Characterization of the effects on the growth kinetics of *Listeria monocytogenes* in solid culture in contact with caseinate base edible films added with antilisterial activity from *Streptococcus* sp. ABMX isolated from *Pozol*, an indigenous Mexican beverage

[4]Mendoza-Mendoza, B., [1]Rodríguez-Hernández, A.-I., [1]Vargas-Torres, A., [2]Díaz-Ruiz, G., [3]Montiel, R., [3]Ramos-Aboites, H.–E., [5]Castro-Rosas, J. and [1*]Chavarría-Hernández, N.

[1]*Cuerpo Académico de Biotecnología Agroalimentaria. Instituto de Ciencias Agropecuarias, Universidad Autónoma del Estado de Hidalgo. Av. Universidad km 1, Rancho Universitario, Tulancingo de Bravo, Hidalgo, C.P. 43600, MEXICO*

[2]*Departamento de Alimentos y Biotecnología. Facultad de Química, Universidad Nacional Autónoma de México. Cd. Universitaria, México D.F., C.P. 54000, MEXICO*

[3]*Langebio, CINVESTAV-IPN. Km. 9.6 Libramiento Norte, Carretera Irapuato-León. Irapuato, Guanajuato, C.P. 36821, MEXICO*

[4]*Instituto Tecnológico Superior del Oriente del Estado de Hidalgo, Carretera Apan-Tepeapulco Km. 3.5, Las Peñitas, Apan, Hidalgo, C.P. 43909, MEXICO*

[5]*Centro de Investigaciones Químicas, Instituto de Ciencias Básicas e Ingeniería, Universidad Autónoma del Estado de Hidalgo, Centro Universitario, Carretera Pachuca-Tulancingo Km. 4.5, Mineral de la Reforma, Hidalgo, C.P. 42183, MEXICO*

Abstract

The antilisterial activity of edible films that contained 4% w/v sodium caseinate, 30% w/v glycerol, dry-based, and different amounts of antimicrobial substances (AM) produced by *Streptococcus* sp. ABMX, a lactic acid bacterium isolated from Mexican *Pozol*, was characterised. The AM were incorporated into the films as concentrated supernatant of culture broths, giving concentrations expressed as gentamicin, for the control-film, treatment 1 (T1), T2, T3 and T4 of 0, 0.5, 0.7, 0.9 and 1.25 $mg_{gentamicin}/g_{film}$, respectively. *Listeria monocytogenes* was grown in Oxford agar in contact with the tested films, at 35°C during 72 h, rendering the following ranges for the colony growth kinetic parameters: 19<colony lag phase, λ_c (h)<∞; 0<maximum specific colony growth rate, $\mu_{c\text{-}max}$ (h^{-1})<0.74, and 0<maximum biomass accumulation [proportional to the maximum value (colony growth area at any time)/(initial colony growth area) ≡ $(A/A_0)_{max}$ (dimensionless)]<460. There were important differences among treatments, being the *L. monocytogenes* growth more affected as the AM concentration increased within the films. Best antilisterial results were obtained in T4 condition, where *L. monocytogenes* could not grow.

Introduction

The use of both biobased edible films and coatings has been proposed as reliable alternatives to reduce the deterioration rate of processed foods which would be driven by oxygen and vapour interchange, biochemical reactions and microbiological growth, among other factors (Bourtoom, 2008). Regarding microorganisms, they are very important since can affect the quality of foods by inducing changes in flavour, colour, odours and texture due to spoilage organisms; even, food can play the role of vector for pathogens (v. gr., *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus*) or the produced toxins, with the consequent great health concern and economic implications

for the companies. In this sense, the food industry is interested in the availability of food packaging materials with convenient mechanical and gas transfer properties, biodegradability, eatable and harmlessness, as well as incorporating additional functions like the antimicrobial activity. This is the case of the biopolymer-based edible films incorporating the antimicrobial activity from different sources, being these the polymers themselves like chitosan (Fernández-Saiz, 2012), plant/fruit extracts (Deng and Zhao, 2011; Ponce *et al*., 2008), whole microorganisms (Gialamas *et al*., 2010), microbial products like bacteriocins (Cao-Hoang *et al*., 2010) or combinations among them (Razavi-Rohani *et al*., 2011; Basch *et al*., 2012). There are reports concerning the elaboration and characterization of

*Corresponding author.
Email: norberto@uaeh.edu.mx
Tel.: +52 (771) 7172000, ext 2425

edible films, based on different biopolymers, which incorporates bacteriocins to give the antimicrobial function to the system, also testing the antimicrobial activity against different indicator microorganisms (Bastarrachea *et al.*, 2010). Specifically, concerning investigations on the use of functional-edible films with activity against *L. monocytogenes*, there are an important number of published papers, where most of the research groups did evaluate the antilisterial effects in terms of the variations in the concentrations of listeria-colony forming units (i.e., CFU/g, CFU/mL or CFU/cm^2) (Ko *et al.*, 2001; Kristo *et al.*, 2008; Cao-Hoang *et al.*, 2010) or by measuring the inhibition zones of the bacterium growth in diffusion assays in agar (Li *et al.*, 2006; Jin and Zhang, 2008; Min *et al.*, 2010; Pintado *et al.*, 2010; Bastarrachea *et al.*, 2010), depending on the treatments in different systems. Nonetheless, according to our knowledge there are no reports where the antilisterial effects of functional-edible films are evaluated in terms of the impacts on the growth kinetic parameters of *L. monocytogenes*, and more specifically within the solid culture which is a frequent and very important case for many food systems (i.e., cheese (Cao-Hoang *et al.*, 2010); frankfurters (Ercolini *et al.*, 2006); poultry (Min *et al.*, 2010), among others).

Based on the previous information, the present work deals with the characterization of the antimicrobial activity of edible films against *L. monocytogenes* as indicator microorganism, through microphotographic-recording of the *L. monocytogenes* surface-colony growth in Oxford agar plates in depth contact with the films, evaluating the antimicrobial effects in terms of the colony-growth kinetic parameters (i.e., colony lag phase, maximum specific colony growth rate, and maximum biomass concentration). The films were elaborated based on sodium caseinate with the incorporation of antimicrobial substances produced by a lactic acid bacterium (LAB, *Streptococcus* sp. ABMX) isolated from a Mexican traditional fermented food (i.e., Pozol from maize; Díaz-Ruiz *et al.*, 2003). Many efforts are necessary to have a really strong ecosystem-friendly food-packaging material industry which can work together with the petroleum based plastic industry, that besides continue supplying a great extent of the food packaging materials required in the food industry (Marsh and Bugusu, 2007; US-EPA, 2012).

Materials and Methods

Specimens

A lactic acid bacterium (LAB-ABMX) previously isolated by the group of C. Wacher, FQ-UNAM, Mexico, from *Pozol*, a Mexican traditional fermented beverage. This gram positive-LAB was analysed by the 16S rDNA sequence methodology to be identified (Escalante *et al.*, 2001). Amplification of 16S gene was done by colony PCR, using pF27 (5'-AGAGTTTGATCMTGGCTCAG-3') and pR1492 (5'-TACGGYTACCTTGTTACGACTT-3') primers. Amplified DNA was purified with PureLink PCR kit (Invitrogen) and sequenced in both strands using amplification primers. After discarding low quality bases at both ends, forward and reverse strands were aligned to obtain a 1436 bp consensus sequence that was submitted to GeneBank. The sequence was submitted to the classifier application (Wang et al., 2007). 16S homologous sequences were retrieved from GeneBank (*http://www.ncbi.nlm.nih.gov/*) and SILVA (*http://www.arb-silva.de/*) databases. The final dataset comprised 48 16S sequences (Table 1). An alignment considering RNA secondary structure was constructed with Aligner at SILVA. The best-fit model of nucleotide substitution was selected with jModelTest 2 (Darriba *et al.*, 2012). The selected model was used for Maximum Likelihood phylogenetic reconstruction using PhyML (Guindon and Gascuel, 2003; Tamura *et al.*, 2011). Robustness of the phylogeny was assessed by the bootstrap method with 100 pseudoreplications.

On the other hand, previous work (Tavera-Montes, 2010) have shown that LAB-ABMX strain is a bacterium that consistently exhibits important antimicrobial activity against some pathogenic microorganisms in plaque assays, being *L. monocytogenes* one of them. The LAB-ABMX strain was conserved at -70°C in blends of 20% glycerol and 80% LAB-ABMX-culture broth in BHI (Fluka), 24 h old, 35°C. *L. monocytogenes* (culture collection FQ-UNAM) was used as the indicator organism. It was conserved at -70°C in blends of 20% glycerol and 80% listeria-culture broth in BHI (Fluka), 24 h old, 35°C.

Production of antimicrobial activity-concentrated supernatant from culture broth (AMC)

A volume of 50 μL was taken from a LAB-ABMX conservation vial to be inoculated into 5 mL of MRS broth (BD Difco) and incubated during 42 h at 30°C. Then, this culture broth was used to inoculate fresh MRS (inoculation ratio, 1%) which was then incubated at 30°C in orbital agitation fashion (130 rpm) during 24 h. Thereafter, the culture broth was centrifuged (5,000 rpm, 4°C, 30 min) to separate the cells. The supernatant was collected; then pH adjusted to 6.5-7.0, and concentrated into a vacuum concentrator

Table 1. Sequences used for phylogenetic reconstruction of a lactic acid bacterium (LAB-ABMX) isolated from *Pozol*, a Mexican traditional fermented food

Species/strain	Accession number
Streptococcus agalactiae	HV111801
Streptococcus alactolyticus	EU728776
Streptococcus anginosus SK52	AFIM01000033
Streptococcus anginosus 1_2_62CV	ADME01000009
Streptococcus australis ATCC 700641	AEQR01000024
Streptococcus bovis MPR1	AY324610
Streptococcus bovis MPR2	AY324611
Streptococcus cristatus ATCC 51100	AEVC01000028
Streptococcus equinus W29	AB563245
Streptococcus equinus W2	AB563226
Streptococcus gallolyticus UCN34	FN597254
Streptococcus hyointestinalis	EU728763
Streptococcus infantarius infantarius ATCC BAA-102	ABJK02000017
Streptococcus infantarius infantarius CJ18	CP003295
Streptococcus infantis ATCC 700779	AEVD01000030
Streptococcus lutetiensis clone PP060	JN713319
Streptococcus lutetiensis strain 844	EU16347
Streptococcus macedonicus	Z94012
Streptococcus pasteurianus ATCC 43144	AP012054
Streptococcus pasteurianus 035	JN581989
Streptococcus peroris ATCC 700780	AEVF01000016
Streptococcus pyogenes ATCC 10782	AEEO01000001
Streptococcus pyogenes MGAS10394	CP000003
Streptococcus salivarius M18	AGBV01000004
Streptococcus salivarius 57.I	CP002888
Streptococcus sanguinis SK340	AFQB01000012
Streptococcus sanguinis SK355	AFFN01000033
Streptococcus suis ST1	CP002651
Streptococcus suis D12	CP002644
Streptococcus thermophilus LMD-9	CP000419
Streptococcus thermophilus ND03	CP002340
Streptococcus urinalis 2285-97	AEUZ02000001
Streptococcus vestibularis	AY188353
Streptococcus sinensis strain HKU4	AF432856
Streptococcus pneumoniae	AF003930
Streptococcus constellatus strain ATCC27823	AF104676
Streptococcus anginosus strain ATCC33397	AF104678
Streptococcus gordonii	AF003931
Streptococcus ursoris	AB501126
Streptococcus dentapri	AB469560
Streptococcus dentirousetti	AB259061
Streptococcus phocae	AJ621053
Streptococcus dysgalactiae ATCC 43078	AB002485
Streptococcus iniae ATCC29178	AF335572
Streptococcus mutans ATCC 25175	AY188348
Streptococcus orisuis	AB182324
Enterococcus avium	AF133535

(BÜCHI R-215) until 75% of the present water was evaporated. Finally, the concentrated supernatant (AMC) was pasteurised at 110°C with vapour during 10 min, and stored at -20°C until use.

Determination of the antimicrobial activity

Prior to define the examined AMC concentrations within films, it was characterised the AMC antilisterial activity by the well-diffusion assay (Mendoza-Mendoza, 2012; Tavera-Montes, 2010). Taking into account that listeria species are frequently inhibited by gentamicin, especially those strains isolated from food samples (Zamora *et al.*, 2006), it was firstly determined the listeria growth inhibition as a function of gentamicin concentration (Eq. 1) by well-diffusion assays in BHI-agar plates.

$$y = a + b \cdot \left(1 - e^{-b \cdot x}\right) \qquad (1)$$

In Eq. 1, y = Δ(*listeria*-growth-inhibition radius), mm, and x = (gentamicin mass), mg; a, b and c are regression parameters. The experimental data were fitted to the model (Eq. 1) by non-linear regression using the SigmaPlot 11.0 software package. Thereafter, when tested the antilisterial activity of AMC samples, the recorded growth inhibition radius was correlated to the corresponding gentamicin mass that yielded the same extent of listeria inhibition, based on Eq. 1 (i.e., the AMC antilisterial activity was expressed as gentamicin concentration).

AMC-film elaboration

Each 100 mL-basal filmogenic solution contained 4 g sodium caseinate (Lacbase tonat EN-US Lactoprot) and 1.2 mL glycerol (ACS, Reactivos Química Meyer) as plasticizer. The caseinate was dissolved in water with constant agitation at lab temperature during 1 h. Then, glycerol was added until homogenization. At this point the previously defined volumes of AMC were added to give the desired final antimicrobial concentrations in the tested films, involving four treatments: T1, 1.6; T2, 2.24; T3, 2.88 and T4, 3.84 mL$_{AMC}$ per 20 mL of filmogenic solution. Each filmogenic solution was homogenized at 11,000 rpm during 5 min (Polytron-Aggregate Luzerrerstrasse 147 CH-6014 Littau/luzern); then they were subjected to a reduced pressure to eliminate air bubbles in a vacuum chamber during 30 min. After that, samples of 20 mL were casting in 10 cm × 10 cm moulds, to be dried in a chamber with a HEPA filtered air flow during 24 h (Purifier® Delta® Series Class II, Type A2 LABCONCO). Then, the films were turned out to be conditioned within a desiccator during 48 h at 50-55% RH and 25°C. The final average thickness of the films was 0.09 mm (standard deviation, SD, 0.02 mm) measured with a digital micrometer (Truper, Mexico) and exhibited an oxygen-permeability, PO$_2$, from 2.27 to 13.2 (10^{-14} g$_{O2}$ m s^{-1} Pa^{-1} m^{-2}) at 25°C, depending on the treatment. The PO$_2$ values were determined through a VAC-V2 permeability tester (Labthink, China).
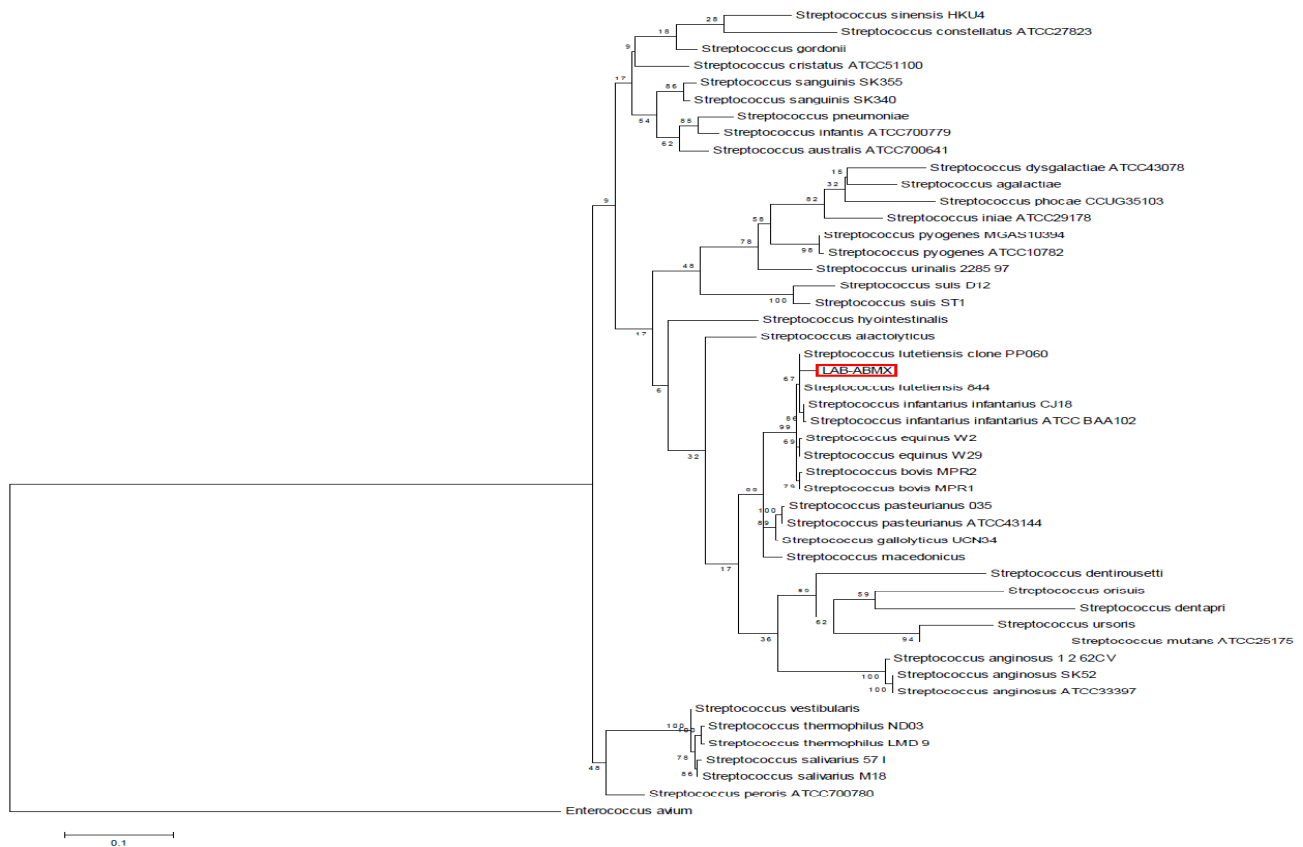
Figure 1. Maximum Likelihood phylogeny of 16S *Streptococcus* sequences, reconstructed using the HKY+I+G substitution model (Tamura *et al.*, 2011). The sequence of *Enterococcus avium* was used as the outgroup to root the phylogeny. Accession numbers of the sequences used are listed in Table 1. Numbers in branches are bootstrap values. LAB-ABMX, lactic acid bacteria tested in this study.

Determination of AMC-films' effects on the growth of Listeria monocytogenes in agar plates

Firstly, a 50 µL sample was taken from a listeria conservation vial to be inoculated in 5 mL of BHI broth and incubated during 24 h at 35°C. Then, 50 µL of this culture broth were transferred into 5 mL of fresh BHI to be incubated during 6 h at 35°C. Thereafter, decimal dilutions of this culture broth were done to take 600 µL of the proper dilution that were thoroughly spread onto the surface of Oxford agar plates (BD Difco) giving 60 CFU per Petri dish. Once the liquid was well absorbed into the agar, the surface was aseptically covered with an AMC-film (2 dishes each treatment) and incubated at 35°C. The tested films were previously surface sterilized with UV radiation during 24 h (12 h each side) in a safety cabinet (Purifier® Delta® Series Class II, Type A2 LABCONCO).

In each Oxford agar plate in depth contact with an AMC-film, the growth of five well isolated-listeria colonies, randomly selected, was monitored under the light microscope (Nikon Eclipse 80i), taking photographs every two hours during 72 h to calculate the area (A) of each monitored colony with the software package Motic images 2000 1.3. The
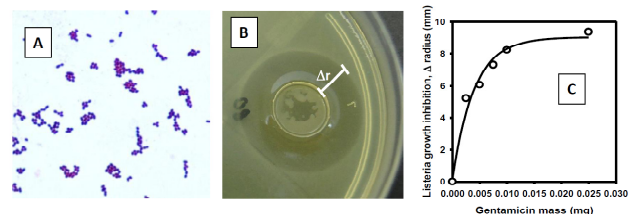

Figure 2. A) Microphotograph of the gram positive-lactic acid bacterium (LAB), *Streptococcus* sp. ABMX, originally isolated from Mexican *Pozol*, which produces antimicrobial substances that were then incorporated into caseinate films (magnification, 1000×). B) Well-diffusion assay showing *Listeria monocytogenes* growth inhibition due to the activity of the concentrated fermentation broth (AMC) of the tested LAB-ABMX. BHI-agar plates incubated at 35°C during 24 h; 80 µL of AMC to diffuse. It is shown a radii variation, Δr, of 9.36 mm. C) Listeria-growth-inhibition as a function of the gentamicin mass in well-diffusion assays (○); Best fit (—) to Eq (1),

$$y = 0.27 + 8.79 \cdot \left(1 - e^{-248.58 \cdot x}\right), \quad R^2 = 0.98.$$

A values were considered to be proportional to the present listeria biomass per colony at each sampling time. Then, the following growth kinetic parameters of *L. monocytogenes*-colonies were determined: a) colony lag phase (λ_c, h) (time when the colonies were first observed). b) Maximum specific colony growth
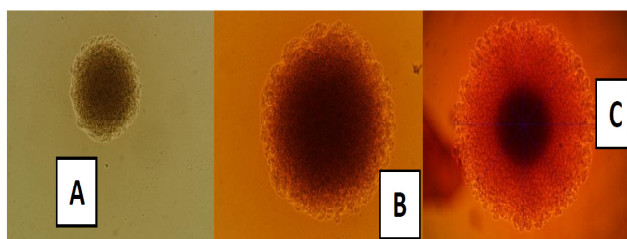
Figure 3. Light microphotographs of a *Listeria monocytogenes* colony growing in Oxford agar with no film at different incubation times: a) 24 h (magnification, 100×), b) 33 h (magnification, 100×) and c) 52 h (magnification, 40×). The colony growth kinetics would be determined on the basis of the colony-size evolution.

rate ($\mu_{c\text{-max}}$, h^{-1}) which is the maximum value of the function (Eq. 2),

$$\mu_c = \frac{d(A/A_0)}{dt} \times \frac{1}{(A/A_0)} \quad (2)$$

in the exponential growth region of the plot $(A/A_0) = f(t)$, where A_0 is the corresponding A value to a colony when it is first observed, and t is time (h). c) The maximum listerial biomass that was accumulated within each monitored colony was considered to be proportional to the corresponding maximum (A/A_0) value recorded (i.e., $(A/A_0)_{max}$, dimensionless).

Results and Discussions

Firstly, the 16S sequence of the LAB-ABMX strain, a bacterium isolated from Mexican *Pozol*, was classified as belonging to the *Streptococcus* genus by classifier (Wang *et al.*, 2007) with 100% probability. Homologous 16S sequences were retrieved and used to reconstruct a Maximum Likelihood phylogeny (Figure 1). The position of LAB-ABMX in this tree is well supported by bootstrap analysis and indicates LAB-ABMX strain might belong to *S. lutetiensis* species. However, the branch length of LAB-ABMX also leaves open the possibility that this strain would represent a new species or subspecies for which additional research would be carried out. That is why we do refer to the used LAB specimen as *Streptococcus* sp. ABMX.

Furthermore, the obtained concentrated supernatants of culture broth fermentations (AMC) conducted by *Streptococcus* sp. ABMX in BHI exhibited important antilisterial activity involving growth inhibition zones till $\Delta r = 9.36$ mm beyond the well diameter in 24 h old-cultures of *L. monocytogenes* in BHI agar at 35°C (Figure 2 B); in fact, it was found that 1 mL AMC has the same inhibitory effect against listeria as 0.3125 mg$_{gentamicin}$ according to the observed growth inhibition of listeria

Table 2. Kinetic parameters of the *Listeria monocytogenes* colony growth in Oxford medium, during the evaluation of the antimicrobial activity of caseinate films which contained antimicrobial substances (AM) produced by *Streptococcus* sp. ABMX, a lactic acid bacterium isolated from Mexican *Pozol*.

Treatment[a]	Colony lag phase, λ_c (h)	Maximum specific colony growth rate, $\mu_{c\text{-max}}$ (h^{-1})	Maximum biomass accumulation per colony [proportional to maximum (A/A_0) value $((A/A_0)_{max}$, dimensionless)]
B-SP	19	0.77 (0.07)[c]a[d]	1592.6 (684.1)a
B-CP	19	0.74 (0.08)a	459.8 (172.7)b
T1	35	0.16 (0.05)b	9.1 (3.3)c
T2	35	0.10 (0.02)c	3.3 (0.6)d
T3	44	0.09 (0.03)c	3.0 (0.6)d
T4[b]	-	-	-

[a]Key: (B-SP) plaque with no film. Plaques with film: (B-CP) film with no AM; T1 (0.5 mg$_{gentamicin}$/g$_{film}$); T2 (0.7 mg$_{gentamicin}$/g$_{film}$); T3 (0.9 mg$_{gentamicin}$/g$_{film}$) and T4 (1.25 mg$_{gentamicin}$/g$_{film}$).
[b]T4 treatment did not present any listeria colony growth during the experiments.
[c]Standard deviation is indicated in parenthesis.
[d]Different letters (a, b, c and d) in columns indicate significant differences among treatments, based on a t_0 test statistic, on means of normal distributions, variance unknown, $\alpha = 0.05$ (Montgomery, 2001)
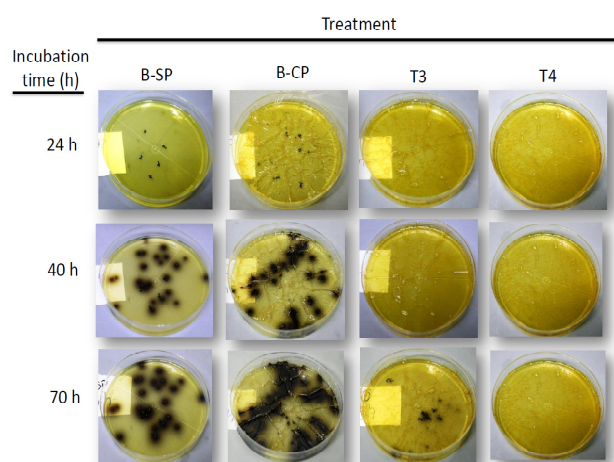

Figure 4. Aspect of the growth of *Listeria monocytogenes* in Oxford agar at 35°C and different times, in contact with caseinate films that incorporate antimicrobial substances (AM) produced by *Streptococcus* sp. ABMX isolated from Mexican *Pozol*. Treatment key: (B-SP), plaque with no film; (B-CP), film with no AM; (T3) and (T4), films with 0.9 and 1.25 mg$_{gentamicin}$/g$_{film}$, respectively.

in presence of gentamicin in well-diffusion assays in BHI agar (Figure 2 C). Therefore, considering the AMC antilisterial activity, in the rest of the paper the used AMC-quantities were expressed in milligrams of gentamicin (i.e., 3.2 mL$_{AMC}$ = 1 mg$_{gentamicin}$).

Antilisterial activity of the AMC-films

In the present investigation it was evaluated the antilisterial activity of edible films, based on the assessment of the colony growth kinetics of *L. monocytogenes* by recording the growth evolution of listeria-colonies in Oxford agar without any films as well as in depth contact with films with/without AMC coming from the *Streptococcus* sp. ABMX-fermented culture-broth (Figure 3). On the other hand, Figure 4 exhibits some aspects of the colony growth of *L. monocytogenes* in Oxford agar, in depth contact with some of the tested films in the present
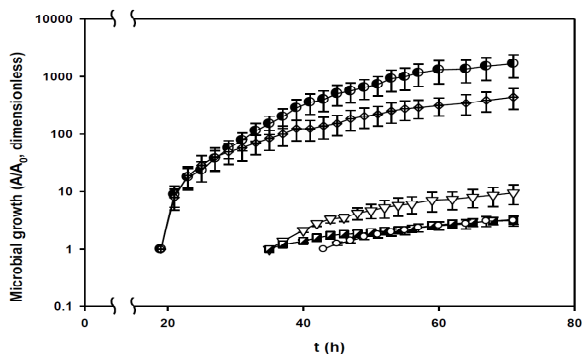
Figure 5. *Listeria monocytogenes*-colony growth curves in Oxford agar at 35°C, in contact with caseinate films that incorporate antimicrobial substances (AM) produced by *Streptococcus* sp. ABMX, isolated from Mexican *Pozol*. Key: (●) plaque with no film. Plaques with film: (◆) film with no AM; (▽) T1 (0.5 $mg_{gentamicin}/g_{film}$); (■) T2 (0.7 $mg_{gentamicin}/g_{film}$) and (○) T3 (0.9 $mg_{gentamicin}/g_{film}$). T4 did not exhibit any listeria growth. The error bars represent the standard deviation of 10 data (5 colonies/ plaque×2 plaques each condition).

work, where there were important differences among the effects of the four treatments examined (i.e., T1, 0.5; T2, 0.7; T3, 0.9 and T4, 1.25 $mg_{gentamicin}/g_{film}$). The bacterium growth was better in both Oxford plaques with no films (i.e., B-SP) as well as with films without any antimicrobial substances from *Streptococcus* sp. ABMX (i.e., B-CP). Nonetheless, *L. monocytogenes* grew with some difficulty when AMC was incorporated within the caseinate films (v. gr. in T3 (0.9 $mg_{gentamicin}/g_{film}$) and T4 (1.25 $mg_{gentamicin}/g_{film}$) listeria had not yet exhibited any growth at t = 40 h and t = 70 h, respectively).

Figure 5 presents the colony growth curves of *L. monocytogenes* in Oxford medium for all the studied conditions in this work. It is observed that for B-SP and B-CP conditions, *L. monocytogenes* colonies exhibited a lag phase λ_c = 19 h, while the corresponding values to T1 and T2 (i.e., 0.5 and 0.7 $mg_{gentamicin}/g_{film}$, respectively) were both equal to 35 h, whereas treatment T3 (i.e., 0.9 $mg_{gentamicin}/g_{film}$) was associated with a value of 45 h. Furthermore, in T4 condition (i.e., 1.25 $mg_{gentamicin}/g_{film}$) there was not detected any growth of the indicator bacterium during the 72 h that the experiment lasted. In Table 2 there is presented the kinetic parameters corresponding to *L. monocytogenes* colony growth in Oxford agar for all the conditions here studied.

The colony lag phase (λ_c) indicates the period that takes listeria cells to be adapted to a certain medium before the colony growth starts as a consequence of the cell reproduction. According to the obtained results, the λ_c value increased when the antimicrobial concentration within films increased too, suggesting that the presence of substances

produced by *Streptococcus* sp. ABMX, can hinder the listeria reproduction at least during 72 h at the tested conditions. In the cases where no AMC was present in the films (i.e., B-SP and B-CP conditions), λ_c values were the minimum (i.e., 19 h). On the other hand, λ_c was the maximum ($\lambda_c \to \infty$) when the AM concentration was the maximum as well (i.e., T4 treatment, 1.25 $mg_{gentamicin}/g_{film}$).

Concerning the results for *L. monocytogenes*-maximum specific colony-growth rate (μ_{c-max}) it is observed a tendency for this kinetic parameter to decrease when the antimicrobial substances concentration does increase within the caseinate films. The μ_{c-max} values for the conditions B-SP and B-CP were 0.77 and 0.74 h^{-1}, respectively, without significant differences. Nonetheless, the mean μ_{c-max} value when no AMC was present (i.e., (0.77+0.74)/2 = 0.76 h^{-1}) was notably higher than the corresponding ones calculated for T1, T2 and T3, being several times greater (i.e., (0.76/0.16) = 4.72, (0.76/0.10) = 7.55 and (0.76/0.09) = 8.39 times, that of T1, T2 and T3 μ_{c-max} values, respectively). The determination of the μ_{c-max} value is of high importance because it indicates how fast the listeria colonies are growing as a result of the cell reproduction rate depending on the environmental conditions. In this work, the maximum μ_{c-max} value was exhibited in B-SP condition (i.e., 0.77 h^{-1}), and the minimum one was calculated in T3 treatment (i.e., 0.1 h^{-1}) where the antimicrobial substances concentration was 0.9 $mg_{gentamicin}/g_{film}$. Moreover, as it has been previously mentioned, at higher AM concentrations there was no listeria-colony growth (i.e., T4=1.25 $mg_{gentamicin}/g_{film}$). In this sense, conditions in T4 treatment would be in such a way that no growth of listeria was allowed; even, they would be killed due to the actual AM concentration in T4 films.

Afterwards, the *L. monocytogenes*-maximum concentration was estimated for all tested conditions, considering that this kinetic parameter is proportional to the dimensionless value $(A/A_0)_{max}$. Both the maximum and minimum values for the parameter $(A/A_0)_{max}$ determined in this work, were 1593 and 3 (-) corresponding to B-SP condition and T3 treatment, respectively (without considering T4, where there was no listeria growth), involving important differences among $(A/A_0)_{max}$ values for the studied treatments. The $(A/A_0)_{max}$ value for B-SP condition was (1593/460) = 3.5 times the corresponding one for the B-CP condition, and this important difference would be due to oxygen limitations in the B-CP condition for the listeria growth, where molecules of molecular oxygen, in order to reach the Oxford agar-film interface, should travel across the film, 0.09

mm in length of a solid material (i.e., the film) with a low oxygen permeability value (i.e., $P_{O_2} = 4.24 \times 10^{-14}$ $g_{O_2}/(s \, Pa \, m^2)$) from the high oxygen partial pressure zone (i.e., gas space within the Petri dishes) to the low oxygen partial pressure region (i.e., Oxford agar-film interface) where listeria is growing. At the end, listeria grew more efficiently in B-SP condition where there was no oxygen transfer resistance due to any caseinate film. In the past some authors have reported the direct relationship between oxygen availability and listeria growth. Noriega *et al.* (2008) have found that *L. innocua*, growing in BHI broth at 25°C, achieved 2.5×10^9 CFU/mL in aerobic conditions in a period of 24 h, in contrast with the 1×10^9 CFU/mL obtained in anoxic conditions in the same time, involving a ratio of 2.5/1 = 2.5 which is relatively similar to the value 3.5 that was here obtained in solid processes that lasted 72 h.

Furthermore, the $(A/A_0)_{max}$ value for B-CP condition was notably higher than the corresponding ones determined for T1, T2 and T3, being (459.8/9.1) = 50.5, (459.8/3.3) = 139.3 and (459.8/3) = 153.3 times, respectively. Taking into account that both thickness and PO_2 values of the films were approximately constant among them, consequently there would be similar conditions concerning the oxygen availability for the *L. monocytogenes* development among the film systems studied. Having this in mind, the observed differences among $(A/A_0)_{max}$ values in the tested films would rely on the presence of antilisterial substances produced by *Streptococcus* sp. ABMX.

On the other hand, taking into account that the AMC used for film formulations was previously subjected to both pH adjustment and heat treatment, it would be hypothesized that the AMC's antimicrobial activity does not rely on the action of organic acids (i.e., lactic acid) nor enzymes, and it would be explained by the presence of certain bacteriocins or similar heat resistant substances produced by the bacterium *Streptococcus* sp. ABMX. In this sense, some bacteriocins like those of Class II, Subclase IIa, are in fact thermostable peptides with the specific conserved N-terminal sequence Tyr-Gly-Asn-Gly-Val-X-Cys, where X denotes any amino acid. Usually, they contain from 37 (i.e., leucocin A and mesentericin Y105) to 48 (i.e., carnobacteriocin B2 and enterocin SE-K4) amino acids and exhibit high activity against *Listeria* spp. (De Vuyst and Leroy, 2007; Stoyanova *et al.*, 2012). Furthermore, some authors have already found that members of the *Streptococcus* genus are able to produce pediocin-like molecules (Heng *et al.*, 2007). In this sense, nowadays our group is conducting some experiments to separate and identify the biochemical nature of the antimicrobial substances present in AMC from *Streptococcus* sp. ABMX.

As it was already mention, in the literature authors have tested different approaches to evaluate the antimicrobial activity of bacteriocin-added-edible films against *L. monocytogenes*. For example, Li *et al.* (2006) reported the antimicrobial effects of konjac glucomannan films with chitosan and nisin, against some indicator bacteria, including *L. monocytogenes*, by measuring the diameters of inhibition zones induced by film-disks ($10^3 \, IU_{nisin}/g_{film}$) placed on plates previously inoculated with the tested bacterium. After 1 day incubation at 37°C, they reported listeria inhibition zones ranging from 19 to 34 mm. On the other hand, Kristo *et al.* (2008) examined the effects of caseinate films with one of three antimicrobials agents, including nisin (0.05 and 0.50 mg/film), against *L. monocytogenes*. The films were placed on listeria surface inoculated Tryptose soy agar-3% NaCl plates and incubated at 10°C for 10 days. These authors aseptically removed the agar to determine the listeria viable count (CFU/cm²), founding lag phase values up to 5 days with maximum listeria counts of 5.5 log CFU/cm² at the end. In contrast, their controls did not exhibit any lag phase and achieved 8.5 log CFU/cm² at 10 days. It is evident the effectiveness of edible-films with bacteriocins to affect the listeria development in different extents in many investigations (Ko *et al.*, 2001; Padgetti *et al.*, 2000; Sanjurjo *et al.*, 2006; Kristo *et al.*, 2008; Basch *et al.*, 2012). Nevertheless, none of these authors did present their microbiological data on the basis of the listeria growth kinetics effects in response to the presence of edible films with antilisterial substances, involving solid-solid interface applications (i.e., film-agar interface, film-meat interface, film-food interface, etc.). This is in fact the main interest of the present contribution. Furthermore, the antilisterial activity of caseinate films with antimicrobial substances produced by *Streptococcus* sp. ABMX studied in the present work was outstanding. Moreover, it was used a concentrated culture-broth supernatant (AMC) with no further purification processing. At the present time, our group is also studying the antilisterial effects of caseinate films with antimicrobials produced by *Streptococcus* sp. ABMX, at lower temperatures (4°C) as well as in certain processed foods previously inoculated with the indicator bacterium.

Conclusion

It is first published the characterisation of the antimicrobial effects of edible caseinate films added with culture broth of *Streptococcus* sp. ABMX,

against *Listeria monocytogene*s growing within the film-Oxford agar interface at 35°C, on the basis of the effects on the listeria colony growth kinetics which did exhibit important changes in response to the specific treatment. The determination of all kinetic parameters (colony lag phase, λ_c; maximum specific colony growth rate, μ_{c-max}, and maximum biomass accumulation [proportional to $(A/A_0)_{max}$]) was useful to estimate the magnitude of the inhibitory effects against *L. monocytogenes*. As a general tendency, when the antimicrobial substances (AM) concentration increased, the λ_c value increased too, whereas both values of μ_{c-max} and $(A/A_0)_{max}$ decreased. Besides, the used antimicrobial substances produced by *Streptococcus* sp. ABMX, isolated from Mexican *Pozol*, exhibited good antilisterial activity which was effectively incorporated into the edible films tested in this investigation.

Acknowledgments

References

Basch, C. Y., Jagus, R. J. and Flores, S. K. 2012. Physical and antimicrobial properties of Tapioca starch-HPMC edible films incorporated with nisin and/or potassium sorbate. Food and Bioprocess Technology doi: 10.1007/s11947-012-0860-3.

Bastarrachea, L., Dhawan, S., Sablani, S. S., Mah, J.-H., Kang, D.-H., Zhang, J. and Tang, J. 2010. Biodegradable poly(butylene adipate-co-terephthalate) films incorporated with nisin: Characterization and effectiveness against *Listeria innocua*. Journal of Food Science 75(4): E215-E224.

Bourtoom, T. 2008. Edible films and coatings: characteristics and properties. International Food Research Journal 15(3): 237-248.

Cao-Hoang, L., Chaine, A., Grégoire, L. and Waché, Y. 2010. Potential of nisin-incorporated sodium caseinate films to control *Listeria* in artificially contaminated cheese. Food Microbiology 27(7): 940-944.

Darriba, D., Taboada, G. L., Doallo, R. and Posada, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9(8):772-772.

De Vuyst, L. and Leroy, F. 2007. Bacteriocins from lactic acid bacteria: Production, purification, and food applications. Journal of Molecular Microbiology and Biotechnology 13(4):194-199.

Deng, Q. and Zhao, Y. 2011. Physicochemical, nutritional, and antimicrobial properties of wine grape (cv. Merlot) pomace extract-based films. Journal of Food Science 76(3):E309-E317.

Díaz-Ruiz, G., Guyot, J. P., Ruiz-Teran, F., Morlon-Guyot, J. and Wacher, C. 2003. Microbial and physiological characterization of weakly amylolytic but fast-growing lactic acid bacteria: a functional role in supporting microbial diversity in Pozol, a Mexican fermented maize beverage. Applied and Environmental Microbiology 69(8): 4367-4374.

Ercolini, D., Storia, A., Villani, F. and Mauriello, G. 2006. Effect of a bacteriocin-activated polythene film on *Listeria monocytogenes* as evaluated by viable staining and epifluorescence microscopy. Journal of Applied Microbiology 100(4): 765-772.

Escalante, A., Wacher, C. and Farrés, A. 2001. Lactic acid bacterial diversity in the traditional Mexican fermented dough pozol as determined by 16S rDNA sequence analysis. International Journal of Food Microbiology 64: 21-31.

Fernández-Saiz, P. 2012. Chitosan and Chitosan Blends as Antimicrobials. In Lagarón, J. M., Ocio, M. J. and López-Rubio, A. (Eds). Antimicrobial Polymers, p. 71-99. Hoboken, New Jersey: John Wiley & Sons, Inc.

Gialamas, H., Zinoviadou, K. G., Biliaderis, C. G. and Koutsoumanis, K. P. 2010. Development of a novel bioactive packaging based on the incorporation of *Lactobacillus sakei* into sodium-caseinate films for controlling *Listeria monocytogenes* in foods. Food Research International 43(10): 2402-2408.

Guindon, S. and Gascuel, O. 2003. A Simple, fast, and accurate algorithm to estimate large phylogenies by Maximum Likelihood. Systematic Biology 52(5):696-704.

Heng, N. C. K., Wescombe, P. A., Burton, J. P., Jack, R. W. and Tagg, J. R. 2007. The Diversity of Bacteriocins in Gram-Positive Bacteria. In Riley, M. A. and Chavan, M. A. (Eds). Bacteriocins. Ecology and Evolution, p. 45-92. Heidelberg: Springer-Verlag.

Jin, T. and Zhang, H. 2008. Biodegradable polylactic acid polymer with nisin for use in antimicrobial food packaging. Journal of Food Science 73(3): M127-M134.

Ko, S., Tanes, M. E., Hettiarachchy, N. S. and Johnson, M. G. 2001. Physical and chemical properties of edible films containing nisin and their action against *Listeria monocytogenes*. Journal of Food Science 66(7): 1006-1011.

Kristo, E., Koutsoumanis, K. P. and Biliaderis, C. G. 2008. Thermal, mechanical and water vapor barrier properties of sodium caseinate films containing antimicrobials and their inhibitory action on Listeria monocytogenes. Food Hydrocolloids 22(3): 373-386.

Li, B., Kennedy, J. F., Peng, J. L., Yie, X. and Xie, B. J. 2006. Preparation and performance evaluation of glucomannan–chitosan–nisin ternary antimicrobial blend film. Carbohydrate Polymers, 65(4): 488-494.

Marsh, K. and Bugusu, B. 2007. Food packaging-roles, materials, and environmental issues. Journal of Food Science 72(3): R39-55.

Mendoza-Mendoza, B. 2012. Caracterización de películas a base de caseinato de sodio, con la inclusión de sustancias antimicrobianas producidas por bacterias ácido lácticas aisladas de pozol. Tulancingo de Bravo, Hidalgo, Mexico: Universidad Autónoma del Estado de Hidalgo, Tesis de Maestra en Ciencia de los Alimentos.

Min, B. J., Han, I. Y. and Dawson, P. L. 2010. Antimicrobial gelatin films reduce *Listeria monocytogenes* on turkey bologna1. Poultry Science 89(6): 1307-1314.

Montgomery, D. C. 2001. Design and analysis of experiments. 5th edn. NY, USA: John Wiley & Sons, Inc.

Noriega, E., Laca, A. and Díaz, M. 2008. Modelling of diffusion-limited growth to predict *Listeria* distribution in structured model foods. Journal of Food Engineering 87(2): 247-256.

Padgetti, T., Han, I. Y. and Dawson, P. L. 2000. Effect of lauric acid addition on the antimicrobial efficacy and water permeability of corn zein films containing nisin. Journal of Food Processing and Preservation 24: 423-432.

Pintado, C. M. B. S., Ferreira, M. A. S. S. and Sousa, I. 2010. Control of pathogenic and spoilage microorganisms from cheese surface by whey protein films containing malic acid, nisin and natamycin. Food Control 21(3): 240-246.

Ponce, A. G., Roura, S. I., del Valle, C. E. and Moreira, M. R. 2008. Antimicrobial and antioxidant activities of edible coatings enriched with natural plant extracts: *In vitro* and *in vivo* studies. Postharvest Biology and Technology 49(2): 294-300.

Razavi-Rohani, S. M., Moradi, M., Mehdizadeh, T., Saei-Dehkordi, S. S. and Griffiths, M. W. 2011. The effect of nisin and garlic (*Allium sativum* L.) essential oil separately and in combination on the growth of *Listeria monocytogenes*. LWT - Food Science and Technology 44(10): 2260-2265.

Sanjurjo, K., Flores, S., Gerschenson, L. and Jagus, R. 2006. Study of the performance of nisin supported in edible films. Food Research International 39(6): 749-754.

Stoyanova, L. G., Ustyugova, E. A. and Netrusov, A. I. 2012. Antibacterial metabolites of lactic acid bacteria: Their diversity and properties. Applied Biochemistry and Microbiology 48(3): 229-243.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony methods. Molecular Biology and Evolution 28(10): 2731-2739.

Tavera-Montes, F.-L. 2010. Producción de bacteriocinas a partir de bacterias ácido lácticas aisladas de pozol para la inhibición de bacterias patógenas. D.F., Mexico: Universidad Nacional Autónoma de México, Tesis de Químico de Alimentos.

US-EPA 2012. Plastics. Downloaded from *http://www.epa.gov/osw/conserve/materials/plastics.htm* on 2/21/2013.

Wang, Q., Garrity, G. M., Tiedje, J. M. and Cole, J. R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73(16): 5261-5267.

Zamora, J. M., Chaves, C. and Arias, M. L. 2006. Comparación del perfil de sensibilidad a antibióticos de cepas de *Listeria monocytogenes* y *Salmonella* spp. aisladas a partir de alimentos con cepas de origen clínico. Archivos Latinoamericanos de Nutrición 56: 171-174.