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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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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.

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1. Introduction

Active packaging is defined as packaging in which subsidiary constituents are deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system (Robertson, 2006). In order to control undesirable microorganisms on 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 (Kuorwel et al., 2011; Seydim & Sarikus, 2006; Tharanathan, 2003). 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; Vásconez, Flores, Campos, Alvarado, & Gerschenson, 2009; Ye, Neetoo, & Chen, 2008). However, the effect of edible film matrix pH on the antimicrobial activity of GRAS organic acids has not examined.

Potassium sorbate (PS) is the potassium salt of sorbic acid that can effectively restrain the activity of mold, yeast and areophilic 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.

The proteins in 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; Javanmard, 2009; Pérez-Gago & Krochta, 2002). Use of whey proteins to manufacture films has received a great deal of attention since they are edible and biodegradable (Krochta, & de Mulder-Johnston, 1997; Ramos, Fernandes, Silva, Pintado, & Malcata, 2012).

Cagri et al. (2001) reported that incorporating 0.5%–1.5% of sorbic acid or p-aminobenzoic acid into acidic whey protein isolate

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films (pH 5.2) can inhibit the growth of *Listeria monocytogenes*, *S. typhimurium* and *Escherichia coli* O157:H7 in trypticase soy agar supplemented with yeast extract (TSAYE) acidified to pH 5.2 but no inhibition was observed in TSAYE adjusted to pH 6.5. These results reveal the importance of the medium pH for a superior effectiveness of the organic acids. Moreover, some restrictions in the pH limit for the elaboration of whey protein edible films are related to the isoelectric point of whey proteins.

Casting films at pH values lower than the isoelectric point proved to be inefficient since the reactivity of SH groups decreases significantly (Ferreira, Nunes, Delgadillo, & Lopes-da-Silva, 2009). In addition, at acidic pH values whey proteins aggregate and films properties could decrease (Pérez-Gago & Krochta, 1999). Hence, changes in the pH of the whey protein/Gly system may affect solution stability, and thus, the antimicrobial characteristics of the films containing PS. To our knowledge, no comparative studies regarding the differences in the pH of the edible films based on whey protein and sorbate have been reported.

Shiga toxin-producing *E. coli* (STEC) is recognized worldwide as one of the most important causes of food-borne infections (Armstrong, Hollingsworth, & Morris, 1996). Clinical presentation of STEC infection varies from an asymptomatic state to bloody diarrhea and life-threatening complications such as hemolytic uremic syndrome (Johnson et al., 1996; Williams et al., 1999). STEC posses the Shiga toxin 1 and/or Shiga toxin 2 (*stx1* and *stx2*) genes considered the critical virulence factors in disease. Environmental conditions present in foods (nutrients, pH, humidity, etc.) are suitable for a rapid colonization by STEC strains to harmful levels for human health. Non-O157 STEC is now recognized as an important group of bacterial enteropathogens (Gilmour et al., 2009; Gyles, 2007). 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 (Gill & Gill, 2010; Hussein, 2007; Hussein & Sakuma, 2005). 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 (Ibarra et al., 2008; Reilly, 1998).

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. One goal of this work was to provide an array of data to support comparative studies on the molecular structure, optical and biocide properties of acidic WPC/Gly films for rational improvement of such films toward their eventual application as edible packaging.

2. Materials and methods

2.1. Materials

WPC 80% (Arla Food Ingredients S.A., Buenos Aires, Argentina) was used to prepare the film forming solutions. Gly (Cicarelli, Santa Fe, Argentina) was added to all film forming solutions as a plasticizer; PS (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was included in the formulations at different concentrations to evaluate the biocide properties of the WPC/Gly films. Trypticase Soy Broth (TSB), Cystine Lactose-Electrolyte-Deficient (CLDE) and Mueller–Hinton culture mediums were purchased from Britania (Buenos Aires, Argentina). 2,3,5-triphenyltetrazolium chloride (TTC) was obtained from Merck (Darmstadt, Germany).

2.2. Film preparation

Edible films (casting solution 11.5% total solid) were obtained with a modification of the method described by Soazo, Rubiolo, and Verdini (2010). Briefly, WPC and Gly (in proportion WPC/Gly 3:1 w/w dry solid basis) were dissolved in distilled water. After mixing, the solution was heated at 90 °C for 30 min in a water bath (TDS-40, Tecno Dalvo, Santa Fe, Argentina). Finally, the solution was homogenized (4 min; 20,000 rpm) with an Omni GLH homogenizer (Omni International Inc., Warrenton, Virginia, U.S.A.). After homogenization, the solutions were placed in an ice bath to prevent further denaturation of the whey proteins and rapidly cooled to room temperature. After incorporating 0.5%, 1.0% or 1.5% (w/w) of PS, the pH was adjusted to 5.2 or 6.0 with 1.0 N HCl using a Metrohm 713 pH-meter (Metrohm Ltd., Herisau, Switzerland). The pH-adjusted film-forming solutions were degassed 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 °C plus 24 h at 25 °C and 48 $\pm$ 4% relative humidity, after which the films were peeled from the plates and stabilized during 24 h at 25 °C and 48 $\pm$ 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 $\pm$ 0.030 mm.

2.4. Transparency

Film transparency was determined according to ASTM D1746 (ASTM, 1997) with modifications of the method described by Ozdemir and Floros (2008). The films were cut into rectangular pieces (10 mm $\times$ 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

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

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.

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 °C in TSB containing 10% (v/v) glycerol and subcultured overnight in CLDE at 37 °C 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 (NCCLS, 2009) as described by Pérez, Balagué, Rubiolo, and Verdini (2011). Briefly, an overnight culture of each bacterial strain was adjusted to McFarland 0.5 standard in saline solution (approximately 5×10^7 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.

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

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

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

In order to study the film performance to prevent microbial contamination a microplate barrier assay was developed. Mueller-Hinton agar adjusted at pH 5.2 was poured into 24 well microtiter plates (Greiner Bio-One, Frickenhausen, Germany). Inocula were prepared from an overnight culture of each bacterial strain adjusted to McFarland 0.5 standard in saline solution and then diluted 1/10,000 (approximately 5×10^3 cfu/ml). Discs of 16 mm diameter WPC/Gly films were aseptically cut from and applied on the surface of the wells filled with Mueller-Hinton agar (pH 5.2). Then, 10 μl inoculum for each strain were seeded on the film discs. Next, 10 μl of TTC (30 mg/ml) were added into each well to color the bacterial colonies. Microplates were incubated at 37 °C for 24 h until naked eye observation. Experiments were performed in triplicate.

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

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

2.11. Statistical analysis

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

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, they 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 more 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 (Hernandez-Izquierdo & Krochta, 2008; Karbowiak et al., 2006). Films showed an average thickness of 0.137 ± 0.030 mm, which is similar to those reported by other authors using similar formulations (Ramos et al., 2013; Soazo et al., 2010).

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 important 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 with 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 (Hamaguchi, Yin, & Tanaka, 2007; Kristinsson & Hultin, 2003). The organic sorbate molecule could interact with the amino or hydroxyl groups on non-crosslinked protein through covalent bonds and thus lead 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). 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 can be seen in Fig. 2 (C and D), the microstructure of WPC/Gly films incorporated with PS shows a smoother surface when compared 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, van Aelst, & van der Linden, 2012). Langton and 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 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. 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

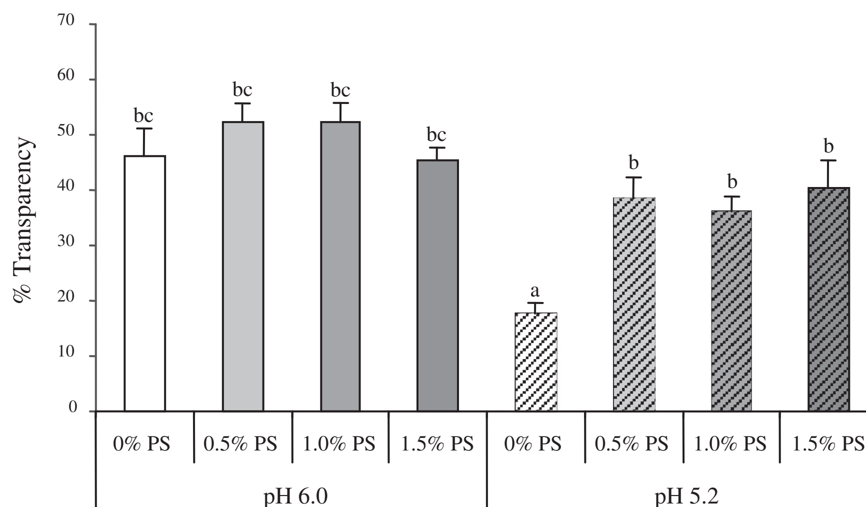

Fig. 1. Effect of potassium sorbate (PS) and pH (6.0 or 5.2) on the transparency of WPC/Gly edible films. Different letters show significant differences ($p < 0.05$).

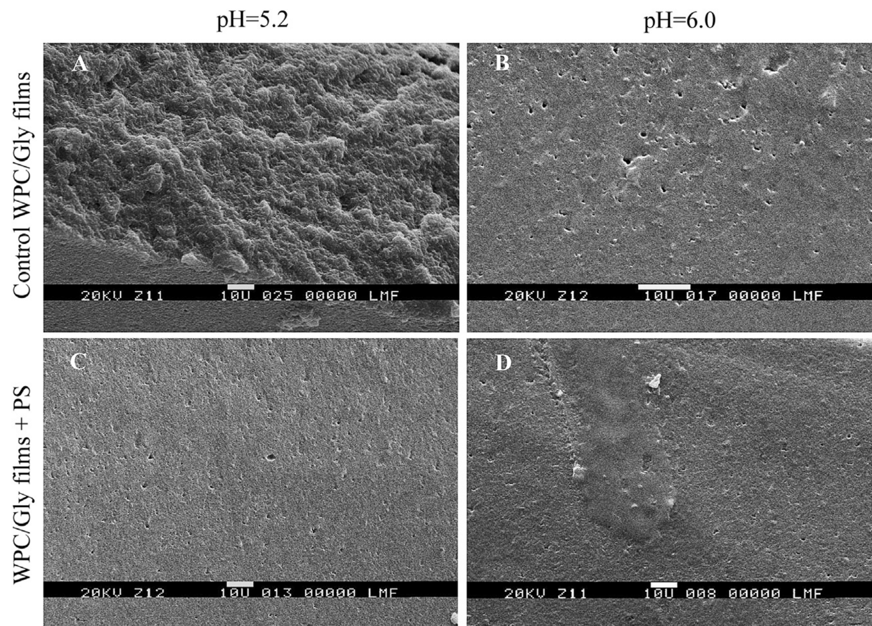

Fig. 2. Scanning electron micrographs of WPC/Gly edible films. Control films without PS are shown in the first row: A) pH 5.2 and B) pH 6.0; films incorporated with 0.5% PS are shown in the second row C) pH 5.2 and D) pH 6.0. Comparison between micrographs (A) and (B) illustrates the greater heterogeneity 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.

the undissociated form due to its increased capability to penetrate the bacterial plasmatic membrane (Luck & Jager, 1997, pp. 255). 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 form at pH 5.2. MIC tests only consider the pH of the media; however, when the organic acid is included in a complex matrix, such as an edible film over a solid media (*e.g.*, agar or food), both the pH of the film matrix and the food model system must be considered in order to improve the effectiveness of the antimicrobial film. Therefore, these antimicrobial-containing films would appear to be best suited for acid foods with $pH < 6$.

3.5. Inhibition zone assay of WPC/Gly films containing PS in agar media

The acidic condition of the media (pH 5.2) for the inhibition zone assay was selected to provide a pH value similar to those reported for several foods, like cheeses and meats (Casp & Abril, 2003, pp. 55), but considering the pH limit for enteropathogens growth and survival (Kaper, Nataro, & Mobley, 2004).

At PS contents of 0.5% only films at pH 5.2 were able to inhibit all STEC analyzed, whereas films at pH 6.0 failed to restrain 5 strains

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

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). However, film discs with PS contents of 1.0% and 1.5%, prepared at both pH (5.2 and 6.0), inhibited all strains with inhibition zones ranging from 5.3 to 13.0 mm and 2.0–9.7 mm, respectively. Control films without antimicrobials were always non-inhibitory.

As expected, in most analyzed strains, inhibition zones were dependent on PS content in films prepared at both pH values (5.2 and 6.0). Taking into account the results in liquid media, we expected an increase in the antimicrobial activity for films prepared at pH 5.2, but this fact was not so clear for all STEC strains (Table 3). This phenomenon may be related to the fact that PS release from film discs proved to be unaffected by the pH of the WPC/Gly matrix (Fig. 3). This observation supports the idea that once liberated from film, [sorbate]/[sorbic acid] ratio and, in this sense, preservative effectiveness, will be governed by the pH of the medium; and the pH of the film may not have any consequence for a superior action of the preservative. Altogether, we suggest that some of the STEC strains analyzed in this study may be simply more resistant to the action of the organic acid, while others more sensitive to growth under acidic conditions. In our opinion, these results reflect the importance of conducting related experiences with bacteria isolated from real samples because, in nature, microorganisms may act quite different than ATCC strains.

3.6. Barrier assay of WPC/Gly films containing PS in agar media

Considering that post-processing surface bacterial contamination is a major issue for the food industry (Cagri, Ustunol, & Ryser, 2004), WPC/Gly edible films formulated with 0.5% PS were evaluated for their ability to inhibit superficial growth of STEC strains in agar media. Results from the barrier test could simulate what would happen if STEC post-contamination occurred on WPC/Gly film-wrapped or coated foods and could be more appropriate in prospects for future applications in real food samples.

Bacterial growth was inhibited equally well at both films pH for almost all STEC strains analyzed with the exception of ARG 4627. Although, in this case, a complete growth inhibition was not reached, a marked reduction of the colonies sizes was clearly observed as shown in Fig. 4 (right). These results strongly suggest

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

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}	$8.7 \pm 1.2b^{c}$	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 ($p < 0.05$).

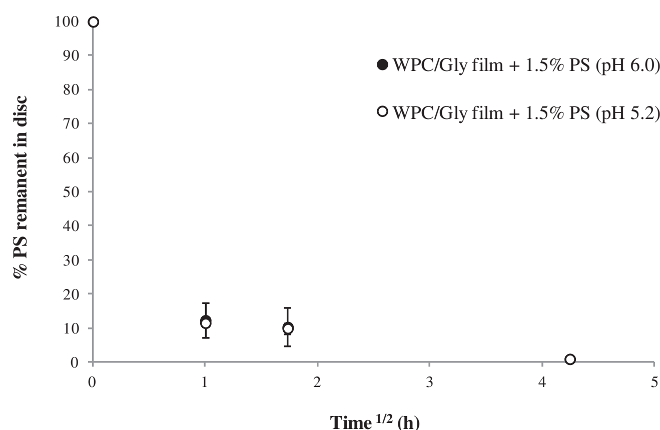

Fig. 3. Potassium sorbate diffusion (PS) from WPC/Gly edible films obtained at pH 5.2 and 6.0. Percentages of PS remaining in films discs at different hours in contact with agar were calculated and plotted as a function of time.

the importance of performing complementary antimicrobial assays, since each test allows a different evaluation perspective. For example, the diffusion agar test did not show inhibition zones around WPC/Gly film discs supplemented with 0.5% PS at pH 6.0 in 5 strains (Table 3) suggesting that those films were not efficient to prevent microbial contamination. However, the barrier test showed inhibition in 4 of those 5 strains demonstrating that sorbate remains chemically active in the film. Although, WPC/Gly edible films obtained in this work were efficient as an antimicrobial barrier, PS release from films was almost completed (~90%) at 1 h in contact with agar medium (see Fig. 3). Similar results were reported by Franssen, Rumsey, and Krochta (2004) when analyzing the migration performance of PS from whey protein films. These observations point out the necessity to optimize the formulations of edible films containing antimicrobials for a controlled preservative release (Guillard, Issoupov, Redl & Gontard, 2009). Several studies have proved that the addition of various lipids or modification of the type and amount of plasticizer used in whey protein films can lower the diffusion coefficients of PS or sorbic acid to release the antimicrobial at a desired rate that would be advantageous in some specific applications (Franssen et al., 2004; Ozdemir & Floros, 2001, 2003). Hence, further research is necessary to determine adequate film coating composition and preservative concentration to obtain films with a satisfactory antimicrobial activity within the accepted policies for food care that ensure microbial control and human safety, in order to reduce the total amount of preservatives in foods.

4. Conclusion

The addition of PS to WPC/Gly-based films prepared at two different pH values (5.2 and 6.0) inhibited the growth of STEC pathogens obtained from food samples. Our results on real pathogenic isolates correlate well with related studies demonstrating that WPC may be a reliable material for the production of acidic antimicrobial edible films. Antimicrobial effectiveness of WPC/Gly film incorporated with PS seems to be more associated with media than with film pH because of the acid–base behavior of the organic acid once liberated from films. Acidic status of edible films and foods where coating will be applied are important parameters to consider for optimizing films as food packages because some

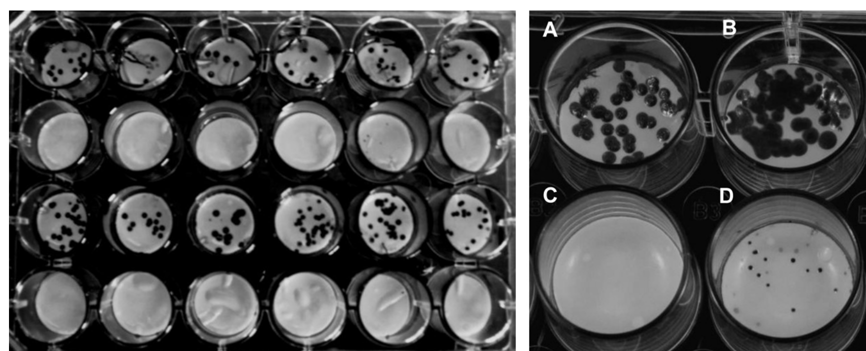

Fig. 4. Barrier assay of WPC/Gly films containing potassium sorbate (PS) in agar media. *Left*, representative photograph of the barrier assay against STEC strains. Control films without PS are shown in the first (pH 6.0) and third (pH 5.2) rows; films with 0.5% PS are shown in the second (pH 6.0) and 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).

restrictions may exist due to changes in the structural, mechanical or optical properties of whey protein film caused by pH. In our study, WPC/Gly films incorporated with PS proved to be transparent and clear enough to be used as see-through packaging. The utilization of whey proteins and low organic acids concentrations to inactivate post-processing contaminants on ready-to-eat foods may be an effective, safe, and ecological alternative. However, formulations of antimicrobial edible films must still be optimized to ensure adequate the preservative content by controlling its release, in order to obtain films with a satisfactory antimicrobial activity within the accepted policies for food care and human health.

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