# Development of bio-based active packaging material evaluating grapefruit seed and grape seed extract as antimicrobials

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# Development of bio-based active packaging material evaluating grapefruit seed and grape seed extract as antimicrobials

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**Abstract.** New trends related to consumers in food packaging include the preference for green and sustainable biopolymers. This study determined a formulation and process flow diagram for the development of an active packaging based on Pullulan and Polylactic acid (PLA), incorporating grape seed and grapefruit seed extract as antimicrobials. Physical, mechanical, and antimicrobial properties of the created film were evaluated. Two completely randomized design with two variables to optimize film formulations was used. The independent variables were grape seed and grapefruit seed extract in two concentrations (1 and 5%). Three replicates were performed for each treatment, for a total of 24 experimental units for each statistical analysis. Evaluations performed were thickness, ultimate tensile strength (UTS), elongation at break (EL), elasticity (YM), color (L*, a*, b*), and antimicrobial activity. Results for mechanical properties indicated that there was an interaction between the extract and concentrations, meaning that, depending the concentration of the extract, the mechanical properties will change. In contrast, antimicrobial properties *Listeria monocytogenes, Salmonella* Infantis and Seftenberg; *Escherichia coli* O26 and O157:H7 are susceptible to films with grapefruit seed extract. However, the inhibitory effect on bacteria growth will remain the same regardless of concentration. This point out that Pullulan with grapefruit seed extract can be used as an active packaging, inhibiting Gram-positive (*Listeria monocytogenes*) and Gram-negative (*Salmonella* spp and *Escherichia coli*) foodborne pathogens.

**Keywords:** Antimicrobial activity, biopolymer, pullulan, PLA, physical properties.

**Resumen.** Las nuevas tendencias relacionadas con los consumidores con los empaques de alimentos incluyen la preferencia por polímeros ecológicos y sostenibles. Este estudio determinó un diagrama de flujo para la formulación y desarrollo de un empaque activo a base de Pullulan y ácido poliláctico (PLA), incorporando extracto de semilla de uva y semilla de toronja como antimicrobianos. Se evaluaron propiedades físicas, mecánicas y antimicrobianas a las películas creadas. Se usaron dos Diseños Completamente al Azar con dos variables para optimizar las formulaciones de las películas. Las variables independientes fueron extracto de semilla de uva y semilla de toronja en dos concentraciones (1 y 5%). Se realizaron tres réplicas para cada tratamiento, para un total de 15 unidades experimentales por cada análisis estadístico. Se evaluó grosor, resistencia máxima a la tracción (UTS), alargamiento a la rotura (EL), elasticidad (YM), color (L*, a*, b*) y actividad antimicrobiana. Los resultados de las propiedades mecánicas indicaron que hubo interacción entre el extracto y sus concentraciones; indicando que, dependiendo de la concentración del extracto, las propiedades mecánicas cambian. En el caso de las propiedades microbiológicas, *Listeria monocytogenes, Salmonella* Infantis y Seftenberg; *Escherichia coli* O26 and O157:H7 resultaron susceptibles a las películas con extracto de semilla de toronja. Sin embargo, la inhibición de las bacterias seguirá siendo el mismo independientemente de la concentración. Los resultados señalaron que las películas de Pullulan y semilla de toronja pueden utilizarse como empaque activo para la inhibición de patógenos transmitidos por alimentos, ya sea Gram-positivo (*Listeria monocytogenes*) o Gram-negativo (*Salmonella* spp y *Escherichia coli*).

**Palabras clave:** Actividad antimicrobiana, biopolímero, pullulan, PLA, propiedades mecánicas.

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# 1. INTRODUCTION

New trends related to consumer trend in food packaging include the preference for green and sustainable biopolymers. Traditionally petroleum packaging has been a convenient, economical, and efficient market but food industries are slowly thinking about sustainable alternatives.

Biopolymers can be classified into natural biopolymers extracted from biomass (e.g. agro resources), synthetic biopolymers from microbial production or fermentation (e.g. polyhydroxy-alkanoates and PHA), synthetic biopolymers conventionally and chemically synthesized from biomass (e.g. PLA) and synthetic biopolymers conventionally and chemically synthesized from petroleum product (e.g. PCL) (Othman 2014). Packagings that are degraded or decomposed by the action of natural organisms are defined as biopolymers. If a packaging also has active functions beyond the inert passive containment and product protection, such as assuring safety and preserving quality, that packaging is defined as active. Among the available biopolymers, Pullulan shows excellent film formability, considerable mechanical strength, the ability to form thin and flexibles layers and unique antimicrobial activity.

Pullulan is a polysaccharide obtained from *Aerobasidium pullulan*. This compound is a maltotriose with high solubility in water and flexibility and insoluble in organic acids. (Trinetta and Cutter 2016). Pullulan is a non-toxic, non-mutagenic, non-carcinogenic, biodegradable, and edible biopolymer (Kumar *et al.* 2012). Another suitable biopolymer is PLA (polylactic acid) obtained by the synthesis of lactic acid, which it can be provide by corn, starch, or other renewable source. This compound is GRAS, generally recognized as safe by FDA (García Ibarra *et al*. 2016). When developing active, antimicrobial, and biodegradable packaging, the GRAS status is a key characteristic for biocide molecules selection. On the other hand, grape seed extract is a natural resource that can provide different benefits for food safety and quality, but with a narrow antimicrobial spectrum. The ideal candidate for food preservation is a natural resource with high yields and low costs of production. It is extracted from grape (*Vitis vinifera*) which is one of the most widespread crops in the world with approximately 75 million tons product per year (FAO and OIV 2016).

Differences in grape seeds extract antimicrobial activity have been observed. According to Jayaprakasha *et al.* (2002) grape seed extract had antimicrobial effect against Gram-positive and Gram-negative bacteria, such as *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Bacillus coagulans, Escherichia coli and Pseudomonas aeruginosa.* According to Delgado Adámez *et al.*(2012) the inhibitory effect of phenolic compounds from seeds extracts were more effective against Gram-positive bacteria, than Gram-negative such as *Staphylococcus aureus*. Additionally, Shoko *et al.* (1999) found that methanol extract from grape seeds combined with gallic acid had antimicrobial activity against *Escherichia coli and Salmonella enteritidis* (Shoko *et al.* 1999), highlighting that its phenolic composition might be responsible for the antimicrobial activity (Delgado Adámez *et al.* 2012).

Another suitable antimicrobial compound for food packaging application is grapefruit seed extract. This extract is obtained from grapefruit (*Citrus paradisi*), the largest grapefruit producing country is United States (FAO 2003) followed by Israel and Cuba. Grapefruit is a rich source of vitamin C, phenolic (flavonoids, phenolic acids and coumarins) and terpenic compounds, such as carotenoids and limonoids (Çiçek Polat *et al*. 2018). All these molecules influence the antimicrobial activity of grapefruit extract. According to Çiçek Polat *et al.* (2018) grapefruit extract has strong antimicrobial activity against *Salmonella enteritidis*. Furthermore, Krajewska-kulak *et al.* (2003) proved that grapefruit extract has antimicrobial activity against yeast. Also, Al–Âni *et al.* (2011), report that *Staphylococcus aureus, Proteus vulgaris, Klebsiella pneumonia and Candida albicans* were reduced with grapefruit extract.

The objectives proposed for this study were:

- Determine a formulation and flow process for the development of an active packaging based on Pullulan and Polylactic acid (PLA), incorporating grape seed and grapefruit seed extract as antimicrobials compounds.

- Evaluate the physical, mechanical, and antimicrobial properties of a film based on Pullulan and Polylactic acid with grape sees and grapefruit seed extract as antimicrobials.

# 2. MATERIALS AND METHODS

The research took place at the Food Microbiology and Safety laboratory at Kansas State University, where film fabrication took place, as well as the physical, mechanical, microbiological properties measurements.

Film fabrication was the first phase of this research. Polylactic acid (PLA) film requires treatment with an organic solvent, while Pullulan was dissolved in water. Both films were dry overnight. The second phase included the measurement of the optical and mechanical properties of films. The third phase consisted of measuring the films activity against different bacteria strains.

**The raw material used for films fabrication.**

- Pullulan powder.
- PLA powder
- Xanthan gum
- Locust bean gum

**Chemical reagents.**

- Chloroform ($CHCl_3$)
- Deionized water
- Glycerol

**Equipment.**

- Texture analyzer
- Convection oven
- Hunter MiniScan EZ spectrophotometer

**Phase 1. Films fabrication protocols**

PLA films were fabricated using 2.5 grams of PLA, then the PLA crystals were mixed with 80 mL of chloroform, the mixture were stirred for 45 minutes until PLA dissolves completely. After the stirred, the solution was poured into glass petri dishes. As final steps, the samples were placed under a chemical hood for a drying process at room temperature an overnight. Allowing to evaporate the organic solvent in films.

For Pullulan films fabrication, firsts were diluted 10 g of Pullulan powder in 100 mL distilled water. The second step was stirring the aqueous solution on a hot plate at 45 °C until powder were dissolved. Then, 3 g/L of glycerol was added as a plasticizer. Additionally, 0.1 g/L of xanthan and locust bean gum were added to stabilize films matrix. The mixture was then homogenized and autoclaved at 121 °C in a cycle of 45 minutes. After been autoclaved, the mixture was cooled down in a water bath at 80 °C. Afterwards 15.5 mL of the mixture was pipetted , poured into sterile plastic weight boats and dried under hood overnight For Pullulan films with grape seeds and grapefruit seeds extracts fabrication, the same protocol previously described was carried out. However, a step was added, which was the addition of 1 and 5% of the extracts during the cooling process (Trinetta *et al*. 2011) (Trinetta *et al*. 2010).

**Phase 2. Optical and mechanical properties.**

The optical and physical properties of the films were measured. The film's thickness was measured with a digital micrometer, testing three random locations and an average measurement for each replicate was reported (McDaniel *et al.* 2017).

Ultimate tensile strength was measured following the ASTM D882-08 standard. First, a load range was identified and measured the cross-sectional area of the sample, films samples were cut with 1 cm of width and 5 cm of length. The speed used for the test was 10 mm/s. The samples were placed in the testing machine grips, the load versus extension recorded. Additionally, the pulling force was recorded until the films were broken. Producing a load-extension curve, between 30 and 60° from the X axis (ASTM D882-08 2002;(McDaniel *et al.* 2017).

The color of the films was evaluated using a Hunter MiniScan EZ spectrophotometer (Model: MSEZ-4500L, 45°/0° geometry, Large viewing area; HunterLab Reston, VA). Each film was sampled five times and averages values were reported for L*, a*, and b* (McDaniel *et al.* 2017).

**Phase 3. Antimicrobial properties.**

The grape seeds and grapefruit seeds extracts were incorporated to aqueous mixture of Pullulan films. The first step was dilute 10 g of Pullulan powder in 100 mL distilled water. Then, was stirred the aqueous solution on a hot plate to reach a temperature of 45 °C until powder was dissolved. After it 3 g/L of glycerol were added as a plasticizer. To stabilize the matrix, xanthan, and locust bean gum (0.1 g/L) were added. Then, the solution was homogenized and autoclaved at 121 °C for 45 minutes. The solution was cooled down with a water bath at 80 °C and added the seed extracts at 1 and 5% (V: V). Aliquots of 15.5 mL solution were pipetted into sterile 10 cm diameter petri dishes and dried under the hood overnight (Trinetta *et al*. 2011) (Trinetta *et al*. 2010). 10 mm diameter disks were cut from each film.

After film fabrication the antimicrobial properties against *Listeria monocytogenes* (7644, B2-323, B-33043, B-33260)*, Escherichia coli* (O121, O26, O157:H7) and *Salmonella* (Infantis, Newport, Thompson and Senftenberg) were tested.

The strains used were kept in CryoCare Organism Preservative System (Key Scientific, Stamford, TX) and stored at -80 °C until experiments. Beads were transferred into 10 mL Tryptic Soy Broth (TSB) (Difco Laboratories), incubated at 37 °C and the streaked for isolation on Tryptic Soy Agar (TSA) (Difco Laboratories, Spark, MD). Plates were incubated for 24 h at 37 °C and kept at refrigeration temperature (4 °C) until used for experiment. First, the isolated colonies were propagated two consecutive days into 10 mL of TSB and incubated for 16 h at 37 °C before use. Afterwards, 150 µL of the culture were transferred to an empty petri dish and 15 mL of TSA were added, the solution was gently mixed and allowed for solidification. After that, circle-shaped film discs of 10 mm diameter were placed onto the inoculated TSA following a modified zone of inhibition Kirby-Bauer test set up (Hudzicki 2009).

Additional tests were performed with the liquid grapefruit seed and grape seed extracts at 33 and 40% concentration, respectively. Where 10 mm diameter holes were craved on the TSA agar and 25 µl of each undiluted extract were added. Then the plates were incubated at 37 °C for 24 h. After that, the inhibition zones (halos) were measured (mm) (Trinetta *et al*. 2010); (McDaniel *et al.* 2017).

**Experimental design for mechanical properties.**

A Completely Randomized Design with two variables was applied to optimize pullulan films formulation. The independent variables were grape seed extract in two different concentrations (1 and 5%) and grapefruit seed extract in two different concentrations (1 and 5%). Also, Polylactic acid (PLA) films formulation without active compound was tested (Table 1). Three replicates were performed for each specific treatment for a total of 24 experimental units. Since the purpose of the package is to preserve food safety, the stability of the package over time and observe color change for each film combination. The following mechanical and optical properties responses were recorded: thickness, ultimate tensile strength (UTS), elongation at break (EL), elasticity (YM), color (L*, a* and b*). A General linear model (GLM) procedure was used, and an ANOVA performed, also, a LS MEANS was applied with 0.05 significant level.

Table 1. Treatment descriptions for testing mechanical properties of film.

| **Material** | **Control** | **GSE 1%** | **GSE 5%** | **GFSE 1%** | **GFSE 5%** | **PLA** |
|---|---|---|---|---|---|---|
| Polylactic acid powder (g) | 0 | 0 | 0 | 0 | 0 | 2.5 |
| Pullulan powder (g) | 10 | 10 | 10 | 10 | 10 | 0 |
| Water (mL) | 100 | 100 | 100 | 100 | 100 | 0 |
| Glycerol (g/L) | 3 | 3 | 3 | 3 | 3 | 0 |
| Chloroform (mL) | 0 | 0 | 0 | 0 | 0 | 80 |
| Xanthan gum (g/L) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0 |
| Locust bean gum (g/L) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0 |
| Grape seed extract (%) | 0 | 1 | 5 | 0 | 0 | 0 |
| Grapefruit extract (%) | 0 | 0 | 0 | 1 | 5 | 0 |

Control: Film without extract, GSE: grape seed extract, GFSE: grapefruit seed extract, PLA: Polylactic acid.

**Experimental design and data analysis of antimicrobial properties.**

A Completely Randomized Design with two variables was used to optimize the inhibitory effect of pullulan film formulations. The independent variables were grape seed extract in two different concentrations (1 and 5%) and grapefruit seed extract in two different concentrations (1 and 5%) (Table 2). Three replicates were performed for each specific treatment, for a total of 15 experimental units. An GLM procedure was used. Also, a LSMEANS mean separation were applied with a significant level of 0.05. Liquid extracts were compared using a T-Test with a significant level of 0.05.

Table 2. Treatment descriptions for testing antimicrobial properties of film.

| **Material** | **Control** | **GSE 1%** | **GSE 5%** | **GFSE 1%** | **GFSE 5%** |
|---|---|---|---|---|---|
| Pullulan powder | 10 g | 10 g | 10 g | 10 g | 10 g |
| Water | 100 mL | 100 mL | 100 mL | 100 mL | 100 mL |
| Glycerol | 3 g/L | 3 g/L | 3 g/L | 3 g/L | 3 g/L |
| Xanthan gum | 0.1 g/L | 0.1 g/L | 0.1 g/L | 0.1 g/L | 0.1 g/L |
| Locust bean gum | 0.1 g/L | 0.1 g/L | 0.1 g/L | 0.1 g/L | 0.1 g/L |
| Grape seed extract | 0% | 1% | 5% | 0% | 0% |
| Grapefruit extract | 0% | 0% | 0% | 1% | 5% |

Control: Film without extract, GSE: grape seed extract, GFSE: grapefruit seed extract.

# 3. RESULTS AND DISCUSSION

**Physical and mechanical properties.**
According to the statistical analysis revealed probabilities ($P < 0.05$) for extract and interaction between extracts and concentrations. Indicating that these aspects presented significant differences in the mechanical properties of pullulan control films and pullulan active films. Thickness is one of the main characteristics that films must comply with. The results indicate that each treatment presented different thicknesses. In Table 3 it can be observed that there is a significant effect of the concentration of the extracts on the thickness of the films. However, the effect of the concentration was not consistent for both extracts. In the case of GSE, the thickness increased as the concentration increased, but for the GFSE, the thickness of the films decreased as the concentration increased. On the other hand, PLA films showed the lowest thickness. It is important to note that these latter films did not contained any extract. These results agreeing with Rodríguez-Hernández (2014) who observed that the films' morphology can be affected by temperature, substrate, solvent type, concentration, chemical composition, and the polymers' molecular weight. (Table 3).

Table 3. Mechanical properties measured in films based on pullulan and PLA.

| Mechanical properties | THK (mm) ± SD | UTS (MPa) ± SD | EL (%) ± SD | YM (MPa) ± SD |
|---|---|---|---|---|
| GSE 1% | $0.38 \pm 0.029^{B}$ | $101.0 \pm 16.06^{A}$ | $79.3 \pm 48.91^{BC}$ | $0.0029 \pm 0.002064^{AB}$ |
| GSE 5% | $0.42 \pm 0.024^{A}$ | $25.3 \pm 3.60^{C}$ | $136.7 \pm 4.23^{B}$ | $0.0003 \pm 0.000023^{B}$ |
| GFSE 1% | $0.37 \pm 0.036^{B}$ | $28.8 \pm 5.47^{C}$ | $203.0 \pm 19.83^{A}$ | $0.0002 \pm 0.000077^{B}$ |
| GFSE 5% | $0.32 \pm 0.027^{C}$ | $4.3 \pm 1.53^{D}$ | $138.7 \pm 47.17^{B}$ | $0.0001 \pm 0.000003^{B}$ |
| PLA | $0.09 \pm 0.014^{D}$ | $56.4 \pm 7.50^{B}$ | $18.7 \pm 8.74^{C}$ | $0.0014 \pm 0.000697^{A}$ |

Control: Film without extract, GSE: grape seed extract, GFSE: grapefruit seed extract, PLA: Polylactic acid, THK: Thickness, UTS: Ultimate tensile strength, EL: Elongation at breaks, YM: Young modulus, A-D: Means followed with different letters within each column are statistically different ($P < 0.05$), SD: Standard deviation.

As is shown in Table 3, none of the films exceeded the 1 mm, which is the thickness limit established for thin films (ASTM D882-08 standard 2002). However, comparing the results obtained by Trinetta *et al.* (2011) who reported a thickness average of 0.054 mm, while PLA and pullulan active films obtained lower thickness values. This may be attributed to films viscosity, according to Bodiguel and Fretigny (2006) they found that when viscosity increase, thickness increase. Additionally, Wu *et al.* 2013 observed that pullulan films have low viscosity dispersion, and it could be easily affected by the environment. In contrast, PLA films' viscosity decreases exponentially with temperature rise (Hamad *et al.* 2010). Taking this into consideration, the relationship between viscosity and thickness is important, since the greater the thickness is, the less interaction between molecules there will be. While the lower the thickness is, the greater the interaction between molecules will be, which can ultimately affect other film mechanical properties

On the other hand, tensile strength results pointed out significant differences between pullulan active films compared to PLA films. This property may be affected by film viscosity, according to the results, the highest UTS value was 101.03 MPa, obtained in Pullulan films with one percent grape seed extract. But, compared to the films with five percent GSE tensile strength values were reduced around four times. As is shown in Table 4, changes in the concentration, produce significant differences in the UTS values. These results followed a linear pattern, where if the concentration of the extract increases, the UTS values of the films will decrease Similar results were obtained by Sogut E and Seydimthe C.A. (2018), who when performing tensile tests in chitosan films with grape seed extract (GSE), reported that the tensile strength significantly decreased in films because of the addition of GSE. Additionally, the statistical analysis indicated a significant interaction between the extracts and concentrations. Due to the interaction of the factors, the UTS of the films decreases.

Moreover, elongation at breaks (EL) expresses the capability of materials to resist changes of shapes without crack formation (Djafari Petroudy 2017). The results obtained for the EL trials indicates that the extracts produce significant differences between pullulan with one percent of GFSE and the other treatments. This could be related to the thickness of the film, since the film of pullulan with one percent of GFSE obtained low values in its thickness allows the molecules to interact more, increasing the viscosity and, therefore its elongation capacity is higher. These results agreeing with the study of Kanmani and Rhim (2014) who observed that the addition of GFSE in films increase the elongation of the films, in general the addition of GFSE into films greatly affected the mechanical properties of the films. But, if the concentration of the extract increases the elongation will decrease.

In contrast, the pullulan film with one percent of GSE obtained a low elongation percentage, since the film's thickness increased, the molecules' dispersion increased reducing their interaction and viscosity. Nevertheless, according to Sogut E. and Seydimthe A.C. (2018) GSE can increase elongation at breaks values. Perhaps the use of a different biopolymer in film formulation, can change the elongation percentage. Furthermore, based on the formulation purpose by Trinetta V *et al.* (2011) pullulan films with: 100 g/L of Pullulan, 10 g/L of glycerol, 1 g/L of xanthan and locust bean gum; this materials provide positive behavior on films elongation values. But Yan *et al.* (2012) observed that pullulan films without active compound had lower percentage in elongation at breaks. Moreover PLA films, obtained low elongation percentages since this polymer is very rigid, apart from considering properties such as thickness, which with low values reduces the interaction of the molecules and more rigid the material becomes, causing it to be brittle compared to pullulan films that are flexible. This agreeing with results obtained by Zhang and Sun (2005), who indicated that the behavior of the PLA films aren't resistance to fracture process because it low flexibility properties and stiffness

In general, in elongation at breaks of active films obtained high standard deviation values, this may be because different factors; one of them is the equipment used for texture analysis; in the food industry, the equipment used depends on the geometry of the product. Also, the texture analyzes do not have intensive quantity characteristics of the materials. According to Nič *et al.* (2009), the intensive quantity is a physical quantity whose magnitude is independent of the extent of the system. However, texture analysis the materials do not always present the same characteristics, causing them to be independent of the system being evaluated.

Young modulus (YM) is a property used to predict the elongation or compression of an object when exposed to a force (Engineering Toolbox 2005). In Table 3, the results of the young modulus

(YM) reveals a similar behavior related to the tensile test. Because as the concentration of the extract increase, the elasticity of the film decrease. However, according to the statistical analysis the concentration of the extract is not significant for this property. Also, the is not an interaction between the extract and concentration. Since the YM test the elasticity in material, this may depend on the properties tested before such as thickness and UTS. For example, UTS obtains high values if the thickness of the film is low, since the molecules interact the viscosity increase and therefore its mechanical properties increase.

Table 4. Physical properties measured in films based on Pullulan and PLA.

| Physical properties | L value ± SD | a* value ± SD | b* value ± SD |
|---|---|---|---|
| GSE 1% | $82.29 \pm 0.893^{C}$ | $2.80 \pm 0.020^{B}$ | $-2.61 \pm 1.089^{C}$ |
| GSE 5% | $79.13 \pm 0.217^{E}$ | $4.22 \pm 0.056^{A}$ | $-4.49 \pm 0.227^{D}$ |
| GFSE 1% | $84.25 \pm 0.281^{B}$ | $1.73 \pm 0.340^{C}$ | $3.05 \pm 0.100^{B}$ |
| GFSE 5% | $87.82 \pm 0.237^{A}$ | $-0.64 \pm 0.110^{E}$ | $6.21 \pm 1.215^{A}$ |
| PLA | $80.85 \pm 0.757^{D}$ | $0.48 \pm 0.078^{D}$ | $-9.22 \pm 0.289^{E}$ |

Control: Film without extract, GSE: grape seed extract, GFSE: grapefruit seed extract, PLA: Polylactic acid, L*: Lightness in a range from 0 to 100, a*: Red/green values (-60 = green, +60 = red), b*: Blue/yellow values (-60 = blue, +60 = yellow), A-E: Means followed with different letters within each column are statistically, different ($P < 0.05$), SD: Standard deviation.

Color is an attribute measured on materials, this test measure three values, the L*, a* and b* value measuring lightness and hue in scale of red, green, yellow, and blue shades. In the study, significant differences were observed in the treatments.

In Table 4, the results obtained for films lightness shows that each treatment presented different lightness values, but the highest L* value was obtained in the film with five percent of grapefruit seed extract, however, according to the analysis carried out, significant differences were detected in all treatments. In the case of a* and b* values presented significant differences between each treatment. Furthermore, the highest mean detected in each value was in films with grape seed extract (GSE) and grapefruit seed extract (GFSE) respectively, both results were obtained with five percent of each extract. For a* value, the film with one percent GFSE it was detected some red shades but with a low hue value, but in the film with five percent of GFSE the shades detected were green. In the case of films with GSE red shades were detected. Also, the results indicate that if the concentration of the extract increased the red shades will intensify. On the other hand, the b* value in the film with GSE presented blue shades and films with GFSE yellow shades were observed. As well, the b* value presented the same pattern of the a* value, which is if the concentration of the extract increases the shades intensify. PLA films obtained high values of lightness. But for a* and b* some red and blue shades were detected. But comparing to pullulan active films, the statistical analysis indicates significant differences for each treatment.

Table 5. Correlation variable analysis for mechanical properties.

| **Properties** | **Thickness** | **UTS** | **EL** | **YM** |
|---|---|---|---|---|
| Thickness | 1.0000 | -0.1058<br>0.8656 | 0.7285<br>0.1627 | -0.1517<br>0.8076 |
| UTS | -0.1058<br>0.8656 | 1.0000 | -0.5700<br>0.3158 | 0.9830<br>0.0027 |
| EL | 0.7285<br>0.1627 | -0.5700<br>0.3158 | 1.0000 | -0.6419<br>0.2429 |
| YM | -0.1517<br>0.8076 | 0.9830<br>0.0027 | -0.6419<br>0.2429 | 1.0000 |

UTS: Ultimate tensile strength, EL: Elongation at breaks, YM: Young modulus.

According to the results indicated in Table 5, UTS and YM have a high correlation, this explains the high YM value of PLA films. Statistically, PLA films are the same as pullulan films with one percent of GFSE. However, even when within the data differences are observed in the values obtained for this property, the statistical analysis indicates that the pullulan treatments with GFSE and GSE films did not show significant differences. These results contrast with the study by Trinetta *et al.* (2011) who obtained a young modulus average of 3304 ± 627 for pullulan films without active compounds. This value is lower than those obtained in this study, which means that the integration of the extracts to the pullulan matrix allows the increase of the elasticity of the films. This increase can be useful since the film is not too rigid, it could withstand more weight and not break easily. Additionally, the study indicated that UTS, Young modulus (YM), and Elongation at breaks requires to be mechanically strong, these are reflected in high values and substantial percent, respectively.

As is shown in Table 5, when correlating the analyzed properties, it was observed that UTS and thickness obtained a medium correlation, this means that one affects the behavior of the other property. Moreover, thickness presented a high correlation with elongation, causing a proportional effect that is, while the thickness of a film changes the elongation at breaks will have a proportional change too. While the relationship between thickness and elasticity is medium, this means if thickness changes, the elasticity of the film could change. In the case of elasticity with UTS, its correlation is high, supporting what has previously been described, where UTS and YM followed the same pattern, where if the concentration of the extract increases, the values of said properties decrease.

**Antimicrobial properties.**
T- test analysis results revealed that for GSEL was not observed inhibition halo. These results were indicating that all strains were resistant to GSEL (40%). This contrast with the results obtained by Prashith *et al.* (2014) which pointed out that GSE's susceptibility was high in the case of Gram-positive bacterium, compared to Gram-negative bacteria. These results may have depended on the amount of extract tested, in the study of Prashith *et al.* (2014) the amount of GSE used was 100 µL compared to 25 µL used in this study (Table 6).

Table 6. T-Test analysis for antimicrobial activity of grapefruit seed at 33% and grape seed at 40% liquid extracts.

| Microorganism strain | Inhibition halo (mm) | | P-value |
|---|---|---|---|
| | GFSEL (33%) ± SD | GSEL (40%) ± SD | |
| *Listeria monocytogenes* | | | |
| 7644 | 27.67 ± 3.51 | 10.00 ± 0.00 | 0.0129 |
| B2-323 | 24.33 ± 3.79 | 10.00 ± 0.00 | 0.0225 |
| B-33043 | 24.00 ± 2.65 | 10.00 ± 0.00 | 0.0117 |
| B-33260 | 28.33 ± 3.79 | 10.00 ± 0.00 | 0.0139 |
| *Salmonella* | | | |
| Newport | 18.33 ± 7.57 | 11.00 ± 1.73 | 0.2318 |
| Infantis | 24.67 ± 4.04 | 10.00 ± 0.00 | 0.0244 |
| Seftenberg | 14.67 ± 2.52 | 10.00 ± 0.00 | 0.0848 |
| Thompson | 20.33 ± 5.51 | 10.00 ± 0.00 | 0.0831 |
| *Escherichia coli* | | | |
| O121 | 17.00 ± 6.56 | 11.67 ± 2.89 | 0.2951 |
| O26 | 19.67 ± 5.51 | 10.00 ± 0.00 | 0.0933 |
| O157:H7 | 17.33 ± 5.51 | 10.00 ± 0.00 | 0.1475 |

GFSEL: Grapefruit seed extract liquid, GSEL: Grape seed extract liquid, SD: Standard deviation.

On the other hand, GFSEL results show in Table 6 revealed that *L. monocytogenes* strains were more susceptible than *Salmonella and E. coli.* Moreover, *L. monocytogenes* strains presented significant differences. Agreeing with Kanmani and Rhim (2014) grapefruit seed extract has a high antimicrobial activity against Gram-positive and Gram-negative foodborne pathogens. Furthermore, Aboaba *et al*. (2006) found that herbs and spices' natural extracts have antimicrobial activity. This high antimicrobial activity presented in GFSE depends upon several factors, including a reduction in pH, ratio of undissociated species of the acid, chain length, and cell physiology and metabolism (Kim *et al*. 2016). Additionally, Xu *et al.* (2007), observed that the inhibitory effect of GFSE has significant differences when the pH of the solution change. For example, when the pH value ups to five inhibitory effect get reduce. But if the pH value is less than five inhibitory effect increases.

Due to the previously described, explain the resistance of the serotypes of *Salmonella* and *E. coli* serotypes. For example, the serotypes tested; *Salmonella* Newport was more resistant than other serotypes. Compared to *Salmonella* Infantis it was the most susceptible serotype. This may be due to the presence of polyphenols in GFSE, these compounds have the property to migrate and have contact with microorganism membranes causing lysis of cells (Kanmani and Rhim 2014)

Moreover, for active films the statistical analysis revealed probabilities (P > 0.05) for extract concentration, repetitions and interaction between extracts and concentrations. Indicating that these aspects does not present significant differences in the control of microorganisms.

Control and pullulan with grape seed extract films do not present significant differences (P > 0.05) regardless of the concentration on all microorganisms tested. However, films with GSE and GFSE statistically are the same; this may be due, that during the addition of the extracts the mixture temperature was 80 °C. According to Suppakul (2011) natural extracts do not tolerate high temperatures, causing active compound loss. Grapefruit seeds and grape seeds extracts contains high amounts of phenolic compounds, which are volatile. According to the method followed by the high temperatures of the mixture, the volatilization of phenols reduced the antimicrobial activity. This may explain the nonsignificant differences between the active films for controlling microorganisms (Table 7).

Table 7. Active compound antimicrobial activity base on inhibition zone measurement (mm).

| **Microorga-nism Strain** | **Control ± SD** | **GFSE 1% ± SD** | **GFSE 5% ± SD** | **GSE 1% ± SD** | **GSE 5% ± SD** |
|---|---|---|---|---|---|
| *Listeria monocytogenes* | | | | | |
| 7644 | $10.7 \pm 0.6^{C}$ | $13.7 \pm 0.6^{AB}$ | $14.7 \pm 1.5^{A}$ | $14.0 \pm 2.0^{AB}$ | $12.3 \pm 1.2^{BC}$ |
| B2-323 | $11.7 \pm 1.5^{B}$ | $15.0 \pm 1.0^{A}$ | $14.3 \pm 1.5^{A}$ | $13.0 \pm 1.0^{AB}$ | $13.3 \pm 1.5^{AB}$ |
| B-33043 | $12.3 \pm 1.5^{C}$ | $17.3 \pm 4.0^{A}$ | $16.0 \pm 2.0^{AB}$ | $13.0 \pm 0.0^{BC}$ | $13.0 \pm 2.0^{BC}$ |
| B-33260 | $12.0 \pm 0.0^{B}$ | $15.0 \pm 0.0^{AB}$ | $17.7 \pm 3.5^{A}$ | $13.3 \pm 2.5^{B}$ | $12.3 \pm 0.6^{B}$ |
| *Salmonella* | | | | | |
| Newport | $12.0 \pm 1.0^{A}$ | $13.7 \pm 1.2^{A}$ | $13.0 \pm 1.0^{A}$ | $12.0 \pm 1.0^{A}$ | $12.3 \pm 0.6^{A}$ |
| Infantis | $11.3 \pm 0.6^{C}$ | $14.3 \pm 1.2^{AB}$ | $15.7 \pm 2.1^{A}$ | $12.7 \pm 1.2^{BC}$ | $12.7 \pm 1.2^{BC}$ |
| Seftenberg | $11.3 \pm 0.6^{C}$ | $14.3 \pm 2.1^{A}$ | $15.0 \pm 2.0^{A}$ | $14.3 \pm 0.6^{AB}$ | $12.0 \pm 1.0^{BC}$ |
| Thompson | $12.3 \pm 1.2^{A}$ | $13.7 \pm 1.5^{A}$ | $14.0 \pm 1.7^{A}$ | $12.0 \pm 1.0^{A}$ | $13.0 \pm 1.7^{A}$ |
| *Escherichia coli* | | | | | |
| O121 | $12.0 \pm 1.7^{A}$ | $15.0 \pm 2.7^{A}$ | $15.0 \pm 2.7^{A}$ | $13.3 \pm 0.6^{A}$ | $12.7 \pm 1.2^{A}$ |
| O26 | $12.0 \pm 1.0^{C}$ | $16.0 \pm 2.7^{AB}$ | $16.7 \pm 2.9^{A}$ | $13.3 \pm 1.2^{ABC}$ | $12.3 \pm 1.2^{BC}$ |
| O157:H7 | $11.7 \pm 1.2^{C}$ | $14.0 \pm 2.7^{AB}$ | $15.3 \pm 0.6^{A}$ | $11.7 \pm 1.5^{BC}$ | $11.3 \pm 0.6^{BC}$ |

Control: film without extract, GSE: grape seed extract, GFSE: grapefruit seed extract, A-C: means followed with different letters within each column are statistically different (P < 0.05), SD: standard deviation.

Films with GFSE inhibited *L. monocytogenes, Salmonella* Infantis, *Salmonella* Seftenberg; *E. coli* O26, and O157:H7. For *L. monocytogenes* strains, it was observed that the Pullulan film control presented a lower antimicrobial activity compared to the treatments with grapefruit seed extract. Agreeing with the result of Wang and Rhim 2016; Kanmani and Rhim, 2014 who studied the incorporation of grapefruit seed extract in films as an antimicrobial compound, exhibits high antimicrobial activity against Gram-positive (*L. monocytogenes*) than Gram-negative (*Salmonella* and *E. coli*). As well, Xu *et al* (2007) observed that GFSE inhibition zone against *L. monocytogenes* were wider than *Salmonella*. But its antimicrobial activity can depend on the type of polymer matrix. In Addition, Trinetta *et al* (2011) demonstrated that Pullulan films do not have antimicrobial properties against microorganisms but had desirable features for active food packaging application.

On the other hand, the results obtained in *Salmonella* are interesting since the Pullulan active films and control tested in *Salmonella* Newport and Thompson serotypes are statistically the same. This may be because phenolic compounds are in a lower proportion, due to the volatilization processes previously described. The reduction of these compounds causes that phenols antimicrobial activity of phenols does not inhibit microorganism as the control films.

Moreover, films with grapefruit seed extract presented significant differences inhibiting *Salmonella* Infantis and Seftenberg serotypes compared to the control films. This susceptibility behavior may be because GFSE is rich in phenolic compounds. Phenols interact with the outer membrane of gram-negative bacteria, releasing lipopolysaccharide, and increasing cytoplasmic membrane's permeability to ATP and potassium ions (Boskovic *et al.* 2016). This points out that the addition of GFSE in Pullulan film formulation allows that phenolic compounds interact with the outer membrane of bacteria, allowing the inhibition of the microorganisms. Additionally, since the GFSE concentration does not present significant differences, the microorganism's inhibition will be the same, regardless of the extract concentration. These results contracts with the study realized by Xu *et al* (2007) who indicated that the inhibitory effect of the GFSE increases proportionally to the increase of the extract concentration.

However, films with grapefruit seed extract presented significant differences inhibiting the growth of *E. coli* O26 and O157:H7 strain. According to Kim *et al* (2016), GFSE contains flavonoids, catechins, minerals, tocopherols, and procyanidins. This allows to GFSE extract to reduce the cell membrane through the microbial uptake by interrupting enzymatic activities. However, *E. coli* serotypes have different mechanisms to adapt and be resistant to antimicrobial compounds. According to Li *et al*. (2019), *E. coli* can present active and passive defenses. For passive defense, the bacteria make itself dormant, reduce the vitality of life, and block the combination of antimicrobials and target to reduce the killing effect of antimicrobials. But, for active defense, they increase the efflux pump's activity to increase the efflux of antimicrobials and reduce the accumulation of antimicrobials in bacteria, thereby reducing the killing effect of antimicrobials on bacteria.

On the other hand, comparing the films with grape seeds and grapefruit seed extract. The result point out, that statistically they are the same. But comparing only pullulan films with grapefruit seed films; it can be determined that the control of microorganisms increases with the grapefruit seed extract film. Moreover, the results obtained from the films with GSE were not satisfactory, their antimicrobial activity is compared with the control films. This agreeing with the study of Corrales *et al* (2009), who observed that GSE does not achieve a high antimicrobial activity against Gram-negative microorganism such as *Salmonella spp.* But for Gram-positive microorganisms such as *L. monocytogenes* GSE have better response to inhibit the microorganisms. One possible reason why GSE did not perform well may be due to flavonols (95%) and proanthocyanidin (82%)

concentrations (Zhu *et al* 2015). Additionally, Zhu *et al* (2015) indicates that if the concentration of GSE increases, the inhibitory effect will increase as well.

However, it is important to emphasize that the results indicate that the integration of extracts such as GFSE allows films based on pullulan to acquire antimicrobial properties, which inhibit the growth of Gram-positive and Gram-negative bacteria. This agreeing with Singh and Saini (2008) who indicates that Pullulan can improve the shelf life of food products.

# 4. CONCLUSIONS

- Two films based on pullulan and PLA were developed. The addition of the antimicrobial compound to the bio-based matrix changed the optical and mechanical properties in the films. In the mechanical properties the Ultimate Tensile Strength, Elongation at Breaks and Young's modulus properties of the pullulan films with active compound decreased as the extract concentration increased. In the case of the physical characteristics, the grape seed and grapefruit seed extract showed different shades, purple and yellow, respectively.

- The formulation of pullulan and grapefruit seed extract can be used as an active packaging, inhibiting the growth of Gram-positive (*Listeria monocytogenes*) and Gram-negative (*Salmonella spp.* and *Escherichia coli*) foodborne pathogens.

# 5. RECOMMENDATIONS

- It is recommended to carry out these tests on food to evaluate the inhibition capacity of microorganisms in food.

- For future studies, it is recommended to cover other types of physical and mechanical tests where puncture, humidity, and even the biodegradation time of polymers are measured.

- Regarding the microbiological property analysis, it is recommended that for future analysis be focused on a specific microorganism, for example L*isteria monocytogenes* strains inhibition since this pathogen is one of the most related to outbreaks in food and according to the results pullulan films with grapefruit seed extract can control it growth.

- Identify and evaluate active compounds for the control of microorganisms that can be integrated with polylactic acid to form active films for food measuring physical, mechanical and antimicrobial properties.

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