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Antibacterial potential of black seed oil and its nanoemulsion against *Listeria monocytogenes* and *Salmonella* Typhimurium in yoghurt

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ABSTRACT

The current study aimed to evaluate the antibacterial efficacy of black seed oil (BSO) and its nanoemulsion (BSO-NE) against two foodborne pathogens, *Listeria monocytogenes* and *Salmonella* Typhimurium, during the processing and storage of yoghurt. Additionally, the sensory properties of the resulting yoghurt were assessed. Firstly, BSO-NE was prepared and characterized using zeta-sizer and Transmission electron microscopy (TEM). Then, the antibacterial activity and minimum inhibitory concentration (MIC) were determined via a resazurin-based microtiter dilution assay. Fresh buffalo's milk was inoculated with *L. monocytogenes* and *Salmonella* Typhimurium (1×10^5 CFU/ml) with the addition of either BSO or BSO-NE. The inoculated milk was then used for yoghurt making. The organoleptic properties and bacterial load of the obtained yoghurt were evaluated during storage in comparison to a control group. This study elucidated that BSO can be successfully produced in a nanoemulsion form, exhibiting acceptable particle size, stability, and morphology. The results demonstrated a strong antibacterial activity of BSO-NE (3.125 mg/mL) against both *L. monocytogenes* and *Salmonella* Typhimurium compared to the control and pure oil groups. Notably, complete eradication of *L. monocytogenes* and *Salmonella* Typhimurium was observed in yoghurt formulated with BSO-NE on the 8th day of storage. Interestingly, the antibacterial effect of BSO and BSO-NE on *Salmonella* Typhimurium was approximately similar throughout the experiment. Moreover, the results indicated that the addition of BSO-NE to yoghurt enhanced its organoleptic properties. Overall, these findings suggest that yoghurt can be fortified with BSO-NE to prevent bacterial contamination by *L. monocytogenes* and *Salmonella* Typhimurium in the dairy industry.

Introduction

Yoghurt is one of the oldest and most popular dairy products worldwide that has gained the consumers' acceptance as a healthy food. Yoghurt is a traditional fermented milk products that produced by the addition of acid forming bacteria (starter culture) into milk to induce fermentation (Shiby and Mishra, 2013). Starter culture of yoghurt includes *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Chen *et al*., 2017) that are responsible for the typical yoghurt flavor. During production of yoghurt, milk sugar (lactose) is fermented and converted into lactic acid (glycolysis) which leads to formation of the curd (Ani *et al*., 2018). Besides, proteolysis and lipolysis are also developed during manufacturing of such food matrix (Chen *et al*., 2017). Yoghurt is a main source of vitamins, minerals, and calcium that are essential for consumer's health (El-Abbadi *et al*., 2014). Furthermore, it could provide health benefits to the consumers as in case of lactose intolerance since starter cultures of yoghurt can enhance lactose digestion (Bichi, 2012; Saborido and Leis, 2018). Yoghurt is highly susceptible to contamination with pathogenic and spoilage microorganisms due to its richness with nutritional substances (Pal *et al*., 2015). Hence, it is crucial to add some additives during manufacturing of yoghurt to improve its quality and positively extend its shelf-life.

Milk and milk products have been implicated in the transmission of several pathogens including *Salmonella* that is a major concern in the dairy industry as it responsible for many outbreaks of illness worldwide. *Salmonella* can contaminate milk products through contaminated raw milk or direct contact with the contaminated utensils and surfaces after pasteurization (Plym and Wierup, 2006; Carrasco *et al*., 2012). While during yoghurt making, the occurrence of *Salmonella* may be due to heavy raw milk contamination or insufficient heat treatment as well as lack of hygiene (Szczawiński *et al*., 2014). Salmonellosis induce gastrointestinal symptoms in human and may develop into bacteremia with systemic complications. Moreover, death may be occurred in children and immuno-compromised persons (Scallan *et al*., 2011; Center for Disease Control and Prevention, 2019). The most common foods that associated with the recorded outbreaks of salmonellosis around the world are non-pasteurized milk, milk products such as powder milk, cheese, ice creams, and butter made from raw milk, beef, chicken, chicken eggs and pork meat (CDC, 2018; Castañeda-Salazar *et al*., 2021). Notably, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most common foodborne pathogens out of more than 2600 *Salmonella* serovars in food sector (Guibourdenche *et al*., 2010; Castañeda-Salazar *et al*., 2021). On the other hand, *Listeria monocytogenes* is of paramount importance as a public health hazardous that raised the global concern for a long time. It can grow easily at a wide range of temperatures (from -0.4 to 45°C) and pH of 4.0 to 9.6, and also tolerate the osmotic stress until 14% of salt (Hamidiyan *et al*., 2018). Listeriosis is the foodborne disease caused by *L. monocytogenes* in human that showed several symptoms such as diarrhea, vomiting, fever, headache, gastroenteritis, and myalgia. Furthermore, septicemia, meningitis and abortion in pregnant women are the most common symptoms (Rahimi *et al*., 2010; Akrami-Mohajeri *et al*., 2018; Bashiry *et al*., 2022). Milk, in particular raw milk, and dairy products are the potential sources of *L. monocytogenes* (Ashraf and Ashraf, 2017; Bashiry *et al*., 2022; Tadesse *et al*., 2024).

In recent decades, using of natural food additives have paid more attention from researchers and consumers in order to increase the food safety and avoid the negative health effects of synthetic antimicrobials (functional food additives) (Fei *et al*., 2018; Guo *et al*., 2019). One of these natural food additives that has been known for centuries as antimicrobial and antioxidant is *Nigella sativa* (Srinivasan, 2018). *Nigella sativa* is commonly known as black seed, blessed seed, black caraway or black cumin

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(Burdock, 2022). Black seeds are rich in protein, carbohydrates, crude fiber and large amounts of minerals as copper, phosphorus, zinc and iron (Tulukcu, 2011). Additionally, black seed is a valuable and popular functional additive that was used in food industry long time ago in a variety of foods such as dairy products (Mediterranean cheeses and yogurt), meat dishes, tea, pickles, sauces, salads and baked goods (Benkaci-Ali *et al*., 2007; Tiruppur Venkatachallam *et al*., 2010; Kıralan, 2014; Burdock, 2022).

Interestingly, black seed oil is a valuable essential oil extracted from black seeds. It contains large amount of polyunsaturated fatty acids (omega-6, omega-9, linoleic and oleic fatty acids) (Tulukcu, 2011). Of note, black seed oil is a stable oil that is not susceptible to oxidative changes (Kıralan, 2014; Górska-Horczyczak *et al*., 2023). Black seed oil showed different activities in vivo and in vitro such as anti-inflammatory, antioxidant, anti-tumor, spasmolytic, immunomodulatory, analgesic, spasmolytic and anti-metabolic disorders (Ali and Blunden, 2003; Salman *et al*., 2008; Górska-Horczyczak *et al*., 2023). In previous studies, BSO showed antibacterial activity against *Salmonella*, *Listeria monocytogenes*, *Shigella* and *Staphylococcus aureus* in food (Burt, 2004; Hassanien *et al*., 2015; Bakal *et al*., 2017). These biological actions induced due to presence of different phytochemicals in such essential oils as thymoquinone, thymohydroquinone, thymol and carvacrol (Kanter, 2008; Mariod *et al*., 2009). In this sense, the functional properties of black seed oil and its good stability make it a useful component in food industry. However, the food application of BSO is still limited due to its strong organoleptic properties, low water solubility and high volatility (Liao *et al*., 2021). To avoid such problems, oil nanoemulsions have been developed and incorporated in food chain (Mahdi Jafari *et al*., 2006; Anton *et al*., 2007).

Nanoemulsions are colloidal dispersions with droplet sizes ranging from 20 to 200 nm (Kasaai, 2024). They offer enhanced stability, bioavailability, and delivery of bioactive compounds compared to conventional emulsions (Lee *et al*., 2024). Nanoemulsions were added to several food matrices due to their unique functional properties in food production (Mahdi Jafari *et al*., 2006; Anton *et al*., 2007). Consequently, nanoemulsions can be easily used in dairy products to increase its nutritive value and safety (Cheong *et al*., 2016). Notably, BSO may be used efficiently in nanoemulsion formulation (ALRashdi *et al*., 2024). For instance, Black seed oil nanoemulsion (BSO-NE) was previously added in ice cream to improve its physical properties and customer acceptance (Mohammed *et al*., 2020). To the best of our knowledge, there are no previous data on studying the antibacterial effect of BSO-NE toward *Listeria monocytogenes* and *Salmonella* Typhimurium in yoghurt. Therefore, the objective of the current study was to produce BSO-NE and determine the functional properties during production and storage of yoghurt.

The purpose of this study was to synthesize and characterize the nanoemulsion prepared from black seed oil using zeta-sizer and Transmission electron microscopy (TEM), as well as investigating the antibacterial effect of black seed oil and its nanoemulsion against foodborne pathogens (*Listeria monocytogenes* and *Salmonella* Typhimurium) in yoghurt. In addition, we evaluated the sensory properties of the formulated yoghurt after addition of the oil and nanoemulsion during storage period.

Materials and methods

Procurement and preparation of black seed oil

Black seed oil was purchased from the National Research Center, Egypt. It was dissolved in sterile dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2650) to prepare the stock solution. Then, two-fold serial dilutions were performed to obtain a concentration ranged from 1% to 0.03125%.

Preparation of nanoemulsion

Oil-in-water nanoemulsion was prepared according to Khairan *et al*. (2024). In brief, 5% (v/v) Tween 80 was thoroughly mixed with 3% Span 20, then the mixture was added to 2% propylene glycol in deionized water at room temperature. The mixture was stirred using a magnetic stirrer for 30 minutes to ensure homogeneity. Subsequently, BSO was added dropwise to the surfactant solution and stirred for 15 minutes using a hotplate stirrer (DAIHAN Scientific Co., Ltd., Korea). The obtained emulsion was then sonicated for 20 minutes using an ultrasonic homogenizer (USH650, max power: 650 watts, frequency: 25 kHz) to get the nanoemulsion. The prepared nanoemulsion were stored at 25°C for further analysis.

Characterization of the essential oil nanoemulsion

Particle size and Polydispersity index (PDI) measurements for BSO-NE

The diameter of BSO-NE droplets was measured using dynamic light scattering (NICOMP 380 ZLS, Dynamic light scattering (DLS) instrument, USA) by photon correlation spectroscopy. Samples were diluted in purified water prior to analysis to avoid multiple scattering effects. Additionally, PDI of the prepared nanoemulsion were measured at 25.0±0.2°C using Zetasizer (3000 HS, Malvern Instruments, Malvern, UK) at the faculty of pharmacy, Al-Azhar University, Assiut, Egypt.

Transmission electron microscope (TEM)

TEM analysis was carried out in the Electronic Microscope Unit, Assiut University to confirm the size measurements and determine the morphological characteristics of the obtained nanoemulsions using JEOL JEM-1230 transmission electron microscope (JEOL, Japan). The sample was diluted with deionized water (100 times), then one drop of the diluted sample was placed on a 200-mesh film grid and dried using Whatman filter paper at room temperature. Further, samples were stained with uranyl acetate and allowed to dry for 10 min before observation with the electron microscope.

Bacterial strain and culture conditions

Listeria monocytogenes ATCC®7644 and *Salmonella* Typhimurium ATCC®14028 were obtained from the Center of Food Safety at the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt. Frozen stock cultures of the examined pathogens were stored at -70°C, then thawed to ambient temperature after which 10 µl of each culture was passed into 10 ml of BHI broth and incubated at 37ºC for 24h. After the first passage, *L. monocytogenes* and *Salmonella* Typhimurium were cultured on *Listeria* agar base (Oxford ISO 11290-1) and xylose lysine desoxycholate agar (XLD), respectively, and incubated at 37ºC for 24h. Further, the cultures were again inoculated into 10 ml of BHI broth and incubated under the same conditions as above. Before inoculation in milk, the inoculum was washed twice in phosphate buffer saline (PBS) (Oxoid), then re-suspended in skim milk and kept at refrigerator temperature until be used for the experiments to determine the antimicrobial effects of BSO and BSO-NE in yoghurt.

Preparation of resazurin solution

Resazurin powder (337.5 mg) was dissolved in 50 ml sterile distilled water to prepare Resazurin solution. Then, the mixture was thoroughly mixed using a sterile vortex mixer for 1 h in dark to ensure homogeneity and the obtained solution was then kept in a brown bottle to prevent exposure to light (Teh *et al*., 2017).

Determination of the Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MICs) values of BSO and BSO-NE against *L. monocytogenes* and *Salmonella* Typhimurium were calculated using resazurin-based microtiter dilution assay according to

Abdelaziz *et al*. (2015). Briefly, ten wells in a vertical row were filled with 100 µL of BHI broth. The first well of each vertical row contained 100 µL of BSO (500 mg/mL) or BSO-NE (50 mg/mL) for *L. monocytogenes* and *Salmonella* Typhimurium. Then, 100 µL of the mixture in the first well was transferred into the second well (2^{-2}) using a separate and sterile pipette and mixed then thoroughly. The serial dilution was repeated from the second well into the third well (2^{-3}) and so on until the tenth well (2^{-10}). The final concentration of the essential oil and its nanoemulsion was one-half of the original concentration in previous well. Then, 100 µL of the bacterial culture (10^5 CFU/ mL) was added into all wells and mixed thoroughly. Plates were then overnight incubated at 37°C (Chan and Mat Don, 2013). After incubation, 20 µl of resazurin dye was added to each well, and the plates were incubated in an incubator shaker at the same temperature for another 1 h. The change of purple color into pink to red indicated the active bacterial cells, while the presence of dark blue color pointed out to the inhibition of the bacterial growth. The MICs were determined as the lowest concentrations of BSO or BSO-NE in the wells that still in blue color. All experiments were conducted in triplicates.

Antibacterial activity of BSO and BSO-NE against *L. monocytogenes* and *Salmonella* Typhimurium during manufacturing and storage of yoghurt

Fresh milk was pasteurized at 85°C for 5 min in water bath, and then suddenly cooled to 38-40°C. The prepared inoculums were added to the warmed milk (41°C) in a count of 10^5 CFU/ mL. The inoculated milk was divided into 6 equal portions for further use as follows: (1) plain sample contained BSO or BSO-NE for sensory evaluation (used as a negative controls), (2) samples inoculated with *L. monocytogenes* or *Salmonella* Typhimurium without BSO or BSO-NE (considered as a positive control), (3) *L. monocytogenes* + BSO, (4) *L. monocytogenes* + BSO-NE, (5) *Salmonella* Typhimurium + BSO, (6) *Salmonella* Typhimurium + BSO-NE. After inoculation of the different treatments, yoghurt was manufactured according to Sarkar (2016) by adding 2% yoghurt starter culture (*Streptococcus thermophilus* and Lactobacillus delbrueckii subsp. bulgaricus) to milk at 41°C. The prepared yoghurt was placed in a stable-temperature incubator at 40°C until pH reached 4.6 to 4.5. Finally, the obtained products were stored at refrigeration temperature (4.0±1.0°C) for 10 days. Samples were collected at different time points such as just after manufacturing of yoghurt, and after one, three, five, eight and ten days of storage, then tested for the count of *L. monocytogenes* using *Listeria* agar base media (Park *et al*., 2014), and *Salmonella* Typhimurium using XLD (Park *et al*., 2012).

Organoleptic assay of yoghurt

Yoghurt was processed with the addition of BSO or BSO-NE, and without inoculation of pathogens, then used for sensorial evaluation just after processing and during storage of yoghurt (after 1, 3, 5, 8 and 10 days). Thirty-five panelists were selected in teams of different ages, gender and education. Sensorial attributes such as color, flavor, mouthfeel, appearance, and overall acceptability of the prepared yoghurt samples were assessed. The scale points were excellent (5); very good (4); good (3); acceptable (2); and poor (1) (Lawless and Heymann, 2010).

Statistical analysis

Experiments were done in triplicate to better capture microbial variability. Descriptive statistics were calculated from the observed data with MS Excel (Microsoft Corporation). The statistical analysis performed consisted of means comparison tests, univariate Analysis of variance (ANOVA) followed by Tukey post-hoc test ($P < 0.05$) to evaluate significant differences in the levels of *L. monocytogenes* or *Salmonella* Typhimurium in yoghurt. The SPSS v22.0 software (Chicago, Illinois, USA) was used for statistical analyses.

Results

Characterization of the black seed oil nanoemulsion

In the present study, nanoemulsion was characterized through determining the droplet size and polydispersity index (PDI) using Zeta sizer. The mean droplet size of the BSO-NE was measured to be 65.8 nm with a PDI of 0.063. The current findings showed that reasonable small-sized particles of BSO-NE were obtained that indicated a good stability of the prepared NEs.

On the other hand, transmission electron microscopy (TEM) was carried out to determine the morphology and size of the biosynthesized nanoemulsion (BSO-NE). TEM analysis was conducted on negatively stained samples. The TEM image of NE revealed that the obtained particles are clearly visible and spherical droplets stained with phosphotungstic acid. Additionally, the mean particle size determined by TEM was 32.083 nm (Fig. 1).

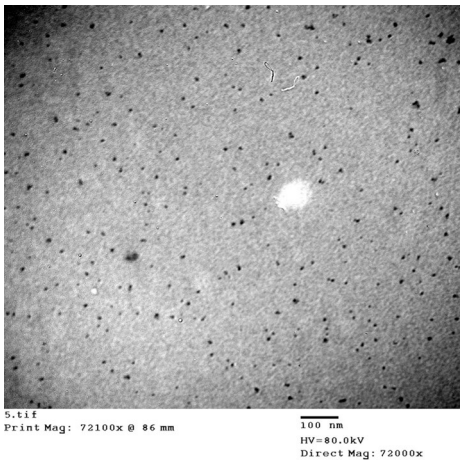

Figure 1. Transmission electron microscope (TEM) for the images of the BSO nanoemulsion with average size of 32.083 nm.

Determination of the Minimum inhibitory concentrations

MICs were examined to evaluate the antibacterial properties of black seed oil and its nanoemulsion against *Listeria monocytogenes* and *Salmonella* Typhimurium. After determining the antibacterial effect of different concentrations, 0.968 mg/mL and 3.125 mg/mL were the MICs of BSO and BSO-NE that could inhibit the *Listeria monocytogenes* strains, respectively. On the other hand, the MICs of BSO and BSO-NE against *Salmonella* Typhimurium was observed at the first well (2^{-1}= 250 mg/ml) for the oil and at the fifth well which was equivalent to 3.125 mg/ml. Notably, the inhibitory effect of BSO toward *Listeria monocytogenes* was higher than that induced against *Salmonella* Typhimurium. While BSO-NE exert similar antibacterial activity against the examined pathogens in the current study.

Antibacterial activity of black seed oil and its nanoemulsion against Listeria monocytogenes and Salmonella Typhimurium *during manufacturing and storage of yoghurt*

Herein, Fig. 2 presented the antibacterial effect of BSO and BSO-NE on *Listeria monocytogenes* and *Salmonella* Typhimurium during making and storage of yoghurt. There was a significant reduction ($P < 0.05$) in the mean count of *Listeria monocytogenes* in BSO-NE treated yoghurt in comparison to control and BSO groups. Notably, the inhibitory effect of BSO on the studied pathogen was approximately similar to the control group at all of the examined time points except at the end of the experiment (day 10) in which we could not detect *Listeria monocytogenes* in BSO treated yoghurt (Fig. 2). While in yoghurt formulated with BSO-NE, the decrease in the average count of *Listeria monocytogenes* was obvious from the beginning of the experiment (zero time) until the 5th day of ex-

amination (3 $\log_{10}$ CFU/g). Then, there was sharp reduction in the level of *Listeria monocytogenes* when compared with control and BSO yoghurts (2.83±0.90 and 2.87±0.27 $\log_{10}$ CFU/g, respectively) (Fig. 2). Interestingly, BSO-NE could induce the highest inhibitory effect ($P < 0.05$) against *Listeria monocytogenes* as the organism failed to be detected after 8 days of storage in comparison to other yoghurt formulations (Fig. 2).

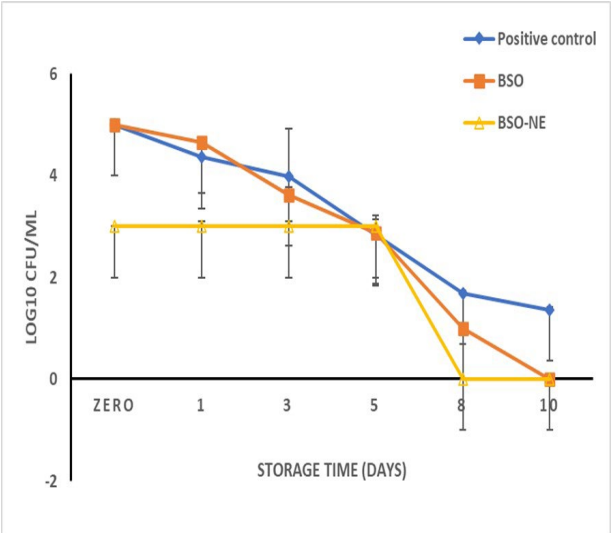

Figure 2. Antibacterial effect of black seed oil (BSO) and its nanoemulsion (BSO-NE) on *Listeria monocytogenes* during manufacturing and storage of yoghurt.

On the other hand, the periodical examination of yoghurt supplemented with BSO or BSO-NE for their antibacterial activity on *Salmonella* Typhimurium showed that there was a drastic decrease in the number of bacterial cells during manufacture and storage of yoghurt versus to control samples ($P < 0.05$) (Fig. 3). The obtained findings were the same in case of BSO and BSO-NE in comparison to control group. In other words, *Salmonella* Typhimurium counts were reduced directly after manufacturing (few hours after getting the finished product) of BSO and BSO-NE formulated yoghurt (Fig. 3). After one day of storage time, the population of *Salmonella* Typhimurium decreased by about 1.2 $\log_{10}$ CFU/g (3.87±0.07 $\log_{10}$ CFU/g) in BSO yoghurt, while around 1 log reduction (4.06±0.06 $\log_{10}$ CFU/g) was observed in case of BSO-NE group in comparison to the initial time and control yoghurt (5.46±0.04 $\log_{10}$ CFU/g). Additionally, *Salmonella* Typhimurium rapidly decreased ($P < 0.05$) during yoghurt storage until became undetectable (< 10 CFU/g) at the 8th of storage BSO and BSO-NE groups in comparison to control yoghurt (1.90±0.17 $\log_{10}$ CFU/g) (Fig. 3).

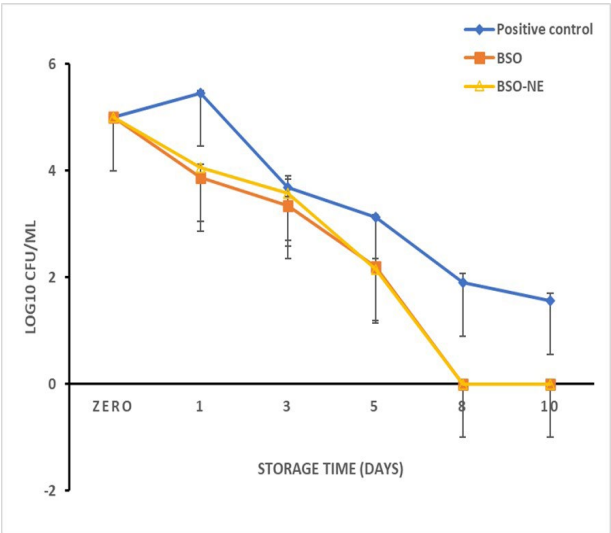

Figure 3. Growth of *Salmonella* Typhimurium during yoghurt manufacturing and storage for 10 days at 7±2 °C. Yoghurt was treated with pure essential oil (BSO) or its nanoemulsion (BSO-NE) in comparison to control group (inoculated with *Salmonella* Typhimurium only).

Overall, the microbial kinetics of *Listeria monocytogenes* and *Salmonella* Typhimurium in control yoghurt compared with the examined essential oil (BSO) and its nanoemulsion (BSO-NE) yoghurts revealed that pure oil and nanoemulsion could decrease the bacterial load of the examined pathogens shortly after manufacturing of yoghurt with undetectable levels at 10 days of storage. Moreover, there was a significant log reduction ($P < 0.05$) in the level of both *Listeria monocytogenes* and *Salmonella* Typhimurium in presence of BSO and BSO-NE when compared to control during making and storage of yoghurt as shown in Fig. 4.

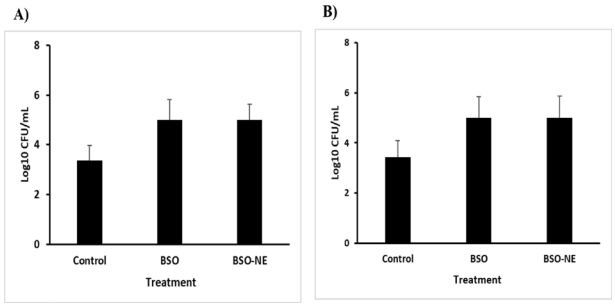

Figure 4. Comparison between the log reduction in the bacterial levels of *Listeria monocytogenes* (A) and *Salmonella* Typhimurium (B) during manufacturing and storage of yoghurt in presence of BSO and BSO-NE versus control.

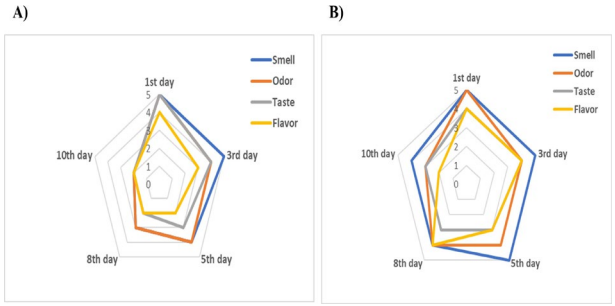

Figure 5. Sensorial evaluation of plain yoghurt (A) and formulated yoghurt with BSO-NE (B) after one, three, five, eight and ten days of storage.

Discussion

Natural food additives have gained researchers' attention for their unique advantages as they could reduce the risk of using antibiotics in food, and had no potential negative health impacts on consumers as expected with the synthetic food additives. Hence, the present study elucidates for the first time in Egypt the inhibitory effect of essential oil (BSO) and its formulated nanoemulsion (BSO-NE) on *Listeria monocytogenes* and *Salmonella* Typhimurium during manufacturing and storage of yoghurt. Interestingly, BSO-NE exhibited the highest antibacterial activity toward the examined pathogens during the initial steps of yoghurt making in comparison to control and pure BSO. Moreover, the formulated nanoemulsion did not induce changes in the sensorial properties of yoghurt (Fig. 5). Consequently, BSO-NE could play a crucial role as a biopreservative against *Listeria monocytogenes* and *Salmonella* Typhimurium in dairy industry.

Herein, we prepared and characterized the formulation of BSO-NE through determination of droplet size and PDI using a Zeta sizer, as well as the morphology was detected by transmission electron microscopy (TEM). The current findings (droplet diameter: 65.8 nm; PDI: 0.063) are significantly smaller than those reported by Jufri and Natalia (2014), Sharif *et al.* (2017) and Mohammed *et al.* (2020). However, a lower particle size of BSO-NE (59.2 nm) was reported by Khorami *et al.* (2024). On the other hand, PDI is a main indicator for detecting the particle size distribution of nanoemulsions. The low values of PDI indicate the narrower distribution (Sharif *et al.*, 2017). There are several factors that significantly affect the mean droplet size of nanoemulsions, such as surfactant type and concentration. Tween 80 effectively reduces the droplet size and enhances the

stability of nanoemulsions (Sugumar *et al*., 2016). When nanoemulsion formulated using non-ionic surfactant, the size of the obtained droplets will be under 70 nm (Sugumar *et al*., 2016). TEM analysis was conducted on the negatively stained samples and the mean particle size of BSO-NE was 32.083 nm that was smaller than the values recorded by Mohammed *et al*. (2020) who observed that the size of *N. sativa* nanoparticles in nanoemulsions was more than 100 nm. In contrast, Meshksar *et al*. (2024) investigated that the nanoemulsion of *Nigella sativa* seed extract has a spherical shape with an average size of 10 nm.

Several studies have examined the antimicrobial effect of some natural antimicrobial compounds of plant origin against foodborne pathogens and clinical isolates. In the present study, Gram-positive bacteria (*Listeria monocytogenes*) was more susceptible to the antibacterial activity of black seed oil in comparison to Gram-negative bacteria (*Salmonella* Typhimurium). The current results were in line with those previously reported with clinical isolates (Kokoska *et al*., 2008; Ugur *et al*., 2016). In addition, Nair *et al*. (2005) and Abo-Neima *et al*. (2023) revealed that the black seed oil expressed a strong antimicrobial impact against *L. monocytogenes*. However, there was no inhibitory activity against *S.* Typhimurium ATCC 14028 (Abo-Neima *et al*., 2023). The potent antimicrobial effect of black seed oil is due to presence of thymoquinonethat gives the oil its medical properties (Abo-Neima *et al*., 2023). Herein, the MIC of black seed oil against *Listeria monocytogenes* (0.97 mg/mL) was much lower than those recorded toward *Salmonella* Typhimurium (250 mg/mL). Ugur *et al*. (2016) noted that the MICs of *Nigella sativa* oil against *S. aureus*, Enterococcus faecalis, E. coli and Pseudomonas aeruginosa strains were 0.5 µg/mL, 2 µg/mL, 64 µg/mL, and 64 µg/mL, respectively. Moreover, the MICs for MRSA and methicillin-resistant coagulase-negative Staphylococci were very low with an average of <0.25–1.0 µg/mL (Ugur *et al*., 2016). However, Abo-Neima *et al*. (2023) found that 50% of black seed oil was the MIC against *L. monocytogenes*. The discrepancy between the MICs obtained in the current study and the previous ones may be due to differences in several factors such as species of the examined microorganisms, extraction methods, level of the active components in the oils and the antibacterial assay methods (Karakaya *et al*., 2011; Mith *et al*., 2014; Ugur *et al*., 2016).

Although there are many studies, with different outcomes, evaluating the antimicrobial effect of pure essential oils on foodborne pathogens, little is known about the inhibitory activity of NEs on spoilage and pathogenic microorganisms. The present study suggests that the antibacterial potential of BSO-NE was higher than the pure oil in vitro. These findings were reinforced by the investigations of Sharif *et al*. (2017) who noticed that the negatively charged black cumin essential oil nanoemulsions exhibited prolonged bactericidal activities when compared with the pure oil due to the better stability of the formulated nanoemulsions. Moreover, Majeed *et al*. (2016) and Ferreira *et al*. (2022) determined slightly lower or equal MIC values for pure oils and their encapsulated form.

Interestingly, the cytotoxic effects of black seed extracts on murine macrophages and liver or kidney function was previously evaluated in several studies (Zaoui *et al*., 2002; Khader *et al*., 2009; Dollah *et al*., 2013), and it was concluded that black seed and its oil are safe as a dietary supplement (Liao *et al*., 2021). During processing and storage of yoghurt in the present study, the inhibitory effect of BSO against *Listeria monocytogenes* and *Salmonella* Typhimurium was higher than the control yoghurt. Application of BSO induces a sharp reduction in the concentration of the studied microorganisms after five days of storage until reaching the undetectable levels (≤ 10 CFU/g) at day 8th of yoghurt storage. Similarly, the inhibitory effect of black seed oil against different pathogens have been proven in other food products. For instance, the addition of black cumin oil during processing of soft cheeses showed a stronger antimicrobial effect than the spices of its seeds (Badawi *et al*., 2009; Ibrahim and Abdel-Hakiem, 2015; Saláková *et al*., 2019). Furthermore, Hassanien *et al*. (2015) evaluated the inhibitory effect of black cumin seeds oil on several foodborne pthogens such as *L. monocytogenes*, *Salmonella* enteritidis, *S. aureus* and *Escherichia coli* during manufacturing and cold storage of soft cheese. The authors found that the best antibacterial activity was reported with *S. aureus* strains (Hassanien *et al*., 2015). In addition, Abd ElmontalebAbd Elmontaleb *et al*. (2020) incorporated black seed oil in Edam cheese and investigated that the total viable count, lipolytic bacteria and, yeast & molds were reduced at the end of cheese ripening, besides the growth of coliform was completely inhibited in comparison to control group. While in the traditional sheep's curd cheese, Voşgan *et al*. (2024) investigated the effect of adding different forms of *Nigella sativa* (seeds, powder, alcoholic extract, and oil) on the quality of such cheese. They determined the *Enterobacteriaceae* colony counts, *S. aureus* and other coagulase-positive staphylococci (CPS) counts, and concluded that the highest antimicrobial effect was recorded in cheese treated with cold-pressed oil (1%) (Voşgan *et al*., 2024). The current results and the findings obtained in the previous studies revealed that the essential oil extracted from black seeds contains several chemical and functional components that characterized by their antimicrobial properties. This enforces the possibility of using black seed oil as natural biopreservative in food industry (Shafodino *et al*., 2022).

It is worth mentioning that the current study represents the first record of utilizing black seed oil nanoemulsion in vivo food bio-preservatives in the dairy industry. Herein, we found that the addition of BSO-NE to yoghurt could reduce the average counts of *L. monocytogenes* and *Salmonella* Typhimurium after yoghurt manufacturing and the drastic decrease was continued till became below the detection limit after 8 days of storage at 7.0±2.0°C. Similarly, Elsherif *et al*. (2024) investigated that the addition of nisin NPs (0.125 mg/mL) during processing of yoghurt showed a strong antibacterial activity against MRSA and E.coli O157:H7 in comparison to control and pure nisin groups. Additionally, nisin NPs could extend the shelf life of the formulated yoghurt than those manufactured without addition of such nanoparticles. Using NEs in the food industry is still elusive; however, our findings provide a theoretical possibility that BSO-NE can be used as a natural antimicrobial to reduce the food safety problems caused by such foodborne pathogens in the dairy industry. In the present study, the sensory evaluation of the yoghurt samples revealed that the yoghurt formulated with BSO-NE was the most preferred by the panelists other than the formulated yoghurt treated with the pure oil. Similarly, Mohammed *et al*. (2020) investigated that BSO-NE improved the physical properties and consumer acceptability of ice cream. On the other hand, Abdol-Samad *et al*. (2016) reported that the high content of microencapsulated BSO induced a bitter taste in yogurt and no significant differences were observed in the color between the control and the formulated samples. Overall, these results suggest that the application of BSO-NE has the potential to be utilized in the yogurt industries for extending its shelf life and other functional properties.

Conclusion

Small-sized particles with low PDI of BSO-NE with good stability were developed, characterized and applied in yoghurt to evaluate their antibacterial activity toward *L. monocytogenes* and *Salmonella* Typhimurium. This study confirms that BSO-NE could exert high antibacterial effects against such pathogens than BSO in yoghurt stored at 7±2 °C. Moreover, the sensory properties of yoghurt formulated with BSO-NE was improved in comparison to control and pure oil group. Overall, these insights develope more sustainable and efficient methods for ensuring the safety and quality of yoghurt in the dairy industry.

Conflict of interest

The authors have no conflict of interest to declare.

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