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Investigating microbial properties of traditional Iranian white cheese packed in active LDPE films incorporating metallic and organoclay nanoparticles

Parisa Abdolsattari [a], Seyed Hadi Peighambardoust[b, *], Sajad Pirsa[a, *], Seyed Jamaleddin Peighambardoust [c], Seyedeh Homa Fasihnia[b]

[a] *Department of Food Science and Technology, College of Agriculture, University of Urmia,Urmia Iran*
[b] *Department of Food Science and Technology, College of Agriculture, University of Tabriz, Tabriz, Iran*
[c] *Faculty of Chemical & Petroleum Engineering, University of Tabriz, Tabriz, Iran*

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ABSTRACT

Iranian white Cheese is a traditional type of cheese in Iran which is popular and widely consumed because of its pleasant organoleptic properties. To manufacture this cheese, raw milk is heated below pasteurization temperature, therefore, pathogenic and spoilage bacteria remain and cause several diseases in public health. In this research three kinds of nanocomposite films based on LDPE incorporated 1) Ag, CuO, ZnO, 2) cloisite 15A, cloisite 20A, cloisite 30B that produced by extrusion method were used for packaging of the cheese and kept in 4 °C during 28 days. Data analysis carried out using SPSS statistical software based on a completely randomized design. The results showed the nanocomposite films incorporated with metal nanoparticles had a significant decrease in the growth of *Staphylococcus aureus*, coliform, mold and yeast after 28 days, while, the growth of lactic acid bacteria decreased but less than control film. Organoleptic properties of traditional Iranian Cheese packed in mentioned nanocomposite films were better. Also, physicochemical characteristics such as pH and moisture were affected significantly by active films. Overall migration of packaging active films in food simulants were within amounts allowed by national and international legislations

1. Introduction

Iranian White cheese is a close textured brined cheese made from cow's milk, sheep's milk, or a mixture of them [1]. Because of its pleasant organoleptic properties, this type of cheese is popular and widely consumed all over Iran and is enjoying high economical and nutritional value [2]. For the preservation of these valuable properties, in the manufacture of this cheese, milk is heated to approximately 23°C [3], so pathogenic bacteria especially *Staphylococcus aureus* can be transmitted through the product to public health.

In recent years, the use of nanotechnology has grown significantly in the production of high-performance plastic materials. The use of nano-fillers is very common in the production of composites and the nano-composites exhibit more unique mechanical properties than pure polymer. It should be noted that nanocomposites exhibit these properties in low amounts of filer (less than 5%). Improvement of nanocomposite properties is due to the high surface ratio of nano-fillers and the uniform distribution of filler particles. Karimi Sania Petroleum polymers (plastics) have some good characteristics, including low cost, good printing, easy plasticity and high chemical resistance, which cause to the increasing application of these polymers [4].

Antimicrobial active packaging is a new generation of food nano-packaging based on metal or organoclay

* Corresponding author. Tel.: +989145454914; e-mail: Pirsa7@gmail.com; s.pirsa@urmia.ac.ir

nanocomposite which is made by incorporating metal or organoclay nanoparticles into polymer films [5]. Nanoparticles of Ag [6, 7], CuO [8], ZnO [9, 10], have antibacterial properties and commonly used in active packaging. Nanoclay is a type of purified clay that at least is the nanometer size in one of the dimensions. The chemical and physical properties of solids depend heavily on the size and shape of the microscopic particles that make them up. Nanoparticles have found many applications in a variety of fields, including medicine, pharmaceuticals, cosmetics, catalysts, food packaging, and the textile industry. In addition to the mentioned applications, Nanoclay is also useful in protecting the environment. Their potential as adsorbents for volatile organic compounds, and organic/mineral pollutants is well documented in wastewater. This diversity in applications can be attributed to the adaptability of Nanoclay to change/modify. Nano-clays in combination with biodegradable polymers are very useful for modifying the mechanical properties of these polymers [11, 12]. Besides, organoclays such as different types of cloisite can incorporate into synthetic polymers and show antimicrobial effects on microorganisms [13]. The release of nanoparticles from the surface of these active films causes bacterial death due to binding to the cell membrane [14]. However, the mechanism of toxicity is only partially understood.

The main objective of this study is to evaluate the capabilities of LDPE nanocomposite packaging loaded by different antimicrobial nanoparticles as a new approach to decrease the microbial growth of traditional Iranian cheese.

2. Experimental

2.1.Nanocomposite film preparation

As it was described by Deghnagi et al. (2019) and Fasihnia et al. (2018), food-grade LDPE granules (LH0075 grade) (Bandar-Imam, Iran) and nanoparticles (Tables 1 and 2) were mixed with mineral oil (polyolefin of C17-C30) in a ratio of 0.1% (w/w), extruded in a co-rotating twin-screw extruder (SMPLATEK, South Korea) and then shaped like a film by another extruder (Castiny Ghioldys, Italy) to produce the final nanocomposite films (in 45±5 µm thickness) with the desired nanoparticle concentrations as shown in Table 3 [19,28]. [15, 16]

2.2 Traditional Iranian Cheese manufacture

Traditional Iranian cheese samples were made in small cheese-making factories in Basmenj (Tabriz, Iran). For this purpose, raw milk was filtered and its temperature was adjusted to 23°C. The milk was later poured into a stainless steel milk vat. Calf rennet (1gr/100 kg) was added to the milk and homogenized. The coagulum after a lapse of 60 to 120 min was usually put into a cheesecloth of 80×80 cm^2, the cloth corners were turned inwards and the cheese inside was squeezed in a way that in 60 to 90 min it would lose most of its whey. Later a smoothly-cut flat piece of plank weighing 6 to 12 kilograms was put over the cheesecloth for 60 to 90 min so that the maximum amount of whey would ooze out. Then, the resulting curd was cut into pieces of 10 ×10 cm^2, and after a time-lapse of 10 min, it was immersed in brine (18 to 24%) for 4 to 6 hours [17]. At the following stage, 50±2 g sample of cheeses packed in different kind of the nanocomposite films (Table 3) with 220×200 mm^2 and sealed by seal heater and then transferred to a refrigerator at 5±0.5˚C. Then, during 0, 7, 14, 21, and 28 days physicochemical, sensorial and microbial tests were assayed.

Table 1. Typical properties of nanometals

Nanoparticles	Size	Purity	Company
Ag nanoparticles	35 nm	99.50%	(TECNAN co., Spain)
ZnO nanoparticles	20-30 nm	99.90%	(TECNAN co., Spain)
CuO nanoparticles	50 nm	99%	Neutrino co. (Tehran, Iran)

Table 2. Typical properties of nanoclays

Nanoparticles	Density (g/ml)	Size	Company
Cloisite15A	1.66	10% less than 2 µm, 50% less than 6 µm, 90% less than 13 µm	Neutrino co. (Tehran, Iran)
Cloisite 20A	1.77	10% less than 2 µm, 50% less than 6 µm, 90% less than 13 µm	Neutrino co. (Tehran, Iran)
Cloisite 30B	1.98	10% less than 2 µm, 50% less than 6 µm, 90% less than 13 µm	Neutrino co. (Tehran, Iran)

Table 3. Different kinds of the nanocomposite films used in packaging of traditional Iranian cheese

Nanocomposite film	Composition
Film A	Ag-NP 0.33 %, CuO-NP 0.33 %, ZnO 0.33 %
Film B	Ag-NP 0 %, CuO-NP 0.67 %, ZnO 0.33 %
Film C	Cloisite15A 2%, Cloisite 20A 2%, Cloisite 30B 2 %
Film D	Cloisite15A 4%, Cloisite 20A 1%, Cloisite 30B 1 %
Film E	Cloisite30B-Ag 1.33%, Cloisite30B-CuO 2.67 %
Film S	Pure LDPE

2.3. Physicochemical properties

The pH was evaluated by pH meter (Methrom, Germany) at 25±1°C [18]. Dry matter content in cheese was determined by heating in 102°C oven (Memert, Germany) until constant weight [19].

2.4. Sensorial properties

A simple unstructured sensory evaluation was performed by 7 trained panelists every time before the samples were used for laboratory analyses, and the color, flavors & odor, texture, and total acceptance were considered. The tasters evaluated 4 parameters on a 5 point hedonic scale of 1 to 5 (1=very poor, 2=poor, 3= fair, 4= good, 5= very good) [20].

2.5.Antimicrobial activity test

50 grams of the cheese packaged in the different nanocomposite films dispersed with 450 ml of a sterile Ringer (Merck, Darmstadt, Germany) in a stomacher bag and mixed for 1 min with a stomacher (Velp, Italy). Decimal dispersion of cheese homogenates performed, and microbiological counts of total coliform, *S.aureus*, lactic acid bacteria, yeast and mold were determined. The number of total coliforms monitored as follows: pour plating in violet red bile agar (Scharlau Chemie, SA., Spain), with a covering layer of the same medium, incubated at 35˚C for 24 hours. The number of *S.aureus* monitored by surface cultures in mannitol salt agar (Scharlau Chemie, SA., Spain), incubated at 35˚C for 24-48 hours. For lactic acid bacteria MRS agar (Scharlau Chemie, SA., Spain), was used and the plates were maintained in anaerobic jars at 35˚C for 24-48 hours. Finally, the diluted solution of the cheese was cultured on YGC agar (Scharlau Chemie, SA., Spain), following incubation at 25˚C for 3-5 days.

2.6. Overall Migration assay

In current European food packaging legislation (EN 1186-1, 2002), various food simulants can be used for migration testing identified. These include water (simulant A), 3% (v/v) acetic acid in water (simulant B), 15% (v/v) ethanol in water (simulant C), olive oil, sunflower oil, and synthetic fat stimulant HB 307 (simulant D) where each simulant is representative of a particular type of food [21,22,23]. Overall migration test of Ag-NP, CuO-NP, and ZnO-NP tested using stimulant B at 40˚C for 10 days. The nanocomposite films with 220×200 mm^2 filled with a 200 ml of stimulant solution and the migration results normalized to metal migrated/cm^2. The calibration graph for zinc was obtained by making 10 standard solutions in the proper range and absorbance measuring by using flame atomic absorption spectrometry. Absorbance measurements of silver and copper elements were done by electrothermal atomic absorption spectrometry and an autosampler system was used for making standard solutions of calibration graph from a stock solution with a proper concentration. Standard solutions of metal ions were prepared by diluting of stock standard solution (Merck Darmstadt, Germany) with acetic acid 3% (Merck, Darmstadt, Germany) as the solvent. Three repetitions were done at each measurement and the average of them was used for following studies. An Analytikjena atomic absorption spectrometer model Nova 400 (Jena, Germany) was used for determination ions [16,24]

2.7. Statistical Analyses

The results were processed by a statistical method based on a completely randomized design using the General Linear Model (GLM) procedure, SPSS (version 19) Statistical differences among the mean values were determined by one-way analysis of variance (ANOVA) and Duncan′s multiple rang test (p=0.05). All analyses were carried out in triplicate.

3. Results and Discussion

3.1. Physicochemical properties

The pH value of traditional Iranian cheese is in the range of 4.22 to 5.50 [3], which decreases during storage. The value of pH in traditional Iranian cheese measured immediately after packaging was 5.13. According to Fig. 1, a significant decrease is observed in pH values of nanocomposite films even pure LDPE. Interestingly, film A acted as the control film and pH value fell to 4.66. Film B was a little different and decreased to 4.70. Nanocomposite films of C and E acted the same and there was no significant difference between them (4.69). But the pH value of traditional Iranian cheese in film D was more than other (4.86). A decrease in pH value is related to an increase of lactic acid bacteria for producing lactic acid [25]. The lower pH values contribute not only in the taste of cheese but also it helps the cheese to maintain its convenient texture and protects it against microbiological spoilage [26].

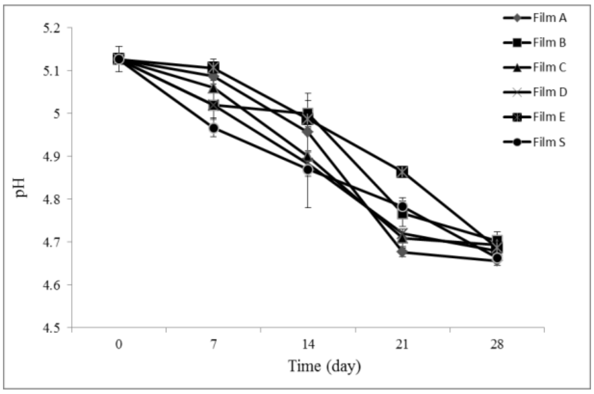

Figure 1. Changes in the pH values of the Iranian Traditional cheese packaged in nanocomposite films during 28 days storage period at 5°C

The moisture content of traditional Iranian cheese was 62.67% on the first day. As can be seen in Fig. 2, during the first seven days the moisture content of cheese samples in all packaging materials decreased on average from 61.19% to 58.52%. The moisture of the cheese decreased to 59.94 in the control film at the end of storage time. It seems that the declining trend in the moisture content of traditional Iranian cheese is an ordinary fact because of the permeability of films. Film C and D had the least moisture content changes in the cheeses (55.98 % and 55.92%, respectively). Also, films A and B could decrease the moisture content of the cheese to 54.71% and 54.94%, respectively and there is no significant difference between them after 28 days of storage. The moisture content of traditional Iranian cheese in film E was 55.32%. The differences between samples are because of the type of cheese and there is no standard for cheese manufacturing method. Institute of Standards and Industrial Research of Iran (ISIRI) suggests that industrial white ripened cheese should not contain lower than 40% dry matter [27].

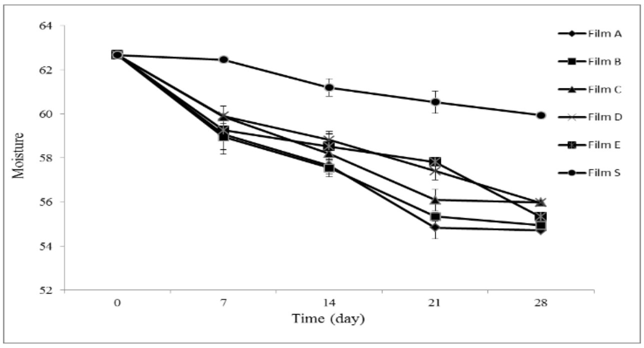

Figure 2. Changes in the moisture content of the Iranian Traditional cheese packaged in nanocomposite films during 28 days storage period at 5°C

3.2. Sensory evaluation

Based on fig. 3, it can be inferred that all nanocomposite films investigated in this study exerted good effects on terms of overall acceptability. Although the control samples became unacceptable after about 7 days, the traditional Iranian cheese packaged in the active films and stored under the same storage condition received an acceptable sensory score for 28 days (more than 4 score). Fig. 3 show the changes of sensory attributes of traditional Iranian cheese packed in different nanocomposite films. The effect of the type of film was investigated on flavor, texture, color and total acceptance of the cheese. According to fig. 3 film B could obtain the highest score for all above factors and control film had the lowest score. Film A and film E were in the next steps. The lowest scores were related to film C and film D.

3.3. Antimicrobial activity

The mean initial population was determined immediately after packaging traditional Iranian cheese which was 5.06 log CFU/g for coliform, 8.03 log CFU/g for lactic acid bacteria, 4.16 log CFU/g for *S. aureus* and 3.29 log CFU/g for yeast and mold. The variations in the population of these microorganisms are shown in Table 1. In pure LDPE packages, the mean population of the mentioned microorganisms had an incremental process during 28 days of storage and did not display any antimicrobial effects.

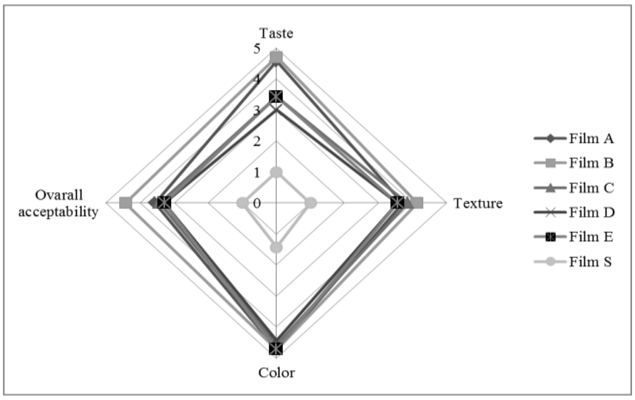

Figure 3. Changes in the organoleptic scores of the Iranian Traditional cheese packaged in nanocomposite films during 28 days storage period at 5°C

Based on Table 1, the results showed that the level of population of coliform in different kinds of nanocomposite films was different. According to Japanese Industrial Standard (JIS Z 2801: 2000), from which ISO 22196: 2007 drives, antimicrobial activity of R>2.0 log CFU/cm^2 is required for the nano food packaging to demonstrate antimicrobial efficacy, as R is the difference in bacteria concentration (expressed in log CFU/cm^2) between the non-treated and treated test specimens [28]. Therefore, films A and B and maybe film E had an antibacterial effect on coliform, while films C and D increased this microorganism (R=0.32) and they did not have a significant difference in this respect. The antibacterial effect of metal nanoparticle had been proven in the packaging of cheese, previously [15,24] Gumiero et al. (2013) investigated the antimicrobial effect of TiO_2 photocatalytic activity in HDPE-based food packaging on coliform growth of a short-ripened cheese during 4 weeks and concluded active films did not increase the microbial growth while control film caused to 1.8 log CFU increase in coliform load [29]. Incoronato et al. (2011), who worked on Fiordilatte packaged in an active system based on silver ions release, in the first days of storage yeasts remained very low and then followed up, without reaching the levels of the other spoilage microbial groups as *Enterobacteriaceae* and *Pseudomonas* spp [30]. *S. aureus* is often found in raw milk and the environment of the cheese plants (equipment and personnel). This organism is salt-tolerant and can grow under a wide range of conditions, low acid production may allow *Staphylococci* to grow and produce enterotoxin [31]. There are no national standards for microbiological and chemical characteristics of traditional Iranian cheese in the country. Iranian Standards described for industrial white ripened cheese (ISIRI 2406, 1994), suggests that white cheese should not contain *S. aureus* [32]. Fortunately, it is affected by nanoparticle and decreased during the cheese storage. However, the only film A had antimicrobial effect according to the above regulation and could decrease bacterial load to 2.15 log CFU/g. Film B decreased *S. aureus* to 3.10 log CFU/g, film E was a little different with 3.16 log CFU/gr at the end of storage period. There was not a significant difference between film C and film D (3.53 log CFU/g and 3.61 log CFU/g). It seems metal nanoparticle of Ag, CuO, and ZnO have a synergic antibacterial effect on *S. aureus*. Fernandez et al. (2010) produced polylactic acid-polylactide (PLA) silver zeolite films and showed the antimicrobial activity against *S. aureus* [33]. Unlike other microorganisms, yeast and mold population showed an incremental trend during 28 days of storage. Yeast and mold may contribute to the ripening process by utilizing lactic acid or by their proteolytic and lipolytic activity [34]. Except for film A, that increased fungal load to 4.06 log CFU/g, film C, film D, and film E had the same population and it was 4.08 log CFU/g, 4. 16 log CFU/g and 4.11 log CFU/g, respectively. It appears that organoclay has an antifungal effect [16,35,36] and can be used in nanocomposite films for acidic food such as cheese and similar products. Lactic acid bacteria as functional bacteria in the ripening of traditional Iranian cheese for producing flavor, aroma, texture, etc. should be protected. Regarding the lactic acid bacteria count (Table 1) all samples showed a similar growth rate. In all nanocomposite films the lactic acid bacteria loads of traditional Iranian cheese increased, but they were significantly different from pure LDPE film (10.01 log CFU/g). Film D increased the microbial load to 9.39 log CFU/g and was different from the rest. The sample packaged in film C had 9.19 log CFU/g lactic acid bacteria at the end of 28 days of storage. So organoclay showed a lower inhibitory effect on this useful bacteria. Film E with 8.72 log CFU/g at 28 days of storage had the most inhibitory effect and between film A with 9.08 log CFU/g and film B with 9.03 log CFU/gr was no significant difference at the end of storage.

3.4. Migration assay

The migration results of the nanocomposite films after 28 days of storage into food simulants are given in Table 2. Amoung metallic nanocomposite films, the Ag-NP levels was less than 50 mg/kg, which was in agreement with legislation [21]. The CuO-NP levels in the food simulants comply with the legislation, which allows a maximum of 10 mg/kg [37]. Besides Zn is a GRAS compound an there is no limit for it in literature.

Table 2. Overall migration of metallic nanoparticles

Treatment	Ag-NP(ppb)	CuO-NP(ppm)	ZnO-NP(ppb)
Film A	37.42	7.45	1.55
Film B	-	8.44	1.83
Film E	45.8	9.52	-

Table 1. Effect of nanocomposite films on Coliform, Lactic acid bacteria, *S. aureus,* and Yeast and Mold during 28 days storage period at 4°C.

Film type	Storage time, day	Coliform Log CFU/g	Lactic acid bacteria Log CFU/g	*S. aureus* Log CFU/g	Yeast &Mold Log CFU/g
Film A	0	5.06±0.152^{Aa}	8.03±0.023^{Ae}	4.16±0.041^{Aa}	3.29±0.047^{Ac}
	7	4.70±0.0972^{Ab}	8.15±0.028^{CDd}	3.80±0.073^{Db}	3.30±0.213^{Bc}
	14	4.95±0.107^{Cc}	8.29±0.032^{Dc}	3.44±0.079^{Dc}	3.68±0.090^{Cbc}
	21	3.60±0.115^{Cd}	8.66±0.06^{Db}	3.12±0.115^{Ed}	3.83±0.149^{Cab}
	28	2.97±0.029^{Ce}	9.08±0.023^{Da}	2.15±0.213^{De}	4.06±0.048^{Ca}
Film B	0	5.06±0.152^{Aa}	8.03±0.023^{Ad}	4.16±0.041^{Aa}	3.29±0.047^{Ad}
	7	4.89±0.115^{Aa}	8.21±0.027^{BCc}	3.86±0.034^{CDb}	3.30±0.092^{Bd}
	14	4.20±0.052^{Bb}	8.39±0.02^{Cb}	3.66±0.010^{CDc}	3.92±0.070^{Bc}
	21	3.81±0.149^{BCc}	8.92±0.59^{Ca}	3.88±0.088^{DEd}	4.15±0.032^{Bb}
	28	3.05±0.124^{Cd}	9.03±0.075^{Da}	3.10±0.024^{Ce}	4.40±0.110^{Ba}
Film C	0	5.06±0.152^{Aa}	8.03±0.023^{Ad}	4.16±0.041^{Aa}	3.29±0.047^{Ac}
	7	5.07±0.172^{Aa}	8.30±0.069^{ABCc}	4.03±0.141^{BCab}	3.47±0.132^{Bc}
	14	5.30±0.054^{Ab}	8.38±0.009^{Cc}	3.88±0.096^{BCabc}	3.74±0.010^{BCb}
	21	5.30±0.124^{Aa}	8.81±0.081^{CDb}	3.75±0.252^{BCbc}	3.95±0.038^{BCab}

	28	5.32 ± 0.017^{Ba}	9.99 ± 0.028^{Ca}	3.53 ± 0.17^{Bc}	4.08 ± 0.096^{Ca}
Film D	0	5.06 ± 0.152^{Aa}	8.03 ± 0.023^{Ae}	4.16 ± 0.041^{Aa}	3.29 ± 0.047^{Ad}
	7	5.08 ± 0.231^{Aa}	8.31 ± 0.0413^{ABd}	4.12 ± 0.141^{ABa}	3.58 ± 0.105^{Bc}
	14	5.29 ± 0.122^{Aa}	8.86 ± 0.077^{Bc}	3.99 ± 0.025^{Bab}	3.87 ± 0.956^{BCb}
	21	5.32 ± 0.124^{Aa}	9.17 ± 0.073^{Bb}	3.88 ± 0.053^{Bb}	3.99 ± 0.066^{BCab}
	28	5.38 ± 0.097^{Bb}	9.39 ± 0.014^{Ba}	3.61 ± 0.143^{Bc}	4.16 ± 0.295^{Ca}
Film E	0	5.06 ± 0.152^{Aa}	8.03 ± 0.023^{Ac}	4.16 ± 0.041^{Aa}	3.29 ± 0.047^{Ac}
	7	4.91 ± 0.261^{Aa}	8.05 ± 0.096^{Dc}	4.01 ± 0.015^{BCa}	3.51 ± 0.06^{Bbc}
	14	4.30 ± 0.012^{Bb}	8.26 ± 0.030^{Db}	3.76 ± 0.183^{BCb}	3.70 ± 0.165^{BCb}
	21	3.97 ± 0.187^{Bb}	8.36 ± 0.043^{Eb}	3.54 ± 0.035^{CDb}	3.95 ± 0.109^{BCa}
	28	3.10 ± 0.112^{Cc}	8.72 ± 0.221^{Ea}	3.16 ± 0.021^{Cc}	4.11 ± 0.031^{Ca}
Film S	0	5.06 ± 0.152^{Ac}	8.03 ± 0.023^{Ae}	4.16 ± 0.041^{Ac}	3.29 ± 0.047^{Ad}
	7	5.11 ± 0.109^{Ac}	8.38 ± 0.040^{Ad}	4.24 ± 0.279^{Ac}	4.77 ± 0.078^{Ac}
	14	5.29 ± 0.046^{Ac}	9.01 ± 0.027^{Ac}	4.30 ± 0.008^{Ac}	5.07 ± 0.109^{Ab}
	21	5.59 ± 0.063^{Ab}	9.44 ± 0.003^{Ab}	4.56 ± 0.034^{Ab}	5.80 ± 0.158^{Aa}
	28	5.90 ± 0.126^{Aa}	10.01 ± 0.027^{Aa}	5.14 ± 0.145^{Aa}	6.02 ± 0.061^{Aa}

Each value is the mean ± SD of 3 replicates. Different small and capital letters in superscripts show a statistical difference during the time (in a row) and between samples (in a column), respectively ($P\leq0.05$).

4. Conclusion

In this study, nanocomposite films containing metallic nanoparticles (film A and film B) and organoclay nanoparticles include Cloisite 15A, Cloisite 20A, Cloisite 30B (film C, film D, and film E) were used to the packaging of the traditional Iranian cheese and its characteristics were investigated. Based on the results, the metallic nanoparticle of Ag, CuO, and ZnO had more antimicrobial effect than organoclay nanoparticles on coliform, *S. aureus*, lactic acid bacteria, and yeast and mold without any negative effect on sensorial parameters. Concluding, the application of antimicrobial films in food packaging systems could diminish the harshness of food processing, the amounts of additives and chemical preservatives in food industries

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