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Original Article *Milk Hygiene*

Prevalence of Listeria species in raw milk, ice cream and yogurt and effect of selected natural herbal extract on its survival

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ABSTRACT

Objective: This study aimed to detect the prevalence of Listeria species in raw milk, ice cream and yogurt, and to evaluate the effect of extract of clove, thyme and pomegranate peel on such organism.

***Design*:** Descriptive study.

Procedures: One hundred and fifty samples of milk, ice cream and yogurt were examined for isolation, identification and molecular identification of *Listeria spp*. Extraction of natural plant extract as clove, thyme and pomegranate peels and detection of their inhibitory effect on *Listeria spp.*

Results: The prevalence of *Listeria spp.* in milk was 36% where 14% as *L. monocytogenes, 6% L. innocua and 16%* and other *Listeria spp.* was *16%.* In yogurt, *Listeria spp.* was 6% as *L. innocua* was 2% and other *Listeria spp.* was 4%, while no *L. monocytogenes* was detected. In ice cream, *Listeria spp.* was 8% where *L. monocytogenes* was 2% and other *Listeria spp.* was 6% while no *L. innocua* was detected. The concentration of plant extract was 2.5% which showed high reduction rate on *L. innocua* and *L. monocytogenes* during shelf life of soft cheese.

Conclusion and clinical relevance: Listeria is widely isolated from milk than from ice cream and yogurt. Plant extracts play role in food preservation and consider as a natural antimicrobial agent where most effective one was clove extract.

Keywords: *Listeria monocytogenes*, *Listeria innocua*, Plant extract, Clove, Thyme, Pomegranate peel

1. INTRODUCTION

Milk and dairy products are considered a source of energy, protein and micronutrient **[1]**. Consumption of milk during childhood protects against immune-mediated diseases **[2]**. Yogurt is a smooth and creamy dairy product manufactured from fermenting of lactose into lactic acid. Yogurt is rich in many minerals and vitamins and a good source of calcium **[3, 4].** Ice cream is a fun, delicious and easily digested food where milk has been pasteurized and homogenized before freezing **[5]**. Ice cream becomes a suitable environment for microbial growth because of its nutritional value, neutral pH and long storage periods **[6-8]**.

Listeria species are food borne pathogens, widely distributed in natural environment, and can survive in a wide range of temperature and pH **[9]**.

Many outbreaks of *Listeria monocytogenes* (*L.monocytogenes*) infection were mainly associated with the consumption of milk and dairy products **[10]**. *L. monocytogenes* has many characters that enable *Listeria* to grow and survive at refrigeration temperature and acidic conditions **[11]**. *Listeria innocua* (*L.innocua* can) considered a non-haemolytic saprophyte that is widely distributed in the natural environment. Strains of *L. innocua* can replicate intracellular in mammalian cells causing infection especially in immune-compromised mammals **[12-14]**, described a fatal bacteremia caused by a *L. innocua* strain and this was the first case reported in humans. Many reports found that the presence of *L. innocua* may mask *L. monocytogenes* which could lead to false negative results for the presence of the latter **[15]**.

Plant extracts acts as a natural preservative which is safe, healthy and effective preservative **[16]**. Plant essential oils have antibacterial, antifungal, anti-inflammatory and antioxidant activities **[17]**. Besides, it considere as natural sources of antimicrobial agents **[18, 19]**.

Clove (*Syzygium aromaticum*) has medicinal activities, such as an antiseptic and analgesic in the medical care, also U.S. Food and Drug Administration recognized clove as safe **[20]**. Clove has inhibitory activities against *Listeria* in food at both ambient and refrigeration temperature **[21]**. Thyme (*Thymus vulgaris*) is a flavor enhancer in many foods and has antiseptic, antispasmodic and antimicrobial activities, and it can be used as a medicinal herb and as a preservative for foods **[22, 23]**. Many studies found that pomegranate extracts (*Punica granatum L.)* act as natural alternative for

treatment of wide range of bacterial and viral infections because of their antimicrobial activities **[24]**. Pomegranate peel is known for its health promoting qualities and wound healing properties **[25]**.

The aims of this study was the molecular characterization of *Listeria* species, isolated from milk, ice cream and yogurt, detection of minimal inhibitory concentration of natural extracts of clove, thyme and pomegranate peels on *Listeria* species., detection of the antimicrobial effect of the chosen plant extracts on *Listeria* species implanted in soft cheese with a concentration palatable to consumers according to sensory evaluation, and Detection the reduction rate of the plant extract on *Listeria* during shelf life of soft cheese.

2. MATERIALS AND METHODS

2.1. Collection of samples

A total of 150 samples (raw milk (50), ice cream (50) and yogurt (50)) was randomly collected from different shops and supermarkets in Mansoura city, Dakahlia, Egypt.

2.2. Preparation of samples

Milk samples were agitatedsampling, while ice cream samples thawed to room temperature and yogurt samples where assumed to room temperature and shaken vigorously before examination. A 25 mL of tested samples were added to 225ml of tryptone soya broth and through mixing.

2.3. Isolation and identification of Listeria species

Isolation of *Listeria* species as applied according to standard methods **[26].** Milk samples were examined by direct isolation, 0.1mL of tryptone homogenate was streaked onto selective agar plates of Oxford media supplemented with *Listeria* selective supplement, and then plates were incubated at 37°c for 48 hr.

Ice cream and yogurt samples were examined by the enrichment procedures, where the tryptone homogenate incubated at 37°c for 24 hr, then 10 mL of incubated homogenate added to 90 ml of *Listeria* selective broth base supplemented with *Listeria* selective enrichment supplement and incubated at 37°c for 48 hr. After that 0.1 ml streaked onto Oxford agar supplemented with *Listeria* supplement and incubated at 37°c for 48 hr.

Representative five colonies (olive green colonies surrounded with black zone) were picked up from positive plates and sub-cultured onto tryptone soy agar slopes and incubated at 37°c for 24 hr, then biochemical identification were performed.

Representative colonies were examined microscopically by Gram stain, where *Listeria* appears as non-spore forming coccobacilli. Isolates were tested for catalase, oxidase and umbrella growth in motility test medium at 25°c. Isolates examined for hemolysin production on blood agar base supplemented with 7% sheep blood, CAMP test against *Staphylococcus aureus*. Isolates were also subjected to VITEK2 system using the VITEK GP cards (bioMérieux, Durham, NC) according to the manufacturer's directions **[27]**. For differentiation of haemolysis between *L.monocytogenes* and *L.innocua,* suspected isolates were streaked onto Columbia blood agar **[28]**.

2.4. Molecular characterization of Listeria

Refreshment of suspected colonies on modified Oxford agar supplemented with *Listeria* selective supplement. Then isolates were screened for the presence of Listeria (hly) gene. The sequence of oligonucleotide primer forward F: 5′-ATTTTCCCTTCACTGATTGC-3′ and reverse: 5′CACTCAGCATTGATTTGCCA -3′.

DNA extraction done by cell wall template and screened for the presence of *L. monocytogenes* (16s) using the oligonucleotide primer forward F: 5′-GGACCGGGGCTAATACCGAATGATAA -3′ and reverse: 5′-TTCATGTAGGCGAGTTGCAGCCTA -3′**[29].**

Extraction of DNA was carried out using QLA amp DNA mini kit, according to manufacturer's instructions. A total of 25µL, with mixture of 12.5µl of Emerald Amp GT PCR mastermix (2x premix), 1µl forward primer, 1µl l reverse primer, 5.5 µl PCR grade water and 5µl from DNA template. Thermal conditions were as follow, primary denaturation at 94°c for 5 min, followed by 35 cycles of 94°c for 30 sec, 60°c for 1 min, and 72°c for 1 min, with final extension at 72°c for 12 min. PCR products were visualized using ethidium bromide stained 1.5% agarose gel electrophoresis under UV light and photographed

L.monocytogenes isolates were also screened for the presence of Listeriosin O (hlyA) gene. The sequence of oligonucleotide primer was forward hylA 634F: 5′-ACTTCGGCGCAATCAGTGA-3′ and reverse hylA 770R: 5′-TTGCAACTGCTTTAGTAACAGCTT-3′. Two colonies from refreshed Oxford plates were picked up and suspended into 50 µL distilled water and subjected to heat block at 90°c for 5 minutes. A total of 25 µl, with mixture of 12.5µl of Dream Tag Green PCR Master Mix (fermentas),1µl forward primer, 1ul reverse primer , 9.5 µl RNAs free water and 1µl from DNA thermal conditions were as follow, initial duration at 95°c for 5 min, followed by 40 cycles of 95°c for 30s., 60°c for 30s. and 72°c for 1 min with final extension at 72°c for 5min. PCR products were visualized using ethidium bromide stained 1.5% agarose gel electrophoresis under UV light and photographed.

2.5. Effect of clove, thyme and pomegranate peel extracts against Listeria spp.

Extractions of plants were carried out by soxhlet apparatus at faculty of Science, Mansoura University.

2.5.1 Minimal inhibitory concentration of plant extract against Listeria species

Minimal inhibitory concentration of plant extract was done to determine antibacterial activity of plant extract by agar well diffusion method**[30]**.*Listeria* strains were grown on Müeller–Hinton agar (oxoid) at 37°C for 24 hr. Single colony

of Listeria was picked up and suspended in nutrient broth at 37°C for 24 hr. The infective dose was adjusted as $1*10^7$ for Listeria monocytogenes **[31]**,and $1*10^5$ for Listeria innocua**[32]**,and streaked onto Müeller–Hinton agar. Wellsof 9 mm diameter were made, 50 µl of the extracted plant solution with different concentrations were placed into the wells of inoculated agar plates. The plates were refrigerated at 8°c for 1 hr to allow the diffusion of the extract into the medium, then incubated at 37°C for 24 hr. The diameter of the inhibition zone was then measured to determine the minimal inhibitory concentration.

2.5.*2Manufacture of plant added white soft cheese*

Soft cheese was manufactured from milk as described elsewhere **[33],**where 9 litres of milk were pasteurized for 30 minutes at 63°C, tempering till 40°C, salt was added (15g/L), rennet was added (15ml/L), milk was then distributed into equivalent portions, and each was exposed to the extracted plant clove, thyme and pomegranate peel extracts by concentration of 2.5%, 3.5% and 4.5% for each extract . The treated milk incubated for 45 minutes at 30°C till formation of curd, then the whey was drained and cheese achieved. Cheese samples kept at refrigeration at temperature (4-2°C) till the time of sensory evaluation.

2.5.3 Sensory evaluation

Sensory evaluation was done according to **Ihokoronye and Ngoddy [34].** Samples were examined for color, taste, salt, appearance and overall acceptability using nine point Hedonic scale, where nine refer to extremely like, while one refer to extremely dislike. The test was represented to 10 panelists. Concentrations of plant extracts used in the sensory evaluation were 2.5%, 3.5% and 4.5%.

2.5.4. Preparation of experimental plants-added cheese inoculated with Listeria isolates

In this study *Listeria monocytogenes* and *Listeria innocua* isolates refreshed on tryptone soy broth then streaked on Oxford media supplemented with *Listeria* selective supplement. The infective dose was adjusted as $1*10^7$ for *L.monocytogenes* **[31]**, and $1*10^5$for *L.innocua* **[32].**

Eight liters of milk was divided into equivalent containers where each on contain one liter of milk. Milk was laboratory pasteurized for 30 minutes at 63°c, then tempered to 40°c then. Salt was added (15gm/L) then the *Listeria* was added and rennet then was add (15ml/L). The eight portions of milk were presented in table 1.

The treated milk was incubated at 30°c till curd formation and removing of whey and store at refrigeration temperature (4-2°c) for 15 days and analyzed just after curd (zero time). The final concentration of plant extract was 2.5% for clove, thyme and pomegranate peel according to the sensory evaluation.

The experiment was done for both *L.monocytogenes* and *L.innocua,* where initial dose was $1*10^7$ and $1*10^5$ for *L.monocytogenes* and *L.innocua* respectively. The experiment repeated twice for each bacteria.

2.5.5 Bacteriological examination

Bacterial count was obtained from cheese curd at zero time, one hour, three hrs, six hrs, and twelve hrs, and 24 hrs, then daily to determine the antibacterial effects of plant extract on the bacteria.

A 10 gm of cheese was added to 90 mL of tryptone soy broth and through mixing and homogenization were followed. 0.1ml of broth streaked onto Oxford agar plates and then incubated at 37°c for 24 hrs. Colony counts were performed for the incubated plates.

3. RESULTS

The results of the present study are presented in figures 1-12.

Table 1. Classification of milk samples to different groups of plant extract.

	Bacteria	Plant extract
Control 1	-ve	-ve
Control 2	+ve	-ve
Control 3 Thyme	-ve	+ve
Control 4 clove	-ve	+ve
Control 5 pomegranate	-ve	+ve
Sample1 clove	+ve	+ve
Sample1 clove	+ve	+ve
Sample2 Thyme	+ve	+ve
Sample 3 pomegranate	+ve	+ve

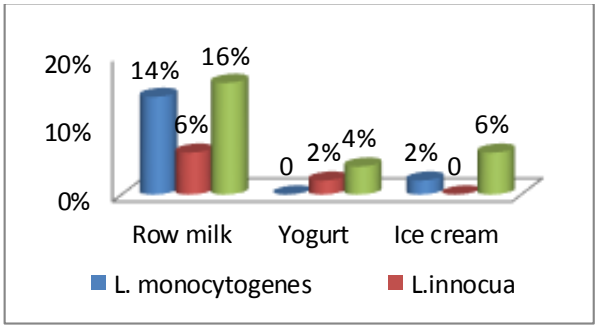

Figure 1. Prevalence of Listeria species in raw milk, yogurt and ice cream.

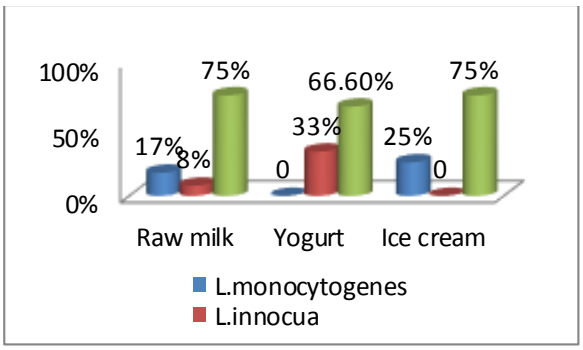

Figure2. Distribution of Listeria species isolates in raw milk, yogurt and ice cream.

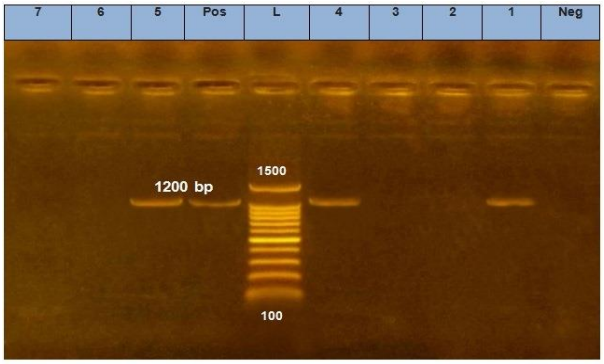

Figure3. PCR of *Listeria monocytogenes* (*16S rRNA*)*L.monocytogenes* appear at 1200bp.

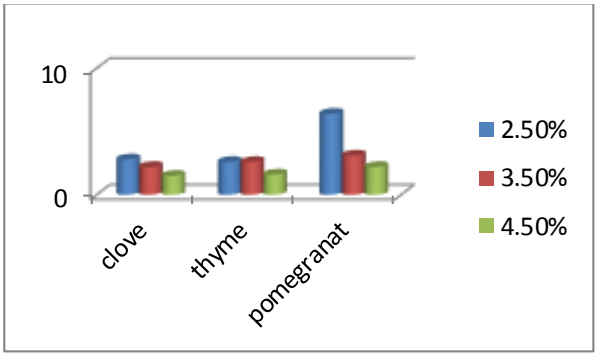

Figure4. Evaluation of taste as one of sensory criteria.

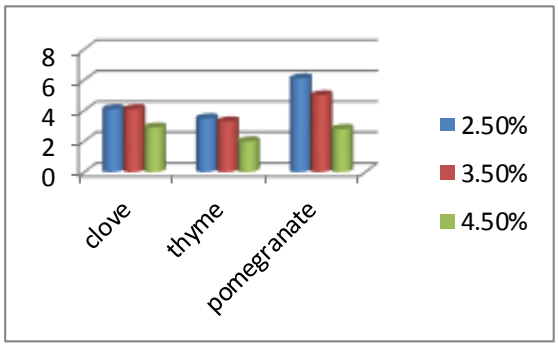

Figure 5. Evaluation of the odor as one of sensory criteria

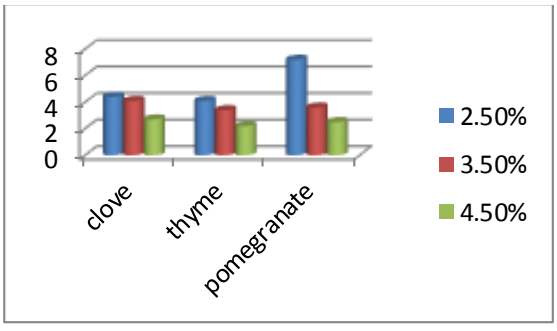

Figure 6. Evaluation of salt as one of sensory criteria

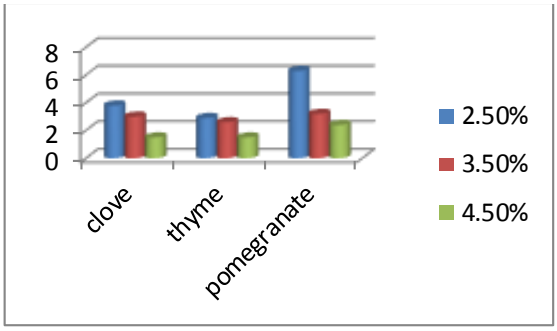

Figure7. Evaluation of appearance as one of sensory criteria.

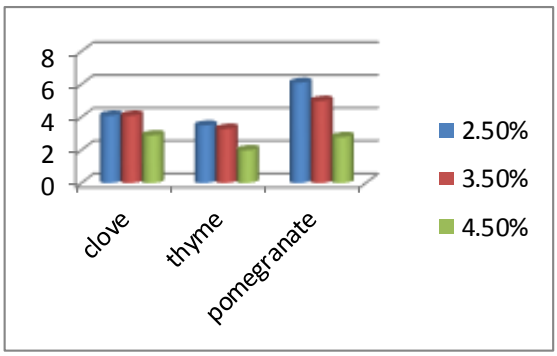

Figure8. Evaluation of overall cceptability as one of sensory criteria

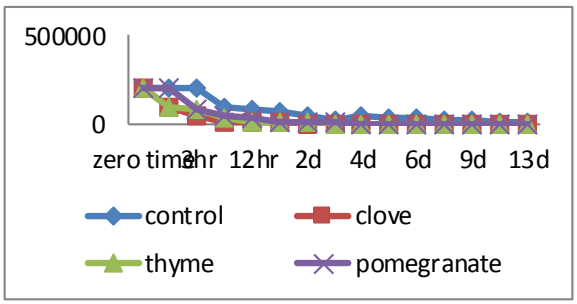

Figure9. Effect of some natural plants extract on *L.innocua* in manufactured white soft cheese.

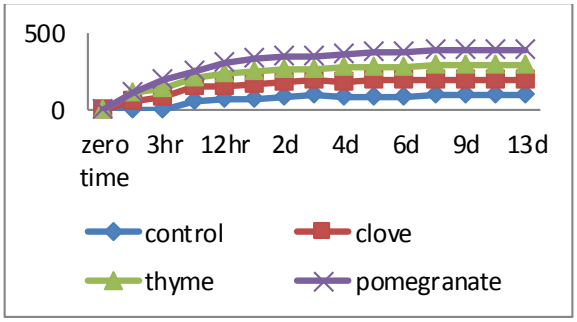

Figure10. Reduction percent of plant extract on *L.innocua* in manufactured white soft cheese.

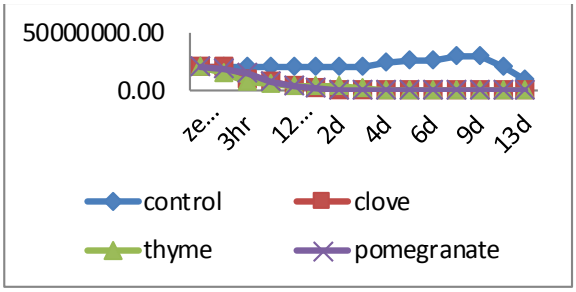

Figure11. Effect of some natural plants against *Listeria monocytogenes* count in manufactured white soft cheese.

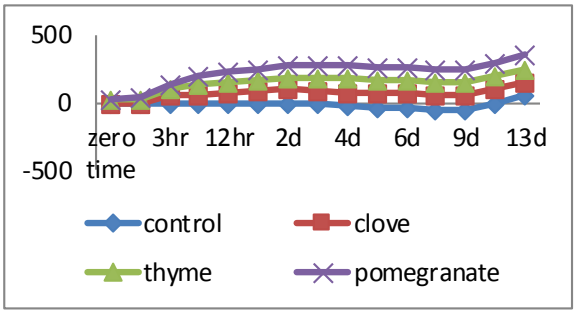

Figure12. Reduction percent of plant extract on *L.monocytogenes* in manufactured white soft cheese.

4. DISCUSSION

Milk can be contaminated through many ways in the milk production process neither through the dairy animal which shed the pathogen in milk or during collection, transportation or subjected to time-temperature abuse **[35]**.

In the present study, from 50 raw milk samples 18 samples (36%) were suspected to have *Listeria* species, of which 7 (14%) were *L.monocytogenes*, 3(6%) were *L.innocua* and 8 (16%) were other *Listeria* species. These results are in agreement with that of previous report **[36]**, where *Listeria* species were found in raw milk as 29.2% and *L. monocytogenes*was 7.8%, while *L.innocua* was15%. Also Gebretsadik et al. **[37]** isolated *Listeria* species from 22% of raw milk with *L. monocytogenes* of 13%. Carlos et al. **[38]** studied the prevalence of *Listeria* species in 1300 milk samples and found that *L. innocua* was 1% while *L.monocytogenes* was 13%. However, Yakubu et al. **[39]**found *Listeria* species in milk as 39.58% but incidence of *L.monocytogenes* was higher than our study as it was 22.4% and *L.innocua* was 51.3%. Also Al-Mariri et al. **[40]**found that the incidence of *Listeria* species was 16.23% from row milk where *L.monocytogenes* was 65% and *L.innocua* was 73%.While our result was higher than that in earlier report **[41]**, as they isolated only 2% of *Listeria* species from milk with *L.innocua* rate of 0.19%, while no *L.monocytogenes* was found.

This study declared that *Listeria* spp. in yogurt was low, where it was 6% as 2% was *L. innocua* while no *L. monocytogenes* was detected, and other *Listeria* species was about 4%, fig.1. Our result matches with that of previous study **[42] w**ho examined the prevalence of *Listeria* species in yogurt and it was 5%. While, *L. monocytogenes* has been found at a higher rate (8%)in yogurt than that of our result **[43]**. On another hand **[44, 45]**found no *Listeria* species in yogurt samples. Survival of *Listeria* species disappear when pH of acidic media fall to 3.5 **[46]**. Also, bacteriocins produced by the lactic acid bacteria can inhibit the presence of *L. monocytogenes* in yogurt **[47]**.

In the current study, the isolated *Listeria* species from ice cream was 8% where *L.monocytogenes* was 2%, other *Listeria* species were 6% while no *L.innocua* was found, fig.1. These results matched with that of previous study **[48]**, who found that *L.monocytogenes* was found only in 0.8% in ice cream samples in Eastern Spain. According to WHO reports, the incidence of *L.monocytogenes* in ice cream varied from 0-5.5% **[49]**.In the contrary, Jalali et al. **[50]** showed high incidence of *Listeria* species in ice cream samples in Iran, with 19% *L. innocua* , and no detection of *L.monocytogenes*.

The repeated application of chemical preservatives in food resulted in accumulation of chemical residues in food, microbial resistance to the used chemicals and bad side effects on human health **[51, 52]**.The antimicrobial activity exhibited by plant extracts against food poisoning bacteria has been demonstrated by several researchers **[53-56]**.

The sensory evaluation was done to evaluate the sensory properties of cheese after addition of clove, thyme and pomegranate peels extract on 2.5%, 3.5% and 4.5% concentrations. The experiment was done to 10 panelists who analyzed the cheese for tastefig.4, odor fig.5, salt fig.6, appearance fig.7 and overall acceptability fig.8. The panelists decided that the concentration 2.5% was more acceptable than other concentrations.

Two *Listeria* species, *L.monocytogenes* and *L.innocua*, were chosen to study the inhibitory effect of some plant extract on them during storage life of white soft cheese. Consistently, the reduction rate of clove *(Syzygium aromaticum*) on *L.innocua* and*L.monocytogenes* was 99% where decreased the bacterial log to 4 log. Hoque et al. **[57]** found that treatment of *L.monocytogenes* with 10% clove provided no viable count could be found after one day of incubation period. Menon **et al. [58]**examined the antilisteric effect of clove oil in cheese and revealed that the concentration of 1% had a pronounced inhibitory effect on *Listeria* count as treated samples were 1-3 $\log_{10}$ cfu/g less compared to controls at different intervals during storage. While Leuschner et al. **[59]** found that clove had bactericidal effect on *L. monocytogenes* in broth system. While no antimicrobial effect of 1% clove powder on *L.monocytogenes* in cheese has been recorded. Clove can induce its antibacterial effects on *L. monocytogenes* via its effect on cell membrane, respiratory metabolism and interaction with DNA **[60]**.

The reduction rate of thyme (*Thymus vulgaris*) on *L. innocua* was 98% fig10 and *L. monocytogenes* was99% fig.12 where decreased the bacterial log to 3 log for *L. innocua* fig. 9and 4 log for *L. monocytogenes*. Abdollahzadeh et al. **[61]**

studied the role of thyme to control *L.monocytogenes*. They showed that using thyme at 0.8% and 1.2% reduced *Listeria* count to 2 log cfu/g. e Carvalho et al. **[62]** proved that addition of 1.25 µL/g of thymus vulgaris oil in the semi-solid cheese model did not reduce the viable counts of *Listeria*. At 2.5 µL/g, thymus vulgaris slightly decreased the viable counts of *L. monocytogenes* and in the semi-solid cheese model over 72 h. El Abed et al. **[63]** showed a gradual decrease in the *Listeria* count with the increase of thyme oil concentration. They added that *Thymus capitata* can exhibit strong antioxidant and antimicrobial activity, probably due to its particular chemical composition, mainly the high amounts of carvacrol.

As observed, the reduction rate of pomegranate peel (*Punica granatum L.)* extract on *L. innocua* and *L. monocytogenes* was 98% and 99%, respectively fig.10, 12 where decreased the bacterial log to 3 log for *L. innocua* and 4 log for *L. monocytogenes*. *Zhaojun* et al. **[32]** proved that 20% pomegranate juice had decreased the level of *L. innocua* to undetectable level. Hayrapetyan et al. **[56]** reported that no *L.monocytogenes* were viable after using 7% of pomegranate peel extract where the extract inhibited the growth by 4.1 logs CFU/g compare with the control.

Conclusion

Listeria species are widely distributed in nature as *L.monocytogenes* and *L. innocua* which play a critical role in food poisoning. Plant extracts are a key features in food preservation and Listeria inhibition with alleviation of the chemical preservatives side effects. Besides, the addition of these extracts gave palatable taste and acceptable texture and appearance to the consumer.

Conflict of interest statement:

The authors declare that there is no any conflict of interest in the current research work.

Research ethics committee permission

This study was permitted by Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University.

Authors' contribution

Mona Yousef performed the experiment and drafted the M.S., Hazem Ramadan performed the molecular experiment in Bacterial Epidemiology and Antimicrobial Resistance Research Unit, US National Poultry Research Center, USDA-ARS, Athens, GA, USA. Maha Al-Ashmawy supervised the whole research work and revised the M.S.

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