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Seasonal Variations of Bacterial Populations in Refrigerated Minced Meat and The Role of Different Essential Oils in Extending Shelf life

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THE LEVEL of bacterial contamination in two essential oil-treated or untreated minced meat samples collected from different butcher shops in Cairo Governorate in July 2019 (sample I) and January 2020 (sample II) was evaluated in this study. The samples were subjected to sensory, bacteriological, and chemical analysis. The counts of anaerobic, aerobic, coliform bacteria, *Escherichia coli*, and *Staphylococcus aureus* were increased in the untreated portions of both samples. Generally, the total bacterial contamination in sample I, which was collected in the summer, was greater than that in sample II, which was collected in winter. Adding 2% marjoram or thyme essential oils to the minced meat samples significantly increased their shelf life at 2ºC up to 7 and 8 days, compared with the untreated portions of the two samples, whose shelf life was 4 and 5 days, respectively. These results indicate that treatment of minced meat with essential oils, especially marjoram or thyme, extends its shelf life at 2ºC by reducing the bacterial contamination without affecting the quality and sensory properties of the meat. This study was carried out to compare the different bacterial counts in minced meat at two different seasons of the year (summer and winter), with the goal of extending the shelf life by reducing bacterial counts using different essential oils.

Keywords: Antibiotic sensitivity, Antimicrobial activity, Bacteriological analysis, Chemical analysis, Essential oils, Sensory properties.

Introduction

Meat and meat products provide an ideal growth medium for bacteria because of their high moisture content, richness in nitrogenous compounds (essential amino acids and proteins), and abundance of minerals, vitamins, and other growth factors. Furthermore, their pH is favorable for the growth of microorganisms (Alahakoon et al., 2015). Meat quality can be defined as a set of characteristics that reflect what we value in meat when we buy, eat, or choose it as a raw material for making meat products (Egyptian Organization for Standards and Quality (EOS, 2005). Traditionally, the quality of meat intended for consumption as whole meat rather than meat products is defined by properties associated with our sensory perception: appearance, color, flavor, texture (especially tenderness), juiciness/water-holding capacity, and odor. Meat has a short shelf life of one day or less at 15°C–30°C and a maximum of seven days at refrigerated temperatures (0°C–4°C) due to microbial spoilage and/or lipid oxidation (EOS, 2005). To ensure food safety and extend the shelf life of food products, chemical preservatives or other physical, chemical, biological treatments, or their combinations, have been used (Sridhar et al., 2020; Huang, 2021). Essential oils are natural products obtained from plants with proven antimicrobial properties against a wide range of microorganisms (Abd El-Zaher et al., 2019; Raveau et al., 2020; Puvača et al., 2021). Hence, essential oils have traditionally been used to protect food against microbial deterioration (Li et al., 2022). This study was carried out to compare the different bacterial counts in two minced meat samples collected during two different seasons

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of the year (summer and winter), with the goal of extending the shelf life of meat by reducing bacterial counts using different essential oils.

Materials and Methods

Bacteria used in this study

Escherichia coli ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Salmonella typhimurium* ATCC 14028 were purchased from TCS Biosciences Ltd., Botolph Claydon, Buckingham, United Kingdom for routine performance testing as positive and negative controls. To make working bacteria cultures, we used the reference stock to create a pure stationary phase culture in a non-selective broth, e.g., tryptone soya broth, according to the International Organization for Standardization, ISO 11133 (2014).

Culture media and kits

The following media were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England): peptone salt water, plate count agar, violet red bile lactose agar, tryptone–bile–glucuronic medium, buffered peptone water, nutrient agar, xylose lysine deoxycholate agar, triple sugar iron agar, MacConkey agar, and egg yolk emulsion. The following media were purchased from Sigma-Aldrich (Darmstadt, Germany): brilliant green lactose bile broth, Rappaport–Vassiliadis medium with soya, Müller–Kauffmann Tetrathionate Novobiocin broth, urea agar (Christensen), Baird–Parker agar medium, L-lysine decarboxylation medium, brain–heart infusion broth, tryptone/DL-tryptophan medium, and yeast extract. Hektoen enteric agar was purchased from HiMedia Laboratories Ltd. (Mumbai, India). The Anti-*Salmonella* I and the Anti-*Salmonella* Poly-H Phase 1 and 2 were purchased from Sifin Diagnostics GMBH company (Berlin, Germany). *Shigella* broth and L-ornithine decarboxylase medium were purchased from Codalab (Madrid, Spain). *Shigella* antiserums were purchased from Bio-Rad laboratories in the USA.

Essential oils

Marjoram, cinnamon, thyme, and lemongrass essential oils were all obtained from the International El-Sedek Center on October 6, Giza, Egypt. They were all in a pure state with a final concentration of 100% and had no preservatives or antioxidants added. These four oils were stored in dark bottles at 4°C until further use.

Collection of minced meat samples

Two samples of minced beef meat were collected according to ISO 17728 (2015) in July 2019 and January 2020 and designated as the first (I) and second (II) samples, respectively. Each sample weighed 16kg and was collected from different butcher shops in Cairo Governorate. All the meat (16kg) was taken directly after the animal was slaughtered, mixed, minced, and packed in sterile polyethylene bags. Then, it was immediately sent to the Acerta Laboratory (Acerta Middle East Company, Maadi, Cairo, Egypt) for sensory, bacteriological, and chemical tests.

Preparation of minced meat samples with essential oils

In each of the two different seasons, a 16kg minced meat sample was collected and divided into 13 portions (each weighing about 1.2kg) to investigate its sensory, bacteriological, and chemical properties after either treating it with oils or leaving it untreated as a control. The experiment was designed as follows: four sets of three portions each were separated. They were labeled as M, T, C, and L, for marjoram oil, thyme oil, cinnamon oil, and lemongrass oil, respectively. The three portions in each set received the corresponding oil at a concentration of 1%, 1.5%, and 2% v/w, and they were labeled as (M1, M2, and M3), (T1, T2, and T3), and so on, respectively. One portion was used as a control and it did not receive oil.

Sensory analysis of minced meat samples

According to Mohammed (2013), panelists evaluated the sensory properties of the minced meat samples in terms of the tenderness, juiciness, color, and overall acceptability (Table 1).

Bacteriological study of minced meat samples
Pretreatment and preparation of initial suspension

In both seasons, 10g from each oil-treated and untreated portion was aseptically weighed and mixed with 90mL sterilized maximal recovery diluent according to ISO 6887 (2017). For suspension preparation, tissues were homogenized in the Stomacher (Bag Mixer Interscience, France) in the dilution fluid for only 60 s to homogenize the sample. This was the initial suspension of each portion, from which eight serial dilutions (12 in the case of aerobic plate count (APC) and five in the case of both *E. coli* and *S. aureus*) were prepared.

TABLE 1. Evaluation form for description of sensory properties according to Mohammed (2013)

Overall acceptability	Color	Juiciness	Tenderness
(1) Rejected	(1) Very light	(1) Very dry	(1) Very hard
(2) Unacceptable	(2) Light	(2) Dry	(2) Hard
(3) Middle	(3) Acceptable	(3) Middle	(3) Middle
(4) Acceptable	(4) Dark	(4) Juicy	(4) Soft
(5) Very acceptable	(5) Very dark	(5) Very juicy	(5) Very soft

Enumeration of contaminating bacteria

For anaerobic bacteria, the count was determined according to NMKL No. 189 (2017), while the aerobic bacteria count was determined according to ISO 4833-1 (2013). Additionally, the coliform bacteria count, *E. coli* count, and *S. aureus* count were determined according to ISO 4832 (2006), ISO 16649-2 (2001), and ISO 6888-1 (1999), respectively. The count was expressed as log CFU/g using the following general equation: CFU per gram = C/v (n1 + 0.1 n2)d, where C was the sum of colonies counted on all the plates, n1 was the number of plates counted for the first dilution, n2 was the number of plates counted for the second dilution, d was the dilution from which the first count was obtained, and v was the volume of inoculum. *Salmonella* and *Shigella* were detected according to ISO 6579-1 (2017) and ISO 21567 (2004), respectively, in 25g of the tested minced meat portions.

Chemical analysis of minced meat samples

According to ISO 2917 (1999), the pH of all the minced meat portions (treated and untreated) was recorded over the eight days of the experiment in both the seasons. Additionally, the amount of total volatile nitrogen (TVN) was determined according to the to Food and Agriculture Organization of the United Nations (FAO, 1980) method and calculated using the following equation: TVN-N mg/100g = (2-t1) 26.088, where t1 was the volume of NaOH exhausted in titration. The amount of thiobarbituric acid (TBA) was determined according to Kirk & Sawyer (1991) and calculated by the following equation: mg malonaldehyde/kg of sample = D × 7.8, where D was the absorbance of the sample against the blank.

Antibiotic susceptibility of bacteria isolated from oil-treated and untreated minced meat portions

The pattern of antibiotic susceptibility exhibited by bacteria isolated from the 2% marjoram oil-treated, 2% thyme oil-treated, and untreated minced meat portions from both samples (I and II) was determined according to the Clinical and Laboratory Standards Institute, CLSI (2019). Overall, 32 *E. coli* isolates, 32 *S. aureus* isolates, and five *Salmonella* spp. isolates were identified. Their susceptibility was tested toward different antibiotics from different chemical groups, selected according to CLSI (2019), by the disc diffusion method, according to Bauer et al. (1966). An inoculum (100µL) containing 4.0×10^8 CFU ($OD_{600} \approx 0.5$) was prepared from 24h-old isolates and streaked individually on the surface of Müller–Hinton agar plates by a sterile glass rod. Different antibiotic discs containing ampicillin (10µg), tetracycline (30µg), chloramphenicol (30µg), ciprofloxacin (5µg), nitrofurantoin (300µg), sulfamethoxazole-trimethoprim (25µg), and penicillin (10µg) were purchased from Oxoid and placed, under aseptic conditions, on the inoculated agar surface using sterile forceps. The experiment was carried out in duplicates and the discs were placed a distance apart. Finally, the plates were incubated at 37°C for 24h and examined for the presence or absence of a clear zone around each antibiotic disc. The clear zone diameter was determined in millimeters according to CLSI (2019).

Screening of essential oils for their antibacterial activity against E. coli isolates

Marjoram and thyme essential oils were screened for their antibacterial activity against *E. coli* isolates from 2% thyme oil-treated minced meat portions from both the samples (I and II) by an agar well diffusion assay, according to Balouiri et al. (2016). Three dilutions (1, 1.5, and 2%) were prepared for each essential oil using analytical grade ethanol. An overnight bacterial suspension (200µL) containing 4.0×10^8 CFU ($OD_{600} \approx 0.5$) of *E. coli* isolates was spread individually on the surface of Müller–Hinton agar plates. Wells were cut in the center of inoculated plates using a sterilized cork borer 6mm in diameter and 100µL of each concentration of the essential oils was added to each well. Plates were carefully incubated at 37°C for 24h. Both absolute ethanol and chloramphenicol (30µg/disc) were used as negative and positive controls, respectively.

Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) according to the software programs in SPSS 2006 (SPSS Base 15.0 User's Guide. SPSS Inc., Chicago, USA) and GraphPad Prism version 9.1.2.

Results

Sensory analysis of the oil-treated and untreated minced meat

The sensory properties, including the color, juiciness, tenderness, and overall acceptability, of the oil-treated and untreated minced meat portions under cold storage (2°C) were recorded at day 0, 2, 4, 5, 7, and 8. Sample I was acceptable up to day 7 when treated with 2% thyme oil. This was the best result compared with the untreated portion on the same day, which was deemed unusable (Supplementary Table S1). Sample II was acceptable up to day 8 when treated with 2% marjoram or thyme oil in comparison with the untreated portion. Also, when minced meat was treated with 2% cinnamon or lemongrass essential oils, the overall acceptability was within the permissible limit up to day 7 compared with the control, which was only acceptable up to day 5 (Supplementary Table S1).

Bacteriological analysis of minced meat

Enumeration of anaerobic bacteria

On day 8, the anaerobic count of sample I was significantly acceptable as recommended by the Egyptian Organization Standard, EOS (2005) in portions M3, T3, and L3, which were treated with 2% of marjoram, thyme, and lemon oils, respectively. The recorded bacterial count was found to be 1.86 ± 0.02, 1.88 ± 0.02, and 1.87 ± 0.02 log CFU/g, respectively (Table 2). In sample II, treatment with marjoram and cinnamon essential oils (M3 and C3) significantly reduced the anaerobic bacterial count to 1.60 ± 0.02 and 1.55 ± 0.02 log CFU/g, respectively (Table 2). Additionally, all the oil-treated portions showed overall acceptability for anaerobic bacterial count, as recommended by EOS (2005), up to day 8 relative to the untreated portion (Table 2).

Enumeration of aerobic bacteria

Treatment of minced meat portions (sample I) with 1.5%, 2% marjoram oil (M2 and M3), or 2% thyme oil (T3) resulted in a significant ($P< 0.05$) reduction in the APC compared with the untreated meat portion on day 8 of the experiment (Table 2). In case of sample II (January 2020), the M3 and T3 treatments resulted in acceptable APCs of 5.84± 0.03 and 5.67± 0.02 log CFU/g, respectively on day 8, compared with the untreated portion, which had an APC of 8.56± 0.02 log CFU/g (Table 2).

TABLE 2. Effect of different concentrations of essential oils on anaerobic plate count and aerobic plate count (APC) in minced meat from Sample I (July 2019) and sample II (January 2020) maintained at 2°C

Samples		Sample I (July 2019)				Sample II (January 2020)			
Type of count		Anaerobic plate count		Aerobic plate count (APC)		Anaerobic plate count		Aerobic plate count (APC)	
Experiment days		Day 0	Day 8	Day 0	Day 8	Day 0	Day 8	Day 0	Day 8
Essential oil treatments	Control	**1.40±0.02**	**4.74±0.02a**	**5.19±0.03**	**10.43±0.03a**	**1.20±0.03**	**2.25±0.03a**	**3.98±0.03**	**8.56±0.02a**
	M1(1%)	1.40±0.02	3.36±0.03c	5.19±0.03	8.91±0.03g	1.20±0.03	1.90±0.02c	3.98±0.02	8.12±0.03c
	M2(1.5%)	1.40±0.02	2.40±0.02ef	5.19±0.03	7.39±0.03m	1.20±0.03	1.75±0.03e	3.98±0.03	7.16±0.04j
	M3 (2%)	1.40±0.02	**1.86±0.02i**	5.19±0.03	7.79±0.03k	1.20±0.03	1.60±0.02h	3.98±0.02	**5.84±0.03k**
	T1 (1%)	1.40±0.02	3.56±0.03b	5.19±0.03	8.36±0.03i	1.20±0.03	1.75±0.01e	3.98±0.01	7.91±0.02d
	T2 (1.5%)	1.40±0.02	2.39±0.02f	5.19±0.03	8.11±0.03j	1.20±0.03	1.65±0.03g	3.98±0.03	7.53±0.01f
	T3 (2%)	1.40±0.02	**1.88±0.02i**	5.19±0.03	7.53±0.03l	1.20±0.03	1.80±0.02d	3.98±0.03	**5.67±0.03l**
	C1 (1%)	1.40±0.02	2.60±0.02d	5.19±0.03	9.91±0.03c	1.20±0.03	1.94±0.02b	3.98±0.04	8.11±0.04c
	C2 (1.5%)	1.40±0.02	2.43±0.03e	5.19±0.03	9.19±0.03e	1.20±0.03	1.75±0.03e	3.98±0.03	7.77±0.02e
	C3 (2%)	1.40±0.02	2.13±0.02h	5.19±0.03	8.68±0.03h	1.20±0.03	1.55±0.02i	3.98±0.02	7.53±0.02f
	L1 (1%)	1.40±0.02	3.59±0.03b	5.19±0.03	10.1±0.03b	1.20±0.03	1.83±0.02d	3.98±0.03	8.28±0.01b
	L2 (1.5%)	1.40±0.02	2.30±0.02g	5.19±0.03	9.35±0.03d	1.20±0.03	1.69±0.01f	3.98±0.01	8.27±0.04b
	L3 (2%)	1.40±0.02	**1.87±0.01i**	5.19±0.03	9.01±0.03f	1.20±0.03	1.63±0.03gh	3.98±0.03	7.80±0.03e

Results are shown as mean ± standard error (SE). Means with different letters in the same column are significantly different ($P< 0.05$) by Duncan's post-hoc test. P-Values were calculated by ANOVA (unit: log CFU/g).

Enumeration of coliform bacteria

Treatment of minced meat portions (sample I) with 2% lemongrass or thyme essential oils resulted in a significant ($P< 0.05$) reduction in the coliform count up to 3.18± 0.02 and 3.22± 0.01 log CFU/g, respectively, on day 8, compared with the untreated portion, whose coliform count increased from 3.25± 0.04 log CFU/g on day 0 to 4.33± 0.03 log CFU/g on day 8 (Table 3). In sample II, the treatment of minced meat portions with 2% marjoram (M3) or thyme (T3) essential oils resulted in a significant reduction in the coliform count to 3.11± 0.02 and 2.89± 0.02 log CFU/g, respectively, on day 8, compared with the untreated portion, whose coliform count increased from 2.11± 0.02 log CFU/g on day 0 to 4.11± 0.02 log CFU/g on day 8 (Table 3).

Enumeration of E. coli

Treatment of minced meat portions (sample I) with 2% marjoram or thyme essential oils significantly ($P< 0.05$) reduced the *E. coli* count up to 2.63± 0.03 and 2.43± 0.02 log CFU/g, respectively, (Table 4). In sample II, the treatment of minced meat portions with 2% marjoram or thyme essential oils also significantly decreased the *E. coli* count up to 2.91± 0.02 and 2.30± 0.02 log CFU/g, respectively, (Table 4).

Enumeration of S. aureus

Treating meat portions (sample I) with 2% marjoram or thyme essential oils significantly reduced *S. aureus* count to 1.82± 0.02 and 1.69± 0.02 log CFU/g, respectively, on day 5 (Supplementary Table S4). In sample II, the *S. aureus* count in the untreated meat portion increased from 1.19± 0.02 log CFU/g on day 0 to 3.65± 0.02 log CFU/g on day 8. Treatment of the minced meat portions with 1.5% and 2% marjoram or thyme oils, and 2% cinnamon oil significantly reduced *S. aureus* count to 1.87± 0.02, 1.71 ± 0.02, 1.91± 0.02, 1.75 ± 0.02, and 1.89± 0.02 log CFU/g, respectively, by day 8 (Table 4).

Detection of Salmonella

In sample I, no *Salmonella* isolates were detected over the 8 days in both treated or untreated meat portions. In contrast, *Salmonella* was detected in the untreated meat portion of sample II on day 5, and in all treated portions on day 8 (Table 6).

Detection of Shigella

No *Shigella* isolates appeared in either sample in all minced meat portions during the period of the experiment.

Chemical analysis of essential oil-treated and untreated minced meat samples

pH values

In sample I, the pH of the untreated meat portion increased from 5.77± 0.01 on day 0 to 6.92± 0.02 on day 8. Portions treated with 2% cinnamon or lemongrass essential oils recorded significantly low pH values of 6.50± 0.02 and 6.47± 0.02, respectively, relative to the untreated portion on day 8 (Supplementary Table S6). In sample II, the pH values of the portions treated with 2% marjoram or lemongrass essential oils on day 8 were significantly ($P< 0.05$) reduced to 6.39± 0.02 and 6.20 ± 0.02, respectively, in comparison to the untreated meat portion, which had a pH of 6.75 ± 0.03 on the same day (Supplementary Table S7).

TVN values

Treatment of minced meat portions (sample I) with 2% marjoram or thyme essential oils significantly ($P< 0.05$) reduced the TVN to 17.43± 0.02 and 17.87 ± 0.02mg/100g, respectively, on day 5 compared with the TVN in the untreated meat portion on the same day (Supplementary Table S6). Treatment of minced meat portions (sample II) with 2% marjoram, thyme, or lemongrass oils significantly reduced the TVN to 16.82± 0.02, 15.77± 0.02, and 16.10 ± 0.02 mg/100 g, respectively, on day 8 compared with the TVN in the untreated meat portion on the same day (34.75± 0.02mg/100 g, Supplementary Table S7).

TBA values

The TBA value of minced meat portions (sample I) treated with 2% marjoram oil, or 1.5% and 2% thyme oil remained significantly acceptable till day 7. In contrast, the TBA value of the untreated meat portion remained acceptable only until day 4 (Supplementary Table S6). In sample II, the TBA value in the untreated meat portion increased from 0.13± 0.02mg malonaldehyde/kg on day 0 to 0.74± 0.02mg malonaldehyde/kg on day 4, and it became unacceptable, as recommended by EOS (2005), on day 5. In contrast to minced meat portions treated with 1.5 and 2% marjoram oil and 1.5 and 2% thyme oil, the TBA values remained significantly within the acceptable limit until day 7, and the values were 0.81± 0.02, 0.76± 0.02, 0.86± 0.02, and 0.82± 0.02mg Melanoaldehyde/ Kg, respectively (Supplementary Table S7).

TABLE 3. Effect of different concentrations of essential oils on the count of coliform bacteria in minced meat from sample I (July 2019) and sample II (January 2020) maintained at 2°C

Type of count		Coliform count			
Samples		Sample I (July 2019)		Sample II (January 2020)	
Experiment days		Day 0	Day 8	Day 0	Day 8
Essential oil treatments	Control	**3.25±0.04**	**4.33±0.03a**	**2.11±0.02**	**4.11±0.02ab**
	M1(1%)	3.25±0.04	3.79±0.02d	2.11±0.02	3.56±0.04ab
	M2(1.5%)	3.25±0.04	3.53±0.02f	2.11±0.02	3.47±0.02c
	M3 (2%)	3.25±0.04	3.42±0.02g	2.11±0.02	**3.11±0.02d**
	T1 (1%)	3.25±0.04	4.13±0.03b	2.11±0.02	3.47±0.04c
	T2 (1.5%)	3.25±0.04	3.41±0.02g	2.11±0.02	3.21±0.02cd
	T3 (2%)	3.25±0.04	**3.22±0.01h**	2.11±0.02	**2.89±0.02d**
	C1 (1%)	3.25±0.04	4.13±0.04b	2.11±0.02	3.98±0.03ab
	C2 (1.5%)	3.25±0.04	3.86±0.03c	2.11±0.02	3.79±0.02ab
	C3 (2%)	3.25±0.04	3.55±0.04f	2.11±0.02	3.49±0.02c
	L1 (1%)	3.25±0.04	4.12±0.02b	2.11±0.02	3.91±0.03ab
	L2 (1.5%)	3.25±0.04	3.69±0.04e	2.11±0.02	3.88±0.02ab
	L3 (2%)	3.25±0.04	**3.18±0.02i**	2.11±0.02	3.73±0.03ab

Results are shown as mean ± standard error (SE). Means with different letters in the same column are significantly different (P< 0.05) by Duncan's post-hoc test. P-Values were calculated by ANOVA (unit: log CFU/g).

TABLE 4. Effect of different concentrations of essential oils on *E. coli* and *S. aureus* counts in minced meat from sample I (July 2019) and sample II (January 2020) maintained at 2°C

Samples		Sample I (July 2019)				Sample II (January 2020)			
Type of count		*E. coli* count		*S. aureus* count		*E. coli* count		*S. aureus* count	
Experiment days		Day 0	Day 8	Day 0	Day 8	Day 0	Day 8	Day 0	Day 8
Essential oil treatments	Control	**2.63±0.02**	**3.93±0.01a**	**1.83±0.02**	**5.73±0.02a**	**1.30±0.02**	**3.81±0.02a**	**1.19±0.02**	**3.65±0.02a**
	M1(1%)	2.63±0.02	3.19±0.02e	1.83±0.02	5.65±0.03a	1.30±0.02	3.20±0.03e	1.19±0.02	2.40±0.03f
	M2(1.5%)	2.63±0.02	2.93±0.02h	1.83±0.02	5.43±0.02b	1.30±0.02	3.17±0.02e	1.19±0.02	**1.87±0.02i**
	M3 (2%)	2.63±0.02	**2.63±0.03i**	1.83±0.02	4.73±0.04d	1.30±0.02	**2.91±0.02h**	1.19±0.02	**1.71±0.02k**
	T1 (1%)	2.63±0.02	3.13±0.02f	1.83±0.02	5.63±0.02ab	1.30±0.02	3.17±0.02e	1.19±0.02	2.26±0.03g
	T2 (1.5%)	2.63±0.02	3.00±0.02g	1.83±0.02	5.33±0.04b	1.30±0.02	3.00±0.01g	1.19±0.02	**1.91±0.02h**
	T3 (2%)	2.63±0.02	**2.43±0.03j**	1.83±0.02	3.02±0.02e	1.30±0.02	**2.30±0.02i**	1.19±0.02	**1.75±0.02j**
	C1 (1%)	2.63±0.02	3.24±0.02d	1.83±0.02	5.89±0.03a	1.30±0.02	3.60±0.02b	1.19±0.02	3.30±0.01b
	C2 (1.5%)	2.63±0.02	3.15±0.02f	1.83±0.02	5.68±0.02a	1.30±0.02	3.50±0.03c	1.19±0.02	2.60±0.03e
	C3 (2%)	2.63±0.02	3.00±0.03g	1.83±0.02	5.36±0.01b	1.30±0.02	3.00±0.02g	1.19±0.02	**1.89±0.02hi**
	L1 (1%)	2.63±0.02	3.82±0.02b	1.83±0.02	5.90±0.02a	1.30±0.02	3.38±0.04d	1.19±0.02	3.29±0.03b
	L2 (1.5%)	2.63±0.02	3.32±0.03c	1.83±0.02	5.69±0.03a	1.30±0.02	3.10±0.02f	1.19±0.02	3.20±0.02c
	L3 (2%)	2.63±0.02	3.00±0.02g	1.83±0.02	5.28±0.02c	1.30±0.02	3.00±0.03g	1.19±0.02	3.10±0.01d

Results are shown as mean ± standard error (SE). Means with different letters in the same column are significantly different (P < 0.05) by Duncan's post-hoc test. P-Values were calculated by ANOVA (unit: log CFU/g).

Antibiotic susceptibility of E. coli, S. aureus, and Salmonella spp. isolated from oil-treated and untreated minced meat

In this study, 32 isolates of *E. coli*, 32 isolates of *S. aureus*, and five isolates of *Salmonella* spp. were subjected to antibiotic sensitivity test by disc diffusion method using the following antibiotics: ampicillin (AMP, 10µg), tetracycline (TE, 30µg), chloramphenicol (C, 30µg), ciprofloxacin (CIP, 5µg), nitrofurantoin (F, 300µg), sulfamethoxazole-trimethoprim (SXT, 25µg), and penicillin (P, 10µg). In sample I, the *E. coli* isolates obtained from the untreated and 2% marjoram oil-treated meat portions were found to be resistant to C, CIP, and SXT and sensitive to AMP, TE, and F. However, *E. coli* isolates from the 2% thyme oil-treated portion were resistant to AMP from day 7 of the cold storage (Table 5). In sample II, the *E. coli* isolates obtained from the untreated and 2% marjoram oil-treated meat portions were sensitive to TE, C, F and SXT and resistant to AMP and CIP (Table 6).

TABLE 5. Antibiotic susceptibility of the *E. coli* and *S. aureus* isolates from untreated, 2% marjoram oil-treated, and 2% thyme oil-treated minced meat portions from sample I (July 2019)

Isolates from untreated minced meat												
Day of	***E. coli***						***S. aureus***					
isolate	**AMP**	**TE**	**C**	**CIP**	**F**	**SXT**	**P**	**TE**	**C**	**CIP**	**F**	**SXT**
Day 0	S (20)	S (23)	R (11)	R (20)	S (23)	I (13)	S (30)	R (12)	S (20)	R (13)	R (13)	S (18)
Day 2	S (20)	S (23)	R (11)	R (20)	S (23)	I (13)	S (30)	R (12)	S (20)	R (13)	R (13)	S (18)
Day 4	S (20)	S (23)	R (11)	R (18)	S (23)	I (13)	S (32)	R (12)	S (20)	R (13)	R (13)	S (18)
Day 5	**S (20)**	S (25)	R (11)	R (18)	S (20)	I (13)	S (32)	R (12)	S (19)	R (13)	R (13)	S (17)
Day 7	**S (20)**	S (25)	R (11)	R (18)	S (20)	I (13)	S (32)	R (13)	S (19)	R (14)	R (13)	S (17)
Day 8	**S (20)**	S (25)	R (11)	R (18)	S (20)	I (13)	S (32)	R (13)	S (19)	R (14)	R (13)	S (17)
Isolates from minced meat treated with 2% marjoram essential oil												
Day of	***E. coli***						***S. aureus***					
isolate	**AMP**	**TE**	**C**	**CIP**	**F**	**SXT**	**P**	**TE**	**C**	**CIP**	**F**	**SXT**
Day 2	S (19)	S (24)	R (12)	R (20)	S (23)	I (13)	S (30)	R (12)	S (20)	R (14)	R (12)	S (17)
Day 4	S (19)	S (24)	R (12)	R (20)	S (23)	I (13)	S (30)	R (13)	S (20)	R (14)	R (12)	S (17)
Day 5	S (20)	S (24)	R (11)	R (20)	S (23)	I (13)	S (30)	R (13)	S (20)	R (14)	R (13)	S (17)
Day 7	S (20)	S (25)	R (11)	R (18)	S (20)	I (13)	S (30)	R (13)	S (20)	R (14)	R (13)	S (17)
Day 8	S (20)	S (25)	R (11)	R (18)	S (20)	I (13)	S (30)	R (13)	S (20)	R (14)	R (13)	S (16)
Isolates from minced meat treated with 2% thyme essential oil												
Day of	***E. coli***						***S. aureus***					
isolate	**AMP**	**TE**	**C**	**CIP**	**F**	**SXT**	**P**	**TE**	**C**	**CIP**	**F**	**SXT**
Day 2	S (19)	S (22)	R (12)	R (19)	S (23)	I (13)	S (29)	R (13)	S (20)	R (13)	R (13)	S (18)
Day 4	S (18)	S (22)	R (12)	R (19)	S (23)	I (14)	S (30)	R (13)	S (20)	R (14)	R (13)	S (18)
Day 5	**I (16)**	S (24)	R (12)	R (18)	S (23)	I (14)	S (30)	R (13)	S (20)	R (14)	R (13)	S (18)
Day 7	**R (13)**	S (24)	R (12)	R (18)	S (23)	I (14)	S (30)	R (14)	S (20)	R (14)	R (13)	S (18)
Day 8	**R (12)**	S (24)	R (12)	R (18)	S (23)	I (14)	S (30)	R (14)	S (20)	R (14)	R (13)	S (18)

Antibiotic sensitivity test according to Clinical and Laboratory Standards Institute (CLSI 2019).

R= Resistance, S= Sensitive, AMP = Ampicillin, TE= Tetracycline, C= Chloramphenicol, CIP= Ciprofloxacin,

F= Nitrofurantoin, SXT= Sulfamethoxazole-trimethoprim, P= Penicillin. Antibiotic susceptibility was determined as the clear zone diameter (mm).

TABLE 6. Antibiotic susceptibility of the *E. coli*, *S. aureus,* and *Salmonella* isolates from untreated, 2% marjoram oil-treated, and 2% thyme oil-treated minced meat portions from sample II (January 2020)

Isolates from untreated minced meat

Day of isolate	*E. coli*						*S. aureus*						*Salmonella*					
	AMP	**TE**	**C**	**CIP**	**F**	**SXT**	**P**	**TE**	**C**	**CIP**	**F**	**SXT**	**AMP**	**TE**	**C**	**CIP**	**F**	**SXT**
Day 0	R (12)	S (17)	S (20)	R (18)	S (19)	S (18)	S (32)	R (13)	R (12)	S (24)	S (18)	S (18)	-	-	-	-	-	-
Day 2	R (12)	S (17)	S (20)	R (18)	S (19)	S (18)	S (32)	R (12)	R (12)	S (24)	S (18)	S (18)	-	-	-	-	-	-
Day 4	R (12)	S (17)	**S (20)**	R (18)	S (19)	S (17)	S (32)	R (12)	R (11)	S (24)	**I (16)**	S (20)	-	-	-	-	-	-
Day 5	R (12)	S (17)	**S (20)**	R (18)	S (18)	S (17)	S (32)	R (12)	R (11)	S (22)	**R (12)**	S (20)	S (17)	R (10)	R (11)	S (27)	S (20)	S (18)
Day 7	R (12)	S (18)	**S (20)**	R (19)	S (18)	S (17)	S (30)	R (12)	R (11)	S (22)	**R (12)**	S (20)	S (19)	R (11)	R (11)	S (29)	S (20)	S (18)
Day 8	R (12)	S (18)	**S (20)**	R (19)	S (18)	S (17)	S (30)	R (12)	R (11)	S (22)	**R (12)**	S (20)	S (19)	R (11)	R (12)	S (29)	S (20)	S (19)

Isolates from minced meat treated with 2% marjoram essential oil

Day of isolate	*E. coli*						*S. aureus*						*Salmonella*					
	AMP	**TE**	**C**	**CIP**	**F**	**SXT**	**P**	**TE**	**C**	**CIP**	**F**	**SXT**	**AMP**	**TE**	**C**	**CIP**	**F**	**SXT**
Day 2	R (13)	S (17)	S (20)	R (18)	S (19)	S (18)	S (31)	R (13)	R (11)	S (24)	S (18)	S (18)	-	-	-	-	-	-
Day 4	R (13)	S (17)	S (20)	R (18)	S (18)	S (18)	S (31)	R (13)	R (12)	S (24)	**S (19)**	S (20)	-	-	-	-	-	-
Day 5	R (12)	S (18)	S (20)	R (18)	S (18)	S (18)	S (31)	R (13)	R (12)	S (22)	**S (18)**	S (20)	-	-	-	-	-	-
Day 7	R (12)	S (18)	S (20)	R (18)	S (18)	S (18)	S (30)	R (12)	R (12)	S (22)	**S (18)**	S (20)	-	-	-	-	-	-
Day 8	R (12)	S (18)	S (19)	R (18)	S (18)	S (18)	S (30)	R (12)	R (12)	S (22)	**S (19)**	S (20)	S (19)	R (11)	R (12)	S (29)	S (20)	S (18)

Isolates from minced meat treated with 2% thyme essential oil

Day of isolate	*E. coli*						*S. aureus*						*Salmonella*					
	AMP	**TE**	**C**	**CIP**	**F**	**SXT**	**P**	**TE**	**C**	**CIP**	**F**	**SXT**	**AMP**	**TE**	**C**	**CIP**	**F**	**SXT**
Day 2	R (12)	S (17)	S (18)	R (19)	S (18)	S (18)	S (31)	R (12)	R (12)	S (24)	S (18)	S (18)	-	-	-	-	-	-
Day 4	R (12)	S (17)	**I (16)**	R (19)	S (18)	S (17)	S (31)	R (12)	**I (14)**	S (22)	S (19)	S (18)	-	-	-	-	-	-
Day 5	R (12)	S (17)	**R (12)**	R (18)	S (18)	S (17)	S (30)	R (12)	**I (16)**	S (22)	S (19)	S (20)	-	-	-	-	-	-
Day 7	R (12)	S (17)	**R (12)**	R (18)	S (18)	S (17)	S (30)	R (12)	**S (19)**	S (22)	S (19)	S (20)	-	-	-	-	-	-
Day 8	R (12)	S (17)	**R (12)**	R (18)	S (18)	S (17)	S (30)	R (12)	**S (20)**	S (22)	S (19)	S (22)	S (19)	R (11)	R (12)	S (29)	S (20)	S (18)

Antibiotic sensitivity test according to Clinical and Laboratory Standards Institute (CLSI 2019). R= Resistance, S= Sensitive, AMP= Ampicillin, TE = Tetracycline, C= Chloramphenicol, CIP = Ciprofloxacin, F= Nitrofurantoin, SXT= Sulfamethoxazole-trimethoprim, P= Penicillin and (-) Negative *Salmonella.* Antibiotic susceptibility was determined as the clear zone diameter (mm).

Screening of essential oils for antibacterial activity against E. coli isolates

Seven *E. coli* isolates were obtained from the two 2% thyme oil-treated minced meat portions; they were tested for antibiotic sensitivity and showed different patterns. These isolates were screened for sensitivity toward different concentrations of marjoram and thyme oils, using absolute ethanol as a negative control and chloramphenicol (30µg/disc) as a positive control. All the isolates were sensitive to both the oils, and the level of susceptibility increased with increasing oil concentrations. The highest concentrations (1.5% and 2%) consistently showed antimicrobial activity against *E. coli* while the lowest concentrations weakly inhibited the growth of *E. coli* (Fig. 1).

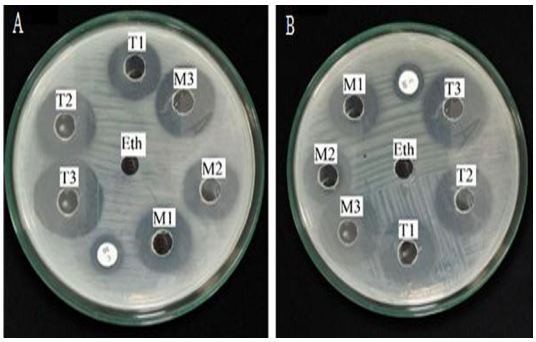

Fig. 1. The diameters of inhibition zones were obtained by well diffusion of different concentrations of thyme and marjoram oils against *E. coli* isolates obtained from minced meat sample treated with 2% thyme essential oil and maintained at 2°C [A Sample I (July 2019), B sample II (January 2020), M: Marjoram, T: Thyme, and Eth: Ethanol]

Discussion

Meat and meat products have a shelf life–the duration they can be stored under certain conditions of temperature and humidity while still being good (Hardin, 2016). They are deemed to be spoiled when the product changes in a way that makes it unpleasant or unappealing to the consumer, for example if it develops off-flavors, off-colors, gas, or slime (Hardin, 2021). Microbial growth is usually implicated in meat spoilage (Hardin, 2016). For raw meat products, safety and quality can be estimated by the use of indicator microorganisms, including total aerobic plate count (APC), coliform count (CC), and *Escherichia coli* count (ECC) (Kim & Yim, 2016). Also, EOS (2005) specified certain parameters to be tested for controlling the quality of the minced meat during cold storage (4°C). These parameters include microbiological tests (anaerobic microorganisms, aerobic microorganisms, coliform bacteria, *E. coli*, *Salmonella*, *Shigella*, *and Staphylococcus aureus*) and chemical tests as (TBA and TVN).

In this study, the mean values of the anaerobic bacterial count in the untreated meat portions peaked on day 8 with 4.74 ± 0.02 and 2.25 ± 0.03 log CFU/g for samples I and II, respectively. The obtained results nearly agree with those of El-Sayed et al. (2020), who reported that the total count of anaerobes was 6×10^3 CFU/g (3.77 log CFU/g) in sheep meat on day 0. In this study, the anaerobic bacterial count decreased as the concentration of essential oils increased and the lowest number was found in meat samples treated with 2% essential oils. Clostridia (*C. perfringens*) and other anaerobic bacteria are responsible for various public health issues. They can withstand high temperatures by producing resistant spores. During slaughtering and subsequent handling, meat can become contaminated with clostridial spores (Smith et al., 2021). De Oliveira Mota et al. (2021) reported that both the total aerobic and coliform count are important indicators of general microbial contamination, and the presence of these bacteria in high numbers indicates a higher potential for spoilage and the presence of pathogens in meat. APC provides an overall estimate of bacterial populations–a higher APC usually translates to poorer quality and reduced shelf life (Kim & Yim, 2016).

Both the coliform count and the *E. coli* count provide an estimate of fecal contamination and poor sanitation during meat processing–high counts generally correlate with high levels of foodborne pathogens originating from feces (Kim & Yim, 2016). In this study, the coliform count decreased in both samples when the minced meat portions were treated with marjoram, thyme, cinnamon, and lemongrass essential oils, compared with the untreated meat portions from the two samples. Similar to the results of Shaltout et al. (2017), we observed the most promising effects at the highest concentration of the essential oils, particularly with M3 and T3 in sample II, and T3 and L3 in sample I. Abu-Salem & Abou Arab (2010) also concluded that 1.5% lemongrass, thyme, and garlic oils had the most

potent effect on the growth of *Enterobacteriaceae* and coliforms, which decreased as the oil concentration increased. Küçükbay et al. (2013) reported that the bactericidal or bacteriostatic effect of thyme oil depends on the phenolic compounds, hydrocarbons, and their effective concentrations in the oil. In the present study, *E. coli* count in the portions treated with T3 and M3 from both the samples was lower than the count in the untreated meat portions.

Generally, an increase in the count of anaerobic, aerobic, coliform bacteria, *E. coli*, and *S. aureus* in the untreated meat portions was obtained from both samples throughout the experiment. Our results agreed with Kim & Yim (2016) and Kim et al. (2018), who discussed the seasonal variations in microbial contamination of meat processing plants. They concluded that the APC of meat was the highest in September and the lowest in February, indicating that the high APC in September might be due to a failure in temperature management in the meat processing plants. Also, they reported that the highest coliform count was obtained in the summer, followed by autumn and then spring.

Conclusion

Our findings showed that adding 2% marjoram and thyme essential oils to the minced meat samples significantly increased their shelf life during cold storage (2ºC) compared with untreated meat samples. In summer (sample I), the untreated sample was found to be unacceptable by day 4 due to *S. aureus* count while the marjoram oil-treated sample was found to be unacceptable by day 7 due to its sensory properties, APC, and *S. aureus* count. The thyme oil-treated sample was also found to be unacceptable by day 7 due to its APC. In winter (sample II), the untreated sample was found to be unacceptable by day 5 due to its APC and the presence of *Salmonella*, but the marjoram and thyme oil-treated samples were found to be unacceptable only by day 8 due to the appearance of *Salmonella*. We demonstrate that marjoram and thyme essential oils can keep minced meat safe during cold storage.

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Authors' contributions: Conceptualization and methodology: H.H.E., M.A.H. and A.M.S., laboratory work: A.M.S., data analysis: M.A.H., A.M.S. and H.H.E., writing - original draft preparation: A.M.S., writing - review and editing: M.A.H. and H.H.E. All authors have read and agreed to the published version of the manuscript.

Ethics approval: Not applicable.

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الاختلافات الموسمية في تعداد البكتريا الموجودة في اللحوم المفرومة المبردة ودور الزيوت الأساسية المختلفة في إطالة مدة الصلاحية.

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هدفت هذه الدراسة إلى تقييم مستوى التلوث البكتيري لعينتين من اللحم المفروم، تمت معالجتهما بالزيوت الأساسية المختلفة. تم جمع عينات اللحوم المفرومة من مختلف محلات الجزارة بمحافظة القاهرة في يوليو 2019 (العينة I) ويناير 2020 (العينة II). تم إجراء التحليل الحسي والبكتريولوجي والكيميائي للعينتين. أوضحت النتائج أن عدد البكتيريا اللاهوائية والهوائية، coliforms، *Escherichia coli* و *Staphylococcus aureus* قد ازداد في الأجزاء الضابطة غير المعالجة للعينتين. كما أظهرت النتائج بشكل عام أن إجمالي التلوث البكتيري في العينة الأولى التي تم جمعها خلال فصل الصيف أكبر من التلوث البكتيري في العينة الثانية التي تم جمعها خلال فصل الشتاء. هذا وقد لوحظ أن إضافة زيت البردقوش وزيت الزعتر، كل على حدة، بتركيز 2٪ إلى عينات اللحم المفروم المبرد، أدى إلى زيادة فترة الصلاحية بدرجة ملحوظة عند درجة الحرارة 2° م حيث امتدت إلى 7 و8 أيام، مقارنة بالأجزاء غير المعالجة من العينتين، والتي سجلت فترة صلاحية 4 و5 أيام على التوالي. وقد يرجع عدم مقبولية الجزء الضابط غير المعالج من العينة I في اليوم الرابع إلى العدد غير المقبول لـ *S. aureus*. أما في العينة II، وجد أن الجزء الضابط غير مقبول في اليوم الخامس وهذا قد يعود إلى العدد غير المقبول للبكتريا الهوائية (APC) ووجود السالمونيلا. تشير هذه النتائج إلى أن معالجة اللحوم المفرومة المبردة بالزيوت الأساسية، وخاصة زيت البردقوش أو زيت الزعتر، تعمل على إطالة مدة الصلاحية عند 2° م عن طريق تقليل مستوى التلوث البكتيري دون التأثير على الجودة والخصائص الحسية للحم.