



## Adsorption of the bacteriocins produced by *Lactobacillus curvatus* CRL705 on a multilayer-LLDPE film for food-packaging applications

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### ABSTRACT

Adsorption of bacteriocins produced by *Lactobacillus curvatus* CRL705, lactocin 705 (whose activity depends upon complementation of two peptides, lac705 $\alpha$  and lac705 $\beta$ ) and bacteriocin/s with strong anti-*Listeria* activity, on a multilayer film was investigated. Lactocin 705 adsorption equilibrium at 30 °C was reached from 1 h of film contact. This bacteriocin exhibited a Langmuir-type adsorption, showing a mass adsorption maximum of  $0.72 \pm 0.05 \mu\text{g cm}^{-2}$  and a minimum inhibition concentration of  $1 \mu\text{g ml}^{-1}$ . The influence of impurities, generated from bacteriocinogenic strains growth, on bacteriocins adsorption to the film was investigated by inhibition area evaluation in semisolid agar. Impurities from LAB growth strongly influenced adsorption and lactocin 705 antimicrobial activity on the film, while antilisterial bacteriocin/s adsorption remained unaffected. To explain these results, a lack of lac705 $\beta$  and lac705 $\alpha$  peptides complementation necessary for antimicrobial activity, while no interactions among impurities and antilisterial bacteriocin/s during adsorption was suggested. Antilisterial bacteriocin/s activity on the film was not influenced by lactocin 705 adsorption; conformational reorganization of adsorbed antilisterial bacteriocin/s in the presence of lactocin 705 could allow the adsorption of both bacteriocins while maintaining antilisterial antimicrobial activity. This study highlights the technological importance of adsorption optimization to obtain effective antimicrobial food packaging systems.

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### 1. Introduction

The use of proper packaging materials to minimize food losses and provide safe and wholesome food products has always been the focus of food packaging. Consumer trends for better quality, fresh-like, and convenient foods have been intensified in recent decades. As a consequence, a variety of active food packaging technologies have been developed, among which antimicrobial

**Abbreviations:** 705, Lactocin 705; AB, Antilisterial bacteriocin/s; ATR-IR, Attenuated total internal reflectance infrared spectroscopy; AU, Arbitrary units; BU, Bacteriocin units; C-AB, Concentrated antilisterial bacteriocin/s; CD, Circular dichroism; CE, Crude extract; CFU, Colony forming units; C-Sac7, Concentrated impurities from Bac-variant growth; FTIR, Fourier Transform Infrared; Imp, Impurities generated from the growth of LAB in MRS broth; LAB, Lactic acid bacteria; LLDPE, Linear low density polyethylene; MIC, Minimum inhibitory concentration; P-AB, Purified antilisterial bacteriocin/s; RSA, Random Sequence Adsorption; S-705, Synthetic lactocin 705.

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containing as well as inherently antimicrobial films offer new opportunities for the food industry (Aider, 2010; Cho, Lee, & Han, 2009). Antimicrobial packaging systems constitute an emerging technology designed to control the microbial population and target specific microorganisms, thus providing higher safety and quality products. A range of chemical preservatives has been used in active-antimicrobial releasing systems among which bacteriocins and particularly nisin was the most commonly incorporated into films (Joerger, 2007).

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria, from which three main classes have been recently proposed for Gram-positive microorganisms (Rea, Ross, Cotter, & Hill, 2011). Among them, Class II bacteriocins encompass pos-transductionally unmodified peptides and include IIa pediocin like (antilisterial bacteriocins) and IIb two-peptide bacteriocins.

The genome sequence of *Lactobacillus curvatus* CRL705, isolated from dry-fermented sausages, revealed the presence of the genes encoding for five bacteriocins production (Hebert et al., 2012). Among them, the bacteriocin lactocin 705, characterized as

belonging to class IIb bacteriocins, and whose activity depends upon the complementation of two peptides lac705 $\alpha$  and lac705 $\beta$  with 33 amino acid residues each (Cuozzo, Sesma, Palacios, Pesce de Ruiz Holgado, & Raya, 2000), exerted antimicrobial activity against some Lactic acid bacteria (LAB) and *Brochothrix thermosphacta* (Castellano & Vignolo, 2006). Although neither lac705 $\alpha$  nor lac705 $\beta$  displayed bacteriocin activity by itself when the growth of sensitive cells was monitored, both peptides showed the ability to interact with a zwitterionic membrane at different bilayer levels (Castellano, Vignolo, Farías, Arrondo, & Cheín, 2007) and bactericidal effect on the indicator strain *Lactobacillus plantarum* CRL691 was exhibited with a 1 to 4 optimal lac705 $\alpha$ /lac705 $\beta$  peptides ratio (Cuozzo, Castellano, Sesma, Vignolo, & Raya, 2003). “Lactocin AL705”, even when it was not yet sequenced, broth and meat slurry assays demonstrated high specific activity against *Listeria* species (Castellano, Holzappel, & Vignolo, 2004; Castellano & Vignolo, 2006), thus it may be ascribed to antilisterial (AB) class IIa bacteriocins. Moreover, since *L. curvatus* CRL705 genome encodes for the production of sakacin P and sakacin X, class IIa bacteriocins, antilisterial activity could be ascribable to those antilisterial bacteriocins (Drider, Fimland, Héchar, McMullen, & Prévost, 2006; Hebert et al., 2012).

The strong amphiphatic nature of proteins and peptides gives them great stability in the adsorbed state. Thus, protein adsorption is a common event that takes place in areas such as medicine, pharmaceutical sciences, analytical sciences, biotechnology, cell biology, or biophysics (Hlady & Buijs, 1996; Rabe, Verdes, & Seeger, 2011). Conformational rearrangements involved in adsorption could cause bacteriocin structure alteration and negatively affect its antimicrobial activity (Drider et al., 2006; Nissen-Meyer, Oppgård, Rogne, Hauguen, & Kristiansen, 2010; Roach, Farrar, & Perry, 2005). Vast investigation on understanding protein adsorption can be found in the literature and techniques to detect adsorbates presence on the surface include ellipsometry, quartz crystal microbalance measurements, and analytical methods such as Lowry method or absorbance determination, among others (Nakanishi, Sakiyama, & Imamura, 2001; Roach et al., 2005; Sarkar & Chatteraj, 1993; Wei, Huang, Hou, Yuan, & Fang, 2007). Techniques that specifically focus on the secondary structure of adsorbed proteins such as attenuated total internal reflectance infrared spectroscopy (ATR-IR) and Circular dichroism (CD) spectroscopy are valuable tools to study conformational changes (Rabe et al., 2011). However, for bacteriocins less information on solid surface interactions is available and techniques used for adsorption determinations include ellipsometry (Bower, McGuire, & Daeschel, 1995; Tai, McGuire, & Neff, 2008) and those taking into account biological activity of the proteinaceous substances, such as turbidimetry or inhibition on semisolid medium (Bower et al., 1995; Guerra, Araujo et al., 2005; Guerra, Macías, Agrasar, & Castro, 2005; Ibarguren, Audisio, Farfán Torres, & Apella, 2010).

Langmuir model is the most basic adsorption model that accounts for the adsorption and desorption of particles at distinct surface sites. Although there is general accordance in the community that this formalism is inadequate to accurately describe protein adsorption, it is a kind of starting point for the development of theoretical descriptions of protein adsorption events, since it has a simple mathematical format (Rabe et al., 2011). Langmuir model application to describe proteins and bacteriocins adsorption behavior has been earlier reported (Daeschel, McGuire, & Al-Makhlafi, 1992; Wei et al., 2007).

Allowing bacteriocins to adsorb to food contact surfaces may have the potential to prevent spoilage and pathogenic colonization of foods. Several works have addressed surfaces activation using bacteriocins accompanied by impurities from the culture medium of production (Bower et al., 1995; Guerra, Araujo et al., 2005;

Guerra, Macías et al., 2005; Ibarguren et al., 2010; Scannell et al., 2000). However, no studies on the interaction of impurities and bacteriocins during adsorption were performed in the above mentioned works. In our previous studies, a CE obtained from *L. curvatus* CRL705 and containing lactocin 705, antilisterial bacteriocin/s and impurities from the producer bacterium was used to adsorb on a multilayer-Linear low density polyethylene (LLDPE) film, to render antimicrobial activity (Blanco Massani, Fernandez, Ariosti, Eisenberg, & Vignolo, 2008) and film surface properties before and after activation treatment were determined (Blanco Massani, Morando, Vignolo, & Eisenberg, 2012). In order to understand and control bacteriocins adsorption on the multilayer-LLDPE film as well as to predict antimicrobial film effectiveness, synthetic lactocin 705 was adsorbed on the multilayer-LLDPE film and the interaction with antilisterial bacteriocin/s and impurities from *L. curvatus* CRL705 growth during the adsorption process was investigated.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*L. curvatus* CRL705, lactocin 705 (705) and antilisterial bacteriocin/s (AB) producer, and *L. plantarum* CRL691, used as an indicator of 705 activity, were isolated from dry-fermented sausages (Vignolo, Suriani, Ruiz Holgado, & Oliver, 1993). Sac7 strain is a derivative from *L. curvatus* CRL705 unable to produce either 705 or AB (Bac-variant) (Cuozzo unpublished results). *L. curvatus* CRL1579, a derivative of CRL705 which only produces AB, was obtained as reported by Castellano and Vignolo (2006). All lactobacilli strains were grown in MRS broth (Britania, Argentina) at 30 °C. *Listeria innocua* 7, used as indicator of AB activity, was obtained from the Unité de Recherches Laitières et Génétique Appliquée, INRA (France) and grown in trypticase soy broth (TSB, Britania) with 5 mg ml<sup>-1</sup> of added yeast extract (YE, Britania) at 30 °C. All strains were maintained and stored at -20 °C in 0.15 g ml<sup>-1</sup> of glycerol.

### 2.2. Sorbent and adsorbates

A 100  $\mu$ m multilayer-LLDPE film composed of an external polypropylene layer, an internal polyamide–polyethylene structure, a barrier layer of ethylene vinyl alcohol copolymer, and a linear low density polyethylene food contact layer (Cryovac; Sealed Air Co, Argentina), was used as sorbent in this study. The bacteriocins lactocin 705, the AB, and impurities from *L. curvatus* CRL705 growth obtained from different sources were used as adsorbates (Table 1).

### 2.3. Adsorbates characterization by Fourier Transform Infrared (FTIR) spectroscopy

Each adsorbate was characterized by FTIR spectroscopy using a Thermo Nicolet 6700 spectrometer equipped with a DTGS KBr detector and a Smart iTR ATR sampling accessory. Sixty four scans were taken for each sample from 4000 to 650 cm<sup>-1</sup> at a resolution of 4 cm<sup>-1</sup>.

### 2.4. Bacteriocins quantification in solution

For lactocin 705 kinetic and equilibrium of adsorption studies (see below), bacteriocin activity was quantified against *L. plantarum* CRL691 with a turbidimetric bioassay (Cabo, Murado, González, & Pastoriza, 1999) based on growth inhibition of the target bacterium caused by serial dilution of bacteriocin samples. Briefly, one volume of S-705 and its two-fold dilutions were mixed in different tubes with one volume of the target bacterium (*L. plantarum*

**Table 1**  
Used adsorbates and their source.

Adsorbates	Source <sup>a</sup>	Producer micro-organisms	Preparation method
Lactocin 705, antilisterial bacteriocin/s and impurities <sup>b</sup>	CE	<i>L. curvatus</i> CRL705	An overnight culture of the producer microorganism was centrifuged (2500 g, 15 min); the supernatant was precipitated using 0.44 g cm <sup>-3</sup> ammonium sulfate, centrifuged (20,000 g, 20 min) and freeze-dried (Blanco Massani et al., 2008).
antilisterial bacteriocin/s and impurities <sup>b</sup>	C-AB	<i>L. curvatus</i> CRL1579	Lac705 $\alpha$ and lac705 $\beta$ peptides were synthesized according to Cuozzo et al. (2000) and Palacios et al. (1999).
Metabolites	C-Sac7	Sac7 strain	
Lactocin 705	S-705	–	C-AB was applied to a solid phase extraction cartridge (C-18) as earlier described (Blanco Massani et al., 2008) and freeze-dried.
antilisterial bacteriocin/s and impurities <sup>b</sup>	P-AB <sup>c</sup>	<i>L. curvatus</i> CRL1579	

<sup>a</sup> CE, crude extract; C-AB, concentrated antilisterial bacteriocin/s; C-Sac7, concentrated impurities from Bac-variant; S-705, synthetic lactocin 705; P-AB, purified antilisterial bacteriocin/s.

<sup>b</sup> Impurities include MRS medium components and bacterial metabolites.

<sup>c</sup> P-AB has lower impurities content than C-AB.

CRL691, 10<sup>5</sup> CFU ml<sup>-1</sup>) suspended in MRS broth (Britania, Argentina). The tubes were then incubated at 30 °C during 16 h, growth inhibition was measured spectrophotometrically at 600 nm (Cuozzo et al., 2003) and dose–response curves were obtained. Controls consisted of tubes in which S-705 was replaced by sterile distilled water. Bacteriocin activity was calculated as bacteriocin units (1 BU: bacteriocin needed to obtain 50% growth inhibition compared with control tubes) (Guerra, Araujo et al., 2005). Active lactocin 705 concentration was determined from a standard curve constructed with BU versus different S-705 concentration ( $\mu\text{g ml}^{-1}$ ).

For bacteriocins adsorption study under different conditions (see below), bacteriocins activity in solution (titer) was determined by a modification of the agar well diffusion method (Pongtharangkul & Demirci, 2004). Fifteen microliter of serial two-fold dilutions of the bacteriocins solutions were added to 5 mm diameter wells cut in semisolid MRS agar plates seeded with *L. plantarum* CRL691 for lactocin 705, and TSB + YE agar seeded with *L. innocua* 7, for AB titration. The agar plates were stored at 4 °C for 24 h to allow pre-diffusion, then incubated for 16–18 h at 30 °C and examined for inhibition zones. Bacteriocins titer, expressed in arbitrary units (AU ml<sup>-1</sup>), was defined as the reciprocal of the highest dilution yielding a visible zone of inhibition on the sensitive strain. All determinations were performed in triplicate.

## 2.5. Bacteriocins quantification on the multilayer LLDPE film

Antimicrobial activity on the multilayer-LLDPE film (see below) was determined as described earlier (Blanco Massani et al., 2008). Film circles (0.95 cm<sup>2</sup>) with and without bacteriocins were placed face down on semisolid agar plates seeded with the sensitive organisms (*L. plantarum* CRL691 for 705 and *L. innocua* 7 for AB). Film activity was evidenced as an inhibition zone of the indicator organisms beneath and around the packaging material and was expressed as relative inhibition area (inhibition zone around packaging film/film area). Four replicates for each sample were run.

## 2.6. Lactocin 705 adsorption on the multilayer-LLDPE film

### 2.6.1. Minimum inhibitory concentration (MIC)

Lactocin 705 MIC necessary to assure uniform inhibition area on the multilayer film LLDPE surface was determined by contacting 0.260 ml of S-705 (0.5, 1, 2, 3, 4, 5, 6 and 8  $\mu\text{g ml}^{-1}$ ) with 0.95 cm<sup>2</sup> of the film. After contact, films were rinsed with sterile distilled water and antimicrobial activity on their LLDPE surface was determined in semisolid agar as earlier described.

### 2.6.2. Adsorption kinetic and equilibrium

To optimize lactocin 705 adsorption temperature, a S-705 bacteriocin solution (1  $\mu\text{g ml}^{-1}$ ) was contacted with the multilayer-

LLDPE film food contact face during pre-established times ranging from 10 to 120 min, at 20, 30 and 40 °C. Lactocin 705 adsorption isotherm was obtained by contacting the film with different concentrations of S-705 solution at 30 °C during 1 h. In all cases, in order to investigate whether a loss of lactocin 705 activity occurred during the active film preparation, control solutions of S-705 were subjected to the adsorption conditions in the absence of the multilayer-LLDPE film (Scannell et al., 2000). Bacteriocin active concentration, of control and film-contacted solutions was examined by BU determination as described above. The amount of active lactocin 705 adsorbed on the multilayer film LLDPE contact face,  $\Gamma$  ( $\mu\text{g cm}^{-2}$ ) at each time/concentration/temperature set, was determined from the difference between bacteriocin concentration in the controls and in the film-contacted solutions, as expressed by equation (1),

$$\Gamma = \frac{(C_c - C_f)v}{A} \quad (1)$$

where  $C_c$ : lactocin 705 concentration in the control solution,  $C_f$ : bacteriocin concentration after the adsorption process,  $v$ : volume of bacteriocin solution to which  $A$  (cm<sup>2</sup>) of multilayer-LLDPE film food contact face were contacted (Sarkar & Chattoraj, 1993). For all experiments lactocin 705 activity on the multilayer film food contact surface was confirmed on semisolid agar. Experiments were run in triplicates.

## 2.7. Bacteriocins adsorption under different conditions

Adsorbates were combined in order to study bacteriocins and impurities interaction during the adsorption process (Table 2). Bacteriocins adsorption curves were constructed from the relative inhibition areas exerted by bacteriocins adsorbed on multilayer-LLDPE film versus bacteriocin titer (AU ml<sup>-1</sup>) after adsorption.

S-705 (8  $\mu\text{g ml}^{-1}$ , 6400 AU ml<sup>-1</sup>) was also contacted (1 h, 30 °C) with the multilayer-LLDPE film surface in the presence of C-Sac7 (0.1, 1, 20 and 40 mg ml<sup>-1</sup>) and P-AB (AB, 12 800 AU ml<sup>-1</sup>) to simulate the conditions presented by 40 mg ml<sup>-1</sup> of the CE (lactocin 705, 6400 AU ml<sup>-1</sup>; AB, 12 800 AU ml<sup>-1</sup>). After contact, S-705 adsorption performance was checked by relative inhibition area evaluation in semisolid agar as earlier described. The same experiment was conducted for antilisterial bacteriocin/s adsorption from P-AB (AB, 12 800 AU ml<sup>-1</sup>), C-AB (AB, 12 800 AU ml<sup>-1</sup>), the combination of P-AB and S-705 (AB, 12 800 AU ml<sup>-1</sup>; 705, 6400 AU ml<sup>-1</sup>), and the CE (40 mg ml<sup>-1</sup>). A sequential adsorption study was also performed; the multilayer-LLDPE film was treated with S-705 (6400 AU ml<sup>-1</sup>, 1 h, 30 °C), rinsed with sterile water, contacted with Sac7 (40 mg ml<sup>-1</sup>, 1 h, 30 °C), rinsed again and

**Table 2**

Combination of adsorbates used in adsorption tests.

Adsorbate I (source)	Adsorbate II (source)	Study developed	Sensitive strain
Lactocin 705, 27 to 4815 AU ml <sup>-1</sup> (S-705)	–	Lactocin 705 adsorption	<i>L. plantarum</i> CRL691
	Impurities (C-Sac7, 1 mg ml <sup>-1</sup> )	Impurities influence on 705 adsorption	
	2000 AU ml <sup>-1</sup> AB (P-AB <sup>a</sup> )	AB influence on 705 adsorption	
AB, 27 to 8717 AU ml <sup>-1</sup> (P-AB <sup>a</sup> )	6400 AU ml <sup>-1</sup> lactocin 705 (S-705)	Lactocin 705 influence on AB adsorption	<i>L. innocua</i> 7
AB, 27 to 8717 AU ml <sup>-1</sup> (P-AB <sup>a</sup> ) compared to AB, 27 to 8717 AU ml <sup>-1</sup> (C-AB)		Impurities influence on AB adsorption	

<sup>a</sup> P-AB has lower impurities content than C-AB, but both adsorbates had the same AB titer.

assayed for antimicrobial activity. The same experiment was performed inverting the adsorbates order.

### 2.8. Statistical analysis

In all experiments data were subjected to analysis of variance (ANOVA), and the Tukey test was applied at the 0.05 level of significance. All statistical analyses were performed using Minitab Statistical Program, release 12 (Pennsylvania, USA).

## 3. Results and discussion

### 3.1. FTIR adsorbates characterization

Bacteriocins and impurities used in this study were obtained from different sources (CE, C-Sac7, C-AB, P-AB and S-705), FTIR spectroscopy was used as a tool to characterize them, looking for molecular groups associated with proteins, fatty acids and polysaccharides (Fig. 1). FTIR bands assignment were carried out according to those reported by Barth (2000), Maquelin et al. (2002), Motta, Flores, Souto, and Brandelli (2008) and Quinteiro Rodriguez (2000) for microorganisms, peptides and amino-acids characterization (Table 3). From these results, the bands exhibited at around 3500 and 3200 cm<sup>-1</sup> as well as those in the amide I (1700–1610 cm<sup>-1</sup>) and II (1520–1500 cm<sup>-1</sup>) regions were present in all analyzed adsorbates (Table 3) and could be associated with hydroxyl groups, proteins or protein compounds which is in agreement with the peptide nature of bacteriocins. On the other hand, the C–H stretching vibration of lipid acyl chains in the spectral region between 2900 and 2800 cm<sup>-1</sup>, C–H deformation of aliphatics at 1450 cm<sup>-1</sup>, stretching vibration from esters at 1740 cm<sup>-1</sup> as well as bands associated with carbohydrates deformation between 900 and 1200 cm<sup>-1</sup> were present in adsorbates from *Lactobacillus* cultures (CE, C-Sac7, C-AB, and P-AB), but were absent in synthetic lactocin 705 (Fig. 1, Table 3). This result suggests the presence of various LAB impurities (metabolites from the LAB growth and MRS medium components) as well as cellular debris, nucleic acids and aliphatic molecules among others. These results are in agreement with those reported by Vodnar, Paucean, Duluf, and Socaci (2010) who were able to fingerprint probiotic LAB using FTIR by the specific bands located around 2845 and 2929 cm<sup>-1</sup>, characteristic to the bacterial wall fatty acids, and a specific absorption peak at 1127 cm<sup>-1</sup>, for lactic acid. The spectrum corresponding to P-AB that was obtained after purification of C-AB (obtained from *L. curvatus* CRL1579) showed to lack a band at 1400 cm<sup>-1</sup> (Fig. 1), assigned to C=O stretching symmetric of COO<sup>-</sup> groups (Table 3); in addition, the bands between 900 and 1200 cm<sup>-1</sup> experienced a marked decrease in P-AB spectrum when compared with that of C-AB. These results suggest that part of acids compounds and polysaccharides from the culture media have been removed after adsorbate purification. On the other hand, the bands at 1438, 1200 and 1122 cm<sup>-1</sup> were present in the synthetic lactocin 705, as well as in the CE spectrum (Table 3). According to Barth (2000), these bands may be assigned to C–N stretching from Histidine (1439 cm<sup>-1</sup>), Tyrosine Tyr–OH bending

(1169–1260 cm<sup>-1</sup>) and C–O stretching from Aspartate (1120–1253 cm<sup>-1</sup>), this being in coincidence with the determined lactocin 705 amino acid sequence in which these amino acids are involved (Cuozzo et al., 2000). The infrared adsorption of amino acids side chain in a protein may deviate significantly from their absorption in solution or in a crystal (Barth, 2000). Although from amino acid side chains infrared bands of Barth's compilation (2000) absorption values for His, Tyr and Asp are in the spectral zone of those found for S-705 differential bands, these values were regarded only as guidelines for spectra interpretation, since experimental conditions used for IR determinations by Barth (2000) were different from that used in our work (amino acids in water in contrast to S-705 as solid powder).

### 3.2. Lactocin 705 adsorption on the multilayer-LLDPE film

#### 3.2.1. MIC determination

Lactocin 705 antimicrobial activity of the multilayer-LLDPE film assayed in semisolid agar after contact with different concentration of S-705 is shown in Fig. 2. Results showed an uneven inhibition area when S-705 at a concentration of 0.5 µg ml<sup>-1</sup> was applied, while uniform areas were observed when the multilayer film LLDPE surface was treated with S-705 concentrations from 1 to 8 µg ml<sup>-1</sup>. Thus, 1 µg/ml was chosen as the lactocin 705 MIC.

#### 3.2.2. Effect of temperature on bacteriocin adsorption

The variation of lactocin 705 active concentration in the activation solution, at three different temperatures, in absence (control) and in presence of the multilayer-LLDPE film was evaluated to determine the influence of temperature on lactocin 705 adsorption (Fig. 3a and b). A decrease of lactocin 705 active concentration as temperature increased from 20 to 40 °C during 120 min was recorded, both in the control solution and when contacted with multilayer-LLDPE film. For the different assayed temperatures (20, 30 and 40 °C), the higher the temperature, the sharper the active lactocin 705 concentration decrease in the absence (control) and in the presence of multilayer-LLDPE film (Fig. 3a and b, respectively). When the adsorbed mass of active lactocin 705 at 20, 30 and 40 °C was evaluated on the multilayer-LLDPE film, it was observed to be maximal at 30 °C (Fig. 3c). Even when thermal resistance of class II peptides is widely accepted, structural changes in the helical region observed at elevated temperatures may account for the loss of activity of these small peptides (Kaur, Andrew, Wishart, & Vederas, 2004; Soliman, Wang, Bhattacharjee, & Kaur, 2010). Besides bacteriocin degradation with temperature, the more pronounced decrease in concentration, when the bacteriocin solution was contacted with the multilayer-LLDPE film, indicated that there was a remaining concentration adsorbed on the film.

Protein adsorption is controlled by many types of interactions, the main constituents being dehydration of the sorbent surface and parts of the protein molecule, electrostatic interactions between the protein and the sorbent, and changes in the conformational entropy of the protein (Norde, 1996). In the adsorbed state, hydrophobic amino acid residues may rearrange its structure in order

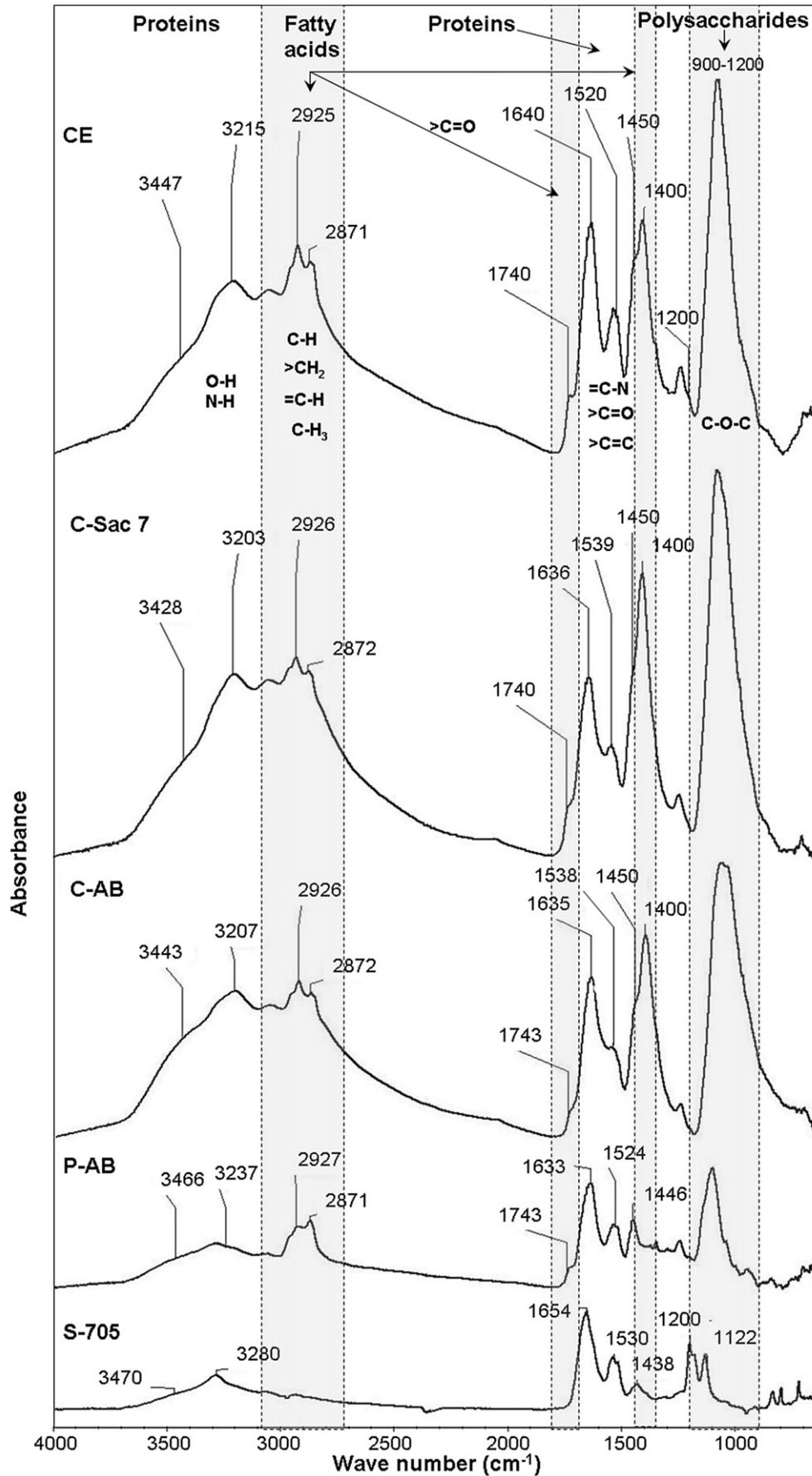


Fig. 1. FTIR spectra for the different adsorbates. For better comprehension the Figure has been divided in spectral zones.

**Table 3**

Tentative assignment of FTIR bands obtained for the different adsorbates, following Barth (2000), Maquelin et al. (2002), Motta et al. (2008) and Quintero Rodriguez (2000).

Wave number (cm <sup>-1</sup> )					Wave numbers obtained from bibliografy	Possible assignment <sup>a</sup>
CE	C-Sac7	C-AB	P-AB	S-705		
3447	3428	3443	3466	3470	~ 3500	O–H stretch of OH <sup>-</sup> groups
3215	3203	3207	3237	3280	3200	N–H stretch (amide A from proteins)
2925	2926	2926	2927	–	2920	CH <sub>2</sub> asymmetric stretch (fatty acids)
2871	2872	2872	2871	–	2870	CH <sub>3</sub> symmetric stretch (fatty acids)
1740	1740	1743	1743	–	1740	C=O stretch of esters (fatty acids)
1640	1636	1635	1633	1654	1700–1610	Amide I (proteins)
1520	1539	1538	1524	1530	1550–1520	Amide II (proteins)
1450	1450	1450	1446	–	1450	C–H def in aliphatics (fatty acids)
1400	1400	1400	–	–	1400	C=O symmetric stretch of COO <sup>-</sup>
1200 <sup>b</sup>	–	–	–	1438, 1200, 1122	1438, 1200, and 1122	C–N, C–O vibrations from amino acids side chains
√	√	√	√	–	900–1200	C–O, C–C stretch and C–O–H, C–O–C def (glycopeptides, phosphodiester, polysaccharides)

<sup>a</sup> Stretch, stretching; def, deformation.<sup>b</sup> Band at 1439, included in band observed around 1450, band at 1198 and 1122 cm<sup>-1</sup> are included in bands between 900 and 1200 cm<sup>-1</sup> (See Fig. 1).

to optimize interaction with the sorbent, preventing contact with water. Such structural rearrangements involve an entropy gain related to an increased rotational mobility along the polypeptide chain. This entropy increase may be sufficiently large to compensate for the positive adsorption enthalpy (Norde, Macritchie, Nowicka, & Lyklema, 1986). The amount of surface adsorbed proteins generally increases at elevated temperatures (Nakanishi et al., 2001). Temperature has an effect on both, the equilibrium state and the kinetics of protein adsorption. At higher temperatures structural arrangements increase significantly, and adsorption rates increases can be expected due to an accelerated diffusivity of proteins toward the sorbent surface (Kondo & Fukuda, 1998; Rabe et al., 2011). In our study, an increase in the active adsorbed mass of lactocin 705 when temperature changed from 20 to 30 °C was observed. However, the active adsorbed mass at 40 °C was lower than that obtained at 30 °C (Fig. 3c). Bacteriocin degradation in the solution as temperature increases from 30 to 40 °C (Fig. 3a), could lead to a decrease in bacteriocin active concentration, available to be adsorbed on the film. This antagonist effect allowed defining 30 °C as an optimal adsorption temperature.



**Fig. 2.** Inhibition areas exerted by multilayer-LLDPE film treated with 0.5; 1; 2; 3; 4; 5; 6; 8 µg ml<sup>-1</sup> of S-705.

### 3.2.3. Effect of contact time in bacteriocin adsorption

The antimicrobial activity of lactocin 705 when adsorption plateau was attained on the film at 20, 60 and 120 min for 40, 30 and 20 °C, respectively determined on semisolid agar is shown in Fig. 3d. A lack of antimicrobial activity on the multilayer-LLDPE film surface up to 120 min of contact at 20 °C and after 20 min at 40 °C were observed, while an activated multilayer-LLDPE film was obtained after contacting 60 min with S-705 at 30 °C. These results are in agreement with the higher bacteriocin adsorbed mass after 60 min of film contact at 30 °C (0.07 ± 0.02 µg cm<sup>-2</sup>) compared with the values obtained at 120 min and 20 min (20 and 40 °C, respectively) (Fig. 3c). Consequently, 60 min was defined as the minimal contact time for lactocin 705 equilibrium attainment in the multilayer-LLDPE film at 30 °C; this result is in coincidence with previously reported exposure time for lactocin 705 and AB adsorption from *L. curvatus* CRL705 CE (Blanco Massani et al., 2008). Greater contact times were necessary to homogeneously adsorb other antimicrobials such as the antilisterial bacteriocin from *Enterococcus faecium* CRL1385 and nisin to silicates and other hydrophilic and hydrophobic surfaces (Guerra, Araujo et al., 2005; Guerra, Macías et al., 2005; Iburguren et al., 2010). This could suggest a higher lactocin 705 bacteriocin affinity for the multilayer-LLDPE film.

### 3.2.4. Isotherm construction

Fig. 4 shows the adsorption isotherm of active lactocin 705 on the multilayer-LLDPE film at 30 °C. Modeling of experimental results from protein adsorption studies often requires the adaptation of different adsorption isotherms models (Hlady & Buijs, 1996). Several reports on nisin adsorption to surfaces with different hydrophobicity degrees showed monolayer (Daeschel et al., 1992), multilayer (Bower et al., 1995; Tai et al., 2008) adsorption isotherms or a combination of both (Guerra, Macías et al., 2005). Different adsorption models have been proposed during the past decades for protein adsorption (Rabe et al., 2011); among them the Random Sequence Adsorption (RSA) model (Talbot, Tarjus, Van Tassel, & Viot, 2000) has been applied for protein adsorption modeling at solid surfaces (Guemouri, Ogier, Zekhnini, & Ramsden, 2000; Ramsden, 1993). Nevertheless, to our knowledge RSA model has not been yet applied for bacteriocins adsorption modeling. Here, for comparative purposes with other studies on bacteriocin adsorption, Langmuir adsorption model was applied. This model assumes a monolayer adsorption, a homogeneous surface and no lateral interaction among adsorbed peptide molecules. Although this theory is too simplistic to explain the complex behavior of bacteriocins adsorption and data are not necessarily well described by the model, lactocin 705 adsorption may be empirically interpreted by Langmuir-type equation (2).

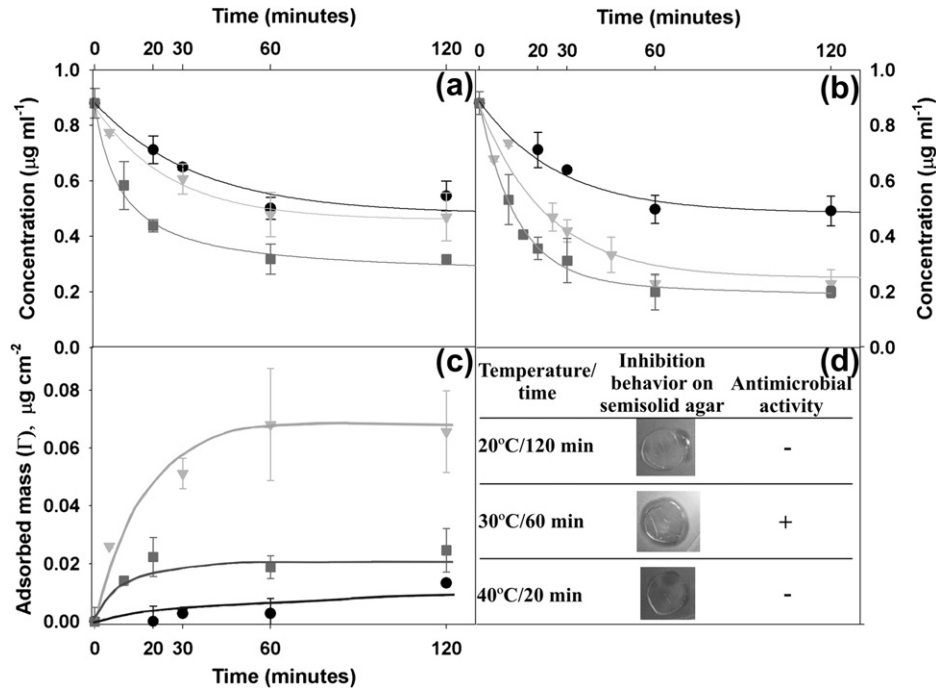


Fig. 3. Active lactocin 705 concentration changes during 120 min at (●) 20, (▼) 30 and (■) 40 °C. (a) S-705 solution; (b) S-705 solution contacted with multilayer-LLDPE film; (c) active lactocin 705 mass adsorbed on the multilayer-LLDPE film and (d) multilayer-LLDPE film 705 antimicrobial activity on semisolid agar. Continuous lines mark tendencies. Error bars indicate standard deviations.

$$\Gamma = \frac{\Gamma_c K C_{eq}}{1 + K C_{eq}} \quad (2)$$

in which,  $\Gamma$ , is the equilibrium concentration in the solid phase,  $C_{eq}$ , concentration in the liquid phase,  $K$ , apparent association constant representing interaction between adsorbate (lactocin 705) and the sorbent film surface (LLDPE multilayer film face).

Adsorption capacity of lactocin 705 on the multilayer-LLDPE film, as calculated from the curve plateau (Fig. 4, eq (2),  $R = 0.9319$ ) showed a value of  $0.72 \pm 0.05 \mu\text{g cm}^{-2}$ , this being similar to those found by Guerra, Araujo et al. (2005) who, using biological methods, determined similar nisin adsorption abilities

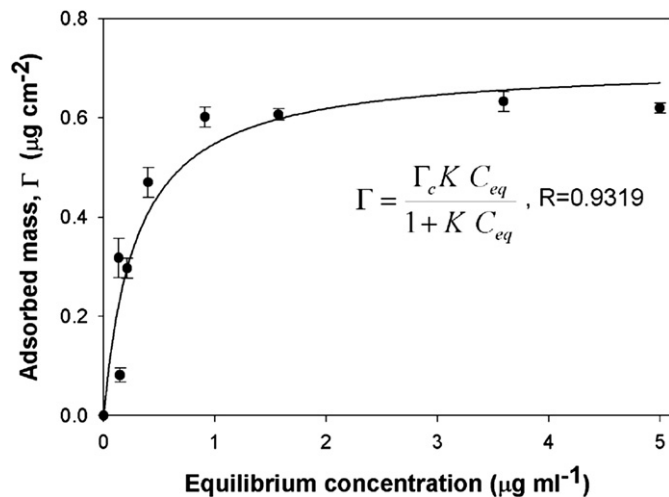


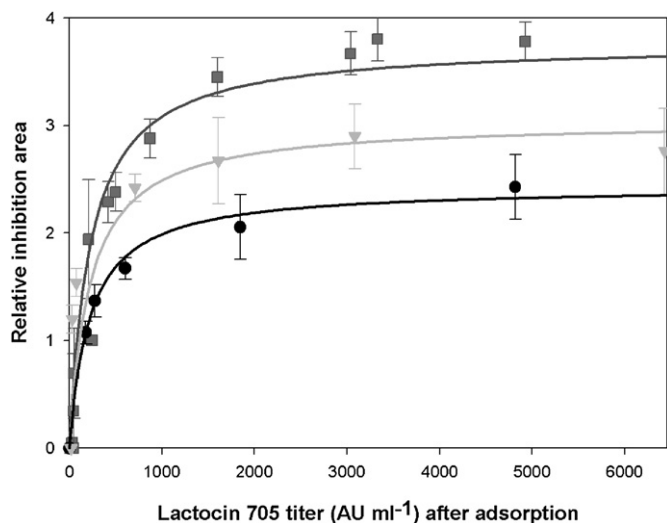
Fig. 4. Lactocin 705 adsorption isotherm on the multilayer-LLDPE film food contact face at 30 °C. The curve drawn through the data follows Langmuir equation (2) ( $R = 0.9319$ ). Error bars indicate standard deviations.

(0.665 and  $0.697 \mu\text{g cm}^{-2}$ ) to polyethylene-terephthalate and rubber, respectively, while a lower value ( $0.396 \mu\text{g cm}^{-2}$ ) was found for stainless steel. However, using ellipsometry, an adsorption capacity of  $0.4 \mu\text{g cm}^{-2}$  was reported for nisin on hydrophobic silicon surface (Daeschel et al., 1992). Lactocin 705 adsorption plateau was reached from an S-705 contact solution with a concentration above  $4 \mu\text{g ml}^{-1}$  ( $\Gamma$ :  $0.61 \pm 0.05 \mu\text{g cm}^{-2}$ ;  $C_{eq}$ :  $0.91 \mu\text{g ml}^{-1}$ ); multilayer-LLDPE film constant inhibition area on semisolid agar was also obtained from this concentration (Fig. 2).

### 3.3. Bacteriocins adsorption under different conditions

#### 3.3.1. Impurities and AB influence on lactocin 705 adsorption

Qualitatively, it can be seen from Fig. 2 that the amount of lactocin 705 antimicrobial activity associated with bacteriocin adsorbed (as indicated by diameters of respective inhibition areas) corresponds to the mass of lactocin 705 actually adsorbed to the respective multilayer films (Fig. 4); i.e., the smallest inhibition zone corresponded with the smallest adsorbed mass of bacteriocin. Base on this trend, adsorption of lactocin 705, checked by relative inhibition area determinations was subjected to an empirical treatment according to Langmuir equation (Fig. 5). Since bacteriocins are produced during bacterial growth, various types and amounts impurities, encompassing metabolites produced during growth of the bacteriocinogenic LAB strains (*L. curvatus* CRL705 and CRL1579) as well as growth medium components, are present in bacteria extracts. Consequently the influence of impurities on lactocin 705 adsorption was investigated. Lactocin 705 relative inhibition area of multilayer-LLDPE film decreased in the presence of impurities from Bac<sup>-</sup> variant (C-Sac7,  $1 \text{ mg ml}^{-1}$ ), from  $3.7 \pm 0.1$  (S-705 alone) to  $2.6 \pm 0.3$  at adsorption equilibrium (Fig. 5). However, a higher relative inhibition area ( $3.0 \pm 0.3$ ) was exerted by lactocin 705 when adsorbed in the presence of P-AB (AB,  $2000 \text{ AU ml}^{-1}$ ). In addition, to simulate the conditions of bacteriocin crude extract (CE); different amounts of impurities (C-Sac7, 0.1, 1, 20 and



**Fig. 5.** Lactocin 705 adsorption on the multilayer-LLDPE film at 30 °C from (■) S-705 alone; in the presence of (▼) P-AB (2000 AU ml<sup>-1</sup>) and (●) C-Sac7 (1 mg ml<sup>-1</sup>). The curves drawn through the data follow Langmuir equation (2). Error bars indicate standard deviations.

40 mg ml<sup>-1</sup>) and P-AB (AB, 12 800 AU ml<sup>-1</sup>) were added to S-705 (705, 6400 AU ml<sup>-1</sup>), and contacted with the multilayer-LLDPE film. Even when lactocin 705 titer in solution did not change upon C-Sac7 addition; as the impurities concentration increased a significant decreasing effect ( $P < 0.05$ ) on lactocin 705 activity on the multilayer-LLDPE film was observed (Table 4). When the film was treated with S-705 in the presence of P-AB, the exerted relative inhibition area ( $3.0 \pm 0.3$ ) was not significantly different ( $P \geq 0.05$ ) from that produced in the presence of Sac7 0.1 mg ml<sup>-1</sup> ( $3.2 \pm 0.3$ ). Multilayer-LLDPE film treated with S-705 added with C-Sac7 (20 and 40 mg ml<sup>-1</sup>), showed the same inhibition area ( $1.6 \pm 0.3$  and  $1.1 \pm 0.5$ , respectively) than that treated with CE ( $P \geq 0.05$ ) (Table 4). The reduction in the adsorption maximum of lactocin 705 added with P-AB and C-Sac7 (containing impurities from LAB growth) may be explained by the presence of proteins, fatty acids and polysaccharides as showed by FTIR analysis of the bacterially produced adsorbates. Lactocin 705 inactivation on the multilayer-LLDPE film surface was previously reported when contacted with sunflower oil (Blanco Massani et al., 2012); lipid acyl chains present in C-Sac7, CE and P-AB may also interfere with lactocin 705 adsorption, contributing to the observed antimicrobial activity

decrease. Since the derivative Sac7 strain differs from the parental *L. curvatus* CRL705 on its ability to ferment sucrose and to produce lactocin 705 and AB, and since the growth MRS medium used do not contain sucrose in its formulation, metabolites produced by both bacteria were assumed to be the same.

When considering protein and peptides mixtures, the adsorption behavior is often a result of an overlap of transport, adsorption and repulsion processes (Rabe et al., 2011). Small proteins diffuse faster than large ones and are the dominating species in the early adsorption stage. However, larger proteins typically bind stronger to the surface because of a larger contact area, and can even repel other pre-adsorbed proteins during spreading on the surface (Lutanie et al., 1992; Nasir & McGuire, 1998). Consequently, the total mass of adsorbed proteins passes through a maximum during the course of adsorption (Andrade & Hlady, 1986). In our study, the pronounced relative inhibition areas reduction for lactocin 705 with increasing C-Sac7 (impurities from LAB growth) concentration might suggest a competitive adsorption between lactocin 705 (synthetic two-peptide bacteriocin) and the molecules present in C-Sac7 adsorbate (fatty acids, peptides or proteins). Similarly, the decreased antimicrobial activity of lactocin 705 on the multilayer-LLDPE film surface in the presence of PAB and from the CE may be ascribed to the presence of such impurities. In order to check a competitive adsorption, a sequential adsorption study was performed and inhibition areas were compared to that of the obtained by CE (Table 4). No significant differences ( $P \geq 0.05$ ) in multilayer-LLDPE film relative inhibition areas were obtained after the sequential adsorption of S-705 and C-Sac7 (40 mg ml<sup>-1</sup>) ( $1.5 \pm 0.1$ ), and from treatment with CE ( $1.4 \pm 0.4$ ). Conversely, relative inhibition area obtained from sequential treatment of multilayer-LLDPE film with C-Sac7 (40 mg ml<sup>-1</sup>) and S-705 ( $3.3 \pm 0.4$ ) showed no significant difference ( $P \geq 0.05$ ) from that of the film treated with S-705 alone ( $3.7 \pm 0.1$ ). These results could suggest that impurities from C-Sac7 are not being adsorbed directly on multilayer-LLDPE film.

### 3.3.2. Impurities content and 705 influence on AB adsorption

AB adsorption on the multilayer-LLDPE film was studied on semisolid agar (Fig. 6, Table 4). No differences in AB relative inhibition areas on the film were observed ( $P \geq 0.05$ ) when the bacteriocin was adsorbed from C-AB, P-AB alone or combined with S-705 ( $8 \mu\text{g ml}^{-1}$ ) (Fig. 6). The effect of the impurities content on lactocin AB adsorption was analyzed by comparing relative inhibition areas of the multilayer-LLDPE film contacted with P-AB and C-AB. No significant differences ( $P \geq 0.05$ ) were found when AB adsorbed from these adsorbate sources (P-AB,  $2.1 \pm 0.2$  and C-AB,

**Table 4**

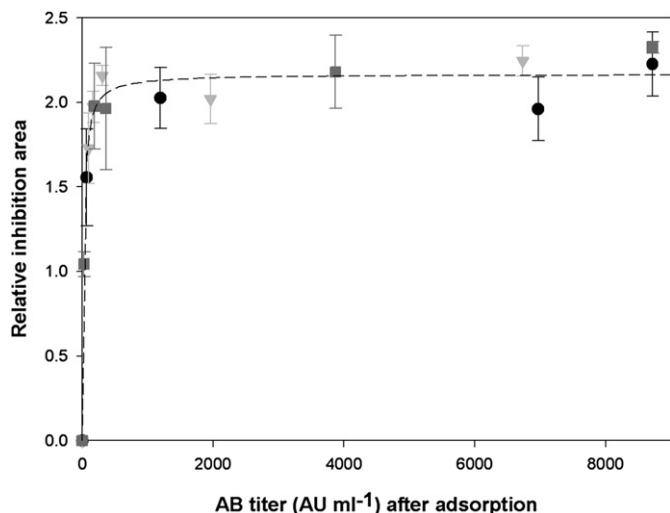
Antimicrobial activity of the multilayer-LLDPE film contacted (1 h, 30 °C) with different adsorbates combination. Values in a column followed by different uppercase letters are statistically different ( $P < 0.05$ ).<sup>a</sup>

Adsorbate I	Adsorbate II	Relative inhibition area exerted by	
		Lactocin 705 <sup>a</sup>	AB <sup>a</sup>
S-705 (6400 AU ml <sup>-1</sup> lactocin 705)	–	3.7 <sup>A</sup> ± 0.1	–
	C-Sac7 (0.1 mg ml <sup>-1</sup> )	3.2 <sup>B</sup> ± 0.3	–
	C-Sac7 (1 mg ml <sup>-1</sup> )	2.6 <sup>C</sup> ± 0.3	–
	C-Sac7 (20 mg ml <sup>-1</sup> )	1.6 <sup>D</sup> ± 0.3	–
	C-Sac7 (40 mg ml <sup>-1</sup> )	1.1 <sup>D</sup> ± 0.5	–
	P-AB (12, 800 AU ml <sup>-1</sup> AB)	3.0 <sup>B</sup> ± 0.3	2.1 <sup>A</sup> ± 0.2
CE (6400 and 12, 800 AU ml <sup>-1</sup> , respectively 705 and AL705)	–	1.4 <sup>D</sup> ± 0.4	2.3 <sup>A</sup> ± 0.2
P-AB (12, 800 AU ml <sup>-1</sup> AL705)	–	–	2.1 <sup>A</sup> ± 0.2
C-AB (12, 800 AU ml <sup>-1</sup> AL705)	–	–	2.2 <sup>A</sup> ± 0.1
S-705 (6400 AU ml <sup>-1</sup> 705) <sup>b</sup>	C-Sac7 (40 mg ml <sup>-1</sup> ) <sup>b</sup>	1.5 <sup>D</sup> ± 0.1	–
C-Sac7 (40 mg ml <sup>-1</sup> ) <sup>b</sup>	S-705 (6400 AU ml <sup>-1</sup> 705) <sup>b</sup>	3.3 <sup>A</sup> ± 0.4	–

<sup>a</sup> Mean of four replications ± standard deviation.

<sup>b</sup> Sequential adsorption (See section 2).





**Fig. 6.** Antilisterial bacteriocin/s (AB) adsorption on the multilayer-LLDPE film at 30 °C from (●) P-AB alone; (▼) in the presence of S-705 (6400 AU ml<sup>-1</sup>) and (■) from C-AB. The curve drawn through the data follows Langmuir equation (2). Error bars indicate standard deviations.

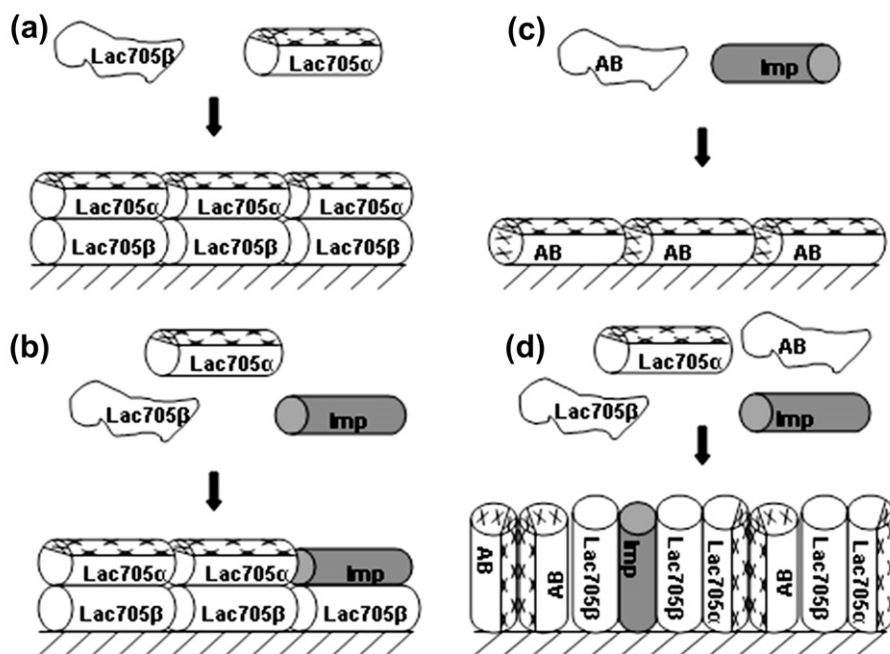
$2.2 \pm 0.1$ ), (Table 4). In addition, no influence of S-705 on AB adsorption was observed, since the obtained relative inhibition area ( $2.1 \pm 0.2$ ) was similar ( $P \geq 0.05$ ) to that of the adsorption from P-AB alone ( $2.1 \pm 0.2$ ).

### 3.3.3. Results rationalization

From the obtained results (Figs. 5 and 6 and Table 4) a rationalized scheme was carried out (Fig. 7) representing the different bacteriocins adsorption processes, on the basis of previously reported interactions between Class II a and b bacteriocins and biological membranes (Castellano et al., 2007; Drider et al., 2006; Nissen-Meyer et al., 2010). A conformational change of lac705β

peptide during the hydrophobic interaction with the multilayer-LLDPE film (Fig. 7a) and a further interaction between this adsorbed peptide and the impurities from C-Sac7, P-AB and CE might have been occurred (Fig. 7b). Thus, a lack of lac705β and lac705α peptides complementation necessary for antimicrobial activity may be suggested, this leading to a decrease of lactocin 705 activity on the multilayer-LLDPE film. Conformational changes upon protein adsorption on hydrophobic surfaces have been earlier reported (Norde et al., 1986; Roach et al., 2005). Moreover, from a previous study reporting the interaction between S-705 and a lipid bi-layer, important conformational reorganization was observed for lac705β; while lac705α interacted with the interfacial region inducing dehydration, lac705β peptide interacted with the hydrophobic core of the bi-layer (Castellano et al., 2007).

For AB adsorption on the multilayer-LLDPE, similar relative inhibition areas were obtained regardless impurities and lactocin 705 presence. Due to its strong antilisterial activity, AB produced by *L. curvatus* CRL705 is believed to belong to class IIa bacteriocins, which are small single-molecule peptides in contrast to class IIb (two-component bacteriocins). From the results, it may be suggested that AB adsorb on multilayer-LLDPE film surface and no further interactions with impurities occur during adsorption (Fig. 7c). In addition, since no differences in relative inhibition areas were observed when P-AB was adsorbed in combination with S-705, no changes in the number of AB molecules neither adsorbed alone nor in the presence of lactocin 705, may be suggested. Therefore, AB adsorption with its long axis parallel to the surface might have been occurred, thus covering the surface with a number of molecules (Fig. 7c). In the presence of S-705, no changes in the number of AB adsorbed molecules would be expected if a rearrangement to a perpendicular orientation were produced, hence lactocin 705 would adsorb on the uncovered surface, maintaining multilayer-LLDPE film AB antimicrobial activity (Fig. 7d). Similar results were reported for fibrinogen adsorption on a hydrophobic surface which in an initial stage adsorbed with its long axis parallel to the surface and then, due to high protein concentration on the



**Fig. 7.** Scheme to allow visualization of bacteriocins adsorption from different sources. (a) lactocin 705 from S-705; (b) 705 in the presence of impurities (Imp); (c) AB in the presence of Imp and (d) 705 in the presence of AB and Imp. X and white parts of the graphs denote respectively hydrophilic and hydrophobic parts of the peptides. For a detailed explanation see text.

surrounding medium, rearrangement of the protein to a perpendicular orientation occurred allowing further protein molecules to adsorb on the uncovered surface (Roach et al., 2005).

#### 4. Conclusions

From the antagonist effect of temperature on lactocin 705 activity and adsorption, 60 min and 30 °C were established as optimal conditions for bacteriocin adsorption on the multilayer-LLDPE film. A Langmuir-type treatment allowed determining lactocin 705 active adsorbed mass. Different bacteriocin sources were characterized and compared regarding their ability to adsorb on the film producing inhibitory activity against food pathogen involving anti-*Listeria* activity. Impurities generated during growth of *L. curvatus* CRL705 and CRL1579, used as bacteriocin-producers, strongly influenced the adsorption and antimicrobial activity of lactocin 705 on the multilayer-LLDPE film, while no evidence of their effect was found for AB adsorption. These results were rationalized and an adsorption mechanism was proposed for the bacteriocins, from which lack of lac705 $\beta$  and lac705 $\alpha$  peptides complementation necessary for lactocin 705 antimicrobial activity, while no interactions among impurities and AB during adsorption was proposed. AB activity on the film was not influenced by lactocin 705 adsorption; conformational reorganization of adsorbed AB in the presence of 705 could allow the adsorption of both bacteriocins while maintaining antilisterial antimicrobial activity.

The study developed in our work contributes to the understanding of bacteriocins adsorption and interactions with metabolites that could negatively affect the adsorption process, decreasing antimicrobial activity on the film. The awareness of these interactions could help to understand the film performance in contaminated food, which is part of current work. Further studies (e.g. circular dichroism) should give detailed information on conformational changes upon adsorption to LLDPE surface of the multilayer film.

The bacteriocins obtained after *L. curvatus* CRL705 growth in MRS medium may be used to be adsorbed to multilayer-LLDPE films offering a promising and simple alternative for anti-*Listeria* packaging development.

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