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Antimicrobial Effects of NaCl and Essential Oils of *Thymus vulgaris* and *Zingiber officinale* against Gram-negative bacteria Inoculated in Fresh Baladi Cheese

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Gram-negative bacteria are the main food borne pathogen that causes different diseases in humans. It may be found in dairy products, especially in fresh-brined cheese. The aim of this study was to investigate the inhibition effect of different substances concentrations (NaCl 3%, 6%, 9%; *Thymus vulgaris* and *Zingiber officinale* essential oils (EOs) in concentrations of 0.25%, 0.5%, 1%, and 2% against the gram-negative bacteria experimentally inoculated in fresh Baladi cheese which were studied at three storage temperatures (-20, 4, and 25) °C for storage intervals (one week, two weeks, three weeks, on month, two months until six months).The highest inhibition effect was obtained at the 9% NaCl concentration, where bacterial count in the contaminated cheese decreased after just one week of storage at 25° C to 6.4 x 10^2 CFU/g, and no bacterial growth was noticed after two months of storage at the same conditions. The storage temperature -20° C had a higher effect on bacterial growth inhibition than other storage temperatures of 4° C and 25 °C; at this temperature, no bacteria growth was revealed after three months of storage at 3% and 6% salt concentrations. In concern to the inhibition effect of EOs, no bacterial colonies were growing on the plates for the experimentally contaminated cheese and its whey that was treated with concentrations of 0.25%, 0.5%, and 1% of *Z. officinale* EO after three months of storage, and after three weeks of storage for those treated with the concentration 2% of *Z. officinale* EO at all storage temperatures. The 2% concentration of *T. vulgaris* EO, led to bacterial growth absence after two weeks of storage at the different temperatures used in the study. Using PCR technique to inquire about bacteria in contaminated cheese samples contributed to fast and highly accurate detection at both levels of genus and type. Fragments with 556 bp, 615 bp, 400 bp, 343 bp, and 373 bp were corresponding to *Escherichia coli, Salmonella typhi, Yersinia enterocolitica, Klebsiella oxytoca and Salmonella* spp., respectively. It was possible to detect genus and species of more than one bacterium in cheese samples in a short time with 100% accuracy.

Keywords: ***Zingiber officinale*, *Thymus vulgaris*, Essential oils, Sodium chloride, Inhibition effect, Gram-negative bacteria, Cheese.**

INTRODUCTION

In ancient times, especially in warm climate regions where goats, cows and sheep were domesticated, contaminating bacteria cause destroyed the milk and form two layers, one is a liquid called whey, and another solid called curd (Barbara et al. 2008). So, the best way for expands the shelf life of milk was by converting it to cheese which is a more storable product than milk (Vedamuthu et al. 1983).

Cheese is a complete nutritious source of many key elements, it is rich in protein, vitamins, fat and minerals, especially calcium, magnesium, zinc and phosphorus (Eichholzer et al. 2005). The main sugar in milk is lactose. Some of this sugar is separated with whey and the other part is fermented to produce lactic acid so the ripened cheese is free of milk sugar (Law, 1984). In addition, cheese is a rich source of amino acids, peptides and proteins. It has all essential amino acids except cysteine and methionine (Tome et al. 2002). Fat is another important component of cheese and consists of 20 to 35% of the dry weight (Sieber, 2001).

There are various types of cheese known around the world, these types can be grouped depending on the used milk type, coagulation method, fat ratio, texture, cheese surface, and salt content (Molimard and Spinnler, 1996). Depending on the procedure of cheese making and its water content, cheese is classified as soft cheese, semi-hard cheese and hard cheese (Pantaleao et al. 1990).

White cheese is the most widespread in the Balkans and Mediterranean (Bintsis and Papademas, 2002), many types of white cheese with different names are known, Domiati in Egypt, Beyaz peynir in Turkey, Feta in Greece and Baladi cheese in Syria (Alichanidis and Polychroniadou, 2008).

In Syria, fresh Baladi cheese is usually manufactured from cow milk in spring. Generally, it is consumed either as salted cheese with 10% brine solution or fresh, so the used milk to produce this type of cheese must be clean, fresh, and milked from healthy animals while the unsuitable milk is discarded (Al-Mariri et al. 2021). Baladi cheese is un ripened white cheese and it is ready to consumed directly after processing.

Dairy products are considered as promoting environment for microorganisms' growth as a result of their sufficient content of water, sugar, protein and other supporting factors (Johnson, 2002). Several types of microbes may contaminate the Baladi cheese due to incorrect procedures and unhygienic handling (Humeid et al. 1990). Dairy product is exposed to contamination by many microbes such as *Staphylococcus aureus* (Castro et al. 2018), *Brucella* spp*.* (Al-Mariri, 2015) and *Enterobacteriaceae* family, which includes *Salmonella*, *Yersinia*, *E. coli*, *Klebsiella* and *Shigella,* most of these gram-negative bacteria cause gastrointestinal diseases in human (Paterson, 2006).

The emergence of bacteria resistant to synthesis antimicrobial materials led to searching for effective natural antimicrobial additives. Essential oils became the alternative antibacterial agents used to inhibit the growth of microbes (Brito et al. 2018).

Plants and their essential oils have been used for different purposes, such as flavor agents, elimination of unpleasant odors, treating some diseases and antimicrobial additives (Franz, 2010).

The genus *Thymus* contains approximately 350 species belonging to the *Lamiaceae* family which are native to the Mediterranean region. One of the most important species is *Thymus vulgaris* L., it is commonly known as thyme, locally known as “zaatar”, and spreads widely in Syria, This herb is an evergreen plant, about 4–20 mm long with small and highly aromatic gray-green oval leaves containing pink, white or purple flowers (Ferreira et al. 2016; Fani and Kohanteb, 2017). It is used for many purposes such as herbal tea, spices and traditional medicine.

Thymus species have strong antibacterial activity, so they can use as an alternative method instead of other traditional additives, especially sugar and salt. Food and Agriculture Organization classified *Thymus* as a safe and natural flavoring additive for the food industry based on the allowed dose (Nieto, 2020).

The essential oil of *T. vulgaris* L. is considered a potential source of antibacterial, antioxidant, anti-inflammatory antifungal, sedative and antiviral activities. The main components of its essential oils are linalool, γ-terpineol, geranial, thymol, carvacrol, and p-cymene. These components differ in their antimicrobial activity and inhibit some pathogenic bacteria such as *Proteus* spp., *Yersinia enterocolitica* O9, *E. coli* O:157 and *K. pneumonia* (Rota et al. 2008; Al-Mariri and Safi, 2014).

Ginger (*Zingiber officinale*) belongs to the *Zingiberaceae* family. The name zingiber derives from a Sanskrit word meaning “horn-shaped” in regard to the juts on the surface of the rhizome. Several names are used for this genus, gengibre, ginger and gingembre (Corrêa et al. 1994). It is used as a medicinal plant in folk therapy, mixed with other foods such as honey or milk, or alone as fresh or dried (Pakrashi, 2003). Ginger has 1 – 3% essential oils (Govindarajan, 1982), the main components of ginger EOs are β-zingiberene, β-sesquiphellandrene, neral, β-bisabolene, citral and geraniol (Mahboubi, 2019). Essential oils of Brazilian ginger inhibited many bacteria including *S. aureus*, *L. monocytogenes*, *S. typhimurium*, *P. aeruginosa*, *E. coli* and *Shigella flexneri* (Mesomo et al. 2013)

The microbial contamination causes deterioration of some sensory and qualitative characteristics of the food, which leads to reducing shelf life. In addition, it constitutes a threat to consumer health (ICMSF, 1996).

Salting of food is a common method to preserve food and maintain its shelf life, salt is low-cost and has antimicrobial effects, and is used as enhancing flavor agent (Silva et al. 2003; Albarracin et al. 2010). The bacterial identification by traditional methods including medium culturing and biochemical tests are still used for different food sources (Bohaychuk et al. 2006).

Thus, the purpose of this study was to estimate the antibacterial effect of different concentrations of NaCl and EOs of *Zingiber officinale* and *Thymus vulgaris* in Baladi cheese experimentally contaminated with gram-negative bacteria including *E. coli*, *S. typhi*, *Y. enterocolitica*, *K. oxytoca* and *Salmonella* spp. at different temperature storage intervals, as well as detecting the bacterial contamination using PCR technique.

MATERIALS AND METHODS

Microorganisms and growth conditions

Five gram-negative pathogens: *E. coli*, *S. typhi*, *Y. enterocolitica*, *K. oxytoca* and *Salmonella* spp*.* were chosen in this study due to their potential existence in Baladi cheese, and were used to contaminate cheese milk. These isolates belonged to the culture collection of the Microbiology and Immunology Division, Department of Molecular Biology and Biotechnology, AECS, Syria. MacConkey agar medium (Sigma Aldrich, St. Louis, MO) was used to refresh the stored bacteria at 37 °C for 24 h. After that, a sterile Phosphate-Buffered saline (PBS) was used to suspend the active culture (Nosir et al. 2014) and adjusted to $1x10^9$ Colony Forming Unit (CFU)/ml by controlling the optical density at 590 nm.

Extraction of EOs

Samples of both *Z. officinale* and *T. vulgaris* were obtained from local markets in Damascus, Syria. The samples were powdered by grounding them using an electrical blender. 100 g of the plant samples were used for EOs extraction which was carried out by the water-steam distillation device (Clevenger-type apparatus, Germany). EOs were collected after steam condensing in a cooling vapor system for 3 h and then dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and stored in resealable vials at 4 °C in the dark, but were allowed to rest at room temperature prior to investigation. Dimethyl sulfoxide (DMSO) with a concentration of 4.4% (v/v) was used for dilution of the extracted EOs and used for antimicrobial activity tests. The EOs extraction yield (%) was 0.5 and 0.8 for *Z. officinale* and *T. vulgaris,* respectively.

Production of fresh Baladi cheese

Milk was filtered by a clean textile piece, then pasteurized at 72 °C for 15 s, and cooled quickly to 39 °C. 20 g of $CaCl_2$ was added per 100 kg of milk, where Ca^{+2} ions are necessary for the curd formation of casein micelles in milk. Rennet enzyme was then added at the rate of 2.5 g to 100 kg of milk which will cause the milk to curdle. The mixture was incubated for 40-60 min at a temperature of 39 °C until the completed coagulation. The curd was destroyed and left for 15 min to allow the whey to separate. Excess whey was then drained off, and the curd was cut off and put into cheesecloth for a few hours and pressed before being turned out.

The experimentally contaminated cheese

The pasteurized milk prepared for the cheese processing was inoculated with 10^9 CFU/ml of gram-negative bacteria used in this study conjugated with adding different concentrations of substances under study (NaCl, EOs of *T. vulgaris* and *Z. officinale*) for comparison of their antimicrobial effect in each of the contaminated cheese and its whey which were stored for six months. As a positive control for microbial growth, experimentally infected cheese samples free of NaCl or EOs were used. While for the negative control, uncontaminated cheese samples were used.

Inhibition activity of NaCl and EOs

To assay the inhibition activity of varying concentrations of NaCl and EOs (*Z. officinale* and *T. vulgaris*) against gram-negative bacteria inoculated in fresh Baladi cheese, three concentrations of NaCl (3, 6 and 9%) and four concentrations of EOs (0.25, 0.5, 1 and 2%) were added to the experimentally contaminated milk during cheese preparation.

The inhibition activity of NaCl and EOs was examined in both cheese and its whey stored at temperatures (-20, 4, 25 °C), for 6 months (one week, two weeks, three weeks, one month, two months until six months). A patch of 25 g of samples (cheese and its whey) was homogenized into sterile strainer/filter-stomaching bags (Seward, stomacher lab system standard bags, England) by adding MacConkey broth and mixing in a pulsed stomacher (Seward, stomacher 80 Biomaster, England) twice for 30s. At different storage times, ten-fold serial dilutions of each sample were cultured on MacConkey agar; then they were incubated at 37 °C for 48 h.

Identification of gram-negative bacteria isolates by polymerase chain reaction (PCR)

Pure isolates of gram-negative bacteria from MacConkey agar were inoculated into LB (Luria-Bertani) broth, and then incubated in a shaking incubator at 37 °C overnight at 140 rpm. PCR technique was used to confirm the identities of the studied isolates. DNA extraction kit (Catalog 732-6030-Biolab, South Africa) was used to extract genomic DNA from the bacterial isolates (*E. coli, S. typhi*, *Y. enterocoltica*, *K. oxytoca* and *Salmonella* spp.) as directed by the manufacturer.

The DNA amplification was managed in a thermocycler (GeneAmp® PCR System 9700, Applied Biosystems). Primers used for the detection of gram-negative bacteria in Baladi cheese were listed in Table 1.

Table 1: Primers used in molecular detection of gram-negative bacteria in Baladi cheese

Bacterial strain	Nucleotide Sequence (5'-3 ')	Amplicon Length (bp)	Reference
E. coli	**F:** CTGGTATCAGCGCGAAGTCT **R:** AGCGGGTAGATATCACACTC	556	(Anbazhagan et al. 2011).
S. typhi	**F:** GAGGAAGGGAAATGAAGCTTTT **R:** TAGCAAACTGTCTCCCACCATAC	615	(Hirose et al. 2002)
Y. enterocolitica	**F:** TTAATGTGTACGCTGGGAGTG **R:** GGAGTATTCATATGAAGCGTC	400	(Momtaz et al. 2013)
K. oxytoca	**F:** GATACGGAGTATGCCTTTACGGTG **R:** TAGCCTTTATCAAGCGGATACTGG	343	(Chander et al. 2011)
***Salmonella* spp.**	**F:** GTATTGTTGATTAATGAGATCCG **R:** ATATTACGCACGGAAACACGTT	373	(Ranjbar et al. 2017).

The PCR reaction contained 10 pmol of each primer, 200 µM of each deoxynucleotide triphosphate (dNTP), 1 U of MaxTaq DNA Polymerase (Vivantis, Malaysia) in a final volume of 50 µl. PCR was carried out under the following conditions: initial denaturation at 95 °C for 3 min; 35

cycles of denaturation at 95 °C for 30 s, annealing at 55-58 °C for 30 s, extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min.

Horizontal electrophoresis was used for the separation of the amplified products, (80 mA, 150 V, 60 min) in agarose gel 1%, pH 8.4. 0.5 µg/ml ethidium bromide was added for staining (Gandra et al. 2016).

Sensory evaluation

The sensory perception of cheese is one of the important keys to being accepted by the consumer. Evaluation of these aspects is an important step in implementation or experiments. So, various sensory characteristics like visual appearance, color, flavor, texture, and, taste for cheese treated with NaCl or with *Thymus and Zingiber* EOs were carried out for overall acceptability evaluation. The sensory quality of processed cheese was judged by people who were familiar with milk products. The sensory assessment was performed at the time of cheese processing and after storage treated cheese with salt or with EOs for six months with intervals (1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months and 6 months).

Statistical analysis

Antibacterial properties of NaCl and oil extracts were analyzed by one-way repeated-measures analysis of variance (ANOVA) to compare the difference between each pair of means. Data were transformed into $\log_{10}$ CFU. All analyses were conducted by using GraphPad Prism Statistical Software V7. For statistical significance, A *p*-value of <0.001 was considered the cutoff level.

RESULTSAND DISCUSSION

Microbial screening of locally marketed Baladi cheese

Syrian standard specification for cheeses requires being free from pathogenic *E. coli* and the Coliform range is between 10^2-10^3 CFU/g. So, in this work, a microbial test was conducted on different samples of Baladi cheese purchased from a local market in Syria. The obtained results indicate that the *Coliform* bacteria count (4.2 x 10^4 CFU/g) exceeded the accepted limit of Syrian standard specification, the *E. coli* count was 1.2 x 10^2 CFU/g, and the yeast count was (2.5 x 10^3 CFU/g), whereas no fungi were found. Furthermore, the studied samples comply with the requirements of Syrian standard specifications where they were free of pathogens (*Salmonella* spp., *Brucella* spp. and *Listeria* spp.).

Effect of different salt concentrations on gram-negative bacteria count in Baladi cheese and its whey

According to the results shown in tables (2, 3 and 4), the bacterial count in unsalted experimentally contaminated cheese stored at -20 °C was more than 10^9 CFU/g or more than 10^9 CFU/ml in their whey for the entire storage period (6 months) as it is evident in table 2. On the other hand, those stored at room temperature (25 °C) were spoiled after three months of storage as shown in table 4. Also, the samples were spoiled in the fourth month of storage when they were stored at 4 °C (Table 3). A significantly greater inhibition effect was obtained at the 9% NaCl concentration against studied bacteria, where bacterial count in the contaminated cheese decreased after just one week of storage at different temperatures storage (-20 °C, 4 °C and 25 °C), at 25 °C it was 6.4 x 10^2 CFU/g in cheese and 6.8 x 10^2 CFU/ml in the whey. More slides were achieved for the bacterial count after two weeks of storage at 25 °C, reached 47 CFU/g in cheese and 51 CFU/ml in the whey (Table 4). After three weeks of storage for contaminated cheese salted with 9% NaCl, the bacterial count was 5, 11, and 17 CFU/g at -20, 4 and 25 °C, respectively. No bacterial growth was detected in contaminated cheese salted with 9% NaCl after two months of storage at three storage temperatures (Tables 2, 3, and 4). No bacterial colonies were detected by selective medium after three months of storage at -20 °C for the samples (cheese and its whey) salted with 3% or 6% NaCl (Table 2).

Table 2: Effect of different salt concentrations on gram-negative bacteria count in Baladi cheese and its whey stored at -20 °C

Storage time At -20 °C	Bacteria count for Cheese (CFU/g)				Bacteria count for Whey (CFU/ml)			
	Sodium chloride (%)				Sodium chloride (%)			
	0	3	6	9	0	3	6	9
At processing time	-	-	-	-	-	-	-	-
first week	$>10^9$	$>10^9$	$>10^9$	$1.4x10^2$	$>10^9$	$>10^9$	$>10^9$	$2.1x10^2$
Second weeks	$>10^9$	$>10^9$	$>10^9$	15	$>10^9$	$>10^9$	$>10^9$	17
Third week	$>10^9$	$>10^9$	$>10^9$	5	$>10^9$	$>10^9$	$>10^9$	9
First month	$>10^9$	$>10^9$	$>10^9$	20	$>10^9$	$>10^9$	$>10^9$	23
Second month	$>10^9$	$>10^9$	151	NG	$>10^9$	$>10^9$	145	NG
Third month	$>10^9$	NG	NG	NG	$>10^9$	NG	NG	NG
Fourth month	$>10^9$	NG	NG	NG	$>10^9$	NG	NG	NG
Fifth month	$>10^9$	NG	NG	NG	$>10^9$	NG	NG	NG
Sixth month	$>10^9$	NG	NG	NG	$>10^9$	NG	NG	NG

NG: No Growth

Table 3: Effect of different salt concentrations on gram-negative bacteria count in Baladi cheese and its whey stored at 4 °C

Storage time At 4 °C	Bacteria count for Cheese (CFU/g)				Bacteria count for Whey (CFU/ml)			
	Sodium chloride (%)				Sodium chloride (%)			
	0	3	6	9	0	3	6	9
At processing time	-	-	-	-	-	-	-	-
first week	$>10^9$	$>10^9$	$>10^9$	$5.9x10^2$	$>10^9$	$>10^9$	$>10^9$	$6.3x10^2$
Second weeks	$>10^9$	$>10^9$	$>10^9$	33	$>10^9$	$>10^9$	$>10^9$	37
Third week	$>10^9$	$>10^9$	$>10^9$	11	$>10^9$	$>10^9$	$>10^9$	15
First month	$>10^9$	$>10^9$	$>10^9$	28	$>10^9$	$>10^9$	$>10^9$	25
Second month	$>10^9$	$>10^9$	$>10^9$	NG	$>10^9$	$>10^9$	$>10^9$	NG
Third month	$>10^9$	$>10^9$	$>10^9$	NG	$>10^9$	$>10^9$	$>10^9$	NG
Fourth month	S	$>10^9$	$>10^9$	NG	S	$>10^9$	$>10^9$	NG
Fifth month	S	$>10^9$	$>10^9$	NG	S	$>10^9$	$>10^9$	NG
Sixth month	S	S	$>10^9$	NG	S	S	$>10^9$	NG

NG: No Growth, S: spoiled

Table 4: Effect of different salt concentration on gram-negative bacteria count in both of Baladi cheese and its whey stored at 25 °C

Storage time At 25 °C	Bacteria count for Cheese (CFU/g)				Bacteria count for Whey (CFU/ml)			
	Sodium chloride (%)				Sodium chloride (%)			
	0	3	6	9	0	3	6	9
At processing time	$>10^9$	-	-	-	$>10^9$	-	-	-
first week	$>10^9$	$>10^9$	$>10^9$	$6.4x10^2$	$>10^9$	$>10^9$	$>10^9$	$6.8x10^2$
Second weeks	$>10^9$	$>10^9$	$>10^9$	47	$>10^9$	$>10^9$	$>10^9$	51
Third week	$>10^9$	$>10^9$	$>10^9$	17	$>10^9$	$>10^9$	$>10^9$	13
First month	$>10^9$	$>10^9$	$>10^9$	29	$>10^9$	$>10^9$	$>10^9$	30
Second month	$>10^9$	$>10^9$	$>10^9$	NG	$>10^9$	$>10^9$	$>10^9$	NG
Third month	S	S	S	NG	S	S	S	NG
Fourth month	S	S	S	NG	S	S	S	NG
Fifth month	S	S	S	NG	S	S	S	NG
Sixth month	S	S	S	NG	S	S	S	NG

NG: No Growth, S: spoiled

It is worth noting that no bacterial inhibition effect was showed up after one month of storage in contaminated cheese salted with 3% or 6% NaCl when they were stored at any storage temperatures (-20, 4 and 25 °C). In the conducted experiment, it is clear that the samples salted with 3% and 6% were spoiled after three months of storage at 25 °C (Table 4).Whereas bacterial growth failed for the same salted cheese when they stored at -20 °C for the same storage period (table 2). On the basis of the collected data in table 3, it was demonstrated that the contaminated cheese salted with 3% sodium chloride maintained its bacterial count ($>10^9$ CFU/g) for five months when stored at 4 °C, and then spoiled after 6 months of storage at the same temperature (4 °C), in comparison with this period of storage at 4 °C, cheese salted with 6% NaCl didn't spoiled and kept their bacterial count (more than 10^9 CFU/g).

Antibacterial activity of *Z. officinale* EO against gram-negative bacteria in both of Baladi cheese and its whey

The data in tables (5, 6 and 7) clarified that no bacterial colonies were seen on MacConkey plates for experimentally contaminated cheese and its whey treated with the 2% concentration of *Z. officinale* EO after three weeks of storage at all conditions of the experiment (-20, 4, and 25 °C). Whilst the same result was achieved after three months of storage at all storage temperatures when experimentally contaminated cheese and its whey treated with 0.25%, 0.5% and 1% of *Z. officinale* EO.

After 3 weeks of storage at -20 °C, the bacterial count in contaminated cheese treated with 0.25% and 0.5% of *Z. officinale* EO were decreased to 60 and 21 CFU/g respectively (Table 5). Also, for the same treated samples and after the same length of storage time, the bacterial count dropped to 10^5 and 52 CFU/g when the cheese was stored at room temperature (25 °C) (Table 7).

In the contaminated cheese treated with 1% of *Z. officinale* EO, no bacterial growth was detected after two months of storage at 4 °C (Table 6), and after three months of storage at 25 °C (Table 7). After one month of storage, using the same *Z. officinale* EO concentration (1%), the bacterial count was 9 CFU/g in contaminated cheese stored at 4 °C (Table 6) and 14 CFU/g in contaminated cheese stored at 25 °C (Table 7).

Table 5: Effect of different *Z. officinale* concentration on gram-negative bacteria count in Baladi cheese and its whey stored at -20 °C

Storage Time At -20 °C	Bacteria count for Cheese (CFU/g)					Bacteria count for Whey (CFU/ml)				
	Z. officinale EOs (%)					*Z. officinale* EOs (%)				
	0	0.25	0.5	1	2	0	0.25	0.5	1	2
At time of processing	ND	-	-	-	-	ND	-	-	-	-
first week	$>10^9$	$>10^9$	$>10^9$	$5.8x10^6$	$2.3x10^3$	$>10^9$	$>10^9$	$>10^9$	$6.4x10^6$	$5.2x10^3$
Second week	$>10^9$	$8.5x10^5$	$7.4x10^4$	11	5	$>10^9$	$0.8x10^6$	$9.1x10^4$	14	7
Third week	$>10^9$	60	21	6	NG	$>10^9$	72	26	10	NG
First month	$>10^9$	23	5	3	NG	$>10^9$	27	8	6	NG
Second month	$>10^9$	NG	NG	NG	NG	$>10^9$	NG	NG	NG	NG

NG: No Growth

Table 6: Effect of different *Z. officinale* concentration on gram-negative bacteria count in Baladi cheese and its whey stored at 4 °C

Storage Time At 4 °C	Bacteria count for Cheese (CFU/g)					Bacteria count for Whey (CFU/ml)				
	Z. officinale EOs (%)					*Z. officinale* EOs (%)				
	0	0.25	0.5	1	2	0	0.25	0.5	1	2
At time of processing	ND	-	-	-	-	ND	-	-	-	-
first week	$>10^9$	$>10^9$	$>10^9$	$3.1x10^7$	$3.8x10^4$	$>10^9$	$>10^9$	$>10^9$	$4.4x10^7$	$6.8x10^4$
Second week	$>10^9$	$7.1x10^6$	$3.6x10^5$	16	9	$>10^9$	$1.1x10^7$	$4.9x10^5$	20	12
Third week	$>10^9$	81	29	16	NG	$>10^9$	93	37	22	NG
First month	$>10^9$	30	13	9	NG	$>10^9$	37	17	10	NG
Second month	$>10^9$	9	4	NG	NG	$>10^9$	12	6	NG	NG
Third month	$>10^9$	NG	NG	NG	NG	$>10^9$	NG	NG	NG	NG

NG: No Growth

Table 7: Effect of different *Z. officinale* concentration on gram-negative bacteria count in Baladi cheese and its whey stored at 25 °C

Storage Time At 25 °C	Bacteria count for Cheese (CFU/g)					Bacteria count for Whey (CFU/ml)				
	Z. officinale EOs (%)					*Z. officinale* EOs (%)				
	0	0.25	0.5	1	2	0	0.25	0.5	1	2
At time of processing	$>10^9$	-	-	-	-	$>10^9$	-	-	-	-
first week	$>10^9$	$>10^9$	$>10^9$	$2.8x10^8$	$5.9x10^5$	$>10^9$	$>10^9$	$>10^9$	$6.6x10^8$	$6.7x10^5$
Second week	$>10^9$	$3.5x10^8$	$1.3x10^6$	23	13	$>10^9$	$5.1x10^8$	$4x10^6$	33	18
Third week	$>10^9$	105	52	17	NG	$>10^9$	125	67	26	NG
First month	$>10^9$	43	24	14	NG	$>10^9$	50	28	17	NG
Second month	$>10^9$	13	8	6	NG	$>10^9$	21	13	11	NG
Third month	S	NG	NG	NG	NG	S	NG	NG	NG	NG

NG: No Growth, S: spoiled

Antibacterial activity of *T. vulgaris* EOs against gram-negative bacteria in Baladi cheese and its whey

For experimentally contaminated cheese and its whey, the 2% concentration of *T. vulgaris* EO led to the absence of bacterial growth after two weeks of storage at the different temperatures used in the study, the concentration of 0.25% caused a decrease in the bacterial count for the contaminated cheese to 53, 71 and 99 CFU/g after three weeks of storage at -20, 4 and 25 °C, respectively (Tables 8, 9, and 10). In similar circumstances, the bacterial count in contaminated whey decreased to 66, 80, and 111 CFU/ml when it was stored at -20, 4 and 25 °C, respectively.

From table 9, the bacterial count decreased to 71 CFU/g after three weeks of storage at 4 °C when it was treated with 0.25% of *T. vulgaris* EOs and to 25 CFU/g for the concentration of 1%.

By using 1% and 2% of *T. vulgaris* EO, the bacterial count in contaminated cheese decreased to 7.6×10^4 and 17 CFU/g, respectively after one week of storage at 4 °C.

From the results in table 10, the concentration of 1% of *T. vulgaris* EO led to a decrease in the bacterial count to 15 CFU/g in the second week of storage at 25 °C, while the concentration of 0.25% decreased the bacterial count to 99 CFU/g in contaminated cheese after three weeks of storage at 25 °C. No bacterial growth was detected after two months of storage at 25 °C when it was treated with 0.5% and 1% of *T. vulgaris* EOs.

Table 8: Effect of different *T. vulgaris* concentration on gram-negative bacteria count of Baladi cheese and its whey stored at -20 °C

Storage Time At -20 °C	Bacteria count for Cheese (CFU/g)					Bacteria count for Whey (CFU/ml)				
	T. vulgaris EOs (%)					*T. vulgaris* EOs (%)				
	0	0.25	0.5	1	2	0	0.25	0.5	1	2
At time of processing	ND	-	-	-	-	ND	-	-	-	-
first week	$>10^9$	$>10^9$	$4.4x10^6$	$1.7x10^4$	NG	$>10^9$	$>10^9$	6.3x106	$2.2x10^4$	NG
Second week	$>10^9$	$6.1x10^5$	$5.7x10^3$	NG	NG	$>10^9$	$7.2x10^5$	$8.8x10^3$	NG	NG
Third week	$>10^9$	53	16	NG	NG	$>10^9$	66	24	NG	NG
First month	$>10^9$	18	NG	NG	NG	$>10^9$	20	NG	NG	NG
Second month	$>10^9$	NG	NG	NG	NG	$>10^9$	NG	NG	NG	NG

NG: No Growth

Table 9: Effect of different *T. vulgaris* concentration on gram-negative bacteria count in Baladi cheese and its whey stored at 4 °C

Storage Time At 4 °C	Bacteria count for Cheese (CFU/g)					Bacteria count for Whey (CFU/ml)				
	T. vulgaris EOs (%)					*T. vulgaris* EOs (%)				
	0	0.25	0.5	1	2	0	0.25	0.5	1	2
At time of processing	ND	-	-	-	-	ND	-	-	-	-
first week	$>10^9$	$>10^9$	$1.1x10^8$	$7.6x10^4$	17	$>10^9$	$>10^9$	$1.6x10^8$	$9.1x10^4$	23
Second week	$>10^9$	$5.5x10^6$	$4.5x10^4$	6	NG	$>10^9$	$9.4x10^6$	$6.6x10^4$	9	NG
Third week	$>10^9$	71	25	5	NG	$>10^9$	80	36	8	NG
First month	$>10^9$	16	7	4	NG	$>10^9$	19	10	8	NG
Second month	$>10^9$	6	NG	NG	NG	$>10^9$	7	NG	NG	NG
Third month	$>10^9$	NG	NG	NG	NG	$>10^9$	NG	NG	NG	NG

NG: No Growth

Table 10: Effect of different *T. vulgaris* concentration on gram-negative bacteria count in Baladi cheese and its whey stored at 25 °C

Storage Time At 25 °C	Bacteria count for Cheese (CFU/g)					Bacteria count for Whey (CFU/ml)				
	T. vulgaris EOs (%)					*T. vulgaris* EOs (%)				
	0	0.25	0.5	1	2	0	0.25	0.5	1	2
At time of processing	$>10^9$	-	-	-	-	$>10^9$	-	-	-	-
first week	$>10^9$	$>10^9$	$>10^9$	$3.5x10^6$	36	$>10^9$	$>10^9$	$>10^9$	$5.2x10^6$	59
Second week	$>10^9$	$4.6x10^7$	$0.9x10^5$	15	NG	$>10^9$	$6.7x10^7$	$3x10^5$	19	NG
Third week	$>10^9$	99	43	14	NG	$>10^9$	111	60	17	NG
First month	$>10^9$	21	18	7	NG	$>10^9$	29	21	11	NG
Second month	$>10^9$	9	NG	NG	NG	$>10^9$	16	NG	NG	NG
Third month	S	NG	NG	NG	NG	S	NG	NG	NG	NG

NG: No Growth, S: spoiled

Sensory appraisal of fresh Baladi cheese

Evaluation of sensory properties (appearance, texture, color, test and flavor) of cheese treated with 0.25%, 0.5%, 1% and 2% of *T. vulgaris* and *Z. officinale* essential oils was carried out for overall acceptability evaluation. None of the appearance or texture was affected by adding different concentrations of EOs, in general, the cheese was evaluated as good texture and smoothness. For color, no change was observed for cheese treated with 0.25%, 0.5% and 1% to any of *Thymus* and *Zingiber* essential oils. At 2% of *Zingiber* EO, the color score was lower compared with the same concentration of *Thymus* EO. Furthermore, at 2% of *Thymus* EO the cheese flavor was more acceptable than 2% of *Zingiber* EO. In general, neither *Thymus* nor *Zingiber* EOs negatively affected the flavor of the cheese.

Identification of gram-negative bacteria isolated from experimentally contaminated cheese using PCR

To detect the existing bacteria in experimentally contaminated cheese, the PCR technique was used. More than one genus of bacteria was possible to detect in the same studied sample. Compared to the traditional method used for bacterial identification, the PCR technique gives more accurate results and requires less time. Figure 1 represents the results of DNA amplification of bacterial DNA isolated from experimentally contaminated cheese samples for the following bacterial genus: *E. coli*, *S. typhi*, *Y. enterocolitica*, *K. oxytoca* and *Salmonella* spp., where the fragments length of bacterial DNA on the agarose gel (1%) were 556 bp, 615 bp, 400 bp, 343 bp, and 373 bp, respectively. While on the left side of figure 1, two DNA fragments appeared, the first with 441 bp was for

Klebsiella genus, and the other with 343 bp for the *K. oxytoca*. The DNA of *Klebsiella* sp. and *K. oxytoca* were used as positive controls.

So, in this study, we could detect bacterial contamination in Baladi cheese by one or more species at the genus and species with 100% accuracy and sensitivity in a relatively short time ranged just from 24 to 48 hours.

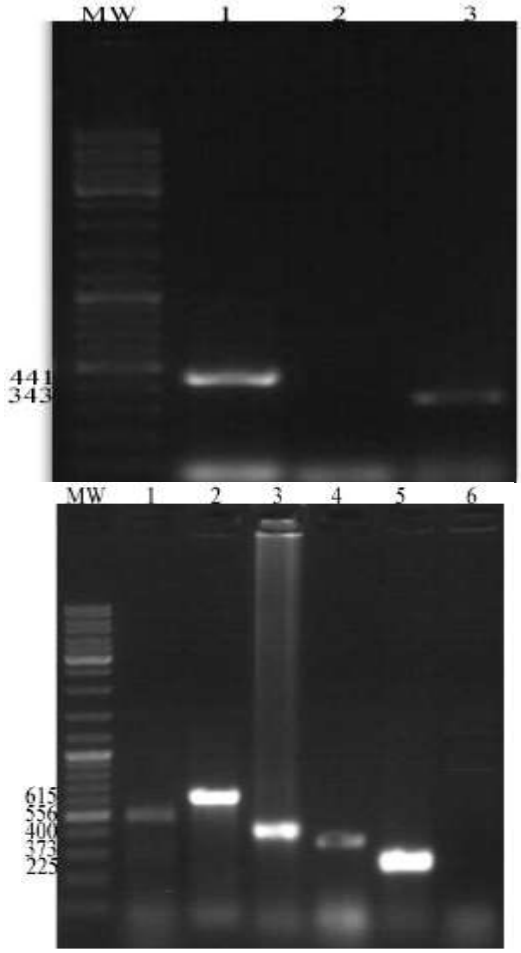

Figure 1: Amplification of bacterial DNA isolated from experimentally contaminated cheese samples visualized on agarose gel (1%) using UV.

MW: molecular weight DNA marker 1kb.

On the right side: 1: *E. coli* (556 bp), 2: *S. typhi* (615 bp), 3: *Y. enterocolitica* (400 bp), 4: *K. oxytoca* (343 bp), 5: *Salmonella spp.* (373 bp), 6: Negative control.

On the left side: 1: *Klebsiella* spp. (441 bp), 2: Negative control, 3: *K. oxytoca* (343 bp).

Baladi cheese is a popular dairy product in Syria, consumed either fresh or in a salt solution with NaCl concentration (5-20%). Microorganisms contaminating this type of cheese limit its shelf life. Microorganisms existing in Dairy products are responsible for pathogens and may cause desirable sensory properties and physical characteristics. Human diseases such as Salmonellosis, Tuberculosis and Brucellosis are transmitted by Dairy products. To avoid having toxins or pathogenic-producing bacteria in cheese, the used milk for cheese production should pasteurize or otherwise treat to inhibit microorganisms. Food-borne diseases associated with *S. aureus*, *E. coli* O157:H7, *S. typhi*, *L. monocytogenes* and *S. enteritidis* are the main concern (Hall, 1997; Farber, 2000).

At high salt concentrations, cheese had a low water activity that may affect the growth of bacteria in addition to other factors such as the pH, temperature, and salt concentration (Valero et al. 2009).

According to Setyawardani et al. (2019), the NaCl content is a crucial factor for determining the sensory properties (texture, shape and test) of cheese. Also, it acted as a preservative and flavor enhancer by enhancing the enzymatic activity of some enzymes responsible for different biochemical mechanisms. (Albarracín et al. 2011; Fox et al. 2017).

Essential oils of different types of plants are hydrophobic liquids consisting of varying compounds that have inhibited effects against microbial organisms (Asli et al. 2017). EOs contain mixtures of components that have antibacterial properties, most of this effect is due to phenylpropanoids, phenolic terpenes, some alcohols and other compounds (Bassole and Juliani, 2012). Due to the hydrophobicity of EOs, they can penetrate the cell membrane of bacteria leading to inhibition as a result of conflict with molecular transport mechanisms (Goni et al. 2009).

Using natural antimicrobial as essential oils were preferred and made it more important than artificial food preservatives, where the indiscriminate use of antibiotics led to boosting the resistant bacterial strains (Bouarab et al. 2019). Against food-borne pathogens generally found in cheese such as *Salmonella, E. coli*, *S. aureus* and *L. monocytogenes*, different essential oils extracted from aromatic plants such as rosemary, oregano, cumin, and pepper were studied, they have shown an obvious antibacterial effect (Carvalho et al. 2018; Moro et al. 2015).

EOs could be added as alternative therapeutics against *Salmonella*. Thereupon, the EO of *Ruilopezia bracteosa* showed effective inhibiter against *S. aureus* and *E. faecalis* in comparison with traditional antibiotics (Alarcon et al. 2015). In a study about the effect of temperatures storage on microbial and sensory properties of untreated white cheese, Eljagmani and Altuner (2020) concluded that the shelf life of white cheese brained with 0.35% salt was from 14 days to 28 days when stored at 5, 15 and 25 °C. In addition, they revealed a high microbial count of *Salmonella*, Coliform and *Staphylococci* in the sample stored at a highest temperature which also led to deterioration the sensory attribution of white cheese samples such as texture, aroma, appearance and test. EOs of *Z. cassumunar* had an inhibitor effect against *A. baumannii*, the effective antibacterial substances in these EOs were, sabinene, terpinene-4-ol, α-terpinene

(Boonyanugomol et al. 2017).

It is well known that gram-negative bacteria are more resistant to EOs than gram-positive ones (Mann et al. 2000). One of the reasons that could explain the high susceptibility to EOs would be the more hydrophobic nature of the outer membrane composed of oligosaccharide chains, while the presence of hydrophilic polysaccharide chains in the outer membrane structure prevents hydrophobic EOs from reaching the bacteria cell membrane (Inouye et al. 2001; Antih et al. 2021).

Another mechanism for EOs antimicrobial effect is the irreversible dissociation of the bacteria cell wall which resulting the leakage of DNA and RNA and proteins (Meng et al. 2016; Montironi et al. 2016). The EOs extracted from oregano clove and thyme can be added as antibacterial agents, where they showed high antibacterial activity against different bacterial strains (Puskarova et al. 2017).

Fournomiti et al. (2015) showed that *K. oxytoca* and *K. pneumonia* were the most sensitive organisms with a mean value of MIC of 8.1µg/mL and 9.5µg/mL for thyme EOs, while *E. coli* was the most resistant to EOs antimicrobial activity. Nedorostova et al. (2009) reported an antimicrobial effect of *T. vulgaris* EO against *S. aureus* with MIC of 17 µL/mL.

Diverse research reported the antibacterial activity of *Z. officinale* EO against various bacteria, highest sensitivity to ginger oil was verified against *L. monocytogenes*. However, *Z. officinale* EO has been shown to have an inhibitor effect on gram-negative bacteria, such as *E. coli* and *Shigella*, active substances including gingerol, zingberene and endoborneol may be the reason for the antimicrobial properties of this oil (Sivasothy et al. 2011; Snuossi et al. 2016).

Results had been confirmed that the essential oil of ginger had a higher inhibition zone diameter for *L. monocytogenes* and *S. aureus*, than for *P. aeruginosa* (Mesomo et al. 2013). Antimicrobial effects of ginger essential oil against *E. coli* O157:H7, *Campylobacter jejuni*, *L. monocytogenes* and *S. enterica* were revealed by (Friedman et al. 2002).

For identification of gram-negative bacteria (*E. coli*, *S. typhi*, *Y. enterocoltica*, *K. oxytoca* and *Salmonella* spp.), many biochemical tests should be performed in traditional phenotypic testing including multiple biochemical tests such as catalase test, oxidase, urease, indole and phenylalanine and other tests which are time-consuming and excessive consumption of test materials (Edwards et al. 1986). Polymerase Chain Reaction (PCR) is fast, sensitive, extremely accurate and safe technique (Momtaz et al. 2013). Studied the diversity and safety of the gram-negative flora naturally present in milk, semi-hard and soft French cheeses using the PCR technique, the results showed the presence of 173 gram-negative bacteria isolates, nearly half of them belonging to the *Enterobacteriaceae* family (Coton et al. 2012).

Our results asserted that a period of 18-24 hours was sufficient to confirm the genus and species of gram-negative bacteria in Baladi cheese, this was referred to (Ramesh et al. 2002; and Tamarapu et al. 2001) who reported that 18 hours was the required time for identification the bacteria.

CONCLUSION

In conclusion, the application of 2 essential oils (EOs) and NaCl as antibacterial in Baladi cheese and its whey gave high efficacy as the bacterial counts decreased when using the concentration of 9% NaCl at all storage degrees and until the sixth month. The storage temperature of – 20 °C had a higher effect on bacterial growth inhibition. The study also showed that the use of *Z. officinale* EO in any of the tested concentrations inhibited the bacterial growth of for three months of storage, while using the concentration 2% of *T. vulgaris* EOs, led to the absence of bacterial growth since the second week of storage at the different temperatures used in the study. The use of PCR technique enables us to detect the bacterial contamination of food samples on the same day compared to other traditional methods.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTION

A. Al-Mariri; conceptualized the study, R. Ismail, R. Alabras and N. Alsahar; collected and performed the practical study; M. Khawajkiah and L. Alhallab; analyzed the results, R. Ismail and R. Alabras; wrote the manuscript, A. Al-Mariri; supervised of the project researchers and workers. All authors have revised and approved the final version of this manuscript.

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