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INHIBITORY EFFECT OF CINNAMON AND BASIL OILS ON *SALMONELLA ENTERITIDIS* DURING WHIPPED CREAM STORAGE AND THEIR IMPACT ON *INVA* GENE EXPRESSION

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ABSTRACT

Antibiotic-resistant bacteria became more prevalent, and medicinal herbs became more well-liked due to their demonstrated advantages and negligible or non-existent side effects. In the current study, the antibacterial effects of cinnamon and basil oils against *Salmonella Enteritidis* were studied *in vitro* and during whipped cream storage, as well as their impact on the *invA* virulence gene. The well diffusion method showed that both oils had a strong antibacterial effect on the pathogen, and the inhibition zones were 23.44±1.76 and 14.22±1.38 mm in the cinnamon and basil oils, respectively. Minimum inhibitory concentration (MIC) was estimated by the broth dilution method, and it was 0.06 and 0.25% in the cinnamon and basil oils, respectively. In addition, the *invA* virulence gene expression of the pathogen was determined by real-time PCR (RT-PCR) using 16S rRNA primers as a housekeeping gene of *Salmonella spp.* and *invA* primers. The results showed downregulation of *invA* gene by both oils, and the fold change in cinnamon oil was 0.1267, and in basil oil was 0.5141. Finally, when the cinnamon and basil oils were added to the whipped cream that was inoculated with *Salmonella Enteritidis*, a complete inhibition of the tested bacteria was observed on the 5th day in the samples supplemented with a mixture of both oils and on the 7th day in the samples supplemented with a separate oil. However, the tested bacteria in the oil-free whipped cream samples (control +ve) were slightly reduced in bacterial count but not completely inhibited till 7th day. The sensory evaluation had shown that the sample with the cinnamon oil was the most acceptable and the sample with mixed oils was the least acceptable.

Keywords: Cinnamon oil, Basil oil, *Salmonella Enteritidis*, *invA* gene, Whipped cream.

INTRODUCTION

Salmonella is a pathogen responsible for foodborne illnesses. It particularly affects sensitive populations such as the elderly, children, pregnant women, and individuals with weakened immune systems (Oh *et al*., 2023). *Salmonella* infection may be transmitted through contaminated dairy products, chicken meat and eggs, as well as during food preparation, transportation or serving processes. After the incubation period

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which ranged from 6 to 48 h, symptoms such as fever, vomiting and diarrhea occur after contaminated foods are ingested (Kubo *et al*., 2020). In 2020, eggs and egg products were considered the most common food items linked to *Salmonella* and responsible for 5.3% of all foodborne *Salmonella* outbreaks (EFSA and ECDC, 2021). In 2021, 5 European Union/ European Economic Area (EU/EEA) nations and the United Kingdom (UK) reported that 272 humans were confirmed to be affected with *Salmonella*, 25 individuals were hospitalized, 2 adult males died, and 60 of the interviewees specifically mentioned consuming eggs or egg-based products (EFSA and ECDC, 2022).

The infective dose of *Salmonella* depends on the serotype. For non-typhoidal salmonellosis, the infective dose is about 10^3 bacilli, while for enteric fever, the infective dose is approximately 10^5 bacilli by ingestion. Elderly people or patients suffering from depressed cell-mediated immunity may become infected with a lower infective dose (Ryan *et al*., 2004 and Bronze & Greenfield, 2005).

Based on many studies, the main serovars of non-typhoidal *Salmonella* infections reported worldwide are *Salmonella Typhimurium* (12%) and *Salmonella Enteritidis* (65%). Because raw or half-cooked eggs are used in food products such as sauces, cream cakes and other types of food, and they are always the source of *Salmonella* contamination (Doulat *et al*., 2018).

Salmonella pathogenicity is expressed in 3 ways: invasion of host cells, intracellular survival and colonization (Foley *et al*., 2013). The invasion A (*invA*) gene is involved in the pathogenicity of *Salmonella* and protein secretion, which is controlled by a set of genes, located on the chromosome or the plasmid (Guo *et al*., 2000 and Ilyas *et al*., 2017). Moreover, many researches have shown that the *invA* gene is an integral component of the type III secretion system and plays an important role in mediating host recognition, resulting in the inducement of invasion of intestinal epithelial cells; it causes colonization in those cells, leading to enteritis disease (Darwin and Miller, 1999). The *invA* gene, which induces the pathogen to invade the intestinal host cell and is considered a universal genetic marker in all *Salmonella* serotypes (Malorny *et al*., 2003).

Essential oils are derived from plant components that have been used to prolong the shelf life of food, and they can reduce infections (Bolouri *et al*., 2022). Due to their antioxidant, antibacterial and anti-carcinogenic characteristics, Cinnamon and basil essential oils may be used in the medical and food industries (Ashfaq *et al*., 2021 and Eid *et al*., 2023).

The present study aimed to explore the antibacterial effect of cinnamon and basil oils against *Salmonella Enteritidis in vitro* during whipped cream storage and their impact on the *invA* gene.

MATERIALS AND METHODS

Essential oils (EOs):
Cinnamon and basil oils were obtained from the National Research Centre, Cairo, Egypt, and were kept at 2 - 8° C in sealed brown vials until use.

Bacterial strain:
Salmonella Enteritidis strain was used in the current study, according to Kamaly *et al*. (2023), with further detection of the *invA* gene (Photo 1).

Bacterial culture:
The bacterial strain was inoculated in a nutrient broth and the concentration was adjusted to 2.5×10^5 cfu /ml according to the McFarland 0.5 standard (Toni, 2021).

Antibacterial effect of cinnamon and basil oils (Sanusi *et al*., 2019):
The agar well diffusion method was used for testing the antibacterial activities of cinnamon and basil oils. A volume of 15 ml of sterilized Müller-Hinton media (Oxoid, England) was poured into sterile Petri-plates and allowed to solidify, then 100 µl of the inoculum bacterial suspension was added and spread on the agar and left to dry for 5 min. A sterile cork borer was used to make holes.

Each oil concentration (8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06 and 0.03%) was dispensed in its respective holes. The plates were then incubated at 37° C for 18 – 24 h. After the incubation period, the diameter of the inhibitory zones was measured and recorded in millimeters using a ruler.

Broth dilution method for determination of MIC and MBC (Mounyr *et al*., 2016):
Two-fold serial dilutions of cinnamon and basil oils (8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06 and 0.03%) were prepared in a nutrient broth using Tween 80 (up to 2% as a solubilizing agent). Each dilution was inoculated with 100 µl from the previously prepared inoculum broth and then incubated at 37° C for 24 h to observe the turbidity. The least concentration showing no turbidity (no visible sign of bacterial growth) was taken as the MIC.

The minimum bactericidal concentration (MBC) was determined by sub-culturing the test tubes onto sterile *Salmonella-Shigella* agar by using a wire loop in a zigzag manner and the plates were incubated at 37° C for about 24 h. The MBC value was recorded as the lowest concentration that destroyed the tested organism totally, which was indicated by the complete absence of its growth.

Effect of cinnamon and basil oils on *invA* gene expression:
Oligonucleotide primers and probes used in SYBR Green real-time PCR were 16S rRNA (Housekeeping gene of *Salmonella spp*.) and *invA* virulence gene, as shown in **Table (1)**.

Table 1: Primers and probes used in SYBR Green real-time PCR.

Gene	Primer sequence (5' - 3')	Reference
16S rRNA	CAGAAGAAGCACCGGCTAACTC	**Yang *et al*. (2014)**
	GCGCTTTACGCCCAGTAATT	
invA	GTGAAATTATCGCCACGTTCGGGCAA	**Oliveira *et al*. (2003)**
	TCATCGCACCGTCAAAGGAACC	

Extraction of RNA according to the RNeasy Mini Kit instructions:
The RNA was extracted from the samples after incubation for 24 h with sub-MIC concentration of the cinnamon and basil oils and control samples by using the Quantitect SYBR green PCR kit.

Real-time PCR (RT-PCR):
In a thermal cycler, the real-time PCR amplification was carried out in a real-time PCR machine (Stratagene MX3005P). The total volume of that reaction was 25 µl. The mixture contained 12.5 µl 2x QuantiTect SYBR Green PCR Master Mix, 0.25 µl reverse transcriptase, 0.5 µl forward primers (20 pmol), 0.5 µl reverse primers (20 pmol), 8.25 µl RNase-free water and 3 µl of template RNA. For normalization of the expression, 16S rRNA total bacteria was used as an internal control.

The thermal profile was done as the following; 1 cycle at 50° C for 30 min for reverse transcription and 94° C for 5 min for primary denaturation; then 40 cycles of 94° C for 15 sec of denaturation, the annealing at 55 - 60° C for 30 sec, and extension at 72° C for 30 sec; then the final extension was 1 cycle (for dissociation

curve) of 94° C for 1 min, annealing at 55 - 60° C for 1 min and final denaturation at 94° C for 1 min.

The expression levels of the *invA* gene were normalized by using 16S rRNA as an internal housekeeping gene of *Salmonella spp.* as a control, and the fold changes of the gene were determined by using Stratagene MX3005P software. For estimating the variation of the gene expression of different samples, the CT of each sample was compared with that of the control group according to the "ΔΔ Ct" method (Yuan *et al*., 2006). To exclude the false positive results, dissociation curves were compared between different samples.

Evaluation of the antibacterial activity of cinnamon and basil oils during the storage of whipped cream:

Preparation of a whipped cream under complete aseptic conditions was done according to Radványi *et al*. (2012) by combining egg white and sugar, then whipped with an egg-whisk at a constant shear rate for 10 min until a hard foaming formation.

The whipped cream was divided into a negative control jar (free from any treatment and the tested strain) and 4 positive control jars which were inoculated with the *Salmonella Enteritidis* strain to obtain a final concentration of 2.5×10^5 cfu /ml as the following; the 1st as a positive control (contain the tested strain without any treatment), the 2nd for MIC of the cinnamon oil, the 3rd for MIC of the basil oil, the 4th for the combination of MIC of mixed cinnamon and basil oils.

The antimicrobial activity of the studied essential oils was evaluated by monitoring the pathogen count in each studied portion (both test and control) by using *Salmonella-Shigella* agar (SS agar) (Himedia, India). The initial count was determined, and the enumeration was then done at 0 day, 1st day, 2nd day, 3rd day, 5th and 7th day of the storage period.

All jars were stored in the refrigerator, and the enumeration was done by ten-fold serial dilutions as follows: 1 g of the whipped cream was added aseptically to 9 ml of normal saline, and 0.1 ml was spread on the *Salmonella-Shigella* agar; and the plates were incubated at 37° C for 24 h.

Sensory analysis (Badmos and Abdulsalam, 2012):

Negative control samples containing separate oils and mixed oils at MIC concentrations were examined by a panel of 86 judges, who can evaluate the sensory qualities of the whipped cream based on 5 attributes of color, odor, taste, texture and overall acceptability (OAA) using 9-point hedonic scale. The most acceptable whipped cream received 9 points, and the most unacceptable received 1 point.

Statistics:

The data for treatment parameters were obtained from 3 experimental repeats and presented as Means± standard error of mean (SEM). The sensory evaluation was made by using Chi-square while comparison in bacterial counts was made using ANOVA and Tukey post-hoc test using SAS 4.9 (2013) software. P <0.0001 was considered statistically significant.

RESULTS

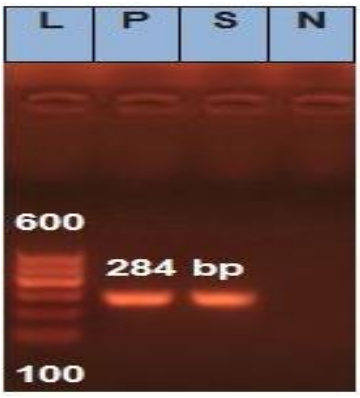

Photo 1. Agarose gel electrophoresis of PCR amplification for *invA* gene (284 bp).
Lane L: 100 bp DNA ladder.
Lane P: Control positive.
Lane S: *Salmonella Enteritidis* strain.
Lane N: Control negative.

Table 2: The inhibition zone, MIC and MBC for EOs against *Salmonella Enteritidis*.

Tested Essential oils (EOs)	Inhibition zone diameter (mm)*	MIC (%)	MBC (%)
Cinnamon oil	23.44±1.76	0.06%	0.125%
Basil oil	14.22±1.38	0.25%	0.5%

*Data were expressed as mean±S.E.

Table 3: Cycle threshold (CT) and fold change of *invA* virulence gene.

Samples	16S rRNA CT	*invA* gene CT	Fold change to control
Treated with cinnamon oil	19.43	24.08	0.1267
Treated with basil oil	19.86	22.49	0.5141
Non-treated sample control (*Sal. Enteritidis* broth without supplemented oils)	19.51	21.18	1

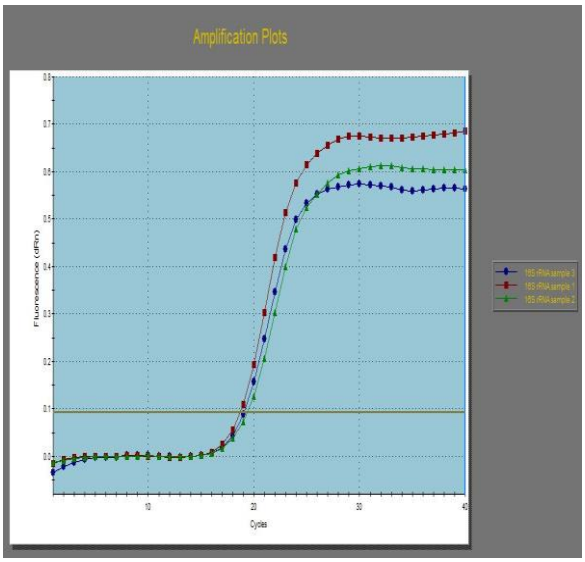

Fig. 1: The amplification plots and cycle thresholds (CT) of 16S rRNA genes.

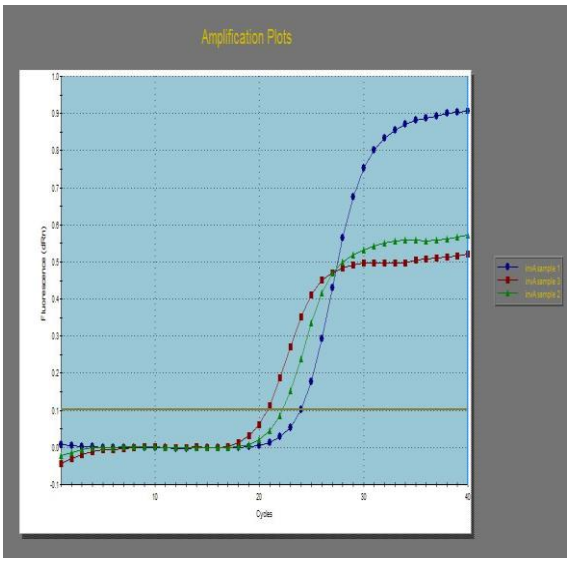

Fig. 2: *invA* virulence gene expression by SYBR green RT-PCR.

Table 4: Survival count (log_{10}) of *Salmonella Enteritidis* in the supplemented whipped cream.

Time	Control -ve	Control +ve	Cinnamon oil	Basil oil	Mixed oils
Initial count	0 ± 0^{B}	5.32 ± 0.01^{A}	5.32 ± 0.01^{A}	5.32 ± 0.01^{A}	5.32 ± 0.01^{A}
1st day	0 ± 0^{D}	5.28 ± 0.01^{A}	4.70 ± 0.01^{C}	4.95 ± 0.00^{B}	4.69 ± 0.01^{C}
2nd day	0 ± 0^{E}	5.18 ± 0.02^{A}	4.62 ± 0.01^{C}	4.85 ± 0.00^{B}	3.95 ± 0.00^{D}
3rd day	0 ± 0^{E}	5.04 ± 0.02^{A}	3.90 ± 0.00^{C}	4.46 ± 0.01^{B}	2.90 ± 0.00^{D}
5th day	0 ± 0^{D}	4.46 ± 0.01^{A}	3.15 ± 0.02^{C}	3.81 ± 0.00^{B}	0 ± 0^{D}
7th day	0 ± 0^{B}	4.40 ± 0.01^{A}	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}

P value < 0.0001

$^{A, B, C, D, and E}$ different letters in the same row refer to significant differences.

Table 5: Reduction% of *Salmonella Enteritidis* count following inoculation in the supplemented whipped cream.

Time	Cinnamon oil (%)	Basil oil (%)	Mixed oils (%)
1st day	76.19	57.14	76.67
2nd day	80	66.2	95.71
3rd day	96.19	86.19	99.6
5th day	99.33	96.95	100
7th day	100	100	100

$$\text{Reduction\%*} = \frac{\textbf{initial count} - \textbf{count of bacteria after treatment}}{\textbf{initial count}} \times 100$$

*Reduction% was calculated upon Ohaegbu *et al*. (2024).

Table 6: Sensory evaluation properties of the manufactured whipped cream.

Sensory traits	Characters	Total number	Control		Cinnamon oil		Basil oil		Mixed oils	
			No.	%	No.	%	No.	%	No.	%
Flavor (**)	Like	86	69	80.23	72	83.72	66	76.74	40	46.51
	dislike		17	19.77	14	16.28	20	23.26	46	53.49
Appearance (**)	Like	86	68	79.07	80	93.02	64	74.42	36	41.86
	dislike		18	20.93	6	6.98	22	25.58	50	58.14
Body and texture (NS)	Like	86	77	89.53	85	98.84	77	89.53	80	93.02
	dislike		9	10.47	1	1.16	9	10.47	6	6.98
Overall acceptance (**)	Like	86	80	93.02	83	96.51	74	86.05	46	53.49
	dislike		6	6.98	3	3.49	12	13.95	40	46.51

(**) P value < 0.0001.
(NS) P= 0. 0608

DISCUSSION

In recent years, bacterial virulence and resistance to most antibiotics have increased, and medicinal herbs have gained popularity due to their proven benefits and minor or absent adverse effects. In confirmation of that, the obtained results of the current study showed that both oils had strong antibacterial effects and the cinnamon oil was more effective than the basil oil against *Salmonella Enteritidis* (Table 2), which showed the inhibition zones were 23.44±1.76 mm in the cinnamon oil and 14.22±1.38 mm in the basil oil using the well diffusion method. The minimum inhibitory concentrations (MIC) were 0.06 and 0.25%, while MBC were 0.125 and 0.5% in the cinnamon and basil oils, respectively. Similar results were obtained by Knezevic *et al*. (2021), who revealed that cinnamon oil was more effective than basil oil on *Salmonella spp.* and the inhibition zone of cinnamon against both *Salmonella Enteritidis* and *Salmonella Typhimurium* was approximately 23.2 mm and the inhibition zone of basil oil ranged from 7-10 mm against *Salmonella Enteritidis* and from 7.17-13 mm against *Salmonella Typhimurium;* while MIC was 14.21–28.41 µl/ml against *Salmonella Enteritidis* and 3.56-28.41 µl/ml against *Salmonella Typhimurium*. Tsai *et al*. (2017) revealed that MIC and MBC for *Salmonella Enteritidis* were 0.05 and 0.1% (v/v), respectively.

A higher result was obtained by Iseppi *et al*. (2024), who revealed that the inhibition zone of cinnamon oil against *Salmonella Typhimurium* was 27±0.9 mm. El-Demerdash *et al*. (2024) recorded that MIC of cinnamon essential oil ranged from 0.125 to 8 µg/mL, and MLC ranged from 0.5 to

32 µg/ml. On the other side, a lower result was obtained by Banik *et al*. (2018), who reported the inhibition zone against *Salmonella typhi* was 18 mm. Ozdemir *et al*. (2021) showed that MICs and MBCs of free basil oil on *Salmonella Typhimurium* were 700 and 800 µg/ml, respectively.

The *invA* virulence gene is required for full *Salmonella* pathogenicity because it improves internalization, which is necessary for deeper tissue penetration (Khan *et al*., 2000). The results obtained showed downregulation of the *invA* gene by both oils by real-time PCR using 16S rRNA primers (Housekeeping gene of *Salmonella spp*.) and *invA* primers. The fold changes of the *invA* gene were 0.1267 and 0.5141 in the cinnamon and basil oils, respectively (Table 3, Figures 1 and 2).

The effect of cinnamon oil on the transcription levels of the *Omp* genes (involved in the metabolism of bacterial substances, such as maintaining cell membrane fluidity, cell structure, ensuring material transport) by q-PCR and had showed significantly increased the expression levels of *OmpA* and *OmpX*, which might be due to the activation of the defense mechanism of *Salmonella Enteritidis* (Zhang *et al*., 2022).

In the study of Ahmed *et al*. (2023) the fold change on *Salmonella typhi* strains treated with thyme and cinnamon extracts showed down-regulation in the *fliC* gene expression and noticeably inhibited to 9-folds in the cinnamon ethyl acetate extracts while a significant decrease in regulation on *invA* gene had occurred by ethanol cinnamon extracts and ethyl acetate and in some strains of *Salmonella typhi* had been banned.

Table (4) showed the effect of cinnamon, basil oil and their mixture on the survival of *Salmonella Enteritidis* in the supplemented whipped cream, and the results had showed that there was a significant decrease in the count of the pathogen during its storage period, and the count was completely inhibited at the 5th day in the sample treated with mixed oils and at the 7th day in the samples treated with the cinnamon or the basil oils separately with reduction rate 100% (Table 5); while in the oil-free whipped cream samples (control +ve) there was a slight reduction in the bacterial count but not completely inhibited. The statistical analysis showed statistically significant differences ($p<0.0001$) with the same storage time between the different treatment groups.

In the study that was performed by Toni (2021), the count of *Salmonella Typhimurium* was completely inhibited after 48 h in rice pudding treated with 0.0625% cinnamon oil. Olaimat *et al*. (2019) could not detect *Salmonella spp.* in hummus treated with 1 – 0.5% cinnamon oil for 1 or 7 days, respectively. Similar results were shown by Gerges and El Asuoty (2022), who recorded that the reduction rate of *Salmonella Typhimurium* count in the chicken sausage samples was 99.81% on the 5th day when treated with basil oil at 0.3% concentration. Stojiljković *et al*. (2015) revealed that the addition of 5% sweet basil oil reduced the number of *Salmonella Enteritidis* by 0.14 log cfu/g.

According to the obtained data in Table (6), the statistical analysis showed a significant difference in taste, flavor and overall acceptability (OAA) between different treatment groups ($P<0.0001$); however, there was no difference in texture and body ($P=0.0608$). It was found that the sample treated with cinnamon oil was the most acceptable and the sample treated with mixed oils was the least acceptable to judges. A parallel result was obtained by Toni (2021) who found the addition of cinnamon oil in a concentration of 0.125% to rice pudding presented the highest panelists' acceptance. Also, Al-Rimawi *et al*. (2020) reported the addition of cinnamon oil to Labneh (concentrated yoghurt) at 600 µl/Kg had increased the shelf life with acceptance of flavor, taste and texture.

CONCLUSION

Cinnamon and basil oils had a strong antibacterial effect against *Salmonella Enteritidis*, as they affected the *invA* virulence gene expression with a significant downregulation. Each oil reduced *Salmonella Enteritidis* count to complete inhibition at the 7th day, while the mixed oils till the 5th day of the experimentally inoculated whipped cream.

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التأثير المثبط لزيتي القرفة والريحان على بكتيريا *السالمونيلا إنتريتيديس* أثناء تخزين الكريمة المخفوقة وتأثيرهما على التعبير الجيني للجين *invA*

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نتيجة لزيادة مقاومة البكتيريا للمضادات الحيوية، فقد إكتسبت الأعشاب الطبية شعبيةً واسعةً حيث فوائدها الكثيرة وأثارها الجانبية الطفيفة أو المعدومة، وبناءا على هذا فقد هدف البحث الحالي إلى دراسة التأثير المثبط لزيت القرفة وزيت الريحان على بكتيريا *السالمونيلا إنتريتيديس (Salmonella Enteritidis)* في المختبر وكذلك أثناء تخزين الكريمة المخفوقة، بالإضافة إلى تأثيرهما على ضراوة الجين *invA*. وقد أظهرت النتائج أن كلا الزيتين لهما تأثير مضاد قوي على العامل الممرض للبكتريا، وكان نطاق التثبيط يتراوح بين ٢٣,٤٤±١,٧٦ مم في زيت القرفة و١٤,٢٢±١,٣٨ مم في زيت الريحان. وقد تم تقدير الحد الأدنى من التركيز المثبط (MIC)، حيث كان 0.06 و0.25%، للزيتين على التوالي. وكذلك تم تحديد التعبير الجيني لضراوة جين *invA* بواسطة تفاعل البلمرة المتسلسل في الوقت الحقيقي (RT-PCR) بإستخدام 16S rRNA primers و*invA* primers، وقد أظهرت النتائج إنخفاضا في تنظيم جين *invA* بواسطة كلا الزيتين، وكان إنخفاض التعبير الجيني في زيت القرفة 0.1267 وفي زيت الريحان 0.5141. وعند إضافة زيتي القرفة والريحان إلى الكريمة المخفوقة الملقحة ببكتيريا *السالمونيلا إنتريتيديس*، فقد تم ملاحظة التثبيط الكامل للبكتيريا المختبرة في اليوم الخامس في العينات المضاف إليها زيت القرفة والريحان معا وأيضا في اليوم السابع في العينات المضاف إليها الزيوت بصورة منفصلة، ومع ذلك فقد إنخفض عدد البكتيريا المختبرة في عينات الكريمة المخفوقة الخالية من الزيوت (المجموعة الضابطة الإيجابية +ve) بشكل طفيف ولكن لم يتم تثبيطها تماما حتى اليوم السابع. وقد أظهر التقييم الحسي أن العينة التي تحتوي على زيت القرفة كانت الأكثر قبولًا حسيا والعينة التي تحتوي على زيت القرفة والريحان معا كانت الأقل قبولًا حسيا.

الكلمات المفتاحية: زيت القرفة، زيت الريحان، *السالمونيلا إنتريتيديس*، جين *invA*، الكريمة المخفوقة.