Master's Thesis of Science

in Agricultural Biotechnology

Development and Characterization of Antimicrobial Films Containing Carvacrol, Thymol and Gallic Acid : Application to Beef Patties Preservation

Carvacrol, thymol, gallic acid를 함유한 필름 개발과 그 특성 규명 : 소고기 패티 저장 시의 적용

February, 2021

Graduate School of Agricultural Biotechnology

Seoul National University

Major of Food Science and Biotechnology

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Submitting a Master's Thesis of Science in Agricultural Biotechnology

February, 2021

The Graduate School
Seoul National University
Department of Agriculture Biotechnology

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Confirming the master's thesis written by Yumin Kim

February, 2021

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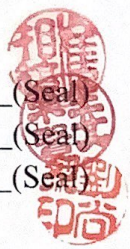

ABSTRACT

Recently, active packaging is regarded as a promising approach to extend the shelf-life of food. In this regard, novel active films, chitosan-starch films containing carvacrol, thymol and gallic acid, were developed and characterized. Our results exhibited that incorporation of gallic acid in chitosan-starch films increased color changes, tensile strength, antioxidant activity and decreased elongation at break, water vapor permeability of the films. The films with carvacrol and thymol showed an increase in ductility, color changes, antioxidant activity, antimicrobial effect and decreasing tensile strength, water vapor permeability. Furthermore, by the addition of both gallic acid and essential oils, films presented the best mechanical, water barrier properties and the strongest antioxidant and antimicrobial activity. Finally, the application of the blending film to the preservation of beef patties was performed. The results showed that the films containing carvacrol and gallic acid effectively prevented lipid peroxidation and reduced total viable cell population of ground beef patties. This study showed the benefits of the

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incorporation of gallic acid, carvacrol and thymol into chitosan-starch films and the potential of their use as active packaging in food products.

Keywords: **Active film, gallic acid, carvacrol, thymol, beef patties**
Student Number: **2019-23944**

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CONTENTS

LIST OF TABLES

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LIST OF FIGURES

`

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I. INTRODUCTION

Major mechanisms involved in the deterioration of processed foods are oxidative rancidity and microbiological spoilage by pathogen development. Oxidative reactions and the growth of pathogens negatively affect the quality and safety of food products. So, preventing these undesirable degradation of food is the major challenge for food industry. Currently, active packaging, which is the packaging with functional properties such as antioxidant, antimicrobial activities, is regarded as a promising solution to extend the shelf-life of foods. Active packaging functionalized with natural compounds is preferred due to health and environmental issues (Sung, Sin et al. 2013).

Carvacrol (C) and thymol (T), which are the main components of essential oil produced by *Origanum* species, are natural sources and classified as GRAS (generally recognized as safe) by the US Food and Drug Administration (Johny, Darre et al. 2010). Essential oils (EOs) and their constituents rich in phenolic compounds show a wide spectrum of antimicrobial and antioxidant activity (Holley and Patel 2005, Gavaric, Mozina et al. 2015). Due to their wide variety of functional properties, films containing EOs have been extensively studied for many years. Ojagh, Rezaei et al. (2010) reported that chitosan film incorporated with cinnamon essential

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oil showed enhanced water barrier properties. Meanwhile, Ma, Zhang et al. (2016) presented opposite results that cinnamon bark oil increased water vapor permeability of chitosan films.

Recently, due to the excessive plastic waste and environmental pollution, demand has risen for biodegradable packaging in the industry of food packaging. Development of the films made from natural and biodegradable materials are needed to replace plastic packaging. Chitosan (CH), derived from the deacetylation of chitin, is one of the most abundant polysaccharide in nature (Jianglian and Shaoying 2013). The application of CH to packaging film is promising due to its good film-forming ability, biocompatibility and non-toxicity. Starch (S) films are biodegradable, low cost and transparent but has disadvantages of water sensitivity and brittleness (Wu and Zhang 2001). The CH and S composite films showed better mechanical, water barrier properties compared to pure S films (Xu, Kim et al. 2005).

Gallic acid (GA) is a naturally occurring plant phenol, which exhibits strong antioxidant activity along with antimicrobial effect. GA is used in food to prevent lipid peroxidation which causes rancidity of food (Cholbi, Paya et al. 1991). When GA is added in polymer films, it shows interactions with polymers and formation of cross-linking (Hager, Vallons et al. 2012, Zarandona, Puertas et al. 2020). Hence, by the addition of GA, chitosan-starch films can show antioxidant effect while at the same time improving

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mechanical, physical properties of the films. To our knowledge, there is no previous studies about chitosan-starch films incorporated with both EOs and gallic acid.

Quality deterioration of beef is correlated with microbial growth and lipid peroxidation due to its high content of unsaturated fatty acids during refrigerated storage. To prevent degradation of beef quality, many strategies have been presented (Jayasena, Jo et al. 2013, Patel and A 2015), such as the direct treatment of essential oils into meat products. However, due to the volatile profiles and strong odors of EOs, evaporation of active compounds of EOs can occur during treatment. So, higher concentration of EOs is needed for effective antimicrobial treatment which can produce undesirable flavors to the food products. On the other hand, incorporation of EOs in the film can reduce the loss of EOs active compounds. Therefore, treatment of films containing EOs can be a more effective method than spraying or dipping the EOs directly on the food (Quintavalla and Vicini 2002, Moradi, Tajik et al. 2011, Han, Wang et al. 2014).

The object of this study was to characterize optical, mechanical, physical properties of chitosan-starch films incorporated with carvacrol, thymol and gallic acid. The effect of C, T and GA on antioxidant and antimicrobial activities of composite films were evaluated. Moreover, this study exhibited

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the effect of films containing GA and C on quality and microbial changes of beef patties during storage.

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II. MATERIALS AND METHODS

2.1. Materials

High molecular weight chitosan (>75% deacetylated, 310-375 kDa) was purchased from Sigma Aldrich Chemical Co. Cassava starch was purchased from the local supermarket. Glycerol (99%, EP) was purchased from DUKSAN. Carvacrol, thymol and gallic acid were purchased from Sigma Aldrich.

2.2 Film preparation

Films were produced by a casting method according to Xu, Kim et al. (2005) with some modifications. Formulations of each film were shown in Table 1. The chitosan (2% w/v) was dissolved in glacial acetic acid (1% v/v) and stirred at 30°C for 4 h to complete dissolve. Starch solution (2% w/v) was heated to 90°C for 30 min until it gelatinized and cooled to 30°C. For preparing chitosan-starch (CH/S) composite films, chitosan solution mixed with starch solution in the ratio of 1:1 (w/w). For CH/S film containing gallic aicd (CH/S-GA), 0.1% (w/v) gallic acid were also added. Afterward, glycerol was added as the plasticizer at the level of 20% (w/w) of the total solid weight in the solution and stirred for 30 min. The carvacrol and thymol were firstly mixed with tween 80 (15% v/v of total carvacrol, thymol) and then carvacrol, thymol emulsion were added to the film solutions in the same ratio presented in Table 1. All of the mixtures were homogenized using homogenizer (IKA-T

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25, IKA-Werke GmbH & Co.) for 4 min at 13,500 rpm and degassed. Then, the mixtures were cast onto patri dishes (9 cm diameter) and dried at 25°C and 50% relative humidity (RH) for 48 h. After drying, films were conditioned at 25°C and 50% RH prior to evaluation.

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Formulation		CH	S	C	T	GA	Glycerol
	CH/S	1	1	-	-	-	0.4
	CH/S-C	1	1	0.8	-	-	0.4
	CH/S-T	1	1	-	0.8	-	0.4
	CH/S	1	1	-	-	0.1	0.4
GA	CH/S-C	1	1	0.8	-	0.1	0.4
	CH/S-T	1	1	-	0.8	0.1	0.4

CH : Chitosan, S : Cassava starch, C : Carvacrol, T : Thymol, GA : Gallic acid

Table 1. Total solids amount (g) per 100 g of the different film-forming dispersions.

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2.3. Film properties

2.3.1. Optical properties

2.3.1.1. Color

The color of the film was determined by measuring the L^*, a^* and b^* parameters using a colorimeter (Minolta CR360, Minolta Camera Co.Ltd., Osaka, Japan). The colorimeter was calibrated using a standard white plate. The measured color parameters were calculated for total color changes (ΔE) and whiteness index (WI), according to the following equations:

$$\Delta E = \sqrt{(L_i^* - L^*)^2 + (a_i^* - a^*)^2 + (b_i^* - b^*)^2} \quad (1)$$

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (2)$$

where L_i^*, a_i^* and b_i^* are the color parameter values of the standard white plate, L^*, a^* and b^* are the measured values of the films in white background.

2.3.1.2. Transparency

The light transmittance of the film was measured at the ultraviolet and visible range (200-800 nm) using an UV-Vis spectrometer (Spectramax M2e; Molecular Devices, CA, USA) according to the method reported by Shiku (2004). Transparency value of the film was evaluated using the following equation:

$$\text{Transparency value} = -\frac{logT_{600}}{x} \quad (3)$$

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where T_{600} is the fractional transmittance at 600 nm and x is the film thickness.

2.3.2. Film thickness

Film thickness was measured by a digital micrometer with an accuracy of ±0.01 mm. Thickness was taken in five random points of the film. Averages were calculated for mechanical properties, water vapor permeability and transparency value.

2.3.3. Mechanical properties

Tensile strength (TS) and elongation at break (E) of the films were determined by Texture Analyzer (TA.XT2-5, Stable micro system, UK) following ASTM standard method D882 (ASTM, 1995). Films were cut into strips (20 mm x 70 mm) and mounted in the grip of the equipment. The initial grip separation was 40 mm and the crosshead speed was 0.83 mm s^{-1}. TS and E were calculated as follows:

$$TS = \frac{F_{max}}{A} \quad (4)$$

where F_{max} is the maximum load (N), A is the initial cross-sectional area (m^2).

$$E\ (\%) = \frac{\Delta L}{L_0} \times 100\% \quad (5)$$

where ΔL is the extension of film strips and L_0 is the initial displacement (40 mm).

`

2.3.4. Physical properties

2.3.4.1. Moisture content

The moisture content was determined according to the method of Jouki (2013) with some modification. The film samples (20 mm x 40mm) were weighed before and after dried in an oven at 50℃ until a constant weight was reached (dry sample weight). The moisture content (MC) was calculated as follows:

$$MC = \frac{(W_i - W_f) \times 100\%}{W_i} \quad (6)$$

where W_i is the initial weight of the film and W_f is the final dry weight of the film.

2.3.4.1. Water vapor permeability (WVP)

Water vapor permeability was determined gravimetrically at 25°C and a RH of 75%, using a modified version of ASTM standard method E96 (ASTM, 1995). Test cups (60 mm diameter) were filled with granular anhydrous calcium chloride (0% RH) and tightly covered with film. After it, cups were located in a chamber at 25°C and 75% RH. The weight gain of the cups was determined after 24 h. The WVP of the film was calculated as follows:

$$WVP = \frac{\Delta m L}{A \Delta t \Delta P} \quad (7)$$

where Δm is the weight of cup increase (g), L is the average film thickness (m), A is the exposed area of film, Δt is the time of permeation (s),

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and ΔP is the water vapor pressure difference between the two sides of the film at test temperature (Pa).

2.3.5. Scanning electron microscopy (SEM)

The microstructure of the films was observed by a Field Emission Scanning Electron Microscope (SUPRA 55VP, Carl Zeiss, Germany). Films were previously dried in a desiccator, immersed in liquid nitrogen, and cryo-fractured. The cross-sections of films were coated with gold and examined at an accelerating voltage of 15 kV (x2000).

2.3.6. Antioxidant activity

The antioxidant effect of the films was determined by using a 2,2-Diphenyl-1-pikryl-hydrazyl (DPPH) reduction method described by Noronha, de Carvalho et al. (2014) with some modifications. The film sample (10 mm x 10 mm) was placed in a centrifugal tube containing 3 ml 0.1 mM DPPH solution and placed in a dark room at 25°C for 30 min with occasional vortexing. Then, the absorbance at 515 nm was measured with a UV-Vis spectrophotometer. The DPPH scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = \left(\frac{1-A_{sample}}{A_{DPPH}}\right)x100 \quad (8)$$

where A_{sample} is the absorbance of the tested sample solution and A_{DPPH} is the absorbance of the control solution.

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2.3.7. Antimicrobial activity

2.3.7.1. Inoculum preparation

S. aureus (ATCC 29213) and *E. coli* (ATCC 43890) was provided from the bacterial cultural collection of the Food Safety Engineering Laboratory at Seoul National University (Seoul, South Korea). The working cultures were maintained on tryptic soy agar (TSA; Difco, Becton, Dickinson and Company, Sparks, MD, USA) and sub-cultured every week. A single colony of working culture was inoculated into 5 ml of tryptic soy broth (TSB; Difco) and incubated overnight at 37°C. The final concentration of each strain of *S. aureus* and *E. coli* was approximately 10^9 CFU/ml and $5x10^8$ CFU/ml, respectively.

2.3.7.2. Disk diffusion assay

The disk diffusion assay was used for assessing the antimicrobial activity of film according to Tepe, Daferera et al. (2005) with some modification. *S. aureus* and *E. coli* culture were adjusted to 10^8 CFU/ml and 0.1 ml of suspension was spread on the solid media plates. Then, films were cut into 6 mm diameter discs and placed on the plates. The plates were incubated for 24 h at 37°C and the clear zones around the discs were measured.

2.3.7.3. Food solid system

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Antimicrobial activity of films in the simulants of solid food was determined according to the method described by Sánchez-González, Cháfer et al. (2011) with some modifications. Tryptic soy agar with NaCl (3%) was used as a model solid food system (TSA-NaCl). 10 ml of TSA-NaCl were poured into petri dishes (6 cm diameter) and solidified. The *S. aureus* and *E. coli* culture were diluted to a concentration of 10^8 CFU/ml and 0.1 ml of aliquots were inoculated on the surface of TSA-NaCl. Each film (6 cm diameter) were placed on the inoculated surface. Inoculated uncoated plates were used as control. TSA-NaCl plates were stored at 25°C for 12 h. *S. aureus* and *E. coli* counts were carried out at 0 h and every 4 h. 10 g of agar were removed aseptically and placed in sterile stomacher bags. 90 ml of peptone water (0.2%) was added to each bag and homogenized for 2 min in a stomacher. The sample solution was serially diluted and diluent was spread onto Baired Parker Agar and Sorbitol-MacConkey Agar. After incubating the plate at 37°C for 48 h for *S. aureus* and 24 h for *E. coli*, colonies were counted.

2.4. Application to beef patties storage

2.4.1. Preparation of beef patties

Ground beef was purchased from a local butcher shop. The beef patties (20 g) were shaped by hand into 6 cm diameter and 1 cm thickness. The patties were randomly divided into four different treatments: 1) control samples without films, 2) samples wrapped with CH/S films, 3) samples wrapped with

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CH/S-GA films, and 4) samples wrapped with CH/S-GA-C films. Four sample groups were individually vacuum packaged in nylon polyethylene pouches. The packaged samples were stored at 4°C for 12 days and analyzed at 3-day intervals (0, 3rd, 6th, 9th, 12th day)

2.4.2. Thiobarbituric acid reactive substances (TBARS)

The degree of lipid peroxidation of the samples was determined by using the TBARS assay according to the method described by Hur, Ye et al. (2004) with some modifications. Briefly, 5 g of each sample were added to 15 ml of trichloroacetic acid (15%). The mixtures were centrifuged (4000 rpm, 10 min) and filtered through Whatman no. 1 filter paper. The 3 ml of filtrate was mixed with 3 ml of 0.mM thiobarbituric acid (TBA) and reacted in water bath at 100°C for 40 min. After the samples were cooled with cold water, centrifuged for 10 min, at 10000 rpm. The absorbance value was measured with a spectrophotometer at 532 nm. The value of TBARS was calculated using a standard curve of malondialdehyde (MDA) and expressed as mg MDA/kg sample.

2.4.3. Color

The color (CIE L^*, a^*, b^*) of beef patties was measured by using a colorimeter (Minolta CR360). The colorimeter was calibrated using a standard white plate.

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2.4.4. Total viable cell count

Total viable cell was determined for the microbiological analysis. 10 g of samples aseptically placed in a sterile stomacher bag with 90 ml of peptone water (0.1%) and homogenized for 2 min using a stomacher. After serial dilutions, 100 ul of diluent was spread on Plate Count Agar (PCA; MB cell) to measure total viable cell. The plates were incubated at 37°C for 48 h. The bacterial colonies on the plate were expressed as the log10 colony-forming units per gram of sample (CFU/g).

2.5. Statistical analysis

The results were obtained from three independently performed experiments. The one-way analysis of variance (ANOVA) was applied for the statistical analysis of data using a SAS (v. 9.4). The significant difference between mean values was evaluated with Duncan's Multiple Range Test ($P < 0.05$) for all statistical analyses.

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III. RESULTS AND DISCUSSION

3.1. Optical characteristic

The color and transparency of the films have an important influence on consumer acceptance of the product (Kunte, Gennadios et al. 1997). Figure.1 shows that films with or without carvacrol, thymol and gallic acid were visually transparent, homogeneous and nearly colorless. The difference in color and transparency of the films was related to the color profiles of the additives and the volume fraction of the dispersed lipids (Villalobos, Chanona et al. 2005). Due to the colorless and pale yellow profiles of carvacrol and thymol, the film containing carvacrol and thymol also showed colorless properties.

The color parameters, total color changes (ΔE), whiteness index (WI) and transparency value of films are shown in Table 2. Incorporation of gallic acid made the film slightly yellow, red and less transparent, resulting significant ($P < 0.05$) changes in WI and ΔE. The ΔE, WI and transparency value of films were also increased with the addition of EOs.

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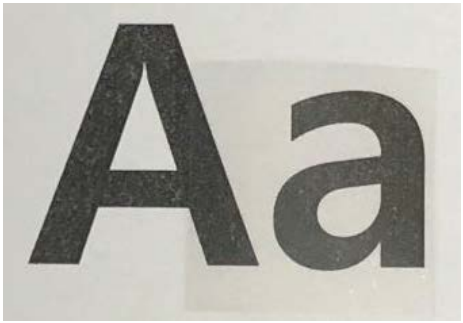
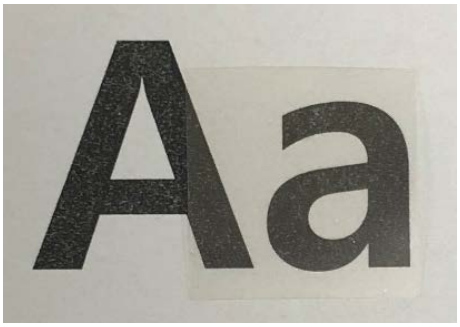
(a)



(b)

(c)



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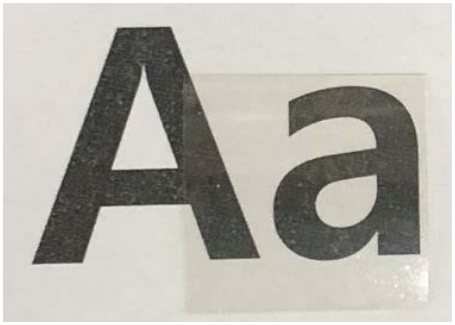
(d)



(e)

(f)

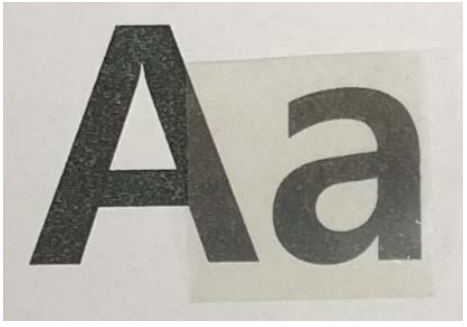


Fig. 1. Appearance of CH/S film (a), CH/S-C film (b), CH/S-T film (c), CH/S-GA film (d), CH/S-GA-C film (e), CH/S-GA-T film (f)

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Formulation		L^*	a^*	b^*	ΔE	WI	Transparency value
	CH/S	96.36 ± 0.45 A	-0.19 ± 0.07 C	3.60 ± 0.55 C	2.11 ± 0.68 A	94.87 ± 0.70 A	1.65 ± 0.10 A
	CH/S-C	96.32 ± 0.33 A	-0.23 ± 0.06 CD	3.92 ± 0.26 CB	2.41 ± 0.32 AB	94.62 ± 0.35 AB	2.11 ± 0.21 ABC
	CH/S-T	95.84 ± 0.13 A	-0.30 ± 0.06 D	4.53 ± 0.19 BA	3.17 ± 0.23 BC	93.84 ± 0.22 BC	2.39 ± 0.21 C
GA	CH/S	95.05 ± 0.46 B	0.13 ± 0.05 B	4.81 ± 0.53 A	3.84 ± 0.69 CD	93.09 ± 0.69 CD	1.78 ± 0.13 AB
	CH/S-C	94.77 ± 0.55 B	0.27 ± 0.09 A	4.69 ± 0.37 A	3.93 ± 0.64 CD	92.97 ± 0.65 CD	2.15 ± 0.36 BC
	CH/S-T	94.80 ± 0.16 B	0.29 ± 0.02 A	5.04 ± 0.25 A	4.18 ± 0.26 D	92.75 ± 0.26 D	3.41 ± 0.44 D

ΔE : total color changes
WI : whiteness index

Table 2. Color difference (L^*, a^*, b^*, ΔE, WI) and transparency value of the films from chitosan-starch in the presence of different essential oils and gallic acid. Data represent means ± standard deviations from three replications. Values followed by the same uppercase letters within columns are not significantly different.

3.2. Mechanical properties

Tensile strength (TS) and elongation at break (E) are the main parameters that represent the mechanical properties of the films. Mechanical properties of the films are dependent on the base polymers, plasticizers and agents interact with polymers (Shit and Shah 2014). Figure. 2 shows mechanical properties of the films. TS and E of CH/S film were 26.56 MPa and 8.06 %, respectively. Notably, TS was significantly ($P < 0.05$) increased and E was slightly decreased by the addition of gallic acid. Since gallic acid enhances structural bonds by cross-linking with polymers which decreases chain mobility (Pasanphan and Chirachanchai 2008), films became stronger and less stretchable. This is similar results by Zhao, Teixeira et al. (2018) for gallic acid cross-linking with chitosan-starch films. In case of the addition of essential oil, E was significantly ($P < 0.05$) increased and TS had no significant changes compared to films without essential oil. The presence of essential oil in the polymer matrix facilitates the movement of the polymer chain which makes the film more flexible. Similar results have been observed by Pelissari, Grossmann et al. (2009) when oregano essential oil was added to chitosan films.

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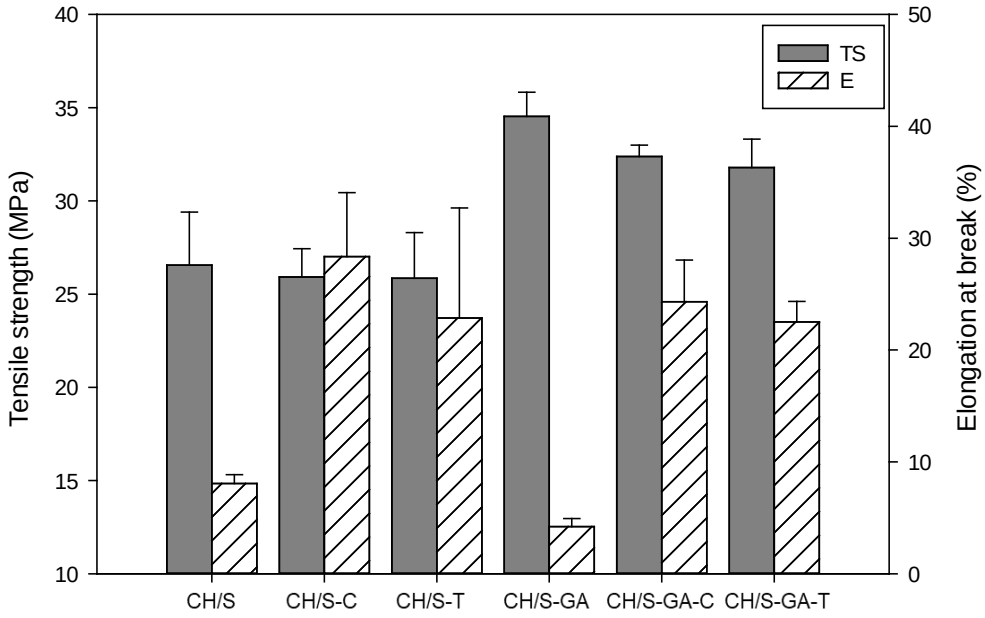

Fig. 2. Tensile strength (TS) and elongation at break (E) of films with or without carvacrol, thymol and gallic acid. The error bars indicate standard deviations.

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3.2. Thickness, water vapor permeability, moisture contents

Table 3 shows the thickness, water vapor permeability (WVP) and moisture contents (MC) of the films. Film thickness increased with carvacrol, thymol and gallic acid, ranging from 0.087 to 0.095 mm. Normally, the addition of other components made the film matrix denser.

WVP is a crucial index of film barrier properties against water vapor. As shown in Table 3, the WVP of CH/S film was 4.05 x 10^{-8} $gm^{-1}s^{-1}Pa^{-1}$. When the GA added, the WVP of the film decreased significantly ($P < 0.05$) which indicated that the incorporation of GA can make the film a better barrier for the vapor. The decrease of WVP is due to the cross-linking ability of GA which makes a tighter structure of the film, as discussed previously. Furthermore, the MC of the film decreased with the addition of GA. The lower MC specified that active sites of polymer which can interact with water decreased owing to the linkage with GA.

In case of EOs addition, the WVP and MC of the film significantly ($P < 0.05$) decreased. Ojagh, Rezaei et al. (2010) reported that the WVP of chitosan film decreased with increasing cinnamon essential oil concentration. It could be the reason that the hydrophobic nature of EOs may act on the hydrophobicity of the film. Hence, the WVP and MC of the film with EOs decreased because the presence of hydrophobic EO droplets in films increases the tortuousness of the vapor diffusing path which makes the vapor less penetrable through the films (Zhang, Zhou et al. 2020). The lowest WVP and MC were achieved when both GA and EOs were added to the film.

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Formulation		Thickness (mm)	WVP (g/m·s·Pa) x 10^{-8}	MC (%)
	CH/S	0.087 ± 0.007 A	4.05 ± 0.20 A	9.48 ± 0.61 A
	CH/S-C	0.093 ± 0.005 BC	3.03 ± 0.12 B	7.75 ± 0.26 BC
	CH/S-T	0.094 ± 0.005 BC	3.00 ± 0.40 B	7.72 ± 0.09 BC
GA	CH/S	0.094 ± 0.006 B	2.25 ± 0.26 C	7.87 ± 0.34 B
	CH/S-C	0.095 ± 0.009 BC	2.14 ± 0.33 C	6.50 ± 0.33 D
	CH/S-T	0.094 ± 0.007 C	2.10 ± 0.43 C	6.9 ± 0.42 CD

WVP : water vapor permeability
MC : moisture content

Table 3. Thickness, water vapor permeability, water content of the films from chitosan-starch in the presence of different essential oils and gallic acid. Data represent means ± standard deviations from three replications. Values followed by the same uppercase letters within columns are not significantly different.

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3.2. SEM

The microstructure of the films is influenced by the organization of the components and by the interaction of the components during the drying process (Sánchez-González, Vargas et al. 2009). SEM micrographs of the cross-section area of films is shown in Fig. 3 The cross-sections of the CH/S film and CH/S film with GA presented a smooth, homogenous appearance and compact structure without any cracks or bubbles which indicates good compatibility of chitosan. starch and gallic acid. The addition of carvacrol and thymol in the films presented increasing heterogeneity and irregularity of the film structures. The cross-section of the films containing carvacrol and thymol shows micro-pores in the film corresponding to the spots of oil droplets. (Song, Zuo et al. 2018) reported similar results. During the drying process of the film, dispersed EOs droplets may aggregate or enlarge, causing flocculation and coalescence.

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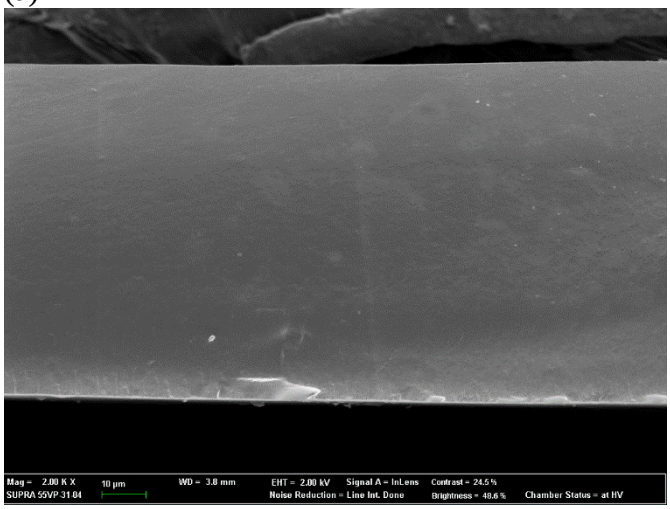
(a)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10 µm
WD = 3.8 mm
EHT = 2.00 kV
Signal A = InLens
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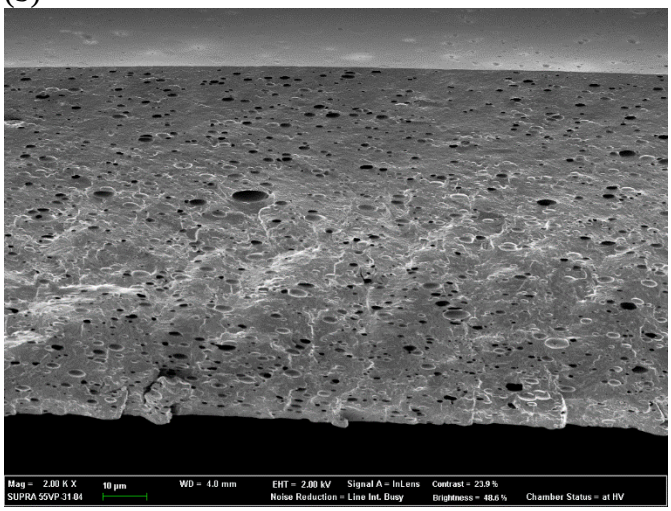
SUPRA 55VP 31 04
Noise Reduction = Line Int. Done
Brightness = 48.6 %
Chamber Status = at HV

(b)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10 µm
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EHT = 2.00 kV
Signal A = InLens
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SUPRA 55VP 31 04
Noise Reduction = Line Int. Busy
Brightness = 48.6 %
Chamber Status = at HV

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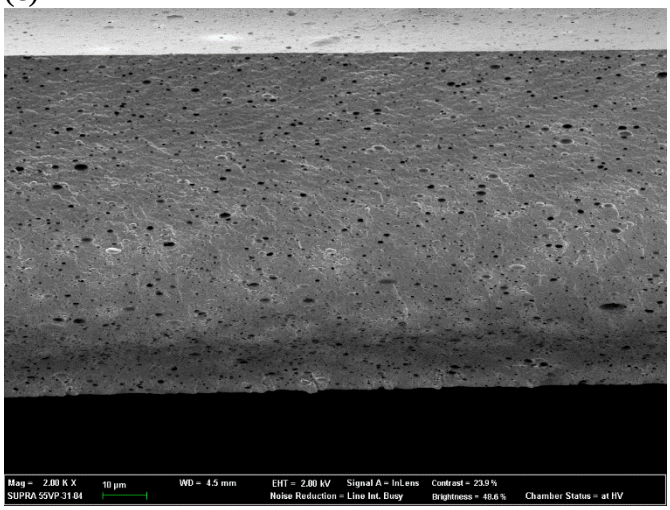
(c)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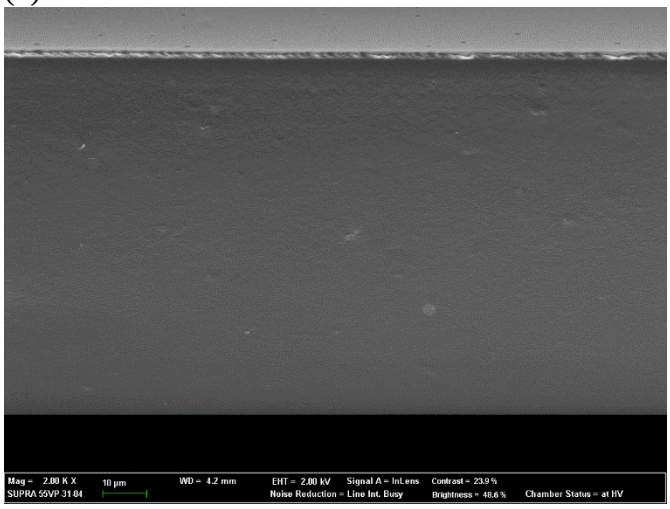
10 µm
WD = 4.5 mm
EHT = 2.00 kV
Signal A = InLens
Contrast = 23.9 %
SUPRA 55VP 31 04
Noise Reduction = Line Int. Busy
Brightness = 48.6 %
Chamber Status = at HV

(d)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10 µm
WD = 4.2 mm
EHT = 2.00 kV
Signal A = InLens
Contrast = 23.9 %
SUPRA 55VP 31 04
Noise Reduction = Line Int. Busy
Brightness = 48.6 %
Chamber Status = at HV

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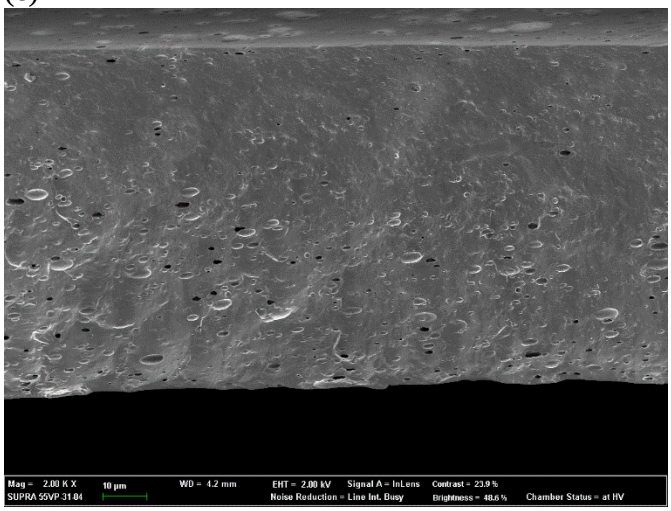
(e)



(f)

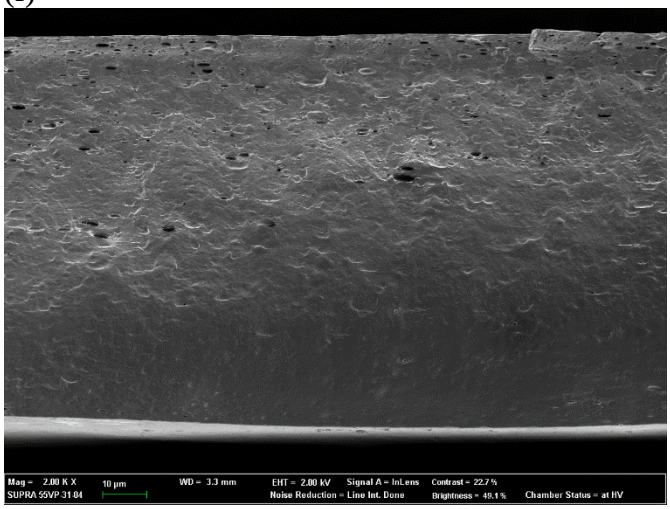


Figure. 3. SEM micrographs of the cross-sections of the films. CH/S film (a), CH/S-C film (b), CH/S-T film (c), CH/S-GA film (d), CH/S-GA-C film (e), CH/S-GA-T film (f).

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3.2. Antioxidant activity

The DPPH scavenging activity is an essential method which can present antioxidant activity of films. Fig. 4 shows DPPH scavenging activity of six films. CH/S film with no addition had a slight antioxidant effect of 8.26% which was related to the radical scavenging activity of chitosan.

The DPPH scavenging activity of films with carvacrol and thymol increased to 33.09%, 19.49%, respectively and films with carvacrol showed a higher antioxidant effect than films with thymol. With the addition of gallic acid, the DPPH scavenging activity significantly ($P < 0.05$) increased to 51.31%. Films incorporated with both GA and EOs show the highest antioxidant effects.

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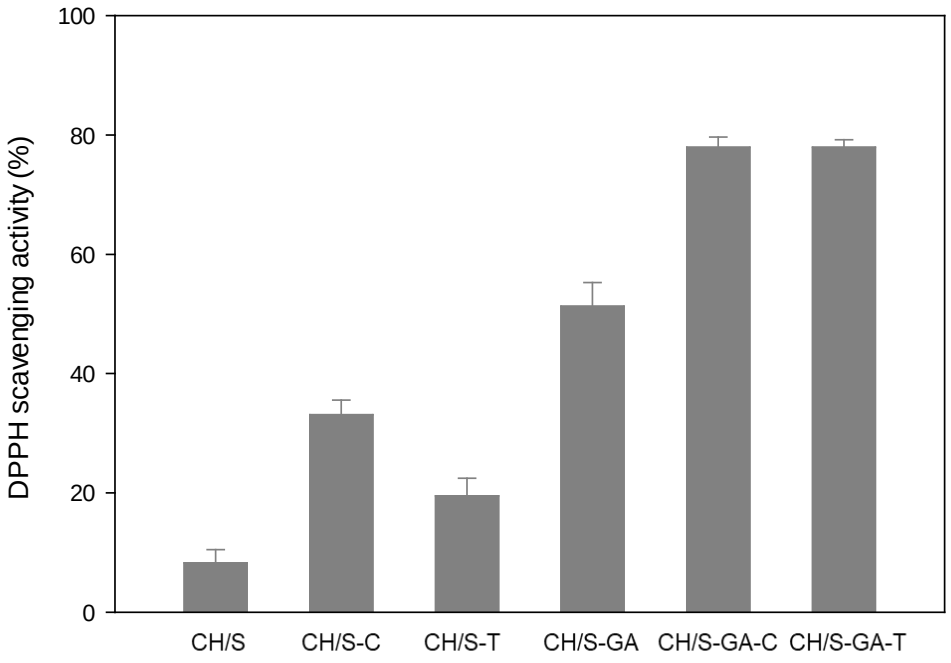

Figure. 4. DPPH-scavenging activities of chitosan-starch films incorporating gallic acid, carvacrol and thymol. The error bars indicate standard deviations.

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3.2. Antimicrobial effect

Table 4 presents the antimicrobial effect of films incorporated with carvacrol, thymol and GA against *S. aureus* and *E. coli*. The CH/S film and CH/S-GA film did not show any antibacterial effect. On the other hand, the films with carvacrol, thymol can inhibit the growth of *S. aureus* and *E. coli*.

To simulate films acting on solid food, the TSA-NaCl was used for a solid food system (Kristo, Koutsoumanis et al. 2008). Population change of *S. aureus* and *E. coli* in TSA plates with or without films are shown in Fig. 5. Pathogens in TSA plates without film grew during storage and increased 1.5 log after 12 hours. The number of *S. aureus* and *E. coli* in TSA plates covered with CH/S film also increased but the growth rate was slower than in TSA plates without film. In the case of films with gallic acid, reduction of bacterial population was observed and the antibacterial effects were maintained over time. After 12 hours, *S. aureus* up to 1.5 log reduction and *E. coli* up to 3.4 log reduction. *E. coli* is more susceptible than *S. aureus* to gallic acid (Borges, Ferreira et al. 2013). Meanwhile, films incorporated with carvacrol and thymol show a great antibacterial effect. After 4 hours of contact time, the number of bacteria reduced to below the detection limit which is more than 5 log reduction.

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Formulation		Inhibition zone (mm^2)	
		S. aureus	*E. coli*
	CH/S	-	-
	CH/S-C	122.2 ± 43.9 A	97.1 ± 34.6 A
	CH/S-T	107.4 ± 43.9 A	87.0 ± 13.9 A
GA	CH/S	-	-
	CH/S-C	143.7 ± 21.2 A	115.1 ± 9.9 A
	CH/S-T	133.9 ± 30.6 A	100.9 ± 5.1 A

Table 4. Antibacterial activity (inhibitory zone) of chitosan-starch films incorporated with gallic acid, carvacrol and thymol against *S. aureus* and *E. coli*. Data represent means ± standard deviations from three replications. Values followed by the same uppercase letters within columns are not significantly different.

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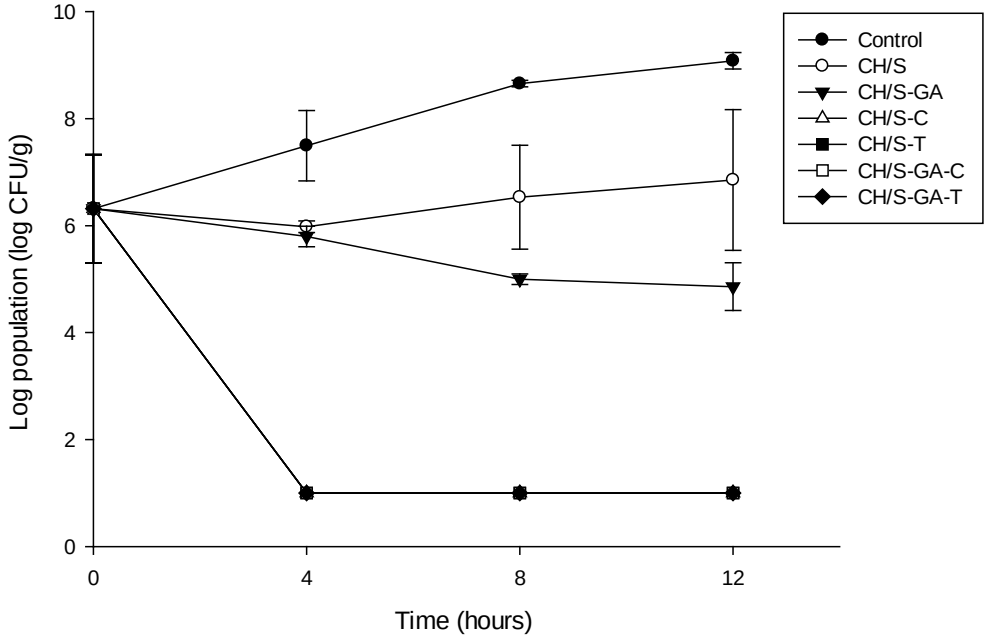
(a)


Log population (log CFU/g)
10
8
6
4
2
0
0
4
8
12
Time (hours)
Control
CH/S
CH/S-GA
CH/S-C
CH/S-T
CH/S-GA-C
CH/S-GA-T

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(b)

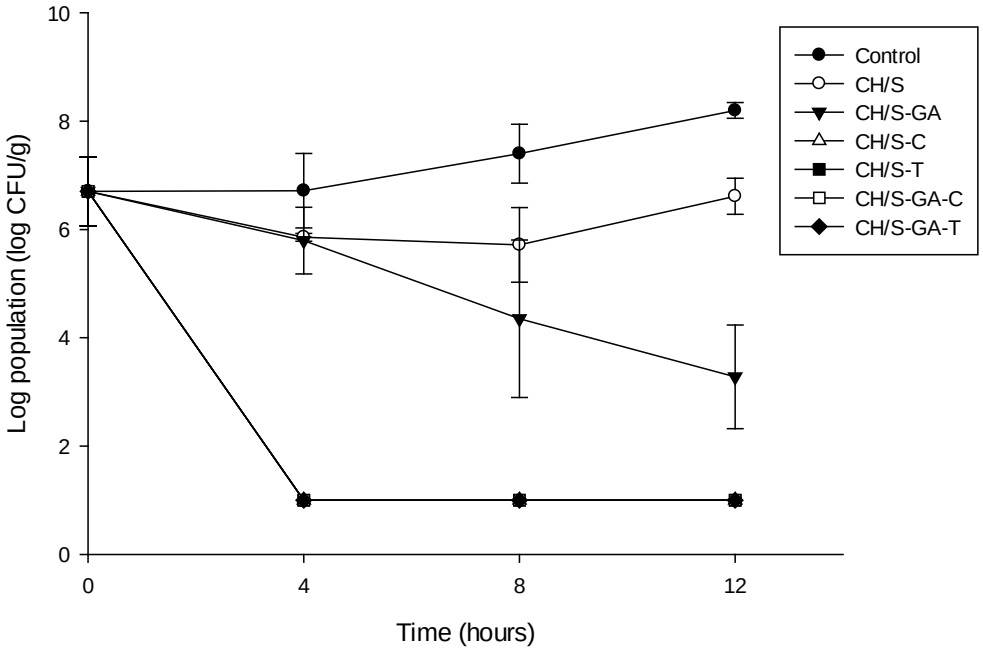

Figure. 5. Antimicrobial activity of chitosan-starch films containing gallic acid, carvacrol and thymol against *S. aureus* (a) and *E. coli* (b) inoculated on TSA-NaCl plates. The error bars indicate standard deviations.

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3.2. Application to beef patties storage

3.2.1. Thiobarbituric acid reactive substances (TBARS)

TBARS is a crucial parameter that indicates the degree of lipid peroxidation in meat. Fig. 6 exhibited the TBARS values of beef patties stored with or without different films. Significant increases ($P < 0.05$) of TBARS value were presented in beef without films at day 3 and increased over time. On the other hand, TBARS value of beef patties with CH/S film slightly increased during storage time ($P < 0.05$) due to the antioxidant activity of chitosan itself and also films work as oxygen barrier. Notably, Films incorporated with GA and both GA and C showed excellent antioxidant properties, resulting no changes in TBARS values of beef patties ($P > 0.05$) for 12 days. The TBARS values of control samples did not exceed the TBARS value of 0.9 mg MDA/kg for 12 days, which may be due to vacuum packaging that prevent oxidation.

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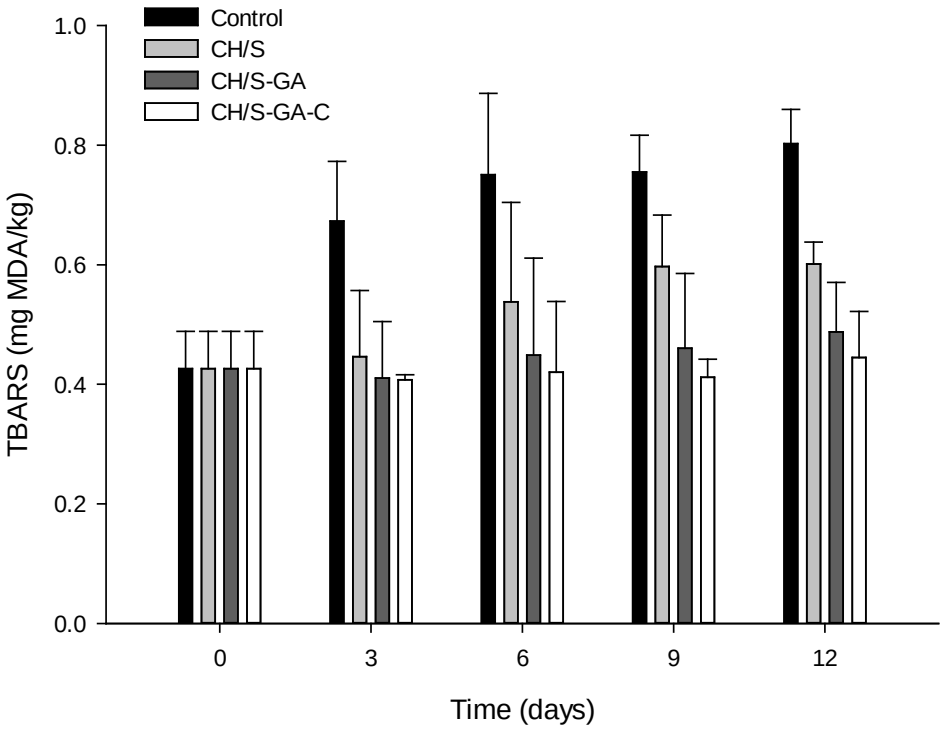

Figure. 6. Effects of antioxidant films incorporating gallic acid and carvacrol on changes in TBARS value of beef patties during refrigerated storage. The error bars indicate standard deviations.

3.2. Color

The color of meat is one of the important factors that influenced consumers choices for meat and meat products. The color change of beef covered with or without films are presented in Table 5. The longer storage duration, the increase of L* value and decrease of a* value were shown. The color difference between beef without film and beef with all three films was not significant ($P < 0.05$). Hence, Films with GA and carvacrol had no significant effect on preventing the color changes of beef patties.

Color attribute	Storage day	Control	CH/S	CH/S-GA	CH/S-C-GA
L* value	0	39.71 ± 2.92 Aa	39.71 ± 2.92 Aa	39.71 ± 2.92 Aa	39.71 ± 2.92 Aa
	3	42.46 ± 2.70 Ba	39.15 ± 1.63 Aa	40.25 ± 2.12 Aa	39.58 ± 1.15 Aa
	6	43.76 ± 1.63 Aa	41.94 ± 2.35 ABa	40.00 ± 2.15 Aa	40.19 ± 1.85 Aa
	9	44.57 ± 1.12 Ba	44.10 ± 2.69 Ba	42.71 ± 2.51 Aa	42.41 ± 2.03 Aa
	12	44.01 ± 0.92 Ba	43.14 ± 1.49 Ba	42.79 ± 1.69 Aa	42.59 ± 1.00 Aa
a* value	0	17.10 ± 0.88 Aa	17.10 ± 0.88 Aa	17.10 ± 0.88 Aa	17.10 ± 0.88 Aa
	3	16.32 ± 1.01 Aa	16.30 ± 0.78 ABa	16.34 ± 1.59 Ba	16.45 ± 0.77 ABa
	6	16.01 ± 0.77 Aa	15.99 ± 0.58 ABa	15.60 ± 0.22 ABa	15.36 ± 0.31 BCa
	9	15.81 ± 1.03 Aa	15.23 ± 0.91 Ba	15.68 ± 0.45 ABa	15.26 ± 0.24 BCa
	12	15.61 ± 1.52 Aa	15.33 ± 0.45 Ba	15.31 ± 0.20 Ba	15.11 ± 0.88 Ca
b* value	0	10.41 ± 0.59 Aa	10.41 ± 0.59 Aa	10.41 ± 0.59 Aa	10.41 ± 0.59 Aa
	3	7.79 ± 0.45 Ba	8.13 ± 0.26 Ba	8.36 ± 0.65 Ba	8.38 ± 0.65 Ba
	6	8.14 ± 0.73 Ba	8.65 ± 0.97 Ba	7.54 ± 0.39 Ba	8.38 ± 0.71 Ba
	9	8.04 ± 0.44 Ba	7.95 ± 1.30 Ba	7.61 ± 0.49 Ba	8.24 ± 0.64 Ba
	12	7.96 ± 0.14 Ba	8.74 ± 0.29 Ba	7.88 ± 0.78 Ba	8.39 ± 1.22 Ba

Table 5. Color values ((L^*, a^*, b^*) of the beef patties packaged with chitosan-starch films containing gallic acid and carvacrol during refrigerated storage. Data represent means ± standard deviations from three replications. Values followed by the same uppercase letters within columns and lowercase letters within rows are not significantly different.

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3.2. Total viable cell count

Fig. 7 showed total viable cell counts (TVC) of the beef patty samples packaged with or without films. TVC of the samples without films had no significant differences ($P > 0.05$) during 12 days of storage. A slight decrease of TVC was presented in beef with CH/S film but no significant changes ($P < 0.05$) compared to beef samples without film. In case of the samples with CH/S-GA films, 1.47 log reduction was exhibited at 3 days and the cell population continued to increase over time. Beef patties with CH/S-GA-C film resulted in 2.27 and 2.32 log reduction of TVC at 3 and 6 days, respectively while the number of viable cells increased again at 9 days. Although films with GA and carvacrol showed strong antimicrobial activity in food simulant as more than 5 log reduction of *S. aureus* and *E. coli* in 4 hour (Fig. 5), the lower antimicrobial effect exhibited when applied in beef patties due to the thickness and complicated structure of ground beef.

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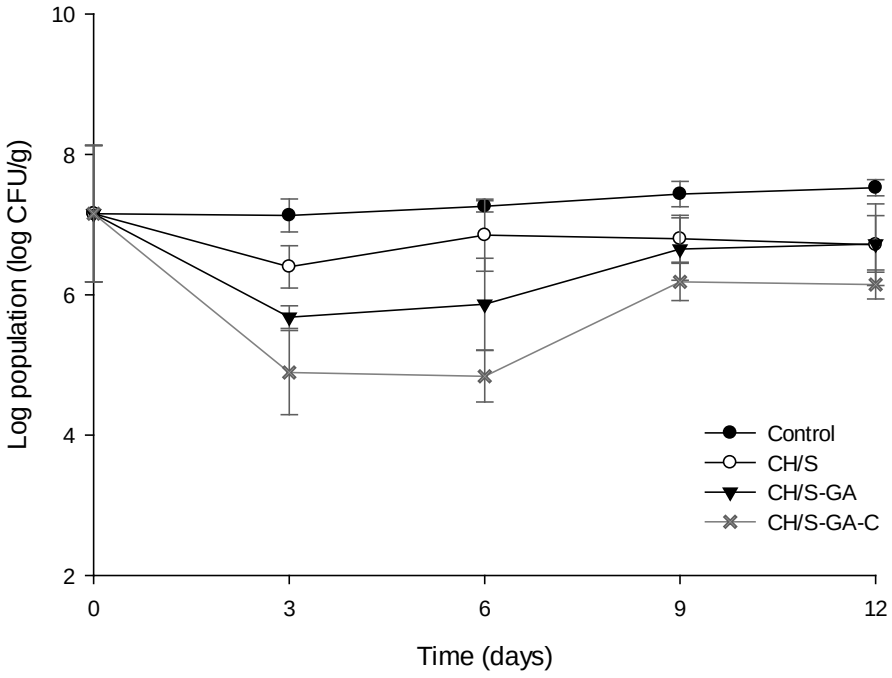

Figure. 7. Total viable cell counts of ground beef patties packaged with or without chitosan-starch films containing gallic acid and carvacrol. The error bars indicate standard deviations.

IV. CONCLUSION

Our results demonstrated that the addition of gallic acid in chitosan-starch films improved tensile strength, water barrier properties of films as a result of the cross-linking. Besides, films with gallic acid showed high antioxidant activity. Films containing essential oils showed strong antimicrobial activity against to gram-positive bacteria (*S. aureus*) and gram-negative bacteria (*E. coli*) . Also, the addition of carvacrol and thymol enhanced elongation of break, water barrier property of films but reduced transparency and tensile strength. Further, incorporation of both gallic acid and essential oils not only shows strong antioxidant, antimicrobial activity but also improves mechanical property, water barrier property of chitosan-starch films. When applied to beef patties preservation, the films containing both GA and carvacrol prevented lipid peroxidation and increased log reduction of total viable cell of ground beef patties. Our work provides novel chitosan-starch films containing GA and EOs and the potential as a packaging film to extend food shelf-life.

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VI. 국문초록

최근 활성 포장이 식품의 유통기한을 연장할 수 있는 유망한 방법으로 간주 됨에 따라 새로운 활성 포장 필름의 개발이 중요해지고 있다. 이에 따라 새로운 활성 필름인 카바크롤, 티몰, 갈산을 첨가한 키토산-전분 필름을 개발했고 그 특성을 규명하였다. 실험 결과를 통해 키토산-전분 필름에 갈산을 첨가했을 때, 갈산이 폴리머와 결합할 수 있는 성질에 의해 필름의 인장 강도가 증가하고 연신율과 수증기 투과도는 감소하는 것을 확인했다. 카바크롤과 티몰을 키토산-전분 필름에 첨가했을 경우 연신율, 색 변화, 항산화, 항균 효과는 증가하고 인장 강도, 수증기 투과도는 감소하는 경향을 보였다. 더 나아가, 필름에 갈산과 에센셜 오일을 같이 넣었을 때 필름은 가장 뛰어난 기계적 성질과 수증기 장벽으로서의 성질을 보일 뿐만 아니라 가장 강력한 항산화, 항균 효과를 보였다. 마지막으로, 필름을 소고기 패티 보관에 적용하여

그 효과를 확인했다. 결과를 통해 갈산과 카바크롤을 같이 첨가한 필름은 소고기 패티 저장 시 높은 항산화, 항균 효과를 보이는 것을 확인하였다. 이 연구를 통해 키토산-전분 필름에 갈산, 카바크롤, 티몰을 첨가함으로써 얻는 다양한 이점을 알 수 있었고, 실제 식품에 적용할 수 있는 가능성을 확인하였다.

주요어: 활성 필름, 갈산, 카바크롤, 티몰, 소고기 패티

학번: 2019-23944

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