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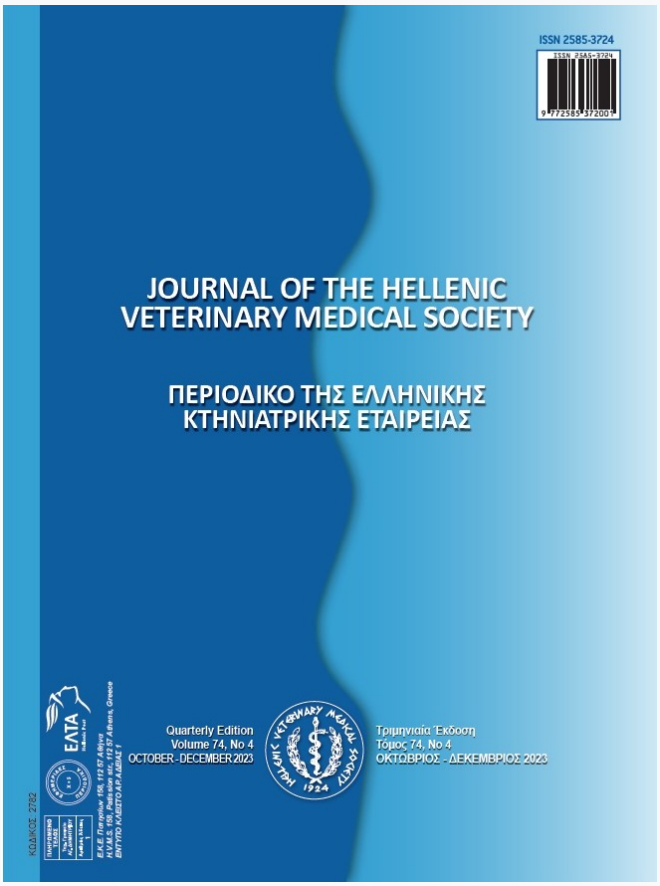

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Research article
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The antimicrobial effect of edible film packaging in instant meatballs on *Escherichia coli* O157:H7

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ABSTRACT: In this study, it is aimed to produce both antimicrobial and edible films that can be used as alternatives to the plastic films that are mostly used in the market. The usability of whey protein isolate in making edible natural packaging material (film) by formulating with additional components (zein, glycerol, thyme essential oil, hibiscus tea and hibiscus extract) was investigated. The antimicrobial effects of the films on *Escherichia coli* O157:H7 were tested, and the effects of edible and plastic films on the shelf life of meatballs were investigated. Analyses (microbiological, chemical and sensory) of the prepared hamburger meatballs were performed on different days (0, 3rd, 5th, 7th, 10th and 12th) of storage (4^{o}C). It was determined that all the edible films produced had an antimicrobial effect, the thyme groups extended the shelf life of meatballs by 7 days compared to the control group, and the other film groups provided a 5 days longer shelf life, again, compared to the control group. It was observed that especially the thyme-added groups degraded *Escherichia coli* O157:H7 at a rate of two logarithms (10^2) and the other groups at a rate of one logarithm. It has been determined that all natural films (except for zein 1% water) preserve their physical structure during the storage days (0, 3rd, 5th, 7th, 10th and 12th) and do not add any undesirable quality to the meatballs.

***Key words*:** *Escherichia coli* O157:H7; hibiscus; thyme; meatballs; whey.

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INTRODUCTION

Food packaging is defined as the process of wrapping, sheathing, or using containers made from materials such as plastic, glass, and paper. These materials serve to protect food from external factors and facilitate marketing and consumption. There is a significant relationship between the environment and packaging materials. Since natural resources originating from packaging are consumed unconsciously, packaging wastes are scattered in the environment, harming the nature, causing extra workload for people, and wasting a large amount of energy (Keleş, 2002). New food packaging technologies are being developed to ensure that the shelf life of industrial products is long, preserves the freshness and the delicious taste of the food, is of high quality, and fulfils the demands of consumers. These technologies have been defined in various ways, including terms like active, smart, interactive, functional, and so on (Dainelli et al., 2008; Kerry et al., 2008).

Recently, there has been an increase in consumer interest in edible packaging, driven by their growing concerns about issues such as food safety, health, and environmental problems (Kayaardı and Akkara, 2010). Edible packages can be consumed along with the food. It can help prolong the shelf life of food by providing barrier (water vapor, oil, moisture, aroma, etc.) properties. Moreover, it can help eliminate quality defects (sensory, chemical, physical) in food (Yang, 1994; Pavlath and Orts, 2009). In addition, antimicrobial and antioxidant components used in film production can prevent unwanted color changes and microbial contamination in foods (Umaraw and Verma, 2017). Edible packages can be evaluated under four headings. These titles include films, coatings, bags, and sheets (Krochta and De Mulder- Johnston, 1997). Edible films are defined as thin layers of material that can be consumed as part of food (Gontard and Guilbert, 1994).In the production of edible packaging, polysaccharides, lipids and proteins are used as the main components (Dursun and Erkan, 2009; Kalkan, 2014; Bolivar et al., 2019). Additionally, different components can be added to improve the properties of the films. These include plasticizing agents, antioxidants, antimicrobials, and other additives (Guilbert et al., 1995; Mkandawire and Aryee, 2018). Meat, poultry, seafood, cereals, frozen foods, fresh foods and fruits and vegetables are the foods most preferred for use with edible packaging (Debeaufort et al., 1998).

In this study, alternative natural edible packaging materials with antimicrobial properties were produced using edible materials. This material can serve as a preferable substitute for plastic packaging within the food industry. The objective of this study was to produce edible films with antimicrobial effects against *Escherichia coli*O157:H7 (*E. coli* O157:H7). These films are intended to be used as an intermediate layer in commercially available meatballs. The materials utilized included whey, a cheap and easily accessible dairy waste, as well as natural herbal teas (hibiscus) and oils (thyme).

MATERIALS AND METHODS

Hibiscus (*Hibiscus sabdariffa* L.) and thyme (*Thymus vulgaris* L.) were obtained from Bağdat Spice company. The medium-fat ground beef (fresh)was obtained from a local market in the city center of Kars.

E. coli O157:H7 (ATCC 43895) used as a reference strain in the study was obtained from the laboratory of Kafkas University Faculty of Veterinary Medicine Department of Food Hygiene and Production.

Additionally, whey protein isolate (Davisco Foods International Inc-USA Hardline Nutrition, 96%), zein (Acros Organics-179315000), glycerol (15524; Sigma-Aldrich, St. Louis, MO, USA) Tween 80 (822187; Merck KGaA, Darmstadt, Germany) were used in the production of the edible films.

Methods

Preparation of plant extract, essential oil and tea

To extract essential oil from the thyme plant, an average of 175 grams of thyme is weighed and crushed into small pieces using a grinder (Waring Commercial Blender-32BL80). An average of 1575 mL of distilled water (1:9 ratio) is added to the Clevenger (WisdWiseTherm, Korea) device along with the thyme. The mixture is then subjected to distillation for approximately 3-5 h. The essential thyme oil (TO) collected during this process is carefully transferred into 10 mL screw cap dark-colored bottles and stored at 4°C (Aksoy et al., 2011).

The study utilized tea and alcohol extracts derived from hibiscus dissolved in water. Firstly, hibiscus tea is prepared by infusing hibiscus plant (5 g) in hot water. To accomplish this, the hibiscus plant is crushed into a powder using a blender. Fifty mL of hot water (95-100 °C) were added to the mixture (1:10 w/v). The mixture is then cooled to room temperature and filtered using coarse filter paper. For each trial, fresh

hibiscus herbal tea (HT) is prepared (Sağdıç and Özcan, 2003). To prepare the alcohol extract from ground hibiscus, 5 g of the plant sample is weighed and mixed with 50 mL of ethyl alcohol (30%) in an open beaker. The mixture is stirred using a magnetic stirrer at 75°C for 1 h. The mixture is subsequently cooled to room temperature and filtered using coarse filter paper. The hibiscus plant extract (HE) is freshly prepared for each trial (Sirag et al., 2013).

Determination of edible film solutions combinations

A modification of the method developed by (Kim and Ustunol, 2001) was used in film production. Various concentrations of the whey protein isolate (WPI) (3%, 5%, 7%), different ratios of glycerol as a plasticizer (4%, 5%), and different pH values within the range of pH 4 to 9 were investigated, as they have a significant impact on the film's structure. Additionally, different heat treatment applications (70°C, 75°C, 85°C) were explored, which influenced the properties of the film solution. In these experiments, while WPI served as the base, different concentrations of zein protein (ZP), solution, essential thyme oil (TO), hibiscus tea (HT), and hibiscus alcohol extract were incorporated into the film material. At this stage, the primary objective is to determine the optimal concentration of additives that do not cause excessive drying, breakage, loss of flexibility, and maintain the usability of the film material.

Preparation of films used in antimicrobial effects trials

Plain (without additive) film solution

An aqueous solution of WPI (5% w/v) by weight was prepared. After mixing in a magnetic stirrer, the pH of the solution was adjusted to a range of 7-8 using 4 N NaOH. Glycerol was then added to the solution, and mixing was continued. The solution was subjected to heat treatment in a water bath at 85°C for 25 min. After cooling to room temperature, the solution was poured into sterile plastic petri dishes (90 x 17 mm) with an approximate volume of 25 mL. The dishes were left to dry for 24-36 h at room temperature.

Thyme added film solutions

5 g of WPI was weighed and dissolved in 85 mL of distilled water. After mixing with a magnetic stirrer, the pH of the solution was adjusted using 4 N NaOH to maintain a pH range of 7 to 8. Glycerol was then added to the solution, and the mixing process was continued. The solution was heat treated in a water bath at 85°C for 25 min. Solutions containing different concentrations of thyme essential oils (0.75%, 1%, 1.5%, and 2%) were added to the solution. After cooling the mixture to room temperature, the mixing process was continued. An average of 25 mL of the mixture was poured into sterile plastic petri dishes and allowed to dry for 24-36 h at room temperature.

Preparation of hibiscus alcohol extract and hibiscus tea added film solutions

An aqueous solution of WPI (5% w/v) by weight was prepared. After mixing in a magnetic stirrer, the pH of the solution was adjusted to 8.5 using 4 N NaOH. Then, 5 mL of glycerol was added to the solution, and the mixing process was continued. The solution was heat treated in a water bath at 85°C for 25 min. Different concentrations of HT (2%, 4%, 6%) or HE (2%, 4%, 6%) were gradually added to the solution, which had been cooled to room temperature, and the mixing process was continued. The solution was stirred slowly for 30 secs using a magnetic stirrer. It was then centrifuged at 4100 rpm for 5 min. After centrifugation, the supernatant was transferred into sterile plastic petri dishes with an average volume of 25 mL. The dishes were left to dry for 24-36 h at room temperature.

Zein (in water) added film solution

Aqueous solutions of zein were prepared with different concentrations by weight (1% and 2% w/v). After adjusting the pH of the solution to a pH of 11-12 using 4 N NaOH and thoroughly mixing it on a magnetic stirrer, the solution was filtered through coarse filter paper. Distilled water and WPI were added to the filtrate, and the mixture was stirred on a magnetic stirrer until the WPI concentration reached 5% w/v. The pH was adjusted to a range of 7-8 using 4 N NaOH. 5 mL of glycerol was added to the solution and mixing was continued. The solution was subjected to heat treatment in a water bath at 85°C for 25 min. The solution, which was cooled to room temperature, was poured into plastic petri dishes with an average volume of 25 mL. The dishes were left to dry at room temperature for 24-36 h.

Zein (alcohol) added film solution

Zein was dissolved in a mixture of 50% alcohol and 50% water by weight (1% and 2% w/v) at the desired concentrations. After thoroughly mixing with a

magnetic stirrer, the solution was subjected to alcohol evaporation in an oven at 75°C for 15 min. The solution was then filtered through filter paper. Distilled water and WPI were added to the filtrate, and the mixture was stirred on a magnetic stirrer until the WPI concentration reached 5% w/v. The pH of the solution was adjusted to a range of 7-8 using 4 N NaOH. Then, 5 mL of glycerol was added to the solution, and the mixing process was continued. The solution was subjected to heat treatment in a water bath at 85°C for 25 min. The solution, which was cooled to room temperature, was poured into plastic petri dishes with an average volume of 25 mL. The dishes were left to dry at room temperature for 24-36 h.

Preparation of bacterial active culture

To activate the *E. coli* O157:H7 lyophilized culture, it was incubated for 24 h at 37 °C in Brain Heart Infusion (BHI) Broth (Fluka-53286). After incubation, the active culture was inoculated onto Sorbitol MacConkey (SMAC)agar (Neogen-NCM0167A) to check for purity. Following the incubation at 37°C for 24 h, the culture was stored at 4°C. For the experiments, a fresh culture was prepared by adding 100 µl of the stock culture to 10 mL of Tryptic Soy Broth (Neogen-004) medium and incubating at 37°C for 24 h. The density of the bacterial culture, obtained from a fresh culture, was adjusted to McFarland 0.5 using physiological saline (FTS). This culture was used for determining the minimum inhibition concentration (MIC) (Yaman, 2010). To determine the bacterial load of the culture adjusted to McFarland 0.5, appropriate dilutions were prepared and inoculated using the SMAC agar smear method. The petri dishes were then incubated at 37°C for 24 h. Colony count was made after incubation and bacterial density was calculated as cfu/mL.

Determination of antimicrobial effects of edible films and additives (hibiscus/zein/thyme essential oil)

The groups used in the determination of trial concentrations and their characteristics are given in Table 1. The agar diffusion method was used in MIC determination studies. The active culture, with a density adjusted to McFarland 0.5, was spread onto the surface of Mueller-Hinton agar (Oxoid-CM0337; Thermo Fisher Scientific Inc., Waltham, MA, USA) using a spreading method. A maximum of 4 sterile blank discs (Oxoid CT998B; Thermo Fisher Scientific Inc.) were placed on the surface of the medium. Next, 100 µL of TO, HT, HE, and zein (alcohol and water) solu-

Table 1. Groups used in determining trial concentrations and their characteristics

Group	Content
1	Plain (without additive) film
2	Film with added hibiscus tea (2% in water)
3	Film with added hibiscus tea (4% in water)
4	Film with added hibiscus tea (6% in water)
5	Film with added hibiscus extract (2% in alcohol)
6	Film with added hibiscus extract (4% in alcohol)
7	Film with added hibiscus extract (6% in alcohol)
8	Thyme (0.75%) added to the film
9	Thyme (1%) added to the film
10	Thyme (1.5%) added to the film
11	Thyme (2%) added to the film
12	Film with added zein (1% in alcohol)
13	Film with added zein (2% in alcohol)
14	Film with added zein (1% in water)
15	Film with added zein (2% in water)
16	Thyme essential oil (0.75%)
17	Thyme essential oil (1%)
18	Thyme essential oil (1.5%)
19	Thyme essential oil (2%)
20	Hibiscus tea
21	Hibiscus extract
22	Zein solution (2% in water)
23	Zein solution (2% in alcohol)

Table 2. Minimum inhibition concentration values for edible films

Group	Content	Impact Status
1	Plain (without additive) film	Less effective
2	Film with added hibiscus tea (2% in water)	Ineffective
3	Film with added hibiscus tea (4% in water)	Ineffective
4	Film with added hibiscus tea (6% in water)	Less effective
5	Film with added hibiscus extract (2% in alcohol)	Ineffective
6	Film with added hibiscus extract (4% in alcohol)	Ineffective
7	Film with added hibiscus extract (6% in alcohol)	Less effective
8	Thyme (0.75%) added to the film	Ineffective
9	Thyme (1%) added to the film	Ineffective
10	Thyme (1.5%) added to the film	Effective
11	Thyme (2%) added to the film	Effective
12	Film with added zein (1% in alcohol)	Ineffective
13	Film with added zein (2% in alcohol)	Less effective
14	Film with added zein (1% in water)	Less effective
15	Film with added zein (2% in water)	Less effective
16	Thyme essential oil (0.75%)	Effective
17	Thyme essential oil (1%)	Effective
18	Thyme essential oil (1.5%)	Very effective
19	Thyme essential oil (2%)	Very effective
20	Hibiscus tea	Less effective
21	Hibiscus extract	Less effective
22	Zein solution (2% in water)	Less effective
23	Zein solution (2% in alcohol)	Less effective

tions, prepared at various concentrations, were carefully pipetted onto the discs. Additionally, pre-prepared edible films were cut into 1x1 cm pieces and then placed on the surface of the medium containing the reference strain. A maximum of 4 pieces per petri dish were placed. All the prepared petri dishes were incubated at 37°C for 24 - 48 h. At the end of the incubation period, the antimicrobial activity of the test groups and their corresponding concentrations were determined in the petri dishes (Table 2).

Preparation of meatball groups and packaging with film

Ground beef (15% -20% fat) obtained from a local market was mixed with 1% -2% salt. After setting aside a portion of homogeneous ground meat for the control group meatballs, the remaining minced meat was inoculated with the *E. coli* O157:H7 strain. The strain was added to the ground meat by calculating 10^5-10^6cfu/g from the active culture adjusted according to McFarland 0.5. The ground meat prepared for all of the groups was kneaded and shaped to a weight of 50-60 g and a diameter of 10 cm. Microbiological analyses (including the presence and count of *E. coli* O157:H7, total psychrophilic count, total mesophilic count, coliform group count, and fecal coliform count) as well as chemical analyses (oil and salt content) were performed on both the salted ground beef and the ground beef inoculated with the strain. The samples were kept at 4°C until analysis. Control groups and experimental groups to be used in the study were prepared. The groups were formed as specified in Table 3 and named according to the components used in the production of the edible film.

The meatballs were prepared and then placed in meatball boxes, with appropriate films assigned to each group. The meatballs were arranged in the following order: edible film + meatball + edible film. They were then placed in lidded packaging boxes (13 x 11 cm/polypropylene) with a total of 6 meatballs (7 films) in each box. The appropriate films were used for packaging. In the control-positive (CP) and control-negative (CN) groups, instead of using edible film, plastic film cut into the size of edible film was placed between the meatballs. All the lidded boxes containing the meatballs were stored in the refrigerator at 4°C. Microbiological, chemical, physical, and sensory analyses were conducted for each group on various storage days. The whole study was carried out in 3 repetitions.

Table 3. Control and experimental groups used in the study

Group	Content			
	Basic film ingredient	Additive	Reference microorganism in meatballs	Lable
1	Plastic film	Not	Not	CN (Control Negative)
2	Plastic film	Not	*E. coli* O157:H7	CP (Control Positive)
3	Plain (without additive) film	Not	*E. coli* O157:H7	PEF Plain (without additive) Film
4	Plain (without additive) film	Thyme (2%) added to the film	*E. coli* O157:H7	TEF-A (Thyme Edible Film 2%)
5	Plain (without additive) film	Thyme (1.5%) added to the film	*E. coli* O157:H7	TEF-B (Thyme Edible Film 1.5%)
6	Plain (without additive) film	Film with added hibiscus tea (6% in water)	*E. coli* O157:H7	HEF (Hibiscus Edible Film)
7	Plain (without additive) film	Film with added zein (2% in alcohol)	*E. coli* O157:H7	ZEF-A (Zein Edible Film 2%)
8	Plain (without additive) film	Film with added zein (1% in water)	*E. coli* O157:H7	ZEF-B (Zein Edible Film 1%)

Microbiological analysis

Samples of meatballs weighing 25 g were collected from each group on different storage days under aseptic conditions. These samples were then homogenized in 225 mL of sterile peptone water (0.1%). Next, appropriate dilutions were prepared, and the media were incubated by inoculating them with the respective microorganisms. The prepared dilutions were inoculated onto plate count agar (PCA, Oxoid CM 325; Thermo Fisher Scientific Inc.) using the spread plate method. The petri dishes were then incubated for 18-24 h at 37°C for the mesophilic group and 5-7 days at 4°C for the psychrophilic group. After incubation, the colonies that developed on the medium were counted. Violet red bile lactose agar (VRBL, Oxoid CM 107; Thermo Fisher Scientific Inc.) was used for the coliform bacteria count, and it was incubated for 18-24 h. For the fecal coliform bacteria count, the VRBL agar was incubated for 18-24 h at 37°C. Furthermore, for the fecal coliform bacteria count, an additional incubation step of 18-24 h at 44.5°C was performed. At the end of the incubation, the dark red colonies surrounded by a reddish precipitate zone that grew on the medium were evaluated and counted. To investigate the presence of *E. coli* O157:H7, 25 g of ground meat was weighed and homogenized in 225 mL of EC broth (10765; Merck KGaA). The mixture was then incubated at 42°C for 18 h. After the incubation, the petri dishes were inoculated on SMAC agar without enrichment and incubated for 18-24 h at 42°C. At the end of the incubation, colorless sorbitol-negative colonies exhibiting typical characteristics that grew on the petri dish were evaluated. For the enumeration of *E. coli* O157:H7 bacteria in the meatball groups with specific strains, the prepared dilutions were plated on SMAC agar using the smear plate method. The petri dishes were then incubated at 37°C for 18-24 h, and the resulting colonies were counted (Harrigan, 1998).

Chemical analysis

The modified Babcock method was employed for determining the fat content in the fresh ground beef. First 9 g of ground meat sample was placed in a poley bottle, followed by the addition of 10 mL of hot water (90 - 95°C). The ground meat was then homogenized using a glass rod. Then, 15 mL of concentrated (95%-97%) H_2SO_4 (07208; Sigma-Aldrich, St. Louis, MO, USA) was added to the bottle. The bottle stopper was closed, and the bottle was held by the neck while shaking it gently on a soft surface. This process ensured that the ground meat had been completely disintegrated, facilitating the subsequent combustion. The fat content in the ground meat was determined as a percentage by reading the level of oil collected in the calibrated neck of the poley bottle (Gökalp et al., 1999).

The ground meat was mixed and kneaded, and during this process, 1% -2% salt was added to transform it into hamburger meatballs. Afterward, a 5g sample was taken and crushed with the assistance of hot water in a stomacher bag. Subsequently, the aqueous portion was filtered through filter paper and transferred to a 500 mL flask. After cooling, the filtrate in the flask was topped up to the volume line with distilled water. Then, 25 mL of the filtrate was measured and 2-3 drops of a 5% potassium chromate (K_2CrO_4) solution (104952; Merck KGaA) were added to it. Then, the solution was titrated with a 0.1 N silver nitrate ($AgNO_3$) solution (101512; Merck KGaA) until a brick-red color was formed. The amount of silver nitrate used in the titration was recorded, and the salt content in ground meat was calculated as a percentage using the formula put forth by Case et al. (1985). The pH of the fresh ground meat and prepared meatballs was measured using a pH meter. For this purpose, a 10 g sample was weighed and 100 mL of distilled water was added to it. After homogenizing the samples, pH measurements were taken using the pH meter (Gökalp et al., 1999).

For the putrefaction test, 3 mL of Eber reagent was transferred into a test tube. Nut-sized pieces were taken from the meatballs using forceps on the days of analysis, and they were carefully brought close to the Eber separator without touching the inner walls of the test tubes. Contact between the separator and the meatballs was prevented. The smoke output and putrefaction, caused by the potential formation of NH_3 due to putrefaction in meatballs, were evaluated as positive (Vural, 1992). Eber reagent was prepared fresh on the days of analysis from 1 part ether (100859; Merck KGaA), 3 parts ethanol (96%) (32221; Sigma-Aldrich), and 1part HCl (d: 1.125) acid.

Sensory analysis

Sensory analyses were conducted for both hamburger patties and edible films. Color, odor, and texture were assessed for both meatballs and films on each analysis day. The analysis data table from Ruiz et al. (2001) was utilized with necessary modifications.

Statistical analysis

The Repeated Measures procedure of General Linear Models analysis was applied to the data obtained in the analysis of the experimental and control groups at different storage days. The difference between the groups was tested by Duncan's multiple comparison method. Statistical analyses were performed using the SPSS 20 package program **(Çimen, 2015).**

RESULTS

Differences in the WPI concentration added to the film solution affected the solubility of the protein in water and the amount of glycerol required for the structure. The ratio of protein to glycerol was prepared as 1:1. It was observed that the films were more acceptable in terms of transparency, drying time, and strength aspects (Figure 1).

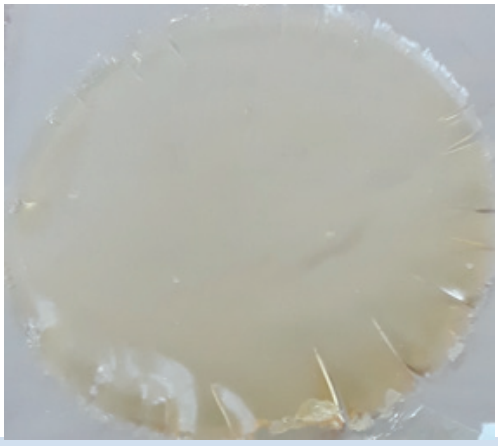

Figure 1. Plain (without additive) edible film (PEF)

The pH values of the natural films were tested as acidic (<7), neutral (7), and alkaline (>7). The change in pH had an effect on the color and structure of the film solutions. The addition of the TO solution caused light turbidity in the color of the film solutions. Increasing the amount of TO further increased the turbidity level compared to the plain edible film (without additive) PEF solution and reduced the drying time of the resulting films (Figure 2).

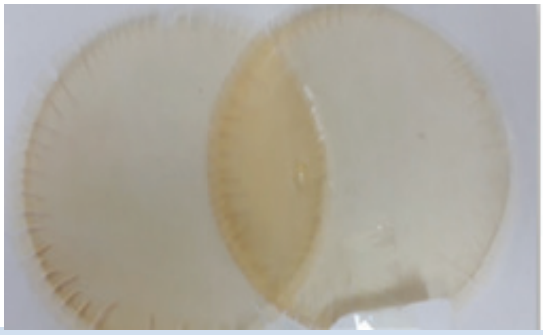

Figure 2. Thyme essential oil added edible film (TEF)

The tea and extract obtained from the hibiscus plant had different effects on the pH. Adding HT (pH: 2.60) and HE (pH: 3.09), which are acidic and dark red, to neutral natural film solutions resulted in the solution turning violet. Increasing the amount of HT and HE intensified the degree of coagulation. For the

hibiscus edible film (HEF), a pH range of 8-8.5 and heat treatment at 85 °C for 25 min were found to be more successful. Adding HT and HE after the heat treatment further enhanced the success of the process (Figure 3).

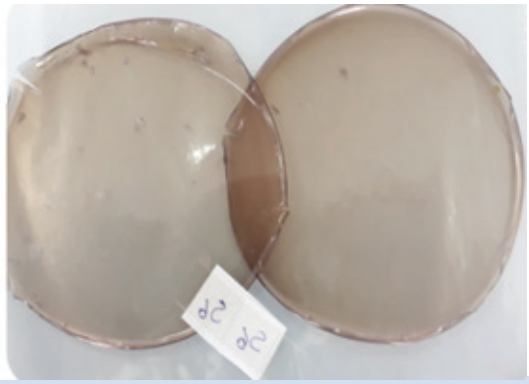

Figure 3. Hibiscus tea added edible film (HEF)

ZP was prepared at different concentrations (1%, 2%, 4%, and 5%) in different solvents (alcohol and water), and then added to the film solution. Concentration determination was performed based on the total protein amount (WPI + ZP) to be used in the film solution. The solubility of the ZP and WPI varied across the different solvents and pH values. The solubility of ZP in alcohol at pH 7-8 and its solubility in water at pH 11-12 were found to be successful. Films prepared by considering a total protein amount of 5% (WPI + ZP) resulted in films obtained using ZP in alcohol (2%) and water (1%). Although zein edible film (ZEF)-B, one of the obtained films, was more transparent than ZEF-A, it was found to be unsuccessful in terms of strength. The antimicrobial effect of TO, HT, and HE on *E. coli* O157:H7 was investigated using the disk diffusion method.

As a result of directly impregnating TO, HT, and HE onto the blank paper disc instead of adding them to the film content, the direct contact of TO, HT, and HE with bacteria, unlike the films, strengthened the antimicrobial effect. It was determined that all the films used in the experimental groups exhibited antimicrobial effects. When compared with the CP and CN groups, there was typically a difference of approximately 10^2 (2 logarithms) in all the investigated microorganism groups across the different days. It was determined that the thyme groups extended the shelf life of meatballs by 7 days compared to the CP and CN groups, while the other film groups provided a shelf life that was 5 days longer than the control group. Thyme groups were more effective in reducing the count of coliform and fecal coliform bacteria and/or slowing their growth compared to the other groups ($p<0.05$) (Tables 4 and 5).

When considering the effect of the groups on microorganism inhibition during storage, the duration of storage (days) had a significant impact ($p<0.01$). When evaluating the antimicrobial effect of the films on *E. coli* O157:H7, the thyme groups exhibited a reduction rate of 10^2 (2 logarithms), while the other groups showed a reduction rate of approximately 1 logarithm. There was also a statistically significant difference between the groups in relation to the duration of the days ($p<0.01$) (Table 6). The mesophilic bacterial count in the groups was generally found to be 10^7, and the sensory tests also indicated positive results for putrefaction. The microbial effect between the PEF, HEF, ZEF, and TEF groups and the CP was statistically significant ($p<0.05$) (Table 7). The relationship between the CP and all other groups in terms of the psychrophilic microorganisms was statistically

Table 4. Coliform bacteria count in meatballs during storage (log cfu/g ± sd)

Groups	0 day	3rd day	5th day	7th day	10th day	12th day	Total
TEF-A	6.63±0.05	5.49±0.14	5.52±0.16	5.66±0.12	5.73±0.15	6.30±0.11	**5.89±0.08^{DE}**
TEF-B	6.63±0.05	5.16±0.14	5.47±0.16	5.64±0.12	5.40±0.15	6.33±0.11	**5.77±0.08^{E}**
HEF	6.63±0.05	5.13±0.14	5.32±0.16	5.44±0.12	6.69±0.15	7.50±0.11	**6.12±0.08^{CD}**
PEF	6.63±0.05	5.60±0.14	5.56±0.16	5.71±0.12	6.67±0.15	7.45±0.11	**6.27±0.08^{BC}**
ZEF-B	6.63±0.05	5.33±0.14	5.48±0.16	5.60±0.12	6.62±0.15	8.30±0.11	**6.33±0.08^{BC}**
ZEF-A	6.63±0.05	5.52±0.14	5.60±0.16	5.74±0.12	6.50±0.15	8.29±0.11	**6.38±0.08^{B}**
CP	6.63±0.05	5.40±0.14	6.75±0.16	7.55±0.12	8.49±0.15	8.61±0.11	**7.24±0.08^{A}**
CN	3.62±0.05	4.54±0.14	6.59±0.16	7.47±0.12	7.77±0.15	8.43±0.11	**6.40±0.08^{B}**

Group (p): 0.000
Day (p): 0.0001
TEF-A: Thyme edible film (2%), TEF-B: Thyme edible film (1.5%), HEF: Hibiscus edible film, PEF: Plain (without additive) film, ZEF-B: Zein edible film (1%), ZEF-A: Zein edible film (2%), CP: Control positive, CN: Control negative.
The letters (A, B, C, D, E) in the total column show the significant difference between the groups at the $p < 0.05$ level.
sd: standard deviation

different ($p<0.05$). While the psychrophilic microorganism counts in the control groups reached levels of 7 log and above on day 5, these levels were reached on day 12, in the thyme groups and on day 10 in the other groups. The effect of the groups on the psychrophilic microorganisms showed a statistically significant difference across the storage days ($p<0.01$) (Table 8).

The fat content and salt content were determined in the fresh ground beef used for making the meat-

Table 5. Fecal coliform group bacteria count in meatballs during storage (log cfu/g ± sd)

Groups	0 day	3rd day	5th day	7th day	10th day	12th day	Total
TEF-A	6.63±0.05	5.17±0.08	5.29±0.08	5.41±0.08	5.48±0.10	6.55±0.16	**5.76±0.07^{F}**
TEF-B	6.63±0.05	5.26±0.08	5.54±0.08	5.69±0.08	5.73±0.10	6.65±0.16	**5.92±0.07^{EF}**
EF	6.63±0.05	5.27±0.08	5.47±0.08	5.55±0.08	6.71±0.10	6.47±0.16	**6.02±0.07^{DE}**
PEF	6.63±0.05	5.36±0.08	5.50±0.08	5.67±0.08	6.70±0.10	7.33±0.16	**6.20±0.07^{CD}**
ZEF-B	6.63±0.05	5.45±0.08	5.79±0.08	5.85±0.08	6.36±0.10	7.36±0.16	**6.24±0.07^{C}**
ZEF-A	6.63±0.05	5.39±0.08	5.66±0.08	5.76±0.08	6.26±0.10	7.35±0.16	**6.18±0.07^{CD}**
CP	6.63±0.05	6.52±0.08	6.89±0.08	7.49±0.08	8.45±0.10	8.93±0.16	**7.48±0.07^{A}**
CN	2.63±0.05	4.91±0.08	6.88±0.08	7.87±0.08	7.92±0.10	8.74±0.16	**6.49±0.07^{B}**

Group (p): 0.000
Day (p): 0.0001
TEF-A: Thyme edible film (2%), TEF-B: Thyme edible film (1.5%), HEF: Hibiscus edible film, PEF: Plain (without additive) film, ZEF-B: Zein edible film (1%), ZEF-A: Zein edible film (2%), CP: Control positive, CN: Control negative.
The letters (A, B, C, D, E, F) in the total column show the significant difference between the groups at the $p < 0.05$ level.
sd: standard deviation

Table 6. *E. coli* O157:H7 count in meatballs during storage (log cfu/g ± sd)

Groups	0 day	3rd day	5th day	7th day	10th day	12th day	Total
TEF-A	6.63±0.06	4.36±0.12	4.68±0.10	5.24±0.13	5.43±0.09	6.41±0.10	**5.46±0.05^{F}**
TEF-B	6.63±0.06	5.35±0.12	5.57±0.10	5.45±0.13	5.62±0.09	6.72±0.10	**5.89±0.05^{D}**
HEF	6.63±0.06	5.30±0.12	5.40±0.10	5.69±0.13	6.55±0.09	6.70±0.10	**6.05±0.05^{C}**
PEF	6.63±0.06	5.40±0.12	5.51±0.10	5.73±0.13	6.38±0.09	7.35±0.10	**6.17±0.05^{C}**
ZEF-B	6.63±0.06	5.20±0.12	5.26±0.10	5.45±0.13	7.72±0.09	8.58±0.10	**6.47±0.05^{B}**
ZEF-A	6.63±0.06	5.48±0.12	5.33±0.10	5.39±0.13	6.28±0.09	7.52±0.10	**6.10±0.05^{C}**
CP	6.63±0.06	6.43±0.12	6.68±0.10	7.68±0.13	7.54±0.09	8.67±0.10	**7.27±0.05^{A}**
CN	0.59±0.06	4.68±0.12	6.22±0.10	6.54±0.13	7.67±0.09	8.80±0.10	**5.75±0.05^{E}**

Group (p): 0.000
Day (p): 0.0001
TEF-A: Thyme edible film (2%), TEF-B: Thyme edible film (1.5%), HEF: Hibiscus edible film, PEF: Plain (without additive) film, ZEF-B: Zein edible film (1%), ZEF-A: Zein edible film (2%), CP: Control positive, CN: Control negative.
The letters (A, B, C, D, E, F) in the total column show the significant difference between the groups at the $p < 0.05$ level.
sd: standard deviation

Table 7. Total mesophilic bacteria count in meatballs during storage (log cfu/g ± sd)

Groups	0 day	3rd day	5th day	7th day	10th day	12th day	Total
TEF-A	6.63±0.06	5.21±0.11	5.35±0.11	5.51±0.07	6.25±0.10	6.34±0.12	**5.88±0.07^{D}**
TEF-B	6.63±0.06	5.39±0.11	5.61±0.11	6.62±0.07	6.68±0.10	6.74±0.12	**6.28±0.07^{C}**
HEF	6.63±0.06	5.27±0.11	5.56±0.11	5.84±0.07	7.17±0.10	7.57±0.12	**6.34±0.07^{C}**
PEF	6.63±0.06	5.60±0.11	5.66±0.11	5.78±0.07	7.30±0.10	7.39±0.12	**6.39±0.07^{C}**
ZEF-B	6.63±0.06	5.46±0.11	5.58±0.11	5.65±0.07	7.38±0.10	7.69±0.12	**6.40±0.07^{C}**
ZEF-A	6.63±0.06	5.55±0.11	5.75±0.11	5.86±0.07	7.38±0.10	7.61±0.12	**6.46±0.07^{C}**
CP	6.63±0.06	6.77±0.11	7.70±0.11	8.40±0.07	9.35±0.10	9.54±0.12	**8.07±0.07^{A}**
CN	4.67±0.06	6.55±0.11	7.86±0.11	7.95±0.07	8.23±0.10	9.39±0.12	**7.44±0.07^{B}**

Group (p): 0.000
Day (p): 0.0001
TEF-A: Thyme edible film (2%), TEF-B: Thyme edible film (1.5%), HEF: Hibiscus edible film, PEF: Plain (without additive) film, ZEF-B: Zein edible film (1%), ZEF-A: Zein edible film (2%), CP: Control positive, CN: Control negative.
The letters (A, B, C, D) in the total column show the significant difference between the groups at the $p < 0.05$ level.
sd: standard deviation

Table 8. Total psychrophilic bacteria count in meatballs during storage (log cfu/g ± sd)

Groups	0 day	3rd day	5th day	7th day	10th day	12th day	Total
TEF-A	6.63±0.06	4.43±0.13	5.48±0.11	5.69±0.13	6.39±0.16	6.34±0.14	**5.83±0.05^{E}**
TEF-B	6.63±0.06	4.52±0.13	5.52±0.11	5.63±0.13	6.40±0.16	7.46±0.14	**6.03±0.05^{D}**
HEF	6.63±0.06	4.74±0.13	5.67±0.11	7.31±0.13	7.55±0.16	8.39±0.14	**6.72±0.05^{B}**
PEF	6.63±0.06	5.55±0.13	5.62±0.11	7.30±0.13	7.51±0.16	8.59±0.14	**6.87±0.05^{B}**
ZEF-B	6.63±0.06	4.18±0.13	5.51±0.11	6.51±0.13	7.11±0.16	7.53±0.14	**6.24±0.05^{C}**
ZEF-A	6.63±0.06	5.44±0.13	5.61±0.11	7.54±0.13	7.46±0.16	8.45±0.14	**6.86±0.05^{B}**
CP	6.63±0.06	5.71±0.13	7.80±0.11	8.33±0.13	9.38±0.16	9.66±0.14	**7.92±0.05^{A}**
CN	3.61±0.06	5.09±0.13	7.41±0.11	7.58±0.13	8.33±0.16	8.47±0.14	**6.75±0.05^{B}**

Group (p): 0.000
Day (p): 0.0001
TEF-A: Thyme edible film (2%), TEF-B: Thyme edible film (1.5%), HEF: Hibiscus edible film, PEF: Plain (without additive) film, ZEF-B: Zein edible film (1%), ZEF-A: Zein edible film (2%), CP: Control positive, CN: Control negative.
The letters (A, B, C, D, E) in the total column show the significant difference between the groups at the $p < 0.05$ level.
sd: standard deviation

balls. The fat content was measured at 15%, while the salt content was determined to be 1.6% in the ground beef purchased as medium fat. When examining the pH values, those in the groups generally exhibited a slight increase initially, followed by a rapid decrease in the subsequent days. However, the pH value began to increase again during the subsequent days of storage ($p<0.05$). On the days with a pH increase (although it varied according to the groups), the putrefaction test generally gave a positive result. The days with a pH increase coincided with day 7 in the CN group and day 5 in the CP group. In the TEF and ZEF groups, the pH started to increase from day 10, while in the HEF and PEF groups, the pH increased from day 7. The variability of the pH values according to days was statistically significant ($p<0.01$) (Table 9). In the putrefaction test (Eber), it was determined that putrefaction was positive starting from day 5 of storage in the control groups, from day 7 in the HEF, PEF, and ZEF-A groups, from day 7 in the thyme groups, and from day 10 in the ZEF-B group. According to the sensory analysis data, the meat retained its distinctive shade of red in the TEF-A, TEF-B, PEF, ZEF-A, and ZEF-B groups. However, in the HEF (from day 7) and CN and CP groups (from day 7), differences in the meat color were observed, with some meat appearing green or purple. The meatballs started to exhibit signs of rancidity, particularly from day 5 in the control groups (CN and CP), and from day 10 in the other groups (except for TEF-A and TEF-B) (Table 10).

DISCUSSION

The main objective of this research was to develop an edible film containing natural components that could effectively inhibit *E. coli* O157:H7, which is commonly associated with foodborne illnesses, particularly as a contaminant in hamburger meatballs. Thymol and carvacrol in the essential oil of the thyme plant are responsible for their antimicrobial proper-

Table 9. pH values of meatballs during storage

Groups	0 day	3rd day	5th day	7th day	10th day	12th day	Total
TEF-A	6.12±0.05	6.19±0.06	6.16±0.06	6.11±0.18	6.43±0.19	6.63±0.14	**6.28±0.07^{ABC}**
TEF-B	6.12±0.05	6.21±0.06	6.12±0.06	6.01±0.18	6.44±0.19	6.61±0.14	**6.25±0.07^{ABC}**
HEF	6.12±0.05	5.92±0.06	5.57±0.06	5.73±0.18	5.83±0.19	6.04±0.14	**5.87±0.07^{D}**
PEF	6.12±0.05	6.02±0.06	5.93±0.06	5.94±0.18	6.09±0.19	6.34±0.14	**6.07±0.07^{CD}**
ZEF-B	6.12±0.05	6.15±0.06	5.98±0.06	5.85±0.18	6.36±0.19	6.87±0.14	**6.22±0.07^{ABC}**
ZEF-A	6.12±0.05	6.18±0.06	5.90±0.06	5.57±0.18	6.27±0.19	6.54±0.14	**6.10±0.07^{BCD}**
CP	6.12±0.05	5.95±0.06	6.05±0.06	6.18±0.18	6.41±0.19	7.24±0.14	**6.32±0.07^{AB}**
CN	6.12±0.05	6.00±0.06	5.90±0.06	6.41±0.18	6.58±0.19	7.24±0.14	**6.37±0.07^{A}**

Group (p): 0.004
Day (p): 0.0001
TEF-A: Thyme edible film (2%), TEF-B: Thyme edible film (1.5%), HEF: Hibiscus edible film, PEF: Plain (without additive) film, ZEF-B: Zein edible film (1%), ZEF-A: Zein edible film (2%), CP: Control positive, CN: Control negative.
The letters (A, B, C, D) in the total column show the significant difference between the groups at the $p < 0.05$ level.

Table 10. Eber test results of edible film and control groups

Groups	0 day	3rd day	5th day	7th day	10th day	12th day
TEF-A	-	-	-	-	+	+
TEF-B	-	-	-	-	+	+
HEF	-	-	-	+	+	+
PEF	-	-	-	+	+	+
ZEF-B	-	-	-	+	+	+
ZEF-A	-	-	-	-	+	+
CP	-	-	+	+	+	+
CN	-	-	+	+	+	+

+: Putrefaction positive, -: Putrefaction negative
TEF-A: Thyme edible film (2%), TEF-B: Thyme edible film (1.5%), HEF: Hibiscus edible film, PEF: Plain (without additive) film, ZEF-B: Zein edible film (1%), ZEF-A: Zein edible film (2%), CP: Control positive, CN: Control negative.

ties as well as the aromatic smell of the plant (Tanker and Tanker, 1990). On the other hand, hydrosols or teas derived from the hibiscus plant have been recently utilized as natural antimicrobial agents. These hydrosols contain anthocyanins such as cyanidin, cyanidin-3-sambubioside, delphinidin, delphinidin-3, and malvidin, which contribute to their antimicrobial properties (Fullerton et al., 2011; Gweyi-Onyango et al., 2021).

In this study, whey proteins and plasticizer (glycerol) were preferred. Different concentrations of WPI (3%, 5%, 7%) and glycerol (4%, 5%) were tested. Success was achieved by utilizing both components in equal proportions (1:1). Anker et al. (2000) explained this with two theories: the Less Plasticizer - Less Protein interaction theory and the More Plasticizer-More Protein interaction theory. A clear perspective was achieved by adjusting the pH value of the films within the range of 7-8. Fitzsimons et al. (2007) noted that the heat has a denaturing and clouding effect on the serum proteins in WPI. However, the solutions regain clarity at a neutral pH. The change in pH with the effect of heat treatment accelerated protein denaturation and affected the strength of the edible films. Anker et al. (1998) and Anker et al. (2000) reported that the structure of edible films undergoes significant changes when exposed to various temperatures (60 - 70°C) and pH levels (7 - 9).

As the pH values increased, the protein molecules formed a more compact network structure, leading to an increase in their strength.

In the film production process, prior to the addition of TO, an emulsion of TO was prepared. For this, an oil-in-water emulsion was prepared. All components were mixed thoroughly in a falcon tube to form an emulsion. Gönül (2000) emphasized the importance of using an effective emulsion type to achieve homogeneity when working with liquids of two different properties (polar and nonpolar). However, Çelebi (2009) emphasized that homogeneous mixing is an important step in ensuring emulsion stability. Increasing the amount of TO shortened the drying time and caused the films to crack. This was due to the structural properties of protein-based edible films and essential oils, as well as the drying properties of oils. Seydim and Sarıkuş (2006) reported that edible films obtained from milk proteins have poor moisture barrier properties. Additionally, Zivanovic et al. (2006) found that thyme essential oils added to edible films increased the water vapor permeability of the films.

HT and HE were added in different ratios (2, 4, 6, 8, and 10) to the film solutions after heat treatment in a controlled manner. At levels of 8% and 10%, the solution was observed to form a fully coagulated and solidified clot. The increase in HT proportionally further reduced the pH values of the solution. The protein particles that settled at the bottom as a result of coagulation and precipitation were separated through centrifugation (4100 rpm for 5 min). The zein was prepared using 2 different solvents: 50% alcohol (2%) and water (1%). While zein is hydrophobic and insoluble in water, it exhibits solubility in alcohol and alkaline environments. Shukla and Cheryan (2001) found that due to its high content of apolar amino acids, zein exhibits reduced solubility in water. However, it can be easily dissolved in solvents with a pH of 11 and above, as well as in ethanol.

MIC values were determined to measure the antimicrobial activity of the prepared edible films (thyme, hibiscus, zein, plain) and solutions. For this study, the films were prepared by adding herbal teas and extracts to HT and HE in different solvents and amounts.

TO impregnated discs were used in various ratios, while ZEF was prepared in different ratios and solvents. ZP impregnated disc solutions were prepared using alcohol and water. Finally, the antimicrobial effects of PEF were tested against *E. coli* O157:H7. Concentrations of TEF at 1.5% and 2%, as well as all of the TO-impregnated discs (0.75%, 1%, 1.5%, and 2% mL), exhibited varying levels of inhibitory effect against the *E. coli* O157:H7 strain. In parallel with the increase in the amount of thyme, the antimicrobial effect against the microorganism also increased. This can be attributed to the high concentration of compounds with antimicrobial properties present in TO. Marino et al. (2001) discovered that thyme and its various varieties contain essential oil contents that exhibit antimicrobial activity. They further revealed that the phenolic compounds present in thyme penetrate the cell membrane of the target microorganism and interact with its metabolic reactions. Additionally, it was emphasized that the cell structure of the target microorganism, along with the concentration of spices and herbs used, played a crucial role in determining their effectiveness (Pandit and Shelef, 1994; Marino et al., 2001; Sağdıç, 2003). According to the MIC data of the films containing HT and HE (2.4%), the tea and extracts impregnated on the discs, as well as the films with the addition of HT and HE at 6%, exhibited reduced effectiveness. Effective and powerful extraction processes also significantly affect the amount and type of phenolic components, indirectly, the structural and antimicrobial effect of the extract (Fullerton et al., 2011). Jung et al. (2013) reported that utilizing hot water in the plant extraction method is superior to ethanol in terms of anthocyanin content and the quantity of phenolic components. Furthermore, they noted that higher concentrations have a synergistic effect, enhancing the antimicrobial efficacy. No significant antimicrobial differences were observed between the ZEF (except 1% alcohol) and zein solutions. According to the MIC data, it exhibited a minor antimicrobial effect on the target microorganism. This can be attributed to the specific characteristics of ZP. In fact, Marino et al. (2001) stated that zein exhibits antimicrobial effects through the formation of a protective layer when used in moisture and oxygen barrier applications such as films and coatings.

pH is one of the crucial parameters for detecting spoilage in meat and meat products. pH is used as an indicator to determine the level of changes/deteriorations in meat and meat products as a result of microbial activity. Pektaş (2013) stated that the biochemical and microbial processes that take place during the storage of meat, can significantly affect its pH levels. The pH value of fresh normal meat typically ranges between 5.5 and 6. However, nitrogenous compounds tend to alkalize food and cause the pH to increase to the range of 7-8, while acidic compounds lower the pH of food, making it more acidic. (Ray and Bhunia, 2016; Aksan, 2017). Generally, the pH values of the groups initially decreased and then gradually increased. Ray and Bhunia (2016) explained that the decrease in pH is due to the breakdown of carbohydrates present in food by microorganisms. In the absence of sufficient carbohydrates, secondary nitrogenous compounds, such as nitrogenous compounds and small peptides, are utilized as an energy source. The variation in the pH values resulting from the microbial activities was identified as an indicator of undesirable putrefaction reactions in the meatballs. The Eber method, commonly employed as a putrefaction test, is typically able to detect early-stage putrefaction based on sensory analysis. In this study, while the CN and CP groups showed positive results on day 5, the HEF, PEF, and ZEF-A groups exhibited positive results on day 7. On day 10, the TEF-A, TEF-B, and ZEF-B groups also tested positive. According to the sensory analysis data, the CN and CP groups displayed signs of putrefaction starting from day 5. The other groups, except for the TEF groups, exhibited indications of putrefaction from day 10. The thyme groups, however, showed signs of putrefaction on day 12. Although the pH values varied among the groups, the putrefaction test generally yielded positive results on the days when the pH levels increased.

Many studies have been conducted utilizing various doses of thyme and hibiscus plant components, such as hydrolysates or essential oils, to investigate their effectiveness against pathogenic microorganisms, particularly *E. coli* O157:H7. These studies have yielded successful results, demonstrating the antimicrobial properties of these plant components (Marino et al., 1999; De Feo V et al., 2003; Dadalıoğlu and Evrendilek, 2004; Olaleye, 2007; Govaris et al., 2011; Paım et al., 2017). In this study, the TEF groups extended the shelf life of the meatballs by 7 days, while the other film groups increased it by 5 days compared to the CN and CP groups.

Fullerton et al. (2011) conducted research to assess the antimicrobial activity of *Hibiscus sabdariffa* against *E. coli* O157:H7 strains present in various food samples, including spinach, vegetables, and

ground meat, obtained from a local market. It was reported that plant extracts applied at different doses (2.5%, 5%, 10%) exhibit antimicrobial effects against the pathogen. A high dose, particularly, is more effective than the other doses.

Li et al. (2021), in their research on the development of antibacterial nano-emulsions containing TO, discovered that a 1% (v/v) concentration of TO exhibited inhibitory effects against both *S. aureus* and *E. coli* during prolonged storage.

CONCLUSION

The antimicrobial properties and structural properties of edible films were utilized. The films were employed as an intermediate material to prevent the meatballs from sticking together and provide overall coverage for the meatballs. Successful results were obtained in terms of durability, solubility, odor and color changes of the films. No degradation in the structure of the films was detected during storage, except for in the ZEF-B group. As a result of the contact between the films and the meatballs, the films absorbed the moisture from the meatballs. Although this situation caused softening in the structure of the films, no conditions such as deformation, rupture, or dissolution were observed. While the red color of the meat caused the films in the TEF and PEF groups to exhibit redness, the films retained their original color in the HT and HE (magenta) and ZEF groups (yellowish). Thanks to the phenolic compounds it contains, hibiscus is able to color the environments it is in. The increase in pH and microorganism load resulted in the release of nitrogenous compounds, causing a color change (green) in the areas where the film came into contact with the meatballs. According to these results, the volatility of thyme plant oil and the color properties of hibiscus draw attention. While the antimicrobial property of thyme is concealed within its volatile components, for hibiscus, this property becomes apparent when it is in direct contact with food. It can be observed that films obtained by utilizing natural plants rich in anthocyanins, such as hibiscus, can be utilized as intelligent pH degradation indicators while enhancing their antimicrobial properties. Additionally, it is recommended to utilize whey, which contains valuable components that are often wasted, successfully for the production of edible films.

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CONFLICT OF INTEREST

None declared.

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