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Enhancing the Quality and Shelf Life of Soft Cheese Using Zinc Oxide and Chitosan Nanoparticles

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Abstract

SOFT CHEESE, an early food introduced into the human diet for its nutritional value, is highly susceptible to microbial contamination by spoilage and/or pathogenic microorganisms during storage. This study evaluated the effects of Zinc oxide (ZnO) nanoparticles (10 mg/ml, G1), chitosan nanoparticles (500 µg/ml, G2), and their combination (G3), compared to a control group (C), on the coagulation time, sensory profile, acidity percentage, and microbiological quality of soft cheese. Results showed significant differences in coagulation time among the groups, with mean times (hour: minute) of 04:20 for C, 03:05 for G1, 02:10 for G2, and 00:51 for G3. Sensory evaluation revealed that treated groups, particularly the combination group (G3), received the highest scores, maintaining acceptability up to 21 days for the control, 39 days for G1, 42 days for G2, and 48 days for G3 under refrigeration. Acidity percentages at 21 days were 0.55 for the control and 0.19 for G3, indicating a slower increase in acidity in treated samples. Microbiologically, treated cheeses showed significant improvement compared to the control. Coliform and E. coli were undetectable in all samples during refrigeration. Mould appeared at 21, 36, 42, and 48 days for C, G1, G2, and G3, with mean counts of 1.6±0.05, 1.5±0.2, 1.4±0.02, and 1.2±0.08 log10 CFU/g, respectively. Yeast was detected at 15, 27, 30, and 36 days with mean counts of 1.7±0.05, 1.9±0.1, 1.6±0.03, and 1.7±0.08 log10 CFU/g, correspondingly. In conclusion, the soft cheese treated with a combination of chitosan and ZnO nanoparticles extended shelf life to 48 days with improved sensory, acidity, and microbiological profiles compared to 21 days for the control group.

Keywords: Chitosan Nano-Particles, Zinc Oxide Nano-Particles, Soft cheese.

Introduction

Soft cheese varieties have long been a cornerstone of human nutrition, providing essential nutrients like calcium, potassium, and vitamins such as B complex and D. In addition, they serve as critical sources of protein and bioactive compounds that support bone health, metabolic function, and immune response [1, 2].

Soft cheese is highly susceptible to microbial contamination throughout its production and distribution. Contaminants can originate from raw milk, especially if unpasteurized, introducing pathogens like *Staphylococcus aureus*, and coliforms [3]. Environmental sources such as cheese vats, molds, wooden tables, packaging materials, and handlers are significant contributors [4]. Poor sanitation, inadequate heat treatment, and high moisture and pH levels in soft cheese one of favorable media for the growth of spoilage and pathogenic microorganisms [5]. Even when pasteurized milk is used, post-processing contamination remains a risk, highlighting the need for strict hygiene and control measures to ensure the microbiological safety of soft cheese [6].

Nano-preservation has emerged as a transformative approach in dairy science, leveraging materials at the 1-100 nm scale to enhance food safety and longevity. Unlike conventional preservatives, nanoparticles can provide targeted antimicrobial action while reinforcing product structure. This technology shows particular promise for soft cheeses, where even minor improvements in shelf life yield significant economic and food safety benefits [7].

Chitosan nanoparticles have emerged as an effective natural preservative in cheese preservation due to their potent antimicrobial and antioxidant properties. Their application, either as coatings or additives, helps extend cheese shelf life by inhibiting the growth of spoilage microorganisms such as

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bacteria, moulds, and yeasts, which commonly contaminate cheese during storage [8]. Besides that, zinc oxide (ZnO) nanoparticles are valuable in cheese preservation due to their effective antimicrobial properties that help inhibit the growth of spoilage microorganisms, thereby extending shelf life and maintaining product quality. Moreover, ZnO nanoparticles are thermally stable, non-toxic, and do not cause discoloration, making them safe and practical for use in food preservation, particularly in active packaging systems to enhance cheese safety and freshness [9].

Chitosan-Zinc oxide (CS-ZnO) nano-combination represent a breakthrough in this field, where chitosan, a biopolymer derived from crustacean shells, exhibits inherent antimicrobial properties through electrostatic interactions with microbial cell membranes. When combined with ZnO nanoparticles which generate reactive oxygen species lethal to bacteria and fungi, the combination treatment creates a synergistic preservation system. Research indicates these nanocomposites can be integrated into edible coatings that simultaneously inhibit spoilage organisms and regulate moisture migration [10].

The application of CS-ZnO nanotechnology in soft cheese preservation addresses multiple challenges simultaneously. Early studies demonstrated 30-50% reductions in microbial population colonization when applied as surface coatings, extending shelf life by 2-3 weeks while maintaining original texture profiles [11, 12]. Therefore, the current study was planned to investigate the impact of chitosan; Zinc oxide nanoparticles, and (CS-ZnO) combination on the coagulation time, sensory profile, acidity % and microbiological quality of low-salt soft cheese during refrigerated storage.

Material and Methods

Materials

Chitosan (CS) of low molecular weight (less than 100 kDa), zinc acetate and Sodium hydroxide (NaOH) as precursors for zinc oxide nanoparticles preparation were obtained from Nakaa Nanotechnology Company, Egypt. Buffalo's milk, was provided by Faculty of Agriculture, Benha University, Egypt. Rennet powder, sodium hydroxide (NaCl) and calcium chloride ($CaCl_2$) (food grade) were used during the manufacturing of soft cheese and were obtained from Al-Alamya Chemical Company, Egypt.

Pathogenic strains

Both strains *Escherichia coli* (ATCC® 25922TM) and *Staphylococcus aureus* (ATCC43300) were provided by Animal Health Research Institute, Benha Lab.

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by ball milling processaccording to Inkyo *et al.* [13]. A total of 25 g from chitosan powder was ball milled in a vessel with 13 numbers of zirconia beads in a range from 0.5 to 1.5 mm diameter (75 beads 0.5 mm diameter, 30 beads 1.0 mm diameter and 25 beads 1.5 mm diameter) and milling at 4000 rpm with a high-energy ball mill was performed. Samples were prepared by varying the milling duration, and then the dried chitosan powder was pulverized for 30, 60 and 90 min to obtain fine powder. A Ball milling process was used as a dispersing agent to prevent particle agglomeration.

Preparation of Zinc oxide NPs

Zinc oxide nanoparticles were synthesized by the precipitation method according to Suntako [14]. In the typical procedure, 5 g of zinc acetate were dissolved in 75 ml of deionized water and 2 Molar NaOH (5 gm of NaOH /50 ml of deionized water) were added dropwise under magnetic stirring at 60^{o}c. After the addition was completed, the stirring was continued for 30 min, then the solution was left to cool then, the precipitates were washed with deionized water three times. Then the obtained precipitates were dried at 100^{o}c then kept at room temperature 37°C.

Characterization of chitosan and ZnO nanoparticles

For TEM "Transmission Electronic Microscopic" analysis, a drop of the Zinc oxide nanoparticles solution and chitosan nanoparticles solution was placed on the carbon-coated copper grids (CCG) and dried by allowing water to evaporate at 37°C. Electron micrographs were obtained using JEOL JEM-1010 transmission electron microscope at 80 kV at the Regional center for Mycology and Biotechnology, (RCMB) Al- Azhar University [15].Determination the minimum inhibitory concentration (MIC) of chitosan and Zinc Oxide Nanoparticles against *E. coli* and *S. aureus* in broth.

Activation of the pathogenic strains

The pathogenic strains of (Escherichia coli and Staphylococcus aureus) were cultured in tryptone soya broth for 24 h at 37 °C for activation, followed by serial dilution to obtain 6 log10 CFU/ml working culture. The method was carried out according to Kim and Kim [16].

Determination of MIC by tube dilution technique

Different prepared double-fold serial dilutions of the used nanomaterials; (1000, 500, 250, 125 and 62.5 µg/ml for chitosan NPs), (40, 20, 10, 5, 2.5 and 1.25 µg/ml for ZnO NPs); were added to the required pathogenic bacterial concentration (1x106 CFU/ml). The tubes were incubated at 37 °C for 24 hours. After incubation, 0.1ml of the incubated culture was spreading on the appropriate agar media (TBX agar for E. coli, and Baird Parker agar for S. aureus) for

determining their count (CFU/ml). The lowest concentration of the tested material that completely inhibited bacterial growth was recorded as the minimum inhibitory concentration (MIC) [17].

Preparation of soft cheese groups

Fresh buffalo's milk was pasteurized at 62.7°C for 30 min, cooled and adjusted to 37°C, then Calcium chloride and Sodium chloride were added at the ratios of 0.02% and 3% (w/v), respectively. Then, chitosan and ZnO nanoparticles were added. Rennet was added at the ratio of (11 ml of rennet per 45.5 kg milk, then incubated at 39°C until curdling occurred [18].

Cheese groups were classified into (4 groups): where, control" untreated soft cheese samples"(C), soft cheese fortified with 10 mg ZnO NPs/ml (G1), soft cheese fortified with 500 µg chitosan NPs/ml (G2), and soft cheese fortified with 10 mg ZnO NPs/ml in combination with 500 µg chitosan NPs/ml (G3). Then cheese groups put into small cylindrical metal containers lined with cheese cloth overnight to get rid of the whey. Then the curd was cut into small cubes and stored in the collected whey at 4°C [19]. Then examined at zero day and every 3 days' intervals till signs of spoilage were occurred. The trials were repeated 3 times.

Coagulation time

Coagulum firmness time was assessed by an experienced cheese manufacturer by tactile and visual inspection according to Johnson et al. [20]. Results were stated and presented as hours and minutes.

Sensory evaluation of cheese samples

Sensory characterization of cheese samples was performed and recorded. Sensory flavor (60), texture (30) and appearance (10) with overall score (100) were performed by well-experienced analysts [21].

Determination of titratable acidity (TA)

It was determined according to AOAC [22] by the standard method of titratable acidity. To determine titratable acidity in cheese, a measured volume of the cheese sample is mixed thoroughly and then pipetted into a container. A few drops of phenolphthalein indicator are added to the sample, which is then titrated with a standardized sodium hydroxide (NaOH) solution while stirring. The titration continues until a faint pink color that persists for about 30 seconds appears, indicating the endpoint. The volume of NaOH used, known as the titer, is recorded and used to calculate the titratable acidity, typically expressed as a percentage of lactic acid, providing a measure of the acid content in the cheese.

Microbiological assessment of cheese samples

Cheese samples were prepared according to ISO [23] using aseptic technique, 10 grams of soft cheese sample were transferred by sterile spatula to sterile polyethylene bag then adding 90 ml sterilized Sodium citrate 2%, bags were placed in stomacher for shaking at 100 beats for two min, then tenfold serial dilutions were prepared using sterilized peptone water. Coliform counts were performed using violet red bile agar (VRBA) and incubation at 37 ^{0}c for 24h [24]. *Escherichia coli* count was performed using Tryptone-Bile-X-Glucuronate (TBX) agar and incubation at 44 ^{0}c for 24h [25]. Total mould and yeast counts were performed using Sabouraud Dextrose agar (SDA) and incubation at 25°C for 5 days according to ISO [26]. Aerobic spore former count was performed using nutrient agar and incubation at 32 ^{0}c for 48 hours [27]. Microbiological counts were repeated three times and recorded as mean ± standard error.

Statistical Analysis

The collected data were statistically analyzed according to Feldman et al. [28] using two-way ANOVA within each group regarding the treatment conditions and storage time. In addition, an independent sample t-test was used to compare the results of the two sample groups. SPSS V. 20 software was used for the data analysis.

Results

Regarding to TEM size-characterization of the prepared nanomaterials, chitosan nanoparticles' size ranged from 13.3 nm to 27.2 nm, while was 9.27 to 18.9 nm for ZnO NPs; which revealed that all of the used nanomaterials were within the nano-size (Fig. 1 A & B).

Referring to the recorded results of coagulation time (Fig. 2), longer coagulation time was recorded in the control group (4 h and 20 min.), followed by G1 (3 h and 5 min.), G2 (2 h and 10 min), and G3 (51 min.)" the lowest coagulation time".

Regarding the *in vitro* determination of the MIC of the used nanomaterials. the examined concentrations of chitosan NPs, 1000, 500, 250, 125 and 62.5 µg/ml, could reduce *E. coli* count to 100, 100, 100, 63.3 and 43.3 %, respectively; while they reduced *S. aureus* counts to 100, 100, 100, 100, and 66.7%, respectively. On the other hand, 40, 20, 10, 5, 2.5 and 1.25 mg/ml ZnO NPs showed total reduction of *E. coli* and *S. aureus* counts up to 5 mg/ml; whereas, 2.5 and 1.25 mg/ml ZnO NPs reduced the counts of *E. coli* by 43.3 and 28.3%, while, reduced *S. aureus* by 41.7 and 28.3%, respectively; which means that the MIC of chitosan NPs against *E. coli* and *S. aureus* were 250 and 125µg/ml, respectively while it was 5 mg/ml for ZnO NPs against both *E. coli* and *S. aureus* (Fig. 3& 4).

Regarding the sensory characters of the examined groups (Fig. 5), higher acceptability scores were

recorded in the treated groups, particularly the chitosan-ZnO NPs treated group (G3); where it showed a decrease in their scores along the storage period up to 48 days of storage. Results showed that C, G1, G2 and G3 kept their acceptability up to 21st, 39th, 42nd, and 48 days of storage, respectively.

Referring to the recorded results in Figure (5A), G3 recorded higher appearance acceptability scores since the zero day of examination (9.9), followed by chitosan nanoparticles treated group (G2: 8.3), ZnO nanoparticles treated group (G1: 7.5), while control group showed the lower acceptability score (6.9), that started gradual decrease in the appearance acceptability scores up to record the spoilage at the 21st, 36th, 42nd and 48th days of storage for C, G1, G2 and G3 with mean values of 2.1, 2.2, 3.0 and 3.2, respectively.

Referring to the recorded results , The higher texture acceptability score since the zero day of examination was 29.8 G3 for , followed by chitosan treated group (G2: 27.8), ZnO treated group (G1: 24.4), while control group showed the lower acceptability score (21.6), that started gradual decrease in the appearance acceptability scores up to record the spoilage at the 21st, 36th, 42nd and 48th days of storage for C, G1, G2 and G3 with mean values of 10.7, 11.2, 12.8 and 13.2, respectively, (Fig, 5B).

While in Figure (5C), G3 recorded higher flavour acceptability scores since the zero day of examination (59.8), followed by chitosan treated group (G2: 57.8), ZnO treated group (G1: 53.8). while control group showed the lower acceptability score (49.7), that started gradual decrease in the appearance acceptability scores up to record the spoilage at the 21st, 36th, 42nd and 48th days of storage for C, G1, G2 and G3 with mean values of 21.7, 24.1, 27.5 and 29.0, respectively.

Regarding to TA values, the examined soft cheese groups were 0.26, 0.24, 0.20 and 0.19 for C, G1, G2and G3, respectively at the zero time, followed by gradual increasing in the TA of the examined groups. While with fast raising ~~up~~ in the TA values was recorded in C (control group), slower increasing was recorded in the treated groups, where the highest TA mean values were recorded in G1, G2 and G3 in the 36th (0.56), 42nd (0.52), and 48th (0.52) days of storage, respectively indicating longer acceptability criteria in the chitosan-ZnO NPs treated group (G3) (Table 1).

The recorded results of the microbiological quality of the examined cheese groups (Table 2 and 3) indicated improvement in the microbiological criteria of the treated cheese samples appeared as slower microbial multiplication compared with the control group, which reflected on the keeping quality and shelf life of the examined soft cheese samples.

Table (2) showed that the aerobic spore former could not be detected in the first 12 days of storage in all the examined groups, while it could be firstly detected in the 15th, 30th, 39th and 45th day of storage in C, G1, G2 and G3, with mean counts of 1.47±0.03, 2.0±0.07, 2.2±0.07 and 1.9±0.05 $\log_{10}$ CFU/g, respectively. Aerobic spore former counts continued to increase until the appearance of spoilage characters at the 21st, 36th, 42nd, and 48th days of storage for C, G1, G2, and G3 with mean counts of 2.17, 2.3, 2.3, and 2.2 $\log_{10}$ CFU/g, respectively.

Regarding both bacteria, coliform and *E. coli* could not be detected along the experimental period, either in the control or the treated groups.

Regarding the total yeast and mould counts, Table (3) declared that mould was firstly found at the 21st, 36th, 42nd, and 48th day of refrigerated storage for C, G1, G2 and G3, respectively, with mean counts of 1.6±0.05, 1.5±0.2, 1.4±0.02, and 1.2±0.08 $\log_{10}$ CFU/g, respectively. On the other hand, yeast was firstly detected in the 15th, 27th, 30th and 36th day of storage with mean counts of 1.7±0.05, 1.9±0.1, 1.6±0.03, and 1.7±0.08 $\log_{10}$ CFU/g, respectively; whereas, yeast mean counts still within the acceptable limit until appearing the signs of spoilage at the 15th, 36th, 42nd and 48th days of storage for C, G1, G2 and G3; where the mean counts ($\log_{10}$CFU/g) were 2.2±0.07, 2.3±0.1, 2.3±0.2 and 2.3±0.1, respectively.

Discussion

Soft cheese is obtained after the coagulation of fresh or concentrated milk, or a mixture of its fresh or dried products, which has been pasteurized or subjected to any heat treatment equivalent to pasteurization [29]. Microbial contamination in soft cheese is often from raw milk, inadequate pasteurization, and unsanitary handling [30]. Studies have detected coliforms, *E. coli, S. aureus*, and Salmonella in commercial soft cheese samples, linked to contaminated udders, unhygienic processing environments, or post-pasteurization exposure [30, 31, 32]. Nanotechnology, recently, offers innovative preservation methods for dairy products such as soft cheese, particularly through the use of chitosan and zinc oxide (ZnO) nanoparticles. Chitosan nanoparticles, alone or combined with essential oils or zinc oxide nanoparticles, exhibit strong antimicrobial effects against spoilage and pathogenic bacteria, as well as yeasts and mould, which are major contributors to cheese spoilage [33, 34].

Regarding the size characterization of the used chitosan and ZnO nanoparticles, chitosan nanoparticle's size ranged from 13.3 nm to 27.2 nm, while was 9.27 to 18.9 nm for ZnO NPs (Fig. 1A and B). These results were lower than those of Yusof *et al.* [35] who revealed that the synthesized chistosan – ZnO NPs of a mean size value of 70 nm have displayed an antibacterial inhibition zone against *S.*

aureus and *E. coli*, which were 16.0 and 13.3 mm, respectively. Also, the current result is lower than those of Abdeltwab *et al.* [36] who revealed that the synthesized chistosan NPs size reached to 93.7nm showed maximum antibacterial activity against *S. aureus* and *L. monocytogenes* with inhibition zone of 30mm for both and 23 mm for *E. coli* at a concentration of 23µg/ml.

The antibacterial effect of nanoparticles (NPs) is strongly influenced by their nanoscale size, with smaller particles exhibiting enhanced antimicrobial activity. This is primarily because as the size of NPs decreases, their surface-to-volume ratio increases significantly, providing a larger active surface area for interaction with bacterial cells. This increased surface area facilitates stronger adhesion to bacterial membranes, greater penetration into cells, and more effective generation of reactive oxygen species (ROS), which collectively disrupt cell membranes, induce oxidative stress, and interfere with intracellular components like DNA and proteins [37]. Figures (3) and (4) revealed that MIC of chitosan NPs against *E. coli* and *S. aureus* were 250 and 125µg/ml, respectively, while it was 5 mg/ml for ZnO NPs against both strains. This came in agreement with Alekish *et al.* [38] who recorded that MIC of ZnO NPs against the selected strains was 31.25 µg/ml and 3.9 µg/ml, respectively. Also, Mubarak Ali *et al.* [39] stated MIC of chitosan NPs against the same strains was 40 and 80 µg/ml, respectively. In addition, Abdeltwab *et al.* [36] recorded that MIC of chitosan nanoparticles against the same strains were 184.32 and 23.04 µg/ml, respectively.

The highest efficacy against S. *aureus*. returned to the chitosan nanoparticle with positive charge can interact with the cell surface of *S. aureus* or essential nutrients, leading to inhibition of its growth [40]. Also, due to Gram-positive bacteria as *S. aureus* have a thick peptidoglycan layer in their cell wall, which is easily penetrated by chitosan nanoparticles. Gram-negative bacteria, like *E. coli*, have a thinner peptidoglycan layer and an additional outer membrane composed of lipopolysaccharides (LPS) and proteins. This outer membrane acts as a barrier, making it harder for chitosan nanoparticles to reach the cell membrane and exert their antibacterial effect [41]. Coagulation time is a critical parameter in cheese manufacture because it determines the end point of the enzymatic phase and the start point of the aggregation, so it is used as a reference to determine the cut-time, which plays an important role in firmness or softness of cheese [42]. The current study showed that chitosan-ZnO NPs treated group (G3) showed the shortest coagulation time and the best firmness and texture in comparison to with control group, which has the longest coagulation time and the lowest firmness and texture (Fig., 2).

The current study nearly came in agreement with Ausar *et al.* [43], who stated that the addition of chitosan, a cationic polymer, to milk causes destabilization and coagulation of casein micelles. This is returned to the electrostatic interactions between positive charges of chitosan NPs and negative charges of casein micelles. This hydrophobicity relationship helps in the coagulation of casein micelles with chitosan molecules. The efficiency of interaction of casein with chitosan depends on the concentration of chitosan molecules, with increasing concentrations of chitosan, the amount of coagulating casein is growing rapidly in a short time due to negatively charged casein micelles interacting with cationic polysaccharide – chitosan, resulting in coagulation [44]. Consumer acceptance of cheese is strongly influenced by sensory attributes-texture, appearance, and taste-as well as acidity. Studies showed that texture and taste are key drivers of preference, with consumers favoring cheeses that match their expectations for creaminess, firmness, or saltiness, while attractive appearance enhances perceived quality and willingness to buy [45].

Fig. 5 (A, B, C) declared that higher sensory scores were stated in the treated groups, particularly the combined chitosan-ZnO NPs treated group (G3) regarding the appearance, texture and flavor criteria, where it showed a gradual decrease in their scores along the storage period up to 48 days of storage. Results showed that C, G1, G2 and G3 kept their sensory up to 21st, 39th, 42nd, and 48 days of storage, respectively. Previous records confirmed that such nanocomposite coatings reduced harmful microbes (including coliforms and spore-formers) to undetectable levels, while preserving the cheese's organoleptic qualities Hassan *et al.* [46]. Youssef *et al.* [47] recorded that a significant enhancement in the texture of the treated soft cheese with CS-ZnO NPs.

Acidity % of cheese samples not only impacts flavor and freshness, but also signals proper fermentation and safety, contributing to consumer trust and satisfaction [48]. The acidity values of the examined groups at the zero day were 0.26±0.01, 0.24±0.01, 0.20±0.01 and 0.19±0.01 for C, G1, G2 and G3, respectively, followed by increasing in the TA of the examined groups especially in C (control group), while slower increasing was stated in the treated groups, where the highest TA mean values were 0.56 ± 0.03 , 0.52±0.03 and 0.52±0.04 for G1, G2 and G3 in the 36th, 42nd and 48th days of storage, respectively indicating longer acceptability criteria in the chitosan-ZnO NPs treated group (G3) (Table, 1).

The recorded results came in line with Salama *et al.* [49] who recorded a gradual increase of TA accompanied with gradual decrease in the microbial counts in the chitosan treated yoghurt groups (TA= 0.81 and 0.83 at zero day while were 0.92 and 0.90

for 2.5 and 5.0 mg chitosan NPs/ml milk, respectively); moreover, sensory characteristics of the treated groups showed significant improvement in the flavor, texture and appearance in comparing with control group after 21 days of refrigerated storage; and Mikky *et al.* [34] who showed that pickled cheese samples coated with chitosan NPs (0.01, 0.02 and 0.04%) fortified with different essential oils exhibited a gradual increase in acidity during refrigerated storage; where TA started around 0.6 in the control and treated groups and increased to be 1.47 in the 35th day of storage for control group; while the treated groups kept their acceptability up to 91, 98 and 105 days of storage, where TA equaled 1.22, 1.28 and 1.55, respectively. Addition of ZnO NPs and chitosan, both separately and in combination, significantly reduced the count of aerobic spore formers in the treated cheese groups as compared to the control group. For C, G1, G2, and G3, they were initially detected on days 15, 30, 39, and 45 of storage, respectively, with mean counts of 1.47±0.03, 2.0±0.07, 2.2±0.07, and 1.9±0.07 (log_{10} CFU/g) (Table, 2).

Against aerobic spore-forming bacteria, ZnO nanoparticles inhibit spore germination by damaging cell membranes and interfering with metabolic pathways [50]. Also, this result nearly came in agreement with Hassan *et al.* [51] who detected antibacterial activity of chitosan nanoparticles on *Bacillus subtilis* at concentration 8000, 4000, 2000, 1000, 500, 250, 125, 62.5 and 31.25 (µg/ml), respectively with inhibition % reached to 100, 100, 90.68, 87.66, 70.17, 56.75, 45.02, 16.36 and 7.65%, respectively. In addition, Alarfaj *et al.* [52] showed inhibition zone of chitosan nanoparticles against Bacillus species at concentrations of 50 µg, 100 µg and 150 µg, respectively was 10, 14 and 18 mm, respectively. Furthermore, the current findings also concurred with those of Djearamane *et al.* [53], who confirmed a significant inhibition of ZnO NPs on *B. subtilis* growth for all the tested concentrations of ZnO NPs from 5 to 150 µg/mL at 24 h. The percentages of growth inhibition for 5, 10, 25, 50, 100, 150 (µg/mL) were reported to be 11.88 ± 0.04, 16.66 ± 0.80, 28.55 ± 0.36, 33.57 ± 0.38, 47.52 ± 1.53, 70.09 ± 1.5%, respectively. Meanwhile, the positive control showed 92.96 ± 0.19% of inhibition. Further, the results detected that the percentage of growth inhibition of *B. subtilis* increased with the increasing concentrations of ZnO NPs. This is returned to surface interaction of ZnO NPs on the bacterial cell wall, and also the morphological alterations in *B. subtilis* induced by ZnO NPs [53].

Regarding mould and yeast findings in the examined groups, results revealed that mould was firstly detected at the 21st, 36th, 42nd and 48th days of refrigerated storage for C, G1, G2 and G3, respectively with mean counts of 1.6±0.05, 1.5±0.2, 1.4±0.02 and 1.2±0.08 (log_{10} CFU/g), respectively (Table, 3). These mean values were more than the acceptable limit (1 log_{10} CFU/g) mentioned by EOS [29]. On the other hand, yeast was firstly detected in the 15th, 27th, 30th and 36th days of storage for C, G1, G2 and G3, respectively with mean counts of 1.7±0.05, 1.9±0.1, 1.6±0.03 and 1.7±0.08 (log_{10} CFU/g) ,–respectively; whereas, yeast mean counts still within the acceptable limit (40 log_{10} CFU/g) according to EOS [29] until appearing the signs of spoilage at the 21th, 36, 42nd and 48th days of storage for C, G1, G2 and G3; where the mean counts (log_{10} CFU/g) were 2.2±0.07, 2.3±0.1, 2.3±0.2 and 2.3±0.1, respectively (Table, 3).

The recent recorded results came in line with the recorded findings of Sayed-Elahl *et al.* [8] who applied chitosan NPs (0.25 and 0.5%) to kareish cheese to evaluate its antimicrobial activity, and they recorded a significant reduction in the fungal counts (4.9 and 3.67 log_{10} CFU/g for 0.25 and 0.5% treated samples, respectively) in comparing with the control group (5.38 log_{10} CFU/g) after 20 days of storage revealing longer shelf life of the treated samples. The current findings also concurred with those of Mohamed *et al*. [54], who recorded that the mould count in the cheese sample dropped by 1 log_{10} CFU/g after 15 days of storage at high concentrations of chitosan NPs (10%) in contrast to our study (500 µg/ml chitosan NPs), also with those of Abdeltwab *et al.* [36] who reported that chitosan NPs have a fungistatic effect reached to 90 and 100 % against *Penicillium roqueforti* at concentrations 3.0 and 4.5 g/L, respectively. This fungistatic value may be returned to nano-chitosan particles that diffuse into fungal cells, disrupting the synthesis of genetic materials [55].

Antifungal activity in the current study came in line with that of Yien *et al*. [56] who indicating that the chitosan NPs were observed to be natural antifungal agents when used in concentrations of 1-3 mg/ml against *Candida albicans* and *Aspergillus niger*. Also, the current results came in line with the recorded results of Awaad *et al.* [33] who applied chitosan nanoparticles (0.25 and 0.5%) to kareish cheese and found that *C. albicans* counts were significantly lowered at day 12 of storage to be 4.12 and 3.66 log_{10}CFU/g after using 0.25 and 0.5% chitosan nanoparticles compared with that for control group (5.43 log_{10} CFU/g). However, *C. albicans* was totally not detected after 18 days of storage for both treatments. The antimicrobial activity of chitosan NPs can be attributed primarily to their electrostatic interactions between their positively charged amino groups and the negatively charged microbial cell walls and membranes. This interaction disrupts membrane permeability, leading to leakage of intracellular components and cell death [57]. Besides that, low-molecular-weight chitosan nanoparticles can penetrate cells, interfering with DNA, RNA, and protein synthesis, and impairing mitochondrial

function, which enhances their antimicrobial efficacy [58]. Additionally, chitosan acts as a chelator of essential metal ions, depriving microbes of nutrients needed for growth. The antimicrobial activity is stronger in acidic conditions due to increased protonation of amino groups, which enhances binding to microbial surfaces [59].

Regarding coliform and *E. coli*, they could not be detected along the experimental period, either in the control or the treated groups, that may be due to good hygienic conditions during the manufacture of cheese and efficient pasteurization of milk. Al-Nabulsi *et al.* [60] declared that chitosan coatings with ZnO nanoparticles (≥0.0125%) decreased *E. coli* O157:H7 counts in white brined cheese by 2.5–2.8 $\log_{10}$ CFU/g at 4°C over 28 days, compared to 2.6 $\log_{10}$ CFU/g for chitosan alone (2.5%); Olaimat *et al.* [61] assessed the antimicrobial efficacy of chitosan-based coatings containing 1.0% ZnO NPs against *L. monocytogenes* on the surface or within packaged white brined cheese at 4°C. They reported that chitosan coatings containing 1.0% ZnO NPs decreased *L. monocytogenes* numbers by 1.5 and 3.7 $\log_{10}$ CFU/g on the surface or by 0.9 and 1.5 $\log_{10}$ CFU/g inside vacuum-packed cheese kept at 10 or 4◦C, respectively. Also, Youssef *et al.* [47] reported a potent antimicrobial effect of chitosan-ZnO nanocomposite film coating, with 1.0% chitosan concentration to 2, 4 and 8% of ZnO NPs concentration, to package Egyptian soft white cheese, resulting in total reductions in total bacterial counts, moulds, yeasts, and coliforms, while also extending shelf life up to 30 days of storage at 7°C.

Additionally, ZnO NPs treated samples, also, revealed significant antimicrobial effect which may be attributed to generation of reactive oxygen species (ROS) such as hydrogen peroxide that damage cell membranes, proteins, and DNA; they release Zn^{2+} ions that disrupt enzymatic functions; and their small size allows direct interaction and penetration of microbial cell walls, causing structural damage [62]. Combination of ZnO with chitosan nanoparticles enhance antimicrobial efficacy through synergistic effects, improving membrane disruption and ROS generation while maintaining biocompatibility and preventing nanoparticle migration into food matrices [63].

Conclusion

The current study revealed that combination of chitosan and ZnO NPs at a concentration of (500 µg/ml and10 mg/ml) achieved prolonged shelf life up to 48 days, and the highest sensory characteristics (preferable creamy taste). Also characterized by slow increasing in titratable acidity value till the end of refrigerated storage period and the highest microbiological quality which reflect good hygienic quality of cheese. So finally, the combination treatment could be recommended as a strong antimicrobial agent in the cheese industry could. It alleviated the microbial status of cheese by inhibiting the growth of pathogenic and spoilage organisms over storage time.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

Not applicable

TABLE 1. Effect of chitosan and Zinc oxide nanoparticles on the titratable acidity (TA) in the examined cheese samples

Storage time	Control (C)	Zn10 (G1)	Ch500 (G2)	Mix (G3)
0	0.26 ± 0.01^{Ad}	0.24 ± 0.01^{Bg}	0.20 ± 0.01^{Cg}	0.19 ± 0.01^{Cg}
3	0.26 ± 0.01^{Ad}	0.24 ± 0.01^{Bg}	0.20 ± 0.01^{Cg}	0.19 ± 0.01^{Cg}
6	0.26 ± 0.02^{Ad}	0.24 ± 0.01^{Bg}	0.20 ± 0.01^{Cg}	0.19 ± 0.01^{Dg}
9	0.26 ± 0.02^{Ad}	0.24 ± 0.02^{Bg}	0.20 ± 0.01^{Cg}	0.19 ± 0.01^{Dg}
12	0.26 ± 0.03^{Ad}	0.24 ± 0.03^{Bg}	0.20 ± 0.01^{Cg}	0.19 ± 0.01^{Dg}
15	0.35 ± 0.03^{Ac}	0.24 ± 0.02^{Bg}	0.20 ± 0.02^{Cg}	0.19 ± 0.02^{Dg}
18	0.45 ± 0.03^{Ab}	0.24 ± 0.02^{Bg}	0.20 ± 0.02^{Cg}	0.19 ± 0.01^{Dg}
21	0.55 ± 0.02^{Aa}	0.25 ± 0.01^{Bf}	0.20 ± 0.03^{Cg}	0.19 ± 0.02^{Dg}
24	S.	0.29 ± 0.01^{Ae}	0.20 ± 0.01^{Bg}	0.19 ± 0.02^{Cg}
27	S.	0.36 ± 0.02^{Ad}	0.21 ± 0.03^{Bf}	0.19 ± 0.01^{Cg}
30	S.	0.41 ± 0.03^{Ac}	0.28 ± 0.02^{Be}	0.20 ± 0.02^{Cg}
33	S.	0.46 ± 0.03^{Ab}	0.34 ± 0.03^{Bd}	0.25 ± 0.03^{Cf}
36	S.	0.56 ± 0.03^{Aa}	0.39 ± 0.04^{Bc}	0.30 ± 0.03^{Ce}
39	S.	S.	$0.44\pm0.04^{*b}$	$0.36\pm0.02^{*d}$
42	S.	S.	$0.52\pm0.03^{*a}$	$0.40\pm0.03^{*c}$
45	S.	S.	S.	0.45 ± 0.04^{b}
48	S.	S.	S.	0.52 ± 0.04^{a}

ABC Different superscript letters within the same row mean significant difference when ($P\leq0.05$), abc Different superscript letters within the same column mean significant difference when ($P\leq0.05$), *-. Superscript star within the same row means significant difference between less than three groups of variance when ($P\leq0.05$), S: spoiled, Zn10 (G1): zinc oxide nanoparticles at concentration 10 mg/ml, Ch500 (G2) : chitosan nanoparticles at concentration 500 µg/ml, Mix (G3) : combination treatment between zinc oxide nanoparticles at concentration 10 mg/ml and chitosan nanoparticles at concentration 500 µg/ml

TABLE 2. Effect of chitosan and Zinc oxide nanoparticles on the aerobic spore-forming count (log_{10} CFU/g) in the examined cheese samples

9	Control (C)	Zn10 (G1)	Ch500 (G2)	Mix (G3)
0	N.D	N.D	N.D	N.D
3	N.D	N.D	N.D	N.D
6	N.D	N.D	N.D	N.D
9	N.D	N.D	N.D	N.D
12	N.D	N.D	N.D	N.D
15	**1.47 ± 0.03^{C}**	N.D	N.D	N.D
18	**1.77 ± 0.1^{B}**	N.D	N.D	N.D
21	**2.17 ± 0.1^{A}**	N.D	N.D	N.D
24	S.	N.D	N.D	N.D
27	S.	N.D	N.D	N.D
30	S.	**2.0 ± 0.07^{B}**	N.D	N.D
33	S.	**2.1 ± 0.3^{B}**	N.D	N.D
36	S.	**2.3 ± 0.2^{A}**	N.D	N.D
39	S.	S.	**2.2 ± 0.07^{A}**	N.D
42	S.	S.	**2.3 ± 0.05^{A}**	N.D
45	S.	S.	S.	**1.9 ± 0.05^{B}**
48	S.	S.	S.	**2.2 ± 0.2^{A}**

ABC Different superscript letters within the same column means significant difference when ($P\leq0.05$), ND Not Detected, S. spoiled, Zn10 (G1): Zinc oxide nanoparticles at concentration 10 mg/ml, Ch500 (G2): chitosan nanoparticles at concentration 500 µg/ml, Mix (G3): combination treatment between zink oxide nanoparticles at concentration 10 mg/ml and chitosan nanoparticles at concentration 500 µg/ml

TABLE 3. Effect of chitosan and Zinc oxide nanoparticles on the mould and yeast counts (log_{10} CFU/g) in the examined cheese samples

Storage time	Control (C)		Zn10 (G1)		Ch500 (G2)		Mix (G3)	
	Mould	Yeast	Mould	Yeast	Mould	Yeast	Mould	Yeast
0	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
3	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
6	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
9	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
12	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
15	N.D	1.7 ± 0.05^{c}	N.D	N.D	N.D	N.D	N.D	N.D
18	N.D	1.9 ± 0.07^{b}	N.D	N.D	N.D	N.D	N.D	N.D
21	1.6±0.05	2.2 ± 0.07^{a}	N.D	N.D	N.D	N.D	N.D	N.D
24	S.	S.	N.D	N.D	N.D	N.D	N.D	N.D
27	S.	S.	N.D	1.9 ± 0.1^{b}	N.D	N.D	N.D	N.D
30	S.	S.	N.D	$2.1\pm0.05^{ab*}$	N.D	$1.6\pm0.03^{d*}$	N.D	N.D
33	S.	S.	N.D	$2.2\pm0.07^{a*}$	N.D	$1.8\pm0.05^{c*}$	N.D	N.D
36	S.	S.	1.5±0.2	2.3 ± 0.1^{Aa}	N.D	2.0 ± 0.1^{Bb}	N.D	1.7 ± 0.08^{Cc}
39	S.	S.	S.	S.	N.D	$2.1\pm0.08^{b*}$	N.D	$1.8\pm0.05^{c*}$
42	S.	S.	S.	S.	1.4±0.02	$2.3\pm0.2^{a*}$	N.D	$2.0\pm0.03^{b*}$
45	S.	S.	S.	S.	S.	S.	N.D	2.2 ± 0.01^{b}
48	S.	S.	S.	S.	S.	S.	1.2±0.08	2.3 ± 0.1^{a}

abc Different superscript numbers within the same column, for yeast counts, means significant difference when ($P\leq0.05$), ABC Different superscript letters within the same row, for yeast counts, means significant difference when ($P\leq0.05$), *-. Superscript star within the same row means significant difference, for yeast counts, between less than three groups of variance when ($P\leq0.05$), ND Not detected, S. spoiled, Zn10 (G1): zinc oxide nanoparticles at concentration 10 mg / ml, Ch500 (G2): chitosan nanoparticles at concentration 500 µg/ml, Mix (G3): combination treatment between zinc oxide nanoparticles at concentration 10 mg / ml and chitosan nanoparticles at concentration 500 µg/ml

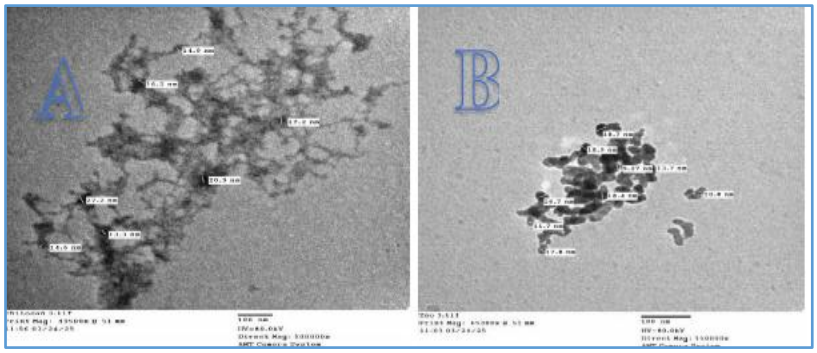

Fig. 1. TEM picture for chitosan and zinc oxide nanoparticles

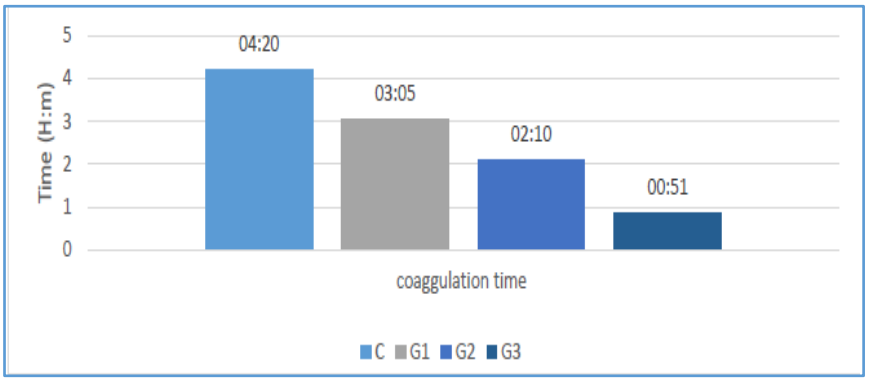

Fig. 2. Coagulation time of the soft cheese samples (Hour: Minute)

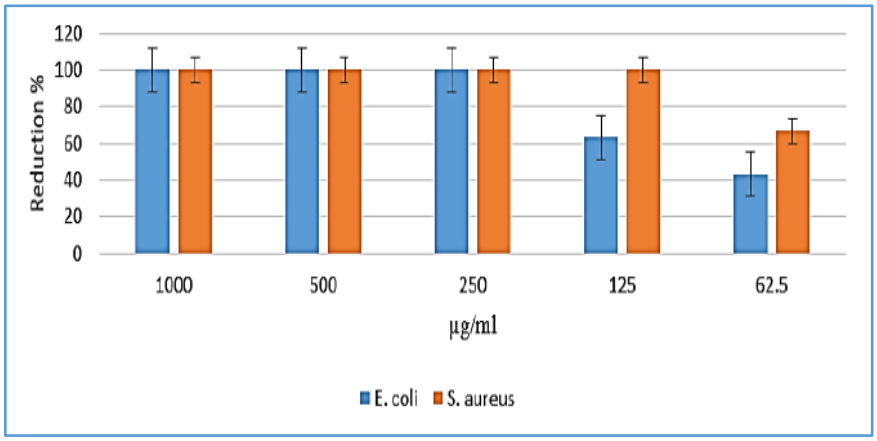

Fig. 3. Reduction rate of *E. coli* and *S. aureus* treated with different chitosan NPs concentrations in broth (6 $\log_{10}$ CFU/ml).

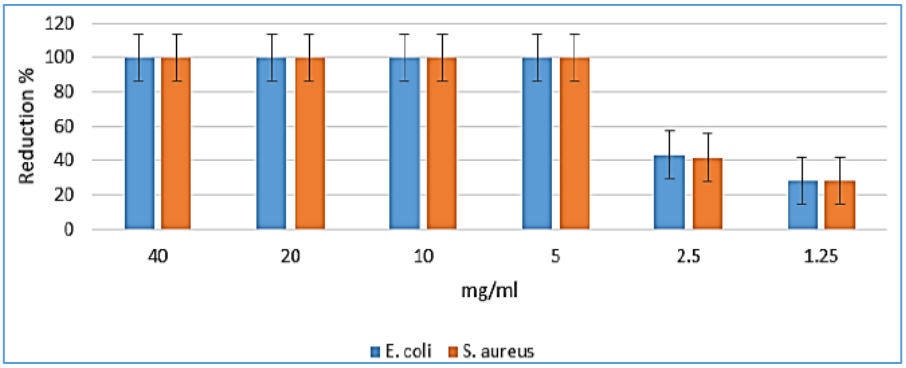

Fig. 4. Reduction rate of *E. coli* and *S. aureus* treated with different ZnO NPs concentrations in broth

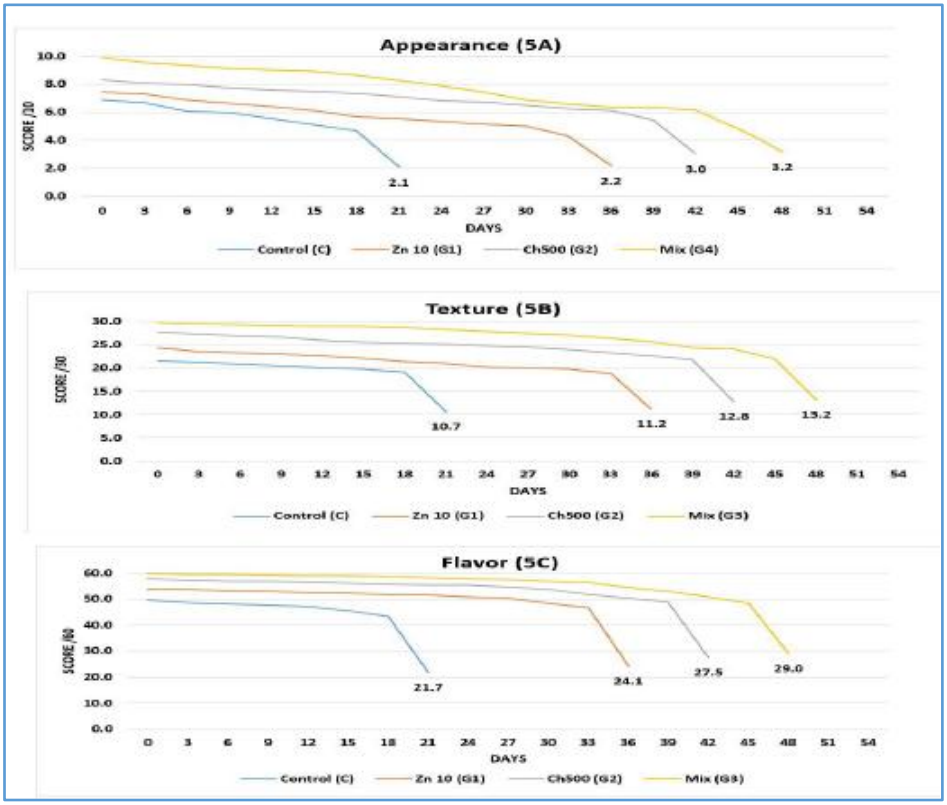

Fig. 5. Sensory evaluation of examined soft cheese samples during storage period (A: Appearance, B: Texture, C: Flavour)

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تأثير جسيمات أكسيد الزنك والكيتوزان النانوية على جودة الجبن الطري

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[1] قسم الرقابة الصحية علي الأغذية، كلية الطب البيطري، جامعة بنها، مصر.

الملخص

يُعد الجبن الطري من أقدم الأطعمة التي أُدرجت في النظام الغذائي البشري ويتميز بقيمته الغذائية العالية، إلا أنه شديد القابلية للتلوث الميكروبي سواء من الميكروبات المسببة للفساد أو الميكروبات الممرضة أثناء التخزين. استهدفت الدراسة الحالية تقييم تأثير جسيمات أكسيد الزنك النانوية (ZnO) بتركيز 10 ملغ/مل (م1) وجسيمات الكيتوزان النانوية بتركيز 500 ميكروغرام/مل (م2) ومزيجهما (م3)، مقارنةً بمجموعة ضابطة غير معالجة، على زمن التخثر والصفات الحسية، ونسبة الحموضة، والجودة الميكروبيولوجية للجبن الطري. أظهرت النتائج وجود اختلاف معنوي في زمن التخثر بين المجموعات، حيث بلغت المتوسطات (بالساعة: الدقيقة): 04:20 في المجموعة الضابطة، 03:05 في (م1)، 02:10 في مجموعة الكيتوزان (م2)، و00:51 في المجموعة المشتركة (م3). أما من حيث الصفات الحسية، فقد حصلت العينات المعالجة، خاصة مجموعة 3 ، على أعلى تقييم حسي. كما أظهرت النتائج أن الجبن الطري احتفظ بجودته وقبوله حتى اليوم 21 في المجموعة الضابطة، واليوم 39 في م 1، واليوم 42 في م 2، واليوم 48 في م 3. من ناحية أخرى، بلغت نسبة الحموضة في اليوم 21 لكل من المجموعة المشتركة (م3) (0.19) والمجموعة الضابطة (0.55)، مع ارتفاع أسرع في الحموضة في المجموعة الضابطة. أما من حيث الجودة الميكروبيولوجية، فلم يتم الكشف عن البكتريا القولونية او الايشرشيا كولاي في جميع العينات خلال فترة التخزين، بينما تم الكشف عن الفطريات في الأيام 21، 36، 42 و48 للمجموعات الضابطة و م1، م2 و م3 على التوالي، وبمتوسطات أعداد أقل في العينات المعالجة. كما تم الكشف عن الخمائر في الأيام 15، 27، 30 و36 مع أعداد أقل في المجموعات المعالجة. خلصت الدراسة إلى أن استخدام مزيج جسيمات الكيتوزان وأكسيد الزنك النانوية أدى إلى إطالة العمر الافتراضي للجبن الطري حتى 48 يومًا مع تحسين الصفات الحسية ونسبة الحموضة والجودة الميكروبيولوجية مقارنةً بـ 21 يومًا فقط في المجموعة الضابطة.

الكلمات الدالة: الجبن الطري، اوكسيد الزنك النانوي، نانوكيتوزان.