ISSN: 2356-7767

Effect of thyme extract on *Bacillus cereus* secretes toxins in poultry meat

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Research Paper

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Received in 18/2/2025
Accepted in 17/3/2025

Abstract

Bacillus cereus (*B.cereus*) possess a significant concern for both food safety and public health, but utilizing herbal extracts can enhance the microbial quality and preservation of poultry meat and giblets. Therefore, the most recent study examined the occurrence of *B.cereus* in poultry and the presence of emetic and hemolytic genes (*hbl, ces*) of *Bacillus cereus* in samples of poultry meat, liver, lung, and gizzard, along with the effects of thyme extract on positive meat samples.

Out of 100 poultry samples contained 18 *Bacillus cereus* strains were isolated. The meat, liver, lung, and gizzard samples had isolation rates of 20%, 28%, 8%, and 12% respectively. Molecular analysis revealed a link between *B. cereus* and the genes *hbl* and *ces*. The strains displayed resistance to gentamycin (55.5%), doxycycline (33.3%), streptomycin (27.7%), and amoxicillin/clavulinic (27.7%) out of the seven antibiotics examined. The findings indicated that the thymthyme extract had a notable effect on *B. cereus* strains, resulting in a 15 mm inhibition zone when assessed with the agar well diffusion technique. 600 grams of fresh chicken meat, liver and gizzard were divided equally into 3 groups, each weighing 200 grams. 1st group was the control, the 2nd group was given a 0.5% thyme extract, and the 3rd group received treatment with 1% thyme extract. All the groups were kept in a fridge at a temperature of 4˚C. Teams evaluated them every 48 hours for general acceptance and levels of *Bacillus cereus*.

Thyme extract in doses of 0.5% and 1% decreased the count of *Bacillus cereus* in meat, liver, and gizzard from their original levels to lower levels, with different reduction rates for each type of sample. The preservation time of meat in the refrigerator is prolonged by the thyme extract, which slows down meat spoilage. In the first group the count was 5.64±0.13, 6.54±0.17, and spoilage on the day six. The initial load and spoilage were both reduced by treatment with 0.5% thyme to 5.57±0.12, 4.31±0.34, 4.14±0.12, and 4±0.09 on the day 1,3,6,9 of the storage, and decreased to 5.60±0.20, 4.14±0.35, 4.04±0.03, 3.94±0.12, 3.64±0.23 on the days 1,3,6,9 ,12 and spoilage happened on the 15th days in samples treated with 1% thyme. The discovery of *B. cereus* in poultry in Egypt highlights their influence on food safety and public health, indicating the need for additional research in this area.

Keywords*: Thyme extract; antibacterial agent; B. cereus*

Introduction

The diverse Bacillus genus includes both beneficial and harmful environmental species,the closely related *B. cereus* group, comprising *B. anthracis*, *B. thuringiensis, B. mycoides*, and *B. cereus*, are recognized as harmful or potentially harmful to humans **(Logan *et al*., 2011**). Poultry is a perishable food that is susceptible to spoilage microorganisms and foodborne pathogens, which can cause outbreaks with significant consequences for public health and the economy (**Li *et al*., 2020**).

It is important for food safety to consider the presence of Bacillus spp. Contaminating the environment which possess at least one enterotoxin gene is considered a risky agent, but the assessment of toxin gene in Bacillus spp. apart from *B. cereus* has not been extensively researched. The main poisons responsible for causing food poisoning in *B. cereus* are Cytotoxin K (derived from the *cytK* gene), enterotoxin FM (from the *entFM* genes), hemolysin hbl (from the *hbl* operon), emetic genes (*ces* gene), and non-hemolytic enterotoxin (from the *nhe* genes) **(Granum *et al.,* 2013 and Walker-York-Moore *et al*., 2017).**

Finding new ways to increase the shelf life of fresh chicken cuts and preserve their quality is crucial due to the financial losses caused by spoilage, a major issue in the poultry processing sector and valued by customers **(Burt, 2004)**. The spotlight has been on herbal and spice extracts, traditionally utilized to enhance the taste and preservation of food products. **(Fernandez-Gines *et al*., 2005)**.

Thyme extract is viewed as a compelling option for preserving food. They have been formally approved as Generally Recognized As Safe (GRAS) for consumption as food additives. Moreover, there are multiple benefits like antibacterial, antifungal, antioxidant, and anti-inflammatory qualities. Previous research discovered that thyme extract possess antimicrobial characteristics effective against various microorganisms found frequently in poultry, meat, fish, dairy products, vegetables, and fruit. The antimicrobial power of Thyme extract originates from compounds like carvacrol, thymol, and γ-terpinene **(Pourhosseini *et al*., 2020)**.

The antimicrobial properties of thyme extract, are closely linked to the bioactive volatile compounds they contain. Herbs and spices in general have been used to improve the taste, characteristics, over acceptability and shelf life of food **(Gibbons, 2008)**. Compounds containing phenolic groups are the most successful resistance against microbial contaminations. Thyme extract have the ability to prevent food from spoiling and extend the shelf life of food products (**Mandal and DebMandal, 2016**). Thyme extract protects food from spoilage (**Valizadeh *et al*., 2016**).

The objective of this research was to investigate the existence of *B. cereus* in chickens and identify the genes associated with the toxicity of *B. cereus* strains discovered in chickens. Identification of antibiotic resistance patterns in *B. cereus* and the impact of thyme extract in controlling *B.cereus*.

Material and Methods

1. Preparation of samples: 100 chicken samples (comprising of meat, liver, lung, and gizzard) were collected from poultry meat and giblets in El-Menoufia governorates and placed in sterile polyethylene bags for storage. The positive samples were divided into 3 equal parts: one served as the control group (control positive) while the other two groups were exposed to Thyme oil (0.5% - 1%).

2. Isolation and identification of *B. cereus* (Rahimi *et al*., 2013), (ISO, 2006): 25 grams of fresh chicken meat and giblet were added to 225 ml of sterile 0.1% buffered peptone water and blended for 2 minutes to create a homogenate, followed by creating decimal serial dilution. 10^{4} dilution was plated on a Bacillus cereus agar medium, spread with a bent rod, and incubated at 37°C for 48 hours to count colonies of *B. cereus* (sized at 5mm and blue in color). Selected colonies were transferred to nutrient broth for sub culturing, cultured at 37°C for 48 hours, and subsequently stored in a refrigerator at 4°C for additional biochemical analysis.

3. Antimicrobial Susceptibility Test: Each *B.cereus* isolate underwent testing for antibiotic susceptibility through the Kirby-Bauer disk diffusion technique, with interpretations

made based on the CLSI guidelines ,2017. The antibiotics penicillin G (P,10 mcg), ampicillin (Amp,10µg), amoxicillin clavulinic acid (Amc20/10 µg), streptomycin (S,10 µg), gentamicin (CN,10 µg), enorfloxacin (Enr,5 µg) and doxycycline (Do,10 µg) were utilized in the study (Oxoid, Biogram). In brief, about 100 -200 µL of the overnight bacterial culture was added to 5 mL of normal saline to reach a concentration equivalent to 0.5 McFarland standard (0.5 x 10^8 cfu/mL). Subsequently, 100 µL was evenly spread on Mueller-Hinton agar plates from Himedia, India using a sterile glass spreader, followed by placing the mentioned antimicrobial discs onto the plates, and incubating at 37°C aerobically for a period of 24 hours. Afterwards, the sizes of the inhibition zone were evaluated and interpreted according to the instructions outlined by the **CLSI, (2017).**

4. Antibacterial effect of thyme extract on Muller Hinton agar: Every identified *B.cereus* isolate underwent an antibacterial test using thyme extract (0.5% and 1%) following the Kirby-Bauer disk diffusion method. Briefly, approximately 100 µL of bacterial overnight culture was transferred into 5 mL of normal saline solution prepared to match the 0.5 McFarland standard (0.5 x 10^8 cfu\mL). Later, 100 µL was spread evenly on Mueller-Hinton agar plates (Himedia, India) using a sterile glass spreader. The plates were then loaded with filter paper in a size resembling te antibiotic tablets and saturated with two different concentrations of thyme extract (0.5%, 1%) and incubated aerobically at 37°C for 24 hours. Afterward, the inhibition zone, measured and computed for each dilution level.

5. Molecular identification of *Bacillus cereus* toxin: The CLQP-PCR unit at the Animal Health Research Institute received five confirmed *B.cereus* strains for molecular detection of genes *(hbl,ces*).

1) DNA Extraction The procedure was carried out in accordance with the guidelines of the QIAamp DNA mini kit (cat.no.). 51304).

2) Amplification program of the examined genes was adjusted according to the kit instruction. The suspected isolates were validated using polymerase chain reaction methods (PCR) and the resulting PCR samples were observed using agarose gel electrophoresis (AGE) **(Sambrook *et al*., 1989).** Oligonucleotide primers used and the reaction protocols are summarized in table A:

Table (A). Sequences of primers, genes being targeted, sizes of amplicons, and conditions for cycling.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
hbl	GTA AAT TAI GAT GAI CAA TTTC AGA ATA GGC ATT CAT AGA TT	1091	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 1 min.	72˚C 10 min.	**Ehling-Schulz *et al* ., (2006)**
ces	GGTGACAC ATTATCAT ATAAGGTG GTAA-GCGAACCT GTCTGTAA CAACA	1271	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 1.2 min.	72˚C 10 min.	

6. Thyme extract preparation: Thyme extract was purchased from botany department, National research center.

7. Experimental application: Three groups of positive samples (meat, giblets): first group as Control positive , second group treated with thyme 0.5%, and third group treated with thyme 1%. Each sample group was put in separate sterile polyethylene bags and kept in a household refrigerator at around ±4 °C. Each sample underwent testing on Day zero, 3rd, 6th, 9th, 12th, and 15th during storage.

8. Sensory examination: The method outlined by **Patsias *et al* . (2006)** was used to evaluate the color, smell, and overall likability of each sample. System for evaluating the senses using a scoring system was top-notch with a rating of (9) indicating a vibrant red color, aromatic odor, and a solid, delicate texture. Ranking as very good at (8), good at (7), acceptable at (6), unsatisfactory at (5), and spoiled at (4) indicates a grey to green hue, foul and decaying smell, and a mushy and thin texture.

Results

Table (1). Occurrence of *Bacillus cereus* in samples of poultry meat, liver, lung, and gizzard (n=100).

Samples (n=25)	No. of isolates	Incidence %
Meat samples	5	20 %
Liver	7	28%
Lung	2	8%
Gizzard	4	16%
Total	18	18%
X^2	4.181	
***p*-value**	>0.05	

X^2The chi-square statistic. The result is *not* significant at $p > .05$

Table (2). Antibiotic susceptibility pattern of *Bacillus cereus* isolates (n=18):

Antimicrobial agent	Symbol	Conc.	Sensitive		Intermediate		Resistant		AA
			No.	%	No	%	No.	%	
Penicillin G	P	10	11	61.1%	4	22.2%	3	16.6%	S
Ampicillin	Amp	10	12	66.6%	3	16.6%	3	16.6%	S
Amoxicillin clavulinic acid	Amc	20/10	9	50%	4	22.2%	5	27.7%	S
Streptomycin	S	10	7	38.8%	6	33.3%	5	27.7%	S
Gentamicin	CN	10	5	27.7%	3	16.6%	10	55.5%	R
Enorfloxacin	Enr	5	14	77.7%	2	11.1%	2	11.1%	S
Doxcycline	Do	10	3	16.6%	9	50%	6	33.3%	S

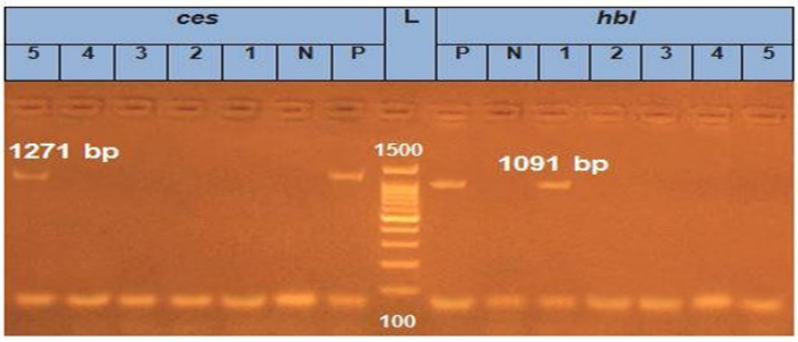

Fig. (1). Agarose gel electrophoresis revealed analysis of PCR, the amplification products of *ces* and *hbl* genes for *B. cereus* isolates at 1271 and 1091 bp.

Table (3). Susceptibility pattern of Bacillus *cereus* isolates treated by thyme (0.5%, 1%) by agar diffusion method:

Antimicrobial agent	Sensitive		Resistant	
	No.	%	No.	%
Thyme 0.5%	5	27.7%	13	72.2%
Thyme 1%	8	32%	10	55.5%

Table (4). (Mean ± SE) of the effect of thyme 0.5%, thyme 1% on *Bacillus cereus* count in examined samples

Samples	Control	Thyme 0.5%	Thyme 1%	P value
Meat	5.64±0.23 [a]	5.50±0.34 [a]	5.11±0.15 [b]	<0.005
Liver	5.76±0.31[a]	5.40±0.19[b]	5.04±0.18[c]	<0.001
Gizzard	5.54±0.16[a]	5.10±0.11[b]	4.47±0.09 [c]	<0.001

Significant difference is indicated by means of count of no. of Bacillus in the same row with different letters; a p value of less than 0.05 is considered significant.

Table (5). Reduction ratio of thyme extract (0.5% and 1%) on examined sample %

Samples/treatment	Thyme 0.5%	Thyme 1%
Meat	2.48%	9.39%
Liver	2.77%	12.5%
Gizzard	7.94%	19.31%

Table (6). Effect of thyme 0.5% and thyme 1% on *B.cereus* count on positive samples within different days of preservation at 4°C:

Groups / storage period	Control (Mean ±SE)	Thyme 0.5% (Mean ±SE)	Thyme 1% (Mean ±SE)	P value
1st day	5.64±0.13 [a]	5.57±0.12 [a]	5.60±0.20 [a]	>0.05
3rd day	6.54±0.17 [a]	4.31±0.34 [b]	4.14±0.35 [c]	<0.001
6th day	S	4.20±0.12 [a]	4.04±0.03 [b]	<0.001
9th day	S	4 ±0.09 [a]	3.94±0.12 [a]	>0.05
12th day	S	S	4.99±0.23	-
15th day	S	S	S	-

Significant difference is indicated by means in the same row with different letters; p value of less than 0.05 is considered significant. S mean spoiled.

Table (7). Assessment of thyme extract 0.5% and 1% on samples through sensory evaluation.

Group	Days	Color	Odor	Appearance	Over all acceptability	consistency	Grade
Control	**Zero day**	8	8	8	9	8	8
	3rd day	7	7	8	8	7	7
	6th day	5	6	5	6	5	5
	9th day	4	4	4	4	4	4
	12th day	4	4	4	4	4	4
	15th day	4	4	4	4	4	4
Thyme 0.5%	**Zero day**	8	9	8	8	9	8
	3rd day	7	8	8	8	7	8
	6th day	7	7	7	7	6	7
	9th day	6	6	7	7	6	6
	12th day	6	6	6	6	6	6
	15th day	4	4	4	5	4	4
Thyme 1%	**Zero day**	8	9	8	9	8	8
	3rd day	8	8	8	8	8	8
	6th day	8	8	7	8	7	7
	9th day	7	7	7	7	7	7
	12th day	6	6	5	6	5	6
	15th day	4	5	4	4	4	4

Abb. means: 9=excellent, 8=very good, 7=good, 6=acceptable, 5=unacceptable, 4=spoiled

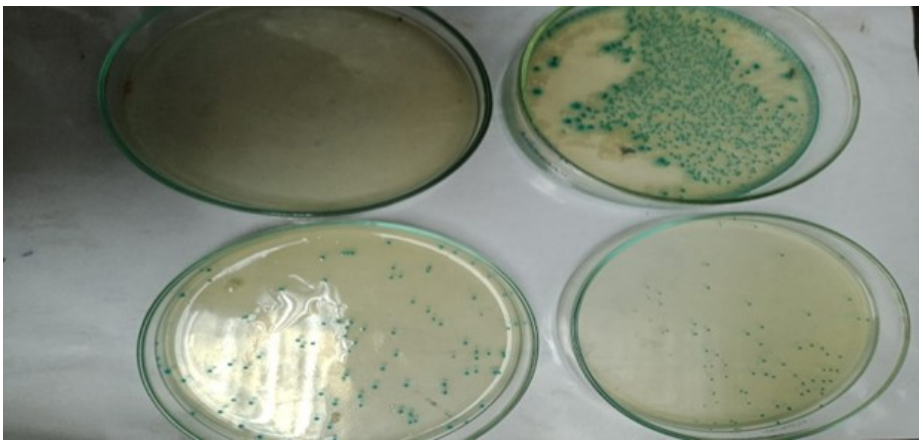

Fig. (2). The effect of thyme 0.5%, thyme 1% on *bacillus cereus* isolated from examined samples

Discussion

Borge *et al*. (2001) reported that *B. cereus* often associate with cases of food borne diseases. *B.cereus* food poisoning can affect anyone. Some types of *B. cereus* can thrive in low temperatures **(Valero *et al*., 2007)** and their spores are resistant to high temperatures. Food technologists are increasingly embracing thyme oil due to its enhanced sensory evaluation and antimicrobial properties (**Fischer and Phillips, 2006).**

The current research retrieved 18 *B. cereus* isolates from a total of 100 poultry samples. Meat, liver, lung, and gizzard samples with isolation rates of 20%, 28%, 8%, and 16%, respectively (table 1) **Rather *et al*. (2012**) found a higher occurrence of *B. cereus* in chicken at 33.33%, while **Zaki *et al*. (2019**) reported a lower incidence of 8.6% of *B. cereus* in chicken meat. Therefore, it is crucial to manage the contamination of chicken meat in slaughterhouses by *B. cereus* in order to decrease the occurrence of foodborne illnesses in humans.

The molecular analysis in the study showed that the *B. cereus* group is associated with the genes *hbl* and *ces* (fig. 2). This finding aligned with **Ehling-Schulz *et al* (2006**) research, which recorded that toxin-producing *B. cereus* is a significant factor in causing both diarrhea and emesis in food poisoning cases. *B. cereus*-induced vomiting syndrome is characterized by vomiting hours after eating contaminated food, while diarrhoeal poisoning is caused by heat-sensitive enterotoxins released by *B. cereus* during vegetative growth in the small intestine. The diarrhea-causing toxins are fully recognized at a molecular and transcriptional level. Less data is accessible regarding the vomiting-causing poison cereulide, which has been connected to the fatality of a child two times as a result of liver failure. The *ces* gene cluster is made up of 7 coding sequences: *cesH, cesP, cesT, cesA, cesB, cesC*, and *cesD* (**Dommel *et al*., 2010**).

It has also been proven that *B. cereus* spores bind to the hydrophobic surfaces of epithelial cells. Around 80% of the protein found in the koilin layer of the gizzard is abundant in leucine and arginine. These proteins may be involved in the formation of a biofilm that can withstand both gastric acid and mechanical digestion (**Majed *et al*., 2016**). Moreover, the digestion of nutrients by *B. cereus* and the possible formation of toxins could damage the lining of the gizzard. Both Hbl and Cytk are harmful substances that can harm the koilin layer of the gizzard, leading to ulceration with the passage of time. Furthermore, Hbl contributes to the development of diarrhea by damaging the digestive system **(Ehling-Schulz *et al*., 2006).** The structure of Hbl resembles that of *Escherichia coli*'s pore-forming haemolysin, causing dermonecrotic effects and altering blood vessels' permeability (**Wijnands *et al*., 2002**).

In this study (table 2), testing for antimicrobial resistance against seven antibiotics showed different isolates were resistant to gentamycin (55.5%), doxycycline (33.3%), streptomycin (27.7%), and amoxicillin/clavulinic (27.7%), but sensitive to enrofloxacin 77.7%, ampicillin (66.6%) and penicillin G (61.1%). The results supported **Luna *et al*. (2007**) in showing *B. cereus* is sensitive to ampicillin, but disagreed with **Turnbull *et al*. (2004**) in finding *B. cereus* isolated was highly susceptible to gentamicin, ciprofloxacin, chloramphenicol, and streptomycin **Whong and Kwaga (2007)** found that 82% and 95% of *B. cereus* had developed resistance to Penicillin G, which aligns with current results showing 91.75% resistance. Different authors have reported a range of resistance levels of *B. cereus* to antibiotics.

In the current research (table 4), the thymus extract showed a significant impact on *B. cereus* with an inhibition zone of 15 mm. Thyme extract at 0.5% reduced the *bacillus cereus* count in meat, liver, and gizzard samples (table 5,6) with reduction percentages ranging from 2.48%.2.77% and 7.94% respectively, and thyme extract and 1% reduced the bacillus cereus count to 9.39%,12.5% and 19.31% respectively. These findings support previous research indicating that thyme and other herbs have antioxidant properties that enhance the color and flavor stability of meat. In this current research, (table 4) the thyme extract enhances the shelf life of meat by delaying spoilage and extending the preservation time of meat in the refrigerator. The initial loads were 5.64±0.13, 6.54±0.17 and spoilage on the 6^{th} day of storage. In (table 4) treatment with 0.5%

thyme reduced the initial load to 5.57±0.12, 4.31±0.34, 4.14±0.12, 4±0.09 on the different storage days and spoilage occurred at the day 12 of storage. In the samples treated with 1% thyme, The number of *B. cereus* reduced to 5.60±0.20, 4.14±0.35, 4.04±0.03, 3.94±0.12, 3.64±0.23, and spoilage occurred on the day 15 of storage**.** The taste, color, odor and overall acceptability of samples improved with using thyme extract (table 7) this results agreed with **Salem *et al*. (2010)** discovered that varying levels of thyme oil (0.5%, 1%, 1.5%) enhanced the taste and smell of minced beef kept at 4°C when compared to samples that were not treated. The most noticeable improvement was seen with a 1.5% concentration of thyme oil. **Shaltout *et al*. (2017)** found that meat samples containing 2% thyme extract showed the most significant improvement in sensory attributes compared to meat samples with 1% thyme oil, which showed less improvement. Nevertheless, **Solomakos *et al*. (2008**) **and Giatrakou *et al*. (2010**) discovered that thyme extract was deemed satisfactory in terms of odor and taste when used at 0.2-0.6% in minced beef, but became unacceptable at 0.9%.

Conclusion

The results of the current study represented the effect of thyme extract (0.5%, 1%) improve the quality and sensory characteristics of chicken meat and giblets under chilled storage (4°C) for the economic and public health importance viewpoint.

Acknowledgments:
Unable to apply.
Statement regarding financial support
There was no financial backing for this research.

Potential Conflict of Interest Declaration

The authors declare they do not have any conflicts of interest.

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