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Impact of olive leaf extract coating chitosan on *Bacillus cereus* and its toxins in refrigerated beef burger

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ABSTRACT

This study aimed to examine the impact of olive leaf extract (OL-E) and olive leaf extract coating chitosan (OL-Ch) on *Bacillus cereus* and its toxin genes, as well as, the sensory qualities of refrigerated beef burgers. The primary phenolic components of olive leaf extract were oleeuropein and protocatechuic acid, identified by HPLC-DAD analysis. The concentrations of OL-E and OL-Ch that were in vitro effective against *B. cereus* were 62.5 mg/mL and 125 mg/mL, respectively. In burgers treated with OL-E and OL-Ch, viable *B. cereus* dramatically decreased ($p < 0.05$) over time during cold storage. The count decreased to 2 log CFU/g in the OL-E group and 1.3 log CFU/g in the OL-Ch group after thirteen days of preservation. The entFM gene expression levels on day thirteen were 0.09 in the OL-Ch group and 0.2 in the OL-E group. Conversely, the *cyt*K gene expression levels in the OL-E and OL-Ch groups were 0.09 and 0.15, respectively. Both supplements could control physicochemical characteristics (pH, volatile basic nitrogen (TVBN), and thiobarbituric acid (TBA)) to be 6.12±1.55, 16.1±2.98, and 0.869±0.28, respectively, in the OL-E group during a storage period. Moreover, they were 5.79, 14.3±2.79, and 0.816±0.31 in the OL-Ch group. They also did not appear to affect the average sensory score of burgers. Thus OL-E and OL-Ch could be used as natural food additives to prevent *B. cereus* contamination in the meat industry.

Introduction

Beef burgers can be a potentially risky endeavor due to the presence of harmful microorganisms that can thrive and produce toxins if not stored appropriately. As a result, the meat industry has implemented several measures to ensure beef burgers' safety, including using additives with antimicrobial and antioxidant properties. These additives serve to prevent spoilage and enhance the shelf life of beef burgers (Bagheri *et al*., 2021).

Bacillus cereus has been a bacterium that can produce toxins. It can survive with or without oxygen and up take gram stain (gram-positive-bacteria). This type of microorganism is found all over the environment, and it can make spores that allow it to survive in extremely hot or cold conditions for a long time. Therefore, it has been detected as a contaminant in various foods, such as red meat, white meat, rice, beans, and vegetables (McDowell *et al*., 2024). According to the EFSA (European Food Safety Authority), *Bacillus cereus* is responsible for 16-20% of food poisoning cases caused by bacterial toxins, making it the leading cause of foodborne illnesses. *B. cereus* was designated as a human pathogen belonging to risk group 2 (RG2) by the Health and Safety Executive (HSE) in 2013. Pathogens in this group have the potential to cause diseases in humans and pose a risk to workers.

B. cereus is known for its ability to cause illness through the production of two primary toxins: diarrheal and emetic toxins. These toxins can cause symptoms such as vomiting and diarrhea. The diarrheal toxins are believed to include hemolysin BL (*Hbl*), non-hemolytic enterotoxin (Nhe), enterotoxin FM, and cytotoxin K (*Cyt*K), while the emetic toxin is called cereulide (Dietrich *et al*., 2021).

Olive leaf extract (OL-E) is a widely recognized natural plant-based source of phytochemical substances, particularly phenolics (Zhang *et al*., 2022). It has been demonstrated to possess antibacterial properties against various foodborne pathogens, including *Salmonella* spp., *E. coli*, *L. monocytogenes, B. cereus,* and *Staphylococcus aureus* (Borjan *et al*. 2020). Studies have also shown that OL-E can extend the shelf life and improve the quality of beef products (Al-Rimawi *et al*., 2017).

Chitosan is a biopolymer consisting of randomly dispersed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), which is the deacetylated derivative of chitin. It is a type of polysaccharide that has a positive charge. It has several useful properties, such as being environmentally friendly, biodegradable, non-toxic, and having a range of physical and chemical characteristics, as well as functional specifications such as antioxidant, antifungal, and antibacterial activities. It is effective against a broad variety of microorganisms, including molds and gram-positive and gram-negative bacteria (Bagheri *et al*., 2021). After treatment with chitosan, certain microbes release lipopolysaccharides or other membrane lipids, as shown by the chemical analysis and electrophoresis results of the supernatants.

Numerous research endeavors have been conducted to enhance the antibacterial properties of chitosan by incorporating plant extracts, to increase its ability to inhibit the growth of microorganisms (Valipour Kootenaie *et al*., 2017; Farokhzad *et al*., 2023).

There is no information available on the effectiveness of using olive-leaf-extract and chitosan-coated-olive-leaf extract as additives to beef burgers, despite their potential advantages in extending the burgers' shelf-life and their antimicrobial activity. Therefore, the purpose of this study was to assess the effectiveness of olive-leaf-extract and chitosan-coated-olive-leaf extract against *B. cereus* bacteria in refrigerated beef burgers. Additionally, the study will also examine the impact of these extracts on the expression of enterotoxin FM and cytotoxin K (*Cyt*K) genes.

Materials and methods

Bacterial strain

The *Bacillus cereus* strain used in this experiment was obtained from the Department of Bacteriology, AHRI, ARC and stored at -80°C. The frozen (glycerol stock) *B. cereus* culture was activated by inoculation into Brain heart infusion broth (BHIB; Lab M), and incubated overnight at 35°C for 24 h to reach a final concentration of approximately 10^7 CFU/mL, as

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determined by plating serial dilutions on Mannitol Egg Yolk Polymyxin (MEYP; BioLife). Serial dilution was performed on sterile saline to give approximately 10^6 CFU/mL in the inoculated solution.

Preparation of OL-E

The olive leaves were first cleaned with distilled water and then dried at 50°C in an oven. Next, a blender was used to powder the dried leaves. An organic solvent called 100% pure ethanol was employed to extract the desired ingredients. The extraction procedure was carried out using Soxhlet equipment.

A total of 25 grams of olive leaves was used for the process. The leaves were placed inside the main chamber of the apparatus in a thimble holder lined with filter paper. The distillation flask was filled with 200 mL of freshly extracted solvent, which was gradually condensed. A syphon was used to remove the solutes from the thimble holder and return them to the distillation flask whenever the solvent level exceeded that of the container. The process was carried out for ten hours at a temperature of 70°C (Abdel-Daim *et al*., 2020).

Identification of phenolic compounds in OL-E by HPLC

Prior to HPLC (UV–vis) (Waters, USA) analysis, 10 mg OL-E was diluted in 1 mL of 80% methanol and filtered through 0.45 µm filters. Phenolic compound separation was carried out at 40°C using a Wakosil C18HG (5 µm, 4.6×150 mm). A binary solvent mixture consisting of 50/50 methanol and acetonitrile (solvent B) and water acidified with 0.2% phosphoric acid (solvent A) was used for the gradient mode elution. After readjusting for 12 minutes to the original composition, a linear gradient was run from 96% (A) and 4% (B) to 50% (A) and 50% (B) over 40 minutes; it then changed to 40% (A) and 60% (B) for 5 minutes; and finally, it changed to 0% (A) and 100% (B) for 15 minutes. Each sample had an injection volume of 20 µL, and the mobile phase used a flow rate of 1 mL/min. By comparing retention times to standards (Sigma) (Gallic acid, Protocatechuic acid, p-hydroxybenzoic acid, Gentisic acid, Catechine, Chlorogenic acid, Caffeic acid, caffeine, Syringic acid, Vanillic acid, Ferulic acid, Sinapic acid, p-coumaric acid, Rutin, hisperdin, naringin, Oleuropein, apegnin-7-glycoside, apegnin, Cinnamic acid, quercetin, Kaempferol, Chrysin, epigallocatechin gallate, epicatechin gallate), all phenolic components were identified (Hamad *et al*., 2023).

Preparation of chitosan coating solution

A 1.5% chitosan (Sigma) coating (w/v) was prepared in 100 mL of 1% acetic acid solution (v/v). The mixture was stirred on a magnetic stirrer for 3 hours at room temperature for complete dissolution. Then, 0.75 mL of glycerol was added as a plasticizer and stirred with a magnetic stirrer for 10 minutes. Finally, a filter paper (Whatman no. 3, UK) was used to remove impurities from the solution. For uniform distribution, the extract was mixed with tween 80 in the chitosan solution (Meherpour *et al*., 2020).

The antimicrobial effectiveness of OL-E and OL-Ch

The standard broth dilution method was used to determine the antimicrobial activity of OL-E and OL-Ch using thiazolyl blue tetrazolium bromide (MTT) assay (Raquena *et al*., 2019). The MIC was determined by serial double dilutions for each material examined, which was obtained in Brain Heart Infusion Broth (BHIB) medium using a sterile 96-well round-bottom polystyrene microtiter plate (12 columns x 8 rows). Two-fold serial dilutions were made from OL-E and OL-Ch to obtain final dilutions of 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128 with a *B. cereus* concentration of 10^6/ml CFU (adjusted by 0.5 McFarland's) in brain heart infusion broth (BHIB), while the control comprised only inoculated broth. Plate incubation lasted 24 hours at 35°C. After incubation, each well received 10 µL of MTT solution, and the plates were incubated again for 4 hours. The color switch from colorless to blue signified the presence of bacteria, and the minimum inhibitory concentration (MIC) was identified as the lowest concentration of the drug that prevented this color shift.

Beef patties preparation / experimental design

As per the Egyptian standard specification (ES 1688/2005) for burgers, we made beef burgers with the following ingredients: 65% minced meat, 20% fat, 5% soybean, 0.3% black pepper, 1.8% salt, and 10% cool iced water. This recipe ensures good manufacturing practices for beef burgers.

To prepare the burger, one kg. of freshly prepared beef chuck, which was 24 hours postmortem, was purchased from a local butcher shop in Giza Market, Egypt. The beef was brought to the laboratory in an icebox and minced in an electrical mincer (5mm). Fresh beef fat was purchased from a local slaughterhouse, washed, and frozen at -18°C for use the next day. Before processing the burgers, the beef fat was minced using an electrical mincer with a 3mm diameter. The frozen minced beef was transferred to a paddle mixer, where common salt, black pepper, and cold water were added. The mixture was thoroughly mixed for five minutes, inoculated with *B. cereus* at levels of 6 log CFU/g, and divided into three groups:

Control group: contaminated with *B. cereus* only (not supplemented); OL-E -group: contaminated with *B. cereus* and supplemented with 150 µg/g OL-E; and the OL-Ch -group contaminated with *B. cereus* and supplemented with 300 µg/g OL-Ch.

Each group was packaged in sterile plastic bags aseptically. For *B. cereus* enumeration, samples were collected from each group following inoculation (zero time) and relative entFM and cytK expressions, chemical analyses, and then each group was saved at 4ºC in a refrigerator. The samples were examined every three days until spoilage. The experiment was repeated three times.

Bacillus cereus viable count

Preparation of sample homogenate

Twenty-five grams of the examined samples were aseptically transferred to a sterile bag homogenized with 225 ml of sterile buffered peptone water (0.1%) (Oxoid) for 30–60 seconds to give an initial dilution of 1/10. Transfer, 1 mL of the initial suspension into a 9-mL sterile saline tube. Mix thoroughly by using a vortex for 5–10 seconds to obtain a 1:100 dilution. Repeat this operation to obtain dilutions of 1:1000, 1:10000, etc. (ISO 6887-3/2017).

Bacillus cereus count

One ml of the food sample homogenate was streaked to two each of MYP agar plates, and let to dry at ambient temperature for 15 min., the inoculated plates were inverted and incubated at 30ºC for 18-24hrs. The bacterial counts were given in log CFU/g (ISO,7932/2004).

Gene expression assessment

The FastPure® DNA/RNA Mini kit was used to extract mRNA in accordance with the manufacturer's instructions. A 1.5-mL RNase-free tube was filled with 200 µL of the sample and 500 µL of Lysis Solution. The tube was then agitated firmly. Once in the adsorption column, the mixture was rapidly centrifuged for a minute. After disposing of the filtrate, the adsorption column was filled with 600 µL of washing buffer and centrifuged for 30 seconds at a high speed. The washers went through one more cycle. The column of adsorption was moved to a new 1.5-mL centrifuge tube, 50 µL of elution buffer was added, and the mixture was allowed to

Table 1. Primers used in this study.

Gene	Primer sequence (5' to 3')	Thermal cycling conditions	Reference
16S *rRNA*	F: TCGAAATTGAAAGGCGGC R: GGTGCCAGCTTATTCAAC	Reverse transcription at 55°C for 30 min followed by activation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 10 s, and annealing 60 °C for 30 s and extension at 60°C for 30 s.	Priha *et al*. (2004)
*ent*FM	F: AAAGAAATTAATGGACAAACTCAAACTCA R: GTATGTAGCTGGGCCTGTACGT		
*cyt*K	F: GTAACTTTCATTGATGATCC R: GAATACTAAATAATTGGTTTCC	Reverse transcription at 55°C for 30 min followed by activation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 10 s, and annealing 48°C for 30 s and extension at 60°C for 30 s.	Park *et al*. (2008)

sit at room temperature for one minute before being rapidly centrifuged for one minute. Up to its utilization, the extract was stored at -80°C.

The expression levels of *B. cereus* toxin genes (*ent*FM and *cyt*K) (Table 1) were determined by qPCR using a HERA SYBR® Green RT-qPCR Kit (Willowfort) and an Applied Real-Time PCR Detection System (Applied Biosystem). Amplification was performed in 10 µL reaction volumes containing 0.5 µL of each primer and 1 µl of RNA with cycling conditions (Table 1).

Chemical analyses

pH determination

Ten grams of homogenized burger samples were added in 100 ml of pre-boiled cooled distilled water at 25°C, checked for 30 min, and left aside for 10 min then, apart from the aqueous layer was added to a beaker, and pH was measured by digital pH meter that was previously calibrated using standard buffers of pH 4 and 7 at room temperature (EOS, 2006).

Total Volatile Basic Nitrogen (TVB-N) determination

Approximately ten grams of previously prepared and homogenized burger sample was put on a conical flask with 300 mL of D.W. Then, two grams of MgO were added followed by an antifoaming agent as some glass beads. The distillate was received in an Erlenmeyer-flask containing 25 mL of 2% boric acid solution until the final distillate reached 125 mL volume. Titration of the distillate occurred by using H_2SO_4 solution (0.1N) till the natural red endpoint. The same procedures occurred by using D.W. instead of the samples for blank detection (EOS, 2006). TVB-N (mg N/100g) of the burger sample was calculated as follows:

TVB-N (mg N/100g) = (S – B) × 14

Where:

S = H_2SO_4 volume that was used for sample titration

B = volume of H2SO4, which was titrated for blank

Thiobarbituric acid (TBA) determination (EOS, 2006)

In a distillation flask about ten grams of the burger, sample were blended with distilled water, HCl solution (4 N), and anti-pumping stones. The distillation process was continued until 50 mL of distillate was collected in an empty flask. In a test tube placed in boiling water for 35 minutes, five mL of distillates and five mL of TBA reagent were combined. Instead of utilizing the sample with the 5 ml of TBA reagent, 5 ml of D.W. was used to complete the blank. On the spectrophotometer, the sample optical density (D) was measured at a wavelength of 538 nm in comparison to the blank.

Calculation: TBA (mg MDA /kg (= D × 7.8

Sensory evaluation of burger supplemented with OL-E and OL-Ch

The investigators in this study were 15 specialists from the Animal Health Research Institute's Food Hygiene Department, ARC staff. The study evaluated ready-to-eat burgers supplemented with OL-E and OL-Ch, and blank (control) samples that did not contain OL-E and OL-Ch. The most frequently cited descriptors included color, taste, aroma, and acceptance. The specialists were asked to rank samples according to their intensity for each of the four descriptors. There was a 9-point hedonic scale where 9 indicated an extreme aversion to fillets compared to fillets without chitosan or lysozyme (Kim *et al*., 2015). The examiner considered the sample rejected if the scores were less than five.

Statistical analysis

Microsoft Excel software was utilized for the statistical and imaging analyses. Using the $2^{-\Delta\Delta Ct}$ method, the relative-expression of target genes was analyzed and compared with the control group. The average Ct values of the target genes were deducted from those of the endogenous control gene 16srRNA to obtain the ΔCt values.

Results and Discussion

Phenolic compounds in OL-E

The HPLC-DAD analysis of olive leaf extract allowed the identification of twelve phenolic compounds (Fig. 1): Oleuropein, Protocatechuic acid, Catechine, Gallic acid, Rutin, Syringic acid, p-coumaric acid, quercetin, Vanillic acid, p-hydroxybenzoic acid, Kaempferol and Cinnamic acid.

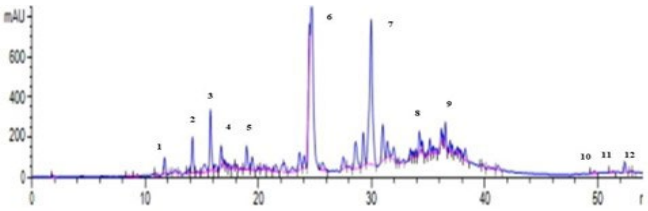

Fig. 1. HPLC profile, visualized at 280 nm, of the OL-E analyzed in this study. Phenolic compounds tentatively identified (text in Table 1 for peak list and details): (1) Gallic acid; (2) Protocatechuic acid; (3) p-hydroxybenzoic acid; (4) Catechine; (5) Syringic acid; (6) Vanillic acid; (7) p-coumaric acid; (8) Rutin; (9) Oleuropein; (10); Cinnamic acid; (11) quercetin; (12) Kaempferol.

The phenolic compounds detected in the analyzed samples were: in total, 12 individual phenolic compounds were identified, which were oleuropein, protocatechuic acid, catechin, and rutin. A similar compound was previously reported by Guebebia *et al.* (2022) and Khelouf *et al.* (2023).

The main compound detected was oleuropein; this result was in line with the previous finding of Šimat *et al.* (2022). In general, the differences in oleuropein and flavonoid ratios between olive leaf varieties can be explained by several factors, such as the type of solvent and the particle size of the extracted leaves, which influence the extract composition. In addition to the extraction method and conditions used, harvest period, storage conditions, and length were other factors that influenced the extraction yield (Šimat *et al*., 2022).

The antimicrobial effectiveness of OL-E and OL-Ch against B. cereus

OL-E and OL-Ch showed antibacterial effectiveness against *B. cereus* after being incubated for 24 hours at 37ºC in an aerobic environment at

dilutions of 1/8 and 1/16 (conc. of 62.5mg/mL and 125 mg/mL), respectively. The present study showed in vitro antimicrobial efficacy of OL-E against *B. cereus* at conc. (62.5 mg/mL). This result was similar to the findings of Pereira *et al*. (2007) and Liu *et al*. (2017). Although, the efficacy of OL-Ch was 125 mg/mL and was in line with the study of Kazan and Demirci (2023).

Bacillus cereus viable count

Food processing facilities are often surrounded by areas where foodborne pathogens can thrive. These pathogens pose a serious risk to human health. *B. cereus* is a common pathogen found in the environment and can contaminate many types of raw and processed foods. Fig. 2 displays the effect of OL-E and OL-Ch on *B. cereus* in burgers stored in a cold environment. The results are presented as log CFU/g. The initial counts (after 1 hour on day 0) of the control group, burgers treated with OL-E, and burgers treated with OL-Ch were 5.7, 5.5, and 5.3 log CFU/g, respectively. During cold storage, viable *B. cereus* reduced significantly ($p < 0.05$) over time in burgers treated with OL-E and OL-Ch. On the thirteen days of preservation, the count went down to 2 log CFU/g in the OL-E group and 1.3 log CFU/g in the OL-Ch group.

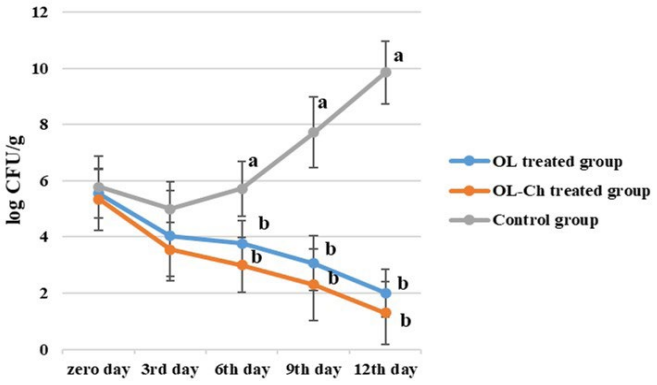

Fig. 2. *B. cereus* viable count (measured in log CFU/g) in burgers treated with OL-E and OL-Ch and the untreated control, during preservation at low temperatures was recorded. The values are presented as mean ±SD, and there are significant differences (P≤0.05) between the points labeled with different letters on the same day of preservation.

It's interesting to note that using OL-E and OL-Ch not only increased the shelf life of burgers but also reduced the risk of *B. cereus* (100 and 20 colony-forming units, respectively). This reduction brought the levels below the *B. cereus* infective dose (10^4 in emetic food poisoning and 105 in diarrheal food poisoning) (Milojevic *et al*., 2019).

The antimicrobial mechanism was reported to be that phenolic compounds have the ability to denature proteins, damage cell membranes, break down cell peptidoglycans, adversely affect cell membrane permeability, and thus create leakage of cytoplasmic elements such as potassium, glutamate, or inorganic phosphate, which delays and inhibits the growth rate of microorganisms (Lee and Lee, 2010; Sanchez *et al*., 2007).

OL-E exhibits greater efficacy against gram-positive bacteria than gram-negative bacteria due to the structural characteristics of the bacteria themselves. The outer layer in gram-negative bacteria is more resistant to antimicrobial molecules compared to the peptidoglycan layer in gram-positive bacteria (Antunes *et al*., 2020). Additionally, the antibacterial activity of chitosan is thought to stem from its ability to bind to negatively charged bacterial cells through electrostatic and non-electrostatic interactions. When chitosan permeabilizes Gram-positive and Gram-negative bacterial cells, it can cause the release of internal substances such as K ions and nucleotides. This release can occur in hyperosmotic settings, where the osmotic gradient causes cell water to follow, leading to a decrease in cell membrane turgor and dehydration of the cells (Mellegård *et al*., 2011).

Gene expression entFM gene and cytK gene assay

The results of the study on the relative expression of *ent*FM and *cyt*K genes in burgers containing the OL-E and OL-Ch, when chilled preserved, are presented in Fig. 3. The study found that the expression of both genes was significantly reduced ($p < 0.05$) in the treated groups compared to the untreated (control) group. Moreover, the OL-Ch group showed more significant downregulation of gene expression than the OL-E group ($p < 0.05$). Particularly on day 0, the relative expression of the entFM was 0.85 and 0.80 in the OL-E and OL-Ch groups, respectively, while on the thirteenth day, the expression levels were 0.2 in the OL-E group and 0.09 in the OL-Ch group. On day 0, the relative expression of the *cyt*K gene in the OL-E group was 0.84, while in the OL-E group, it was 0.76. It further decreased until the thirteenth day, reaching 0.15 in the OL-E group and 0.09 in the OL-Ch group.

It is suggested that the mechanism by which OL-E affects gene expression in bacterial cells involves the interruption of biochemical pathways responsible for DNA and protein synthesis. This interruption is similar to how alkaloids in olive leaf extract work. Additionally, the hydroxyl groups present in phenolic substances can bind to enzymes and alter their function, which enhances their ability to combat bacteria (Abdullah *et al*., 2018). The mechanism by which chitosan could affect the expression of *B. cereus* genes is that chitosan has the potential to bind with DNA intracellularly, leading to an interaction with mRNA and protein synthesis. This has significant implications for cellular processes and functions (Fernandes *et al*., 2009).

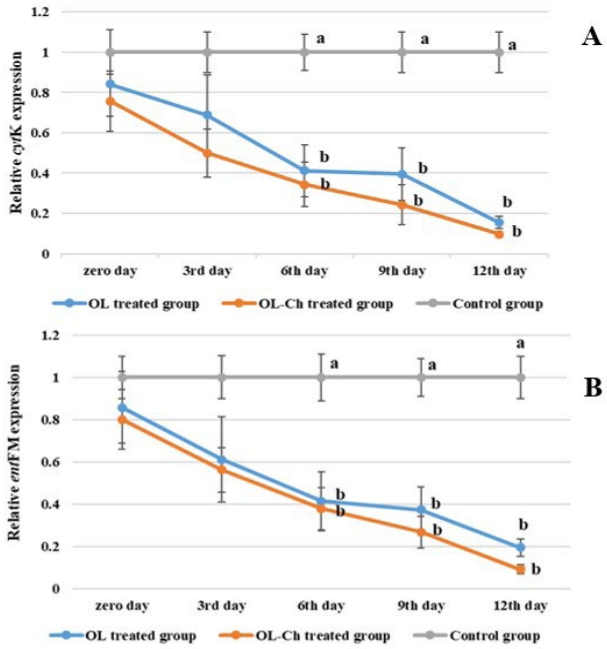

Fig. 3. (A). Relative *cyt*K gene expression of *B. cereus* in burgers that were treated with OL-E and OL-Ch and the untreated control, during preservation at low temperatures was recorded. The values are presented as mean ±SD, and there are noticeable differences (P≤0.05) between the points labeled with different letters on the same day of preservation. (B). Relative *ent*FM gene expression of *B. cereus* in burgers treated with OL-E and OL-Ch and the untreated control, during preservation at low temperatures was recorded. The values are presented as mean ±SD, and there are significant differences (P≤0.05) between the points labeled with different letters on the same day of preservation.

Chemical Analyses

pH

The pH value indicates the extent of protein breakdown that occurs during storage (Wereńska and Okruszek., 2023). The results presented in Figure 4.a. indicated that the pH values in the group treated with OL-E ranged from 6.22±1.23 to 6.12±1.55 from the zero-day to the 12th day. On the other hand, the OL-Ch-treated group showed pH values ranging

from 5.57±1.43 to 5.79±1.12 during the same period. The control group had pH values ranging from 6.2±1.87 to 7.95±1.77. It was noticed that there was no significant difference ($p < 0.05$) between both treatments from day 0 to day 3. Table 2 shows that on day 3, the control group started to become unacceptable, nevertheless the OL-E and OL-Ch groups were accepted until the 12th day of refrigerated storage.

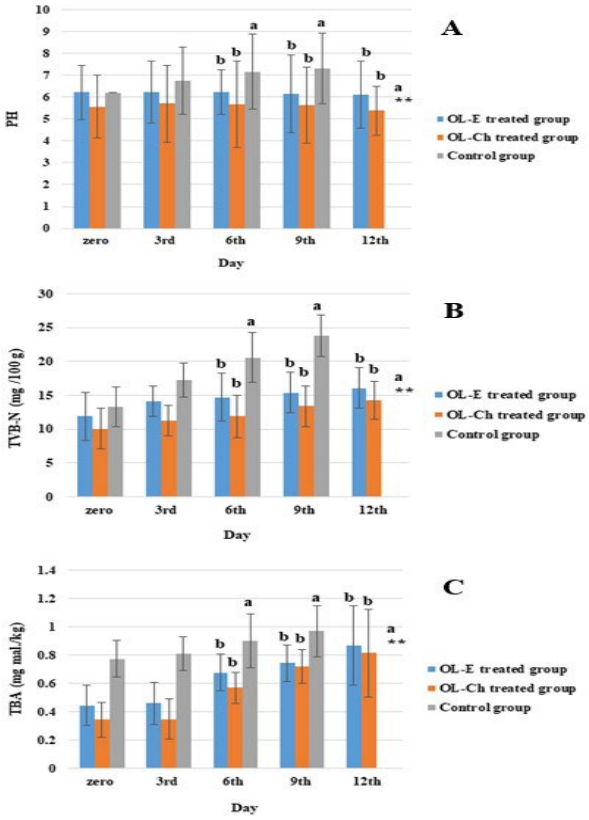

Fig. 4. B. pH, B. TVB-N (mg /100 g), C. TBA (mg mal./kg) values in burgers treated with OL-E and OL-Ch and the untreated control, during preservation at low temperatures was recorded. The values are presented as mean ±SD, and there are noticeable differences (P≤0.05) between the points that are labeled with different letters on the same day of preservation.
** spoiled

During 12 days of refrigeration, the group treated with OL-Ch exhibited an increase in pH values from 5.57 to 5.79. These results are consistent with the findings of Fiorentini *et al.* (2023). It has been found that OL-E has the potential to decrease the pH levels in meat, which can make the environment unfavorable for the growth of most bacterial populations present on the meat surface (Gunenc, 2007). On the other hand, an increase in pH levels during storage is an indication of protein breakdown, which leads to the production of free amino acids and results in the formation of more alkaline NH3 and amines (Tabatabaee Bafroee *et al.*, 2020).

According to our research, the treated group with OL-Ch exhibited lower pH values compared to the OL-E treated group. This could be attributed to the excellent coating properties, high antimicrobial activity, and compatibility with substances such as vitamins, minerals, and antimicrobial agents that are present in Chitosan. This information was reported by Maghsoudlou and Maghsoudlou (2013).

Total Volatile Basic Nitrogen (TVBN)

TVBN values are connected to protein degradation due to various microorganisms' activity and their proteolytic enzymes Pérez-Palacios *et al.* (2008). The findings shown in Figure 4.b., revealed that the TVBN values (measured in mg/100gm) within the control group ranged from 13.3±2.95 to 23.8±3.11 between day 0 and day 9. In the OL-E group, the values ranged from 11.9±3.56 to 16.1±2.98 between day 0 and day 12. While, in the OL-Ch group, the values ranged from 10.1±3.05 to 14.3±2.79 between day 0 and day 12, respectively.

On the 6th day of the experiment, the control group exhibited spoiled results, while the OL-E and OL-Ch treated groups showed a slow increase of values and remained below 20mg/100g. A similar finding was reported by Saleh *et al.* (2020), who discovered that increasing the OL-E concentration to 1% could decrease the TVBN values up to 15 days of storage. The OL-Ch treated group showed the lowest mean values of TVBN, especially on the 3rd, 6th, and 9th days of storage. These results are consistent with Meherpour *et al.* (2020) study, where the addition of olive leaf extract to chitosan coating reduced the total volatile basic nitrogen amount. It is believed that this ability to reduce protein hydrolysis is due to the extract's efficacy, as per Ezz El-Din Ibrahim *et al.* (2022).

Our findings revealed that OL-E and OL-Ch could reduce protein decomposition and decrease the TVBN values at day 12 of storage and OL-Ch had the higher effect.

Thiobarbituric acid (TBA)

Thiobarbituric acid (TBA) is a commonly used method for measuring secondary oxidation products The primary product of MDA is believed to be the root cause of oxidative rancidity and may also be responsible for the off-flavor of oxidized fat Zhang *et al.* (2021). Based on the data presented in Figure (4.c), it can be observed that the control group had TBA values ranging from 0.775±0.13 to 0.97±0.18 on day zero and day 9, respectively. Conversely, the OL-E treated group had values ranging from 0.445±0.14 to 0.869±0.28, while the OL-Ch treated group had values ranging from 0.343±0.12 to 0.816±0.31 on day zero and day 12, respectively.

The spoilage of the control group occurred on the 6th day, meanwhile, the OL-Ch-treated group had the lowest mean values of mg MDA/kg meat, particularly on the 6th and 9th days of storage. These findings were consistent with those of Fiorentini *et al.* (2023), who reported that the OL-Ch coating showed lower mean values of mg MDA/kg meat, significantly different from the other samples, especially on the 7th and 9th days of storage.

Sensory evaluation

The findings of the sensory evaluation of burgers enhanced with OL-E and OL-Ch are displayed in Figure 5.

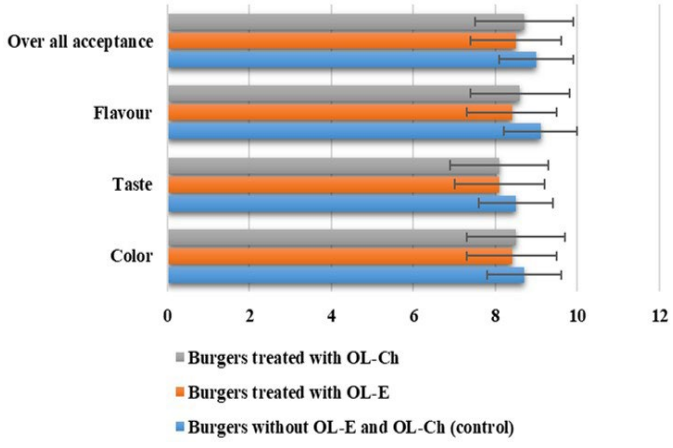

Fig. 5. The average score of taste, color, flavor, and overall acceptability of burgers treated with OL-E and OL-Ch, and the control group at chilling preservation. Values are expressed as the mean ±SD.

The assessment covered color, taste, aroma, and overall acceptability, with OL-E-supplemented burgers receiving high scores (8.4, 8.1, 8.4, and 8.5, respectively) and OL-Ch-supplemented burgers also performing well (8.5, 8.1, 8.6, and 8.7, respectively). Moreover, OL-E and OL-Ch to burgers did not affect sensory attributes ($P > 0.05$). These outcomes demonstrate

that meat containing OL-E and OL-Ch can be safely consumed without compromising its sensory quality. Similar conclusions were reached by Aouidi *et al*. (2017), and Meherpour *et al*. (2020), regarding meat product supplementation with OL-E and OL-Ch, respectively.

Conclusion

Burgers can benefit from the significant antibacterial effect of OL-E and OL-Ch against vegetative cells of *B. cereus*. Our findings propose that OL-E and OL-Ch could be utilized as natural food additives to prevent *B. cereus* contamination in the meat industry. However, suppose OL-E and OL-Ch are to be used as bactericidal substances added to meat products. In that case, it is recommended that further studies, including safety experiments, dosage optimization, and analysis of the sensory characteristics of foods, should be undertaken.

Conflict of interest

The authors have no conflict of interest to declare.

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