 and AL705 carrier to avoid contamination in RTE meat products such as cooked sausages.

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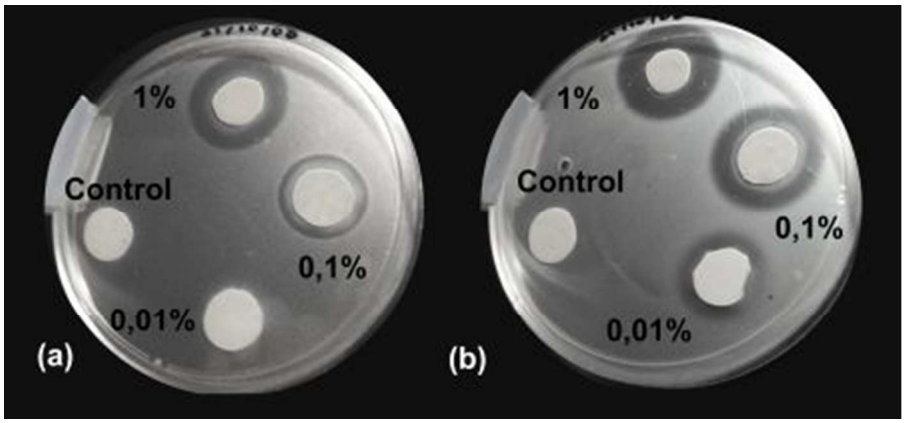
Figure legends

Figure 1 Lactocin 705 (a) and lactocin AL705 (b) antimicrobial activity on WG-films doped with BCE (0.01, 0.1 and 1%). Control film had no antimicrobials in its formulation.

Figure 2 Mechanical properties, of the active WG-film (▼) and a control without bacteriocins (●) as a function of storage time. Lines illustrate trends.

Figure 3 Lactocin 705(●) and AL705 (○) antimicrobial activity on the WG-film as a function of storage time. Lines illustrate trends.

Figure 4 Lactocin 705 and AL705 activity of the control (a), (d), and active WG-films (b), (e) after water contact, and sunflower oil (c), (f) contact. C+ is the inhibition area exerted by a spot of BCE.



Lactocin 705 (a) and lactocin AL705 (b) antimicrobial activity on WG-films doped with BCE (0.01, 0.1 and 1%). Control film had no antimicrobials in its formulation.
118x54mm (96 x 96 DPI)

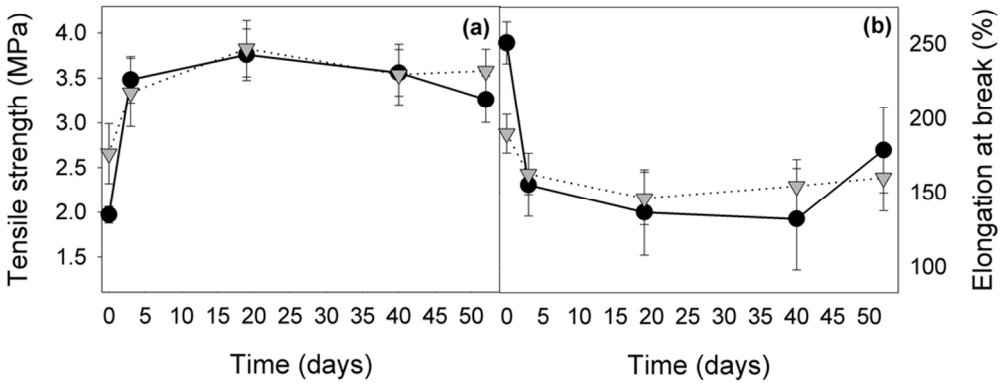


Figure 2 Mechanical properties, of the active WG-film (▼) and a control without bacteriocins (●) as a function of storage time. Lines illustrate trends.
46x21mm (600 x 600 DPI)

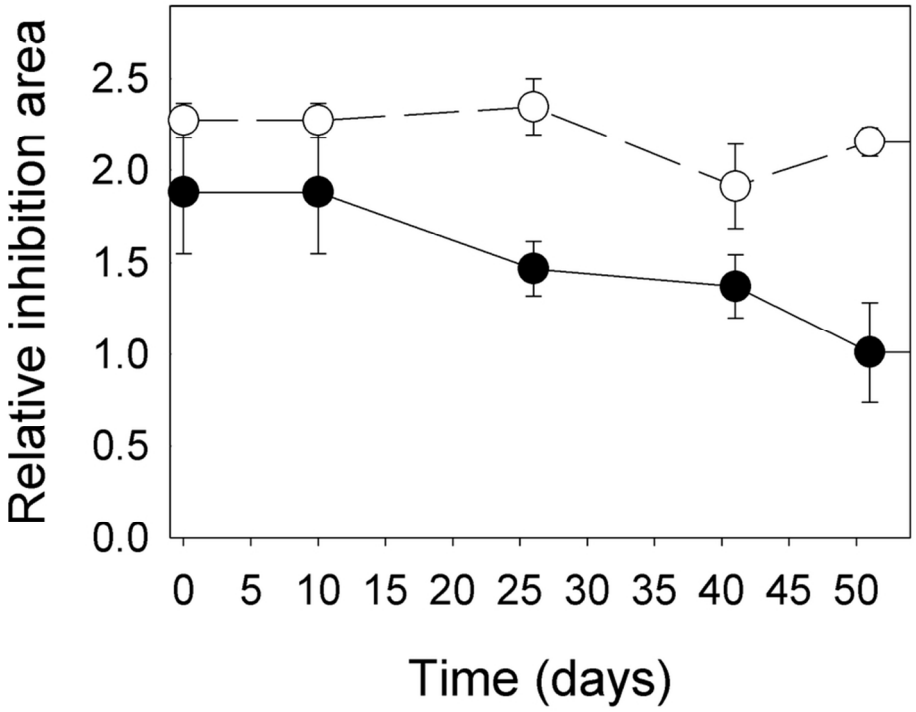


Figure 3 Lactocin 705(●) and AL705 (○) antimicrobial activity on the WG-film as a function of storage time. Lines illustrate trends.
43x37mm (600 x 600 DPI)

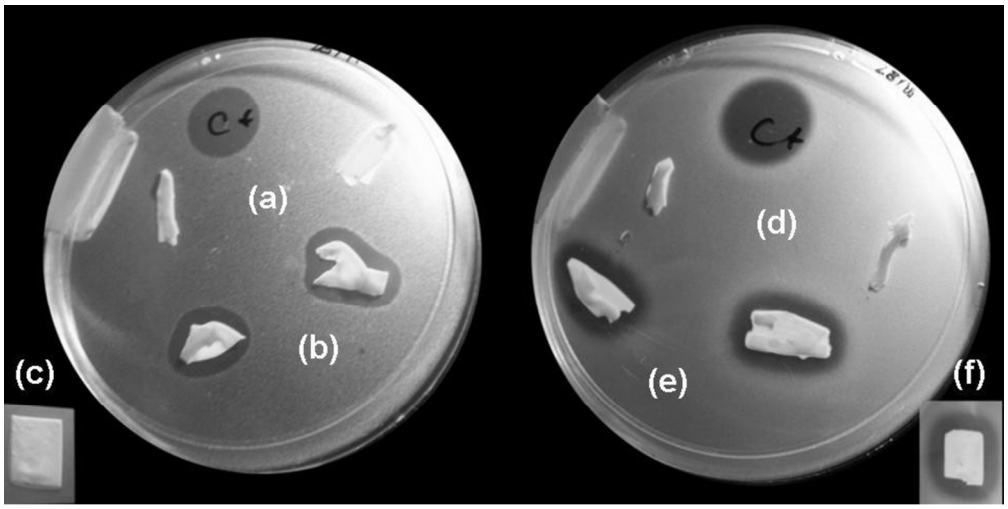


Figure 4 Lactocin 705 and AL705 activity of the control (a), (d), and active WG-films (b), (e) after water contact, and sunflower oil (c), (f) contact. C+ is the inhibition area exerted by a spot of BCE.
254x127mm (96 x 96 DPI)

Table 1. Residual antimicrobial activity in water and sunflower oil after WG-film direct contact.

Storage time (days)	Activity in the media (AU cm^{-3})			
	Water		Sunflower oil	
0	19*	122**	ND*	ND**
15	ND	32	ND	ND
30	ND	ND	ND	ND
44	ND	ND	ND	ND
50	ND	ND	ND	ND

ND: not detected.

* Lactocin 705 activity

** Lactocin AL705 activity