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CHARACTERIZATION OF EDIBLE FILMS FROM WHEY PROTEINS TREATED WITH HEAT, ULTRASOUNDS AND/OR TRANSGLUTAMINASE. APPLICATION IN CHEESE SLICES PACKAGING

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Abstract

Application of high power ultrasound and microbial transglutaminase addition, and their combination, have been studied for the modification of protein structure and crosslinking in order to change the properties of whey protein concentrate edible films. They were compared to untreated and heat-treated films (from heat-treated solutions). Ultrasound treatment modified some film properties. Water vapor permeability of ultrasound-treated films was slightly lower than those of untreated and heat-treated films. Ultrasound treatment increased tensile and puncture strengths of films in comparison with untreated samples. Transglutaminase addition only increased puncture deformation values and made films less green, more yellow and darker than non-added films. Heat-treated films showed better mechanical properties and were selected for a preliminary evaluation as separation material of cheese slices; they exhibited similar results regarding slice separation easiness and slice wholeness after separation that the commercial material, without modifying cheese color and odor.

Keywords

Ultrasound; microbial transglutaminase; edible films; whey protein; cheese packaging

47 **1. Introduction**

48

49 Among natural biopolymers, whey proteins can produce films suitable for food
50 packaging (Schmid & Müller, 2019) with good gas barrier properties (6.8-9 $cm^3.m^{-2}.d^{-1}.bar^{-1}$
51 oxygen permeability) and better tensile strength (6-14 MPa) than polysaccharide-
52 based (potato and rice starches, 3.5 and 1.6 MPa, respectively) and other protein-based
53 films (corn zein, 0.4-11 MPa; wheat gluten, 0.9-3.1 MPa) (Khwaldia, Perez, Banon,
54 Desobry, & Hardy, 2004; Guerrero & de la Caba, 2016; Zink, Wyrobnik, Prinz, &
55 Schmid, 2016; Mauri, Salgado, Condés, & Añón, 2017; Menegalli, 2017). Their
56 excellent oxygen barrier properties have been applied to the manufacture of plastic
57 laminates with improved characteristics (Schmid et al., 2012). However, their water
58 vapor barrier properties are lower than those of synthetic polymer materials (Ramos,
59 Fernandes, Silva, Pintado, & Malcata, 2012).
60 The characteristics of whey protein films are determined by protein-protein interactions
61 which in turn are influenced by many factors (composition, type of bonding, etc.).
62 Protein structure and interactions (and, as a consequence, protein film properties) can be
63 modified by physical, chemical and enzymatic treatments (Zink et al., 2016). Heat
64 treatment is one of the most common methods to modify proteins for film formation and
65 improves film characteristics (Perez-Gago & Krochta, 2002). Heat treatments at
66 temperatures above 70ºC cause unfolding of whey protein chains, which exposes SH
67 and hydrophobic groups that can react forming inter- and intra-molecular bonding
68 during film drying (Shimada & Cheftel, 1989; McHugh & Krochta, 1994). During heat-
69 induced gelation of proteins, other bonds are also formed, such as hydrogen and ionic
70 bonds (Havea, Alistair, & Creamer, 2004). Disulfide bonds formation is considered very
71 important in the mechanical properties and insolubility of whey protein films (Pérez-

72 Gago, 2012). Native whey proteins can give rise to films although, due to their globular
73 structure that maintains SH groups buried inside the molecule, the main interactions that
74 promote film cohesion are hydrogen and hydrophobic bonds (Perez-Gago & Krochta,
75 2002).
76 However, other physical treatments can also be applied. High power ultrasound (20-100
77 kHz) can induce chemical modifications in foods mainly due to the process of
78 cavitation, but also other mechanical, physical and chemical effects are involved (Zink
79 et al., 2016; Schmid & Müller, 2019). The effects of ultrasounds in edible protein films
80 properties and in the interactions among protein molecules have hardly been studied
81 (Banerjee, Chen, & Wu, 1996; Liu, Tellez-Garay, & Castell-Perez, 2004; Marcuzzo,
82 Perssini, Debeaufort, & Sensidoni, 2010). Heating produces the increase in the
83 concentration of free sulfhydryl groups, while after ultrasound treatment the amount of
84 these groups remains unchanged or even decreasing. For this reason, it is considered
85 that there is not disulfide bonds formation during drying. The decrease of free SH
86 groups after sonication has been attributed to their oxidation due to the generation of
87 free radicals that react and form hydrogen peroxide during cavitation (Gülseren, Güzey,
88 Bruce, & Weiss, 2007). Ultrasound (US) modifies the secondary and tertiary structures
89 of proteins that lead in protein aggregation in bovine serum albumin solutions with a
90 treatment at 20 kHz, 90 min, (ultrasonic intensity of the generated ultrasonic wave of 20
91 W/cm^2) (Gülseren et al., 2007). However, Marcuzzo et al. (2010) reported that US
92 treatment at 24 kHz for 3-12 min (the ultrasonic wave intensity is not described) could
93 improve the appearance of gluten films by the breakdown of protein aggregates; this
94 disparity of results could be due to the type of protein and treatment conditions. High
95 power ultrasounds have been used for whey protein properties modification; US of 20
96 kHz and, in a lesser extent, of 40 kHz frequency (ultrasonic intensity of 43-48 W/cm^2

97 and 1 W/cm^2, respectively) for 15 min increased solubility and foaming ability of whey
98 proteins in whey protein concentrates and isolates (Jambrak, Mason, Lelas, Herceg, &
99 Herceg, 2008). Nevertheless, the information about the effect of ultrasounds in whey
100 protein films and coatings properties is scarce. Banerjee et al. (1996) found that low
101 power US treatment at 168 and 520 kHz frequencies for 60 min and 1.90-5.22 W
102 acoustic power improved tensile strength in whey protein concentrate films. Rodriguez-
103 Turienzo, Cobos, & Diaz (2012) reported that high power US treatment at 35kHz (20.8
104 W acoustic power) for 15 and 60 min of whey protein-based coatings significantly
105 delayed the lipid oxidation of frozen salmon pieces. Sonication has also been used to
106 improve nanoparticles distribution in whey protein isolate film matrix and, as a
107 consequence, the water vapor barrier and mechanical properties of films (Kadam et al.,
108 2013).
109 The most widely enzyme used to improve protein film properties is microbial
110 transglutaminase (MTGase), which catalyzes the formation of ε-(γ-glutamyl)-lysine
111 cross-links in proteins via an acyl transfer reaction, resulting in intra- and inter-
112 molecular covalent bonding development between protein molecules (Wihodo &
113 Moraru, 2013; Zink et al., 2016). MTGase has been used for promoting cross-linking of
114 proteins in films based on whey proteins and whey protein mixtures. The tensile
115 strength of whey protein-soy protein films and whey protein-chitosan added with
116 MTGase increased by 200% and 45-63%, respectively (Yildirim & Hettiarachchy,
117 1998; Di Pierro et al., 2006). It also improved water vapor barrier properties (Di Pierro
118 et al., 2006; Schmid, Sängerlaub, Wege, & Stäbler, 2014b) and decreased the solubility
119 of films (Yildirim & Hettiarachchy, 1998).
120 Native whey proteins are poor substrates for MTGase due to their globular structure and
121 have to be denatured before enzyme aggregation. The combination of high power

ultrasound as denaturation treatment and transglutaminase addition could be interesting for the obtaining of protein-based materials useful for food packaging. This approach has only been used for the development of whey protein-based coatings for frozen fish preservation (Rodriguez-Turienzo, Cobos, & Diaz, 2013), but it has not been investigated for the formation of stand-alone edible films.

In cheeses, one of the main problems in the commercialization of sliced products is the break of the slices during separation just before consumption. Commonly, slice separator films of different materials (paper, plastics and other polymers) are used in order to avoid sticking, such as oriented polypropylene, PET, or paper coated with PVDC dispersions or PE (Schneider, Kluge, Weiß, & Rohm, 2010). The information about the application of edible films or coatings as anti-sticking layers is very limited. Warwick (1975) and Arenson (1976) patented coatings made from starch, with or without gums, and from anhydrous milk fat powder, respectively, applicable onto cheese slice surfaces. Lecithin preparations are commercialized anti-sticking agent for cheese slices also as coating material (LECICO GmbH, 2019). Zein edible pouches have been developed for the individual packaging of processed cheese slices (Ryu et al., 2005) that not affected negatively the preservation of the product. Edible films based on milk proteins could be a good alternative to commercial separators due to the lack of negative interactions with the product and the probable good acceptance of consumers; however, no information about the use of whey protein films for cheese slices packaging has been found in the literature.

The objective of this work was to develop edible films from whey protein concentrates applying different treatments (heating, ultrasounds and addition of transglutaminase) and to evaluate their properties (water vapor permeability, solubility, color and

146 mechanical properties). The potential as cheese slices separators of selected films was
147 also assessed.
148
149 **2. Material and methods**
150
151 *2.1. Film preparation*
152 Whey protein concentrate (WPC), containing 80% protein was purchased from Armor
153 Proteines (Protarmor 800; Saint-Brice en Coglès, France). WPC composition and
154 detailed preparation of film-forming solution prior to treatments (pH adjustment,
155 plasticizer addition, mixing conditions) are described by Díaz, Candia, & Cobos (2016).
156 Briefly, 8% protein (w/w) WPC film-forming solutions were preparing by stirring for
157 30 min at 20ºC, glycerol (2:1 protein/plasticizer) was added and the pH was adjusted to
158 7.0.
159 Whey protein dispersions were divided in eight portions (120 g each) and were poured
160 into 250 ml Erlenmeyer flasks in order to prepare eight types of films; the treatments of
161 each film-forming solution are detailed in Table 1. Two batches were used as controls:
162 films from untreated solutions with (UTMT) or without (UT) microbial
163 transglutaminase addition, in order to compare the effect of the physical treatments and
164 the addition of MTGase. Any protein denaturation degree can be expected in untreated
165 WPC solutions due to the thermal treatments (pasteurizations, spray drying) applied
166 during WPC manufacture. Heat-treated solutions (HT and HTMT before MTGase
167 addition) were subjected to heating at 82ºC for 30 min in a circulating water bath. The
168 sonicated solutions were subjected to ultrasound treatment (15 or 60 min; US15 and
169 US60, respectively) in a 35 kHz ultrasound bath Sonorex Digital 10P, power 820 W
170 (Bandelin Electronic, Berlin, Germany). The solutions were treated at a power setting of

50%; the ultrasonic power of the generated ultrasonic wave was 20.8 W, as measured by calorimetry (Rodriguez-Turienzo et al., 2012). 10 units MTGase/g protein (TGT-PROQ, Proquiga, A Coruña, Spain) were added to the UTMT and HTMT solutions and the ultrasound-treated solutions (US15MT and US60MT); the microbial transglutaminase had a nominal activity of 108 U/g of power, according to the information provided by the manufacturer. The solutions with enzyme were stirred for 30 min at 20ºC. The ultrasound treatment conditions and the amount of MTGase added were selected in the range of US power and enzyme concentration reported in the literature (Yildirim & Hettiarachchy, 1998; Di Pierro et al., 2006; Gülseren et al., 2007; Jambrak et al., 2008; Marcuzzo et al., 2010; Schmid et al., 2014b) and according to previous research carried out by the research group (Rodríguez-Turienzo et al., 2012, 2013).

Afterwards, all solutions were poured into Plexiglas Petri dishes of 9 cm diameter (1.2 g total solids per dish) and were dried in an air convection heat oven (Indelab, model IDL.FI 80, Navarra, Spain) at 35ºC for 18 h. The films were stored in a desiccator at 50% relative humidity for 96 h (Hygrometer Testo, model 645, Lenzkirch, Germany) and after they were peeled from the dishes and kept at 20ºC and 50% relative humidity for 48 h. Film storage time is in the range reported in the literature (Schmid, Krimmel, Grupa, & Noller, 2014a; Schmid et al., 2014b; Silva, Fonseca, Amado, & Mauro, 2018) and all samples were in the same stage of the crosslinking changes that happens after film preparation (Schmid, Merzbacher, & Müller, 2018; Schmid, Reichert, Hamman, & Stäbler, 2015).

All experiments were performed in triplicate.

2.2. Dry matter content, solubility, thickness, density, color and water vapor barrier properties of films

196 Film characteristics were determined according to the methods described by Díaz et al.
197 (2016). In brief, dry matter content was determined by weighing after drying for 24h at
198 105ºC. The solubility in water was defined as the film dry matter percentage solubilized
199 after 24 h immersion in water. Film thickness was assessed using an electronic digital
200 micrometer (0.001 mm resolution) at 9 points of three films selected randomly.
201 Film density (ρ^s) was calculated from the film dimensions and weight (Ramos et al.,
202 2013) and expressed as the dry matter density according to the formula:
203 ρ^s (g cm^{-3}) = m/A x δ
204 where m is the dry mass (g), A is the film area (6.25 cm^2), and δ is the film thickness
205 (cm). Samples were taken at 2 points of three films selected randomly.
206 Color parameters (CIE *L*a*b** color space) and opacity were determined using a X-Rite
207 spectrophotometer (Michigan, USA), with a D65 illuminant; the difference in color
208 *(ΔE**) was calculated according to the formula:
209 $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$
210 where *ΔL**, *Δa** and *Δb** represent the differentials between the sample color parameter
211 and the color parameter of a standard white tile used as background in the other color
212 measurements (L* = 96.04, a* = -0.36, b* = 1.62).
213 The opacity of each sample was calculated as the ratio between the opacity on a black
214 background (*Yb*) and opacity on a white background (*Yw*). These parameters were
215 measured in three random positions of three films. Opacity was expressed in
216 percentage:
217 *Opacity* (%) = (*Yb / Yw) x 100*
218 Water vapor barrier properties (transmission and permeability) were measured
219 according to Gontard, Guilbert & Cuq (1992), based on the ASTM E96-93 method
220 (ASTM, 1993). Film samples were attached to glass cups containing silica gel and were

placed into a desiccator maintained at 100% RH with distilled water and at 20 ± 0.5ºC. The water transmission rate (WVT) was calculated as follows:

$$WVT\ (g/h\ m^2) = w/A$$

where w is the weight gain of the cell (g) after 1 h and A is the area of the exposed film. The water vapor permeability (WVP) transferred through the film was calculated as the weight gain of the cups, measured every two hours until constant weight:

$$WVP = WVT * L/\Delta P$$

where WVT is the water vapor transmission (g/h m^2); L is the film thickness (mm) and ΔP is the partial water vapor pressure difference (Pa) across the two sides of the film (2.339 kPa at 20ºC).

2.3. Mechanical properties

An EZTest texturometer and the Trapezium2 Data Processing System software (Shimadzu Corporation, Tokyo, Japan) version 2.22E (2004) were used for mechanical properties determination of films, that were carried out at 20ºC and 50% RH. Tensile strength was determined according to the ASTM D882 method (ASTM, 2000). Elongation at break, puncture strength and puncture deformation were measured according to the methods described by Muñoz, Aguilera, Rodriguez-Turienzo, Cobos, and Diaz (2012).

2.4. Evaluation of films as packaging material for the separation of cheese slices

2.4.1. Composition and textural properties of cheese slices

Manchego cheese was used for the tests; it was a semi-hard cheese from pasteurized ewes' milk inoculated with mesophilic lactic acid bacteria and coagulated with recombinant chymosin; the pressed curd was ripened for 3 months. Cheese composition

246 was determined by the methods of AOAC (2006a,b) for dry matter and ash, Hanson and
247 Olley (1963) for fat and AOAC (1995) for protein contents.
248 The EZTest texturometer and the Trapezium2 Data Processing System software were
249 also used for cheese texture measurements. Texture profile analysis was carried out at
250 23ºC in a two-cycle compression of cheese samples (cylinders of 6 mm x 20 mm;
251 twelve replicates) using a cylindrical probe (20 mm diameter). Compression tests were
252 performed until 50% sample deformation at 10 mm/min speed with 2 s of delay
253 between the first and second cycles. Force (N) was recorded during the compressions.
254 The most related textural properties with separators adhesion [adhesiveness (N s) and
255 cohesiveness (dimensionless)] were quantified according to Bourne (2002). Tensile
256 strength determination was performed by setting cheese slices (3 mm x 20 mm x 100
257 mm; twelve replicates) in metal grips with an initial separation of 80 mm and stretched
258 at a crosshead speed of 20 mm/min. Tensile strength of cheese samples was calculated
259 by dividing maximum load by sample cross-sectional area.
260
261 *2.4.2. Preparation and sensory evaluation of films as cheese slices separators*
262 Films selected according their physical and mechanical properties were used for the
263 evaluation of their potential as separation material for cheese portions.
264 Cheese slices (5 cm long, 3 cm wide, 0.3 cm thickness) and edible films were
265 alternately piled; twelve piles formed by four cheese slices with three separators (three
266 evaluable surfaces) were packaged in heat-sealed polyamide-polyethylene copolymer
267 bags (60 μm thickness; Plastinal S.L., Arrubal, La Rioja, Spain). Other twelve piles
268 were made and packaged in the same way but films of a commercial material
269 (Cellophane, Vapta S.L., Madrid, Spain) were used as separators; these piles were
270 utilized as control (Fig. 1). Tensile strength of the control separators, measured by using

271 the ASTM D882 method (ASTM, 2000), was 30.68 ± 7.79 MPa. The packages were
272 stored at 4ºC for 7 days. Then, they were left at room temperature (23ºC) for 2 hours
273 before opening and extraction of the piles. The experiments were repeated three times.
274 Sensory evaluation was performed by three analysts individually. Each judge evaluated
275 four slices packages with edible films as separators (108 slices) and four control
276 packages (108 slices), and the responses were recorded in questionnaires that also
277 included a space for observations about changes of color or odor in cheese slices or
278 separators that could affect the commercialization of the product. Three analyses were
279 carried out:
280 a) Slice separation easiness, according to a scale of three points comparing with control
281 slices: more sticky, more difficult to separate (1); similar (2); less sticky, easier to
282 separate (3). The evaluation was carried out for each surface in contact with the
283 separation film (three surfaces per package). The results were expressed as percentage
284 of each evaluation related to the total of evaluated surfaces.
285 b) Slice wholeness after separation, by determining the number of broken slices during
286 separation. The results were expressed as percentage of broken slices of the total
287 number of slices.
288 c) Wholeness of the separation material, comparing the number of broken separators
289 during slice separation or when they were eliminated from the slice surface.
290
291 *2.5. Statistical analysis*
292 Data were evaluated using the SPSS version 19.0.0 for Windows (2010; SPSS Inc.,
293 Chicago, USA). Data were checked for normal distribution using the Shapiro-Wilks
294 test. The statistical significance of differences among treatment means was evaluated by
295 analysis of variance (one-way ANOVA), and the means were compared using the

296 Duncan's multiple range test at $P < 0.05$. The comparison of the means of the films
297 from untreated solutions and ultrasound-treated solutions and the comparison of the
298 means of samples without the addition of transglutaminase (except heat-treated
299 samples) (UT, US15 and US60) and with the addition of transglutaminase (UTMT,
300 US15MT and US60MT) were done using a t-test for independent samples.
301
302 **3. Results and discussion**
303
304 *3.1. Film formation*
305 Film formation was possible in all samples except for HTMT films, in which the
306 homogeneous distribution of film-forming solution in Petri dishes was infeasible, due to
307 its high viscosity that avoid a correct flux on the support surface. For this reason,
308 HTMT films were not produced. Probably the addition of lower amount of MTGase to
309 protein heated solutions could enable them to be correctly distributed, but in order to
310 carry out the comparison among the different types of films, the amount of enzyme
311 added was not modified.
312
313 *3.2. Water vapor permeability, solubility, thickness, density and dry matter*
314 Fig. 2 shows the values of water vapor permeability, water solubility, thickness, density
315 and dry matter of WPC films from heat-treated solutions and from untreated and
316 ultrasound-treated solutions with or without the addition of MTGase. These parameters
317 could be related to chemical and structural characteristics of film components, mainly
318 the proteins, that influence their interaction with water and are modified by film-
319 forming solution treatments.

320 Water vapor transmission of films ranged from 20.53 to 24.41 g h^{-1} m^{-2} and was not
321 significantly affected by the different treatments (data not shown). Water vapor
322 transmission values were higher than those reported in heat-treated WPI films with or
323 without the addition of transglutaminase (Schmid et al., 2014b; 0.30-0.39 g h^{-1} m^{-2};
324 Schmid, Pröls, Kainz, Hammann, & Grupa, 2017; 0.5 g h^{-1} m^{-2}), and for WPI-coated
325 polyethylene terephthalate and polytetrafluoroethylene films (Schmid et al, 2014a; 5.2 g
326 h^{-1} m^{-2}), mainly due to differences in relative humidity conditions of the test (0 to 85%
327 in the formers or 0 to 50% in the latter), actual film thickness (4.34-184 μm) and
328 composition of whey protein product that influenced the results. The gradient of RH
329 used in this research is frequently applied and generally regarded as the "worst case"
330 conditions (Greener Donhowe & Fennema, 1994).
331 On the contrary, significant differences in water vapor permeability (WVP) were found.
332 HT films showed significantly higher values than the other samples, except UTMT
333 films. The ultrasound-treated films showed significantly lower values than the UTMT
334 films and HT samples. Only US60 films were significantly different from untreated
335 films. Using t-test, significant differences ($p<0.001$) between untreated films and
336 ultrasound-treated films were found. MTGase addition did not significantly affect
337 WVP. Permeability values were lower than those found in heat-treated and ultrasound-
338 treated whey protein films by Banerjee et al. (1996), that were 10.6 and 12.4 g mm/kPa
339 m^2, respectively, and the values for heat-treated WPI films (1.5-5.0 g mm/kPa m^2)
340 described by Krochta (2002) and Osés et al. (2009).
341 The effects of ultrasounds in WVP could be explained by a better distribution of WPC
342 lipids (3.5% lipid content) in the film. Sonication is used in milk homogenization
343 because improves lipid distribution in the product and reduces fat globule size
344 (Villamiel & Jong, 2000). Film WVP decreases as lipid concentration increases

345 (McHugh & Krochta, 1994) but this effect is enhanced when emulsion particle size
346 decreases. Ultrasounds application also improves the emulsifying properties of whey
347 proteins, that would cause a better distribution of lipids inside the films (Pérez-Gago,
348 2012). Water vapor permeability is also generally lower in denser film networks,
349 depending of cross-linking degree or film modification (Schmid & Müller, 2019). The
350 density of US films was significantly higher ($p<0.001$; t-test) than that of untreated and
351 heat treated films (Fig. 2).
352 Solubility was significantly influenced by heat treatment of film-forming solution. HT
353 films showed significantly lower water solubility than UT and sonicated films. Water
354 solubility values of HT films were close to those reported by Ramos et al. (2013)
355 (63.91%) and Silva et al. (2018) (66.84%) and those of UT films similar than those
356 reported by Pérez-Gago, Nadaud, & Krochta (1999) (99%). The intermolecular
357 disulfide bonds of heat denatured whey proteins increase the insolubility of films, while
358 unheated whey proteins form soluble films (Pérez-Gago, 2012).
359 Significant differences ($p<0.01$) in solubility between untreated films and ultrasound-
360 treated films were observed. Some authors consider that ultrasound application causes
361 the exposition of hydrophilic groups of proteins outside the molecules and a certain
362 degree of hydrolysis appears (Kresic, Lelas, Jambrak, Herceg, & Brncic, 2008;
363 Jambrak, Lelas, Mason, Kresic, & Badanjak, 2009; Jambrak et al., 2011; Marcuzzo et
364 al., 2010). Besides, the enhancement of whey protein solubility after ultrasound
365 treatment has been attributed to the increase in the amount of charged groups of
366 proteins, that improves electric conductivity of the solutions and may lead to the
367 increase of electrostatic forces and high interaction among water and protein molecules
368 (Jambrak et al., 2008).

369 Thickness values of ultrasound-treated films were lower than those of the other films.
370 The values of UTMT and HT films were higher than those of the other films. Thickness
371 of films was significantly influenced (p<0.001; t-test) by the ultrasound treatments in
372 relation to untreated samples, but not by MTGase addition. However, UTMT films
373 showed significantly higher thickness values than untreated films. The reduction of the
374 thickness of ultrasound-treated films could be attributed to the formation of a more
375 compact structure, as can be observed in the results of film density (Fig. 2), which was
376 higher in these films and has been reported by some authors in the microstructure of
377 heat-induced gels from whey proteins previously subjected to ultrasounds (Zisu et al.,
378 2011).
379 Dry matter content showed the highest values in HT samples and the lowest in UT
380 films. HT films were significantly different from the other samples except from
381 US15MT and USMT60 films. Ultrasound treatment increased significantly the dry
382 matter of films (p<0.05). The significant increase of dry matter content of US films in
383 relation to untreated films, that has been described by other authors (Banerjee et al.,
384 1996), could also be due to the formation of a denser structure that retained a lower
385 amount of water molecules. In addition, it has been observed that ultrasound application
386 breaks protein aggregates in film-forming solutions of gluten (Marcuzzo et al., 2010)
387 and in gel-forming solutions of whey proteins (Zisu et al., 2011); this effect could
388 facilitate the formation of cross-links between protein molecules and the generation of a
389 thinner and more compact structure.
390 Thickness, water vapor permeability and transmission, solubility, density and dry matter
391 of WPC films were not significantly influenced by sonication length (15 or 60 min).
392 When comparing films without MTGase versus MTGase-added films, enzyme addition
393 only significantly increased the dry matter content of films (p<0.01). Tang, Jiang, Wen

394 and Yang (2005) described similar results in soy protein isolate films. They related
395 them to the cross-linking induced by MTGase that decreases the capacity of proteins to
396 bind water via the participation of amino groups (mainly from lysine) in cross-links; in
397 these conditions, these groups are not available for binding water through hydrogen
398 bonding. Modifications in other parameters (such as water vapor barrier properties,
399 thickness and solubility) in films from heated or chemically-treated proteins added of
400 MTGase have been observed by other authors (Tang et al., 2005; Chambi & Grosso,
401 2006; Di Pierro et al., 2006). Chemical nature of macromolecules, crystallinity,
402 molecular mass, orientation and cross-linking degree affect film permeability (Yi, Kim,
403 Bae, Whiteside, & Park, 2006). Ultrasound treatment did not sufficiently modify the
404 globular structure of whey proteins so that the enzyme catalyzed cross-links that could
405 affect these film properties.
406
407 *3.3. Color*
408 CIEL*a*b* color values, total color difference (ΔE) and opacity of WPC films from
409 heat-treated, untreated and ultrasound-treated solutions, with or without MTGase
410 addition, are shown in Fig. 3. According to the relation of ΔE values in the CIE L*a*b*
411 spaces and the differences in color that a standard observer can see described by
412 Mokrzycki and Tatol (2011), a clear difference in color can be noticed in heat-treated
413 films (ΔE between 3.5 and 5.0) and two different colors can be detected for all the other
414 films (ΔE > 5.0). For heat-treated films, a* values were lower and b* values higher than
415 those found by Osés et al. (2009) and Galus and Kadzinska (2016), while luminosity
416 presented intermediate values. ΔE results were lower than those reported by Ramos et
417 al. (2013) and Pérez, Piccirilli, Delorenzi and Verdini (2016), and higher than those
418 described by Galus and Kadzinska (2016).

419 Opacity was not significantly modified by the treatments and its values varied from 7.74
420 to 10.42 %.
421 HT samples showed significantly lower a*, b* and ΔE values than UT films. No effects
422 of ultrasounds on WPC films color in relation to untreated films were found. Liu et al.
423 (2004) also observed no effects of ultrasound treatment applied to solutions on the color
424 of peanut protein films.
425 Color of films was significantly affected by the addition of transglutaminase. The films
426 with this addition showed significant lower values of luminosity (L*) (p<0.001) and
427 higher a* (p<0.001), b* (p<0.01) and ΔE (p<0.001) values than the films without
428 enzyme. So, transglutaminase action caused that the films were less green and more
429 yellow and darker than non-added films.
430 The information about the effects of the addition of MTGase in protein films color is
431 variable and not very abundant. Tang et al. (2005) observed that the transglutaminase
432 treatment decreased the transparency of soy protein isolate films and they indicated that
433 this decrease was probably due to the cross-linking or aggregation induced by the
434 enzyme, which could make the film-forming dispersion more turbid. In fish gels, Uresti,
435 Ramirez, López-Arias and Vázquez (2003) also reported that the action of
436 transglutaminase induced covalent crosslinking between adjacent proteins, promoting
437 the formation of stronger gels and decreasing their lightness. This crosslinking between
438 proteins could also explain the decrease of L* observed in our WPC films. However, no
439 significant changes were observed in the lightness of fish gelatin films when
440 transglutaminase was added (Yi et al., 2006; Weng & Zheng, 2015).
441 Yi et al. (2006) observed that the addition of transglutaminase decreased a* and
442 increased b* values of fish gelatin films with sorbitol as plasticizer. Contrary to that
443 observed in WPC films, the color of gelatin films became more greenish as the

444 transglutaminase reaction occurred. These authors indicated that transglutaminase
445 reaction changes the crystallinity or molecular structure of the protein matrix, leading to
446 a different response to light. They observed that the opacity of films increased with the
447 reaction time probably due to the cross-linking or aggregation induced by
448 transglutaminase. A decrease of a* values and no significant changes of b* values were
449 observed by Weng and Zheng (2015) in fish gelatin films when transglutaminase was
450 added. In fish gels, Uresti et al. (2003) reported that a* and b* values were affected by
451 MTGase concentration, although the behaviour was not equal for both parameters; at
452 low enzyme concentration both values increased, while at high enzyme concentration,
453 a* increased and b* decreased. The different protein concentration and composition and
454 the plasticizer (sorbitol or glycerol) used in protein films could influence to the different
455 effect of transglutaminase on color values (García & Sobral, 2005; Tang et al., 2005).
456
457 *3.4. Mechanical properties*
458 Mechanical properties of WPC films from heat-treated, untreated and ultrasound-treated
459 solutions, with or without MTGase addition, are shown in Fig. 4. Films from HT
460 solutions have significantly higher values than those from UT and US solutions; these
461 results can be attributed to the disulfide covalent bonding among protein chains
462 (McHugh & Krochta, 1994). Tensile strength (TS) and elongation at break (EB) values
463 of HT films were higher than those reported by Ramos et al. (2012, 2013) (TS 0.65-0.75
464 MPa; EB 18-20%) and Silva et al. (2018) (TS 2.18 MPa; EB 11.24%), probably due to
465 differences in whey protein product characteristics.
466 Ultrasound treatment significantly increased tensile strength (p<0.01) and puncture
467 strength (p<0.001) of films in comparison with untreated films according to t-test
468 results, while elongation at break and puncture deformation did not change. The length

469 of ultrasound treatment did not significantly affect mechanical properties. The increase
470 of tensile and puncture strengths in films from ultrasound-treated solutions was also
471 observed by Banerjee et al. (1996); these authors attributed it to the increment of the
472 molecular order due to ultrasounds and to a more rigid structure. Sonication produced
473 significant changes in mechanical properties probably due to the improvement of non-
474 covalent bonding among protein molecules. As it was mentioned above, several authors
475 have pointed out that ultrasounds could produce an increase in the exposition of
476 hydrophilic and hydrophobic groups because of the unfolding of protein chains; it is
477 possible that these exposed groups could form new bonds and modify mechanical
478 properties of films. Besides, the increase of the amount of charged groups, that made
479 possible that more water molecules interact with proteins (Jambrak et al., 2008), could
480 also favor the formation of electrostatic bonds among protein molecules.
481 In relation to elongation at break (property associated to film flexibility), no significant
482 differences due to ultrasound treatment were found, although its values were higher in
483 treated than in untreated samples. However, other authors (Banerjee et al., 1996; Liu et
484 al., 2004), in dairy and peanut proteins, observed a reduction in the elongation. The fact
485 that in our work the values did not decrease could be due to the higher lipid content of
486 the whey protein product that was used in the experiments. Lipids reduce the
487 intermolecular attraction forces and increase the mobility of protein chains, improving
488 film extensibility (Chambi & Grosso, 2006).
489 The addition of MTGase only significantly increased puncture deformation values
490 (p<0.01). Rises of tensile and puncture strengths have also been described in egg protein
491 films treated with dithiothreitol and transglutaminase (Yildirim & Hettiarachchy, 1998)
492 and in films of egg proteins and chitosan (Di Pierro et al., 2007). Bae, Darby, Kimmel,
493 Park and Whiteside (2009) reported that an increase in the degree of cross-linking in

494 polymer matrices enhances the rigidity of the polymer molecules and leads in the
495 improvement of the tensile strength of films. Regarding to elongation at break, the
496 observations are variable, and increases (Chambi & Grosso, 2006) and decreases (Tang
497 et al., 2005; Di Pierro et al., 2006) of the values in protein films have been reported. It
498 has been pointed out that the effect of the enzyme is strongly conditioned to the type of
499 protein used in film formation (Chambi & Grosso, 2006).
500
501 *3.5. Evaluation of films as packaging material for the separation of cheese slices*
502 According to the results of film properties described above, HT films were selected for
503 a preliminary evaluation of their capability as separation material in cheese slices
504 packaging due to their low solubility and the best mechanical properties. Cheese
505 composition was: moisture 37.0 ± 1.11 g/100g; fat 31.9 ± 0.43 g/100g; protein 22.8 ±
506 3.26 g/100g; and ash 4.1± 0.29 g/100g. Adhesiveness of cheese samples was -0.403 ±
507 0.051 N s, and cohesiveness was 0.384 ± 0.023. Tensile strength was 0.005 ± 0.001
508 MPa.
509 Slice separation easiness was similar for HT films and Cellophane control films (60.18
510 % of slices), or even was easier (39.81 % of slices) when HT films were used, probably
511 due to the protein films showed higher thickness, flexibility and deformation capacity.
512 In the evaluation of slice wholeness after separation, no important differences in the
513 number of broken cheese slices between both types of films were found (25.00% for HT
514 films and 20.37% for control films). Related to wholeness of the separation material,
515 14.81% of HT films broke during separation, while control films remained intact. The
516 evaluators did not find any changes of cheese color and odor caused by the separation
517 materials.
518

4. Conclusions

Ultrasound treatment of whey protein concentrate film-forming solutions produced less water vapor permeable, thinner and higher mechanical resistant films compared to those from untreated proteins. Water vapor permeability of ultrasound-treated films was lower than that of heat treated films. The addition of microbial transglutaminase to ultrasound treated solutions did not affect the properties of films except their color. Films from heat-treated solutions exhibited the best mechanical properties. So, transglutaminase addition and ultrasound treatment are not useful methods for improving the mechanical properties of films. For this reason, heat-treated films were selected as potential separators of cheese slices. In relation to the preliminary study of whey protein films as separation material of cheese slices, the results are promising and deserve further investigation.

Declarations of interest

None.

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840 **Figure captions**

841 **Fig. 1.** Cheese-separator piles for the evaluation of WPC films as packaging material for
842 the separation of cheese slices. Right: pile with Cellophane separators; left: pile with
843 WPC film separators.

844

845 **Fig. 2.** Water vapor permeability, thickness, dry matter content, solubility and density of
846 WPC films from film-forming solutions subjected to different treatments: untreated
847 (UT); untreated with the addition of MTGase (UTMT); heat-treated (HT); ultrasound
848 treated for 15 min (US15); ultrasound treated for 60 min (US60); ultrasound treated for
849 15 min with the addition of MTGase (US15MT); ultrasound treated for 60 min with the
850 addition of MTGase (US60MT). Columns with different letters are significantly
851 different (p<0.05).

852

853 **Fig. 3.** Color parameters and total color difference of WPC films from film-forming
854 solutions subjected to different treatments: untreated (UT); untreated with the addition
855 of MTGase (UTMT); heat-treated (HT); ultrasound treated for 15 min (US15);
856 ultrasound treated for 60 min (US60); ultrasound treated for 15 min with the addition of
857 MTGase (US15MT); ultrasound treated for 60 min with the addition of MTGase
858 (US60MT). Columns with different letters are significantly different (p<0.05).

859

860 **Fig. 4.** Mechanical properties of WPC films from film-forming solutions subjected to
861 different treatments: untreated (UT); untreated with the addition of MTGase (UTMT);
862 heat-treated (HT); ultrasound treated for 15 min (US15); ultrasound treated for 60 min
863 (US60); ultrasound treated for 15 min with the addition of MTGase (US15MT);
864 ultrasound treated for 60 min with the addition of MTGase (US60MT). The thickness

865 values of films samples (the mean values of each type of film) used in tensile strength
866 and elongation at break determinations were: UT: 0.109 mm; UTMT: 0.120 mm; HT:
867 0.112 mm; US15: 0.087 mm; US60: 0.088 mm; US15MT: 0.091 mm; US60MT: 0.086
868 mm. Columns with different letters are significantly different ($p<0.05$).

Fig. 1

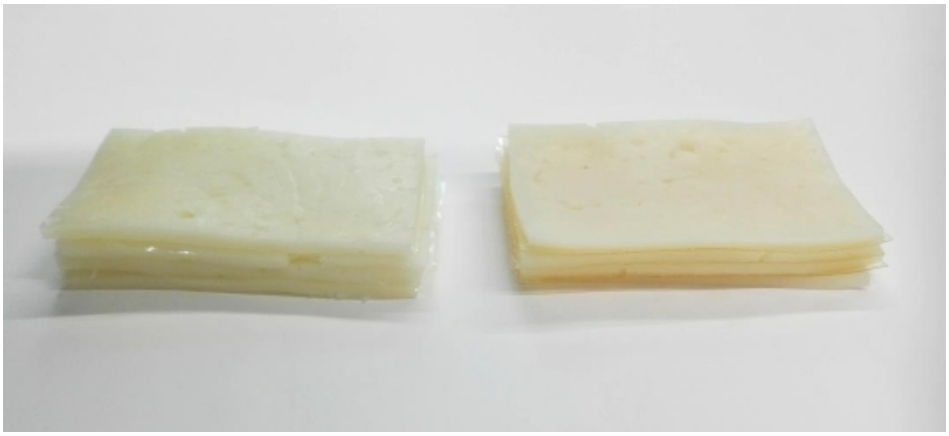

Fig. 2

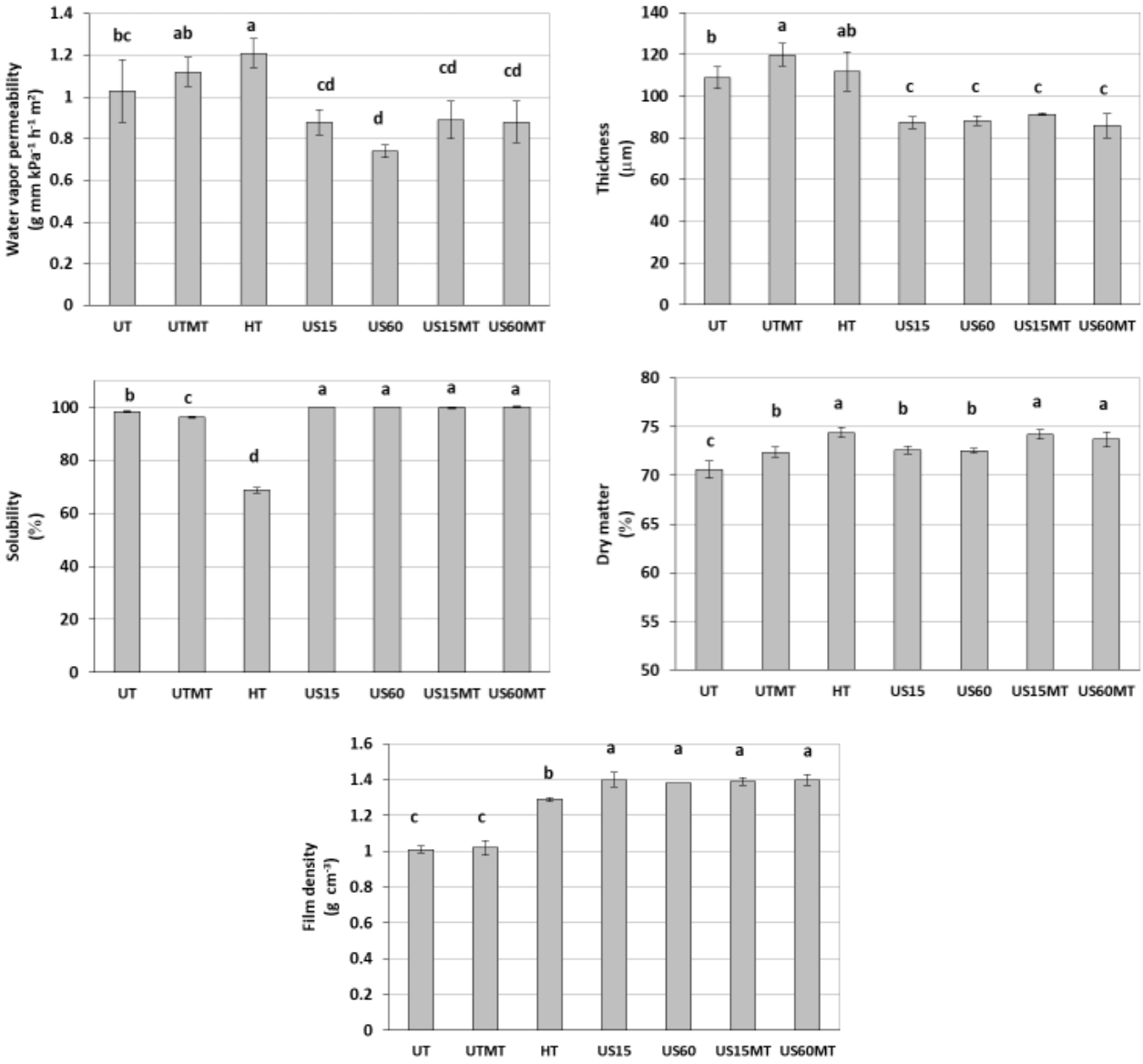
Water vapor permeability (g mm kPa⁻¹ h⁻¹ m²)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
bc
ab
a
cd
d
cd
cd
UT
UTMT
HT
US15
US60
US15MT
US60MT
Thickness (μm)
140
120
100
80
60
40
20
0
b
a
ab
c
c
c
c
UT
UTMT
HT
US15
US60
US15MT
US60MT
Solubility (%)
100
80
60
40
20
0
b
c
d
a
a
a
a
UT
UTMT
HT
US15
US60
US15MT
US60MT
Dry matter (%)
80
75
70
65
60
55
50
c
b
a
b
b
a
a
UT
UTMT
HT
US15
US60
US15MT
US60MT
Film density (g cm⁻³)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
c
c
b
a
a
a
a
UT
UTMT
HT
US15
US60
US15MT
US60MT

Fig. 3

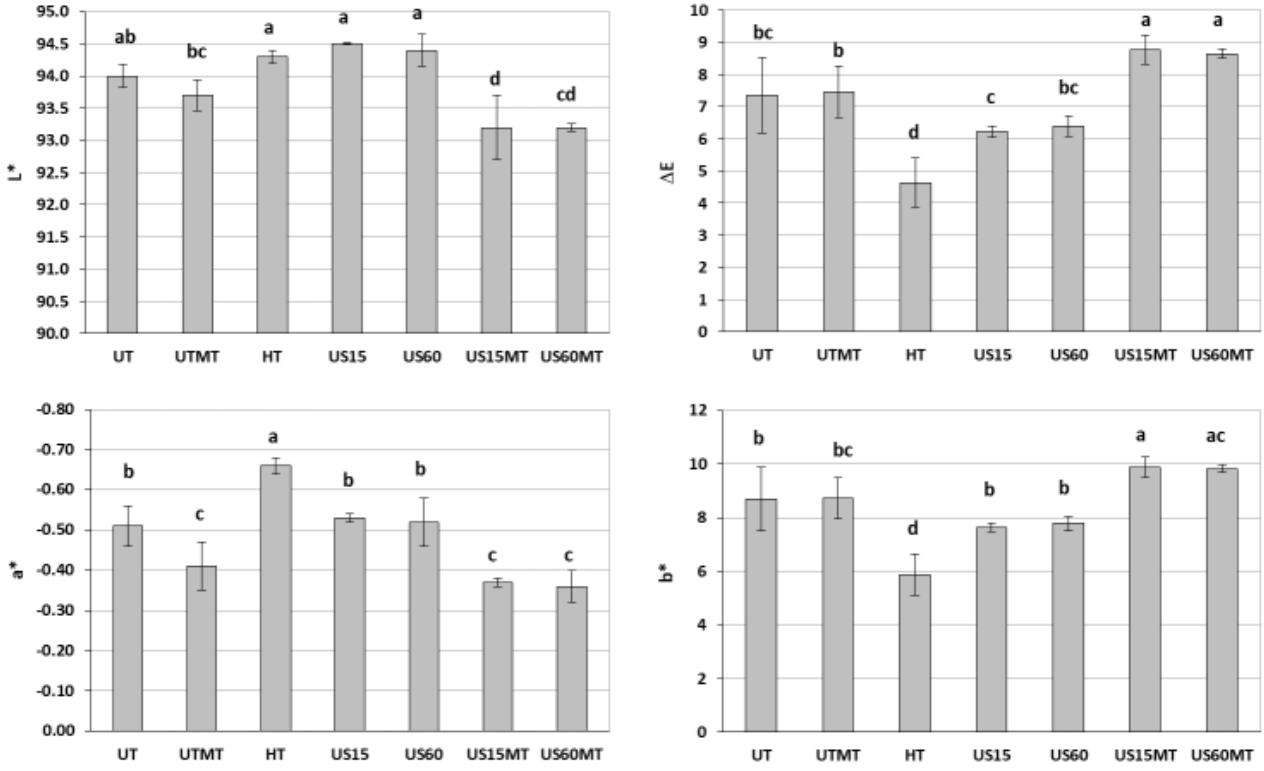
L*
95.0
94.5
94.0
93.5
93.0
92.5
92.0
91.5
91.0
90.5
90.0
ab
bc
a
a
a
d
cd
UT
UTMT
HT
US15
US60
US15MT
US60MT
ΔE
10
9
8
7
6
5
4
3
2
1
0
bc
b
d
c
bc
a
a
a*
-0.80
-0.70
-0.60
-0.50
-0.40
-0.30
-0.20
-0.10
0.00
b
c
a
b
b
c
c
b*
12
10
8
6
4
2
0
b
bc
d
b
b
a
ac

Fig. 4

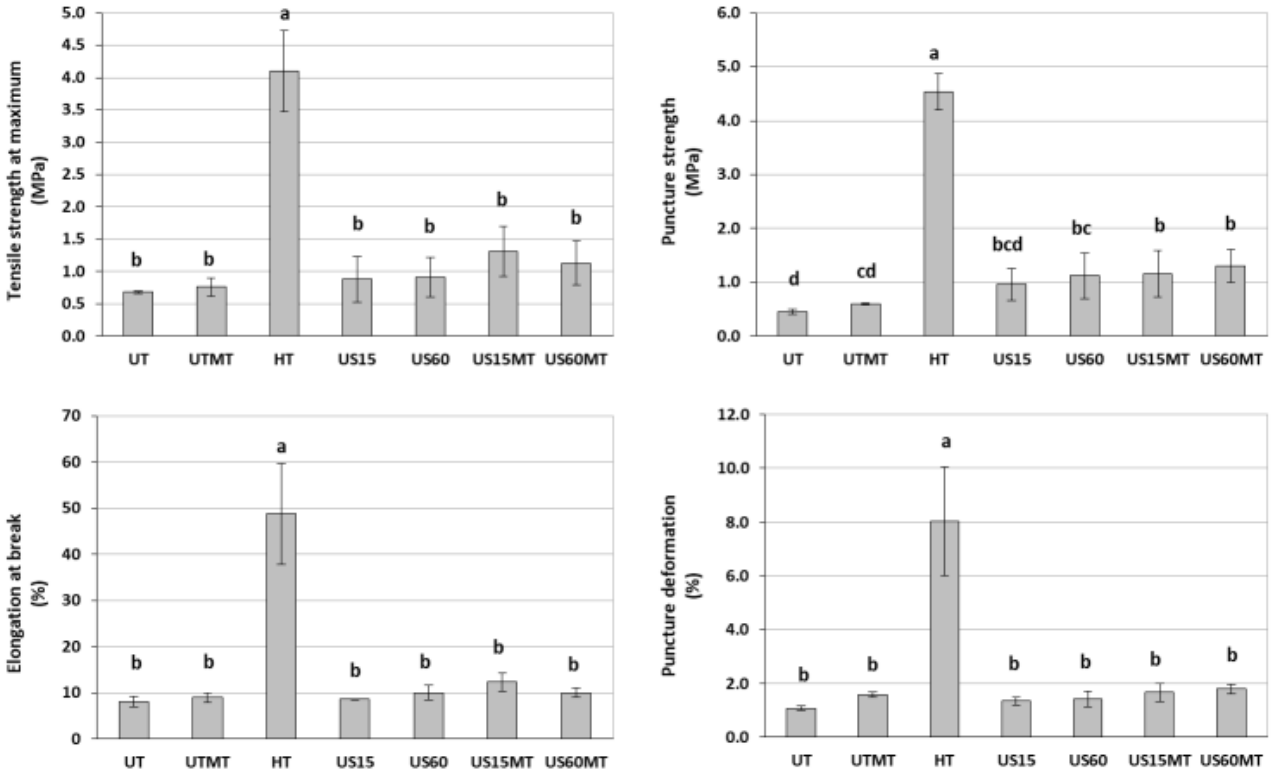
Tensile strength at maximum (MPa)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b
b
a
b
b
b
b
UT
UTMT
HT
US15
US60
US15MT
US60MT
Puncture strength (MPa)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
d
cd
a
bcd
bc
b
b
UT
UTMT
HT
US15
US60
US15MT
US60MT
Elongation at break (%)
70
60
50
40
30
20
10
0
b
b
a
b
b
b
b
UT
UTMT
HT
US15
US60
US15MT
US60MT
Puncture deformation (%)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
b
b
a
b
b
b
b
UT
UTMT
HT
US15
US60
US15MT
US60MT

Table 1. Film-forming solutions treatments

Film abbreviation	Heat treament (82ºC 30 min)	Ultrasound treatment		Microbial transglutaminase (10 units /g protein)
		15 min	60 min	
UT*	-	-	-	-
UTMT*	-	-	-	+
HT	+	-	-	-
HTMT	+	-	-	+
US15	-	+	-	-
US60	-	-	+	-
US15MT	-	+	-	+
US60MT	-	-	+	+

* Films used as controls