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Additional Information

1   **PHYSICAL AND BIOACTIVE PROPERTIES OF CORN STARCH -**

2   **BUTTERMILK EDIBLE FILMS**

3   Olga Moreno*, Clara Pastor, Justine Muller, Lorena Atarés, Chelo González, Amparo Chiralt.

4   Departamento de Tecnología de Alimentos – Instituto de Ingeniería de Alimentos para el

5   Desarrollo. Universitat Politècnica de València, Camino de Vera s/n 46022 Valencia, Spain.

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7

8   **Abstract:**

9   The effect of incorporating different ratios of both non-heated and heated (95 ºC) buttermilk

10   (BM) to corn starch (CS) films was analyzed in terms of its structural, mechanical, barrier,

11   optical and bioactive properties. The properties of the film forming dispersions (particle size

12   distribution, ζ-potential and rheological behavior) were also analyzed. As the BM increased in

13   the blend, both the average particle size and the apparent viscosity of the film forming

14   dispersions were reduced. The low degree of compatibility between both materials resulted in

15   heterogeneous structures, where an interpenetrated protein phase in the starch matrix was

16   observed as a result of the protein gelation when BM was heated. This affected the mechanical

17   and barrier properties giving rise to more resistant and extensible, and less permeable films

18   than in non-heated BM. Only films formulated with heated BM exhibited antioxidant activity,

19   probably due to the release of the antioxidant peptides during thermal treatment of proteins. BM

20   did not have any effect on the growth of *Listeria innocua*.

21

22   Keywords: edible films, corn starch, buttermilk, heat treatment.

23

24   **1. Introduction**

25   Currently, most of the plastics used are petroleum-derived (Saiah et al., 2009) and

26   about a third of the world's plastic production goes into packaging applications (Wiles, 2005).

27   The use of these non-biodegradable materials represents a huge worldwide environmental

28   problem (Azeredo, 2009), since these materials are highly polluting and their recycling implies a

29   great expense (Sánchez-García et al., 2008). To face up to this situation, much research work

*corresponding author: Olga Moreno. Fax: 0034 96 387 73 69 e-mail: olmomar1@upvnet.upv.es

30   has been focused on the substitution of synthetic plastics by biodegradable polymers

31   (biopolymers) obtained from renewable resources (Saiah et al., 2009), the use of which would

32   reduce the environmental impact of petroleum plastics (Sánchez-García et al., 2008).

33   Of the renewable sources with film-forming ability, polysaccharides are the most

34   abundant (Carvalho, 2008). Starch is often used due to its low cost and easy availability (Cuq et

35   al., 1997, Carvalho, 2008). Native starch becomes thermoplastic after heat treatment with

36   plasticizers, with properties similar to those of common synthetic polymers. The material

37   obtained is called thermoplastic starch (TPS).

38   The development of biodegradable packaging materials with adequate physical

39   properties (mechanical and water and gas barrier), with antimicrobial or antioxidant activity, is

40   especially relevant for food preservation. This stems both from reasons related to environmental

41   aspects and from consumer demand for safe and high quality products. The incorporation of

42   active compounds into food packaging increases the efficiency of food preservation. Packaging

43   becomes the vehicle for preservatives or compounds of interest from a nutritional point of view,

44   such as nutraceuticals.

45   Several authors have developed and characterized edible films based on starch of

46   different origins containing diverse bioactive ingredients (Pyla et al., 2010; Kechichian et al.,

47   2010; Shen et al., 2010; Mathew & Abraham, 2008). Buttermilk is a by-product of the butter-

48   making process, which is spray-dried to obtain a commercial powder, whose principal

49   compounds are lactose, proteins, fat and mineral salts. The breaking of the fat globule

50   membrane during the process releases a high quantity of proteins and membrane peptides with

51   bioactive properties, such as antioxidants and others with physiological effects (Affolter et al.

52   2010; Michalski & Januel, 2006). Previous studies have pointed out the antioxidant role of

53   buttermilk, (Wong & Kitts, 2001, 2003), and of different dairy peptides (Pihlanto, 2006). Both

54   lactoferrin and its derived peptide lactoferricin have been reported to have bactericidal,

55   fungicidal, and antiviral activities (Van der Kraan et al, 2004). The heat treatment of buttermilk

56   leads to the inactivation of its native flora and could release antimicrobial peptides from milk

57   proteins (Mills et al, 2011).

58   No previous studies have been found into the use of buttermilk to form films, despite

59   their bioactive properties and high protein content (whey protein) with film-forming ability. The

60   other compounds, lactose and minerals, would act as plasticizers which can reduce the

61   requirements of other agents to this end. Likewise, the blend of buttermilk with other film-

62   forming compounds, such as starch, might improve the functional properties of the film and its

63   bioactivity.

64   The objective of this work was to analyse the effect of buttermilk incorporation on the

65   properties of the film-forming dispersions and the physical (mechanical, barrier, optical) and

66   microstructural characteristics of corn starch films. The impact that heat treatment has on films

67   containing buttermilk was analyzed. The antioxidant and antimicrobial activities of the films were

68   also tested.

69

70   **2. Materials and methods**

71

72   2.1. Raw materials

73   Corn starch (CS) and buttermilk (BM), supplied respectively by Roquette Laisa España,

74   SA (Valencia, Spain) and Lactotecnia, S.L. (Barcelona, Spain), were used to obtain the films.

75   BM composition was: lactose (51%), proteins (31%), fat (7%) and salts (7%). Glycerol and

76   magnesium nitrate were purchased from Panreac Química S.L.U. (Barcelona, Spain). The

77   reactants for the antioxidant capacity assay – Trolox (6-hydroxy- 2,5,7,8-tetramethylchroman-2-

78   carboxylic acid), $K_2S_2O_8$ and ABTS (202-azino-bis-[3-etilbenzotiazol-6-sulfonic acid]) – were

79   supplied by Sigma-Aldrich (Madrid, Spain). For the antimicrobial activity analysis, stock culture

80   of *L.innocua* (CECT 910) was supplied by the Spanish Type Culture Collection (CECT,

81   Burjassot, Spain). Tryptone Soy Broth, Agar bacteriological and tryptone phosphatewater were

82   provided by Scharlab, (Barcelona, Spain). NaCl was purchased from (Panreac, Barcelona,

83   Spain).

84

85   2.2. Preparation of film forming dispersions (FFD)

86   CS was dispersed at 3% (w/w) in distilled water and stirred for 5 min at room

87   temperature. Then the dispersion was heated at 95ºC for 30 min to induce starch gelatinization

88   and cooled down under running water to reach room temperature. Glycerol was added as a

89   plasticizer in a CS:glycerol ratio of 1:0.25 (Jiménez et al., 2012, Talja et al., 2007, Teixeira et al.,

90   2007). Distilled water was added to adjust the concentration, and homogenization was carried

91   out in a rotor-stator ultraturrax (DI25, Janke and Kunkel, Germany) at 13,500 rpm for 4 min. CS

92   dispersion was degasified for 15 min at room temperature by means of a vacuum pump (MZ 2C

93   NT, Vacuubrand GMBH + CO KG, Wertheim, Germany). BM (3% w/w) was dispersed in

94   distilled water and stirred for 5 min at room temperature. Glycerol was added in a BM:glycerol

95   ratio of 1:0.25, and the dispersion was stirred at room temperature for another 10 min. Finally,

96   both suspensions were mixed in four different CS:BM w/w ratios (1:0, 0.75:0.25, 0.50:0.50,

97   0.40:0.60) and kept under stirring at room temperature for 10 min.

98   A second series of FFDs was prepared with the aim of testing the effect that heat

99   treatment had on buttermilk. In this case, both dispersions were mixed prior to heating them for

100   30 min at 95ºC. The resulting formulations were referred to as $CS_{0.75}:BM_{0.25}$ Q, $CS_{0.50}:BM_{0.50}$ Q

101   and $CS_{0.40}:BM_{0.60}$ Q.

102

103   2.3. Characterization of the film-forming dispersions

104

105   2.3.1. Particle size, pH and ζ-potential

106   The particle size analysis of the FFDs was carried out by using a laser scattering

107   instrument (MasterSizer 2000, Malvern Instruments,UK). The samples were dispersed in

108   distilled water at 2,000 rpm until an obscuration rate of 8-10% was obtained. The Mie theory

109   was applied by considering a refractive index of 1.52 and absorption of 0.1. Three samples of

110   each FFD were measured at 25ºC. Two average diameters were obtained: the area-volume

111   mean diameter (d_{32}), which is related to the average surface area of droplets exposed to the

112   continuous phase per unit volume of emulsion, and the volume-length diameter (d_{43}), which is

113   the sum of the volume ratio of droplets in each size-class multiplied by the mid-point diameter of

114   the size-class.

115
$$d_{3,2} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}$$
(Eq. 1)

116
$$d_{4,3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$$
(Eq. 2)

117

118   The pH of the FFDs was measured in triplicate at 25ºC by using a pH-meter

119   (SevenEasy, Mettler-Toledo, S.A.E, Barcelona, Spain). Prior to the measurement of Zeta

120   potential (ζ-potential), FFDs were diluted to a droplet concentration of 0.02% (w/v) using distilled

121   water. ζ-potential was determined in triplicate by measuring the electrophoretic mobility of the

122   dispersed particles in a charged field by using ZetaSizer equipment (Nano-Z, Malvern

123   Instruments, UK). The Smoluchowsky mathematical model was used by the software to convert

124   the electrophoretic mobility measurements into ζ-potential values.

125

126   2.3.2. Rheological behaviour

127   The rheological behaviour of the FFDs was analysed in triplicate at 25ºC by means of a

128   rotational rheometer (HAAKE RheoStress 1, Thermo Electric Corporation, Germany) with a type

129   ISO 3219 Z34DIN sensor system of coaxial cylinders. Rheological curves were obtained after a

130   stabilization time of 5 min at 25ºC. Shear stress (σ in Pa) was measured as a function of shear

131   rate ($\dot{\gamma}$ in s^{-1}) from 0 to 512s^{-1} in the following way: 5 min to reach the maximum shear rate and

132   5 min to attain zero shear rate. The power law model (Eq. 3) was applied to determine both the

133   consistency index (K in Pa.s^n) and the flow behaviour index (n). Additionally, the apparent

134   viscosity (η_{ap}) at 100 s^{-1} was determined.

135    $$\sigma = K \cdot \dot{\gamma}^n \qquad\qquad\qquad (\text{Eq. 3})$$

136

137   2.4. Film preparation

138   FFDs were poured onto framed and levelled polytetrafluorethylene (PTFE) plates (15

139   cm diameter) and were dried for at least 24 h under natural convection at 25ºC and 45(± 2)%

140   relative humidity (RH). Film thickness was controlled by pouring the amount of FFD onto the

141   PTFE plate that would provide a surface density of solids of 56 g/m^2. Dry films were peeled off

142   the casting surface and preconditioned for 14 days in desiccators at 25ºC and 54% RH, by

143   using an oversaturated $Mg(NO_3)_2$ solution.

144

145   2.5. Film characterization

146

147   2.5.1. Film thickness

148   A hand-held digital micrometer (Electronic Digital Micrometer, Comecta S.A.,

149   Barcelona, Spain) was used to measure film thickness to the nearest 0.0001mm. This was

150   measured in triplicate for samples submitted to mechanical tests and water vapour permeability

151   analyses.

152

153   2.5.2. Microstructure

154   Cross-section images of the films were obtained by using Scanning Electron

155   Microscopy (SEM) with a JEOL® microscope, model JSM-5410. The samples were immersed

156   in liquid nitrogen and cryofractured. After gold coating, the samples were observed using an

157   accelerating voltage of 10 kV.

158   The surface morphology of studied films, previously dried with P_2O_5, was observed by

159   using Atomic Force Microscopy (AFM), with an 8 multimode microscope, Bruker AXS, (Santa

160   Barbara, California), under V NanoScope® electronic control. Three 1x1 cm square samples

161   per formulation were cut and the surface scanning was carried out by using the tapping mode,

162   on a 50x50 μm area and with a maximum vertical limit of 6 μm. According to the ASME B46.1

163   (1995) method, the following statistical parameters, related to the surface roughness of each

164   sample, were calculated:

165   Average roughness (R_a): arithmetic average of the absolute values of height deviations

166   from a mean surface (Eq. 4)

167
$$R_a = \frac{1}{N}\sum_{J=1}^{N}\left|Z_j\right| \qquad \text{(Eq. 4)}$$

168   Root-mean-square roughness (R_q): root-mean-square average of height deviations

169   taken from the mean data plane (Z_j) (Eq. 5)

170
$$R_q = \sqrt{\frac{\sum_{J=1}^{N}Z_J^2}{N}} \qquad \text{(Eq. 5)}$$

171   The percentage image surface area difference (%ISAD) was also calculated. This

172   parameter represents the difference between the image's three-dimensional surface area and

173   the two-dimensional projected surface area. Surface images were also obtained by using the

174   Phase Imaging mode, which allows surface variations of the composition, adhesion, friction,

175   viscoelasticity and other properties to be detected.

176

177   2.5.3. Mechanical properties

178   A texture analyser (TA-XTplus, Stable Micro Systems, Surrey, United Kingdom) was

179   used to measure the mechanical properties of films equilibrated at 54% RH and 25ºC. Film

180   strips (25.4 mm wide and 100 mm long) were mounted in the tensile grips (A/TG model) and

181   stretched at a rate of 50 mm/min until breaking. The elastic modulus (EM), tensile strength at

182   break (TS) and percentage of elongation at break (%E) were determined from stress-Henky

183   strain curves, obtained from force-deformation data. The experiments were carried out at 25ºC

184   on twelve replicates per formulation.

185

186   2.5.4. Moisture content

187   In order to determine the moisture content of the films, six samples of each formulation

188   were dried at 60ºC for 24 h in a natural convection oven, and for another 24 h in a vacuum oven

189   (60ºC). Afterwards, the samples were placed into desiccators with P_2O_5 at room temperature,

190   until constant weight was reached.

191

192   2.5.5. Water vapour permeability

193   The water vapour permeability (WVP) of films was measured with a modification of the

194   ASTM E96-95 (ASTM, 1995) gravimetric method, using Payne permeability cups (Elcometer

195   SPRL, Hermelle/s Argenteau, Belgium) of 3.5 cm in diameter. For each formulation,

196   measurements were replicated six times and WVP was calculated following the methodology

197   described by Gennadios et al. (1994), at 25ºC and a 54-100% relative humidity gradient, which

198   was generated by using an oversaturated $Mg(NO_3)_2$ solution and pure water, respectively. To

199   determine WVP, the cups were weighed every 2 h, for 10 h. After the steady state was reached,

200   the slope obtained from the weight loss *vs.* time was used to calculate WVP, according to

201   ASTM (1995).

202

203   2.5.6. Optical properties

204   The optical properties of the films were determined in film samples previously

205   equilibrated at 25ºC and 54% RH. CIE-L*a*b* coordinates: lightness (L_{ab}*), chrome (C^*_{ab}) and

206 hue (h^*_{ab}) of the films were obtained through the surface reflectance spectra determined by

207 means of a spectrocolorimeter (CM-3600d, Minolta Co., Tokyo, Japan) with a 10-mm diameter

208 window, using D_{65} illuminant/10º observer. Measurements were taken on black and white

209 backgrounds and the reflectance infinite (R_∞) was determined. The whiteness index (WI) was

210 calculated using equation 6:

$$WI = 100 - \sqrt{(100 - L*) + a*^2 + b*^2}$$ (Eq. 6)

212

213 The internal transmittance (T_i) of the films was determined by applying the Kubelka–

214 Munk theory (Hutchings, 1999) for multiple scattering to the reflection spectra, following the

215 methodology described by Pastor et al. (2010).

216

217 The gloss was measured on the film side in contact with air during drying, at a 60º

218 incidence angle according to the ASTM standard D-523 (ASTM, 1999), using a flat surface

219 gloss meter (Multi-Gloss 268, Minolta Co., Tokyo, Japan). Six replicates were obtained per

220 formulation. All the results are expressed as gloss units, relative to a highly polished surface of

221 black glass standard with a value near to 100.

222

223   2.5.7. Antioxidant activity

224 The antioxidant capacity of BM and of the films was determined through a

225 spectrophotometric method, as described by Re et al. (1999). The objective of this method is to

226 compare the antioxidant activity of the analyzed substance with that of an antioxidant standard,

227 trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a vitamin E analogue.

228 ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]) was dissolved in water

229 to a concentration of 7 mM, and allowed to react with a 2.45 mM potassium persulfate solution

230 (final concentrations) for 16 h in the dark. ABTS radical cation ($ABTS^{\cdot+}$), a blue chromophore,

231 was produced during that period. The $ABTS^{\cdot+}$ solution was diluted with ethanol until an initial

232 absorbance of 0.70 (±0.02) at 734 nm (A_0). 10μl of the test solution was added to 1 mL of this

233 solution, and the percentage of absorbance reduction at 6 minutes was registered. All

234 absorbance measurements were taken with a Beckman Coulter DU 730 spectrophotometer,

235 using ethanol as blank. The test solutions (BM or film extracts) were prepared by completely

236 dissolving 0.5 g of the BM or film in 10 ml of bidistilled water, and stirring for 16 h. Tests were

237 performed in triplicate.

238 A calibration curve (% absorbance reduction *vs*. concentration of Trolox) was obtained

239 with different dilutions (0 mg/l to 50 mg/l) of trolox as standard antioxidant agent. The Trolox

240 equivalent antioxidant capacity (TEAC) of BM and films was defined as the concentration of BM

241 or dry film (g BM or dry film /l) producing the same perceptual absorbance reduction as 1mM

242 Trolox.

243

244 2.5.8. Antimicrobial activity

245 The antimicrobial properties of the films were analyzed through the agar disk diffusion

246 method adapted from Kristo et al. (2008). Stock culture of *L.innocua* (CECT was kept frozen (-

247 18ºC) in Tryptone Soy Broth (TSB) supplemented with 30% of glycerol. To regenerate the

248 culture, a loopful was transferred into 10 mL of TSB, the tube was incubated at 37ºC overnight

249 and 10μL were again transferred into 10 mL of TSB. The tube was kept at 37ºC for 48h to reach

250 the exponential phase of growth. Finally, the culture was adequately diluted for the inoculation

251 of the agar plates in order to obtain 10^2 UFC/cm^2 target inocula.

252 Tryptone Soy Agar - TSB with Agar bacteriological with 3% NaCl- was used as a model

253 solid food system (TSA-NaCl) (Sánchez-González et al., 2011). Under sterile conditions, 20 g of

254 TSA-NaCl were poured into each Petri dish and left to solidify. The culture of *Listeria innocua*

255 was properly diluted and inoculated on the surface of the culture medium. Then, a maximally

256 concentrated solution of heated buttermilk (10% w/w) was homogenously poured onto the

257 inoculated surface to provide the same surface density of BM as the films (44.8 g/m^2), and the

258 plates were dried under sterile conditions for 2 hours. Inoculated and uncoated TSA-NaCl Petri

259 dishes were used as control. Plates were then covered with parafilm to avoid dehydratation and

260 stored at 10ºC for 13 days. Counts were made periodically during the storage period. To this

261 end, the agar was removed aseptically from the Petri dishes and placed in a sterile plastic bag

262 with 100 mL of Tryptone phosphate-water. Homogenization was performed for 2 minutes in a

263 Stomacher blender (Bag Mixer 400, Interscience). Then, serial dilutions were made and poured

264 onto TSA. Plates were incubated at 37 ºC for 24 h before colonies were counted. All tests were

265 run in triplicate.

266

267   2.6. Statistical analysis

268   A statistical analysis of data was performed through analyses of variance using

269 Statgraphics® Plus for Windows 5.1. Homogeneous sample groups were obtained by using

270 LSD test (95% significance level).

271

272   3. Results and discussion

273

274   3.1. Properties of the film-forming dispersions

275

276   3.1.1. Particle size, pH and ζ-potential

277   Figure 1 shows the particle size distributions of FFDs. The dispersions showed similar

278 distributions with a main peak at about 10-30 μm. The fact that the formulation without

279 buttermilk ($CS_1:BM_0$) presents a particle size distribution which is detectable in the range of the

280 equipment used, suggests the formation of amylose aggregates. The amylose concentration in

281 the system is lower than the critical concentration for gel formation, since the dispersions did not

282 form gels at rest when cooling. Nevertheless, this does not limit the possible association of the

283 amylose chains, probably through the helical conformation zones. The addition of BM resulted

284 in a displacement of the main peak towards slightly smaller particles, although distributions

285 were wider. The samples with the lowest ratio of BM submitted to heat treatment exhibit a more

286 similar distribution to the one containing only starch. Table 1 shows the results obtained for

287 diameters $d_{3\,2}$ and $d_{4,3}$ for every formulation. Coherently with the distributions shown in Figure 1,

288 an increase in the BM ratio led to smaller average $d_{3,2}$ and $d_{4,3}$ diameters. This could be

289 attributed to an inhibition effect of the amylose aggregation in the more complex composition

290 system, while other smaller particles, such as fat globules from BM, contribute to the light

291 dispersion pattern. The heat treatment of the buttermilk led to a slight increase in the average

292 diameters, probably because heat induces whey protein denaturation and aggregation, thus

293 increasing particle size. In this sense, Nicolai et al, 2011 report that the rate at which protein

294 aggregation takes place increases as the temperature rises. The major whey protein in milk is β-

295 lactoglobuline, and its aggregation kinetics is known to govern that of the whole whey protein

296   (Ndoye et al., 2013). Two factors, namely heat and calcium concentration, affect whey protein

297   aggregation. The presence of calcium divalent ions enhances heat-induced whey protein

298   aggregation and strongly influences the size of whey protein aggregates (Ndoye et al, 2013,

299   Nicolai et al, 2011).

300   The values of ζ-potential are shown in Table 1. The corresponding values for the

301   sample without BM ($CS_1:BM_0$) were slightly negative, as a result of the adsorption of negative

302   ions from the aqueous medium onto neutral starch chains. When adding BM, lipid particles

303   appear in the medium probably coated by whey proteins, as well as whey protein aggregates

304   when BM is heat treated. At the pH of the system (about 6), which is higher than the isoelectric

305   point of the proteins, the carboxylic groups of amino acids are dissociated. Therefore, the

306   adsorbed proteins induced a greater negative net charge on the particles present (Pelegrine &

307   Gasparetto, 2005). Heating BM provokes a significant pH increase, probably due to the partial

308   depolymerization of protein and the release of aminoacids into the medium. The pH increase

309   did not have a significant impact on ζ-potential, due to the fact that it is still higher than the

310   protein isoelectric point.

311   Particle size and ζ-potential have a great impact on both the FFD stability and on the

312   changes which take place during their drying step in the film formation process. The progressive

313   water loss can promote flocculation of the particles and coalescence and creaming phenomena,

314   which will affect the film's microstructure and functionality. The viscosity of the FFDs also plays

315   an important role in the described phenomena, since it greatly contributes to the stabilization of

316   the dispersion, thus limiting the coalescence and creaming which may imply a more

317   homogeneous film structure.

318

319   3.1.2. Rheological behaviour

320   Figure 2 shows the flow curves for the different film-forming dispersions, and the

321   rheological parameters resulting from fitting the Ostwald de Waale law (Eq. 3) are shown in

322   Table 1, together with the apparent viscosity at 100 s^{-1}. The FFDs exhibited shear thinning

323   behaviour in the range of the shear rate considered, but tend towards Newtonian behaviour

324   when the BM ratio increases. The formulation without BM ($CS_1:BM_0$) showed the lowest flow

325   behaviour index (n), which tended towards 1 as the BM ratio increased in the mix. The

326 formulations with the highest quantity of BM ($CS_{0.40}:BM_{0.60}$ and $CS_{0.40}:BM_{0.60}$ Q) exhibited

327 Newtonian behaviour and the lowest values of viscosity. No notable effect of the thermal

328 treatment of BM was observed on the sample rheological behaviour. This indicates that the

329 possible protein aggregations did not have an impact on the flow properties.

330   As the BM ratio increased in the mix, a progressive decrease in apparent viscosity (η_{ap})

331 was observed, both with heated and non-heated BM. This can be explained by to the lower

332 average molecular weights of the components (the highest molecular weight compound of BM

333 is whey protein, whose molecular size is lower than that of starch chains) and the subsequent

334 decrease in the hydrodynamic volume of polymeric chains. For macromolecules, the higher the

335 molecular weight, the higher the hydrodynamic volume and the higher thickening power.

336 Additionally, the incorporation of ionic and polar solutes from BM makes the solvent poor for the

337 macromolecules, which, in turn, reduces their hydrodynamic volume and intrinsic viscosity

338 (Dickinson and Stainsby, 1982). Greater hydrodynamic volumes are more sensitive to shear

339 rate since they can be easily deformed during shear giving rise to the shear-thinning effects. As

340 commented on above, as the BM ratio increased, the formation of amylose aggregates seemed

341 to be limited, which could also contribute to the drop in viscosity.

342

343   3.2. Film Properties

344

345   3.2.1. Microstructure of the films

346   Figure 3a shows the cross-section SEM images of CS films and CS:BM blend films with

347 non-heated BM, while Figure 3b shows the corresponding images of CS:BM blend films with

348 heated BM. For films without buttermilk ($CS_1:BM_0$), a rather homogenous structure was

349 observed, with linear formations in the direction of the water flow during film drying, which could

350 be attributed to amylose crystalline associations. Different authors (Gelders et al. 2004; Famá et

351 al. 2005), have reported the formation of V type crystalline shapes of oriented amylose helixes,

352 which are formed by complexing lipids or other non-polar molecules, which can be endogens of

353 starch. The formation of these helicoidal complexes and their aggregates is coherent with the

354 results commented on in section 3.1.1. On the other hand, the continuous matrix fractures more

355 irregularly when the BM content is high, due to a heterogeneous distribution of components with

356 areas of different mechanical resistance. This suggests the coexistence of two phases: a

357 starch-rich phase and a protein-rich phase, resulting from the lack of compatibility of both

358 polymers. This occurs for both film series, with and without BM heating.

359 In films with a higher BM content, small irregularly-shaped lipid particles are clearly

360 observed corresponding to solid dairy fat during film formation (in many cases, the hole that

361 these left as the film is cryofractured is observed). In films with heated buttermilk, a great deal

362 fewer holes from irregular lipid particles were observed. When comparing the two films with the

363 highest buttermilk ratio, it was observed that films with heated BM showed a better inclusion of

364 the lipids in the matrix and a more cohesive structure, which could be explained by the

365 formation of protein gel during the film drying, as reported by Nicolai et al. (2011). Zuniga et al.

366 (2010) also observed curved linear aggregates (strands) of milk whey protein caused by heating

367 at pH 6.8, which at a high enough concentration, and in the presence of salts, would form gel.

368 Figure 4 presents the topographic images of the surface of the films obtained by AFM

369 (*Phase Imaging* mode). In these images, the differences in the mechanical response (or other

370 properties) at different points on the surface of each sample can be seen. In the $CS_1:BM_0$

371 sample, the heterogeneous response of different surface zones can be observed, which could

372 be explained by the presence of crystalline areas of amylose. In films with BM, the surface

373 heterogeneity increases along with the ratio of BM, which agrees with the formation of two

374 phases in the polymer matrix: one rich in starch and the other in proteins, each one of them with

375 different properties. The area of zones with different surface properties increased when the BM

376 proportion rose, which confirms the phase separation process with the formation of a great

377 amount of protein-rich fraction.

378

379 3.2.2. Mechanical properties and equilibrium moisture content

380 Table 2 shows the thickness, the mechanical parameters and the equilibrium moisture

381 content of the films equilibrated at 54% RH. Thickness was significantly affected by the heat

382 treatment ($p<0.05$) but not by the CS:BM ratio ($p>0.05$). Apparently, the changes induced by the

383 heat treatment on the structure of buttermilk greatly affect the interactions with corn starch and

384 the molecular arrangement of the film components, causing an increase in film thickness.

385   The mechanical parameters obtained for the $CS_1:BM_0$ films are similar to those reported

386   by Jiménez et al. (2012). The addition of BM to the formulation significantly affected the elastic

387   modulus and the tensile strength at break (p<0.05). For both series, as the BM proportion

388   increased the films became less rigid and less resistant, which is coherent with the appearance

389   of a dispersed phase of protein-rich polymer which limits the cohesion forces of the starch

390   matrix. The incompatibility of both materials results in an interrupted starch matrix, leading to the

391   reduction of its mechanical resistance. In addition, BM incorporation is associated with a high

392   proportion of non-polymeric solids, such as lactose and salts, which do not contribute to the

393   strength of the polymeric network and that promote the plasticization of the matrix. However, in

394   films with the highest amount of buttermilk, heating had a positive impact on the mechanical

395   behaviour, since EM and TS were much higher than those of the non-heated sample. As

396   commented on above, in films with heated BM, aggregates may form a gel during the film drying

397   when the critical concentration for gel formation is reached, which seems to have a positive

398   impact (increased EM and TS) on the mechanical properties. This gel is formed by cross-linked

399   protein strands above a critical concentration and this aggregation progresses when the film

400   loses the remaining water till it is totally dry. Moreover, the presence of calcium salts in the

401   medium reduces the critical concentration for gel formation, having a positive impact on the gel

402   strength (Nicolai et al., 2011). According to Baussay et al. (2004), the critical gelation

403   concentration at pH 7 is less than 1g l^{-1} in 1mM $CaCl_2$ (0.111g l^{-1}). In the films studied, the

404   protein concentration for $CS_{0.4}:BM_{0.6}$ Q sample was 5.4 gl^{-1} at the beginning of the film drying,

405   while BM provided the medium with a substantial amount of salts (1.3 g l^{-1}) including $CaCl_2$.

406   Consequently, it can be concluded that a protein gel was formed during film drying when

407   heating was applied to BM formulations, which contributes to the cohesion of the film structure,

408   having a positive impact on the stiffness and resistance of the films.

409   The stretchability of the films depended on the BM ratio and the heat treatment. For

410   films with only corn starch, the value was in the order of that obtained by Jimenez et al. (2012)

411   for films with the same composition. In films with non-heated BM, a significant elongation

412   increase was found when the BM ratio was 60 %. The $CS_{0.4}:BM_{0.6}$ formulation was the most

413   extensible, while showing the lowest EM and TS values (Table 2). This behaviour is explained

414   by both the high proportion of lactose in the film coming from BM (which acts as a plasticizer),

415   and the high equilibrium moisture content of these films (Table 2). The high ratio of ionic and

416   low molecular weigh solutes (salts and lactose) contributed to such a high moisture content. So,

417   the results suggest that the highest BM proportion is critical, since it introduces a notable

418   amount in this type of solids which favours the water sorption capacity of the film by solvent

419   effects when they equilibrate at 54%RH (Fabra et al., 2010). The plasticizing effects of the

420   adsorbed water and lactose make these films both very soft and poorly resistant, even though

421   they are very extensible. However, at the same BM proportion, films with heated BM appeared

422   less hygroscopic and extensible (Table 2). It is likely that salts and lactose were trapped in the

423   formed protein gel network, rendering them less available for water interaction, thus reducing

424   the water retention capacity of the film. Heating the BM greatly reduced the film extensibility in

425   all cases, which agrees with the formation of the above- mentioned protein network after the

426   gelling process. This reduces the possibility of the polymer chain slippage during the extension

427   test, in line with the cross-linking effect induced by gel formation.

428

429   3.2.3. Water vapour permeability

430   Values of water vapour permeability (WVP) at 25°C and 54-100% RH gradient are

431   shown in Table 2. The WVP found for films without BM is comparable to that reported in

432   previous studies (Jiménez et al., 2012). The addition of non-heated BM up to 50% did not entail

433   significant changes in the water vapour barrier properties of the films. The presence of lipid

434   compounds incorporated with BM (which, in principle, could favour the barrier effects) is actually

435   mitigated because of the lower structural cohesion of the films caused by the non-compatible

436   compounds, which favours the mass transfer across the film. In this sense, the film formulation

437   with the highest content of non-heated BM, presented the highest WVP value ($p<0.05$), which

438   can be attributed to its remarkable structural heterogeneity and to its higher moisture content,

439   which plasticizes the matrix, promoting molecular mobility and mass transfer through the film.

440   Nevertheless, the equivalent films with heated BM showed lower WVP values, in agreement

441   with the previously commented on protein network formation; this contributed to the greater

442   cohesion of the film components, limiting mass transfer processes.

443

444   3.2.4. Optical properties

445   Table 3 shows the colour parameters of all formulations: lightness (L*), chrome (C_{ab}*)

446 and hue (h_{ab}*), together with the whiteness index (WI). The incorporation of BM into CS films,

447 provoked a drop in the lightness of films with non-heated BM, which was statistically significant

448 for $CS_{0.40}$:$BM_{0.60}$ samples ($p<0.05$). On the contrary, the films with heated BM experienced a

449 significant increase in lightness ($p<0.05$) as compared to the corn starch films. This difference in

450 the lightness behaviour can be attributed to the different film structure, which affect film-light

451 interactions and final colour parameters. The saturation of yellowness of the films notably

452 ($p<0.05$) increased for both series along with the BM ratio. This is due to the natural colour of

453 the BM (slightly yellow) which affect the overall matrix colour. The whiteness index (WI) was

454 coherently modified, and it decreased as the BM proportion increased. These effects were less

455 sensitive to the BM ratio when it was heated due to the heat induced structural differences

456 commented on above.

457   Figure 5 shows the spectral distribution of the internal transmittance (T_i) of the films, as

458 an indicator of the translucency level. The internal transmittance decreased as the BM content

459 increased, mainly at low wavelengths, which indicates a rise in the film opacity. This is coherent

460 with the formation of a more heterogeneous structure with changes in the refraction index

461 through the film structure, which promotes light dispersion. As the ratio of dispersed phase (BM

462 components) becomes higher, a loss of transparency occurs. The films with heated BM showed

463 smoother curves as compared to non-heated, and were less transparent. This also agrees with

464 the formation of a more compact and complex structure, in line with the formation of the protein

465 gel and network (as shown in Figures 3 and 4), which caused the loss of film transparency.

466   The gloss values of the films are shown in Table 4. The measurements were taken at a

467 60° angle, for the requirements of the standard to be fulfilled, according to the gloss intensity of

468 the samples. Every film had average gloss values ranging between 10 and 40, meaning that

469 they could be considered mostly matt. The incorporation of non-heated buttermilk caused the

470 starch films to lose gloss, whereas the opposite was observed when they contain heated

471 buttermilk. The generation of a different structure due to heating and the promotion of the

472 protein network favours gloss development, in all likelihood due to the fact that the surface

473 formed is smoother.

474   Table 4 also shows the surface roughness values (R_a, R_q and Image Surface Area

475   Difference %), obtained from the AFM images. It was observed that, despite the high variability

476   of the roughness values, a tendency can be established: the rougher the surface, the lower the

477   gloss, as found by other authors (Villalobos et al. 2005; Fabra et al. 2009). The $CS_{0.40}:BM_{0.60}$

478   films showed the highest roughness and the lowest gloss ($p<0.05$) and at the highest buttermilk

479   ratio, heat treatment led to a gloss increase (as previously commented on) and a decrease in

480   roughness.

481

482   3.2.5. Antioxidant activity

483   The antioxidant capacity of BM (heated and non-heated) was analyzed and expressed

484   as the Trolox Equivalent Antioxidant Capacity (TEAC), or the concentration of BM (g/L) that

485   produces the same inhibition percentage of absorbance as 1mM trolox solution. The TEAC

486   values were 76 and 70 g BM/L, respectively for heated and non-heated BM. This means that

487   the thermal treatment did not notably affect the antioxidant capacity of the components.

488   The antioxidant potential of proteins derived from dairy products has been repeatedly

489   reported in literature (Allen and Wrieden, 1982; Colbert and Decker, 1991; Stuchell and

490   Krochta, 1995; Maté et al., 1996). Lactoferrin, present in the whey fraction of milk, has affinity

491   for iron and inhibits iron-catalyzed oxidation in iron-supplemented infant formulas (Satué-Gracia

492   et al., 2000) and liposomal containing phospholipid systems (Wakabayashi et al., 1999). Wong

493   & Kitts (2003) concluded that the reducing activity in buttermilk was mainly attributed to the

494   sulfhydryl content.

495

496   Table 4 shows the TEAC values of the films, expressed as the concentration of dry film

497   (g of dry film/L) that produces the same inhibition percentage of absorbance as 1mM trolox

498   solution. Films without buttermilk and those with non-heated BM did not exhibit antioxidant

499   activity, as opposed to those with heated BM. Assuming that the activity was due to BM

500   components, the TEAC values can be referred to as g BM/L. In this case, the values were 62,

501   36 and 27, respectively, for samples with 25, 50 and 60% of BM. These values are lower than

502   that obtained for isolated BM, which indicates a greater antioxidant capacity of BM when it is

503   embedded in the film after heating; the higher the BM ratio in the film, the more activity.

504   Mills et al., 2011 report that the antioxidant components in the BM are released by heat

505   treatment. Although it was not observed for isolated samples of BM, it was shown for the BM in

506   the films, which could be due to the action of both thermal treatment and shear applied for

507   homogenization of the film forming dispersions. In this sense, it is remarkable that BM exhibited

508   a greater antioxidant capacity in the film than when isolated. This could be attributed to a certain

509   degree of protein depolymerization during the combined homogenization thermal treatment

510   when obtaining film forming dispersions, with the subsequent release of some active peptides.

511

512   3.2.6 Antimicrobial activity

513   Figure 6 shows the growth curves of *Listeria innocua* over a period of 13 storage days

514   at 10ºC. The two sets of data represent the bacterial growth on control plates (without coating)

515   and on those coated with BM (44.8 g/m^2). Both sets of data showed a bacterial growth starting

516   at 2 log and reaching 8.8 logs UFC/cm^2 after 13 days. The similarity between the two curves

517   reveals that BM had no effect on the growth of *L. innocua* under the conditions tested. This

518   apparently contradicts the results found in literature, pointing to the antibacterial effect of some

519   buttermilk components, such as lactoferrin and its derived peptides (Farnaud & Evans, 2003;

520   Jenssen & Hancock, 2009; Mishra et al., 2013). This was probably caused by the low content of

521   lactoferrin (bovine milk contains between 0.02 - 0.35 mg/mL according to Madureira et al,

522   (2007). It can therefore be stated that, at this low concentration, and accompanied by high

523   proportions of nutritional compounds, the antimicrobial effect of these agents could not be

524   detected.

525

526   **4. Conclusions**

527   Microstructural analysis of starch-buttermilk blend films revealed a reduced compatibility

528   between the starch and milk proteins, leading to phase separation and a heterogeneous

529   structure where lipid droplets can also be observed. Heating the buttermilk implied structural

530   differences and the protein phase interpenetrated the starch matrix when there are 60% of BM

531   in the film. Incorporation of BM to starch films provoke a significant decrease in film stiffness

532   and resistance to break without notable changes in film stretchability, except for 60 % non-

533   heated BM, when films become more extensible but very soft. BM slightly promote WVP of the

534 starch films and imparted them a more saturated yellowness, reducing their gloss when not

535 heated, but increasing it when heated. Only films containing heated BM showed antioxidant

536 activity, which is attributed to the active peptides released during thermal-homogenization

537 treatments. Buttermilk did not exhibit antimicrobial activity against *Listeria innocua*, probably

538 due to the low proportion of antimicrobial compounds or to the difficulties involved in their

539 release into the culture medium. Despite its high protein content, with potential antioxidant or

540 antimicrobial properties, BM is not appropriate to formulate starch film due to the negative effect

541 of the other solids present in the commercial powder on the film properties.

542

543

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