

1 Active whey protein edible films and coatings  
2 incorporating *Lactobacillus buchneri* for  
3 *Penicillium nordicum* control in cheese

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20 **Abstract**

21 Fungal contamination of food is responsible for health issues and food waste. In  
22 this work, the incorporation of a lactic acid bacteria (LAB) with antifungal properties  
23 (*Lactobacillus buchneri* UTAD104) into whey protein-based films and coatings was  
24 tested for the control of an ochratoxigenic fungi (*Penicillium nordicum*) in a cheese  
25 matrix. The incorporation of *L. buchneri* cells resulted in thicker films with less  
26 luminosity than control films and colour alteration. Nevertheless, cells inclusion did not  
27 alter moisture content, water vapour permeability, mechanical properties,  
28 hydrophobicity and chemical structure of the films. Whey protein films were able to  
29 maintain the viability of *L. buchneri* UTAD104 cells in 10<sup>5</sup> CFU/mL after 30 days of  
30 storage at 25 °C. When applied in cheese, films and coatings containing *L. buchneri*  
31 cells prevented fungal contamination for at least 30 days, while control cheeses with  
32 films and coatings either without LAB or with *L. casei* UM3 (a strain without antifungal  
33 ability) showed fungal contamination during that period. Ochratoxin A (OTA) was not  
34 found in cheeses treated with films and coatings containing the bacteria. Results showed  
35 that the inclusion of a LAB with antifungal properties in edible films and coatings can  
36 help to reduce or eliminate *P. nordicum* contamination in cheeses.

37 **Keywords:** bioactive film; lactic acid bacteria; antifungal activity; *Penicillium*  
38 *nordicum*; cheese; ochratoxin A

## 39 **1. Introduction**

40 Moulds are the primary spoilage organism in various food products, such as  
41 cereals, bread and fermented dairy products (Schnurer and Magnusson 2005). Cheeses  
42 are particularly vulnerable to the growth of filamentous fungi due to their pH, water  
43 activity, and nutritional profile (Hymery et al. 2014). Fungal contamination can produce  
44 discolouration, off-flavours, alterations in texture and appearance, leading to loss of  
45 quality, waste and consequently, economic losses. Additionally, some of the spoilage  
46 fungi can produce toxic secondary compounds named mycotoxins. These toxins are a  
47 serious concern to public health because they cause mutagenic, carcinogenic, cytotoxic,  
48 neurotoxic and immunogenic effects, among others (Hymery et al. 2014).

49 The use of lactic acid bacteria (LAB) in a perspective of biopreservation  
50 (inhibition of one microorganism by another) is considered as an alternative to chemical  
51 methods for the control of fungal contamination (Reis et al. 2012). LAB present several  
52 advantages for this biological strategy. They occur naturally in many foods, have a long  
53 history of usage in the manufacture and fermentation of dairy products, are generally  
54 regarded as safe (GRAS), and have, in some cases, probiotic properties (Giraffa et al.  
55 2010; Rhee et al. 2011; Varsha and Nampoothiri 2016; Prema et al. 2010). LAB  
56 antifungal properties are the result of a synergetic effect of several factors combined,  
57 such as the decrease of environmental pH, competitive growth and production of  
58 antifungal substances (Guimarães et al. 2018b; Guimarães et al. 2018c; Gerez et al.  
59 2013; Corsetti et al. 1998; Bianchini and Bullerman 2009; Özcelik et al. 2016; Niku-  
60 Paavola et al. 1999; Lavermicocca et al. 2000; Ström et al. 2002; Rizzello et al. 2011;  
61 Prema et al. 2010). However, in some particular cases, LAB may lose their metabolism  
62 and viability, which impairs their ability to exert antifungal effects (Burgain et al. 2011).  
63 The use of edible films and coatings is a strategy that can overcome this problem since  
64 the viability of microorganisms can be protected and maintained above  $10^6$  CFU/g to  
65 keep an adequate concentration of active compounds in the surface of the food products  
66 (Soukoulis et al. 2014c).

67 Edible films and coatings can be competent carriers of LAB, but can also improve  
68 foods quality by acting as semipermeable barriers to gases by controlling lipid  
69 oxidation, loss of volatile aromas and flavours; and by improving the mechanical  
70 integrity of the food product (Guimarães et al. 2018a). Also, edible films and coatings  
71 meet environmental concerns since they are usually produced with food-grade by-

72 products from agricultural and marine sources (Tharanathan 2003).  
73 Protein-based films and coatings have been gaining interest due to their functional and  
74 nutritional properties (Ramos et al. 2016; Ozdemir and Floros 2008; Ramos et al.  
75 2012a). Whey protein is a by-product of cheese production, which represents 20% of  
76 the total protein content in milk (Ramos et al. 2012a; Campos et al. 2010). It is a suitable  
77 material for the production of edible films and coatings with transparent, flavourless  
78 and flexible characteristics, as with good mechanical, oxygen and aroma barrier  
79 properties (Pérez-Gago and Krochta 2001; Pérez-Gago et al. 1999; Khwaldia et al.  
80 2004; Ramos et al. 2013; Campos et al. 2010). Previous studies reported that whey  
81 protein films, in conjugation with soluble prebiotic fibres, promote the stability of  
82 *Lactobacillus rhamnosus* GG (Soukoulis et al. 2014b) and can be effective carriers of  
83 this microorganism in pan bread (Soukoulis et al. 2014c). Additionally, several works  
84 have combined edible films and coatings with probiotic LAB strains (Tapia et al. 2007;  
85 Kanmani and Lim 2013; Piermaria et al. 2015). Regarding the antimicrobial activity of  
86 films and coatings incorporating LAB, some studies have shown positive results in the  
87 inhibition of spoilage bacteria even if their application in real food conditions is still  
88 scarce (Gialamas et al. 2010; Sánchez-González et al. 2013; Sánchez-González et al.  
89 2014; Pereira et al. 2018; Dai et al. 2018; Settier-Ramírez et al. 2019; Ye et al. 2018;  
90 Pavli et al. 2017). Nonetheless, to our knowledge, only a few works approached the  
91 incorporation of living bacteria in edible films and coatings to control fungal  
92 contamination (Pereira et al. 2018; Bambace et al. 2019), and none has evaluated these  
93 films in cheese products and their influence in the production of mycotoxin yet.

94 The present work aimed at investigating the antifungal properties of whey  
95 protein-based films containing LAB and at studying the influence of cells addition on  
96 the films physicochemical and structural properties. The produced antifungal coatings  
97 were also tested in a cheese matrix against *Penicillium nordicum*, and their influence in  
98 the production of ochratoxin A by this fungus was evaluated.

## 99 **2. Materials and Methods**

### 100 **2.1. Biological Materials and Growth Conditions**

101 *Penicillium nordicum* MUM 08.16 (CBS 112573) was revived in yeast extract  
102 sucrose (YES) agar in the dark at 25 °C. YES agar formulation consisted in 150 g/L of  
103 sucrose, 20 g/L of yeast extract, 15 g/L of agar, 0.5 g/L of MgSO<sub>4</sub>, 0.01 g/L of ZnSO<sub>4</sub>  
104 and 0.005 g/L of CuSO<sub>4</sub>. After 7 days of growth, fungal spores were suspended in a  
105 0.1% peptone solution with 0.05% Tween 80 (Fisher Chemical, USA). The spore  
106 concentration of inocula was counted in a microscope using a Neubauer cell chamber.  
107 Spore concentration was adjusted to 10<sup>6</sup> spores/mL, and aliquots of spore solutions  
108 were stored at -20 °C during the experimental work. *Lactobacillus buchneri* UTAD104  
109 and *Lactobacillus casei* UM3 were isolated from milk and silage and posteriorly stored  
110 in glycerol stocks in our private collection. Strains were molecularly identified by  
111 STAB VIDA laboratory (Portugal).

112 *L. buchneri* UTAD104 was previously selected for its antifungal ability against  
113 *P. nordicum* MUM 08.16 (Guimarães et al. 2018c) and *L. casei* UM3, which showed  
114 no antifungal activity (Guimarães et al. 2018b), was used as a negative control. For the  
115 production of antifungal films and coatings, LAB were cultivated in MRS broth (Oxoid,  
116 UK) supplemented with 20% of tomato juice (Fluka, Switzerland) and 1 g/L of cysteine  
117 (Fisher Chemical, USA) at 30 °C for 48 h. A 5% aliquot from the overnight culture was  
118 transferred into the 30 mL of modified MRS broth and grown again at 30 °C for 48 h.  
119 The bacteria cultures were concentrated by centrifugation (10 min at 7000 rpm), and  
120 cells number was adjusted with the MRS culture broth for a concentration of  
121 approximately 1×10<sup>11</sup> CFU/mL.

## 122 2.2. Production of Films and Coatings

123 Whey protein concentrate (WPC) (kindly supplied by Armor Proteines, Saint-  
124 Brice-en-Coglés, France) was used as the base material for film and coating  
125 formulations. Glycerol (87% purity, Panreac, Spain) as a plasticizer, guar gum (Sigma-  
126 Aldrich, USA) as a thickener, and Tween 20 (Acros Organic, Belgium) as a surfactant  
127 were also used. All the materials, except WPC, were previously sterilized at 121 °C for  
128 15 min.

129 Film-forming solutions were prepared by adapting the protocol reported by  
130 Ramos et al. (2012b). Briefly, WPC (10%, w/w) was dissolved in distilled water, and  
131 then glycerol (5%, w/w) was added to the solution, which was magnetically stirred for  
132 2 h. The solution was then heated in a water bath at 80 °C under agitation for

133 approximately 5 min. The solution was allowed to cool down at room temperature to  
134 approximately 30 °C. Then, Tween 20 (0.2%, w/w) was added, and the solution was  
135 magnetically stirred for another 20 min. After this, guar gum (0.7%, w/w) was added,  
136 and the solution was again magnetically stirred for approximately 16 h. This agitation  
137 period was necessary to hydrate the guar gum thoroughly in order to improve the films'  
138 homogeneity. Afterwards, film-forming solutions were adjusted to pH 6 using pure  
139 glacial acetic acid (Fisher Scientific, USA).

140 *L. buchneri* UTAD104 and *L. casei* UM3 were individually added at a  
141 concentration of 10, 20 or 30% (w/w) to the film-forming solution and homogenized.  
142 This corresponds, respectively, to  $1 \times 10^{10}$ ;  $2 \times 10^{10}$  and  $3 \times 10^{10}$  CFU/mL in the film  
143 solution. Finally, the film-forming solution was poured onto Teflon coated plates. The  
144 same quantity of solution (approximately 1 g for 5 cm<sup>2</sup>) was poured onto each Teflon  
145 plate to obtain similar film thickness. Film-forming solutions were allowed to dry in an  
146 oven with ventilation at 25 °C for 24 h. Once dried, films were peeled off and stored at  
147 room temperature in a sealed compartment.

148 For the antifungal coatings, *L. buchneri* UTAD104 was added at a 30% (w/w)  
149 and the solution was applied directly onto the cheese surface with the aid of a sterile  
150 syringe, to ensure that the entire surface was covered. The residual coating solution was  
151 allowed to drip off.

### 152 2.3 *In vitro* Antifungal Activity of Films against *Penicillium nordicum*

153 Three sets of films were tested to assess its activity against *P. nordicum*: i) films  
154 incorporating *L. buchneri* UTAD104, ii) films incorporating *L. casei* UM3, and iii)  
155 films without any LAB (the two last formulations were tested as negative controls).  
156 Films antifungal activity was evaluated by the ability of *P. nordicum* to grow on the  
157 film surface. Films circles (20 mm of diameter) were placed in Petri plates with YES  
158 agar and 10 µL of a spore solution ( $10^6$  spores/mL) of *P. nordicum* was center  
159 inoculated in the film's surface. Plates were incubated at 25 °C for 7 days. Visual  
160 inspection was conducted daily until 7 days of growth. All experiments were done in  
161 duplicate.

### 162 2.4 Enumeration of Lactic Acid Bacteria in the Films

163 The viability of incorporated *L. buchneri* UTAD104 and *L. casei* UM3 in the  
164 formed films was analysed. Each film was cut into circular discs with 20 mm of  
165 diameter (approximately 0.1 g) and placed in a sterile falcon with 9.9 mL of PBS. PBS  
166 was prepared in the following way: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na<sub>2</sub>HPO<sub>4</sub> and  
167 0.24 g of KH<sub>2</sub>PO<sub>4</sub> were added to 1 L of distilled water, and the final pH set to 7.4. The  
168 falcons were strongly vortexed until the film was dissolved. Sequential 10-fold  
169 dilutions were done with PBS and plated on MRS agar. Plates were incubated at 30 °C  
170 until colonies were visible. Films were stored in sterile Petri plates sealed with parafilm  
171 at room temperature and analysed every 3 to 5 days. Films were analysed until 30 days  
172 after casting.

## 173 2.5. Film Characterization

174 Physicochemical and mechanical properties of control films without LAB and  
175 films incorporating *L. buchneri* UTAD104 at 30% (w/w) (films that demonstrate a  
176 stronger *P. nordicum* inhibition) were fully characterized as detailed below. Before  
177 characterization, films were conditioned at approximately 54 ± 1% relative humidity  
178 (RH) and 25 ± 2 °C for at least 24 h.

179

### 180 2.5.1. Thickness

181 Film thickness was measured with a hand-held digital micrometer (Mitutoyo,  
182 Japan) with 0.001 mm accuracy. Thickness was measured randomly at five different  
183 points in each sample. Two samples for each film were analysed. Mean values were  
184 used for further calculations.

### 185 2.5.2. Moisture Content and Water Solubility

186 Moisture content was determined gravimetrically by drying film samples (discs  
187 of 20 mm diameter) at 105 °C for 24 h. Moisture content was given by the percentage  
188 of water removed from the initial film sample. For films' water solubility evaluation,  
189 film samples were placed in beakers with 40 mL of distilled water, sealed with parafilm  
190 and shaken at 60 rpm at 25 °C for 24 h. The undissolved film was recovered and  
191 desiccated at 105 °C for 72 h. The non-soluble part of each film was weighted and  
192 solubility, expressed as a percentage, was calculated by the equation:

193 
$$F_s(\%) = \left( \frac{W_i - W_f}{W_i} \right) \times 100$$

194 where  $F_s$  was the film solubility,  $W_i$  was the initial weight of film as dry matter, and  $W_f$   
195 was the weight of the undissolved film residue. All experiments were performed in  
196 triplicate for each film sample, and two samples of each film were tested.

### 197 2.5.3. *Water Vapour Permeability*

198 Water vapour permeability (WVP) was determined gravimetrically according to  
199 the American Society for Testing and Materials (ASTM) E 96-92 method, as described  
200 by Cerqueira et al. (2012). Three samples from each film were cut and placed in a  
201 permeation cell (a cup with water at 100% RH;  $2.337 \times 10^3$  Pa vapour pressure at 20 °C).  
202 Cups with a diameter of 4 cm and depth of 7 cm were filled with 50 mL of distilled  
203 water. The films were mounted with the upper side facing the controlled environment  
204 (desiccator with silica gel 0% RH, 0 Pa, 20 °C). The weight loss of the cups was  
205 measured at 2 h intervals for a total of 10 h, to determine water-transferred through the  
206 samples films. Water vapour transmission rate (WVPR) was calculated by dividing the  
207 slope of the linear regression of weight loss versus time by the film area ( $\text{g}/(\text{day m}^2)$ ).  
208 Afterwards, WVTR was multiplied by film thickness and divided by vapour pressure  
209 differential across the sample film to obtain WVP ( $\text{g mm}/(\text{day m}^2 \text{ kPa})$ ).

### 210 2.5.4. *Mechanical Properties: Tensile Strength and Elongation at Break*

211 Tensile strength (TS) and elongation at break (EB) were determined with a texture  
212 analyser TA.XT Plus (Model TA.HD Plus, Stable Micro Systems, Surrey, UK)  
213 according to the ASTM Standard Method D 882-91. Film samples were cut into  $120 \times 20$   
214  $\text{mm}^2$  strips and placed between the tensile grips. A load cell capacity of 5000 g was  
215 used. The initial grip separation and crosshead speed were set to 100 mm and 5  
216 mm/min, respectively. Tensile strength (MPa) was determined by dividing maximum  
217 load (N) by the cross-sectional area of the film ( $\text{m}^2$ ), and elongation at break (%) was  
218 calculated as a ratio of the final length at the point of sample rupture to the initial length  
219 of the sample. At least eight strips of each film sample were analysed.

### 220 2.5.5. *Optical Properties*

221 The colour of the films was measured using a colorimeter CR 400 (from Konica  
222 Minolta, Japan). Colour coordinates  $L^*$  (black to white hue component),  $a^*$  (red to  
223 green hue component) and  $b^*$  (yellow to blue hue component) of CIELab colour scale  
224 were measured in 5 random points of each sample, and two samples from each film  
225 were tested. Films were measured against the surface of a white standard plate with  
226 defined colour coordinates ( $L_{\text{standard}}=97.7$ ,  $a_{\text{standard}}=0.04$  and  $b_{\text{standard}}=1.47$ ).

227 The total colour difference ( $\Delta E$ ) was calculated according to the following  
228 equation:

$$229 \quad \Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

230 where  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the differentials between the film colour parameter and  
231 the colour parameter of a standard used as the film background.

232 The opacity of each sample was determined according to the Hunterlab method,  
233 as a relationship between the opacity of the sample on a black standard and the opacity  
234 of the samples in a white standard. Five measurements were made for each sample, and  
235 two samples of each film were tested.

#### 236 2.5.6. *Surface Hydrophobicity*

237 Surface hydrophobicity of the films was evaluated by measuring the contact angle  
238 of a water droplet with the surface of the film, with an optical contact angle measuring  
239 system (OCA20, DataPhysics, Germany) equipped with the C20 software and with  
240 CCD video camera (resolution of 752×582 pixel) at 23 °C. Measurements were  
241 performed according to sessile drop methods using a 500  $\mu\text{L}$  syringe filled with ultra-  
242 pure water. Film samples were tape-glued to the centre of the equipment stand, and a  
243 drop of 2  $\mu\text{L}$  was placed on the film surface. Measurements were made from 0 to 60 s,  
244 and 10 replicates (5 from each sample) were analysed for each film type.

#### 245 2.5.7. *Fourier Transform Infrared Spectroscopy*

246 The Fourier transform infrared spectroscopy (FTIR) spectra of the films with and  
247 without cells were recorded with a Bruker FT-IR VERTEX 80/80v (Boston, USA)  
248 using Attenuated Total Reflectance mode (ATR) with a platinum crystal accessory.

249 Film spectra result from 16 scans in the wavelength range 4000-400 cm<sup>-1</sup>, at a resolution  
250 of 4 cm<sup>-1</sup>. Before analysis, an open bean background spectrum was recorded as a blank.

#### 251 2.5.8. X-Ray Diffraction

252 Crystallinity phases of the sample films were evaluated by X-ray diffraction  
253 (XRD). XRD patterns of each film were analysed between 2 $\theta$ =10° and 2 $\theta$ =50° with a  
254 step size 2 $\theta$ =0.02° and recorded 14 pts/s with a Cu source, X-ray tube ( $\lambda$  = 1.54056 Å)  
255 at 45 kV and 40 mA in an X-ray diffraction instrument (X'Pert<sup>3</sup> Powder, Panalytical,  
256 Almelo, Netherlands).

#### 257 2.6. Antifungal activity of films and coatings against *Penicillium nordicum* in cheese

258 The cheese used for this study was a sliced, semi-hard “Flamengo” cheese,  
259 obtained commercially and manufactured by “Terra Vento” (São Miguel, Portugal).  
260 This cheese is produced with pasteurized bovine milk, with the addition of calcium  
261 chloride and packed in a protective atmosphere. It requires refrigeration at low  
262 temperature (0 to 10 °C). The typical composition of this cheese is 23.6% of protein,  
263 27.8% of fat and 1.4% of salt. For the following experiments, samples of approximately  
264 4 cm of diameter and 0.5 cm of thickness were used.

265 Films were placed on top of the cheese surface and centre inoculated with  
266 *P. nordicum* (10<sup>6</sup> spores/mL) to determine their antifungal activity. Films incorporating  
267 *L. buchneri* UTAD104 and *L. casei* UM3 at 30% (w/w), and films without cells and  
268 untreated cheese were tested in duplicate.

269 The same WPC solutions were used to directly coat the cheese samples, thus  
270 avoiding the film-forming step to simulate better the application conditions of the  
271 industry. Cheese samples were left to dry in a vertical flow chamber for 1 h at room  
272 temperature. After that period, they were transferred to an incubator with air circulation  
273 at 25 °C for approximately 24 h, to ensure that the coating was completely dry. Cheeses  
274 samples were then centre inoculated with *P. nordicum* (10<sup>6</sup> spores/mL).

275 All cheese manipulation, and films and coating application were performed in a  
276 sterile environment. After inoculation, cheeses samples were placed in an incubator at  
277 25 °C and approximately 90% RH. The growth of the fungi and other microbial  
278 contaminations were observed regularly for a storage period of 30 days.

### 279 2.6.3. *Mycotoxin analysis*

280 Production of OTA in cheeses samples inoculated with *P. nordicum* was  
281 evaluated after 30 days of the inoculation. Cheese samples with 4 cm of diameter and  
282 approximately 0.5 mm of thickness were cut in small pieces (approximately 1 cm<sup>2</sup>) to  
283 facilitate the extraction, placed into falcons and extracted with 20 mL of an  
284 acetonitrile/methanol/acetic acid (78/20/2, v/v/v) solution. Then, falcons were  
285 subjected to vigorous vortexing and placed in the dark at room temperature overnight.  
286 Samples were centrifuged (3000 rpm, 10 min), to deposit cheese residues, and  
287 supernatants were filtered using a 0.45 µm nylon syringe filters (Whatman, USA) into  
288 amber vials and analysed by high-performance liquid chromatography (HPLC) with  
289 fluorescence detection as previously reported (Guimarães et al. 2018c). The HPLC  
290 system consisted of a Varian Prostar (USA) 210 pump and 410 autosampler, connected  
291 to a Jasco (Japan) FP-920 fluorescence detector ( $\lambda_{exc}$ =333 nm and  $\lambda_{em}$ =460 nm). A  
292 Varian 850-MIB data system was used to establish the interface between the HPLC  
293 system and a computer, and the chromatographic data were analysed by Galaxy  
294 software. The column used to perform chromatographic separation was a C<sub>18</sub> reversed-  
295 phase YMC-Pack ODS-A analytical column (250 x 4.6 mm I.D., 5 µm) that was  
296 protected by a pre-column with the same stationary phase. The mobile phase used to  
297 elute OTA was a mixture of water/acetonitrile/acetic acid (99/99/2, v/v/v) that was  
298 previously filtered with 0.2 µm GHP membrane filter (Pall, USA) and degassed for 20  
299 min in ultrasounds. The flow rate was set to 1.0 mL/min for a 15 min isocratic run, and  
300 the injection volume was 50 µL. Calibration curves were done with a certified OTA  
301 standard solution (CRM46912, Sigma-Aldrich, USA) in the mobile phase at  
302 concentrations between 0.05 and 100 ng/mL. The concentration of OTA was obtained  
303 by comparing peak areas with the calibration curves and limits of detection and  
304 quantification for OTA were 0.18 ng/mL and 0.54 ng/mL, respectively.

### 305 2.7. *Data Analysis*

306 At least three independent replicates were performed in all experiments and  
307 results were expressed as mean values  $\pm$  standard deviation. Data were compared by  
308 mean analysis (t-test) at  $p < 0.05$  using the software Microsoft Excel version 14.6.0  
309 (Microsoft Corporation, USA).

### 310 3. Results and Discussion

#### 311 3.1. Antifungal Activity of WPC-based Films

312 A preliminary assay was used to evaluate the antifungal activity of WPC based  
313 films incorporating LAB. Figure 1 shows the antifungal capacity of the WPC based  
314 films after 7 days of inoculation with a *P. nordicum* spore solution. As expected, the  
315 films incorporating the strain *L. casei* UM3 showed no inhibitory effect on *P. nordicum*  
316 growth (Fig 1 a, b and c) at all concentrations tested (10, 20 and 30% (w/w)). On the  
317 other hand, *P. nordicum* was not able to grow on the surface of WPC based films  
318 incorporating *L. buchneri* UTAD104, whatever the cell concentration used (Fig. 1 d, e  
319 and f). Additionally, in an agar diffusion assay, films containing 30% of *L. buchneri*  
320 UTAD104 exhibited more pronounced inhibition halos than those incorporated with 10  
321 and 20% of the bacteria (data not shown), thus the 30% concentration was selected for  
322 the following studies.

323 The antifungal capacity of *L. buchneri* UTAD104 has been reported previously  
324 and associated with the production of acetic, lactic and phenyllactic acids (Guimarães  
325 et al. 2018c). These organic acids already demonstrated antifungal ability in other  
326 studies (Prema et al. 2010). In films, the produced organic acids are probably inhibiting  
327 *P. nordicum* growth. This is possibly the primary inhibitory mechanism involved, since  
328 others, such as the competitive growth, would also produce an inhibitory effect in films  
329 incorporating the strain *L. casei* UM3. On the contrary, films incorporated with *L. casei*  
330 UM3 did not show antifungal activity.

#### 331 3.2. Lactic Acid Bacteria Survival in Films

332 Viability of *L. buchneri* UTAD104 and *L. casei* UM3 incorporated in WPC based  
333 films was assessed throughout a storage period of 30 days at room temperature in order  
334 to evaluate films capacity to maintain cell survival. Counts of viable LAB over that  
335 period are shown in Fig. 2.

336 For both strains, there is a consistent decrease in the number of viable cells in the  
337 films throughout the 30 days of storage. In the first 15 days, there was a more  
338 accentuated decrease in viability, of approximately 5 log CFU/mL. After that period  
339 and until 30 days of storage, the decrease in viable LAB was less accentuated

340 (approximately 2 to 3 log). Between day 22 and 30, the number of viable cells stabilized  
341 around  $1 \times 10^5$  CFU/mL for both LAB.

342 In edible films, the presence of relatively high amounts of solutes combined with  
343 their rubbery physical state leads to the occurrence of enzymatic and chemical  
344 reactions, which can cause damage in essential cellular structures (Soukoulis et al.  
345 2014b), and consequently affect cell survival within those films. Still, nutrients present  
346 in the films and in the culture media that were added together with the cells can, usually,  
347 support cell viability (Soukoulis et al. 2014a). Furthermore, whey proteins and glycerol  
348 are described to have a positive effect on LAB viability (Mohammadi et al. 2011).  
349 However, reduction of viable LAB throughout time can still occur due to film  
350 dehydration, temperature, presence of oxygen and further depletion of nutrients (Pereira  
351 et al. 2016; Sánchez-González et al. 2014).

352 Similar results for cell survival in films were obtained by Fan et al. (2009), which  
353 reported the capability of sodium alginate films to maintain half of the *Cryptococcus*  
354 *laurentii* population after a 20-day storage period. Sánchez-González et al. (2013) also  
355 reported the ability of films made of pea protein, methylcellulose and sodium caseinate,  
356 to maintain at least half of the initial viable counts of *L. plantarum*, after 30 days of  
357 storage at 5 °C. Differences in cell survival in film matrix may be attributed to the type  
358 of entrapped strain, composition of the film and drying and storage conditions.

### 359 3.3. Film characterization

#### 360 3.3.1 Thickness, moisture content, water solubility and water vapour permeability

361 Table 1 shows the values obtained for thickness, moisture content, water  
362 solubility and WVP of films with and without the incorporation of *L. buchneri*  
363 UTAD104 (30% w/w). In general, incorporation of LAB did not affect significantly  
364 ( $p < 0.05$ ) the moisture content and WVP values in comparison to the plain WPC films  
365 (control films). However, the addition of cells decreased the thickness of the films  
366 significantly and slightly increased their solubility in water (Table 1).

367 The thickness of the WPC based films was significantly affected by the  
368 incorporation of cells with values decreasing from 0.23 mm (control film) to 0.17 mm  
369 (film incorporating *L. buchneri* UTAD104). The thickness of the obtained control films

370 was, in general, higher than those reported by Pérez-Gago et al. (1999), Ramos et al.  
371 (2013) and Pérez et al. (2016) for similar whey protein films, i.e. 0.115, 0.170 and 0.126  
372 mm, respectively. Still, other works reported thicker whey protein films (0.31 mm)  
373 (Bahram et al. 2014) and others reported similar values (0.229 mm) to those obtained  
374 in this work (Reinoso et al. 2008). These differences in film thickness are probably due  
375 to different film formulations and film making procedures, even though examples given  
376 are whey protein-based.

377 The moisture content of edible films is an important parameter to estimate other  
378 film properties, such as films dissolution ability during human consumption and their  
379 capacity to withstand probiotic survival (Kanmani and Lim 2013). The incorporation  
380 of cells has not affected the moisture content of the films significantly ( $25.0\% \pm 1.2$  and  
381  $26.2\% \pm 0.9$  for control and *L. buchneri* films, respectively). Similar values of moisture  
382 content (27.9%) for WPC based films with glycerol as a plasticizer were obtained by  
383 other authors (Pérez et al. 2016).

384 Both films (with and without cells) swelled and maintained their integrity during  
385 the dissolution procedure for 24 h. Control films presented solubility in water of  $43.7 \pm$   
386  $1.6\%$ , while films incorporating cells showed a significantly higher ( $p < 0.05$ ) solubility  
387 of  $45.1\% \pm 0.9$  (Table 1). The solubility of the control films was relatively similar to  
388 other films in the literature (Ramos et al. 2012c; Pérez-Gago et al. 1999; Pérez et al.  
389 2016). The partial insolubility of the films can be attributed to strong intermolecular  
390 bonds existing between the protein molecules within the film matrix (Ramos et al.  
391 2013). The higher content of water that resulted from the incorporation of the cell  
392 culture can explain the slightly higher solubility of films containing *L. buchneri*  
393 UTAD104. It may also indicate lower cohesion of the film matrix, due to the presence  
394 of cells.

395 The WVP is a critical characteristic of edible films once low WVP is generally  
396 necessary for the control of detrimental reactions (such as lipid oxidation, browning  
397 and vitamin reactions) and the structural decay, which can facilitate microbial spoilage  
398 and result in a weight loss of the food product. The incorporation of cells in the film did  
399 not significantly alter the WVP of the films, with the WVP of control films and films  
400 with *L. buchneri* UTAD104 being 9.6 and 10.0 g mm/(day m<sup>2</sup> kPa), respectively. For  
401 the control films, similar WVP were obtained by Ramos et al. (2012c). Alike, Aloui et  
402 al. (2015) and Gialamas et al. (2010) reported that the incorporation of cells did not  
403 affect the barrier properties of edible films significantly. In contrast, Sánchez-González

404 et al. (2013) reported that the incorporation of *L. plantarum* increased WVP due to the  
405 discontinuities introduced in the film matrix.

### 406 3.3.2 Mechanical properties

407 Edible films should be able to maintain their integrity during processing, storage,  
408 shipping and handling. To do so, they must possess adequate tensile strength and  
409 elongation (Falguera et al. 2011). Table 1 presents the results of the tensile strength and  
410 elongation at break for control films and films with *L. buchneri* UTAD104. Tensile  
411 strength indicates the maximum stress that the films sustain during stretching, while  
412 elongation at break indicates the increase in film length from the initial point to the  
413 breakpoint, giving a measure of the film extensibility.

414 In general, the results indicate that the presence of cells did not significantly  
415 influence the mechanical properties of the films, which indicate that when incorporated  
416 in the WPC films, LAB do not interfere in the structure of the proteinaceous network.  
417 This is in agreement with results obtained by other authors (Soukoulis et al. 2016;  
418 Kanmani and Lim 2013; Gialamas et al. 2010). So, films incorporating *L. buchneri*  
419 UTAD104 should be able to stand the same procedures as control films. Obtained  
420 values for tensile strength in whey protein films are similar to those observed by Ramos  
421 et al. (2012c) and Bahram et al. (2014) (around 0.7 and 0.8 MPa, respectively);  
422 however, these values are relatively low when compared with other reported by Pérez-  
423 Gago et al. (1999), Reinoso et al. (2008) and Aziz et al. (2018) with values around 6.5,  
424 6.1 and 7.1 MPa, respectively. It is known that the content of plasticizer can influence  
425 the tensile strength of films, with high contents of plasticizer (such as the used in this  
426 work) resulting in low tensile strength values (Fang et al. 2002). Both produced WPC  
427 films had an elongation at break of almost 50%, which indicate that films have high  
428 extensibility. These values are similar to the one reported by Pérez-Gago (1999) for  
429 other whey protein films (43%), even though these films consisted of only 5% WPC.  
430 Other authors using 10% of WPI reported a considerably lower elongation at break  
431 (19.5%) (Reinoso et al. 2008).

### 432 3.3.3 Optical properties

433 Colour and optical properties are important characteristics of edible films since  
434 they can directly influence consumer acceptance. Colour of the films is affected by  
435 several factors that include plasticizer addition, thermal treatments, production process,  
436 and storage conditions and, in the case of protein films, the protein concentration (Su  
437 et al. 2012). The hue values and opacity for both films are shown in Table 2. All  
438 analysed parameters were significantly affected by the incorporation of cells, and all  
439 samples showed a yellowish colour typical of WPC based films, which can be linked  
440 to the presence of fat and phospholipids (Pérez et al. 2016; Ramos et al. 2013). In  
441 general, films incorporating *L. buchneri* UTAD104 have less luminosity ( $L^*$ ) than  
442 control films (90.6 and 94.1, respectively); have a slightly red hue opposed to control  
443 films that show a more “neutral” hue ( $a^*$  values of -0.4 for control films and 0.8 for  
444 films incorporating LAB); and are also more yellowish than control films ( $b^*$  value of  
445 -22.4 for films with LAB higher than -10.5 of control film). The addition of *L. buchneri*  
446 UTAD104 was also related to an increase in the opacity of the films when compared to  
447 control films (13.4 and 12.6%, respectively). The total colour difference ( $\Delta E$ ) that  
448 includes the three colour parameters was also calculated for both films. Values of 9.2  
449 and 21.6 were obtained for films without and with *L. buchneri* UTAD104, respectively.  
450 This difference confirms that addition of cells alters the colour and optical properties  
451 of WPC based films and that the difference can be perceived clearly, as 3 is considered  
452 the threshold of human-perceivable colour differences for  $\Delta E$  values (Mokrzycki and  
453 Tatol 2011). The incorporation of cells within MRS culture media (that presents a  
454 brownish colour) is probably the main responsible for these differences of colour.

#### 455 3.3.4 Surface hydrophobicity

456 Water contact angle measurement is used to determine the hydrophilicity ( $\theta <$   
457  $65^\circ$ ) or hydrophobicity ( $\theta > 65^\circ$ ) of the film surface. This parameter can be used to  
458 estimate the resistance of the film to moisture transfer (Phan et al. 2005). The initial  
459 contact angle (time 0 s) and contact angles after 1, 5, 15, 30, 45 and 60 s of applied  
460 water droplets were measured. The upper side of the films was analysed, and the  
461 obtained values are shown in Table 3. In general, both films demonstrated a hydrophilic  
462 character ( $\theta < 65^\circ$ ) at 0 s, with similar values of  $\theta$  for control films and films with *L.*  
463 *buchneri* UTAD104 ( $51.06^\circ$  and  $51.13^\circ$ , respectively). These  $\theta$  values are similar to the  
464 ones obtained in other works (Kokoszka et al. 2010). Throughout the experiments,

465 contact angles values dropped until reaching 29.5° and 20.8°, for films without and with  
466 *L. buchneri* UTAD104, respectively. The control films showed lower  $\theta$  values until 30  
467 s after the water droplet application; however, the differences were only statistically  
468 significant after 60 s. With time, the swelling of the films (more visible in films with *L.*  
469 *buchneri* UTAD104) and the absorption of the water droplet was noticeable, being  
470 observed for times larger than 30 s.

#### 471 3.3.5. Fourier Transform Infrared (FTIR) spectroscopy

472 FTIR spectrum of films was analysed in the region of 4000 to 400  $\text{cm}^{-1}$  (Fig. 3a).  
473 Similar peaks can be observed for both samples, with and without *L. buchneri*  
474 UTAD104, thus indicating that the presence of the bacteria did not alter the chemical  
475 structure of the films. The strong absorption band at 3271  $\text{cm}^{-1}$  is associated with the  
476 stretching vibration of free and bound OH and N-H groups, which can be intensified by  
477 the addition of glycerol (Ballesteros et al. 2018; Cerqueira et al. 2012). Literature  
478 indicates that the N-H band generally appears at 3253  $\text{cm}^{-1}$  (Ramos et al. 2013). So, the  
479 presence of the other film components, in particular, glycerol, may be responsible for  
480 the band shift seen in the obtained spectra. This band is also characteristic of moist  
481 materials. The bands at 2874  $\text{cm}^{-1}$  and 2930  $\text{cm}^{-1}$  correspond to stretching of -C-H  
482 groups. Bands at 1535  $\text{cm}^{-1}$  and 1628  $\text{cm}^{-1}$  are characteristic of the primary amine group  
483 of proteins (-CO-NH<sub>2</sub>) and the secondary amide group of proteins (-CO-NH), and both  
484 are characteristic of whey proteins. The band around 1035  $\text{cm}^{-1}$  corresponds to the C-  
485 O band stretching and can be related to the addition of glycerol (López-Rubio and  
486 Lagaron 2012; Pérez et al. 2016). Also, bands at 852  $\text{cm}^{-1}$  and 924  $\text{cm}^{-1}$  correspond to  
487 asymmetric and symmetric stretching vibrations of alkoxy groups (C-O-C) and are also  
488 originated by the presence of glycerol in the films (Cerqueira et al. 2012).

#### 489 3.3.7 X-ray Diffraction

490 The X-ray diffraction pattern was used to perceive if the addition of cells to the  
491 film caused alterations in the film crystallinity. Generally, in a crystalline material  
492 spectrum, several sharp peaks are present, while in amorphous substances, a broad  
493 background pattern is shown (Fernandes et al. 2014). XRD spectra of the analysed films  
494 (with and without *L. buchneri* UTAD104) showed similar profiles (Fig. 3b). As

495 expected, both films show a non-crystalline structure, presenting two broad peaks at  
496 approximately  $2\theta=11^\circ$  and  $2\theta=20^\circ$ . This second peak is present in the three raw  
497 materials (whey protein, glycerol and guar gum) that compose the tested films and is  
498 typical for amorphous structures (Martins et al. 2012), while  $2\theta=11^\circ$  is typically present  
499 in whey protein XRD pattern (Le Tien et al. 2000; Li et al. 2011).

#### 500 3.4. Antifungal activity of the bioactive films and coatings against *Penicillium* 501 *nordicum* in cheese

502 The effect of the tested WPC based films on fungal decay of cheese during their  
503 storage at 25 °C and 90% RH are presented in Fig. 4. After 30 days of storage, the total  
504 absence of fungal growth was only observed on cheese covered with films incorporating  
505 *L. buchneri* UTAD104 (Fig. 4d). In control films (without LAB and with *L. casei* UM3),  
506 the fungal colonies became visible with a delay of two days comparing to uncovered  
507 cheeses (data not shown). After 20 days of incubation, their surface was already entirely  
508 covered by *P. nordicum* (Fig. 4b and 4c). For the uncovered cheeses, this occurred soon  
509 after 7 days of incubation (Fig. 4a).

510 Afterwards, the WPC solutions were used to coat the cheese samples directly,  
511 eliminating the film-forming step, thus simplifying the application process. Coated  
512 cheese samples were stored similarly at 25 °C and 90% RH. After 5 days of storage, the  
513 uncoated cheese samples (Fig. 5a) were considerably infected by *P. nordicum*, with the  
514 fungi covering all the surface of the cheese sample. Cheeses coated with WPC without  
515 LAB and coated with WPC incorporating *L. casei* UM3 (Fig. 5b and 5c) also developed  
516 fungal contamination. However, when compared to uncoated samples, there was a  
517 considerable delay in the infection process. Thus, it seems the coating alone affected the  
518 development of the fungus (Fig. 5b), probably, because it created a barrier between the  
519 fungus and the nutritive cheese surface. Cheese samples coated with WPC incorporating  
520 the antifungal strain *L. buchneri* UTAD104 remained free of fungal contamination for  
521 the 30 days test period (Fig. 5d). Other authors reported similar results for different  
522 coatings materials. One work showed that ‘Coalho’ cheese coated with  
523 alginate/lysozyme nano-laminate coatings had no apparent microbiological  
524 contamination after 20 days, while uncoated cheeses showed evident fungal  
525 proliferation (Medeiros et al. 2014). Another work showed that chitosan coatings  
526 reduced infection in strawberries up to three days of storage when compared with non-

527 coated ones (Perdones et al. 2012). On the other hand, another study presented that  
528 sodium alginate and locust bean gum coatings delayed infection in oranges for 5 days  
529 when compared with uncoated fruits (Aloui et al. 2015).

530 When comparing both application strategies, the *P. nordicum* growth was less  
531 accentuated in coated cheese samples (Fig. 5) than in film covered ones (Fig. 4). The  
532 drying step required after the coating of the cheese can be responsible for this effect,  
533 since it lowered samples  $a_w$ , thus making fungal germination and colonization more  
534 difficult.

### 535 3.5. Mycotoxin analysis in cheese covered with films and coated

536 OTA content in cheeses samples is shown in Table 4. OTA in cheese covered  
537 with WPC based films was 83% inferior to the uncoated control, while those covered  
538 with films containing *L. casei* UM3 showed a reduction of 94% of OTA when  
539 compared with the uncoated control. In the cheese samples covered with WPC  
540 containing *L. buchneri* UTAD104, no OTA was detected. Since there was no fungal  
541 growth in those samples, this was expected. In the case of coated cheeses, OTA was  
542 only detected in uncoated samples. The absence (Fig. 5d) and weak growth of  
543 *P. nordicum* (Fig. 5 b and c) in these coats justify the absence of OTA in those samples.  
544 To our knowledge, none of the studies regarding the incorporation of LAB in edible  
545 films analysed the mycotoxin content of the product.

## 546 4. Conclusion

547 This study shows that WPC based films incorporating a LAB with antifungal  
548 properties could potentially be used in food packaging, contributing to an increased  
549 shelf-life of cheese. Moreover, it is demonstrated that the presence of cells did not  
550 significantly affect some essential characteristics of the films such as moisture content,  
551 WVP and mechanical properties. However, the presence of cells had an impact on the  
552 thickness and color of the films. Application of the film with the antifungal LAB strain  
553 to cheeses allowed the inhibition of *P. nordicum* growth for a storage period over 30  
554 days, in which  $1 \times 10^5$  CFU/mL of LAB were still viable. OTA amounts were  
555 substantially lower in cheese protected by the WPC based films and coatings.

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563 **Conflict of interest**

564 The authors declare that they have no conflict of interest.

565 **Bibliography**

- 566 Aloui, H., Licciardello, F., Khwaldia, K., Hamdi, M., & Restuccia, C. (2015). Physical properties and  
567 antifungal activity of bioactive films containing *Wickerhamomyces anomalus* killer yeast and  
568 their application for preservation of oranges and control of postharvest green mold caused by  
569 *Penicillium digitatum*. *International Journal of Food Microbiology*, *200*, 22-30.
- 570 Aziz, S. G.-G., & Almasi, H. (2018). Physical characteristics, release properties, and antioxidant and  
571 antimicrobial activities of whey protein isolate films incorporated with thyme (*Thymus vulgaris*  
572 L.) extract-loaded nanoliposomes. *Food and Bioprocess Technology*, *11*(8), 1552-1565.
- 573 Bahram, S., Rezaei, M., Soltani, M., Kamali, A., Ojagh, S. M., & Abdollahi, M. (2014). Whey protein  
574 concentrate edible film activated with cinnamon essential oil. *Journal of Food Processing and*  
575 *Preservation*, *38*(3), 1251-1258.
- 576 Ballesteros, L. F., Cerqueira, M. A., Teixeira, J. A., & Mussatto, S. I. (2018). Production and  
577 physicochemical properties of carboxymethyl cellulose films enriched with spent coffee  
578 grounds polysaccharides. *International Journal of Biological Macromolecules*, *106*, 647-655.
- 579 Bambace, M. F., Alvarez, M. V., & Moreira, M. d. R. (2019). Novel functional blueberries: Fructo-  
580 oligosaccharides and probiotic lactobacilli incorporated into alginate edible coatings. *Food*  
581 *Research International*, *122*, 653-660.
- 582 Bianchini, A., & Bullerman, L. B. (2009). Biological control of molds and mycotoxins in foods. In M.  
583 Appell, D. F. Kendra, & M. W. Trucksess (Eds.), *Mycotoxin Prevention and Control in*  
584 *Agriculture* (Vol. 1031, pp. 1-16, ACS Symposium Series). Washington, DC.: ACS Symposium  
585 Series, American Chemical Society.
- 586 Burgain, J., Gaiani, C., Linder, M., & Scher, J. (2011). Encapsulation of probiotic living cells from  
587 laboratory scale to industrial applications. *Journal of Food Engineering*, *104*, 467-483.
- 588 Campos, C. A., Gerschenson, L. N., & Flores, S. K. (2010). Development of edible films and coatings  
589 with antimicrobial activity. *Food and Bioprocess Technology*, *4*(6), 849-875.
- 590 Cerqueira, M. A., Souza, B. W. S., Teixeira, J. A., & Vicente, A. A. (2012). Effect of glycerol and corn  
591 oil on physicochemical properties of polysaccharide films - A comparative study. *Food*  
592 *Hydrocolloids*, *27*(1), 175-184.

- 593 Corsetti, A., Gobetti, M., Rossi, J., & Damiani, P. (1998). Antimould activity of sourdough lactic acid  
594 bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco*  
595 CB1. *Applied Microbiology and Biotechnology*, 50(2), 253-256.
- 596 Dai, L., Yuan, Y., Song, Z., Qiu, Y., & Yue, T. (2018). Preparation and characterization of *Lactobacilli*-  
597 loaded composite films with sustaining antipathogenic activity and preservation effect. *Journal*  
598 *of Food Science*, 83(10), 2511-2519.
- 599 Falguera, V., Quintero, J. P., Jiménez, A., Muñoz, J. A., & Ibarz, A. (2011). Edible films and coatings:  
600 Structures, active functions and trends in their use. *Trends in Food Science & Technology*, 22(6),  
601 292-303.
- 602 Fan, Y., Xu, Y., Wang, D., Zhang, L., Sun, J., Sun, L., et al. (2009). Effect of alginate coating combined  
603 with yeast antagonist on strawberry (*Fragaria × ananassa*) preservation quality. *Postharvest*  
604 *Biology and Technology*, 53(1-2), 84-90.
- 605 Fang, Y., Tung, M. A., Britt, I. J., Yada, S., & Dalgleish, D. G. (2002). Tensile and barrier properties of  
606 edible films made from whey proteins. *Journal of Food Science*, 67(1), 188-193.
- 607 Fernandes, R. V. B., Borges, S. V., Botrel, D. A., & Oliveira, C. R. (2014). Physical and chemical  
608 properties of encapsulated rosemary essential oil by spray drying using whey protein-inulin  
609 blends as carriers. *International Journal of Food Science and Technology*, 49(6), 1522-1529.
- 610 Gerez, C. L., Torres, M. J., Font de Valdez, G., & Rollán, G. (2013). Control of spoilage fungi by lactic  
611 acid bacteria. *Biological Control*, 64(3), 231-237.
- 612 Gialamas, H., Zinoviadou, K. G., Biliaderis, C. G., & Koutsoumanis, K. P. (2010). Development of a  
613 novel bioactive packaging based on the incorporation of *Lactobacillus sakei* into sodium-  
614 caseinate films for controlling *Listeria monocytogenes*. *Food Research International*, 43, 2402-  
615 2408.
- 616 Giraffa, G., Chanishvili, N., & Widyastuti, Y. (2010). Importance of lactobacilli in food and feed  
617 biotechnology. *Research in Microbiology*, 161(6), 480-487.
- 618 Guimarães, A., Abrunhosa, L., Pastrana, L. M., & Cerqueira, M. A. (2018a). Edible films and coatings  
619 as carriers of living microorganisms: a new strategy towards biopreservation and healthier  
620 foods. *Comprehensive Reviews in Food Science and Food Safety*, 17(3), 594-614.
- 621 Guimarães, A., Santiago, A., Teixeira, J. A., Venâncio, A., & Abrunhosa, L. (2018b). Anti-aflatoxicenic  
622 effect of organic acids produced by *Lactobacillus plantarum*. *International Journal of Food*  
623 *Microbiology*, 264, 31-38.
- 624 Guimarães, A., Venancio, A., & Abrunhosa, L. (2018c). Antifungal effect of organic acids from lactic  
625 acid bacteria on *Penicillium nordicum*. *Food Additives & Contaminants: Part A*, 35(9), 1803-  
626 1818.
- 627 Hymery, N., Vasseur, V., Coton, M., Mounier, J., Jany, J.-L., Barbier, G., et al. (2014). Filamentous  
628 fungi and mycotoxins in cheese: a review. *Comprehensive Reviews in Food Science and Food*  
629 *Safety*, 13(4), 437-456.
- 630 Kanmani, P., & Lim, S. T. (2013). Development and characterization of novel probiotic-residing  
631 pullulan/starch edible films. *Food Chemistry*, 141(2), 1041-1049.
- 632 Khwaldia, K., Perez, C., Banon, S., Desobry, S., & Hardy, J. (2004). Milk proteins for edible films and  
633 coatings. *Critical Reviews in Food Science and Nutrition*, 44(4), 239-251.
- 634 Kokoszka, S., Debeaufort, F., Lenart, A., & Voilley, A. (2010). Water vapour permeability, thermal and  
635 wetting properties of whey protein isolate based edible films. *International Dairy Journal*,  
636 20(1), 53-60.

- 637 Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., & Gobetti, M. (2000).  
 638 Purification and characterization of novel antifungal compounds from the sourdough  
 639 *Lactobacillus plantarum* strain 21B. *Applied and Environmental Microbiology*, 66(9), 4084-  
 640 4090.
- 641 Le Tien, C., Letendre, M., Ispas-Szabo, P., Mateescu, M. A., Delmas-Patterson, G., Yu, H. L., et al.  
 642 (2000). Development of biodegradable films from whey proteins by cross-linking and  
 643 entrapment in cellulose. *Journal of Agricultural and Food Chemistry*, 48(11), 5566-5575.
- 644 Li, Y., Jiang, Y., Liu, F., Ren, F., Zhao, G., & Leng, X. (2011). Fabrication and characterization of  
 645 TiO<sub>2</sub>/whey protein isolate nanocomposite film. *Food Hydrocolloids*, 25(5), 1098-1104.
- 646 López-Rubio, A., & Lagaron, J. M. (2012). Whey protein capsules obtained through electrospraying for  
 647 the encapsulation of bioactives. *Innovative Food Science and Emerging Technologies*, 13, 200-  
 648 206.
- 649 Martins, J. T., Cerqueira, M. A., Bourbon, A. I., Pinheiro, A. C., Souza, B. W. S., & Vicente, A. A.  
 650 (2012). Synergistic effects between  $\kappa$ -carrageenan and locust bean gum on physicochemical  
 651 properties of edible films made thereof. *Food Hydrocolloids*, 29(2), 280-289.
- 652 Medeiros, B. G. d. S., Souza, M. P., Pinheiro, A. C., Bourbon, A. I., Cerqueira, M. A., Vicente, A. A., et  
 653 al. (2014). Physical characterisation of an alginate/lysozyme nano-laminate coating and its  
 654 evaluation on 'Coalho' cheese shelf life. *Food and Bioprocess Technology*, 7(4), 1088-1098.
- 655 Mohammadi, R., Mortazavian, A. M., Khosrokhavar, R., & da Cruz, A. G. (2011). Probiotic ice cream:  
 656 viability of probiotic bacteria and sensory properties. *Annals of Microbiology*, 61(3), 411-424.
- 657 Mokrzycki, W. S., & Tatol, M. (2011). Colour difference  $\Delta E$  - A survey. *Machine graphics and vision*,  
 658 20(4), 383-411.
- 659 Niku-Paavola, M. L., Laitila, A., Mattila-Sandholm, T., & Haikara, A. (1999). New types of  
 660 antimicrobial compounds produced by *Lactobacillus plantarum*. *Journal of Applied*  
 661 *Microbiology*, 86(1), 29-35.
- 662 Özcelik, S., Kuley, E., & Özogul, F. (2016). Formation of lactic, acetic, succinic, propionic, formic and  
 663 butyric acid by lactic acid bacteria. *LWT - Food Science and Technology*, 73, 536-542.
- 664 Ozdemir, M., & Floros, J. D. (2008). Optimization of edible whey protein films containing preservatives  
 665 for water vapor permeability, water solubility and sensory characteristics. *Journal of Food*  
 666 *Engineering*, 86(2), 215-224.
- 667 Pavli, F., Kovaiou, I., Apostolakopoulou, G., Kapetanakou, A., Skandamis, P., Nychas, G. E., et al.  
 668 (2017). Alginate-based edible films delivering probiotic bacteria to sliced ham pretreated with  
 669 high pressure processing. *International Journal of Molecular Sciences*, 18(9), 1867.
- 670 Perdonés, A., Sánchez-González, L., Chiralt, A., & Vargas, M. (2012). Effect of chitosan–lemon  
 671 essential oil coatings on storage-keeping quality of strawberry. *Postharvest Biology and*  
 672 *Technology*, 70, 32-41.
- 673 Pereira, J. O., Soares, J., Sousa, S., Madureira, A. R., Gomes, A., & Pintado, M. (2016). Edible films as  
 674 carrier for lactic acid bacteria. *LWT - Food Science and Technology*, 73, 543-550.
- 675 Pereira, O. J., Soares, J., Monteiro, M., Gomes, A., & Pintado, M. (2018). Impact of whey protein coating  
 676 incorporated with *Bifidobacterium* and *Lactobacillus* on sliced ham properties. *Meat Science*,  
 677 139, 125-133.
- 678 Pérez, L. M., Piccirilli, G. N., Delorenzi, N. J., & Verdini, R. A. (2016). Effect of different combinations  
 679 of glycerol and/or trehalose on physical and structural properties of whey protein concentrate-  
 680 based edible films. *Food Hydrocolloids*, 56, 352-359.

- 681 Pérez-Gago, M. B., & Krochta, J. M. (2001). Denaturation time and temperature effects on solubility,  
682 tensile properties, and oxygen permeability of whey protein edible films. *Journal of Food*  
683 *Science*, 66(5), 705-710.
- 684 Pérez-Gago, M. B., Nadaud, P., & Krochta, J. M. (1999). Water vapor permeability, solubility, and tensile  
685 properties of heat-denatured versus native whey protein films. *Journal of Food Science*, 64(6),  
686 1034-1037.
- 687 Phan, T. D., Debeaufort, F., Luu, D., & Voilley, A. (2005). Functional properties of edible agar-based  
688 and starch-based films for food quality preservation. *Journal of Agricultural and Food*  
689 *Chemistry*, 53(4), 973-981.
- 690 Piermaria, J., Diosma, G., Aquino, C., Garrote, G., & Abraham, A. (2015). Edible kefir films as vehicle  
691 for probiotic microorganisms. *Innovative Food Science and Emerging Technologies*, 32, 193-  
692 199.
- 693 Prema, P., Smila, D., Palavesam, A., & Immanuel, G. (2010). Production and characterization of an  
694 antifungal compound (3-phenyllactic Acid) produced by *Lactobacillus plantarum* strain. *Food*  
695 *and Bioprocess Technology*, 3(3), 379-386.
- 696 Ramos, Ó. L., Fernandes, J. C., Silva, S. I., Pintado, M. E., & Malcata, F. X. (2012a). Edible films and  
697 coatings from whey proteins: A Review on formulation, and on mechanical and bioactive  
698 properties. *Critical Reviews in Food Science and Nutrition*, 52(6), 533-552.
- 699 Ramos, Ó. L., Pereira, J. O., Silva, S. I., Fernandes, J. C., Franco, M. I., Lopes-da-Silva, J. A., et al.  
700 (2012b). Evaluation of antimicrobial edible coatings from a whey protein isolate base to  
701 improve the shelf life of cheese. *Journal of Dairy Science*, 95(11), 6282-6292.
- 702 Ramos, Ó. L., Pereira, R. N., Rodrigues, R. M., Teixeira, J. A., Vicente, A. A., & Malcata, F. X. (2016).  
703 Whey and whey powders: Production and uses. In B. Caballero, P. M. Finglas, & F. Toldrá  
704 (Eds.), *Encyclopedia of Food and Health* (pp. 498-505). Oxford: Academic Press.
- 705 Ramos, Ó. L., Reinas, I., Silva, S. I., Fernandes, J. C., Cerqueira, M. A., Pereira, R. N., et al. (2013).  
706 Effect of whey protein purity and glycerol content upon physical properties of edible films  
707 manufactured therefrom. *Food Hydrocolloids*, 30(1), 110-122.
- 708 Ramos, Ó. L., Silva, S. I., Soares, J. C., Fernandes, J. C., Poças, M. F., Pintado, M. E., et al. (2012c).  
709 Features and performance of edible films, obtained from whey protein isolate formulated with  
710 antimicrobial compounds. *Food Research International*, 45(1), 351-361.
- 711 Reinoso, E., Mittal, G. S., & Lim, L.-T. (2008). Influence of whey protein composite coatings on plum  
712 (*Prunus domestica* L.) fruit quality. *Food and Bioprocess Technology*, 1(4), 314-325.
- 713 Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012). Lactic acid bacteria antimicrobial  
714 compounds: Characteristics and applications. *Food Engineering Reviews*, 4(2), 124-140.
- 715 Rhee, S. J., Lee, J.-E., & Lee, C.-H. (2011). Importance of lactic acid bacteria in Asian fermented foods.  
716 *Microbial Cell Factories*, 10(1), S5.
- 717 Rizzello, C. G., Cassone, A., Coda, R., & Gobbetti, M. (2011). Antifungal activity of sourdough  
718 fermented wheat germ used as an ingredient for bread making. *Food Chemistry*, 127(3), 952-  
719 959.
- 720 Sánchez-González, L., Saavedra, J. I. Q., & Chiralt, A. (2013). Physical properties and antilisterial  
721 activity of bioactive edible films containing *Lactobacillus plantarum*. *Food Hydrocolloids*,  
722 33(1), 92-98.
- 723 Sánchez-González, L., Saavedra, J. I. Q., & Chiralt, A. (2014). Antilisterial and physical properties of  
724 biopolymer films containing lactic acid bacteria. *Food Control*, 35(1), 200-206.

- 725 Schnurer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food*  
726 *Science and Technology*, 16(1-3), 70-78.
- 727 Settler-Ramírez, L., López-Carballo, G., Gavara, R., & Hernández-Muñoz, P. (2019). Antilisterial  
728 properties of PVOH-based films embedded with *Lactococcus lactis* subsp. *lactis*. *Food*  
729 *Hydrocolloids*, 87, 214-220.
- 730 Soukoulis, C., Behboudi-Jobbehdar, S., Yonekura, L., Parmenter, C., & Fisk, I. (2014a). Impact of milk  
731 protein type on the viability and storage stability of microencapsulated *Lactobacillus*  
732 *acidophilus* NCIMB 701748 using spray drying. *Food and Bioprocess Technology*, 7(5), 1255-  
733 1268.
- 734 Soukoulis, C., Behboudi-Jobbehdar, S., Yonekura, L., Parmenter, C., & Fisk, I. D. (2014b). Stability of  
735 *Lactobacillus rhamnosus* GG in prebiotic edible films. *Food Chemistry*, 159, 302-308.
- 736 Soukoulis, C., Singh, P., Macnaughtan, W., Parmenter, C., & Fisk, I. D. (2016). Compositional and  
737 physicochemical factors governing the viability of *Lactobacillus rhamnosus* GG embedded in  
738 starch-protein based edible films. *Food Hydrocolloids*, 52, 876-887.
- 739 Soukoulis, C., Yonekura, L., Gan, H.-H., Behboudi-Jobbehdar, S., Parmenter, C., & Fisk, I. (2014c).  
740 Probiotic edible films as a new strategy for developing functional bakery products: The case of  
741 pan bread. *Food Hydrocolloids*, 39, 231-242.
- 742 Ström, K., Sjögren, J., Broberg, A., & Schnürer, J. (2002). *Lactobacillus plantarum* MiLAB 393  
743 produces the antifungal cyclic dipeptides cyclo(l-Phe-l-Pro) and cyclo(l-Phe-trans-4-OH-l-Pro)  
744 and 3-phenyllactic acid. *Applied and Environmental Microbiology*, 68(9), 4322-4327.
- 745 Su, J.-F., Yuan, X.-Y., Huang, Z., Wang, X.-Y., Lu, X.-Z., Zhang, L.-D., et al. (2012). Physicochemical  
746 properties of soy protein isolate/carboxymethyl cellulose blend films crosslinked by Maillard  
747 reactions: Color, transparency and heat-sealing ability. *Materials Science and Engineering: C*,  
748 32(1), 40-46.
- 749 Tapia, M. S., Rojas-Grau, M. A., Rodríguez, F. J., Ramírez, J., Carmona, A., & Martín-Belloso, O.  
750 (2007). Alginate- and gellan-based edible films for probiotic coatings on fresh-cut fruits.  
751 *Journal of Food Science*, 72(4), 190-196.
- 752 Tharanathan, R. N. (2003). Biodegradable films and composite coatings: past, present and future. *Trends*  
753 *in Food Science & Technology*, 14(3), 71-78.
- 754 Varsha, K. K., & Nampoothiri, K. M. (2016). Appraisal of lactic acid bacteria as protective cultures.  
755 *Food Control*, 69, 61-64.
- 756 Ye, J., Ma, D., Qin, W., & Liu, Y. (2018). Physical and antibacterial properties of sodium alginate -  
757 sodium carboxymethylcellulose films containing *Lactococcus lactis*. *Molecules*, 23(10), 2645.  
758

759 **Figures captions**

760 **Fig. 1** Antifungal activity of WPC based films against *P. nordicum* by inoculation of a  
761 spore solution in the surface of the films. Films incorporating *L. casei* UM3 at 10% (a),  
762 20% (b) and 30% (w/w) concentration (c); films incorporating *L. buchneri* UTAD104  
763 at 10% (d), 20% (e) and 30% (w/w) concentration (f); samples without film - *P.*  
764 *nordicum* control (g)

765 **Fig. 2** Number of viable LAB (expressed in log CFU/mL) in the films over time (days).  
766 *L. buchneri* UTAD104 represented by the black line and *L. casei* UM3 represented by  
767 the grey line

768 **Fig. 3** FTIR (a) and X-ray diffraction pattern (b) obtained from films without (black)  
769 and with *L. buchneri* UTAD104 (grey)

770 **Fig. 4** Growth of *P. nordicum* after 30 days in uncovered cheese (a); cheese covered  
771 with WPC based film (b); cheese covered with WPC based film incorporating *L. casei*  
772 UM3 at 30% (w/w) (c); cheese covered with WPC based film incorporating *L. buchneri*  
773 UTAD104 at 30% (w/w) (d)

774 **Fig. 5** Growth of *P. nordicum* after 30 days in uncoated cheese (a); cheese coated with  
775 WPC based solution (b); cheese coated with WPC incorporating *L. casei* UM3 at 30%  
776 (w/w) (c); cheese coated with WPC incorporating *L. buchneri* UTAD104 at 30% (w/w)  
777 (d)

778

779 **Table 1**

780 Thickness, moisture content, water solubility, water vapour permeability (WVP), tensile strength  
781 (TS) and elongation at break (EB) of films without and with *L. buchneri* UTAD104.

WPC film	Thickness (mm)	Moisture (%)	Solubility (%)	WVP (g mm/ (day m <sup>2</sup> kPa))	TS (MPa)	EB (%)
Without LAB	0.23 ± 0.02 <sup>a</sup>	26.2 ± 0.9 <sup>a</sup>	43.7 ± 1.6 <sup>a</sup>	9.6 <sup>a</sup>	0.6 ± 0.06 <sup>a</sup>	53.8 ± 25.1 <sup>a</sup>
With <i>L. buchneri</i>	0.17 ± 0.03 <sup>b</sup>	25.0 ± 1.2 <sup>a</sup>	45.1 ± 0.9 <sup>b</sup>	10.0 <sup>a</sup>	0.5 ± 0.11 <sup>a</sup>	48.1 ± 21.3 <sup>a</sup>

782 <sup>a-b</sup> Different letters within the same column indicate significant differences ( $p < 0.05$ ).

783 **Table 2**

784 Colour parameters  $L^*$ ,  $a^*$  and  $b^*$  and opacity of WPC based films with and without  
785 *L. buchneri* UTAD104.

WPC film	$L^*$	$a^*$	$b^*$	Opacity (%)
Without LAB	$94.1 \pm 0.45^a$	$-0.4 \pm 0.03^a$	$10.5 \pm 0.33^a$	$12.6 \pm 0.30^a$
With <i>L. buchneri</i>	$90.6 \pm 0.65^b$	$0.8 \pm 0.56^b$	$22.4 \pm 0.79^b$	$13.4 \pm 0.24^b$

786 <sup>a-b</sup> Different letters within the same column indicate significant differences between film  
787 formulations ( $p < 0.05$ )

788 **Table 3**

789 Measurements of contact angle ( $\theta$ ) in function of time (s) after water droplet application  
 790 on films with and without cells of *L. buchneri* UTAD104.

Contact angle ( $\theta$ )		
Time (s)	Film without LAB	Film with <i>L. buchneri</i>
0	51.1 $\pm$ 2.86 <sup>a</sup>	51.1 $\pm$ 4.78 <sup>a</sup>
1	40.5 $\pm$ 2.24 <sup>a</sup>	41.1 $\pm$ 1.96 <sup>a</sup>
5	35.7 $\pm$ 2.17 <sup>a</sup>	36.6 $\pm$ 1.27 <sup>a</sup>
15	32.6 $\pm$ 2.23 <sup>a</sup>	34.2 $\pm$ 1.14 <sup>a</sup>
30	30.7 $\pm$ 1.69 <sup>a</sup>	31.7 $\pm$ 1.36 <sup>a</sup>
45	29.9 $\pm$ 1.47 <sup>a</sup>	25.8 $\pm$ 7.41 <sup>a</sup>
60	29.5 $\pm$ 1.39 <sup>a</sup>	20.8 $\pm$ 10.35 <sup>b</sup>

791 <sup>a-b</sup> Different letters within the same line indicate significant  
 792 differences between film formulations ( $p < 0.05$ )

793 **Table 4**  
 794 OTA (ng/mL) detected on cheese samples covered with WPC based films and  
 795 coatings.

Cheese	Films		Coatings	
	OTA (ng/mL)	Reduction (%)	OTA (ng/mL)	Reduction (%)
Uncoated	7.5 ± 1.7	-	8.8 ± 1.5	-
WPC film	1.3 ± 0.3	83	0	100
WPC + <i>L. casei</i>	0.4 ± 0.3	94	0	100
WPC + <i>L. buchneri</i>	0.0 ± 0.0	100	0	100

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