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Use of Egg White Protein Powder Based Films Fortified with Basil (*Ocimum basilicum* L.) Essential Oils in the Storage of Çökelek Cheese

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Authors' contributions

This work was carried out in collaboration between both authors. Author NK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Author GK managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Edible film was produced by adding 1.5% sorbitol (w/v) to egg white protein powder (EWPP). The 1th batch of the çökelek cheese samples was coated with film fortified with basil essential oil (EO_B) at concentrations [2% (v/v)]. The 2th batch of the çökelek cheese samples was coated exclusively with non-fortified EWPP and the 3th batch was left uncoated (Control). All of the cheese samples were artificially contaminated with *Escherichia coli* O157:H7*, Listeria monocytogenes* and *Staphylococcus aureus*. All the samples were stored at +4±1℃. Their physicoche mical and microbiological properties were examined on the 1st, 7th, 15th and 30th days of the storage. It was found that addition of 2% (v/v) EO_B caused an increase in film thickness and consequently a decrease in water vapor permeability, weight loss and inner and outer hardness values. The relation between EO_B addition and the increase in fat levels was significant ($p<0.05$). Microbial counts during storage were significant ($p<0.05$). *Escherichia coli* O157:H7 was the most resistant microorganism to the essential oils while *Staphylococcus aureus* was the most sensitive.

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1. INTRODUCTION

Çökelek cheese is a highly consumed type of cheese in the Turkish cuisine, especially in rural areas,where it serves as a substantial sourceof their animal-derived protein needs. In Turkey, it is produced from the acidification of milk or yogurt derived from cow, sheep or goat milk. The composition varies according to the raw materials used. Çökelek cheese, which has high water activity, contains casein and serum proteins, has high protein and calcium in its composition and is low fat [1,2]. The çökelek cheese that is made at home or in rural settings under primitive conditions and sold in local markets is vulnerableto microbial growth due to unhygienic production conditions, unsuitable storage conditions, absence of proper packaging, unsuitable pH levels and high water activity.

Egg white proteins (EWP) possess many functional properties, such as antimicrobial, anti-viral and anti-inflammatory [3]. Egg white protein powder (EWPP) is produced by drying the EWP with conventional spray methods. The heat applied during the drying process does not affect the solubility of the EWPP [4].

The major components of edible films and coatings are polysaccharides, proteins and lipids [5]. In general, the mechanical properties of protein-based films are better than those of polysaccharide and fat-based films. The protein-based films exhibit excellent gas and lipid barrier [6], characteristics at low and moderate relative humidity (RH) and offer certain mechanical advantages, such as gelatinization, thermal stability and foam formation [7]. However, because of their hydrophilic character, they have low water barrier properties. Moreover, theyare weak, brittle and prone to cracking in the drying stage. These problems can be solved by adding plasticizers [8]. Among the plasticizer substances, sorbitol is particularly prominent on account of itbeing less hygroscopic than the others and 100% soluble. It has been reported that while EWP-based films have similar properties to those of other protein-based films [9], they have higher water vapor permeability compared to whey protein- and soy protein-based films [10]. Edible films also function as carriers for antimicrobial agents [11]. Accordingly, vegetable essential oils (EOs), which have antimicrobial properties to control pathogenic bacteria growth and deterioration in food, are used extensively [12,13].

Basil (*Ocimum basilicum L.*), a member of the *Lamiaceae* family, contains 0.5-2.5% essential oil [14]. There are significant differences in the composition of different types of basil essential oil (EO_B). Major components in the species grown in the Aegean Region (Turkey) are linalool, methyl chavicol, 1.8 cineol and eugenol [15,16]. In general, EO_B contains methyl eugenol, eugenol, methyl chavicol, methyl cinnamate, linalool, α-cubebene, 1.8-cineole, nerol, geranial, estragole and β-elemene [17,18] and has antibacterial [19,20,21,22,23] and antifungal [24] effects. Its antimicrobial activity has been variously shown to be associated with linalool and eugenol [25,26] in some studies, estragole [27] in another study and estragole and linalool [28] in one other.

No reports were found on coating çökelek cheese with essential oil-containing protein-based edible film to prolong its shelf life. Hence, the present study aimed to coat çökelek cheese with different concentrations of essential oil (EO_B) obtained from the fresh leaves of basil (*Ocimum basilicum* L.) with EWPP [2% (v/v)], sorbitol and alginate (algae)-based film in order to prolong its shelf life.

2. MATERIALS AND METHODS

2.1 Egg White Protein Powder (Ewpp), Alginate and D-Sorbitol

For the preparation of coating material, Alfasol® egg white protein powder (EWPP) (pH 7.00; total microorganisms <100 cfu/g; Coliform <10 cfu/g; *S. aureus* and Salmonella content: none and humidity ratio 7.10%) was obtained from Kimbiotek Chemical Agents Inc. (İstanbul-Turkey) and D-sorbitol (S1876) was obtained from Sigma-Aldrich and alginate were obtained from Fluka-Norway.

2.2 Essential Oils

Essential oil from fresh leaves of basil (*Ocimum basilicum*) (EOB) was purchased from flora Sultanhisar (Aydın-Turkey). EOB was obtained by hydrodistillation for 3 h using a Clevenger-type apparatus [29]. The authentic chemical standards for GC-MS analysis were obtained from Sigma-Aldrich (Steinheim, Germany)."

2.3 Analysis of Essential Oils and Volatile Compounds by GC/MS

GC/MS analyses were carried out using Agilent 6890 GC system with Agilent 5973 MS system. The column was used DB-5MS 30 m x 0.25 mm x 0.25 um (5% Diphenyl / 95% Dimethylpolysiloxan) Agilent. The oven temperature was initially held at 50℃ /3 min, programmed to 160℃ at 1.5℃/min, then to 315℃ at 3℃/min and the final temperature was held for 30 min. the carrier gas helium with a flow rate of 1.0 ml/min. The split mode 1:20 was used. The injection volume was 1.0 µl. The mass spectrometer was operated in the electron impact mode (70 eV). The ion source temperature was held at 230℃. The transfer-line was maintained at 280℃. The scanned mass range was from 30 to 500 u.

2.4 Çökelek Cheese

Cheese samples were produced from cow milk. After adding water equal to half the volume of the milk and keeping it at room temperature for 24 to 48 hours (h), the milk was separated from its oil. The remainder of the milk was heated up to 95ºC for curdling and cooling (4 to 6 hours) and then filtered using a press cloth (2 to 3 hours). A weight of 50 kg/10 kg was applied to the filtered curd, and filtering at room temperature was continued overnight. The composition of the çökelek cheese was: pH 4.79; dry matter 33.24%; oil 3.5%, titration acidity (LA%) 0.341% and protein 23.03%. The çökelek cheese was divided into three batches: the first batch of the çökelek cheese samples was coated with film fortified with basil essential oil (EOB) at concentrations [2% (v/v)]. The second batch of the çökelek cheese samples was coated with egg white protein powder and the third batch was left uncoated.

2.5 Preparation of Edible Film Solution

Edible films were prepared according to Pintado et al. [30] and Mchugh and Krochta [31], with some modifications. Accordingly, 5% w/v EWPP was prepared, and after the addition of % 1.5 w/v sorbitol to the solution, a homogenization process was carried out in a homogenizer. The mixture was adjusted to pH 8 and kept in a water bath at 45±2℃ for 30 minutes in order to improve the mechanical properties of the film solution. Then, 0.5% w/v alginate was added in the standby stage. The solution was then cooled to room temperature and alginat-sorbitol-amended EWPP was obtained. The cooled solution was filtered and divided into two equal parts; first part was coated eddible film only EWPP, second part was contain was contain 2% (v/v) $EO_{B(2)}$ ($EWPP_{EOB(2)}$). Following the basil essential oil addition, in order to maintain the homogeneous distribution of oil in the solution, Tween 20 (0.5% (v/v)) was added [13]and the solution was centrifuged again at 20,000 rpm for 1 minute (3-16 K Type-Model, Sigma, Germany [32]. As a result, edible film containing EWPP based 2% (v/v) EO_B [$EWPP_{EOB(2)}$] were obtained. Cheese samples coated with these films were left to dry at room temperature for 24 hours (h) (Fig. 1).

2.6 Preparation and Storage of Samples

E. coli O157:H7 (ATCC 43895), *L. monocytogenes* (ATCC 19118) and *S. aureus* (ATCC 6538) strains used for the artificial contamination of çökelek cheese samples were obtained from Hemakim Corporation (Turkey). Yeast-mold enumeration was carried out immediately after the cheese production. For the artificial contamination, 10^6 cfu^{-g} (6 Log cfu^{-g}) inocula of *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* were used. In order to maintain the artificial contamination, çökelek cheese samples were divided into 50 g portions and immersed in *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* inocula separately. Cheese samples were kept in each inoculum for 15 minutes for contamination and bacterial adhesion. Artificially contaminated cheese samples and the samples prepared for yeast and mold enumerations were coated with films by immersing in film solutions containing basil essential oil which were prepared as explained above. Accordingly, çökelek cheese samples were immersed in film solutions for 90 seconds, removed, hold 3 minutes, immersed again in film solution for 60 seconds and removed. Following the immersion process, cheese samples which were coated with EWPP and $EWPP_{EOB(2)}$ based films were left to dry at 10℃ for 4-5 hours (h) .Control (K) samples which were not coated with films were stored at 4±1℃ following the artificial contamination. The prepared samples were stored at 4±1℃ for 30 days and *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and yeast-mold counts of samples were calculated as Log_{10} cfu^{-g} on the 1st, 7th, 15th and 30th days of the storage (Fig. 1).

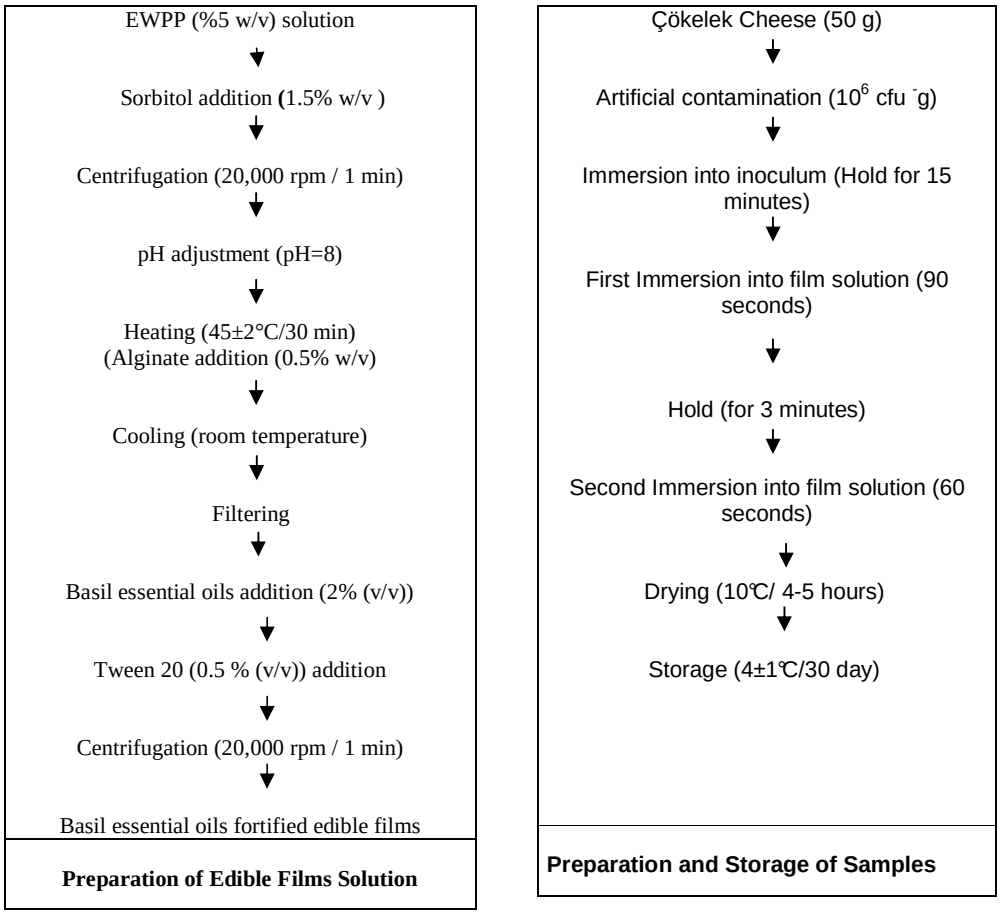

Fig. 1. Preparation of edible films solution and preparation and storage of samples

2.7 Physicochemical Analysis

pH values were examined with a SS-3 Zeromatic pH meter (Beckman Instruments Inc., California, USA). Acidity (°SH) and fat content (%) were analyzed according to AOAC [33].The inner - outer hardness was determined at 3±1ºC with a penetrometer (4500 CT3 texture analyzer Brookfield Made in USA). Film thicknesses were measured with a micrometer at 0.005 precision (Digimatic Micrometer / Japan). Water vapor permeability of films was determined according to a gravimetric method (ASTEM E96-80) at 25℃ [34]. WVP was calculated by finding the slope of weight-time line and substituting it in the following formula.

$$\text{Slope (C)} = \frac{WVP \times A \times \Delta p}{X} \quad WVP = C\ \frac{x}{A \times \Delta p}$$

A: Surface area (m^2)
WVP: Water vapor permeability (g mm m^{-2} h^{-1} kPa^{-1})
Δp: Partial pressure difference of the gases (kPa)
x: Film thickness (mm)

2.8 Microbiological Analysis

E. coli O157:H7 was enriched in selective modified EC Broth at 35-37℃'de for 24-48 hours (h). For enumeration of *E.coli* O157:H7 was used Sorbitol MacConkey Agar containing Cefixime-Tellurite Supplement and incubating at 35-37℃ for 24-48 hours. After incubation, sorbitol negative colonies were counted. *L. monocytogenes* was enriched in Listeria selective Enrichment Broth at 30℃'de for 24 hours(h). For enumeration of *L. monocytogenes*, Palcam Listeria Selective Agar (Base) was inoculeted and incubating at 37℃ for 48 hours. *S.aureus* was enriched in Brain Hearth Infusion Broth at 37℃'de for 48 hours. % 5 Egg Yolk Tellurite emulsion was added to Baird Parker Agar and incubating under aerobic conditions at 35-37℃ for 24-48 hours. Than, colonies were

counted [35]. For yeast and mold enumeration, Yeast-extract-glucose chloramphenicol agar (YGC) (Merck 1.16000) was used with incubation at 25℃ for 3-5 days [36].

2.9 Statistical Evaluation

Four different cheese samples were examined with 3 parallels and 2 repetitions. For this purpose, SPSS version 15 statistical analysis package software was used.

3. RESULTS AND DISCUSSION

The active substances of the EO_B containing; linalool 64.54%, eugenol 16.12%, methyleugenol 5.78%, estragol 3.16%, methylcinnamat 2.11%, α-pinen 1.3% and β-pinen 1.0% was obtained from *Ocimum basilicum* L. species.

The thickness of EWPP based film was 0.166 mm and the thickness of 2% (v/v) EO_B supplemented $EWPP_{EOB(2)}$ respectively 0.172 mm (Table 1). The results obtained in this study regarding film thickness and water vapor permeability are consistent with those reporting that EWPP with the addition of various herbal ingredients can be used in the preservation of foods [10]. However Taqi et al. [5] reported that the film thickness values increased in white albumin based films with the addition of olive oil and oleic acid in a dose-dependent manner. Additionally, it has been reported that film thickness increased and water vapor permeability decreased in edible films containing hydrophobic agents such as wax and vegetable oils [37].

The inner and outer hardness values during storage of the samples coated with $EWPP_{EOB}$ were lower than of those coated with EWPP. The differences observed between coated and uncoated (K) cheese samples during storage were statistically significant in terms of inner and outer hardness values ($p<0.05$). Hardness values determined for the control were higher than those determined for film coated samples. In terms of storage days, the difference between the weight losses of the samples were not significant ($p>0.05$). Also, the weight loss differences between the coated and the non-coated (K) samples were significant ($p<0.05$). A significant relationship was detected between weight loss and inner-outer hardness ($p<0.05$). In this study, it was determined that addition of 2% (v/v) EO_B caused an increase in film thickness and consequently a decrease in water vapor permeability, weight loss and inner and outer hardness values.

Protein based films have been characterized by their low water barrier properties in previous studies. On the contrary, the water barrier property increased in this study. The superior water barrier properties of EWPP were improved (increased) by the addition of 2% (v/v) EO_B to the film. A significant relationship was detected between EO_B addition (2% v/v) and the increase in the water barrier property of the EWPP based film ($p<0.05$). Sarioğlu and Oner [38] reported that film coating caused a decrease in the inner and outer hardness (shell formation) of cheese samples. Additionally, in many studies, it has been reported that film coating prevented water vapor transmission and decreased weight losses [38,39], also using protein based film together with lipids was effective in the prevention of weight losses in the product due to the high water vapor permeability of protein based films [40].

The increase in the acidity of film coated samples prepared by adding essential oil was found to be higher than the increase in the samples coated with EWPP ($p <0.05$), which remained relatively low throughout storage. This result was particularly found in the significant relation determined between the increase in acidity and the EWPP-based film coating applied to the çökelek cheese ($p<0.05$). The highest acidity increase was determined in the control sample and was found to rise in parallel with pathogen microorganism growth. The relationship between the increase in acidity and addition of essential oil was significant ($p<0.05$).

Table 1. Film thicknesses and water vapor permeability of EWPP and EWPPEOB (2), based films

Samples	Thickness /mm ±б	Water vapor permeability (g mm m^{-2} h^{-1} kPa^{-1})
EWPP	0.166 ± 0.001^{A}	5.25 g mm m^{-2} h^{-1} kPa^{-1A}
$EWPP_{EOB(2)}$	0.172 ± 0.009^{B}	5.18 g mm m^{-2} h^{-1} kPa^{-1C}

б :Standard deviation (n=3) ^{A,B}

With the addition of essential oil to EWPP-based film, an increase in fat values was determined during the storage of the cheese samples. The relation between EO_B addition and the increase in fat levels was significant ($p<0.05$). This was associated with an increase in the hydrophobicity of the essential oil due to the acidity increase in the product, and thus the tendency of the cheese to dissolve in the lipid phase [41]. Additionally, it was found that the fat barrier property of film produced with fortification of EWPP based film with 2% (v/v) EO_B was increased. During storage, the relationship between the average fat values of the control group and EWPP based film coated samples was not significant ($p>0.05$). Our study results are compatible with previous studies which have reported that composite films with good mechanical, fat, oxygen and water vapor barrier properties can be produced with addition of different essential oils to protein based films [42,43,44,45] and also with the studies reporting that EWPP based films show similar properties to other protein based films [9].

In our study, cheese samples were artificially contaminated with pathogenic microorganisms at 10^6 Log_{10} cfu^{-g}(6 Log_{10} cfu^{-g}). Yeast-mold enumeration was carried out immediately after the cheese production [10^7 Log_{10} cfu^{-g} (7 Log_{10} cfu^{-g})]. It was determined that coating the cheese samples with film obtained by adding 2% (v/v) $EO_B$to EWPP based film had a bacteriostatic effect at the beginning of the storage and a bactericidal effect as the storage period progressed . In this study, antimicrobial activity observed in EWPP coated samples increased significantly with the addition of 2% (v/v) EO_B($p<0.05$). Therefore, significant relationships were determined between coating the cheeses with EWPP based film and the antimicrobial activity and also between the increase in the antimicrobial activity and the addition of EO_B to the film ($p<0.05$). The relationship between the bactericidal effect and the progression of the storage period was also significant ($p<0.05$).

This result was associated with slower transmission of antimicrobial agent from the film layer to food in the edible film systems, with a high concentration of antimicrobial agent remaining in the film and the surface of the food, thus providing a longer effect against microorganisms [46]. Also, in relation with the pH decrease in cheese samples, the increase in hydrophobicity of essential oil and the easier dissolution of cell membrane in the lipid phase have an effect on the increase in antimicrobial activity [41]. Additionally, it was concluded that linalool (64.54%) and eugenol (16.12%) which was found in high concentrations in the composition of EO_B along with low concentrations of estragol (3.16%) and α-pinen (1.3%) may have contributed to the increased antimicrobial activity. In previous studies, it has been found that that estragole and linalool [28] in the composition of EO_B has antimicrobial properties, linalooland eugenol [25,26] have strong antimicrobial effects. The results regarding the antimicrobial activity of EO_B in our study are compatible with other studies. Also, it was also determined that the antimicrobial effects of EWPP in foods reported in previous studies also apply to EWPP based edible films; using EWPP+ EO_B together increased this effect.

In this study, a bacteriostatic effect of EWPP and $EWPP_{EOB(2)}$ based films against *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* was determined on the 1st day of the storage. A bactericidal effect was determined in EWPP coated samples against *L. monocytogenes* and *S. aureus* on the 30th day. A bactericidal effect was not determined in EWPP and $EWPP_{EOB(2)}$ coated samples against *E. coli* O157:H7. A bactericidal effect was determined in $EWPP_{EOB(2)}$ coated samples against *S. aureus* and *L. monocytogenes* on the 15th day. Microbial growth was highest in control samples throughout the storage. The antimicrobial effect of $EWPP_{EOB(2)}$ coating was higher compared to EWPP coating. This effect increased in the later days of the storage. In fact, the decrease in *E. coli* O157:H7 levels in $EWPP_{EOB(2)}$ samples on the 1st day of the storage was higher than was observed in EWPP. With the increase in antimicrobial effect in $EWPP_{EOB(2)}$ between the 1st and the 30th days, the decrease in *E. coli* O157:H7 advanced (Fig. 2A). In the EWPP-coated samples, the number of *E. coli* O157:H7 was decreased by 6 $\log_{10}$ cfu^{-g} during storage (only on the 30th day 4.45$\log_{10}$ cfu^{-g}). In the $EWPP_{EOB(2)}$ coated samples, the number of *E. coli* O157:H7 was decreased by5 $\log_{10}$ cfu^{-g} during storage (only on the 30th day 4.45$\log_{10}$ cfu^{-g}). The low antimicrobial effect of coating with EWPP, and EWPP containing EO_B, on *E. coli* O157:H7 was associated with the ability of *E. coli* O157:H7 to grow in mediums with a low pH (pH<3.6) and its resistance to acidity [47]. Furthermore, *E. coli* O157:H7 contains an outer phospholipid membrane that acts as a barrier and renders the membrane impermeable to lipophilic compounds [48], and studies have

shown that this membrane protected the bacteria against the essential oils [49]. In the $EWPP_{EOB(2)}$ coated samples similar results (between the 1st and the 7th days)were determined for *S. aureus* (Fig. 2B) and *L. monocytogenes* (Fig. 2C). On the 1st day of the storage, the antimicrobial effect of $EWPP_{EOB(2)}$ for *S. aureus* (5.21 Log_{10} cfu^{-g}) and *L. monocytogenes* (5.96 Log_{10} cfu^{-g}) was higher than that of EWPP. The most sensitive microorganism to EWPP and $EWPP_{EOB(2)}$ coating throughout the storage was *S. aureus*. Actually, *S. aureus* levels were lower than those of *E. coli* O157:H7 and *L. monocytogenes* on all days of the storage in both of the coatings. It was concluded that with the addition of 2% (v/v) EO_B to EWPP based film, a strong bacteriostatic effect was observed from the first days of the storage.

The fungucides effect of 2% (v/v) EO_B addition on yeast-mold was observed on day 7.The results of this study on yeast-mold were in agreement with the results obtained by studies reporting the strong antifungal activity of EO_B[24].

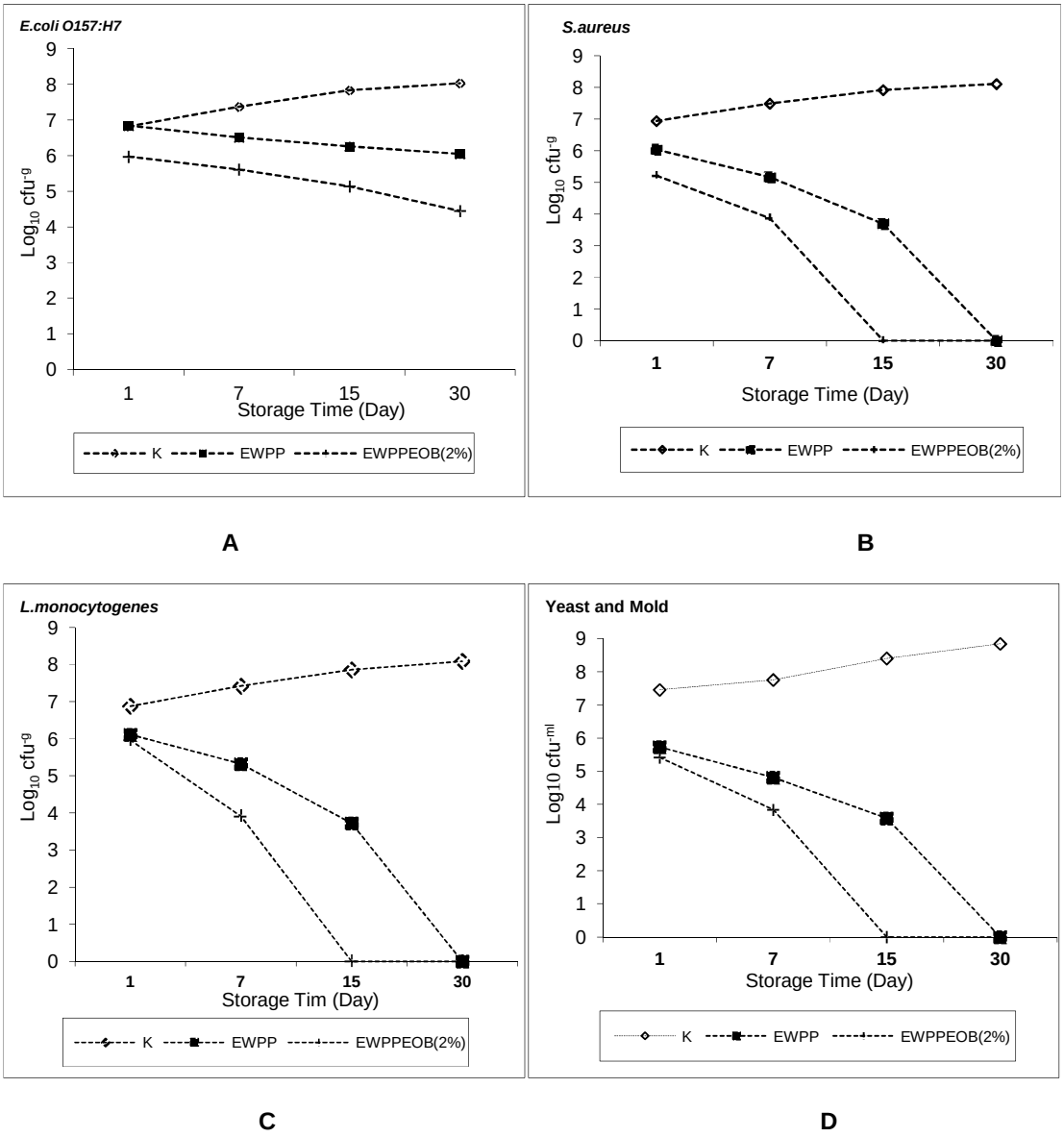

Fig. 2. *E. coli* O157:H7 (A), *S. aureus* (B), *L. monocytogenes* (C) and Yeast and Mold (D) Growth in Samples Coated With Film and K Sample.

The EWPP-coating showed a fungistatic effect on yeast-mold until the 15th of the storage period. In the samples coated with EWPP, yeast-mold was decreased by 4.21 $\log_{10}$ cfu^{-g} at day 7th and 3.80 $\log_{10}$ cfu^{-g} at day 15th. A fungicides effect was determined in EWPP coated samples against yeast-mold on the 30th day. A fungucides effect was determined in $EWPP_{EOB(2)}$ coated samples on the 15th day against yeast-mold. In the samples coated with $EWPP_{EOB(2)}$ yeast-mold was decreased by 5.42 $\log_{10}$ cfu^{-g} at day 1th and 3.84 $\log_{10}$ cfu^{-g} at day 7th. Yeast-mold growth was highest in control samples throughout the storage. Also, the antifungal effect of $EWPP_{EOB(2)}$ coating was higher compared to EWPP coating (Fig. 2D). Microbial growth was found to be highest in the C sample throughout the period of storage.

4. CONCLUSION

It was demonstrated that EWPP based films can be exclusively used in edible film production and the appearance properties are similar to other protein based films. Addition of essential oils to EWPP based film improved both physico-chemical and antimicrobial properties in a manner. In our study, it was determined that S+Alge+EWPP based film was a good water and fat barrier. With the addition of 2% (v/v) EO_B to EWPP based film, the mentioned properties improved along with an increase in antimicrobial activity. In our study, it was found that the antimicrobial effect provided by basil essential oil during storage was in the form of a bacteriostatic effect on *E. coli* O157:H7 and a bactericidal effect on *S. aureus, L. monocytogenes* and yeast-mold. Consequently, $EWPP_{EOB}$ was more effective in the extension of the storage period compared to EWPP.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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