Effect of pH on the effectiveness of whey protein/glycerol edible films containing potassium sorbate to control non-O157 shiga toxin-producing Escherichia coli in ready-to-eat foods

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ABSTRACT

Potassium sorbate (PS) (0.5%, 1.0% and 1.5% w/w) was included into whey protein concentrate (WPC)/glycerol (Gly) edible films at pH 5.2 and 6.0. The films inhibited or retarded the growth of Shiga toxin producing *Escherichia coli* (STEC) pathogens in both diffusion and barrier tests. Bacterial growth inhibition was dependent on PS content at both pH values. PS release was not affected by pH. Scanning electron microscopy (SEM) was used to analyze the microstructure of the films and gain a better understanding of their optical parameters. Acidic control films (pH 5.2) prepared without PS were the least transparent. SEM micrographs confirmed the greater structural heterogeneity of these films, coinciding with opacity. The incorporation of PS into WPC/Gly films improved transparency and produced a smoother surface than acidic control ones. The utilization of active packaging based on whey proteins and organic acids to control and prevent the dissemination of STEC pathogens may be an effective, safe, ecological and relatively inexpensive alternative to be used in the food packaging industry.

Keywords: whey protein edible films; antimicrobial properties; non-O157 *Escherichia coli*; potassium sorbate; film microstructure; transparency.

1. Introduction

Active packaging can be defined as a mode of packaging in which the package, the product, and the environment interact to prolong shelf life or enhance safety or sensory properties, while maintaining the quality of the product (Suppakul, Miltz, Sonneveld & Bigger, 2003). In order to control undesirable microorganisms in food surfaces, natural or synthetic antimicrobial agents can be incorporated into polymer coatings (Appendini & Hotchkiss, 2002; Kuorwel, Cran, Sonneveld, Miltz & Bigger, 2011). Several compounds have been proposed as antimicrobial agents in food packaging, including organic acids, enzymes, fungicides and natural compounds such as spices and essential oils (Tharanathan, 2003; Seydin & Sarikus, 2006; Kuorwel et al., 2011). Sorbic acid, p-aminobenzoic acid, lactic acid, and acetic acid have a long history as generally recognized as safe (GRAS) food preservatives that have been extensively used as fungistatic and bacteriostatic agents for foods. Several studies have proved the effectiveness of some food preservative addition into edible films to control microbial growth (Cagri, Ustunol & Ryser, 2001, 2004; Ye, Neetoo & Chen, 2008; Vásconez, Flores, Campos, Alvarado & Gerschenson, 2009). However, studies involving the effect of edible film matrix pH on the antimicrobial activity of GRAS organic acids were not examined.

Potassium sorbate (PS) is the potassium salt of sorbic acid that can effectively restrain the activity of mould, yeast and aerophile bacteria. The use of PS is effective up to pH 6.5 but effectiveness increases as pH decreases, consequently the pH of the film and the food to which the film is applied are important parameters.

Cheese whey, a by-product in the cheese making process, have excellent nutritional and functional properties in addition to their capacity to form films (Chen, 1995; Pérez-Gago, Serra, Alonso, Mateos & Del Río, 2003; Javanmard, 2009). Use of whey protein to

66 manufacture films has received a great deal of attention since they are edible and
67 biodegradable.
68 Cagri et al. (2001) reported that incorporating 0.5% to 1.5% of sorbic acid or p-aminobenzoic
69 acid into acidic whey protein isolate films (pH 5.2) can inhibit the growth of
70 *L. monocytogenes*, *S. typhimurium* and *E. coli* O157:H7 in trypticase soy agar supplemented
71 with yeast extract (TSAYE) acidified to pH 5.2 but no inhibition was observed in TSAYE
72 adjusted to pH 6.5. These results reveal the importance of the medium pH for a superior
73 effectiveness of the organic acids. Moreover, some restrictions in the pH limit for the
74 elaboration of whey protein edible films are related to the isoelectric point of whey proteins.
75 Casting films at pH values lower than the isoelectric point proved to be inefficient since the
76 reactivity of SH groups decreases significantly (Ferreira, Nunes, Delgadillo & Lopes-da-
77 Silva, 2009). In addition, at acidic pH values whey proteins aggregate and films properties
78 could decrease. Hence, changes in the pH of the whey protein/Gly system may affect solution
79 stability, and thus, the antimicrobial characteristics of the films containing PS. To our
80 knowledge, no comparative studies regarding the differences in the pH of the edible films
81 based on whey protein and sorbate have been reported.
82 Shiga toxin-producing *Escherichia coli* (STEC) is worldwide recognized as one of the most
83 important causes of food-borne infections. Clinical presentation of STEC infection varies
84 from an asymptomatic state to bloody diarrhea and life-threatening complications such as
85 hemolytic uremic syndrome (Johnson, Clarke, Wilson, Read, Rahn & Renwick, 1996;
86 Williams, Boyd, Nutikka, Lingwood, Barnett, Milford & Taylor, 1999). STEC posses the
87 Shiga toxin 1 and/or Shiga toxin 2 (*stx1* and *stx2*) genes considered the critical virulence
88 factors in disease. Environmental conditions present in foods (nutrients, pH, humidity, etc.)
89 are suitable for a rapid colonization by STEC strains to harmful levels for human health. Non-
90 O157 STEC is now recognized as an important group of bacterial enteropathogens (Gyles,

2007; Gilmour, Chui, Chiu, Tracz, Hagedorn, Tschetter, Tabor, Ng & Louie, 2009). Several outbreaks caused by non-O157 STEC were described (Johnson et al., 1996; Williams et al., 1999), although data implicating these STEC in some outbreaks were scanty and the source of infection was not always known. In the United States, most STEC outbreaks were traced to beef containing *E. coli* O157:H7 and for that reason most epidemiological studies have focused on the prevalence of this serotype in beef and beef cattle (Hussein & Sakuma, 2005; Hussein, 2007; Gil & Gil, 2010). Interestingly, undercooked ground beef and other beef products are now considered reservoirs of O157 and non-O157 STEC (Hussein, 2007). However, worldwide, additional STEC serotypes, including members of the recently named big six serogroups (O26, O45, O103, O111 and O121), have been isolated from foods other than beef and caused human illnesses (Mathusa, Chen, Enache & Hontz, 2010). Balagué et al. (2006) reported the phenotypic and genotypic characteristics and virulence properties of non-O157 strains isolated from ready-to-eat food samples obtained from supermarkets and shop selling in Argentina were hemolytic uremic syndrome is endemic (Reilly, 1998; Ibarra, Goldstein, Silberstein, Zotta, Belardo & Repetto, 2008).

Because of the global nature of the food supply, safety concerns and new challenges facing the food industry are increasing both at the production and processing levels. Considering that antimicrobial edible packaging is a novel technology with the potential to help food preservation, the purpose of the present study was to evaluate the inhibitory effects of WPC/Gly edible films incorporated with PS against non-O157 STEC strains isolated from ready-to-eat food samples and its relationship with the pH of the film. However, since pH modifications can negatively alter other relevant properties of protein-based films, influence of both pH and organic acid presence on the optical properties of WPC/Gly films was also analyzed. Hence, one goal of this work was to provide an array of data to support comparative

115 studies on the molecular structure, optical and biocide properties of acidic WPC/Gly films for
116 rational improvement of such films toward their eventual application as edible packaging.
117
118 **2. Materials and Methods**
119 *2.1. Materials*
120 WPC 80% (Arla Food Ingredients S.A., Buenos Aires, Argentina) was used to prepare the
121 film forming solutions. Gly (Cicarelli, Santa Fe, Argentina) was added to all film forming
122 solutions as a plasticizer; PS (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was included in
123 the formulations at different concentrations to evaluate the biocide properties of the WPC/Gly
124 films. Trypticase Soy Broth (TSB), Cystine Lactose-Electrolyte-Deficient (CLDE) and
125 Mueller-Hinton culture mediums were purchased from Britania (Buenos Aires, Argentina).
126 2,3,5-triphenyltetrazolium chloride (TTC) was obtained from Merck (Darmstadt, Germany).
127
128 *2.2. Film preparation*
129 Edible films (casting solution 11.5% total solid) were obtained with a modification of the
130 method described by Soazo, Rubiolo & Verdini (2010). Briefly, WPC and Gly (in proportion
131 WPC/Gly 3:1 w/w dry solid basis) were dissolved in distilled water. After mixing, the
132 solution was heated at 90 ºC for 30 min in a water bath (TDS-40, Tecno Dalvo, Santa Fe,
133 Argentina). Finally, the solution was homogenized (4 min; 20 000 rpm) with an Omni GLH
134 homogenizer (Omni International Inc., Warrenton, Virginia, U.S.A.). After homogenization,
135 the solutions were placed in an ice bath to prevent further denaturation of the whey proteins
136 and rapidly cooled to room temperature. After incorporating 0.5%, 1.0% or 1.5 % (w/w) of
137 PS, the pH was adjusted to 5.2 or 6.0 with 1.0 N HCl using a Metrohm 713 pH-meter
138 (Metrohm Ltd., Herisau, Switzerland). The pH-adjusted film-forming solutions were degassed
139 at room temperature with a vacuum pump. Following degassing, the film forming solutions (8

g/plate) were casted by pipetting the solution into sterile 90 mm diameter plastic plates. The plates were dried for 2 h at 45 ºC plus 24 h at 25 ºC and 48 ± 4% relative humidity, after which the films were peeled from the plates and stabilized during 24 h at 25 ºC and 48 ± 4% relative humidity. The films used in the different tests were selected based on the lack of physical defects such as cracks, bubbles and holes.

2.3. Film thickness

The thicknesses of three replicates of each film formulation were measured with an electronic digital disk micrometer (Schwyz®, China) at nine locations on the film to the nearest 0.001 mm. Average film thickness was 0.137 ± 0.030 mm.

2.4. Transparency

Film transparency was determined according to ASTM D1746 (ASTM, 1997) with modifications of the method described by Ozdemir & Floros (2008). The films were cut into rectangular pieces (10 mm x 30 mm) and placed on the internal side of a spectrophotometer cell. Transparency of films was measured using a spectrophotometer (Model V-530, Jasco International, Tokyo, Japan) at 560 nm. Five replicates of each film were tested. The transparency (% Transparency) was calculated as the percentual relationship between the light intensity with the specimen in the beam and the light intensity with no specimen in the beam.

2.5. Scanning electron microscopy

In order to study the structure of WPC/Gly films and to asses their homogeneity, SEM experiments were carried out. Film samples were cryo-fractured by immersion in liquid nitrogen and mounted on bronze stubs perpendicularly to their surface. The portions were coated with gold during 15 min at 70-80 mTorr. Micrographs of films cross-section were

taken with a scanning electron microscope (AMR 1000, Leitz, Wetzlar, Germany) using an accelerating voltage of 20 kV. Magnifications of 500 and 1000 were used.

2.6. Bacterial strains and growth conditions

Eight non-O157 STEC strains isolated from ready-to-eat food samples obtained from supermarkets and shops selling in Rosario (Argentina) were kindly provided by Balagué et al. (2006) as test microorganisms (Table 1). *E. coli* O157:H7 ATCC 43895 from the American Type Culture Collection which expresses the Shiga toxin genes *stx1* and *stx2* was used as a control. All strains were maintained at –70 ºC in TSB containing 10% (v/v) glycerol and subcultured overnight in CLDE at 37 ºC before use.

2.7. Inhibitory activity of PS in liquid media

Minimum inhibitory concentration (MIC) was estimated according to the National Committee for Clinical Laboratory Standards-recommended macrodilution broth method (2009) as described by Pérez, Balagué, Rubiolo & Verdini (2011). Briefly, an overnight culture of each bacterial strain was adjusted to McFarland 0.5 standard in saline solution (approximately 5 x 10^7 cfu/ml) and used as test inoculum. One ml of the bacterial inoculum was added and mixed with 1 ml of each PS solution in Mueller-Hinton broth adjusted at pH 5.2 or 6.0. The PS concentrations evaluated were 0.3125, 0.625, 1.25, 2.5, 5.0, 10 and 20 mg/ml. A control tube without PS was inoculated to test microbial growth and a tube containing only broth medium was evaluated to discard possible contaminations. All tubes were overnight incubated (~18 h) at 37°C. The MIC values were estimated as the lowest concentration of PS that completely inhibits growth of the microorganism in the tubes as detected by the unaided eye. Growth control tubes to assess MIC end points were also evaluated.

190 *2.8. Inhibition zone assay of WPC/Gly films containing PS in agar media*

191 The inhibition zone assay in solid media was used to determine the antimicrobial potential of
192 films. WPC/Gly films were aseptically cut in 12 mm diameter discs using a sterile cork borer.
193 The discs were then aseptically transferred to pour plates containing 10 ml of Mueller-Hinton
194 agar broth acidified to pH 5.2 with 1.0 N HCl, which had been previously seeded with each
195 bacterial suspension adjusted to McFarland 0.5 standard in saline solution. After overnight
196 incubation (~18 h) at 37 ºC the diameter of the inhibition zone represented by a clear area of
197 non-growth or a decreased growth around the film disc was measured perpendicularly to the
198 nearest millimeter with a caliper. Control films without antimicrobial were also evaluated.
199 Experiments were performed in triplicate.

200

201 *2.9. Barrier assay of WPC/Gly films containing PS in agar media*

202 In order to study the film performance to prevent microbial contamination a microplate barrier
203 assay was developed. Mueller-Hinton agar adjusted at pH 5.2 was poured into 24 well
204 microtiter plates (Greiner Bio-One, Frickenhausen, Germany). Inocula were prepared from an
205 overnight culture of each bacterial strain adjusted to McFarland 0.5 standard in saline solution
206 and then diluted 1/10000 (approximately 5 x 10^3 cfu/ml). Discs of 16 mm diameter WPC/Gly
207 films were aseptically cut from and applied on the surface of the wells filled with Mueller-
208 Hinton agar (pH 5.2). Then, 10 µl inoculum for each strain were seeded on the film discs.
209 Next, 10 µl of TTC (30 mg/ml) were added into each well to color the bacterial colonies.
210 Microplates were incubated at 37 ºC for 24 h until naked eye observation. Experiments were
211 performed in triplicate.

212

213 *2.10. Determination of sorbate release from WPC/Gly edible films*

214 To analyze sorbate release, WPC/Gly films containing 1.5% PS (to enhance detection
215 sensibility) were cut in 12 mm diameter discs using a cork borer. The discs were placed on the
216 surface of Petri dishes containing 10 ml of Mueller-Hinton agar broth acidified to pH 5.2 with
217 1.0 N HCl. After 1 h, 3 h, or 18 h of incubation at 37 ºC films disc were removed and the
218 change in PS concentration with time in the film was determined. The initial amount of PS
219 per disc was also evaluated. Each PS-containing WPC/Gly disc was immersed in 4 mL 0.01 N
220 HCl solution, homogenized (1 min; 20 000 rpm) with an Omni GLH homogenizer (Omni
221 International Inc., Warrenton, Virginia, U.S.A.) and 10 min sonicated. Samples (30 µL) were
222 diluted to 3.0 mL with 0.01 N HCl. Potassium sorbate concentration of the collected samples
223 were measured at 254 nm with a UV-Vis spectrophotometer (Model V-530, Jasco
224 International, Tokyo, Japan). Calibration of the extraction procedure showed that these
225 conditions enabled stable and reproducible recovery (98% with r^2=0.993). Percentage of PS
226 remaining in disc were calculated and plotted as a function of time. Experiments were
227 performed in triplicate.

228

229 *2.11. Statistical analysis*

230 All experiments were replicated using a complete randomized design. Analysis of variance
231 (ANOVA) was used and when the effect of the factors was significant ($p < 0.05$), the test of
232 multiple ranks honestly significant difference (HSD) of Tukey was applied (95% of
233 confidence level). The statistical analysis was performed using Minitab 13.20 (Minitab Inc.,
234 State College, PA).

235

236

3. Results and Discussion

3.1. Film appearance

In general, WPC-based films obtained in this study were rather flexible and clear enough for use as see-through packaging; however, exhibited a slightly yellowish color probably because the presence of contaminants (i.e. lactose, lipids and minerals) in WPC. Their surfaces appeared smooth, without visible pores or cracks. Appearance of the film side facing the casting plate was shiny, while the film side exposed to air was dull; Ramos et al. (2013) reported that this is likely an indication of some phase separation occurring in the solution during drying. Films incorporated with PS, at both pH, were easily separated from the casting plate than control films without preservative. Furthermore, when PS concentration was increased in the film-forming solutions, WPC/Gly films flexibility improved. These observations may be explained because this additive has an additional effect as plasticizer on film matrixes (Famá, Rojas, Goyanes & Gerschenson, 2005). The plasticizer molecules lead to decreases in intermolecular forces along the polymer chains, thus improving flexibility, and allowing an easier removal from the forming support (Karbowiak et al., 2006; Hernandez-Izquierdo & Krochta, 2008). Films showed an average thickness of 0.137 ± 0.030 mm, which is similar to those reported by other authors using similar formulations (Soazo et al., 2010; Ramos et al., 2013).

3.2. Film transparency

It is important to notice that when an edible film is intended to be used as a superficial layer on food surfaces, transparency is an interesting attribute that contributes to consumer acceptability. In general, films obtained in this study were rather transparent. However, in the absence of PS, films obtained at the acidic pH value of 5.2 were substantially less transparent than films obtained at pH 6.0, presumably due to a partial proteins precipitation when pH is

close to the isoelectric point of whey proteins (pI ≈ 5) (Fig. 1). Film formation from proteins is believed to proceed through the formation of a three-dimensional network of protein molecules by ionic, hydrogen, hydrophobic, and disulfide bonds. At pH 5.2 proteins are mainly in the zwitterions form; *i.e.*, a neutral molecule with a positive and a negative electrical charge at different locations within that molecule. At this state, near the pI, the solubility of a protein is negligible because its net charge is zero and any electrostatic repulsion disappears. This fact supports the idea that film transparency could decrease at acidic pH due to protein aggregation. Our results correspond well this phenomenon in WPC/Gly films without PS. Interestingly, the addition of 0.5%, 1.0 % and 1.5% of PS significantly improved the transparency of WPC/Gly films when compared to control films without PS at pH 5.2. Under acidic conditions, protein molecules in film-forming solutions are partially unfolded due to the protein denaturation and their hydrophobic groups are exposed (Kristinsson & Hultin, 2003; Hamaguchi, Yin & Tanaka, 2007). So, the organic sorbate molecule could interact with the amino or hydroxyl groups on non-crosslinked protein through covalent bonds and thus leading to an increase in transparency (Fig. 1). This effect was also observed in analogous experiments performed by our group where incorporation of sodium benzoate or sodium propionate in WPC/Gly acidic film obtained at pH 5.2 improves transparency (David, 2012; unpublished undergraduate thesis data). So, this phenomenon seems to be independent of the organic salt and its concentration, and is probably more related to hydrophobic interactions between the organic chain and the unfolded protein matrix structure.

3.3. Scanning electron microscopy

Our observations on films transparency are supported by SEM images. As we can see in Fig. 2 (C and D), the microstructure of WPC/Gly films incorporated with PS shows a smoother

surface when compare with that of the acidic control films obtained at pH 5.2. These observations correspond well with the fact that whey protein particles formed at pH values near the pI had a cauliflower-like appearance giving rise to macroscopic gels with heterogeneous microstructure formed of larger pores (Sağlam, Venema, de Vries, Aelst & Linden, 2012). Langton & Hermansson (1992) showed that whey protein gels formed between pH 4 and 6 were opaque and had a particulate network which can be described as a network consisting of aggregated particles. These authors also reported that the size of those aggregates changed to denser and well-packed aggregates when the pH increased. So, when pH values are far from the pI the microstructure of whey proteins macroscopic gels present a homogeneous surface with relatively small pore sizes distributed regularly through the protein network (Langton & Hermansson, 1992; Sağlam et al., 2012). SEM images presented in Fig. 2 (A and B) agree with these previous experiences, while changes observed in the microstructure of the WPC/Gly films incorporated with PS suggest that interactions between protein chains and the organic acid contribute to improve the structural arrangement of the compounds, and thus could enhance film transparency.

3.4. Inhibitory activity of PS in liquid media

Our MIC data showed that the PS concentration needed to inhibit microbial growth in a liquid medium at pH 6.0 was 2-4 levels higher than at pH 5.2 for all the STEC analyzed (Table 2). These results confirm that the unprotonated-protonated ratio in the liquid medium was relevant for the antimicrobial ability of the organic acid. PS is a weak acid (pKa 4.76) which is more effective in the undissociated form due to its increased capability to penetrate the bacterial plasmatic membrane (Luck & Jager, 1997). Applying the Henderson-Hasselbalch equation (pH = pKa + log [A-]/[HA]) the resulting [sorbate]/[sorbic acid] ratios were 2.75 at pH 5.2 and 17.4 at pH 6.0. These calculations confirm the expected increase of the protonated

312 form at pH 5.2. MIC tests only consider the pH of the media; however, when the organic acid
313 is included in a complex matrix, such as an edible film over a solid media (*e.g.*, agar or food),
314 both the pH of the film matrix and the food model system must be considered in order to
315 improve the effectiveness of the antimicrobial film. Therefore, these antimicrobial-containing
316 films would appear to be best suited for foods that have acidic pH values.
317
318 *3.5. Inhibition zone assay of WPC/Gly films containing PS in agar media*
319 The acidic condition of the media (pH 5.2) for the inhibition zone assay was selected to
320 provide a pH value similar to those reported for several foods, like cheeses and meats (Casp
321 & Abril, 2003), but considering the pH limit for enteropathogens growth and survival (Kaper,
322 Nataro & Mobley, 2004).
323 At PS contents of 0.5% only films at pH 5.2 were able to inhibit all STEC analyzed, whereas
324 films at pH 6.0 failed to restrain 5 strains (Table 3). However, film discs with PS contents of
325 1.0% and 1.5%, prepared at both pH (5.2 and 6.0), inhibited all strains with inhibition zones
326 ranging from 5.3 to 13.0 mm and 2.0 to 9.7 mm, respectively. Control films without
327 antimicrobials were always non-inhibitory.
328 As expected, in most analyzed strains, inhibition zones were dependent on PS content in films
329 prepared at both pH values (5.2 and 6.0). Taking into account the results in liquid media, we
330 expected an increase in the antimicrobial activity for films prepared at pH 5.2, but this fact
331 was not so clear for all STEC strains (Table 3), This phenomena may be related to the fact
332 that PS release from film discs proved to be not affected by the pH of the WPC/Gly matrix
333 (Fig. 3). This observation support the idea that once liberated from film, [sorbate]/[sorbic
334 acid] ratio and, in this sense, preservative effectiveness, will be governed by the pH of the
335 medium; and the pH of the film may not have any consequence for a superior action of the
336 preservative. Altogether, we suggest that some of the STEC strains analyzed in this study may

337 be simply more resistant to the action of the organic acid, while others more sensitive to
338 growth under acidic conditions. In our opinion, these results reflect the importance of
339 conducting related experiences with bacteria isolated from real samples because, in nature,
340 microorganisms may act quite different than ATCC strains.
341
342 *3.6. Barrier assay of WPC/Gly films containing PS in agar media*
343 Considering that post-processing surface bacterial contamination is a major issue for the food
344 industry (Cagri et al., 2004), WPC/Gly edible films formulated with 0.5% PS were evaluated
345 for their ability to inhibit superficial growth of STEC strains in agar media. Results from the
346 barrier test could simulate what would happen if STEC post-contamination occurred on
347 WPC/Gly film-wrapped or coated foods and could be more appropriate in prospects for future
348 applications in real food samples.
349 Bacterial growth was inhibited equally well at both films pH for almost all STEC strains
350 analyzed with the exception of ARG 4627. Although, in this case, a complete growth
351 inhibition was not reached, a marked reduction of the colonies sizes was clearly observed as
352 shown in Figure 4 (right). These results strongly suggest the importance of performing
353 complementary antimicrobial assays, since each test allows a different evaluation perspective.
354 For example, the diffusion agar test did not show inhibition zones around WPC/Gly film discs
355 supplemented with 0.5% PS at pH 6.0 in 5 strains (Table 3) suggesting that those films were
356 not efficient to prevent microbial contamination. However, the barrier test showed inhibition
357 in 4 of those 5 strains demonstrating that sorbate remains chemically active in the film.
358 Although, WPC/Gly edible films obtained in this work were efficient as antimicrobial barrier,
359 PS release from films was almost completed (~90%) at 1 h in contact with agar medium (see
360 Fig. 3). Similar results were reported by Franssen, Rumsey & Krochta (2004) when analyze
361 the migration performance of PS from whey protein films. These observations point out the

362 necessity to optimize the formulations of edible films containing antimicrobials for a
363 controlled preservative release (Guillard, Issoupov, Redl & Gontard, 2009). Several studies
364 have proved that the addition of various lipids or modification of the type and amount of
365 plasticizer used in whey protein films composition can lowered the diffusion coefficients of
366 PS or sorbic acid to release the antimicrobial at a desired rate that would be advantageous in
367 some specific applications (Ozdemir & Floros 2001; Ozdemir & Floros, 2003; Franssen,
368 Rumsey & Krochta, 2004). Hence, further research are necessary to adequate film coating
369 composition and preservative concentration to obtain films with a satisfactory antimicrobial
370 activity within the accepted policies for food care that ensure microbial control and human
371 safety, in order to reduce the total amount of preservatives in foods.
372
373 **Conclusion**
374 The addition of PS to WPC/Gly-based films prepared at two different pH values (5.2 and 6.0)
375 led to inhibit the growth of STEC pathogens obtained from food samples. Our results on real
376 pathogenic isolates correlate well with related studies demonstrating that WPC may be a
377 reliable material for the elaboration of acidic antimicrobial edible films. Antimicrobial
378 effectiveness of WPC/Gly film incorporated with PS seems to be more associated to media
379 pH than to film pH because the acid-base behavior of the organic acid once liberated from
380 films. Acidic status of edible films and foods where coating will be applied are important
381 parameters to consider for optimizing films as food package because some restrictions may
382 exist due to changes in the structural, mechanical or optical properties of whey protein film
383 caused by pH. In our study, WPC/Gly films incorporated with PS proved to be transparent
384 and clear enough to be use as see-through packaging. The utilization of whey proteins and
385 low organic acids concentrations to inactivate post-processing contaminants on ready-to-eat
386 foods may be an effective, safe, ecological and relatively inexpensive alternative. However,

387 formulations of antimicrobial edible films must still be optimized to adequate the preservative
388 content by controlling its release, in order to obtain films with a satisfactory antimicrobial
389 activity within the accepted policies for food care and human health.
390
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398
399 **References**
400 Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative*
401 *Food Science and Emerging Technologies*, *3*(2), 113-126.
402 Armstrong, G., Hollingsworth, J., & Morris, G. (1996). Emerging foodborne pathogens:
403 *Escherichia coli* O157:H7 as a model of entry of new pathogen into the food supply of the
404 developed world. *Epidemiologic Reviews*, *18*(1), 29-51.
405 ASTM (1997). Designation D 1746-96: Standard test method for transparency of plastic
406 sheeting. In *Annual Book of ASTM standards* (pp. 389-391). Philadelphia: American Society
407 for Testing and Materials.
408 Balagué, C., Khan, A. A., Fernández, L., Redolfi, A. L., Aquili, V., Voltattorni, P., Hofer, C.,
409 Ebner, G., Dueñas, S., & Cerniglia, C. E. (2006). Occurrence of non-O157 shiga toxin-
410 producing *Escherichia coli* in ready-to-eat food from supermarkets in Argentina. *Food*
411 *Microbiology*, *23*(3), 307-313.

412 Cagri, A., Ustunol, Z., & Ryser, E. T. (2001). Antimicrobial, mechanical, and moisture barrier
413 properties of low pH whey protein-based edible films containing p-aminobenzoic or sorbic
414 acids. *Journal of Food Science*, *66*(6), 865-870.
415 Cagri, A., Ustunol, Z., & Ryser, E. T. (2004). Antimicrobial edible films and coatings.
416 *Journal of Food Protection, 67*(4), 833-848.
417 Casp, A., & Abril, J. (2003). *Procesos de conservación de alimentos*. (2nd ed). Madrid:
418 Mundi-Prensa. (pp. 55).
419 Chen, H. (1995). Functional properties and applications of edible films made from milk
420 proteins. *Journal of Dairy Science*, *78*(11), 2563-2583.
421 David, M. G. (2012). Estudio de las propiedades físico-químicas y de la actividad
422 antimicrobiana de películas comestibles elaboradas a base de concentrado de proteínas del
423 suero de quesería. *Unpublished undergraduate thesis, National University of Rosario,*
424 *Argentina.*
425 Fairley, P., Monahan, F. J., German, J. B., & Krochta, J. M. (1996a). Mechanical properties
426 and water vapor permeability of edible films from whey protein isolate and sodium dodecyl
427 sulfate. *Journal of Agricultural and Food Chemistry*, *44*(2), 438-443.
428 Fairley, P., Monahan, F. J., German, J. B., & Krochta, J. M. (1996b). Mechanical properties
429 and water vapor permeability of edible films from whey protein isolate and N-ethylmaleimide
430 or cysteine. *Journal of Agricultural and Food Chemistry*, *44*(12), 3789-3792.
431 Famá, L., Rojas, A. M., Goyanes, S., & Gerschenson, L. (2005). Mechanical properties of
432 tapioca-starch edible films containing sorbates. *Lwt - Food Science and Technology, 38*(6),
433 631-639.
434 Ferreira, C., Nunes, C., Delgadillo, I., & Lopes-da-Silva, J. (2009). Characterization of
435 chitosan–whey protein films at acid pH. *Food Research International*, *42*(7), 807-813.

436 Franssen, L. R., Rumsey, T.R., & Krochta, J.M. (2004). Whey protein film composition
437 effects on potassium sorbate and natamycin diffusion. *Journal of Food Science, 69*(5), 347-
438 350.
439 Gill, A., & Gill, C.O. (2010). Non-O157 verotoxigenic *Escherichia coli* and beef: a Canadian
440 perspective. *Canadian journal of veterinary research*, *74*(3), 161-169.
441 Gilmour, M. W., Chui, L., Chiu, T., Tracz, D. M., Hagedorn, K., Tschetter, L., Tabor, H., Ng,
442 L. K., & Louie, M. (2009). Isolation and detection of Shiga toxin-producing *Escherichia coli*
443 in clinical stool samples using conventional and molecular methods. *Journal of Medical*
444 *Microbiology*, *58*(Pt7), 905-911.
445 Gyles, C. L. (2007). Shiga toxin-producing *Escherichia coli*: an overview. *Journal of Animal*
446 *Science*, *85*(13), E45-E62.
447 Hamaguchi, P. Y., Yin, W.W., & Tanaka, M. (2007). Effect of pH on the formation of edible
448 films made from the muscle proteins of Blue marlin (*Makaira mazara*). *Food Chemistry*,
449 *100*(3), 914-920.
450 Hernandez-Izquierdo, V. M., & Krochta, J. M. (2008). Thermoplastic processing of proteins
451 for film formation - a review. *Journal of Food Science*, *73*(2), 30-39.
452 Hussein, H. S. (2007). Prevalence and pathogenicity of Shiga toxin-producing *Escherichia*
453 *coli* in beef cattle and their products. *Journal of Animal Science*, *85*(13), E63-E72.
454 Hussein, H.S. & Sakuma, T. (2005). Prevalence of shiga toxin-producing *Escherichia coli* in
455 dairy cattle and their products. *Journal of Dairy Science*, *88*(2), 450-465.
456 Ibarra, C., Goldstein, J., Silberstein, C., Zotta, E., Belardo, M., Repetto, H. A. (2008).
457 Síndrome urémico hemolítico inducido por *Escherichia coli* enterohemorrágica. *Archivos*
458 *Argentinos de Pediatría, 106*(5), 435-442.
459 Javanmard, M. (2009). Biodegradable whey protein edible films as a new biomaterials for
460 food and drug packaging. *Iranian Journal of Pharmaceutical Sciences*, *5*(3), 129-134.

461 Johnson, R., Clarke, R., Wilson, J., Read, S., Rahn, K., & Renwick, S. (1996). Growing
462 concerns and recent outbreaks involving non-O157 serotypes of verotoxigenic *Escherichia*
463 *coli*. *Journal of Food Protection*, *59*(10), 1112-1122.
464 Kaper, J. B., Nataro, J. P., & Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nature*
465 *Reviews Microbiology*, *2*(2), 123-140.
466 Karbowiak, T., Hervet, H., Léger, L., Champion, D., Debeaufort, F., & Voilley, A. (2006).
467 Effect of plasticizers (water and glycerol) on the diffusion of a small molecule in κ-
468 carragennan biopolymer films for edible coating application. *Biomacromolecules*, *7*(6), 2011-
469 2019.
470 Kristinsson, H. G., & Hultin, H. O. (2003). Effect of low and high pH treatment on the
471 functional properties of cod muscle proteins. *Journal of Agricultural and Food Chemistry*,
472 *51*(17), 5103-5110.
473 Krochta, J. M., & de Mulder-Johnston, C. D. (1997). Edible and biodegradable polymer films:
474 challenges and opportunities. *Food Technology*, *51*(2), 61-74.
475 Kuorwel, K. K., Cran, J. M., Sonneveld, K., Miltz, J., & Bigger, S. (2011). Antimicrobial
476 activity of biodegradable polysaccharide and protein-based films containing active agents.
477 *Journal of Food Science*, *76*(3), R90-R102.
478 Langton, M., & Hermansson, A. M. (1992). Fine-stranded and particulate gels of [beta]-
479 lactoglobulin and whey protein at varying pH. *Food Hydrocolloids*, 5(6), 523-539.
480 Luck, E., & Jager, M. (1997). *Antimicrobial food additives: characteristics, uses, and effects*.
481 (2nd ed.). Berlin: Springer Verlag. (pp. 255).
482 Mathusa, E.C., Chen, Y., Enache, E., & Hontz, L. (2010). Non-O157 shiga toxin-producing
483 *Escherichia coli* in foods. *Journal of Food Protection*, *73*(9), 1721-1736.
484 McHugh, T. H., Aujard, J. F., & Krochta, J. M. (1994). Plasticized whey protein edible films:
485 water vapor permeability properties. *Journal of Food Science*, *59*(2), 416-419.

NCCLS (2009). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*. Document M07-A8. Vol. 29. Number 2. Pennsylvania: Clinical and Laboratory Standards Institute.

Ozdemir, M., & Floros, J. D. (2001). Analysis and modeling of potassium sorbate diffusion through edible whey protein films. *Journal of Food Engineering*, *47*(2), 149-55.

Ozdemir, M., & Floros, J. D. (2003). Film composition effects on diffusion of potassium sorbate through whey protein films. *Journal of Food Science*, *68*(2), 511-516.

Ozdemir, M., & Floros, J. D. (2008). Optimization of edible whey protein films containing preservatives for mechanical and optical properties. *Journal of Food Engineering*, *84*(1), 116-23.

Pérez, L. M., Balagué, C. E., Rubiolo, A. C., & Verdini, R. A. (2011). Evaluation of the biocide properties of whey-protein edible films with potassium sorbate to control non-O157 shiga toxin producing *Escherichia coli. Procedia Food Science*, *1*, 203-209.

Pérez-Gago, M. B., & Krochta, J. M. (1999). Water vapor permeability of whey protein emulsion films as affected by pH. *Journal of Food Science*, *64*(4), 695-698.

Pérez-Gago, M. B., & Krochta, J. M. (2002). Formation and properties of whey protein films and coatings. In A. Gennadios (Ed.), Protein-based films and coatings (pp. 159-177). New York: CRC Press.

Pérez-Gago, M. B., Serra, M., Alonso, M., Mateos, M, & Del Río, M. A. (2003). Effect of solid content and lipid content of whey protein isolate-beeswax edible coatings on colour change of fresh-cut apples. *Journal of Food Science*, *68*(7), 2186-2191.

Ramos, O. L., Fernandes, J. C., Silva, S. I., Pintado, M. E., & Malcata, F. X. (2012). Edible films and coatings from whey proteins: a review on formulation, and on mechanical and bioactive properties. *Critical Reviews in Food Science and Nutrition*, *52*(6), 533-552.

510 Ramos, O. L., Reinas, I., Silva, S. I., Fernandes, J. C., Cerqueira, M. A., Pereira, R. N.,
511 Vicente, A. A., Fátima Poças, M., Pintado, M. E., & Malcata, F. X. (2013). Effect of whey
512 protein purity and glycerol content upon physical properties of edible films manufactured
513 therefrom. *Food Hydrocolloids*, *30*(1), 110-122.
514 Reilly, A. (1998). Prevention and control of enterohaemorrhagic *Escherichia coli* (EHEC)
515 infections: memorandum from a WHO meeting. *Bulletin of the World Health Organization*,
516 *76*(3), 245-255.
517 Sağlam, D., Venema, P., de Vries, R., van Aelst, A., & van der Linden, E. (2012). Relation
518 between gelation conditions and the physical properties of whey protein particles. *Langmuir*,
519 *28*(16), 6551-6560.
520 Seydim, A.C., & Sarikus, G. (2006). Antimicrobial activity of whey protein based edible
521 films incorporated with oregano, rosemary and garlic essential oils. *Food Research*
522 *International*, *39*(5), 639-644.
523 Soazo, M., Rubiolo, A. C., & Verdini, R. A. (2010). Effect of drying temperature and
524 beeswax content on physical properties of whey protein emulsion films. *Food Hydrocolloids*,
525 *25*(5)*,* 1251-1255.
526 Suppakul, P., Miltz, J., Sonneveld, K., & Bigger, S. W. (2003). Active packaging
527 technologies with an emphasis on antimicrobial packaging and its applications. *Journal of*
528 *Food Science*, *68*(2), 402-420.
529 Tharanathan, R. N. (2003). Biodegradable films and composite coatings: past, present and
530 future. *Trends in Food Science and Technology*, *14*(3), 71-78.
531 Vásconez, M. B., Flores, S. K., Campos, C. A., Alvarado, J., & Gerschenson, L. N. (2009).
532 Antimicrobial activity and physical properties of chitosan-tapioca starch based edible films
533 and coatings. *Food Research International*, *42*(7), 762-769.

534 Williams, J. M., Boyd, B., Nutikka, A., Lingwood, C. A., Barnett, F. D., Milford, D. V., &
535 Taylor, C. M. (1999). A comparison of the effects of verocytotoxin-1 on primary human renal
536 cell cultures. *Toxicology Letters*, *105*(1), 47-57.
537 Ye, M., Neetoo, H., & Chen, H. (2008). Control of *Listeria monocytogenes* on ham steaks by
538 antimicrobials incorporated into chitosan-coated plastic films. *Food Microbiology*, *25*(2),
539 260-268.
540

Table 1. Characteristics of non-O157 STEC isolates from food samples obtained in Rosario, Argentina (data from Balagué et al., 2006).

Table 2. Minimum inhibitory concentration (MIC) for potassium sorbate (PS) against non-O157 STEC and ATCC 43895 strains.

Table 3. Inhibition zone assay of WPC/Gly films containing potassium sorbate (PS) in agar media against non-O157 STEC and ATCC 43895 strains.

Figure 1. Effect of potassium sorbate (PS) and pH (6.0 or 5.2) on the transparency of WPC/Gly edible films. Different letters show significant differences ($p < 0.05$).

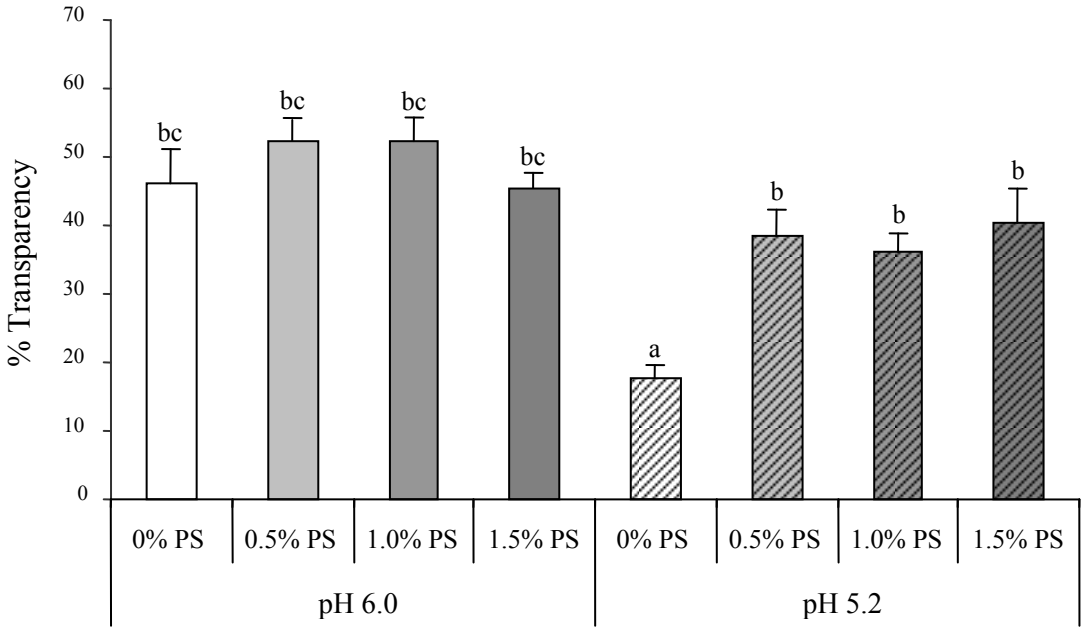
Figure 2. Scanning electron micrographs of WPC/Gly edible films. Control films without PS are shown in the first row: **A)** pH 5.2 and **B)** pH 6.0; films incorporated with 0.5% PS are shown in the second row **C)** pH 5.2 and **D)** pH 6.0. Comparison between micrographs (A) and (B) illustrates the greater heterogeneity in the microstructure of WPC/Gly edible films at pH 5.2. Note that whey protein films containing PS (C and D) shows smoother surfaces at both pH, similar to control films at pH 6.0.

Figure 3. Potassium sorbate diffusion (PS) from WPC/Gly edible films obtained at pH 5.2 and 6.0. Percentages of PS remaining in films discs at different hours in contact with agar were calculated and plotted as a function of time.

Figure 4. Barrier assay of WPC/Gly films containing potassium sorbate (PS) in agar media. *Left*, representative photograph of the barrier assay against STEC strains. Control films

without PS are shown in the first (pH 6.0) and third (pH 5.2) rows; films with 0.5% PS are shown in the second (pH 6.0) and fourth (pH 5.2) rows. Control films were always non-inhibitory. *Right***,** representative photograph of the barrier assay test against ARG 4627 STEC strain. Control WPC/Gly films without PS are shown in **A)** pH 5.2 and **B)** pH 6.0; and films supplemented with 0.5% PS are shown in **C)** pH 5.2 and **D)** pH 6.0. Note the complete absence of bacterial growth in **C)** and the reduced sizes of the colonies in **D)**.

Figure 1


% Transparency
70
60
50
40
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20
10
0
bc
bc
bc
bc
a
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b
b
0% PS
0.5% PS
1.0% PS
1.5% PS
0% PS
0.5% PS
1.0% PS
1.5% PS
pH 6.0
pH 5.2

Figure 2

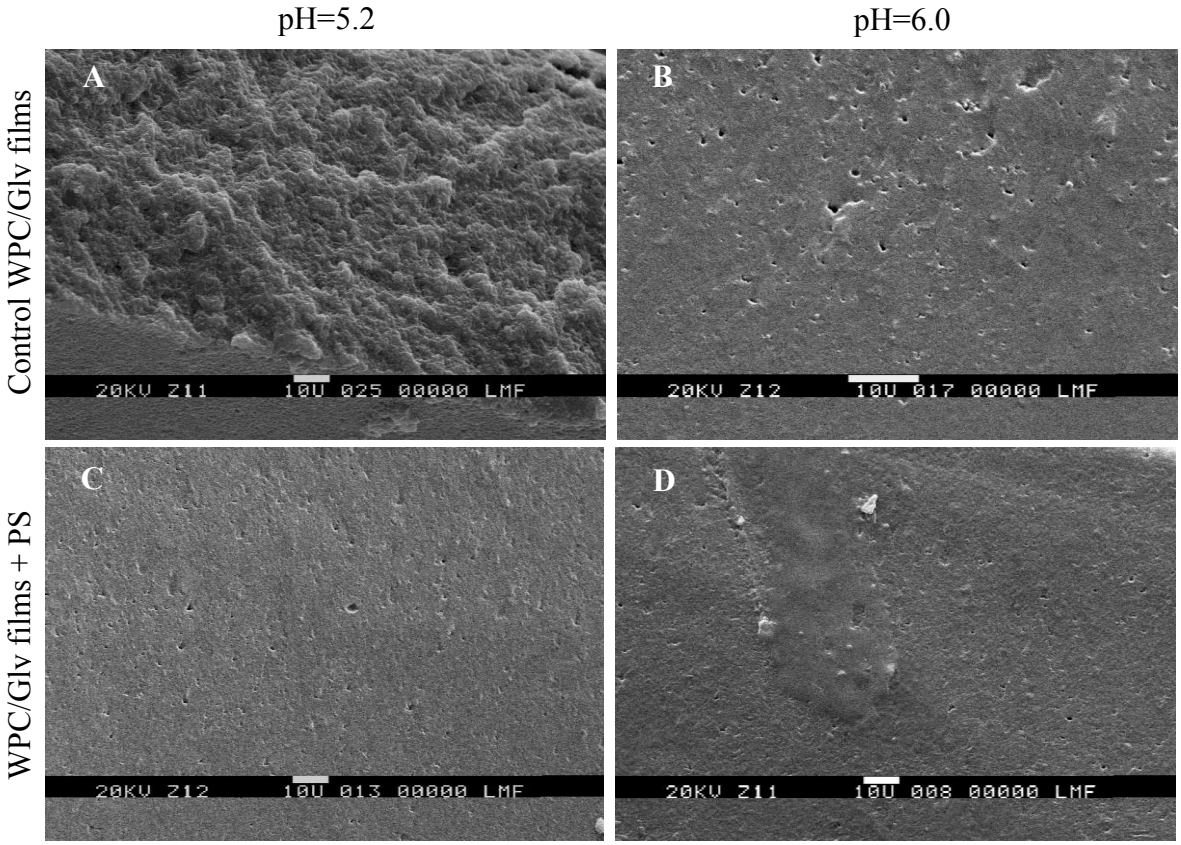
pH=5.2
pH=6.0
Control WPC/Glv films
WPC/Glv films + PS
A
B
C
D
20KV Z11 10U 025 00000 LMF
20KV Z12 10U 017 00000 LMF
20KV Z12 10U 013 00000 LMF
20KV Z11 10U 008 00000 LMF

Figure 3

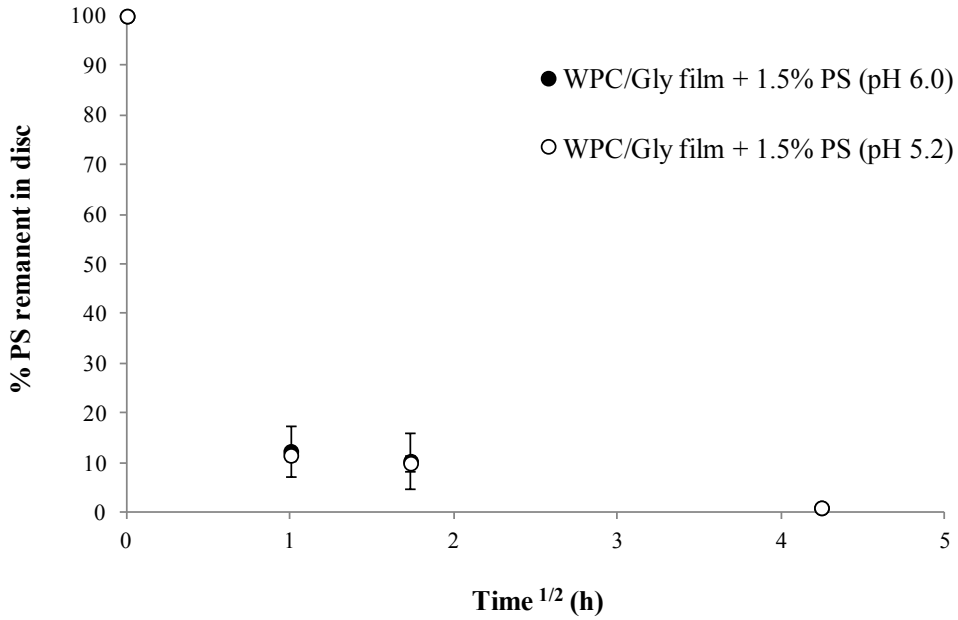
100
90
80
70
60
50
40
30
20
10
0
% PS remanent in disc
0
1
2
3
4
5
Time 1/2 (h)
WPC/Gly film + 1.5% PS (pH 6.0)
WPC/Gly film + 1.5% PS (pH 5.2)

Figure 4

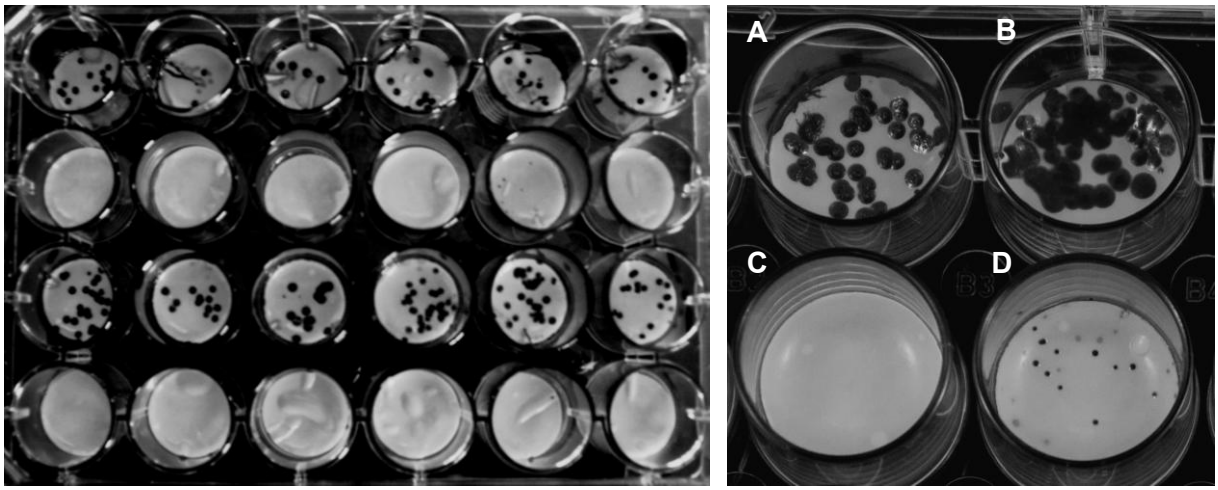

Table 1

Type of food	Strain Number	Origin of sample	PCR results for:		
			Serogroup	*stx1*	*stx2*
Soft cheese	ARG 4827	supermarket	O18	+	+
Soft cheese	ARG 2379	supermarket	O128	+	+
Cottage cheese	ARG 5266	supermarket	O79	+	+
Meat with sauce	ARG 4824	supermarket	ONT	+	+
Soft cheese	ARG 4627	supermarket	O18	+	+
Vegetables with mayonnaise	ARG 2000	food ready-to-eat	ONT	+	+
Chicken with sauce	ARG 4823	supermarket	O57	+	+
Cottage cheese	ARG 5468	food ready-to-eat	O44	+	+

ONT, O serogroup non-typable; *stx*, shiga toxin genes.

Table 2

Strain number	MIC (mg/ml)	
	pH 6.0	pH 5.2
ARG 4827	5.0	1.25
ARG 2379	2.5	0.625
ARG 5266	5.0	1.25
ARG 4824	5.0	1.25
ARG 4627	5.0	2.5
ARG 20	2.5	1.25
ARG 4823	5.0	2.5
ARG 5468	5.0	1.25
ATCC 43895	2.5	1.25

Table 3

Strain number	Diameter of inhibition zone (mm)					
	WPC/Gly films (pH 5.2)			WPC/Gly films (pH 6.0)		
	PS 0.5%	PS 1.0%	PS 1.5%	PS 0.5%	PS 1.0%	PS 1.5%
ARG 4827	4.3 ± 0.6^{a}	8.3 ± 1.5^{cd}	11.3 ± 0.6^{d}	4.7 ± 1.2^{ab}	7.7 ± 1.5^{bc}	9.0 ± 1.0^{cd}
ARG 2379	4.7 ± 1.2^{b}	8.3 ± 1.5^{c}	13.0 ± 1.0^{d}	0^{a}	7.3 ± 1.5^{bc}	9.0 ± 1.0^{c}
ARG 5266	4.0 ± 1.0^{a}	8.7 ± 0.6^{c}	10.7 ± 0.6^{d}	3.7 ± 0.6^{a}	6.3 ± 0.6^{b}	6.3 ± 0.6^{b}
ARG 4824	3.3 ± 1.2^{b}	8.7 ± 0.6^{d}	12.7 ± 1.2^{e}	0^{a}	5.7 ± 1.2^{bc}	7.0 ± 1.0^{cd}
ARG 4627	3.0 ± 1.0^{c}	8.3 ± 1.2^{d}	9.3 ± 1.2^{d}	0^{a}	2.0 ± 1.0^{bc}	7.0 ± 1.0^{d}
ARG 20	4.3 ± 1.5^{a}	8.7 ± 1.2^{bc}	10.3 ± 0.6^{c}	3.0 ± 1.0^{a}	3.7 ± 0.6^{a}	7.7 ± 0.6^{b}
ARG 4823	2.7 ± 1.2^{b}	5.3 ± 1.2^{c}	9.0 ± 1.0^{d}	0^{a}	6.0 ± 1.0^{c}	5.0 ± 1.0^{bc}
ARG 5468	3.0 ± 1.0^{a}	6.7 ± 1.2^{b}	10.3 ± 0.6^{c}	3.3 ± 1.5^{a}	6.0 ± 0.0^{b}	7.0 ± 0.0^{b}
ATCC 43895	6.3 ± 0.6^{b}	9.3 ± 1.5^{c}	11.7 ± 0.6^{c}	0^{a}	9.0 ± 1.5^{bc}	9.7 ± 1.0^{c}

Different letters in same row means significant differences ($p < 0.05$).