Research Article

A New Approach in using Moringa Oil (Mo) and Nano-Mo as a Bio Preservative in White Cheese

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Abstract | Cheese's shelf life may be shortened by pathogenic and spoilage bacteria, which are prone to contaminating food. This suggests that bio-preservatives may be used during the cheese-making process. So, this research was done to determine how well moringa oil (MO) and its nano emulsion (Nano-MO) work on some food poisoning bacteria inoculated into white cheese in vitro and in vivo. Prepared (Nano-MO) had the Z-average diameter of 76.04±51.13 nm and polydispersity index (PDI) of 0.319. The spherical shape of the generated nano-emulsion was revealed by Transmission Electron Microscope (TEM) and the flow of active functional groups was clarified by Fourier-transform infrared spectroscopy (FTIR). Antibacterial activity of MO and Nano-MO was assessed against reference bacterial strains by using agar well diffusion method. The minimum inhibitory concentration (MIC) of MO and Nano-MO was 3% and 2%, on *Listerea monocytogenes*, *Staphylococcus aureus*, *Salmonella typhi* and *E. coli* bacteria, respectively. While, in inoculation of MO and Nano-MO in cheese complete reduction of examined bacteria was recorded at 3rd week of storage except for *L. monocytogenes* Nano-MO killed it at 2nd week. Overall acceptability (OAA) investigations showed better results of Nano-MO than MO. Nano-MO showed a great antibacterial property against different pathogenic bacteria without any effect on the palatability of cheese which make it an excellent choice as a bio preservative.

Keywords | Moringa oil, Nano-MO, Bacterial load, Preservative, Cheese.

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INTRODUCTION

Cheese is considered a rich source of essential nutrients such as protein, vitamins, minerals, short chain fatty acids and some trans fatty acids; it plays an important role in human nutrition in many parts of the world (Khorshidian et al., 2018), but is also prone to contamination by different harmful bacteria; thus the world intended to use preservatives as a critical step in cheese making (Mena and Pamela, 2020).

Several studies have been conducted on many plant-derived essential oils (EO) used to prevent pathogenic bacteria from causing significant human diseases (Basuny et al., 2022), these essential oils are environmentally friendly and bio friendly with antioxidants properties and antibacterial properties extend the shelf life and safety of food (Ekpo et al., 2019).

One of the most important oils is Moringa oleifera, which is a good source of antioxidants and has many uses, including a natural food supplement and preservative due

to its bioactive, antimicrobial , antioxidant properties and has great potential use in functional food formulations that can promote nutritional and health benefits (Anwar et al., 2007; Fabiane et al., 2021).

Moringa oil and its palmitic, stearic, and oleic acids make it a promising new potential anti-infective strategy to combat multi-drug resistant pathogenic bacteria such as *E.coli, Candida albicans, Enterobacter, Streptococcus, Pseudomonas aeroginosa, Salmonella and S.aureus* (Nepolean et al., 2009). However the antibacterial mechanism of MO is still unclear (Haiying et al., 2020).

Recently, nanotechnology has been introduced in food preservation due to the stability of the liquid dispersion, resulting in interfacial tension between the two liquids wherever they come in contact due to different attractive interactions between the molecules of the two liquid phases. This tension is also reduced by the addition of amphiphilic surfactant molecules (Amin and Das, 2019).

Therefore, we tried to prepare a nano-emulsion from MO and investigate the differences in the antibacterial activities of MO and Nano-MO against gram-positive (*L. monocytogenes and S.aureus*) and gram-negative (*Sal.typhi and E.coli*) bacteria *in vitro* and *in vivo* after inoculation in white cheese to see if they have an antibacterial effect and might be used as a natural preservative.

MATERIALS AND METHOD

Nano-emulsion (NE) preparation

Preparation of Nano-MO: Moringa olifera oil was purchased from the National Research Center in Egypt's Moringa oil extraction unit. We bought Tween*80 (polyethylene glycol sorbitan monooleate) from Sigma Aldrich. From the Molecular Biology Unit at Assiut University, deionized water was collected.

To detect how much EO and surfactants are needed to make the most stable NE in an O/W nano-emulsion of one oil at room temperature varied according to the viscosity of the oil. According to (Tirmiara et al., 2019) the Nano-MO was produced but instead of DMSO, Tween*80 and MO were added in a 1:3 (v/v) ratio, and the mixture was then centrifuged at 500 rpm for ten minutes using a magnetic stirrer. Then it was sonicated at 750W for five minutes at 20 kHz in a USH650 ultrasonicator with a 750 watt max output then filter the NEs at 0.22 m (200 nm), (Elsherif and Al Shrief, 2021).

Characterization of NEs

The prepared NEs' mean droplet size and polydispersity index (PDI) were calculated using dynamic light scattering (DLS) in Unit of Nanotechnology, Giza. Animal Health Research Institute, Egypt. Utilizing a Zeta-Sizer (3000HS, Malven Instruments, Malvern, United Kingdom) at a 173 degree fixed dispersed angle. Three measurements were made at a temperature of 25 degrees Celsius. Zeta-sizer® software (version 7) was used to gather and evaluate the data. The Chemistry Program at the Science of Faculty, Assuit University made use of infrared spectroscopy using Fourier transform (FTIR, NICOLET, IS10, and Thermo Scientific) to determine the functional groups as well as their modes of attachments and the fingerprint of the molecules. An appropriate approach, such as the potassium bromide pellet method was employed to get samples ready for FTIR. Nujol ponders in a FTIR spectrometer were scanned in the wave number range of 4000- 500^{-1}cm followed by the sample.

To evaluate the morphology of the produced NEs, the Electronic Microscope Unit at Assuit University used a TEM (JEOL-100CX II).

Small drop of Nano-MO was applied on 200-mesh copper grids coated at room temperature and the grids were then negatively stained for three hours with uranyl acetate. After three minutes diluted with deionized water and the surplus liquid was dried using Whatman filter paper.

Bacterial suspension preparation

E.coli (ATCC:9637), *Sal.typhi* (ATTC:19430), *S.aureus* (ATTC: 29213) and *L.monocytogenes* (NCTC: 13372 ATTC® 7644) are the bacterial strains examined in this study. They were obtained from licensed food lab at (AHRI), Giza, Egypt and cultivated on selection broth according to (BAM, 2022) for *E.coli*, (ISO, 2022) for *Sal. typhi*, (ISO 6888-1: 2021) for *S.aureus* and (ISO 11290-2:2017) for *L.monocytogenes* and incubated before being inoculated into selective agar. Each bacterial strain's pure colonies were injected into 5 ml of saline and evaluation of antibacterial activity of MO and Nano-MO against the examined strains was performed after vortex the bacterial suspension and comparing it to a concentration of 0.5 McFarland Standard as per (McFarland, 1907) then diluted to be justified to 10^7.

Evaluation of the antibacterial activity of MO and Nano-MO against bacterial strains (*in vitro*)

Approximately 0.1 ml of oil was dissolved in 5.0 ml of DMSO (dimethyl sulfoxide) at a concentration of 200 mg/ml, followed by several dilutions in DMSO to give concentrations from 1%, 2% and 3%.

Antibacterial assay for different concentrations of MO and Nano-MO was performed by agar well diffusion method

using nutrient agar medium with minor modifications according to (Dodiya and Amin, 2015).

Nutrient agar was poured into petri dishes and allowed to solidify; pathogenic strains (0.1 ml previously prepared tested microorganisms) were applied to the surface of the agar with a sterile cotton band. The plates were allowed to stand for 2 hours at 37°C to saturate the agar with pathogenic strains. A well was made with a cup drill (0.5 cm) and 50 µL of each concentration of MO and Nano-MO was inoculated directly into the well. Plates were incubated at 37°C for 24 hours. After the incubation the zones of inhibition around every well were measured in millimeters. The test was performed in triplicate.

Anti-bacterial effect of MO and Nano-MO in white cheese (*in vivo*)

Cheese was prepared according to (El-Kholy et al., 2016) with slight modification. Fresh buffalo milk was heated at 80°**C** for 15 seconds, then rapidly cooled to 37°C, 1 ml aliquot of each prepared pathogen suspension was inoculated into 100 ml of pasteurized milk prior to cheese making and initial counting then $CaCl_2$, NaCl and rennet were added with concentrations about 0.02%, 3% and 0,05% (w/v) respectively added. To validate the experiment under perfect a septic conditions cheese lots were divided into negative control (no treatment and tested microorganisms) and positive control (inoculated with *E.coli, S.aureus, Sal. typhi* and *L.monocytogenes* alone without treatment). The groups inoculated for each microorganism were divided into two groups (3% MO and 2% Nano-MO). These concentrations were detected using the MIC assay. All experiment was performed at 4°C. For tenfold serial dilution, 25 gm from cheese was added a aseptically to 225 ml of 0.1% peptone water and 1 ml was plated on EMB, Baird Parker, XLD and ALOA agar plates to enumerate *E.coli, S.aureus, Sal.typhi* and *L.monocytogenes,* respectively. Plates were incubated for 24 hours at 35°C according to (BAM, 2022) for *E.coli,* (ISO 6888-1, 2021) for *S.aureus*, (ISO, 2002) for *Sal.typhi* and (ISO11290-2, 2017) for *L.monocytogenes*.

Organoleptic analysis

Negative control samples containing 3% MO and 2% Nano-MO were examined by a panel of thirty (30) judges familiar with the sensory characteristics of the cheese based on five attributes: color, taste, smell, texture and over all acceptability using a 9 points hedonic scale. The most acceptable cheese received 9 points and the most un-acceptable received 1 point (Badmos and Abdulsalam, 2012).

Statistical analysis

Statistical analysis for all experiment was performed in triplicate. It was performed using GraphPadPrism 5.0 (GraphPad, Inc.,San Diego, USA) and statistical 12.0 (Dell, Inc., Tulsa, USA) to determine the statistical significance of the samples. Bacterial counts were presented as mean ± SE. the data is presented using Microsoft Excel sheet.

RESULTS AND DISCUSSION

Nowadays people think of food not to fulfill starvation only but they want to anticipate nutrition-related illnesses and make strides consumers' physical and mental well-being (Roberfroid, 2000). Most of bio preservatives have appeared its ability to minimize the food contamination as well as minimize the frequency of foodborne infections caused by nourishment deterioration microscopic organisms (Mena and Pamila, 2020).

According to many studies, MO has antimicrobial activity and inhibits many harmful bacteria including *S.aureus, E.coli, Pseudomonas aeruginosa* and *Bacillus subtilis* (Saadabi and Zaid, 2011). Its mechanism of action either kills the microorganisms (bactericidal) or inhibits its growth (bacteriostatic), (Lockett and Louis, 2000; Anwar and Rashid, 2007).

From the data presented in Table (1) it was obvious that the test was done to detect the antibacterial activity of MO and Nano-MO against *E.coli* and *Sal.typhi* as gram negative bacteria and there are significance difference of the antibacterial activity between MO and its Nano emulsion. Zones of inhibition of hydrolysate were measured and recorded. The mean of MO inhibition was 27.75±0.04 and 25±0.3 mm at 3% for *E.coli* and *Sal.typhi*, respectively. While Nano-MO the means was 30±0.11mm and 27±0.08 at 3% while at 2% it was 28±0.05mm and 26±0.22mm. The MIC for MO and Nano-MO were 3% and 2%. There are significance differences ($p < 0.05$) between MO and its Nano emulsion for their antibacterial activity. As a result, the prior concentrations used for inoculation in cheese were studied to see if they had any influence on these microorganisms in the food system.

In Table (2) the data cleared the antibacterial activity of moringa oil and its nano-emulsion against some gram positive bacteria as *S.aureus* and *L.monocytogenes.* Zones of inhibition of hydrolysate were measured and recorded. The mean of MO inhibition was slightly higher; it was 29.75±0.5mm and33±0.6mm, respectively for *S.aureus* and *L.monocytogenes.* In Nano-MO the means were 31±0.7mm and 37±0.7mm at 3% while, 29±0.2mm and 35±0.5mm at 2%. The MIC for MO and Nano-MO were 3% and 2%, which used in inoculation of *S.aureus* and *L.monocytogenes* in white cheese experiment.

Our findings were similar to (Othman and El-Mongy,

Table 1: Antibacterial activity of moringa oil and Nano-MO at different concentrations against *E.coli* and *Sal.typhi* by agar well diffusion method.

Concentrations	Inhibition zone (mm) ± SE			
	Moringa oil		Nano-MO	
	E.coli	*Sal.typhi*	*E.coli*	*Sal.typhi*
3%	27.75±0.04	25±0.3	30±0.11	27±0.08
2%	0	0	28±0.05*	26±0.22
1.5%	0	0	0	0
1%	0	0	0	0

* Significantly different (P < 0.05).

Table 2: Antibacterial activity of moringa oil and its nano-emulsion at different concentrations against *S.aureus* and *L.monocytogenes* by agar well diffusion method.

Concentrations	Inhibition zone (mm) ± SE			
	Moringa oil		Nano-MO	
	S.aureus	*L.monocytogenes*	*S.aureus*	*L.monocytogenes*
3%	29.75±0.5	33±0.6	31±0.7	37±0.7
2%	0	0	29±0.2*	35±0.5
1.5%	0	0	0	0
1%	0	0	0	0

* Significantly different (P < 0.05).

Table 3: Physical properties of formulated Nano-MO.

Type	PDI	z-average (d.nm)	size±SD	% of intensity
Moringa NEs	0.319	48.15	76.04±51.13	100%

2016) who reported that MO had variable antibacterial activity against all tested bacterial strains while (El-Sayed et al., 2017; Ali et al., 2001; El-Gammal et al., 2017) showed that MO had a strong antibacterial activity against gram-positive bacteria than gram-negative one. The diameter zone of inhibition increased about 7 mms against the gram-positive microorganism than the gram-negative ones.

These findings may be due to the biological precursor of glucosinolate glucomoringin the activated framework of MO which hindered gram-positive bacteria such as *L. monocytogenes* and *S.aureus* interfering with cell division and the structure and composition of the layer, actuating oxidative push, influencing cell motility and preventing DNA replication (Wen et al., 2022).

The better antimicrobial action of Nano-MO may be due to rupture of bacterial cell membrane (Linklater et al., 2020), an exacerbated permeability process that affects cellular metabolism destroys genetic material ions leakage leading to pathogen damage (Doost et al., 2020).

Nano-MO was prepared as shown in Figure (1) by adding Tween 80, safe surfactant and used in the pharmaceutical and food industries (D'Agostino et al., 2019). In addition high ultrasound intensity produces massive cavitation of small bubbles and more lipophilic hydrophilic micelles leading to a reduction in nano emulsion size (Kumar et al., 2011; Hassanien et al., 2021b).

Figure 1: difference between MO and its nan-emulsion

Using the zeta sizer, the PDI was (0.319) indicating the stability of the nano product and the dynamic nano diameter was 48.15 (Table 3). As the PDI of the prepared NEs was less than 0.5 it showed stability and good homogeneity and the surfactant ratio was used to prevent from coalescing at room temperature and for long time. Moreover since

PDI indicates droplet size homogeneity in nano-emulsion, the higher value of PDI, the lower uniformity of droplet size of NEs. In addition, DLS analysis is not suitable for samples with a very wide size distribution and a PDI value greater than 0.7 (Nirmala et al., 2020; Elsherif and Al Shrief, 2021).

FTIR used to detect functional groups and their attachment methods and molecular fingerprinting. The energy difference (E) between the excited and ground states of the molecules is used in IR spectroscopy (Tatiana et al., 2013). The interaction between surfactant (Tween 80) and EO to convert it to NE can be seen in the difference of this peak (Shakeel et al., 2008). In addition the aromatic bond is crucial, it determines the stability of the generated NE by saturation or if unsaturated it also shows the aromatic substitution pattern. The inclusion of other functional groups and the differences in the peaks of NEs can be the main reasons for their nano properties, stability and antibacterial activity.

TEM was presented as spherical, dispersed without aggregation or agglomeration or aggregation in nano form. This confirms the effectiveness of Nano-MO against the investigated microorganisms (Figure 2). TEM results of the prepared NEs showed sufficient degradation ability which was consistent with DLS test results. These results were almost similar to (Justina and Syurya, 2018; Tirmiara et al., 2019) who found that loaded Eos of the NEs had a spherical form and were mono or dispersed with a regular distribution. In addition, the shape and size of NEs allowed them to penetrate the bacterial cell wall and increasing the antibacterial inhibition (Athikomkulchai et al., 2021).

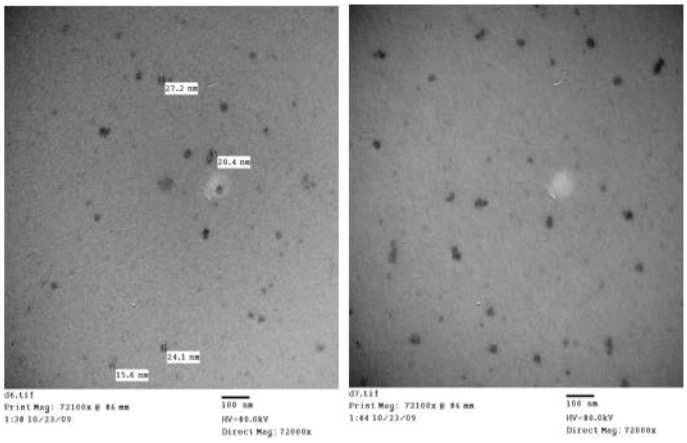

Figure 2: TEM image of spherical Nano-MO with an average size 24.1 nm

The formed NEs in this investigation showed a tiny DP, which was essentially identical to those reported by Hassanien et al (2021a).

Cheese can contain harmful and multi-resistant strains that represent an open health risk such as *E.coli* (Imre et al., 2022) and also *Salmonella spp.* which are the most common pathogenic microscopic organisms in humans and animals and cause salmonellosis (Jones et al., 2004). Also, the listeriosis risk per serving of soft-ripened cheeses made from unpasteurized milk is also estimated to be high (Nüesch-Inderbinen et al., 2021). *S.aureus* was found in many of the soft cream cheese samples tested and its high count may an index of enterotoxin production leading to cases of food poisoning (Carmo et al., 2002; Johler et al., 2015). These findings highlight the need for stricter hygiene practices and using of natural preservatives to prevent microbial contamination, especially for traditional cheese.

White cheese which was manufactured from raw milk after boiling in the laboratory and inoculated with the MIC detected in vitro as following 3% for MO and 2% for Nano-MO to see if these concentrations had any influence in the food system. These concentrations revealed good result in reduction count of *E.coli*, (Figure 3) the reduction started from the 3rd day and the microorganism was killed at 3rd week. Also, for *Sal.typhi,* the reduction appeared obviously from the first week (Figure 4).

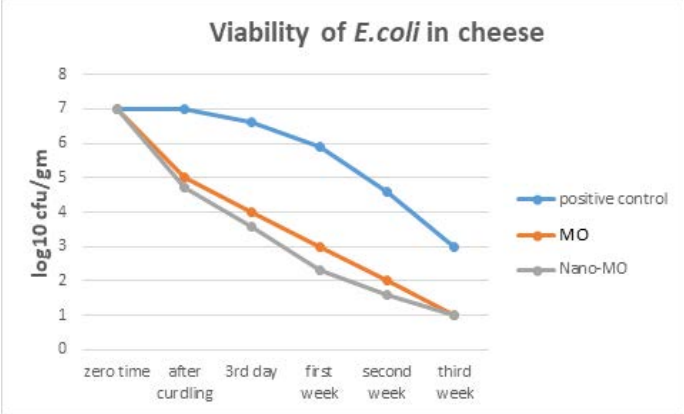

Figure 3: The efficacy of moringa oil and Nano-MO against *E.coli* inoculated in white cheese.

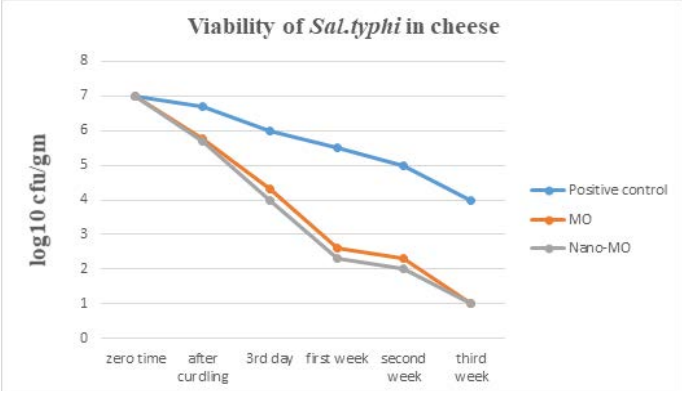

Figure 4: The efficacy of moringa oil and Nano-MO against *sal.tphi* inoculated in white cheese.

Count of *S.aureus* during the storage of cheese in refrigerator completely disappeared at 2nd week in MO treatment and at 3rd week for Nano-MO (Figure 5). While in *L.monocytogenes* the Nano-MO killed it at 2nd week (Figure 6).

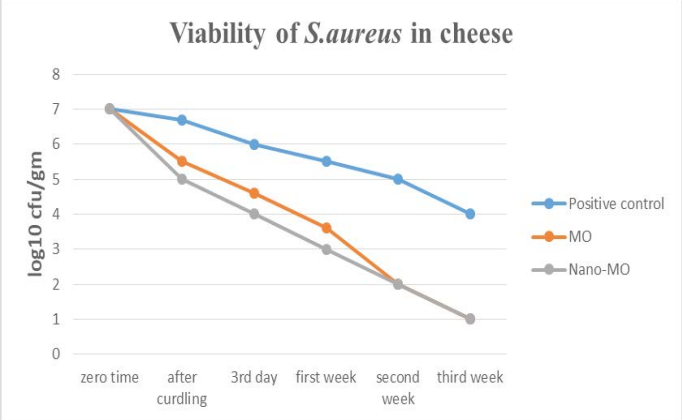

Figure 5: The efficacy of moringa oil and Nano-MO against *S.aureus* inoculated in white cheese.

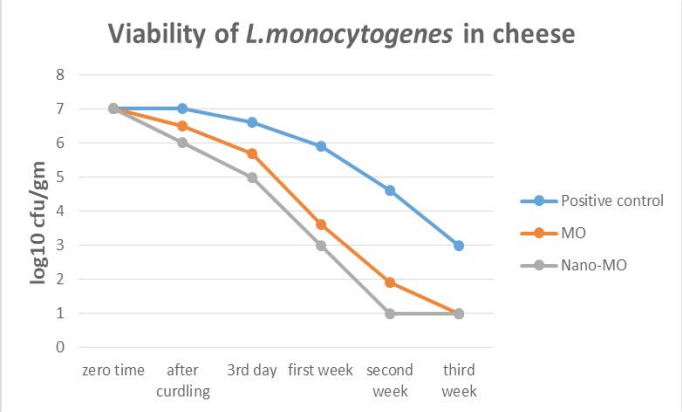

Figure 6: The efficacy of moringa oil and Nano-MO against *L.monocytogenes* inoculated in white cheese.

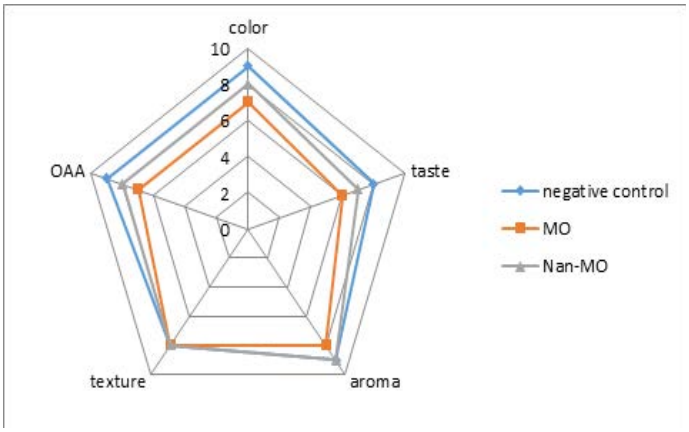

Figure 7: Organoleptic of MO and Nano-MO in laboratory manufactured white cheese.
OAA: Over All Acceptability

The reduction in bacterial count of cheese treated MO may be related to the presence of several bioactive components in moringa plant such as flavonoids, phenolic, alkaloids, terpenoids and steroids. These antibacterial substances inhibiting the enzyme bound membrane activity or disrupt microorganism's DNA for some pathogenic bacteria (Bennett et al., 2003; Li-Weber, 2009).

Several types of nano composites have been classified based on their stability and small droplet sizes of 20-200 nm (Solans et al., 2003). In addition, increasing the ratio of surfactant to Eos increases the surface area and reduces stress, increasing the interaction surface area, thermodynamic stability and bioavailability, all of which NE an effective food preservative. The use of MO for fortification of dairy products such as cheese has been reported at various concentrations up to approximately 3% (He et al., 2010; Hekmat et al., 2015; Kuikman and O'Connor, 2015; Salem et al., 2013) and lead to increase texture, flavor and shelf life of cheese (Fatma et al., 2018).

Moringa oil and its nano emulsion were different in OAA with control one and that may returned to the nature of Nano-MO which is white in color as the color of cheese that made them has no color effect in cheese. In addition, the nano-emulsion nearly have not any taste so; there is no effect on taste of manufactured cheese, that reflect the agreement in OAA with control while the application of MO has slightly nutty flavor and yellow color (Figure 7). In spite that (Haiying et al., 2020) showed that MO addition in cheese could effectively extend shelf life without affecting sensory evaluation.

CONCLUSION AND RECOMMENDATIONS

This study was aimed to detect the efficiency of MO and Nano-MO as anti-bacterial compound against some food borne pathogens and it was found that 2% of Nano emulsion of moringa oil could prevent (*E.coli and Sal.typhi*) as gram negative bacteria and (*S.aureus and L.monocytogenes*) as gram positive bacteria growth with significant zones of inhibition. Also, in cheese could reduce the count of tested organisms as early as the 2nd and 3rd week of storage. The findings revealed that 2% of Nano-MO suspension improved the shelf life and providing good consumer satisfaction; therefore should be used as a natural additive in Egyptian dairy products.

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nano materials and her contribution to this study.

CONFLICT OF INTEREST

No conflict of interest.

NOVELTY STATEMENT

The research sheds light on moringa oil and its nano-emulsion with the perfect concentration and the technique used in preparation of nano emulsion is new.

AUTHORS CONTRIBUTION

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Dina Nour- Eldin Ali ,Sayed el Habtey and Manal M. Amin. The first draft of the manuscript was written by Dina Nour- Eldin Ali and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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