Carboxymethyl Cellulose Film Optimized with Persian Gum, Titanium Dioxide Nanoparticles, and Fennel Essential Oil: Investigation of Chemical, Antimicrobial, and Sensory Properties on Rainbow Trout Fillet

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Abstract

Today, the use of biodegradable packaging containing antimicrobial agents has been approved by the people and industry. In this study, the effect of carboxymethyl cellulose (CMC) film optimized with Persian gum (PG) containing titanium dioxide nanoparticles (TiO_2) and fennel essential oil (FEO) was inspected and the sensory, chemical, and microbial properties were evaluated on refrigerated rainbow trout fillets during the storage period (0, 3, 6, 9, and 12 days). The lowest values for the minimum inhibitory concentration (MIC) (8 mg/mL) and the minimum bactericidal concentration (MBC) (10 mg/mL), and the highest diameter of the inhibitory zone in the disk diffusion (19 mm) for FEO were observed against *Listeria monocytogenes*, *Escherichia coli*.O157:H7, *Pseudomonas fluorescens*, and *Shewanella putrefaciens*, respectively. Moreover, as a microbiological analysis, total viable count (TVC), *Pseudomonas spp*, lactic acid bacteria (LAB), *Enterobacteriaceae*, and psychrotrophic bacteria were determined on rainbow trout fillets covered with different treatments (CMC/PG, CMC/PG/FEO, CMC/PG/TiO_2, and CMC/PG/FEO/TiO_2) during the storage at 4°C. The results showed that three treatments of CMC/PG/FEO, CMC/PG/TiO_2, and CMC/PG/FEO/TiO_2 had a significant effect on growth control in the studied bacteria (P <0.05). In all treatments, the amount of thiobarbituric acid increased over time, but in the treatment containing FEO, this amount was lower. Sensory scores of the samples were very high until day 6. In general, the results of this study showed that the use of FEO and TiO_2 in biodegradable films improves the antimicrobial, chemical, and sensory properties of rainbow trout fillets.

1. Introduction

Foods are always exposed to chemical, microbial, and physical spoilage and food preservation plays an essential role in controlling changes during the storage of different products such as fresh vegetables, fruits, and meats [1]. This would lead to the increased shelf life through inhibiting the oxidative changes and growth of microorganisms [2][3].

Films are thin layers around food that prevent the transfer of moisture and oxygen, as well as solutes to the food. In this regard, active packaging is designed to preserve the packaged material in the best possible manner according to the storage conditions of the package so that the food reaches the consumer in appropriate conditions.

In general, biodegradable films are made from three main components: protein, lipid, or polysaccharide; these materials can be used alone or in combination with each other. The physical and chemical properties of these materials remarkably influence the properties of the films [4].

Carboxymethyl cellulose (CMC) is known as a water-soluble cellulose derivative owing good film-forming properties [5]. It is capable of forming colorless, water soluble, non-toxic, stable, and uniform film-forming solution albeit with poor mechanical properties [6]. Persian gum (PG) is obtained from the wild almond tree (*Amygdalu scoparia* Spach) and is available in white to dark colors, in different shapes and sizes. This gum dissolves easily in hot water [7][8].

Films used in the food industry may contain a variety of substances, including antioxidants, antimicrobials, and flavorings. This type of packaging prevents the activity of pathogenic and spoilage microorganisms during the storage and increases the shelf life [9].

Biodegradable films are supplemented with essential oils to improve the antimicrobial-antioxidant effects, the aroma of the film, and the permeability properties of hydrophilic films [10]. Fennel (*Foeniculum vulgare* Mill) is a biennial or perennial herbaceous plant of the *Umbelliferae* family, and fennel essential oil (FEO) has antiseptic, antifungal, anti-flatulence, and appetizing properties [11].

Silver, zinc oxide, copper, and titanium dioxide nanoparticles (TiO_2) are the most important nanoparticles used in the production of nanocomposites, with many applications, and antimicrobial and optical properties [12]. TiO_2, used in the structure of nanocomposites, prevents the growth of pathogenic microorganisms in addition to improving their properties [13]. It is widely used in the film in the range of 0.5 to 1 w/w, based on dry matter weight [14].

CMC film with PG, TiO_2, and FEO has desirable physical and mechanical properties [15]. Packaging the refrigerated rainbow trout fillet with the biodegradable film of CMC/PG, containing TiO_2 and FEO, and subsequently, the study of microbial, chemical, and sensory properties of the film on rainbow trout fillet at refrigeration temperatures were the objectives of this study.

2. Materials And Methods

CMC (viscosity of 1% solution in water at 20°C is 1500–3500 centipoise, with a pKa of 3.5), PG (Parsian Gam Reyhan, Isfahan, Iran), FEO (Johare Taem Shargh, Mashhad, Iran), glycerol, TiO_2 (anatase, purity>99%), and other consumables were purchased from Merck, Germany.

2.1. Preparation of biodegradable film

To prepare the treatments (CMC/PG, CMC/PG/FEO, CMC/PG/TiO_2, and CMC/PG/FEO/TiO_2), 1.25 g of CMC was added to 100 mL of water at 60°C and mixed with a mixer for 15 min. Then PG solution (0.65% w/w), glycerol (40% w/w), FEO (2% w/v), and TiO_2 (0.5% w/w) were added to the film solution and mixed with a stirrer for 15 min. To remove the air bubbles, an ultrasound bath (Schaper Unique USC 25 KHz) with a frequency of 25 KHz and a field strength of 100 W was used for 15 min. Then it was dried at 37°C for 24 h in a hot air dryer [15].

2.2. Determination of MIC and MBC of FEO

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured in vitro on pathogenic bacteria including *Listeria monocytogenes* ATCC 7644 and *Escherichia coli* O157:H7 NCTC 12900, and the causative agents including *Shewanella putrefaciens* NCTC 10762 and *Pseudomonas fluorescens* NCTC 10038. Bacterial strains were cultured for 24 h at 37°C and 22°C in Müller-Hinton broth, respectively. Specific concentrations of FEO (in DMSO) were prepared and 20 µL of it

was added to 96-well microplates containing 20 µL bacterial suspension (0.5 McFarland) and 160 µL sterile broth. Similar tests were performed for both positive control (culture medium containing bacteria without antimicrobial agent) and negative control (culture medium without bacteria). The microplates were kept in an incubator at 37°C and 22°C (*Pseudomonas* spp.) for 24 h. The lowest concentration of FEO in which no turbidity was observed was reported as the MIC. Ten microliters were taken from all wells and cultured on a plate containing brain heart infusion (BHI) agar. The plates were stored in the incubator at 37°C for 24 h. The lowest concentration of FEO, which was associated with the absence of bacterial colony, was considered as MBC. All these tests were performed in triplicate [16].

2.3. Antimicrobial activity of films by disc diffusion method

Antimicrobial activity of the film was analyzed on *S. putrefaciens*, *P. fluorescens*, *E. coli* O157: H7, and *L. monocytogenes*. To this end, the films were cut into discs (1 cm in diameter) and placed on Müller Hinton agar plates. First, 0.1 mL of the 24-hour bacterial suspension containing the desired bacteria (10^6–10^5 CFU/mL) was cultured freshly. The plates were inoculated at 37°C for 24 h. The diameter of the growth inhibition halo was measured by an accurate caliper and its area was reported in terms of square millimeters [17].

2.4. Preparation of rainbow trout samples and treatments

Rainbow trout were obtained from reputable shopping centers in Mashhad and transferred to the laboratory in cold conditions. Cutting, abdominal emptying, and washing operations were performed. The fish meat was divided into the pieces of about 50 g with an average thickness of 2 cm. The fish pieces were covered with films. The coated samples and controls were placed in sterile plastics and stored at refrigeration temperature (4 to 5°C). All samples were evaluated at intervals of 0, 3, 6, 9, 12, and 15 days.

2.5. Microbial analysis

Ten grams of fish samples were transferred to 90 mL of peptone water 0.1% and mixed with a mixer (Lab Blender 400, Stomacher, USA) for 1 min at room temperature. Peptone water 0.1% was used to prepare serial dilutions and count the desired bacteria by placing them in a specific culture medium and the specific temperature of each bacterium [16].

For total viable count (TVC), nutrient agar medium was used by surface culture at 37°C for 48 h. *Pseudomonas* spp. was determined on cetrimide fusidin cephaloridine agar (supplemented with selective supplements) at 22°C for 48 h. Lactic acid bacteria (LAB) were cultured in deMan Rogosa Sharpe (MRS) anaerobic medium at 25°C for 5 days under anaerobic conditions. For *Enterobacteriaceae* count, 0.1 mg of sample was inoculated in 10 mL of Violet Red Bile Glucose agar (VRBGA) culture medium. Then 10 mL of molten culture medium was poured on them and VRBGA medium was kept in a 30°C oven for 24 h. To determine and count the psychrotrophic bacteria, 0.1 mL of the desired dilution was cultured completely and superficially on Tryptic Soy Agar (TSA). Samples were kept at 7°C for 10 days and then the colonies were counted [14][18]

2.6. pH

Ten grams of each sample were homogenized in 100 mL of distilled water and then filtered. A digital pH meter was employed to measure the pH of the solution (Hanna pH meter, HI 221, Romania).

2.7. Determination of TBA

Thiobarbituric acid (TBA) was calculated according to Kirk and Sawyer (1991) using a spectrophotometer and expressed as mgMDA/kg tissue. The minced fish sample (200 mg) was dissolved in 1-butanol to reach a volume of 25 mL. Then, 5 mL of the solution was mixed with 5 mL of TBA reagent and kept at 95°C for 2 h and then the adsorption value was read at 532 nm [19].

2.8. Sensory evaluation

Sensory evaluation of fish fillet samples with different treatments was done by 5 semi-skilled panelists. Each sample with 3 replicates were placed in separate plates without specifying the type of treatment. Panelists assessed color, odor, texture, and overall characteristics of acceptability using a 9-point rating scale. The scoring values were as follows: bad or unacceptable: 1-3; good: 4-6; very good: 7-8; and excellent: 9 [18].

2.9. Statistical analysis

In this study, SPSS software version 16.0 (IBM; Armonk, N. Y, USA) was applied and a thoroughly randomized factorial design was used to compare the results. All experiments were performed in three replicates and the means were used for statistical analysis. The significance level of 5% was compared with Duncan's test.

3. Results And Discussion

3.1. MIC and MBC values of FEO

The MIC and MBC values of FEO are provided in Table 1. The results depicted that in concentrations between 8 and 20 mg/mL, FEO was active against *L. monocytogenes, Escherichia coli* O157:H7, *P. fluorescens,* and *S. putrefaciens*, respectively. Our results lent support to those of Roby et al. (2013) in evaluating the antimicrobial properties of FEO[20]. Our study also corroborated the study of Ojeda-Sana AM, et al (2013) on rosemary essential oil in that *L. monocytogenes* was the most sensitive bacterium to essential oils [21].

Table 1

The MIC and MBC values of FEO

Bacteria	MIC (mg/ml)	MBC (mg/ml)
Listeria monocytogenes	8.00	10.00
O157:H7*Escherichia coli*	10.00	14.00
Shewanella putrefaciens	16.00	20.00
Pseudomonas fluorescens	14.00	16.00

3.2. Antimicrobial activity of films determined by disc diffusion method

The antimicrobial activities of films were determined by disk diffusion method. Growth inhibition as the zone of inhibition diameter is given in Table 2. The CMC/PG film showed no antimicrobial activity against the tested bacteria. Moreover, based on the results, CMC/PG/FEO/TiO_2 films had the greatest effect on the studied bacteria. As Table 2 shows, CMC/PG/FEO/TiO_2 and CMC/PG/FEO treatments had the highest and the lowest antimicrobial effects, especially on *L. monocytogenes* and *S. putrefaciens*, respectively. Sani et al. (2017) obtained similar results for TiO_2 and rosemary essential oils[18]. Our results also showed that the effect of FEO on Gram positive bacteria was greater than that on Gram negative bacteria; this greater effect is ascribed to the structure of the outer membrane of Gram (-) bacteria, which limits the diffusion of hydrophobic components of FEO to the lipopolysaccharide layer [22]. In general, the antimicrobial effects of TiO_2 and FEO are associated with microbial cell components, destroying the cytoplasmic membrane, affecting cell wall permeability, and affecting biological molecules such as DNA and protein [18]. The antimicrobial effect of FEO is also related to the phenolic compounds called terpenoids [23].

Table 2

Growth inhibition zone of film disks (mm)

Bacteria	CMC/PG	CMC/PG/F	CMC/PG/TiO_2	CMC/PG/F/TiO_2
Listeria monocytogenes	-	15.00±0.34	±0.2316.00	±0.6719.00
O157:H7*Escherichia coli*	-	14.00±0.12	±0.1615.00	±0.3416.00
Shewanella putrefaciens	-	9.00±0.09	±0.079.50	±0.1710.40
Pseudomonas fluorescens	-	12.50±0.13	±0.1113.00	±0.2514.00

3.3. Microbial tests

3.3.1. Total viable count

Total viable count (TVC) indicator for rainbow trout fillets kept in refrigerator during storage is shown in Fig. 1. The initial TVC was 3.01 log CFU/g, which according to the standards, this level of microbial load in fish fillets indicates the good quality of fish fillets; this result substantiated those of Ojagh SM, et al (2010) [24] and Arashisar Ş, et al (2004) [25]. Control and CMC/PG treatments had very high microbial loads due to the lack of antimicrobial agents during refrigeration, and the lower microbial load of CMC/PG films compared to the control samples was probably due to the mere film, preventing secondary contamination, reducing available oxygen, and reducing the water available to microorganisms. Compared to the control samples, CMC/PG/FEO, CMC/PG/TiO_2, and CMC/PG/FEO/TiO_2 treatments exerted a significant effect ($P < 0.05$) in reducing the growth rate of bacteria. According to the International Commission on Microbiological Specifications for Foods (ICMSF), the standard log level of microbial load in fish fillets is 7 log CFU/g. Accordingly, CMC/PG/FEO treatment can be stored for 6 days, CMC/PG/TiO_2 treatment for 9 days, and CMC/PG/FEO/TiO_2 treatment for 12 days in the refrigerator. The lowest bacterial growth was observed in CMC/PG/FEO/TiO_2 treatment, which is due to the synergistic effect of these two compounds (FEO and TiO_2). This result lends support to that of Farshchi E, et al (2019) for the antimicrobial effect of TiO_2-Ag [14], and Maghami M, et al (2019) for FEO [26].

3.3.2. *Pseudomonas* spp. count

Pseudomonas spp. are Gram (-), highly aerobic, and CO_2-sensitive bacteria [27]. As shown in Fig. 2, compared to the control group, the significant effect of treatments with antimicrobial compounds was observed on the growth rate of *Pseudomonas* spp. ($P < 0.05$). The initial *Pseudomonas* spp. value for all treatments was 3.02 log CFU/g on the first day and 7.87 log CFU/g on the 12th day for the control samples. CMC/PG treatment was not a good barrier to the growth of these microorganisms due to the lack of antimicrobial compounds, but treatments containing FEO and TiO_2 significantly reduced the growth rate of these microorganisms ($P < 0.05$); this result substantiates the results of Sani MA, et al (2017) [18] For TiO_2, Maghami M, et al (2019) [26] and Kivanç M, Akgül A (1986) [28] for FEO. A decrease in the antimicrobial effects of CMC/PG/FEO, CMC/PG/TiO_2, and CMC/PG/FEO/TiO_2 treatments in the last days was attributed to the decrease in the release of these substances.

3.3.3. Lactic Acid Bacteria

LAB are optional anaerobic bacteria that grow in low concentrations of O_2. They make up a significant portion of the meat's natural microflora. As Fig. 3 displays, the LAB content for the treatments on the first day was 2.0 log CFU/g, which increased during the maintenance period and for the control sample reached 5.67 log CFU/g. CMC/PG/FEO, CMC/PG/TiO_2, and CMC/PG/FEO/TiO_2 treatments significantly

reduced the growth rate of LAB compared to the control samples (*P<0.05*), Maghami M, et al (2019) for FEO [26] and Sani MA, et al (2017) for TiO_2 [18] achieved the same results on LAB. On the last day, the lowest level of LAB was related to CMC/PG/FEO/TiO_2 treatment, which was due to the synergistic effect of two antimicrobial agents.

3.3.4. *Enterobacteriaceae* count

Enterobacteriaceae microorganisms are health indicators and part of the natural microbial flora of rainbow trout [29]. Based on the results, the initial level of *Enterobacteriaceae* was 2.12 log CFU/g, which indicates the good quality of the meat (Fig. 4) [18]. During the storage period, the number of *Enterobacteriaceae* increased and reached 6.21 log CFU/g on day 15 for the control sample. On the 15th day, the number of *Enterobacteriaceae* for CMC/PG/FEO/TiO_2 treatment was 3.99 log CFU/g which showed the favorable effect of antimicrobial agents. FEO and TiO_2 reduced the growth of *Enterobacteriaceae* compared to the control sample (*P <0.05*); this shares a number of similarities with the results of Österblad M, et al (1999) [30], Kačániová M, et al (2019) [31] and Sani MA, et al (2017) [18]. Furthermore, the synergistic effect of FEO and TiO_2 was quite evident in reducing the growth of *Enterobacteriaceae*.

3.3.5. Psychrotrophic bacteria

Changes in psychrotrophic bacteria for treatments during refrigeration are shown in Fig. 5. The initial number of psychrotrophic bacteria at the beginning of the period was 3.42 log CFU/g. Over time, the number of these bacteria increased in all treatments, and in the control samples reached 8.77 log CFU/g in the end of the period, which was very high. The difference between the control samples and CMC/PG/FEO/TiO_2 treatment in the end of the period was about 2 log CFU/g, which indicates the favorable antimicrobial effect of TiO_2 and FEO (*P<0.05*). These results concurred well with the results of Mazandrani HA, Javadian S, Bahram S (2016) [32] and Bagheri R, et al (2016) [33] for the antimicrobial effect of FEO on silver carp and common kilka, respectively, and the results of Alizadeh-Sani M, et al (2020) [34] for TiO_2.

3.4. pH

The pH changes of fish fillets during the storage period are shown in Fig. 6. The initial pH of the fish fillet was 6.33, which was consistent with the pH levels in the studies of Arashisar Ş, et al (2004) [25] and Gimenez B, et al (2002) [35] for rainbow trout. The pH level decreased until the third day due to the glycolysis process of fish [36]. During this period, the pH increased and bacterial metabolites, and microbial enzymes produced in the fillet tissue [37]. By increasing the pH, the quality characteristics of the

fish fillets decreased. Treatments with antimicrobial agents significantly kept the pH at lower levels than that in the control samples; this was consistent with the results of the microbial part.

3.5. Thiobarbituric acid (TBA)

TBA changes of different treatments of fish fillets during refrigeration are shown in Fig. 7. The initial TBA level of fish fillets was determined to be 0.13 mgMDA/Kg, which was in the same level of that in the study of Ojaq et al. (2010) for rainbow trout. In all treatments, the amount of TBA increased over time (*P<0.05*). Perumalla AVS, Hettiarachchy NS (2011) [38] showed that essential oil reduces the amount of TBA in samples by various mechanisms such as preventing the onset of radical formation, and reducing the transfer of metal ion catalysts, as well as peroxide decomposition and reaction with free radicals [39]. TBA level is about 1-2 mgMDA/Kg in fish fillets, which is the starting point for an unpleasant odor. However, in general, less than 5 mgMDA/Kg is desirable for consumption [40]. The effect of TiO_2 on the reduction of TBA was much less than that of FEO, because TiO_2 has antimicrobial properties and FEO has high antioxidant properties in general, and as a result, these further reduce the TBA level. The film around the fish fillets also reduced the TBA level by reducing the amount of oxygen available. The trend of change is consistent with the results of Ojagh SM, et al (2010) [24] obtained for rainbow trout.

3.6. Sensory evaluation

As depicted in Fig. 8, the quality of sensory indicators decreased over time (*P<0.05*). Odor index under the influence of TiO_2 and FEO treatments scored higher than the control sample because odor is one of the most sensitive indicators in fish fillets that is strongly influenced by storage conditions, microbial load, and chemical reactions. The color index was not significantly affected during the storage and all samples had a relatively high score. Microbial activity and chemical reactions that occur during fillet storage have less effect on the color index. The tissue of fish fillets was severely affected by the storage time and treatments, and in the control samples, due to the bacterial growth and chemical reactions, the surface of the fish fillets became viscous and soft, and had an undesirable texture. TiO_2 and FEO treatments were significantly lower (P <0.05) due to the reduction of microbial growth and chemical reactions. The overall acceptance index decreased during the storage; and considering the acceptance limit of 5, the samples treated with TiO_2 and FEO could be easily used up to day 6. This result is completely consistent with the results obtained in the microbial stage.

4. Conclusion

To determine the microbial properties of the films, disk diffusion test was performed, which showed that the films with TiO_2 and FEO had a significant antimicrobial effect. To determine the antimicrobial effect of the films on food models, 4 film treatments were prepared and rainbow trout were covered with the produced films. During the refrigeration, the TVC, LAB, *Enterobacteriaceae*, *Pseudomonas* spp., and

psychrotrophic bacteria were quantified. The results showed that films containing antimicrobial compounds of FEO and TiO_2 significantly reduced the bacterial growth during the refrigeration. When FEO and TiO_2 were used simultaneously in the film, the growth of microorganisms was severely reduced due to their synergistic properties. Samples treated up to day 6 received acceptable scores in terms of sensory evaluation. Based on the results, in general, the use of FEO and TiO_2 in biodegradable films improves the antimicrobial, chemical, and sensory properties of rainbow trout fillets.

Declarations

Conflict of interest:

The authors declare they have no financial interests.

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Figures

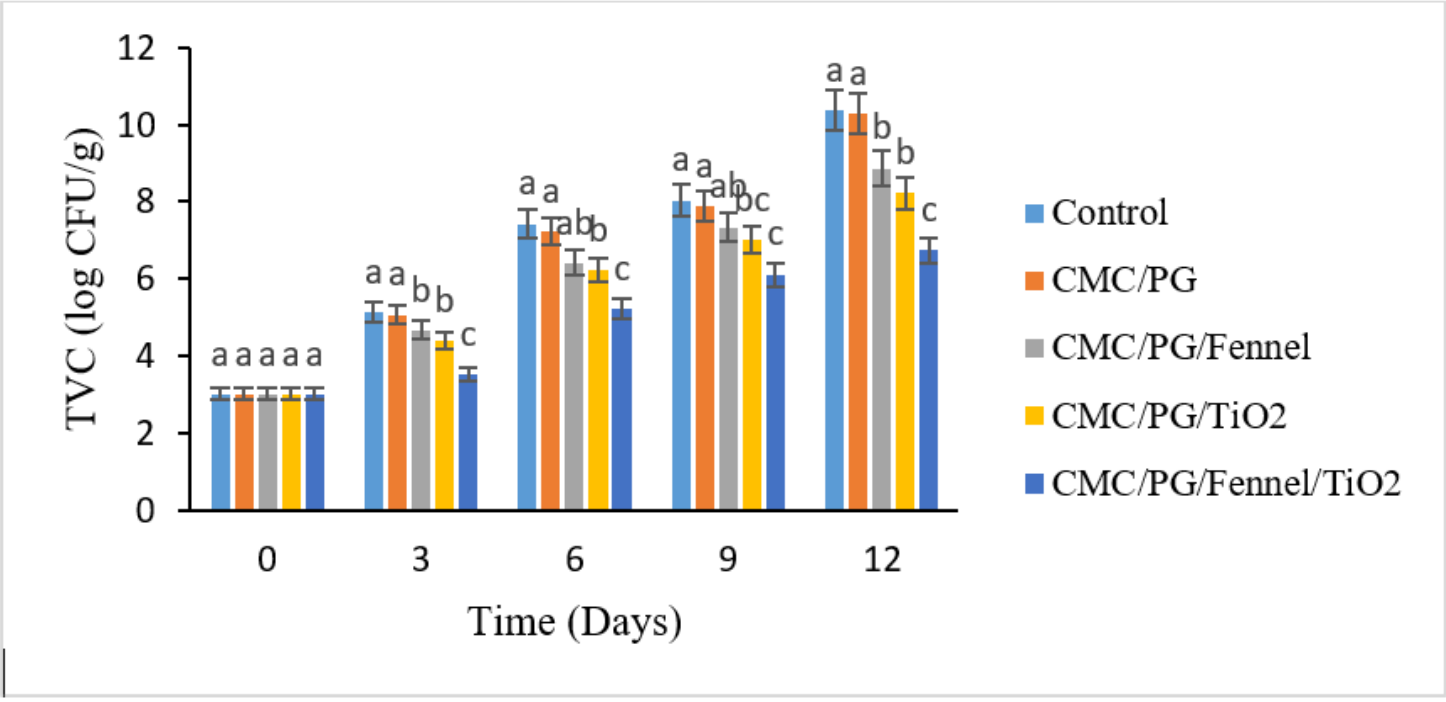

Figure 1

Changes in TVC of different treatments during storage at 4°C for 12 days

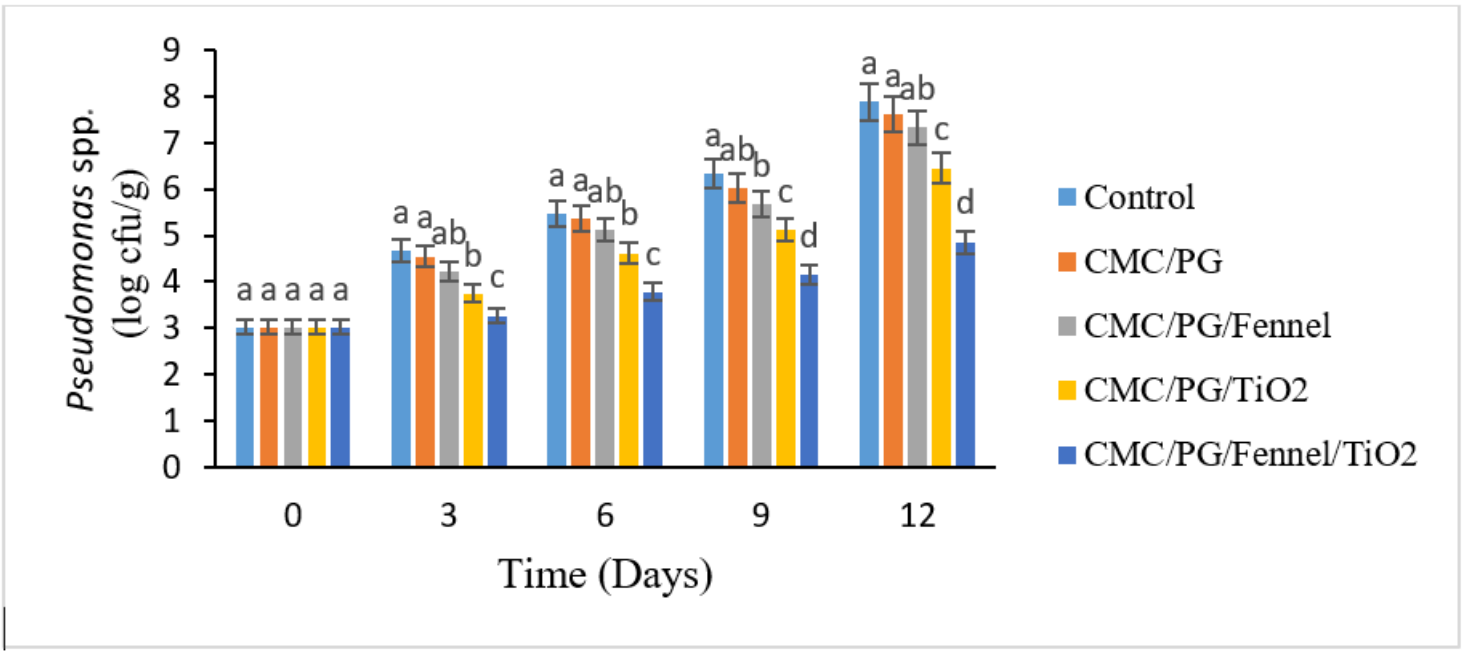

Figure 2

Changes in Pseudomonas spp. Count of different treatments during storage at 4°C for 12 days

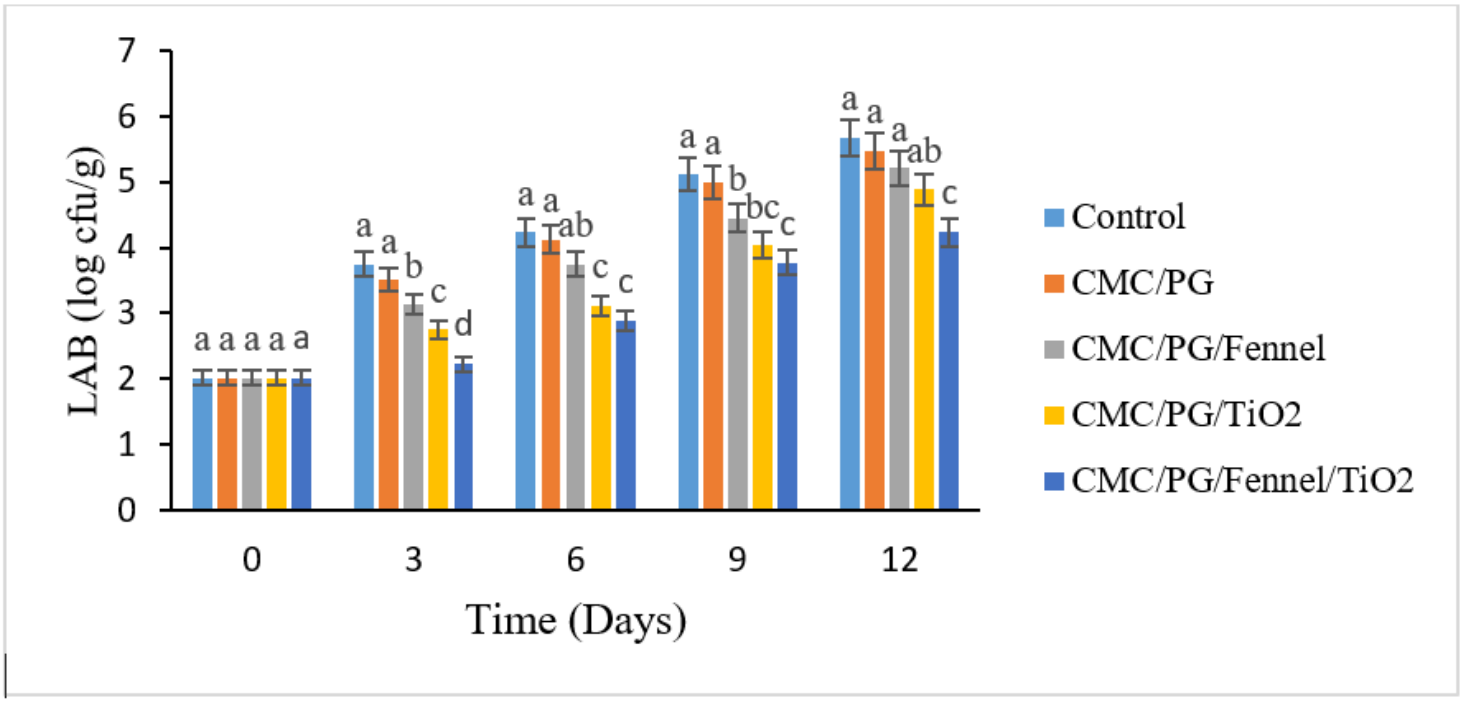

Figure 3

Changes in LAB of different treatments during storage at 4°C for 12 days

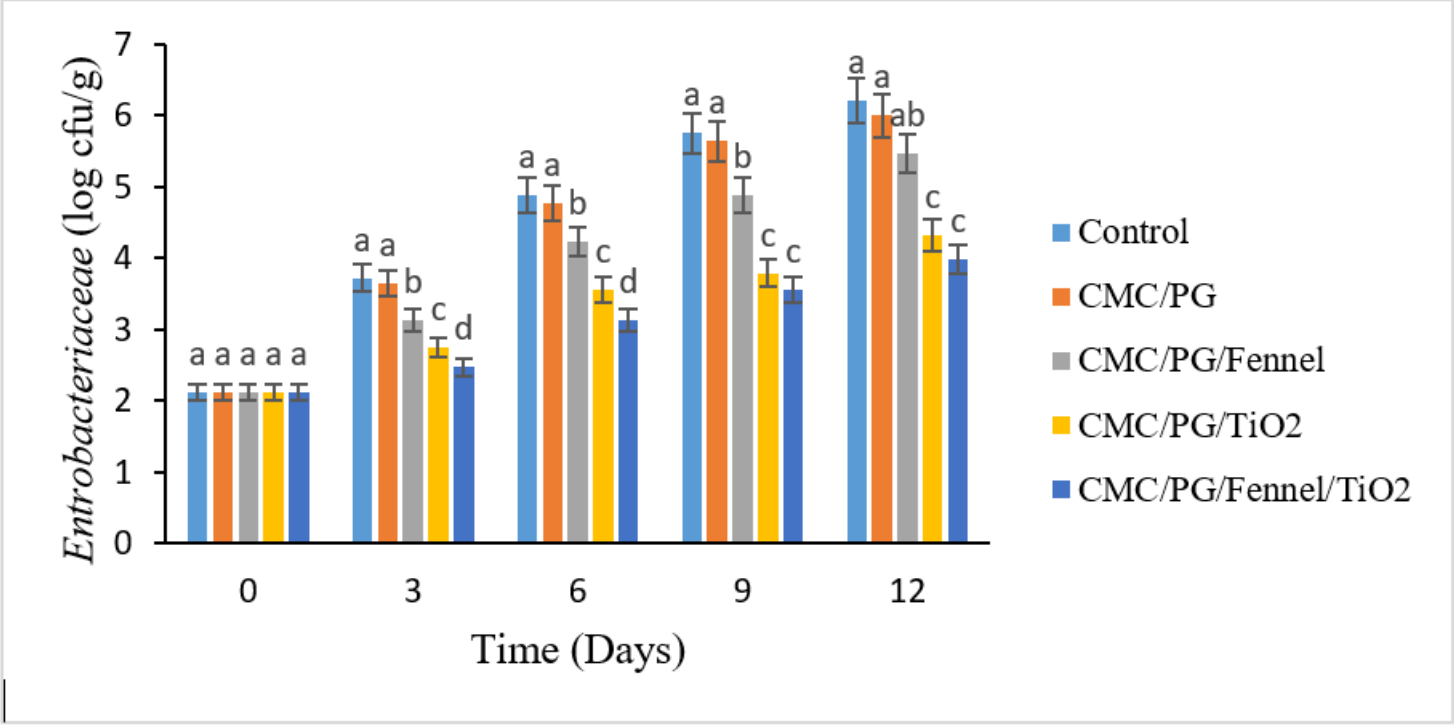

Figure 4

Changes in Enterobacteriaceae count of different treatments during storage at 4°C for 12 days

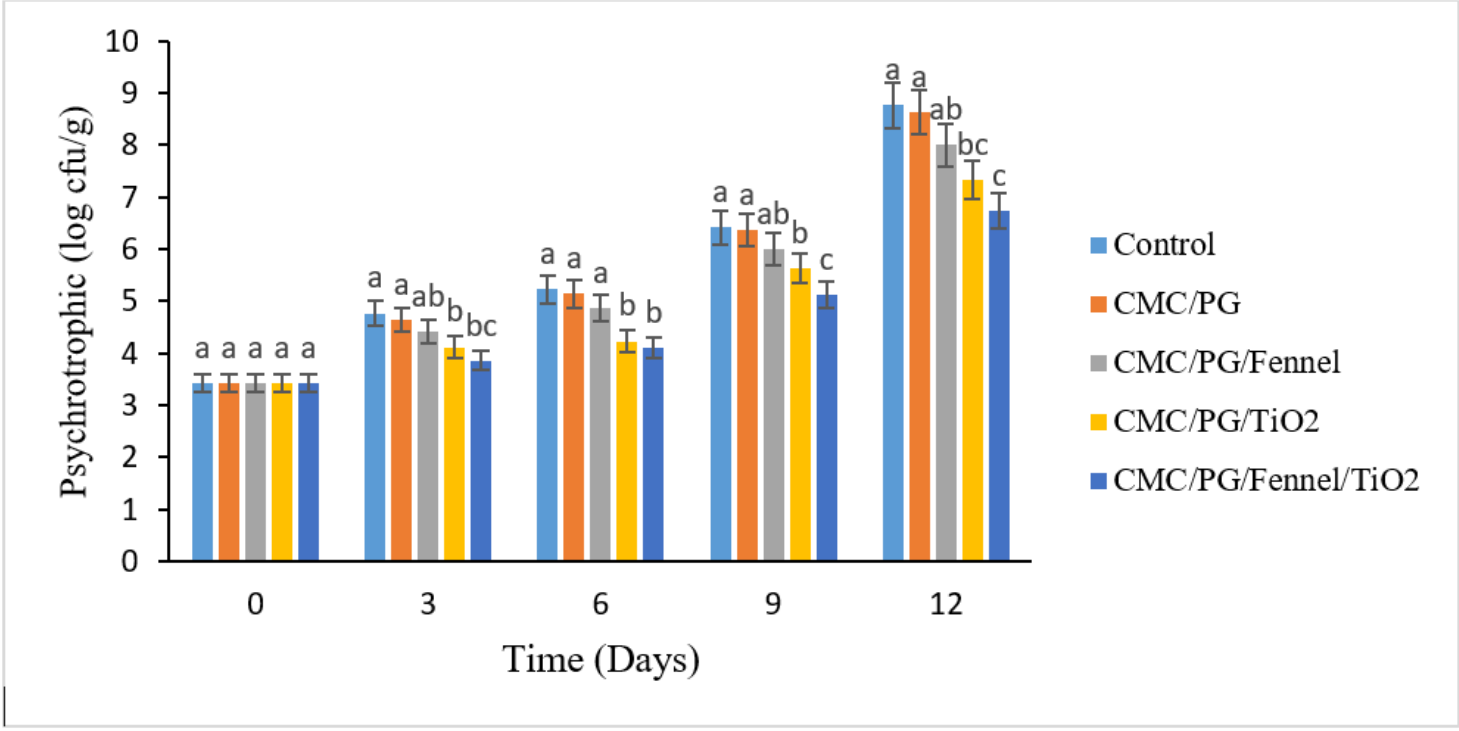

Figure 5

Changes in Psychrotrophic bacteria of different treatments during storage at 4°C for 12 days

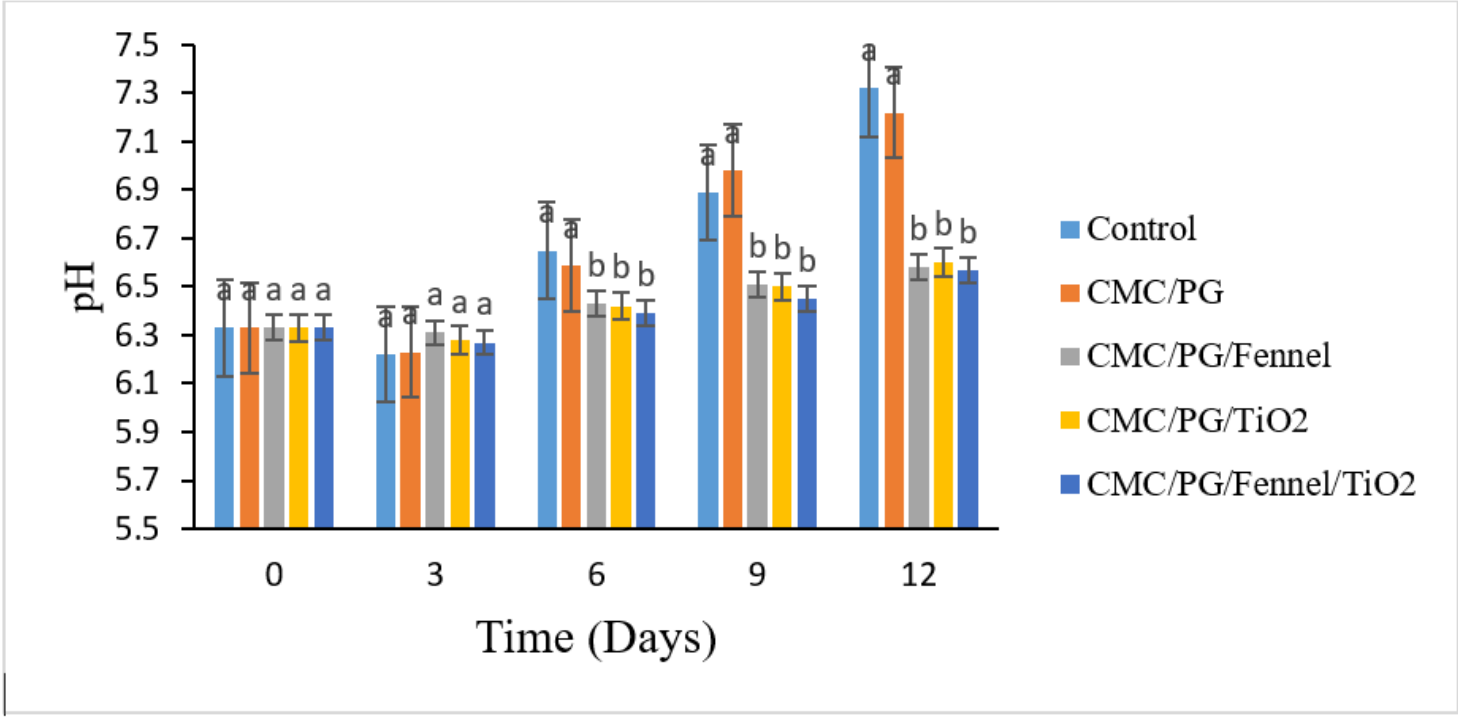

Figure 6

Changes in pH of different treatments during storage at 4°C for 12 days

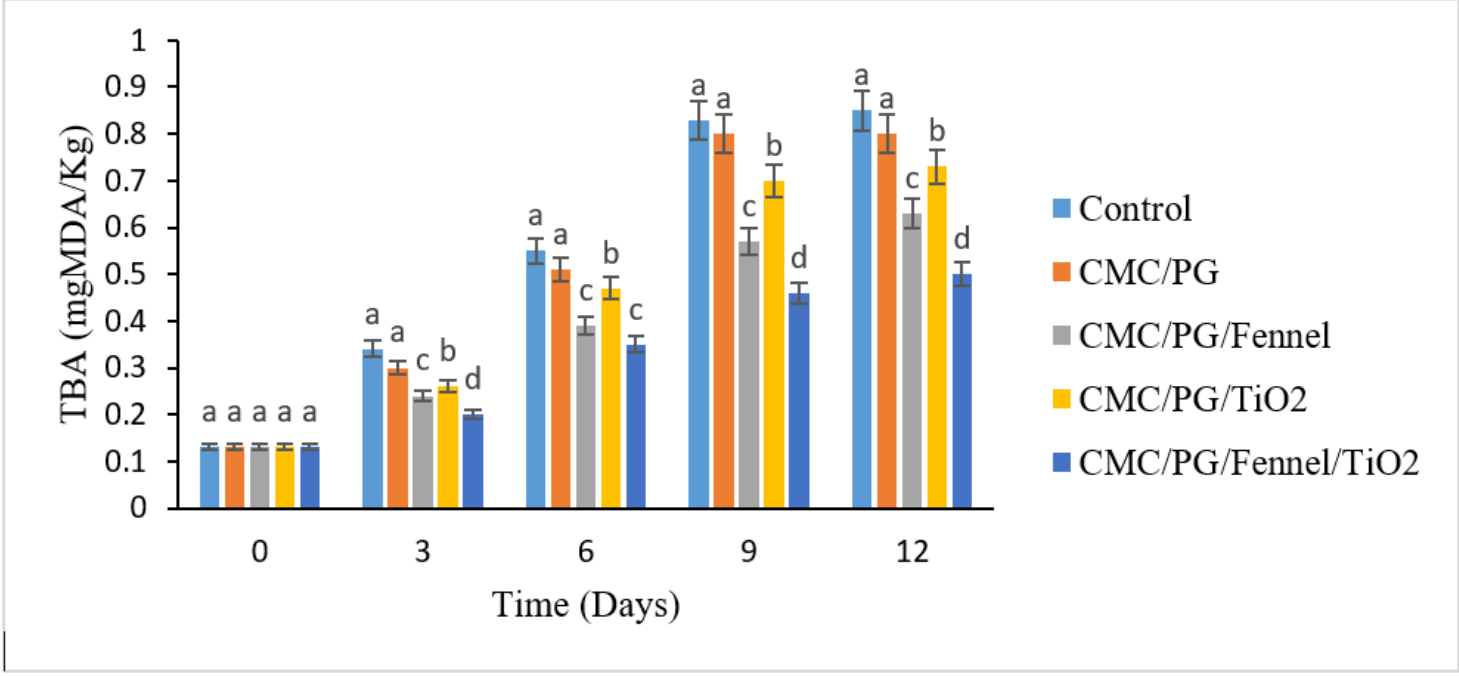

Figure 7

Changes in TBA of different treatments during storage at 4°C for 12 days

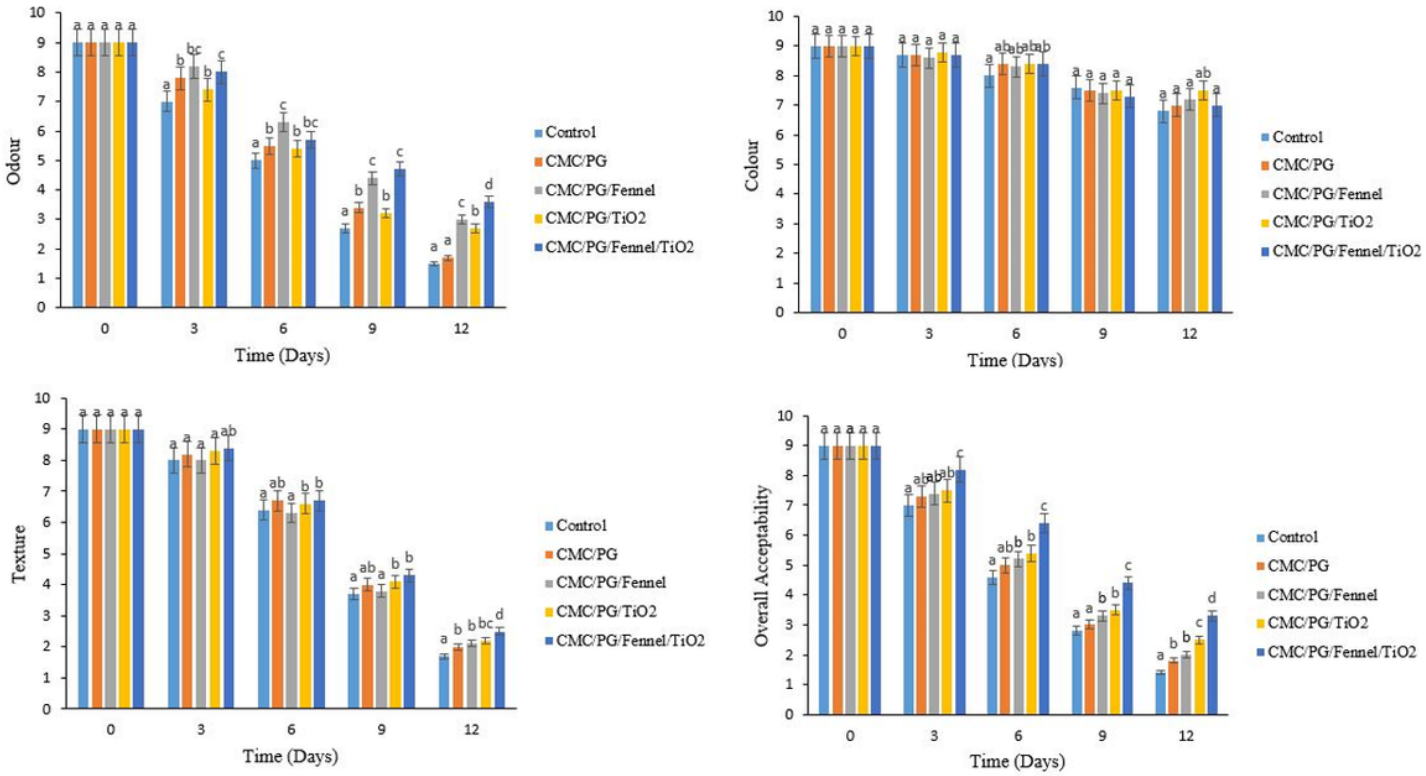

Figure 8

Changes in Sensory evaluation of different treatments during storage at 4°C for 12 days