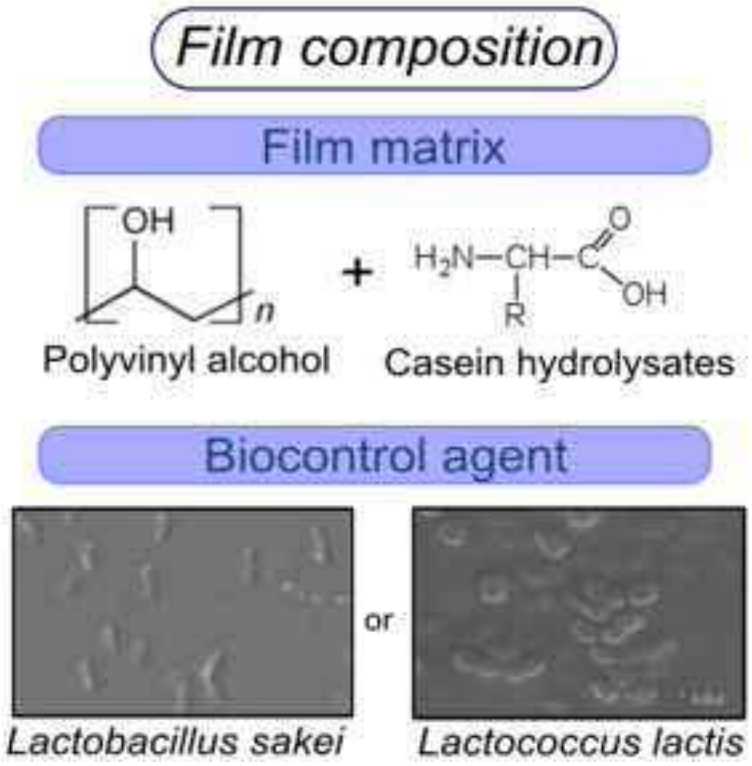
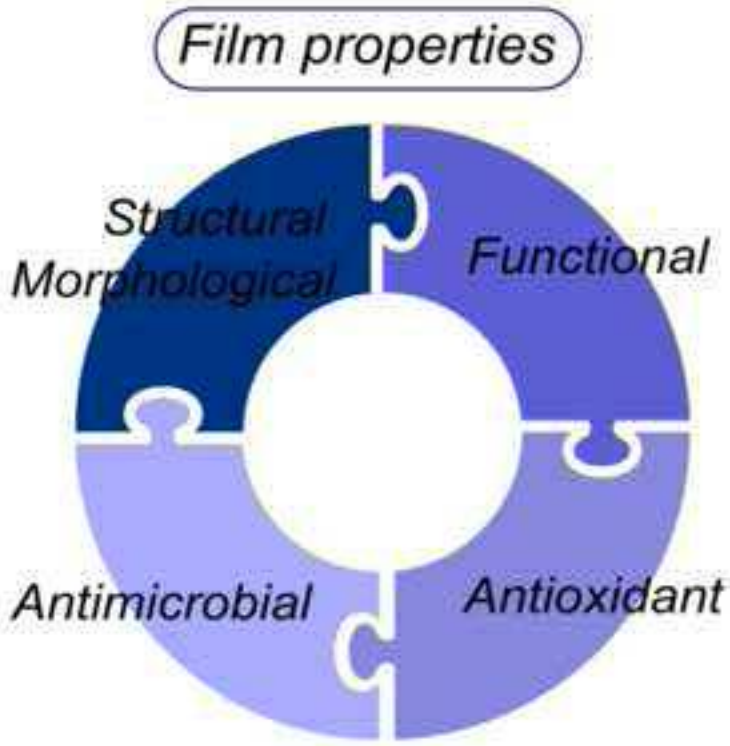
## Highlights

- PVOH films blended with HCas and lactic acid bacteria were successfully developed
- HCas increased cell viability during film drying and in long- term storage
- HCas modified functional properties of PVOH films when blended in a 1:1 mass ratio
- Films exerted antilisterial activity which improved incorporating HCas
- Prepared formulations can be used as films or coating for carrying biocontrol agents



# Effect of casein hydrolysates on the survival of protective cultures of *Lactococcus lactis* and *Lactobacillus sakei* in PVOH films

Laura Settier-Ramírez; Gracia López-Carballo; Rafael Gavara; Pilar Hernández-Muñoz*

Packaging Lab, Instituto de Agroquímica y Tecnología de Alimentos, IATA-CSIC, Av. Agustín Escardino 7, 46980 Paterna, Spain.

*Corresponding author: phernan@iata.csic.es

## ABSTRACT

The aim of this work has been to explore the potential of blending polyvinyl alcohol (PVOH) with casein hydrolysates (HCas) to obtain self-standing films capable to act as carriers of lactic acid bacteria (LAB) as biocontrol agents against food pathogens. For this purpose, PVOH was blended with HCas at different weight ratios and the blends were incorporated with *Lactococcus lactis* and *Lactobacillus sakei*. Blending HCas with PVOH resulted in the modification of some functional properties of the films whereas bacteria did not change them. Moreover, incorporation of HCas resulted in an increase in cell viability after film casting and in long-term film storage, and also in film antilisterial properties. These results could be related to the capacity of bacterial autoaggregation in the films during the drying process when HCas was added, as observed by fluorescence light microscopy. Blends could be used in the active packaging of foods.

Keywords: lactic acid bacteria, biocontrol agents, anti-Listeria films, casein hydrolysates, polymer matrices, bacterial auto-aggregation.

## 1. Introduction

The use of bacteriocins to design active packages to control the growth of foodborne patogens has been greatly explored in the last years. However, antimicrobial packaging based on the use of bacteriocin producing bacteria as biocontrol agents has not been deeply explored. The use of bacteria instead of their bacteriocins presents several advantages. In this regard, commercial bacteriocins preparations have a high price due to the low fermentation yields and high production costs (Musatti et al., 2020), and some bacteriocins are not classified as GRAS.

It is known that foodborne pathogens and spoilage organisms can lose viability during growth in associative cultures with lactic acid bacteria (LAB), which in most of the cases is attributed to the production of bacteriocins and also other antimicrobial compounds

such as organic acids and hydrogen peroxide that help to increment the antimicrobial effect; in addition, bacteria also compete with other bacteria that can cause spoilage in foods or being pathogens (De Vuyst & Leroy, 2007).

The incorporation of LAB as protective cultures into films and coatings is recent and a deeper understanding of the dependency between LAB viability and antimicrobial activity, and the film composition, processing and storage is required (Guimarães et al., 2018). Until now, most of the studies are based on the use of water soluble biopolymers which are incorporated with low molecular compounds with the aim of acting as nutrients or protective agents for bacteria (Bekhit et al., 2018; Gialamas et al., 2010; Guimarães et al., 2018; Ye et al., 2018a). However, the use of water soluble Polyvinyl alcohol (PVOH) has been little explored.

PVOH is a water soluble biodegradable and synthetic polymer with excellent film forming properties. PVOH is widely used in the industry due to its emulsifying and adhesive properties, having an excellent mechanical strength and flexibility. Contrary to water soluble films made from biopolymers, PVOH films are stable during storage without altering their physico-chemical properties; that can be a great advantage for its industrial application as carrier of protective cultures for active food packaging purposes. PVOH is approved by the FDA for use in food contact and as a food additive with INS n°.1203 (Codex alimentarius) (FAO, 2018). In the EU, PVOH is approved by the EFSA as a food additive in food supplements in accordance with Annex II to Regulation (EC) No 1333/2008. The studies focused on the use of PVOH as carrier of protective cultures for active packaging are scarce. Only a couple of works related to the use of PVOH coatings as carriers of antilisterial producing bacteriocin are documented in the bibliography (Degli Esposti et al., 2018; Iseppi et al., 2011).

In previous studies, the authors of the current work have reported that PVOH is capable of maintaining the viability of *L. lactis* and its antilisterial properties. They also found that these properties are improved when a low percentage of proteins or protein hydrolysates are incorporated in the film formulation (Settier-Ramírez et al., 2019). Thus, it is worthy

to optimize the formulation of PVOH incorporated with protein hydrolysates to improve the antimicrobial effectivity of the films without altering some PVOH functional properties that are of great importance when used in the design of food packages.

Therefore, the aim of this work has been to study the effect of incorporating different amounts of casein hydrolysates in PVOH matrices on the viability and antilisterial properties of two LAB strains producers of bacteriocins, nisin-producer *Lactococcus lactis* and sakacin-producer *Lactobacillus sakei* in different testing conditions. The structural and morphological properties of the blend films have been correlated with the microbiological results obtained. Moreover, some important functional properties of the resulting films for its use in the design of antimicrobial food packages (moisture absorption, optical, and mechanical properties) were assayed.

## 2. Materials and methods

### *2.1. Bacterial strains*

*Lactococcus lactis* subsp. *lactis* (CECT 539, ATCC 11454) supplied by the Spanish Type Culture Collection (CECT) and *Lactobacillus sakei* subsp. *sakei* (ATCC 15521) kindly supplied by M. Rollini from *Università degli Sudi* from Milan, were stored in Man, Rogosa, and Sharpe (MRS) broth supplemented with 20 % glycerol at −80 °C. The microbial cultures were re-generated and maintained by regular subcultures at 4 °C on MRS broth. An aliquot from the cultures was subcultured by overnight incubation in 10 mL of MRS prior to the experiments.

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

84 *2.2. Determination of the minimum inoculum of L. lactis and L. sakei active against L.*
85 *monocytogenes*
86 The minimum initial inoculum of *L. lactis* and *L. sakei* able to reduce the microbial growth
87 of *L. monocytogenes* under refrigerated storage in liquid culture medium was adapted
88 from previous work (Laura Settier-Ramírez et al., 2019). *L. lactis* cells were harvested
89 by centrifugation at 2,500 RCF for 15 min at 4 °C and washed twice with peptone water.
90 Then, they were suspended in TSB with 0.3 % of yeast extract (TSB + YE). Appropriate
91 dilutions were made in order to inoculate tubes with 10 mL of TSB + YE with
92 concentrations ranging from 9 to 6 log CFU/mL. After that, all the tubes were inoculated
93 with 4 log CFU/mL of *L. monocytogenes*. The same procedure was repeated with *L.*
94 *sakei*. Three control tubes for each bacterium were prepared to evaluate their growth
95 without any influence.
96 The tubes were stored at 4 °C during 13 days. Aliquots were taken immediately after
97 bacteria inoculation, and after 1, 2, 3, 6, 8, 10 and 13 days of storage. Serial dilutions
98 with peptone water were made and plated in Petri dishes with Polymyxin Acriflavine
99 Lithium Chloride Ceftazidime Aesculin Mannitol agar (PALCAM agar) to study
100 logarithmic reduction of *L. monocytogenes*, and also in MRS to study growth of *L. lactis*
101 and *L. sakei* in contact with *L. monocytogenes*. MRS agar plates were incubated at 30
102 °C during 4 days and PALCAM plates were incubated at 37 °C for 48 h, respectively.
103 After the incubation time, *L. lactis* and *L. sakei* colonies were counted in MRS agar and
104 *L. monocytogenes* colonies were counted in PALCAM agar. Tests were carried out in
105 triplicate.
106
107 *2.3. Film formation*
108 Polyvinyl alcohol (PVOH, Gohsenol GH17, Nippon Synthetic Chemical Company,
109 Osaka, Japan) was used as the polymer matrix for the preparation of the films. Film
110 forming solutions (FFS) were prepared in distilled water dissolving PVOH and
111 incorporating casein hydrolysates (HCas, Peptone from casein, enzymatic digest,

Sigma-Aldrich, France) in a mass ratio of 1:0, 1:0.125 and 1:1 (w:w) obtaining a final concentration of dry solids of 2 % (w/w) in all the cases. *L. lactis* and *L. sakei* cells were harvested by centrifugation at 2,500 RCF for 15 min at 4 °C and washed twice with peptone water. Then they were incorporated into the different FFS in order to obtain 7 log CFU/mL (selected for the results observed in the above section). Next, 15 g of each FFS were cast in Petri dishes (90 mm of diameter) and dried at 20 °C under the air flow of a biological safety cabinet (Biostar Plus, Telstar) for 24 h. Films were stored at 43.2 % RH and 20 °C for two weeks prior to be characterized. PVOH films and their blends with HCas without LAB were used as controls.

### *2.4. Functional properties of the films*

#### *2.4.1. Thickness*

A digital micrometer (Mitutoyo Manufacturing Co., Ltd., Tokyo, Japan) with a sensitivity of 1 μm was used to measure film thickness. Five measurements were taken randomly for each film sample and three samples of each film were measured.

#### *2.4.2. Moisture content*

Films of approximately 0.5 g were placed on aluminum plates and stored at 20 ºC in glass desiccators containing a saturated solution of potassium carbonate anhydrous and potassium acetate (Acros Organics, France) in order to obtain 43.2 % and 23.1 % RH respectively. After two weeks, the weight equilibrium was reached and samples were weighed and placed in desiccators with phosphorus pentoxide (Fluka, Sigma-Aldrich, France) for dehydration until reaching constant weight. Moisture content was calculated based in weight changes (Bittante et al., 2014). Tests were carried out in triplicate.

#### *2.4.3. Optical properties*

Color of the films was measured with a Konica Minolta CM-3500d spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan) set to D65 illuminant/10° observer was

used to determine the color of all the prepared films. The samples were measured against the surface of a standard white plate to acquire the color data and to display them in the CIELAB color space. The parameters L* [black (0) to white (100)], a* [green (−) to red (+)], and b* [blue (−) to yellow (+)] were obtained and the polar coordinates, the chroma C*, and the hue angle h° were calculated. Eight measurements of each sample were taken, and three samples of each film were measured.

A Perkin Elmer Lambda 16 UV–Visible spectrometer was used to obtain the absorption spectrum of the films in the wavelength range of 190–800 nm. The apparent opacity of the films was calculated as the area under the absorption curve (Au × nm) in the UV and visible wavelengths.

*2.4.4. Mechanical properties*

A Mecmesin MultiTest 1-í universal test machine (Landes Poli Ibérica, S.L., Barcelona, Spain) equipped with a 100-N static load cell was used to evaluate the maximum tensile strength ($\sigma_m$), percentage of elongation at break ($\varepsilon_b$) and Young's modulus ($E$) of the films. Films were cut into 25.4 mm × 130 mm strips and conditioned at 43.2 % RH and 20 °C for one week before testing. Sample Grip separation was set at 100 mm and cross-head speed at 25 mm/min. 12 replicates from each sample were tested.

*2.5. Structural and morphological properties of the films*

*2.5.1. Modulated differential scanning calorimetry (MDSC)*

Differential scanning calorimetry was performed using a DSC Q2000 (TA Instruments Inc., New Castle, DE, USA) equipped with Universal Analysis 2000 software. $N_2$ was used as the purge gas at a flow rate of 50 mL/min, and at a heating rate of 10 °C/min from - 50 °C up to 220 °C the modulation period was 60 s, and the amplitude of modulation was 0.32 °C. Temperature calibration of the instrument was performed with indium. Dry samples of approximately 5 mg were weighted in an Tzero aluminum pan and hermetically closed with a TZero lid (TA instruments), punctured three times, and

kept over $P_2O_5$ for two weeks prior to scanning. Experiments were carried out in three cycles to eliminate the thermal history of the samples. Thus, samples were cooled down to -50 ºC and after 2 min of equilibrium, they were heated at a constant 10 ºC/min rate up to 115 ºC, and equilibrated for 2, then cooled to -50 °C, 2 min of equilibrium and a second heating up to 220 ºC. Glass transitions temperature ($T_g$) was taken at the inflexion point of the transition the case of samples PVOH:HCas 1:1, $T_{gs}$ were recorded during the first heating since in this cycle the presence of the transitions of both polymers were observed. Melting temperatures ($T_m$) were taken at the minimum point of the endotherm at the third heating. Melting enthalpy was calculated using the TA Universal Analysis software. From melting enthalpy values, the crystalline fraction ( ) of PVOH blend films was calculated using the following equation:

(1)

where is the enthalpy for melting; is melting for a 100 % crystalline PVOH sample and $m_p$ the weight fraction of PVOH in the sample. The melting enthalpy of 100% PVOH was taken as 161.1 J/g (Faisant, J.B., Aït-Kadi, A., Bousmina, M., L. Deschenes, 1998).

*2.5.2. Scanning electron microscopy*

Cross-sectional images of polyvinyl alcohol films and their blends with casein hydrolysates without and with LAB were observed by scanning electron microscopy (SEM) using a HITACHI S-4100 unit equipped with a BSE AUTRATA detector and an EMIP 3.0 image capture system (HITACHI, Madrid, Spain). Prior to cross-sectional cut of the films, they were immersed in liquid nitrogen to obtain a perfect cut without mechanical damage of the area. Then, samples were mounted on a stub of metal with adhesive, and film surface and cross sectional area coated under vacuum with gold–palladium in a sputter coating unit. Images were captured at 5 kV.

197 *2.5.3. Fluorescence light microscopy*

198 The distribution of bacteria and casein hydrolysates in polyvinyl alcohol matrix was
199 observed using a Nikon Eclipse 90i fluorescence microscope.

200 Prior to the formation of the film, LAB were stained with DNA-intercalating agent DAPI
201 (4',6-diamidino-2-phenylindole, dihydrochloride, Sigma-Aldrich). 1 microliter of a solution
202 of 1 mg/mL DAPI was added to 1 mL of bacterial suspension and incubated for 10 min
203 at 20 °C in the dark. Then, stained LAB cells were resuspended in 10 mL sterile distilled
204 water. Cells were harvested by centrifugation at 2,500 RCF for 15 min at 4 °C and
205 washed twice with distilled water. Then, they were incorporated into the different FFS
206 and films were cast following the same methodology than in section 2.3. The films were
207 observed under a blue filter (DAPI was excited at the wavelength of 405 nm and the
208 emission filter was set at 420–460 nm).

209

210 *2.6. Antioxidant properties of the films*

211 Antioxidant activities of just made films and films stored for one month at 43.2 % RH and
212 20 °C were measured by the ABTS assay following the methodology described by López
213 De Dicastillo et al. (2013), this assay is based on the inhibition of the radical cation 2,2′-
214 azinobis(3-ethylbenzothiazoline-6-sulphonate), ABTS·+, which has a characteristic
215 wavelength absorption spectrum at 715 nm. When this indicator radical is neutralized by
216 an antioxidant substance, its absorption decreases. The percentage inhibition values
217 were calculated using this equation:

218

219 $$I\,(\%) = [(\text{Abs control} - \text{Abs sample})/\text{Abs control}] \times 100 \qquad (2)$$

220

221

222 For the determination, films were dissolved in 10 mL distilled water and were incubated
223 during 1 h with ABTS·+. Results were expressed as ABTS inhibition activity after 1 h
224 reaction.

225

226 *2.7. Microbiological studies of the films*

227 *2.7.1. LAB survival after film processing and in vitro* antilisterial *activity*

228 The survival of *L. lactis* and *L. sakei* after the film was formed, was evaluated according

229 to the equation:

230 — (3)

231 where "$N_0$" represents the number of viable bacteria in the film forming solution (FFS)

232 prior the drying process of the FFS and "N" is the number of viable bacteria in just made

233 films.

234 The bacterial viability in the FFS was evaluated placing 1 mL of each solution in tubes

235 with 10 mL of sterile peptone water. After homogenization, appropriate dilutions were

236 made. Counts of *L. lactis* and *L. sakei* were performed in MRS agar after incubation for

237 4 days at 30 °C. To determine bacteria viability after film drying, circular films of 90 mm

238 of diameter (0.3 g approx.) corresponding to different blend ratios were peeled off from

239 Petri dish after drying and placed in tubes with 10 mL of sterile peptone water. Then,

240 tubes were vigorously stirred at 20 °C until the film was dissolved and the

241 microorganisms released. Later, appropriate dilutions were made and it was proceeded

242 as described above. Tests were carried out in triplicate.

243 Antilisterial activity of just made films incorporating LAB was evaluated against *L.*

244 *monocytogenes,* the survival of LAB after being in contact with the pathogen during the

245 antimicrobial test was also studied. To this end, films of 90 mm of diameter were

246 removed from Petri dishes and immersed in tubes containing 10 mL of Tryptone Soy

247 Broth supplemented with 0.6 % of yeast extract (TSB+YE) which were previously

248 inoculated with 4 log CFU/mL of *L. monocytogenes*. Then, tubes were stored at 37 °C

249 for 24 h, after that time, serial dilutions with peptone water were made and plated in Petri

250 dishes with PALCAM agar and MRS agar. Culture conditions for both LAB and *L.*

251 *monocytogenes* were the same as previously described in section 2.2.

252

253 *2.7.2. In vitro evolution of* antilisterial *activity and growth of LAB after immersion of the*
254 *films in liquid culture medium at 4 °C*
255 The activity of the films against *L. monocytogenes* was evaluated in refrigerated liquid
256 culture medium through the time to simulate the active packaging of a liquid food using
257 the developed films; the survival of LAB in the refrigerated culture medium during that
258 time was evaluated at the same time. To carry out the experiments, films were immersed
259 in tubes with 10 mL of TSB+YE previously inoculated with 4 log CFU/mL of *L.*
260 *monocytogenes* and stored at 4 °C for 15 days. Aliquots were taken immediately after
261 film dissolution, and after 1, 3, 6, 9 and 15 days. Serial dilutions with peptone water were
262 made and plated in Petri dishes with PALCAM agar and MRS agar. Culture conditions
263 for both LAB and *L. monocytogenes* were the same as previously described in section
264 2.2. The tests were done in triplicate.
265
266 *2.7.3. Survival of* LAB *in long-term stored films*
267 To know the survival of bacteria in the films through storage, just made films were stored
268 at 20 °C for four weeks in desiccators conditioned with saturated salt solutions of
269 potassium acetate, and potassium carbonate for reaching relative humidities of 23.1 ±
270 0.3 % and 43.2 ± 0.3 %, respectively. The viability of *L. lactis* and *L. sakei* was evaluated
271 each week as mentioned above. Analyses were performed in triplicate.
272
273 *2.8. Statistical analysis*
274 One-way analyses of variance were carried out. The SPSS computer program (SPSS
275 Inc., Chicago, IL) was used. Differences in pairs of mean values were evaluated by the
276 Tukey b test for a confidence interval of 95 %. Data were represented as the average ±
277 standard deviation.
278
279 **3. Results and discussion**

280 *3.1. Determination of the minimum inoculum of L. lactis and L. sakei active against L.*
281 *monocytogenes*
282 When using LAB as protective cultures to develop antilisterial films for active packaging
283 it is essential to choose an adequate size of the inoculum to be incorporated in the film
284 and also to keep cell viability and antimicrobial properties after film processing.
285 Before the formation of the film, the minimum initial inoculum of *L. lactis* and *L. sakei*
286 necessary to inhibit *L. monocytogenes* was studied in liquid TSB+YE for 13 days at 4 ℃
287 simulating refrigerated storage of perishable foods. Inoculum sizes of 6, 7, 8 and 9 log
288 CFU/mL of *L. lactis* or *L. sakei* were assayed against 4 log CFU/mL of *L. monocytogenes*.
289 Figure 1a shows the evolution of the initial inoculum size of *L. lactis* or *L. sakei* when is
290 confronted with 4 log CFU/mL of *L. monocytogenes* in liquid TSB+YE for 13 days at 4
291 ℃. Independently of the size of the inoculum added to the liquid medium, the population
292 of *L. lactis* was slightly lower than that of *L. sakei*, that is attributable to small differences
293 in the amount of bacteria in the preculture, in any case, these differences were not
294 relevant. When inoculum sizes of 9 and 8 log CFU/mL of both LAB were assayed
295 (differences of +5 and +4 respect to 4 log CFU/mL of *L. monocytogenes*), both LAB had
296 the same growth, reaching values around 9 log for differences of +5, and 8 log CFU/mL
297 for differences of + 4. When the size of the inoculum was lower, 7 and 6 log CFU/mL
298 (differences of +3 and +2 respect to 4 log CFU/mL of *L. monocytogenes*), the growth of
299 *L. sakei* in the liquid medium was faster than for *L. lactis*. In fact, the former reached a
300 stationary phase of around 8 log CFU/mL after six days. However, when the inoculum
301 size was +3, *L. lactis* reached values of 7 log CFU/mL after eight days of storage, it took
302 ten days to reach this stationary phase for an inoculum size of +2.
303 Figure 1b shows the antilisterial activity of different inoculum sizes of *L. sakei* and *L.*
304 *lactis* evaluated at 4 °C for 13 days. All the inoculum sizes tested for both LAB exerted
305 antilisterial activity although was greater for *L. lactis*. The two bacteria strains assayed
306 are effective against Gram-positive bacteria mainly due to the generation of organic acids
307 and bacteriocins, which are products of their metabolism and also due to direct

competition for nutrients (De Vuyst & Leroy, 2007). In this study, TSB+YE is a liquid culture buffered medium so pH was maintained around 7 during all the storage. Thus, the antilisterial activity of *L. sakei* and *L. lactis* could be attributable to the bacteriocins produced. Indeed, *L. lactis* generates nisin, a bacteriocin classified as class I and *L. sakei* generates sakacin classified as class II (Carvalho et al., 2018; Deegan et al., 2006). The greater antilisterial activity of *L. lactis* can be attributable to the different effectiveness of each of the two bacteriocins. Both bacteriocins have a similar mode of action by destabilization of the plasmatic membrane of *L. monocytogenes* but they are not the same molecule. In the same way, the biosynthesis of *L. lactis* and *L. sakei* is not exactly the same (Ibarra-Sánchez et al., 2020; Mapelli et al., 2018). The rate of production of the bacteriocin considering the temperature and time of storage can also affect to the antimicrobial properties of the tested LAB.

The inoculum size of *L. lactis* and *L. sakei* is essential to obtain an effective antilisterial activity through the whole storage time as shown in Figure 1b. Regarding *L. lactis*, depending on the size of the inoculum used the antimicrobial properties changed considerably during the first 10 days of storage, the greater antimicrobial activity through this time was exerted by an inoculum size of 9 log CFU/mL, however, the antimicrobial activity was independent of the inoculum size after 13 days. Related to *L. sakei*, the antilisterial activity was greater for all the storage time when using inoculum sizes of 8 and 9 log CFU/mL. Therefore, a concentration of 8 log CFU/mL was chosen as inoculum size to incorporate in the film forming solution.

### *3.2. Functional properties of the films*

Previous studies carried out by the authors showed that 1:1 was the ratio with the greater concentration of casein hydrolysates (HCas) that being incorporated in polyvinyl alcohol (PVOH) matrix give homogeneous films with good visual appearance and handling. The addition of LAB did not modify the appearance of the films at the naked eye compared to that of PVOH films without bacteria (Figure S1 of Supporting Information).

Table 1 recompiles moisture content and optical properties of PVOH films and its blends with casein hydrolysate (HCas). As other authors pointed out in several studies with different film formulations, LAB incorporation did not change film properties such as thickness (Gialamas et al., 2010; Piermaria et al., 2015), color (Odila Pereira et al., 2016; Ye et al., 2018b), opacity (L. Settier-Ramírez et al., 2019; Soukoulis et al., 2014) or mechanical properties (Abdollahzadeh et al., 2018; Sánchez-González et al., 2014). The results are given for films without carrying bacteria since the tested properties did not alter after LAB incorporation. The thickness of PVOH films was not modified after addition of HCas, being the concentration of dried solids in the film forming solution the same for all the films prepared.

It is important to determine the moisture content (MC) of the hydrophilic films because it can affect the rate of viability of LAB after drying during long storage periods (Kanmani & Lim, 2013). MC of the films stored at 43.2 % and 23.1 % RH, and 20 °C are shown in Table 1. As expected, moisture absorbed by all the films was lower when they were stored at 23.1 % RH, and similar values were obtained for all the blending ratios, ranging from 3.8 for plain PVOH to 4.1 (g water/ 100 g of dry film) for 1:1 blends. When films were stored at a higher relative humidity, they absorbed more water and differences among compositions were more evident.

It is well known that optical properties of food packaging materials are important properties to be considered in packaging design since it has a great impact on the appearance of the package and their commercialization. Color parameters and opacity of the films are represented in Table 1. All the films presented high values of L*, close to the white plate, which indicates a high transparency. Indeed, PVOH is well known for its transparency. The addition of HCas did not modify L* parameter, even at 1:1 ratio which indicated the great compatibility of hydrolysates with PVOH.

Regarding film color, incorporation of HCas resulted in an increase in yellow color which provoked a decrease in the hue angle of the blends, whereas chromaticity of the films increased with the content of HCas. The tendency to yellowness when several proteins

such as whey protein isolate or gelatin hydrolysate protein were added in different film formulation was also reported by other authors (da Rocha et al., 2018; Gonzalez-Cuello et al., 2018; Nuanmano et al., 2015; Soukoulis et al., 2016). However, these differences were not appreciable to the naked eye.

Apparent opacity of the films was measured as the area under the absorbance curve in the visible region (400–800 nm) and in the middle and near UV region (190–400 nm). Low opacity values in the visible region were observed in all the films indicating the high transparency of films to the visible light. Nevertheless, some differences were found in the UV region from 190 to 400 nm. It can be noted that the more HCas was added, the more opacity was reported. It is known that proteins are a good barrier in the UV spectrum region. This is due to certain aromatic amino acids such as tryptophan, tyrosine, and phenylalanine present in HCas, which exerts a great light absorbance in the UV region. Absorbance of UV light by films could be considered as an advantage for packaged foods because they can decrease the undesirable chemical reaction like lipid oxidation that are of importance for maintaining bacterial viability (Ebrahimi et al., 2018).

Mechanical properties of the films are displayed in Table 2. It can be appreciated that casein hydrolysates act as plasticizers on PVOH films, decreasing tensile strength and Young´s modulus and increasing elongation of the films. Nevertheless, films still had good mechanical properties with higher strength when comparing with edible films made from proteins (Sánchez-González et al., 2013). Those results are in line with other authors that found the same behaviour in the mechanical properties of agar films when incorporating protein hydrolysates (da Rocha et al., 2018). In fact, other authors also reported that peptides with short chain can act as plasticizers reducing interactions between polymer chains, thus increasing the free volume between them and leading to a reduction of the tensile strength and Young´s modulus (Nuanmano et al., 2015). The incorporation of LAB did not have a significant effect on the mechanical properties of the films probably due to the relatively insignificant mass of cells added.

392

393 *3.3. Structural and morphological properties of the films*

394 *3.3.1. Thermal properties of the films*

395 Thermal properties of polyvinyl alcohol (PVOH) powder, casein hydrolysates (HCas),
396 and their blends in the form of cast films without incorporating or incorporating *L. lactis*
397 and *L. sakei* are shown in Table 3 whereas DSC thermograms showing thermal events
398 of the different materials, and of its blend films are depicted in Figures S2 to S5 of the
399 Supplementary Information. PVOH powder experienced a glass transition temperature
400 ($T_g$) at 54.1 °C, and the melting temperature ($T_m$) at 184.7 °C which is in line with the
401 results given in the literature (Tang & Alavi, 2011). Casein hydrolysates are amorphous
402 and only present a glass transition temperature at 73.1 °C (Figure S2). Cast PVOH films
403 and those blended with HCas at the ratio 1:0.125 displayed a unique $T_g$ around 68.2 °C
404 and 69.2 °C, respectively. However, two separate $T_{gs}$ were observed at 62.4 °C and 74.3
405 °C in films blended with HCas at 1:1 weight ratio which suggested the occurrence of
406 phase separation between PVOH and HCas. The $T_g$ related to PVOH phase at 62.4 °C
407 was slightly lower respect to plain PVOH films (Figure S3) which can be due to the
408 plasticizing effect of HCas.

409 The melting temperature of plain PVOH films was similar than that for PVOH powder,
410 although the percentage of crystallinity of the films ( %) suffered a slight decrease with
411 respect to the powder. The melting temperature of PVOH blended with HCas moved to
412 lower values, and the melting enthalpy and crystallinity of PVOH films incorporating
413 HCas decreased considerably as the content of protein hydrolysates in the matrix
414 increased. Therefore, inclusion of a great amount of low molecular weight hydrolysates
415 of casein disrupted crystallization of PVOH and also decreased the melting point of the
416 polymer which elucidates an increase in smaller less organised crystallites.

417 When *L. lactis* or *L. sakei* were added to the films, no relevant changes were found in
418 the thermodynamic properties of them (Figures S3-S5).

419

420 *3.3.2. SEM*

421 The morphology of the cross-section surface of polyvinyl alcohol (PVOH) films and their
422 blends with casein hydrolysates (HCas) was examined by SEM and showed in Figure
423 2. The fracture surface of plain PVOH films and of those blended with a small amount
424 of HCas (1:0.125 weight ratio) was uniform and smooth as shown in Figure 2 a) and
425 Figure 2 b) respectively. However, a less smooth cross-section surface with pores and
426 cracks was observed in films with a greater amount of HCas (1:1 weight ratio) as Figure
427 2 c) shows. This topography is due to separation of the blend components in two phases.
428 Meanwhile, the observed cracks are due to the mechanical damage caused when the
429 cross-sectional cut was made using freeze-fracture method in liquid nitrogen since 1:1
430 films did not have a brittle nature as reported when measuring mechanical properties.
431 The differences observed between 1:0.125 and 1:1 films indicate that when the content
432 of HCas in the blends is beyond a certain threshold, the blends are not miscible. SEM
433 observations support the conclusions obtained by means of thermal characterization of
434 the films, where two separate Tg were observed in 1:1 film and was attributed to a phase
435 separation between PVOH and HCas.
436 The cross-section surfaces of films carrying LAB were similar to films without
437 incorporating bacteria (results not shown). These findings along with the MSDC results
438 found for films carrying bacteria support that their incorporation into the films did not
439 modify their mechanical properties.
440

441 *3.3.3. Fluorescence Light microscopy*

442 Fluorescence light microscopy images of polyvinyl alcohol (PVOH) films and their blends
443 with casein hydrolysates (HCas) loaded with bacteria stained with DAPI are shown
444 in Figure 3. Pictures revealed that the arrangement of the entrapped bacteria in the film
445 matrix was different depending on the strain immobilized and the matrix formulation.
446 Regarding the distribution of *L. lactis* in plain PVOH films, dispersed bacterial clusters of
447 different sizes were observed (Figure 3 a). Bacteria clusters tend to agglomerate in large

448 aggregates of undefined shape with a high cell density when a small amount of casein
449 hydrolysates was added to PVOH films (Figure 3 b); when PVOH was blended with HCas
450 at the ratio 1:1, it was found bacterial aggregates of irregular shape and very different
451 sizes (Figure 3 c). In contrast, when *L. sakei* was added to PVOH films, the cells
452 distributed uniformly, and no clusters were observed (Figure 3 d). Small clusters of *L.*
453 *sakei* were formed in 1:0.125 films (Fig 3 e); increasing the amount of HCas in the ratio
454 1:1 gave rise to larger clusters (Figure 3 f). Doherty et al., (2010) studied cell
455 immobilization of *Lactobacillus rhamnosus* GG in native, denatured, and hydrolysed
456 whey protein isolate (WPI), reporting the formation of cell aggregates in hydrolysed
457 proteins together with higher values of cell survival. Many bacteria present the property
458 of auto-aggregate which has been related with a greater survival rate against
459 environmental stresses (Trunk et al., 2018). In the current study, it was observed that
460 after centrifugation of both bacteria, the auto-aggregation of *L. lactis* was much greater
461 than the auto-aggregation of *L. sakei*. Indeed, studies carried out with LAB have shown
462 that bacteria auto-aggregation is strain dependent (Gómez et al., 2016). This may
463 explain why in plain PVOH films small clusters of bacteria were observed when *L. lactis*
464 was added while they were not observed with *L. sakei*.
465 Several authors have found that bacteria strains with auto-aggregation ability present a
466 greater hydrophobic surface compared with autoaggregation-deficient bacteria (Nikolic
467 et al., 2010). Since casein hydrolysates have hydrophobic groups along the unfolded
468 peptidic chains, they are able to stablish hydrophobic interactions with apolar binding
469 sites present in the surface of LAB cells (Léonard et al., 2013). This “preference” for the
470 hydrophobic groups of the HCas instead of polar groups of hydrophilic PVOH may
471 explain clustering or/and aggregation by current bacteria when casein hydrolysates are
472 added to PVOH matrix.
473 Together with the ability of cells to auto-aggregate, the disruption of polymer-polymer
474 interactions in the structure of PVOH by HCas, and the decrease of film crystallinity could
475 also contribute to the auto-aggregation of cells during the processing of the films.

476 The drying time was also a key factor in the formation of these clusters, in the current
477 study when thinner films were prepared and dried in 30 min compared to 18 hours for
478 regular films, clusters were only observed for *L. lactis* immobilized in 1:1 films and were
479 much smaller in size than those observed above (supplementaryInformation, Figure
480 S6). In fact, auto-aggregation is a time dependent process characterized by the creation
481 of a network between the cells and further sedimentation (Arellano-Ayala et al., 2020).
482 The same authors demonstrated that auto aggregation follows a constant increase
483 through the time for all the strains studied (including LAB), reaching the maximum auto
484 aggregation values after 4 h of incubation at 23 °C. This fact is consistent with the current
485 results where no aggregates were found in the films dried in 30 minutes.
486
487 *3.4. Antioxidant properties of the films*
488 ABTS assay was selected to test the antioxidant properties of the films since the radical
489 and the films solubilize completely in water. ABTS radical scavenging results are shown
490 in Table 4. Plain polyvinyl alcohol (PVOH) films did not show ABTS radical scavenging
491 activity regardless the addition of LAB. However, when casein hydrolysates (HCas) were
492 added, the ABTS radical scavenging reaction increased considerably after 1 h reaching
493 almost a 100 % of scavenging for 1:1 blend films. Previous studies conducted by
494 ABTS·+ assay of different amino acids determined that cysteine followed by tryptophan,
495 tyrosine and histidine were the most active scavengers (Gómez-Ruiz et al., 2008). All of
496 those amino acids are present in HCas (Wang et al., 2013). These results are in line with
497 different works reporting the accessibility to the oxidant–antioxidant test systems is
498 greater for small peptides and amino acids than for large peptides and proteins (Gómez-
499 Ruiz et al., 2008; Re et al., 1999). In this case, casein hydrolysates also had the ability
500 to scavenge the ABTS radical cation by hydrogen or electron donation (Díaz & Decker,
501 2004).

As it can be seen in Table 4, no differences in ABTS radical scavenging were found between newly dried films and one-month stored films at 43.2 %. These results seem logical since no film degradation was produced at such low humidity and storage time. Finally, no differences in ABTS radical scavenging were found when LAB were added to the films. Other authors have reported that some LAB strains such as *L. lactis* or *L. sakei* are able to produce antioxidant exopolysaccharides (Bajpai et al., 2016; Guo et al., 2013). Nevertheless, those molecules are products of their metabolism and when LAB were entrapped in films at such low RH, they remain in a latency state (Laura Settier-Ramírez et al., 2021).

### *3.5. Microbiological studies of the films*

#### *3.5.1. LAB survival after film drying and in vitro antilisterial activity at 37 °C*

The viability of *L. lactis* and *L. sakei* in polyvinyl alcohol (PVOH) and their blends with casein hydrolysates (HCas) after film processing is displayed in Figure 4. The survival of LAB after being in contact with the pathogen during the antimicrobial assay and the antimicrobial activity of the films against *L. monocytogenes* after 24 h of incubation at 37 °C also are shown in the figure. In the present study, for the same film formulation and processing parameters, *L. lactis* presented greater viability values than *L. sakei*, being differences more accentuated in plain PVOH films where *L. lactis* had a viability of 77.53 % while that for *L. sakei* was 52.38 %.

The viability of lactic acid bacteria depends on factors that are intrinsic or inherent to the LAB strain and on extrinsic factors such as film processing parameters and composition. Related to extrinsic factors affecting cell viability, it has to be considered that many parameters can injure bacterial cells during film processing by casting. In this regard, the drying step is a critical factor for bacteria survival during film casting since surface proteins, cell wall and membrane can be damaged when bound water is removed during the evaporation of the solvent. As a consequence, desiccation can destabilize the structural integrity of cellular components which entails in loss or damage of cell function (Brennan et al., 1986). However, the parameters used in this work to cast the films, low

temperature for a prolonged time allows low rates of dehydratation and great percentage of bacteria survival after their encapsulation in a film. Therefore, film casting can be considered a low-aggressive technique for bacteria encapsulation in contrast to other more aggressive techniques such as spray drying where greater temperatures and short times are required (Carvalho et al., 2004, Meng et al., 2008).

Besides the drying process, the composition of the film plays a considerable role in the viability of encapsulated bacteria. In the current work, the incorporation of casein hydrolysates to the PVOH matrix increased the viability of both LAB. When HCas were added at the weight ratio 1:0.125 with respect to PVOH, the viability of *L. lactis* increased from 77.5 % to 89.8 %, and from 52.4 % to 78.6 % for *L. sakei.*

In the present study, it has been observed by fluorescence light microscopy that the incorporation of HCas in PVOH films promoted that *L. lactis* clusters observed in PVOH films tended to agglomerate in large aggregates, and *L. sakei* tended to form clusters when HCas were incorporated. Several authors have related high percentages of auto-aggregation with greater resistance to stresses such as acid, salts or dehydration. In the present study, the films with greater bacterial viability were those having a greater content of HCas and consequently, auto-aggregation.

Apart from HCas providing protection to the cells against dehydration by promoting cell auto-aggregation during the step of evaporation of the solvent in the processing of the film, HCas could also supply micronutrients and provide protection to the cells against the dehydratation step by additional ways. In that sense, it has been reported that amino acids and low molecular weight polymers can penetrate the cell wall providing protection to the cell during dehydratation (Carvalho et al., 2004, Meng et al., 2008). The molecular weight of commercial HCas used in this work is around 5 kDa (Laura Settier-Ramírez et al., 2020), therefore they could act in that way although this kind of study has not been the aim of this work.

The antimicrobial capacity of the resulting films was tested against *L. monocytogenes* in liquid medium (TSB+YE) after 24 h of incubation at 37 ℃ which is the optimal growth

558 temperature of the pathogen. TSB+YE tubes inoculated with *L. monocytogenes* without
559 film were used as control since it was previously proved that nor PVOH nor HCas had
560 influence on the growth of the pathogen. The survival of LAB in the culture medium was
561 also determined, and the results are displayed in Figure 4. When films entered in contact
562 with the liquid culture medium, they lost their integrity and readily dissolved, thus
563 releasing the carrying bacteria. The availability of nutrients and the amount of water
564 provided by TSB+YE promoted LAB growth reaching counts up to 8 log independently
565 of the film composition and the type of LAB. This fact proves that although both bacteria
566 had different viabilities, when films were immersed in enriched culture medium, they were
567 able to reach the stationary phase in 24 h. Those results are in accordance with previous
568 works where *L. lactis* population was recovered achieving the stationary phase in contact
569 with *L. monocytogenes* regardless of the initial inoculum using the same incubation time
570 and culture medium conditions (Laura Settier-Ramírez et al., 2019).
571 In general, the antilisterial activity of films carrying *L. lactis* was greater compared with
572 those carrying *L. sakei*, independently of the film composition. Regarding the effect of
573 the film composition on the antimicrobial activity, this increased when films were
574 supplemented with HCas, obtaining the best results when incorporating HCas at 1:1
575 weight ratio. However, only a slight increase in the antimicrobial capacity of the films was
576 observed for 1:1 blend films. Thus. only a small amount of HCas is enough to increase
577 the effectiveness of PVOH films when tested in liquid medium at 37 ℃.
578
579 *3.5.2. Evolution of the antilisterial activity of the films immersed in liquid medium for 15*
580 *days at 4 °C.*
581 The antilisterial activity of PVOH film and its blends with HCas carrying LAB was studied
582 in TSB+YE for 15 days at 4 °C in order to simulate the storage of a refrigerated liquid
583 food. The survival of LAB liberated from the films into the medium previously inoculated
584 with *L. monocytogenes* was also monitored during this time and the results are displayed
585 in Figures 5a and 5b. Figure 5a shows that in spite of *L. lactis* and *L. sakei* being

586 mesophiles they can growth at refrigeration temperatures since they have psychrotrophic
587 behavior as well as the mesophile *L monocytogenes*. However, the growth rate was
588 different depending on the bacteria. *L. lactis* incorporated in plain PVOH films or films
589 blended with HCas maintained their initial population through the whole storage time.
590 However, independently of the initial population of the bacteria and film formulation, *L.*
591 *sakei* only reached 8 log (CFU/mL) counts after 15 days of storage.

592 The antilisterial activity of the films is represented in Figure 5b. It can be observed that
593 this property increased for the two LAB assayed and for all the film combinations during
594 the first 9 days of storage. Films of PVOH blended with HCas and incorporating *L. lactis*
595 exhibited the greater antilisterial activity. After 9 days of refrigerated storage, reductions
596 of 7 logs were obtained for 1:1 PVOH:HCas films and around 6 log for 1:0.125
597 PVOH:HCas films, whereas plain PVOH films achieved reductions of 3 log after this time.
598 Regarding the antilisterial activity exerted by films carrying *L. sakei* after nine days of
599 storage at 4 °C, it was found that film composition had little effect on the antimicrobial
600 properties of the films, and plain PVOH and 1:0.125 blend films achieved reductions of
601 3 log, whereas reductions were of 3.5 log for 1:1 blend films.
602 In the next days of storage reductions began to decrease independently of the bacteria
603 strain and film composition except for PVOH film carrying *L. lactis*. One explanation for
604 this behaviour could be that, after nine days of storage nutrients in the medium are scarce
605 and LAB could decrease their active metabolism and production of bacteriocins.
606 Moreover, *L. monocytogenes* could tolerate better the refrigeration temperature used
607 when there is a lack of nutrients in the medium.
608 Correlating Figure 5a with Figure 5b, it can be appreciated that during the first 9 days of
609 storage there is a slower growth of *L.sakei* coming from PVOH films, whereas the
610 population of *L. sakei* corresponding to films incorporating HCas remained constant and
611 around 7 log through the first 9 days of storage. Regarding *L. lactis*, its population was
612 of 7 log for bacteria coming from plain PVOH films and 8 log for bacteria coming from
613 films incorporating HCas through the whole storage time (Figure 5a). At day 9 the

614 antilisterial activity exerted by plain PVOH films carrying *L. lactis* was the same than that
615 for plain PVOH carrying *L. sakei* and their blends with HCas. However, the antimicrobial
616 activity of PVOH blends incorporated with *L. lactis* was higher (Figure 5b). Thus, it is
617 observed that the greater survival of the cells in the films is accompanied by a superior
618 and rapid recovery of the cells in the refrigerated medium inoculated with *L.*
619 *monocytogenes* and also better antimicrobial activity.
620 These results correlate well with the results obtained when studying the distribution of
621 bacteria in the films by light fluorescence microscopy and concluding that cell auto-
622 aggregation promoted by HCas during the processing of the film, implies higher cell
623 viability and a slight increase of the antimicrobial activity evaluated at 37 °C after 24 h
624 (section 3.5.1). However, when the test is carried out at 4 °C for 15 days immersing the
625 films in the liquid medium at day zero, and monitoring bacterial population and antilisterial
626 capacity of the two LAB strains, it can be clearly appreciated that the evolution of the
627 population of each strain through the time is different and related to the film composition.
628 Moreover, the incorporation of HCas in PVOH films slightly affects the antilisterial
629 properties of films with *L. sakei* compared with films with *L. lactis*.
630
631 *3.5.3. Survival of LAB in long-term stored films*
632 When encapsulating bacteria in a polymer matrix, the knowledge of their survival
633 throughout the time of storage is essential in order to ensure its effectivity when are used.
634 The inactivation of LAB during storage is influenced by several factors such as
635 species/strain dependency, storage environmental conditions, water content in the
636 polymer matrix, presence of protective agents, and oxidative damage of the cells due to
637 oxygen permeation through the encapsulating polymer wall (Tripathi & Giri, 2014).
638 Therefore, the effect of storage time on cell viability of *L. lactis* and *L. sakei* encapsulated
639 in polyvinyl alcohol (PVOH), plain or blended with casein hydrolysates (HCas) and stored
640 at 23.1 % and 43.2 % RH and 20 ℃ for one month is depicted in Figure 6.

Cell viability in plain PVOH films, was reduced from 6.8 log to 5.3 log for *L. lactis* and from 4.5 log to 3.5 log for *L. sakei* after one more of storage at 22 % RH, whereas at 43.2 % RH the viability decreased in the same range but with more abrupt changes.

Residual moisture content in the film can allow in some degree biochemical and enzymatic reactions and metabolic activity. Since the storage environment does not allow reproduction, cells presenting a minor degree of metabolic activity would suffer natural death (Fu & Chen, 2011). In the present study, the water content in the films exposed to the two relative humidities studied is below to the water needed for LAB to start metabolic processes (Romano et al., 2014). Thus, PVOH films were able to sustain viable LAB more than 1 month with high levels of bacterial counts at the humidities studied. Compared with other polymers used to entrap LAB, PVOH is a polymer that achieves considerable maintenance of viability. Different authors have observed reductions from 4 to 5 logs in the viability of LAB immobilized in biopolymer films for a long-term storage (Ma et al., 2019; Ye et al., 2018; Sánchez-González et al., 2018). When PVOH was blended with HCas, the long-term viability of *L. lactis* and *L. sakei* improved considerably. PVOH films blended with HCas at the 1:1 ratio maintained the viability of *L. lactis* in 8 log CFU/ml without suffering any variation through the storage time independently of the environmental moisture used to store the films. This result also was observed for 1:0.125 blend films incorporating *L. lactis* although the viability values were slightly lower. Cell viability of *L. sakei* in 1:1 blend stored at 22.1 % RH also was maintained stable in 7 log, and the viability varied between 6.5 and 6 log for the blend 1:0.125 when films were stored at 22.1 % RH. Storing the films at 43.2 % RH gave rise to some viability variations but always ranging 6-7 log.

It can be stated from these results that long-term viability of *L. lactis* and *L.sakei* in the blends increased compared with that in plain PVOH films, and was higher and less affected by environmental moisture content for *L. lactis*. These findings can probably be attributed to the formation of aggregates in blends carrying *L. lactis* and clusters in blends incorporated with *L. sakei* as observed by fluorescence light microscopy. Cells can better

669 support time and some degree of humidity when auto-aggregation is greater as it has
670 been observed.
671 Oxidative stress is another factor that bacteria have to face to avoid cell damage and
672 death during storage. PVOH presents high oxygen barrier properties at low humidities,
673 and still exhibits great oxygen barrier properties at the humidities assayed in the current
674 work (Labuschagne et al., 2008), thus, PVOH can exert a protective role against oxygen
675 and oxidative processes in LAB such as lipid oxidation of membrane fatty acid that is
676 responsible for cell death during storage (Teixeira et al., 1996). Moreover, it is also
677 deeply studied that proteins can inhibit the peroxidation o membrane cells via free radical
678 scavenging and can maintain the biological activity of LAB. In the current study, it has
679 been demonstrated that the incorporation of HCas into the polymer matrix, increases the
680 antioxidant activity of films reaching values of almost 100 % antioxidant inhibition in 1:1
681 films maintaining the antioxidant activity after one month of storage.
682
683 **4. Conclusions**
684 PVOH has been successfully blended with casein hydrolysates (HCas) for carrying nisin-
685 producing *L. lactis* and sakacin-producing *L. sakei* acting as biocontrol agents against *L.*
686 *monocytogenes*. Cell viability after film drying was strain-dependent and was enhanced
687 with the addition of HCas. Films supplemented with HCas also maintained *L. lactis* and
688 *L. sakei* viability throughout one-month storage. HCas promoted auto-aggregation of
689 cells which was more accused for *L. lactis* strain. Bacterial auto-aggregation in the films
690 could be related with a greater cell viability. The higher antilisterial activity was obtained
691 for PVOH blended with HCas at 1:1 weight ratio, and entrapping *L. lactis*. HCas act as
692 plasticizers for PVOH polymer, and when a great amount of HCas was added to the
693 films, their mechanical strength dramatically decreases improving film elongation; HCas
694 also confers films with UV barrier and antioxidant properties. In spite of the loss of
695 mechanical strength, films incorporating a great proportion of HCas can be formed by

casting and used as self-standing films or coatings to be applied in the antilisterial preservation of refrigerated foods or as carriers of LAB.

## CRediT authorship contribution statement

**Laura Settier:** Methodology, Investigation, Formal analysis, Validation, Data curation, Writing - original draft. **Gracia López**: Data curation, Writing -review & editing, Visualisation, Supervision **Rafael Gavara:** Resources, Data curation, Visualisation, Supervision, Project administration, Funding acquisition. **Pilar Hernández-Muñoz:** Term, Conceptualisation, Methodology, Resources, Data curation, Writing -review & editing, Visualisation, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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924

**Figure captions**

Figure 1. Evolution of the initial inoculum size of *L. lactis* or *L. sakei* (a) and *L. monocytogenes* logarithmic reduction rate (b) when is confronted with 4 log CFU/mL of *L. monocytogenes* in liquid TSB+YE for 13 days at 4 ℃. Mean values and 95 % LSD intervals.

Figure 2. Scanning electron microscopy pictures of cross-section surface of PVOH films blended with HCas, PVOH:HCas, at different weight ratios: a) 1:0, b) 1:0.125 and c) 1:1.

Figure 3. Fluorescence light microscopy images of *L. lactis* (a-c) and L. sakei (d-f) entrapped in PVOH films blended with HCas, PVOH:HCas, at different weight ratios: a & d) 1:0, b & e) 1:0.125 and c & f) 1:1. Blue color corresponds to cells dyed with DAPI. Magnification 20x.

Figure 4. Viability of *L. lactis* and *L. sakei* in just made PVOH films blended with HCas, PVOH:HCas, at different weight ratios (1:0, 1:0.125, 1:1); survival of LAB after being in contact with the pathogen during the antimicrobial assay; and antimicrobial activity of the different blend films against *L. listeria monocytogenes.* Mean values and 95% LSD intervals.

Figure 5. Growth of lactic acid bacteria (a) and *L. monocytogenes* logarithmic reduction value (b) corresponding to PVOH films blended with HCas, PVOH:HCas, at different weight ratios (1:0, 1:0.125, 1:1) and immersed in liquid medium (TSB+yeast) stored at 4 ℃ for 15 days. Mean values and 95% LSD intervals.

Figure 6. Viability of *L. lactis* and *L. sakei* in PVOH films blended with HCas at different weight ratios and stored at 20°C and 22.1 % RH (a) and 43.2 % RH (b). Mean values and 95% LSD intervals.

**FIGURE 1**

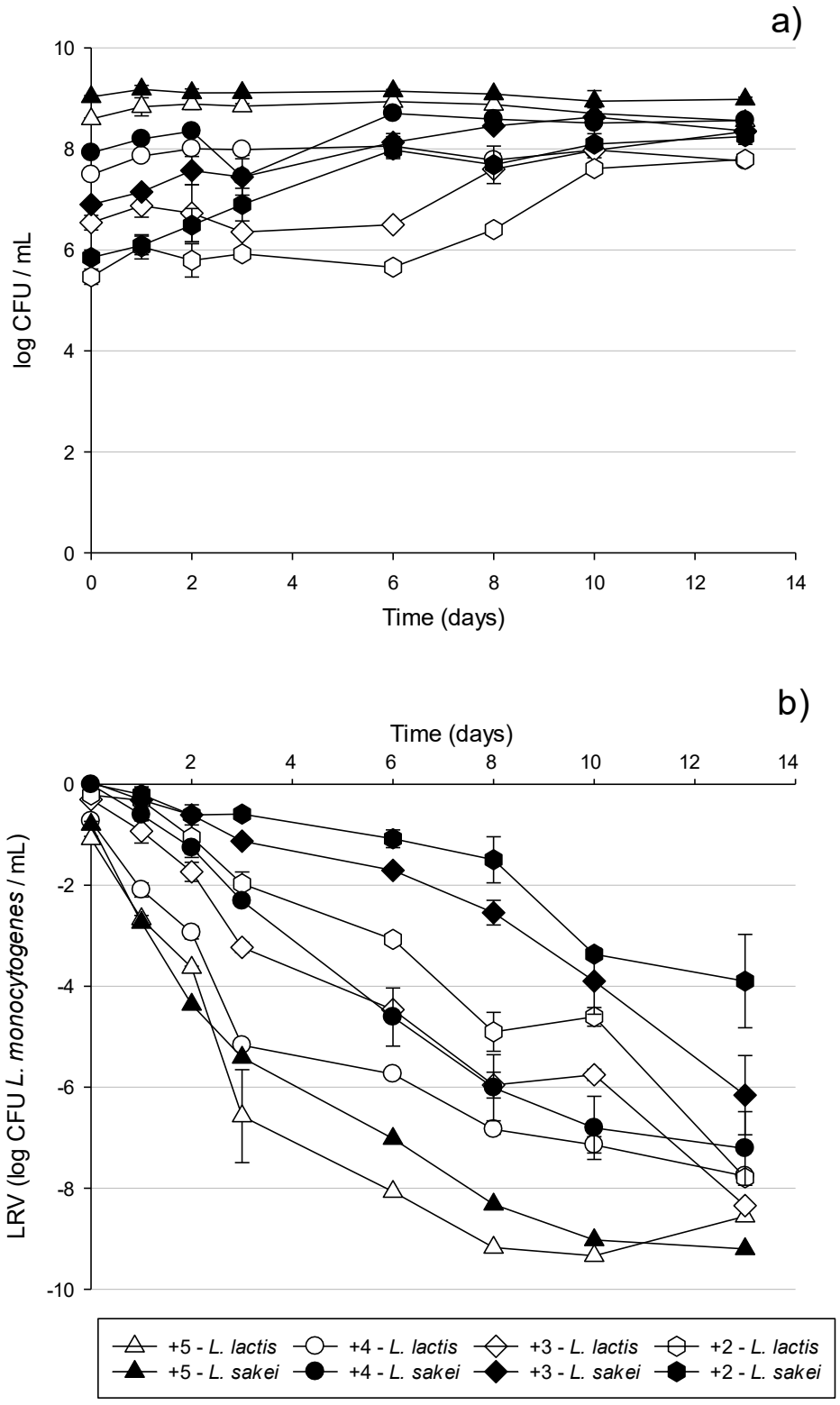
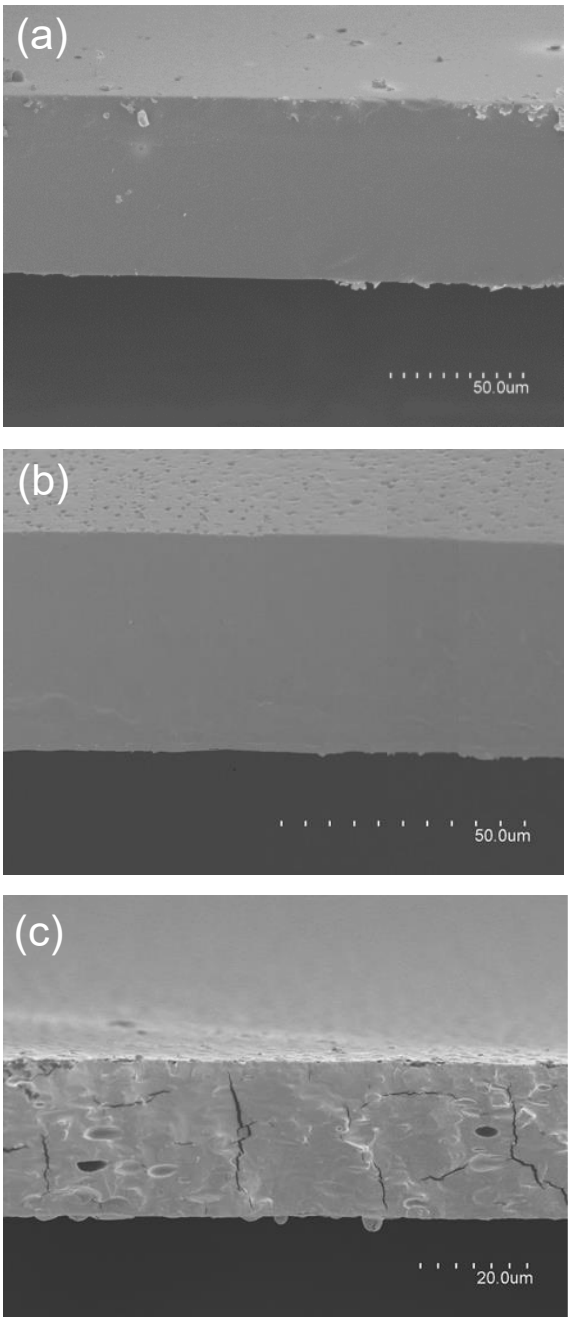


**FIGURE 2**



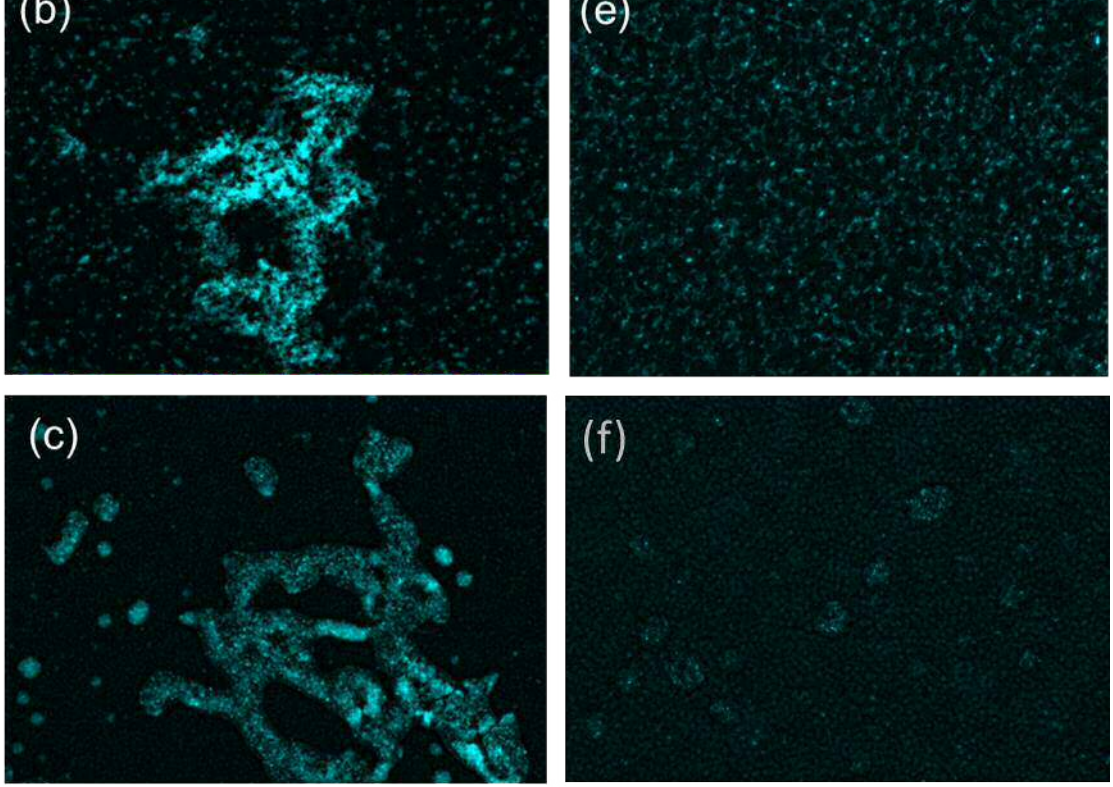

(b)
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**FIGURE 4**

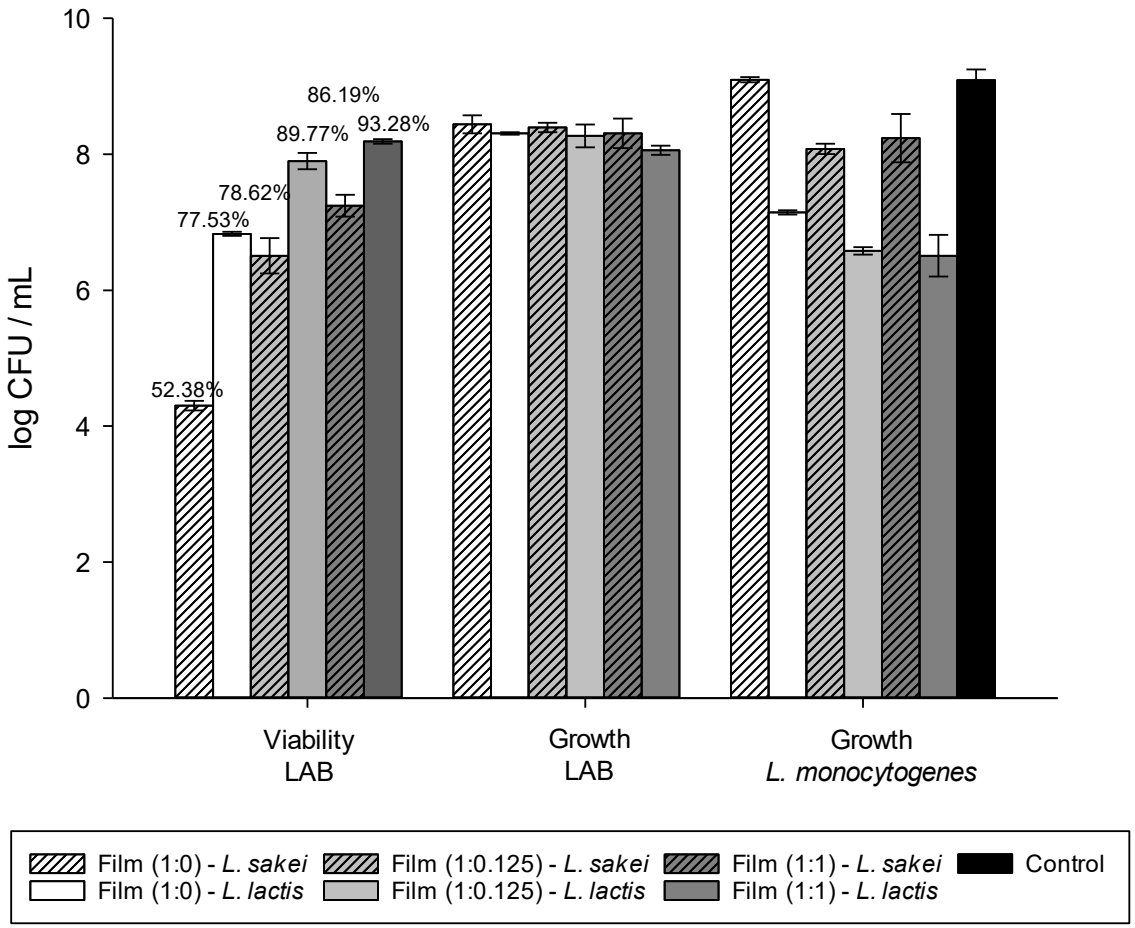
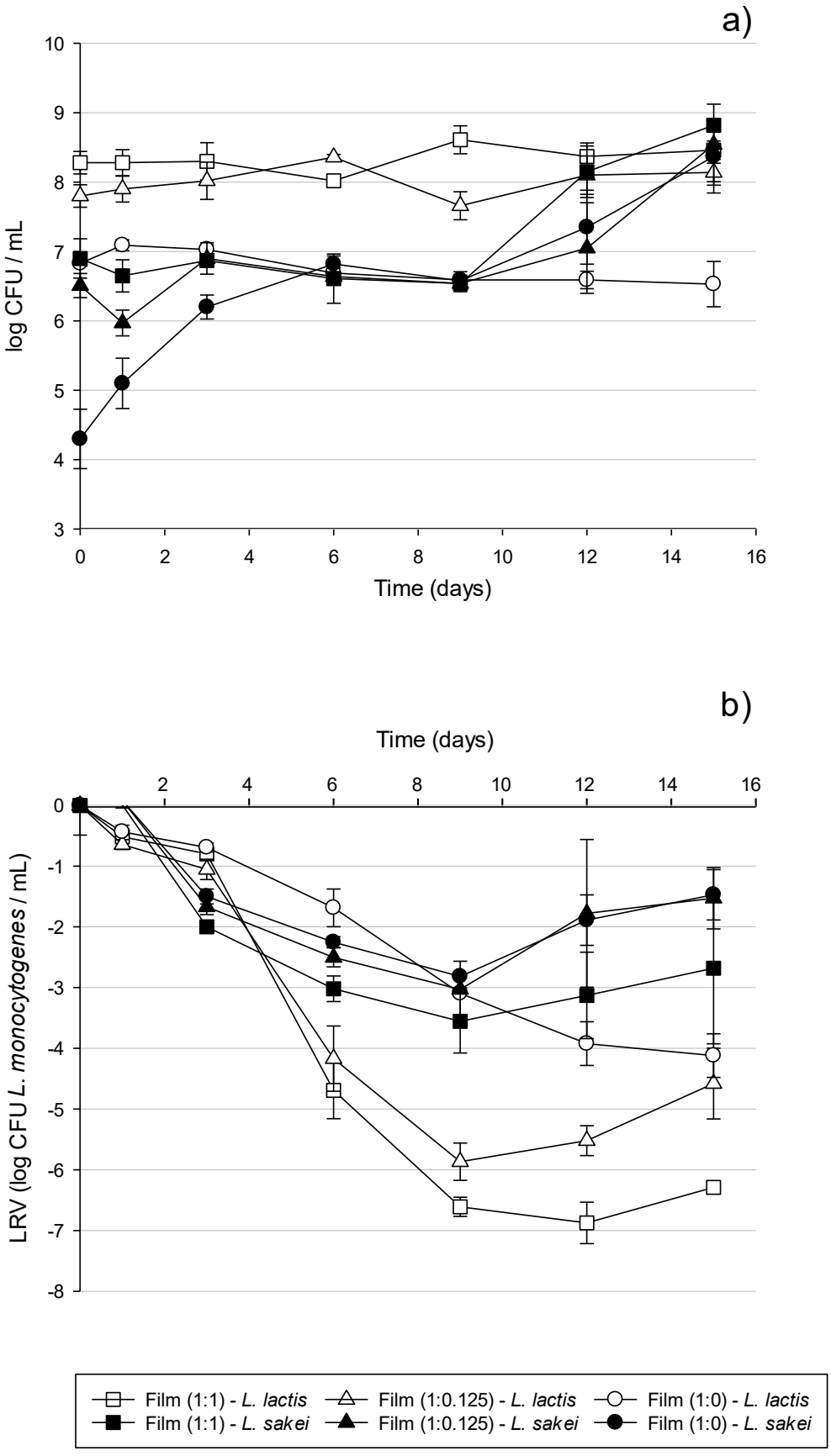
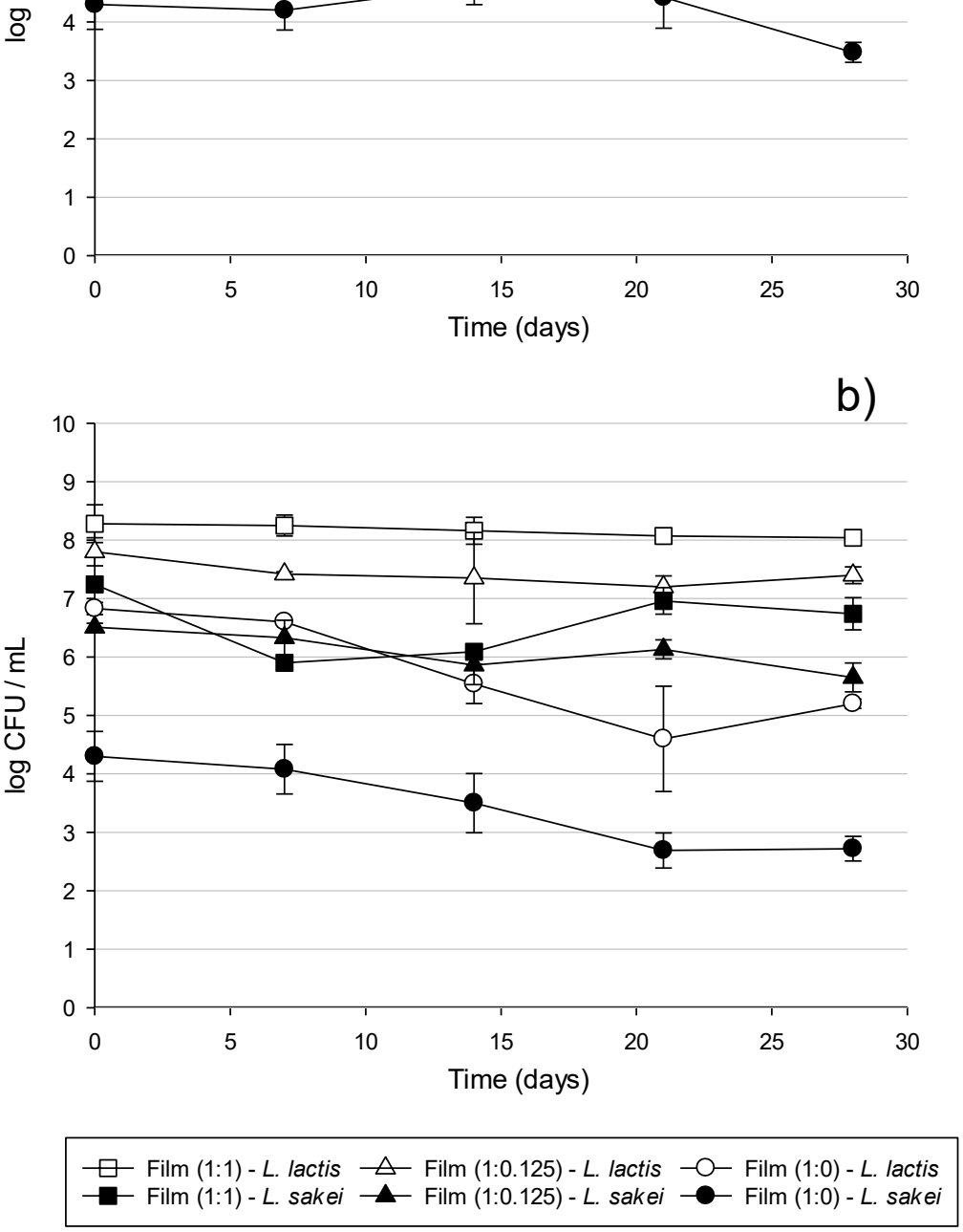


**FIGURE 5**

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Time (days)
Film (1:1) - L. lactis
Film (1:0.125) - L. lactis
Film (1:0) - L. lactis
Film (1:1) - L. sakei
Film (1:0.125) - L. sakei
Film (1:0) - L. sakei

Table 1. Effect of casein hydrolysates (HCas) on some functional properties of PVOH films measured at 20 °C.

| FILMS | Thickness | Moisture content (g/100 g dry film) | | Color Properties | | |
|---|---|---|---|---|---|---|
| | (μm) | 43.2% RH | 23.1 % RH | L* | h* | |
| **1:0*** | 32 ± 3[a] | 5.8 ± 0.1[a] | 3.8 ± 0.1[a] | 89.23 ± 0.16[a] | 0.02 ± 0.00[a] | -0 |
| **1:0.125*** | 32 ± 3[a] | 6.4 ± 0.1[c] | 4.0 ± 0.1[ab] | 89.25 ± 0.31[a] | 0.05± 0.05[a] | 0. |
| **1:1*** | 31 ± 4[a] | 8.4 ± 0.1[d] | 4.1 ± 0.1[bc] | 90.18 ± 0.74[a] | 0.24 ± 0.02[b] | 0. |

[a-d] Different letters in the same column indicate significant differences among formulations ($p < 0.05$).

*PVOH:HCas ratio in grams.

Table 2. Mechanical properties of PVOH: HCas blend films alone, and incorporating bacteria.

| Film | PVOH:HCas | Tensile strength (MPa) | Young's modulus (MPa) | Elongation at break (%) |
|---|---|---|---|---|
| Without bacteria | 1:0 | 60 ± 12$^{b}$ | 2275 ± 115$^{c}$ | 10 ± 4$^{a}$ |
| | 1:0.125 | 17 ± 1$^{a}$ | 1139 ± 41$^{b}$ | 79 ± 11$^{b}$ |
| | 1:1 | 12 ± 5$^{a}$ | 445 ± 273$^{a}$ | 124 ± 44$^{c}$ |
| *L. sakei* | 1:0 | 64 ± 15$^{b}$ | 2371 ± 345$^{c}$ | 9 ± 5$^{a}$ |
| | 1:0.125 | 23 ± 2$^{a}$ | 1103 ± 157$^{b}$ | 78 ± 2$^{b}$ |
| | 1:1 | 11 ± 1$^{a}$ | 453 ± 102$^{a}$ | 104 ± 23$^{b,c}$ |
| *L. lactis* | 1:0 | 64 ± 4$^{b}$ | 2055 ± 144$^{c}$ | 3 ± 6$^{a}$ |
| | 1:0.125 | 27 ± 5$^{a}$ | 1078 ± 154$^{b}$ | 106 ± 24$^{b,c}$ |
| | 1:1 | 11 ± 1$^{a}$ | 464 ± 95$^{a}$ | 123 ± 49$^{c}$ |

$^{a\text{-}c}$ Different letters in the same column indicate significant differences among formulations ($p < 0.05$).

Table 3. Thermal properties of PVOH powder, HCas and PVOH:HCas blend films alone and incorporating bacteria according to MDSC thermograms.

| **Sample** | **Polymer comp. PVOH:HCas** | **$T_g$ (°C)** | **$T_m$ (°C)** | **ΔH (J/g)** | **(%)** |
|---|---|---|---|---|---|
| | | *POWDERS* | | | |
| PVOH | 1:0 | 54.1 | 184.7 | 48.1 | 33.6 |
| HCas | 0:1 | 73.1 | | | |
| | | *FILMS* | | | |
| Without bacteria | 1:0 | 68.2 | 185.3 | 36.9 | 22.9 |
| | 1:0.125 | 69.2 | 182.6 | 18.2 | 12.9 |
| | 1:1 | 62.4/74.3 | 173.3 | 7.9 | 9.8 |
| *L. sakei* | 1:0 | 64.7 | 185.2 | 31.2 | 19.2 |
| | 1:0.125 | 67.9 | 180.3 | 17.5 | 12.4 |
| | 1:1 | 64/74.2 | 168.9 | 6.6 | 8.2 |
| *L. lactis* | 1:0 | 64.2 | 183.1 | 29.8 | 18.5 |
| | 1:0.125 | 69.6 | 184.9 | 27.5 | 19.5 |
| | 1:1 | 69.8/75.1 | 172.0 | 4.3 | 5.3 |

$T_g$ (°C), temperature of glass transition; $T_m$ (°C), temperature of melt; *ΔH (J/g),* melting enthalpy.
* Crystallinity percentage calculated from enthalpy values, considering that **ΔH** of 100% crystalline PVOH is 161.1 J/g.

Table 4. Antioxidant activity of just made PVOH:HCas blend films without and with bacteria, and after one month of storage at 20 °C at 43.2 % HR.

| Film | PVOH:HCas | Just made I (%) | One month stored I (%) |
|---|---|---|---|
| without bacteria | 1:0 | 0 ± 0[a] | 0 ± 0[a] |
| | 1:0.125 | 63.9 ± 3[b] | 64.0 ± 1[b] |
| | 1:1 | 97.3 ± 0.4[c] | 97.1 ± 0.3[c] |
| *L. sakei* | 1:0 | 0 ± 0[a] | 0 ± 0[a] |
| | 1:0.125 | 64.1 ± 2[b] | 65.3 ± 0.1[b] |
| | 1:1 | 96.9 ± 0.4[c] | 91.2 ± 0.1[c] |
| *L. lactis* | 1:0 | 0 ± 0[a] | 0 ± 0[a] |
| | 1:0.125 | 64.8 ± 2.3[b] | 65.1 ± 1.1[b] |
| | 1:1 | 96.9 ± 0.2[c] | 92.7 ± 1[c] |

[a-c] Different letters in the same column indicate significant differences among formulations ($p < 0.05$).

I (%): ABTS inhibition activity after 1 h of reaction.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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**CRediT authorship contribution statement**

**Laura Settier:** Methodology, Investigation, Formal analysis, Validation, Data curation, Writing - original draft. **Gracia López**: Data curation, Writing -review & editing, Visualisation, Supervision **Rafael Gavara:** Resources, Data curation, Visualisation, Supervision, Project administration, Funding acquisition. **Pilar Hernández-Muñoz:** Term, Conceptualisation, Methodology, Resources, Data curation, Writing -review & editing, Visualisation, Supervision, Project administration, Funding acquisition.