Effect of *Lactobacillus acidophilus*

Addition on Mechanical and Barrier Properties of Binary Films During Storage

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Abstract

This study aimed at determining the effect *Lactobacillus. acidophilus* inclusion on mechanical and barrier properties of films based on low acyl gellan (LAG) gum and whey protein concentrate (WPC) storage for 30 days. The counting of probiotic bacteria indicated that WPC facilitates the microbial growth. This behavior may lead to a breaking structure of the films resulting in changes to the barrier and mechanical properties of films. Considering those as mentioned above it seems no convenient used WPC as a probiotic carrier. Thus, the microbial addition on film made from LAG could be viewed as an alternative in the production of active films.

Keywords: Films, low acyl gellan (LAG), microencapsulation, probiotic bacteria, whey protein concentrate

Introduction

Food packaging is a crucial step in food manufacturing due to packaging can act as oxygen and water barriers, thus slowing oxidation reactions and retaining moisture, improving quality and extending product shelf life. Nevertheless, the majority of packaging are based on plastic materials. This tendency has also led to researchers focus on developing new biodegradable packaging materials from *heteropolysaccharides* to get an alternative to plastic packaging uses [1].
Edible and biodegradable films can be produced from various sources (polysaccharide, lipid, protein) [2, 3]. Gellan gum is an anionic extracellular heteropolysaccharide produced by the bacterium *Sphingomonas paucimobilis* and consists of repeating units of a tetrasaccharide (1,3-β-D-glucose; 1,4-β-D glucuronic acid; 1,4 β-D-glucose; and 1,4-α-L-rhamnose). It is available in two forms: High acyl gellan (HAG) and low acyl gellan (LAG). When HAG is exposed to strong alkali treatment at high temperature, the acyl groups are hydrolyzed, and LAG is obtained [4]. Whey protein is an attractive material to produce environmental-friendly packaging. Heat-denatured whey proteins can produce edible films with low oxygen permeability, although they show higher water vapor permeability and lower tensile strength and elongation at break than conventional plastics (LDPE and HDPE) [5]. Tensile strength (TS) and elongation at break (EB) are the most important mechanical properties because they provide an idea of the strength and flexibility of the material to be employed as a film/coating [6].

In the film formulations, the addition of specific compounds to impart active properties such as antimicrobial, antioxidants and probiotics is very common [7]. The use of edible films incorporating viable probiotic cells has been increasing recently [8–11]. For example, Tapia et al. (2007) [12] incorporated *Bifidobacterium lactis* in coating alginate/gellan-based blends. Gialamas et al., (2010) [13] added *Lactobacillus sakei* in sodium/caseinate films to control *Listeria monocytogenes* in culture medium and fresh beef.

Probiotics are live microorganisms which when are administered in specific amount exert a beneficial effect on health [14] and an estimated daily intake of around 106–109 CFU/day is recommended to ensure a therapeutic effect. Benefits associated with the probiotics intake include modulation of the gastrointestinal system, stimulation of the immune system and reduction of lactose intolerance and irritable bowel symptoms [15]. It has been demonstrated that the inclusion of probiotic bacteria in edible films can improve the bacterial resistance to heat, osmotic stress, and survival throughout ingestion and gastrointestinal passage [16]. López de Lacey et al. (2012) [17] found that *Lactobacillus acidophilus* and *B. bifidum* into gelatin coatings remained viable during storage and reduced the population of microorganisms H2S producer. Thus, the specific objective of this work was to evaluate the effect of probiotic inclusion on mechanical and barrier properties of films based on low acyl gellan gum and whey protein concentrate stored for 30 days.

**Materials and Methods**

*Preparation of probiotic cells*
The growth of *L. acidophilus* was carried out at 37 °C for 24 h. Then, 5 mL of culture were aseptically transferred to 250 mL of MRS broth at 37 °C with shaking. After 16 h of incubation, the culture was harvested by centrifugation at 5000 rpm for 20 min, and the supernatant liquid was discarded.
The harvested bacterial cells were re-suspended in 20 mL of deionized water for film application.

**Edible film formation containing L. acidophilus**

The film forming solutions (FFS) of low acyl gellan (1% w/v) and whey protein concentrate (WPC) were prepared separately by dispersing in distilled water containing glycerol as plasticizer under constant stirring at 80°C. Subsequently, different LAG and WPC solutions were mixed in the ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 (v/v). The FFS were cooled at 37°C to be inoculated with the adequate probiotic concentration. Films were prepared using 25 mL of each solution and poured into sterile Petri dishes controlling the film thickness. Finally, all the FFS were dried for 48 h in an incubator at 34 °C. Once formed, the films were peeled off and placed in Petri dishes covered using Parafilm for air-tight storage. The procedure mentioned above was used without probiotic cells to prepare the control samples.

**Bacterial enumeration**

The viability of *L. acidophilus* incorporated into the film was determined as follows: 1 g of the prepared film containing *L. acidophilus* was suspended in 9 mL sterile peptone water and vortexed for 3 minutes to ensure sufficient dissolution of the film. The bacterial quantification was performed in triplicate by the standard plating method employing MRS agar. The total counts of the viable bacteria were expressed as log colony forming units per gram (log CFU/g). It must be highlighted that the viability of incorporated bacteria was studied in the films during 30 days of storage under RH and temperature controlled.

**Water content and thickness measurement**

The water content of each film sample was calculated according to the method of Kanmani and Lim, (2013) [8]. Each sample was cut into rectangles and dried in a hot air oven at 100 °C for 12 h. Then, the weight loss was measured as water content and expressed as a percentage based on the initial weight of the film. The thickness (mm) of each film sample was measured employing a micrometer with an accuracy of 0.01 mm. The average thickness was calculated from seven independent measurements, taken randomly at different locations of the films.

**Water vapour permeability**

Water vapor permeability (WVP) of the edible films containing probiotic was determined by the gravimetric method. All films sample were cut into a circular shape and placed on the top of a cup containing 30 mL water. Cups were covered with each film, the initial weight of the whole setup was measured, and it was subsequently positioned in a relative humidity chamber with a constant relative
humidity (RH) of 75% at 24 °C. The weight loss of each setup was measured at 24 hours intervals for 4 days. All experiments were carried out in triplicate. The WVP (g mm/kPa h m²) of each sample was calculated according to the Equation 1.

\[
WVP = \left( \frac{W}{t} \right) \cdot \left( \frac{x}{\Delta P \cdot A} \right)
\]

(1)

Where: \( w \) = weight change due to evaporate water (g); \( t \) = time (h); \( x \) = film thickness (mm); \( A \) = exposed film area (m²); \( \Delta P \) = partial pressure difference through the film.

**Opacity**

The opacity of the film samples was measured according to the method described by Soukoulis et al., (2017) [20] using a UV-VIS spectrophotometer. Each film sample was cut into rectangles (1.0 x 2.3 cm) and placed into the spectrophotometer cell after calibration with an air blank sample. The wavelength was fixed at 550 nm (A550). The measurements were carried out in four replicates for each film and the average values calculated. The higher the absorbance value, the lower the transparency. The opacity was calculated according to the Equation 2.

\[
Opacity = \frac{\text{A550}}{\text{Thickness (mm)}}
\]

(2)

**Mechanical characterization**

The mechanical properties measured were tensile strength (TS) (Equation 3) and percentage of elongation at break (EB) (Equation 4) using a texturometer Shimadzu model EZ-Test EZ-S. Edible films samples were placed between the tensile grips giving a grip separation distance of 65 mm. A cross-head speed of 30 mm/s.

\[
TS = \frac{\text{Force at break (newton)}}{\text{area (mm²)}}
\]

(3)

\[
\%EA = \frac{L_{\text{max}}}{L_0} \times 100
\]

(4)

Where, \( L_0 \) = initial film length (mm), \( L_{\text{max}} \) = film length at break (mm).
**Statistical analyses**

The experimental data were analyzed to determine whether differences in the properties measured were statistically significant. Statistical comparisons between films samples were made by analysis of variance (ANOVA-one way) followed by Tukey's test using the Statistical Package for the Social Sciences (SPSS for windows). The significance level was set at p<0.05.

**Results and Discussion**

**Enumeration of probiotic bacteria**

The development of edible films is an interesting strategy that could be useful for probiotic cells delivery in target place. Figure 1 shows the behavior of *L. acidophilus* into films based on WPC and LAG mixtures during 30 days of storage, where it can be observed that materials employed for films manufacturing (LAG and WPC) have no toxic effect on probiotic viability. It is therefore assumed that there was not injured effect due to the conditions applied (heating and osmotic) during film drying process. Although it is relevant to consider the ionotropic gelation, which takes place when anionic polysaccharides are mixed with WPC resulting in the formation of stable molecular networks that may stabilize the probiotic viability [18]. Considering those as mentioned above, our results disagree with those reported by Yonekura *et al.* (2014) [19] who stated that *L. acidophilus* strains could be injured by the osmotic and heating conditions employed through film manufacture.

As can be seen in Figure 1, the probiotic viability was significantly influenced by WPC, which indicates that *L. acidophilus* growth using WPC as a substrate. Similar findings were published by Soukoulis *et al.* (2017) [20] who found that supplementation of the FFS with WPC resulted in an enhanced *L. rhamnosus* GG storage stability.

The population of *L. acidophilus* presented a significantly increased (p < 0.05) during the entire storage period (30 days), where the highest (11.5 UFC) amounts of *L. acidophilus* was found inside films made from WPC at the end of the storage period (30 days). Whey protein had significant positive effects on the survival of probiotic microorganisms during storage [21] due to providing nutrition for the microbial cells [22].

It must be noted that all studied films had similar bacterium concentration (8.8 to 9.1 UFC) at the beginning of storage period (1 day). To sum up, films manufactured with higher amounts of WPC were most useful for maintaining biological activity of the probiotic cells compared with those films made from LAG, this behavior also could be explained by the low water vapor permeability.
presented in the binary system. Moreover, glycerol and WPC are known as protectants could help in protecting the viability of probiotic cells [14].

Figure. 1. Changes in the number of *Lactobacillus acidophilus* inside films based on WPC and LAG mixtures during 30 days of storage

*Thickness and Water content*

The thickness is one of the most important parameters, related to transparency, water vapor permeability, and mechanical properties of the films. At the beginning of the storage time (1 day), all studied films showed thickness values ranged from 0.16 to 0.18 mm indicating that there were no significant differences (p<0.05) induced by the ratio LAG /WPC. This result was probably obtained due to the control exerted during films production. Conversely, when the storage time increase, significant differences (p<0.05) were found in all films (Figure 2 A), which could be caused by loss water trapped inside matrix formed by LAG and by the microbial growth previously discussed.
The water content was significantly (p<0.05) affected by the ratio WPC/LAG employed in the preparation of films. Similar results were reported by Soukoulis et al. (2017) [20] who found that water content is affected by the type of material employed for manufacturing film, which in turn is caused by the ability of these materials to form hydrogen bonds with water molecules [23].

Films made from LAG with or without *L. acidophilus* exhibited at the beginning of storage period (1 day) the highest moisture levels with 12.56 and 12.48 % respectively. Films based on WPC with or without *L. acidophilus* showed lowest values (5.21 – 5.23% respectively) and those elaborated with mixtures of WPC/LAG presented intermediate moisture values with 6.98 and 11.52 % at the beginning of storage period (1 day). Its means, that microbial growth does not affect water content property. By applying ANOVA statistical analysis (one way) to the water content during storage, it was appreciated a slight decrease in whole mixture films with or without *L. acidophilus*. In this study the amount of plasticizer was set and remained constant during complete analysis due to glycerol can affect the water content.

**Water vapour permeability (WVP)**

The main objective of applying films on food is to improve the quality and extend the shelf life of food products. Given the above, films with low WVP are desired for food packaging industries. Figure 3 depicts the WVP behavior of films made from WPC and LAG during 30 days of storage. The lowest WVP values (0.05 g mm/kPa h m2) was found in films based on LAG with and without *L. acidophilus*.
at the end of the storage period (30 days) indicating that probiotics presence did not affect WVP values of films based on LAG. Conversely, films made from WPC containing *L. acidophilus* at the end of the storage period (30 days) presented higher values of WVP (1.84 g mm/kPa h m²) compared with those found on films without *L. acidophilus* (1.30 g mm/kPa h m²) at the same storage time. Also, it should be mentioned that films containing *L. acidophilus* with WPC concentrations higher than 50 % (p/v) had mean increases of 0.15 (g mm/kPa h m²) in the WVP values after 15 days of storage. It is therefore assumed that *L. acidophilus* could degrade whey protein, decreasing the protein network, which increases permeability. In general term, the WVP at the beginning of storage period was a descending function of WPC concentration as a result of the reduction of the intermolecular space due to hydrogen bond formation. However, when films made from different WPC ratios are incorporated with *L. acidophilus* an increased in WVP values was presented. This behavior is supported by the moisture data values aforementioned.

Figure 3. Water vapour permeability behavior of films based on WPC/LAG during 30 days of storage

Optical properties
Evaluate the light transmission on films is a key parameter considering that it influences the appearance and taste of packaged food product. Films based on WPC without probiotic bacteria at the beginning of the storage period (1 day) had the highest transparency values (3.63), while films made from LAG storage during 30 days had the lowest value (1.66). All blend films (WPC/LAG) had transparency values ranging from 3.27 to 2.31. It is noteworthy that there were no
significant differences (p<0.05) during the entire storage period in films based on LAG with or without *L. acidophilus* indicating that gellan is not a suitable substrate for microbial growth as can be seen in Figure 1. Film opacity increased significantly (p< 0.05) with the presence of WPC, which could be caused to the Maillard reaction [24] as well as the microbial growth that result in lactic acid production. These results align very well with those reported by Soukoulis *et al.* (2017) [20] who found that WPC addition induced a significant (p<0.05) changes on film color. As illustrated in Figure 4, the opacity values of the films increased significantly (p<0.05) by the presence of *Lactobacillus acidophilus*. These findings are contrary to those reported by Soukoulis *et al.* (2014) [20] who declared that film opacity is not significantly affected by the presence of probiotic cells.

Figure 4. Opacity of films made from WPC, LAG and their blends stored during 30 days

[![Opacity of films made from WPC, LAG and their blends stored during 30 days](image)](image)

**Mechanical properties of films made from low acyl gellan ang whey protein concentrate**

Mechanical properties such as tensile strength (TS) and elongation at break (EB) gives information concerning resistance and flexibility of one material. These characteristics are important to hold out stresses involved in conventional processing, handling and storage conditions. Figure 5A shows as films based on LAG and containing probiotic bacteria had the highest tensile strength with 66.4MPa, followed by the LAG-film without probiotic (65.7 MPa). This behavior means that probiotic addition on LAG-film has not effect on TS. Similar results were achieved by Kannani and Lim (2013), through the addition of probiotic cells into the pullulan/starch film which resulted in a reduction of TS. By the opposite, WPC-films containing *L. acidophilus* and stored during 30 days had a lower value (18.5 MPa) compared with those LAG-films, this behavior could be
attributed to the protein degradation network caused by probiotic growth. Considering the TS reduction values, it can be concluded that films based on WPC may be less useful packaging material where resistance to high mechanical stresses by handling or processing operations is mandatory. Films manufactured with mixtures WPC/LAG resulted in intermediate values ranging from 24.1 to 58.8 MPa. In general term, films based on different amounts of WPC and containing probiotic bacteria had lower TS values than films without *L. acidophilus*.

Figure 5. Values of tensile strength and elongation at break of films based on whey protein concentrate and low acyl gellan gum storage during 30 days

To determinate, the EB on films is of great significance since this parameter let give an idea to the manufacturer about the flexibility of the material employed for film production. The highest EA value (1.74 %) was presented on WPC-films without *L. acidophilus* at the end of the storage period (30 days) (Figure 5B). Most likely, this high EA value was caused by the presence of hydrogen bond formation and expulsion of water during storage. However, when WPC-film has incorporated with *L. acidophilus*, the EA value decrease until reaching values near to 0.55%. Conversely, the lowest percentages of elongation were obtained on films based only in LAG and containing *L. acidophilus* (0.77 %) and those LAG-films without *L. acidophilus* (0.57 %) at the beginning of storage period. As was expected, blend films (WPC/LAG) had intermediate values between those previously mentioned for LAG and WPC films. It should be highlighted that there were no statistically differences (p<0.05) between all films with or without *L. acidophilus* at the beginning of storage period (1 day). In general term, all films without probiotic bacteria had higher EA values than those films with *L. acidophilus*, whose flexibility decreased significantly during storage (p<0.05) as a result of the aforementioned microbial metabolism. Changes in TS and EB values have also been attributed to the formation of a denser, rigid structure that produced stronger and less extensible protein matrices [25].
Conclusion

This study provides an insight into the effect of incorporation of L. acidophilus on mechanical and barrier properties of films made from whey protein isolate and low acyl gellan gum. The findings show an influence of L. acidophilus addition on the barrier and mechanical properties of films containing WPC. This behavior can be attributed to the microbial growth which degrades WPC resulting a breaking structure of the films. Considering the previously premise, it seems no convenient employed WPC as a probiotic carrier. Thus, the microbial addition on film made from LAG could be viewed as an alternative in the production of active films.

References


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