Research Article

Prevalence of *Clostridium perfringens* in Retail Meat and Meat Products with Some Decontamination Trials by some Essential Oils

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Abstract | Meat is a valuable contribution to diets because of its high nutritional values. *Clostridium perfringens* is a commensal inhabitant of animals and human intestinal tract as well as a common foodborne pathogen associated with food poisoning. The present study was carried out to investigate the prevalence of *C. perfringens* in meat and its products at Zagazig city, Sharkia Governorate, Egypt. In addition, toxin genes, antimicrobial susceptibility testing and biofilm formation of *C. perfringens* strains isolated from meat and its products were determined. The achieved results revealed the contamination of meat and meat products by *C. perfringens* with varying degrees. Alpha toxin gene was found in all isolated strains, while enterotoxin gene not detected. By disc diffusion method, *C. perfringens* isolates were found resistant to most of the tested antibiotics with high multiple antibiotic resistance (MAR) indices. Most of *C. perfringens* isolates were able to form biofilms at different temperatures. Finally, marjoram oil 2% was more effective than thyme oil 5% in reduction of *C. perfringens* count in vitro. The results suggested appropriate food safety practices in meat and meat product production and processing facilities in Zagazig city, Egypt.

Keywords | *C. perfringens*, Enterotoxin, Antibiotic, Biofilm, Essential oils

Editor | Asghar Ali Kamboh, Sindh Agriculture University, Tandojam, Pakistan.
Received | November 02, 2020; **Accepted** | November 23, 2020; **Published** | December 27, 2020
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Citation | El-Bayomi RM, El-Mesalamy YM, Hafez AE, Ahmed HA (2020). Prevalence of clostridium perfringens in retail meat and meat products with some decontamination trials by some essential oils. J. Anim. Health Prod. 9(s1): 61-68.
DOI | http://dx.doi.org/10.17582/journal.jahp/2020/9.s1.61.68
ISSN | 2308-2801

INTRODUCTION

Meat and meat products are valuable sources of various nutrients; mainly protein, fat, B-vitamins, iron, zinc and vitamin A, in addition to the essential amino acids. Meat products are preferred by many peoples because they are easily prepared meals of low price (Shaltout et al., 2016).

Clostridium perfringens is a Gram-positive, non-motile, spore-forming, anaerobic bacillus that commensally inhabits the intestinal tract of animals and humans. It is a cytotoxin producing bacterium with an optimum growth temperature of 35°C–40°C (Dawson et al., 2009). *C. perfringens* type A strains are implicated in numerous human diseases such as food poisoning and gastrointestinal illness (Fisher et al., 2005).

The extent use of antimicrobial agents for growth promotion and disease control is the main cause of antimicrobial resistance spread particularly in the normal enteric flora, including *C. perfringens*. Resistance of *C. perfringens* isolates to various antibiotics has been reported in different countries (Slavic et al., 2011).

Biofilm is structured communities of the bacterial cell enclosed in a self-produced extracellular polysaccharide matrix that provides an increased resistance to environmental stresses. Biofilm formed by *C. perfringens* protects it from exposure to the atmospheric oxygen and to the high concentrations of antibiotics (Charlebois et al., 2014).

Essential oils (EOs) are aromatic volatile oily liquids obtained from plant materials, particularly. Thyme (*Thymus vulgaris L.*) is an aromatic plant belongs to family

Labiateae, used mainly for several culinary purposes. Marjoram (*Origanum majorana L.*) is another essential oil belongs to Lamiaceae family and has a broad inhibitory spectrum against wide range of Gram-negative and Gram-positive bacteria (Mohamed and Mansour, 2012).

Thus, the present study was planned for isolation of *C. perfringens* from meat and meat products, as well as detection of *C. perfringens* virulence genes, antimicrobial susceptibility test, and investigation of *C. perfringens* ability to form biofilms at different temperatures. In addition to investigate the antibacterial effect of some essential oils on *C. perfringens*.

MATERIALS AND METHODS

Collection of samples

A total of 40 fresh meat samples and 160 meat products (minced meat, burger, sausage and luncheon, 40 each) were randomly collected from different outlets at Zagazig city, Sharkia Governorate, Egypt.

Isolation of *C. perfringens*

Under complete aseptic conditions, 10 g of each sample were aseptically homogenized in a sterile blender containing 90 ml of 0.1 % sterile Buffered Peptone Water (BPW, OXOID, CM9). The enrichment of each sample was performed by transferring 1 ml of each homogenized sample into a tube containing 9 ml of sterile Cooked Meat Broth (CMB; TM MEDIA) (ISO6887, 2003). The tubes were anaerobically incubated at 37°C for 24 h. For isolation of *C. perfringen*, a loopful from the enriched cultures were streaked on to the surface of Reinforced Clostridial Agar (RCA; Oxoid, CM0151), and were anaerobically incubated at 37°C for 24-48 h in anaerobic jar containing gas generating kits (anaeroGen, OXOID ltd, England). The suspected colonies of *C. perfringens* (shiny, pin headed and translucent) were picked and subjected to Gram staining and biochemical tests (Tizhe et al., 2015).

Molecular characterization of C. perfringens virulence genes

The extraction of the bacterial DNA was carried using QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany, Cataloge no. 51304) according to the manufacturer's instructions. The amplification of *C. perfringens plc* gene encoding phospholipase C (*alpha*-toxin) and *C. perfringens* enterotoxin (*cpe*) was performed using primers from Midland Certified Reagent Company oilgos (USA). The *alpha*-toxin primers were sense 5′GTTGATAGCGCAGG ACATGTTAAG′3 and antisense 5′CATGTAGTCATCTGTTCCAGCATC′3 with a product size of 402 bp (Yoo et al., 1997). The primers used for detection of *cpe* gene were sense 5′ACATCTGCAGATAGCTTAGGAAAT′3 and antisense 5′CCAGTAGCTGTAATTGT TAAGTGT ′3 with a product size of 247 bp. (Kaneko et al., 2011).

Antibiotic susceptibility testing

It was performed by the disc diffusion method according to Bauer et al. (1966). The types of antibiotics and concentrations of each antibiotic disc are listed in a supplementary Supplementary Table 1. The results were used to calculate the Multiple Antibiotic Resistance (MAR) index for the total number of isolates as: MAR index=a/b, where a is the number of antibiotics to which the isolate is resistant, and b is the total number of tested antibiotics.

Detection of biofilm production by microtiter plate assay (MtP)

The biofilm production of *C. perfringens* isolates at different storage temperatures was determined by MtP assay as previously performed by Kırmusaoğlu (2019).

Trials to improve meat quality and decrease C. perfringens count

Nine Essential oils of watercress (*Eruca Sativa*), argan (*Argania spinosa*), marjoram (*Origanum majorana*), black seed (*Nigella sativa*), thyme (*Thymus vulgaris*), cumin (*Cuminum cyminum*), lemon grass (*Cymbopogon citratus*), rosemary (*Rosmarinus officinalis*) and lettuce (*Lactuca sativa*) were purchased from National Research Center, Dokki, Giza. These were used to determine the most effective oil on *C. perfringen*, by the disk diffusion method on Brain Heart Infusion agar (Nuno et al., 2016). The best effective oils in disk-diffusion method were thyme and marjoram essential oils, which were selected for further investigation in different concentrations and to calculate their minimum inhibitory concentration (MIC). The following concentrations: 10, 5, 2, 1, 0.5 and 0.25% v/v were prepared from these two oils. The MIC was taken from the lowest dosed disc concentration showing no growth after 24 h.

For the experimental trails was repeated in triplicates, a total of 1200 g of fresh meat was aseptically minced and divided into four groups (100 g, each). One ml of the *C. perfringens* broth that was adjusted to 0.5 McFarland was inoculated by pipetting over each 100 g of minced meat. The inoculated samples were left for 30 min at room temperature (25°C). Then it was divided in to four groups ;1st group, was inoculated with 1 ml sterile distilled water as a positive control ;2st group, was inoculated with thyme oil 5%; 3ndgroup, was inoculated with marjoram oil 2% and 4rdgroup, minced meat without inoculation of the microorganism as a negative control group. The control as well as the treated groups were examined for determination

of the antimicrobial effect of the above-mentioned oils against *C. perfringens* after treatment for 0.5, 1, 2, 4, 6 and after 24 h.

Statistical analysis

All values of bacteriological analysis are presented as means ± standard error (S.E). Data were analyzed by SPSS and One-Way Analysis of Variance (ANOVA) at 95% level of confidence. Significant differences among the means were determined by DUNCAN test considering $p<0.05$ as significant.

RESULTS AND DISCUSSION

Occurrence of *C. perfringens* in the examined meat and meat products

The bacteriological analysis of meat and meat product samples showed the presence of *C. perfringens* in 29 (14.5%) of the examined samples (Table 1). It was found that all examined *C. perfringens* isolates were positive for alpha toxin, while *C. perfringens* enterotoxin (*cpe*), not detected in all examined *C. perfringens* isolates (Figure 1).

Table 1: Occurrence of *C. perfringens* in the examined meat and meat product samples (N= 40, each).

Samples	Positive samples	
	Number	Percentages
Fresh meat	6	15%
Frozen minced meat	6	15%
Burger	8	20%
Sausage	4	10%
Luncheon	5	12.5%
Total	29	14.5%

N: Number of examined samples (40, each).

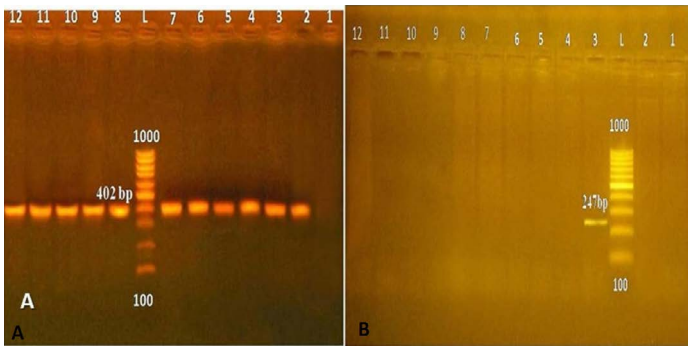

Figure 1: (A) *C. perfringens* Alpha toxin in 1.5% agarose gel (L: 100 bp ladder; 1: negative control; 2: positive control, from 3 to 12: *C. perfringens* positive for Alpha toxin). (B) *C. perfringens cpe* gene (L: 100 bp ladder; 2: negative control; 3: positive control; 4-12: negative *C. perfringens* for *cpe*).

Antimicrobial susceptibility of *C. perfringens*

Data presented in Table 2 shows 100% resistance of the tested *C. perfringens* isolates to oxytetracyclin and 90% resistance to erythomycin, neomycin and amoxicillin. Resistance profile of multidrug resistant of *C. perfringens* isolated from meat and meat product samples revealed that the MAR ranged from 0.1 to 1 with an average of 0.68 (Table 2).

Biofilm formation in *C. perfringens* isolates at 4°C, 25 °C and 35 °C

The results presented in Table 3 show the percentages and degrees of biofilm production by *C. perfringens* isolates. At the three tested temperatures (4 ˚C, 25 ˚C, and 37 ˚C), 50%, 40% and 100% of the isolates were biofilm producers, respectively. At refrigeration temperature (4 ˚C) and at room temperature (25˚C), isolates were classified as weak and moderate producers, while 50 % and 60% of the isolates were unable to produce biofilm. All *C. perfringens* isolates were biofilm producer at 37 ˚C.

Antibacterial effect of some essential oils *C. perfringens*

The obtained results in Table 4 revealed that from the tested nine oils, marjoram and thyme oils were the highest effective antibacterial oils against *C. perfringens*.

The data presented in Table 5 revealed the effect of different concentrations of thyme and marjoram essential oils on *C. perfringens* by the disk diffusion method. The mean inhibition zone diameter from the effect of thyme oil 5% and marjoram oil 2% against *C. perfringens* was 10±2.7 and 15.33±0.34 mm that were moderately inhibitory (10-20 mm) (Nuno et al., 2016). Thus, marjoram oil 2% and thyme oil 5% were chosen to investigate the chicken meat inoculated with *C. perfringens* for 0.5, 1, 2, 4, 6 and 24 h at 4 °C.

After 0.05 h, the mean count of *C. perfringens* in the control group was 5.59±0.36 $\log_{10}$ CFU/g, while the mean values of treated groups with thyme oil 5% and marjoram oil 2% was decreased to 4.87±0.13 and 4.65±0.09 $\log_{10}$ CFU/g. After treatment for 24 h, the mean *C. perfringens* count in control group increased to 5.73±0.30 $\log_{10}$ CFU/g, while the mean counts of treated groups with thyme oil 5% was reduced to 4.99±0.17 $\log_{10}$ CFU/g with a reduction percentage of 81.8% and 4.89±0.17 $\log_{10}$ CFU/gin marjoram oil 2%. Statistical analysis by ANOVA revealed significance differences between control and treated samples at $p<0.05$ (Table 6).

C. perfringens is a normal inhabitant of the animals' intestinal tract which can contaminate the carcass during unhygienic slaughtering process. The prevalence of *C. perfringens* in the current study is higher than 11.2% in India (Gurmu et al., 2013) and 5.6% in Turkey (Yibar et al., 2018).

Table 2: Antimicrobial susceptibility and Resistance profile of *C. perfringens* (N=10).

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Oxytetracyclin (T)	0	0	0	0	10	100%
Erythomycin (E)	1	10%	0	0	9	90%
Neomycin (N)	1	10%	0	0	9	90%
Amoxicillin (AX)	0	0	1	10%	9	90%
Ampicillin (AM)	0	0	2	20%	8	80%
Streptomycin (S)	1	10%	1	10%	8	80%
Novobiocin (NV)	1	10%	2	20%	7	70%
Gentamicin (CN)	4	40%	0	0	6	60%
Norfloxacin (NOR)	3	30%	2	20%	5	50%
Enrofloxacin (ENR)	5	50%	0	0	5	50%
Pattern	**Resistance profile**			**Number of isolates (%)**	**Number of antibiotics**	**MAR**
I	T, E, N, AX, AM, S, NV, CN, NOR, ENR			3(30%)	10	1
II	T, E, N, AX, AM, S, NV, NOR, ENR			2(20%)	9	0.9
III	T, E, N, AX, AM, S, NV, CN			2(20%)	8	0.8
IV	T, E, N, AX, AM, S, NV			1(10%)	7	0.7
V	T, E, N, AX, AM, S			1(10%)	6	0.6
VI	T			1(10%)	1	0.1
Average				0.68		

N: Number of *C. perfringens* isolates; No.: Number of sensitive, intermediate or resistant *C. perfringens* isolates
%: Percentage of sensitive, intermediate or resistant *C. perfringens* isolates; MAR: Multiple Antibiotic Resistance index (a/b), where (a) is the number of antibiotics to which the isolates are resistant. (b): is the total number of tested antibiotics (10).

Table 3: Biofilm formation in *C. perfringens* isolates at 4°C, 25 °C and 35 °C.

Temperature	Non-producer	Degree of biofilm production (No, %, Average OD±SD)			Overall biofilm producers
		Weak	Moderate	Strong	
4 °C	5(50%) 0.025249±0.04891	2(20%) 0.170283±0.06859	3(30%) 0.273894±0.0362	-	5(50%)
25°C	6(60%) 0.09496±0.069	1(10%) 0.26357±0.004	3(30%) 0.62969±0.009	-	4(40%)
35 °C	-	3(30%) 0.241909±0.05810	5(50%) 0.526731±0.173907	2(20%) 0.713631±0.03229	10(100%)

OD: Optical Density; SD: Standard Deviation.

Table 4: Antibacterial effect of the used essential oils.

Pure oil	Mean inhibition zone diameter (mm)±SE
Watercress (*Eruca Sativa*)	-
Argan (*Argania spinosa*)	-
Thyme (*Thymus vulgaris*)	22.67±7.5
Black seed (*Nigella sativa*)	-
Marjoram (*Origanum majorana*)	23.33±6.8
Cumin (*Cuminum cyminum*)	16.66 ±7.4
Lemon grass (*Cymbopogon citratus*)	13.33±4.7
Rosemary (*Rosmarinus officinalis*)	5±0.59
Lettuce (*Lactuca sativa*)	-

Table 5: Antibacterial effect of different concentrations of thyme and marjoram essential oils.

Concentrations	Mean inhibition zone diameter of thyme(mm)	Mean inhibition zone diameter of marjoram (mm)
10%	18±0.59	16±0.59
5%	10±2.7	15.67±0.34
2%	5±0.58	15.33±0.34
1%	5±0.58	5±0.58
0.5%	5±0.58	5±0.58
0.25%	5±0.58	5±0.58

Table 6: Effect of thyme oil 5% and marjoram oil 2% on *C. perfringens* count (log_{10} CFU/g) after 0.5, 1, 2, 4, 6 and 24h at 4°C.

Storage hours		Control	Thyme oil 5%	Marjoram oil 2%
0.5 h	Mean±S.E	5.59±0.36[a]	4.87±0.13[b]	4.65±0.09[b]
	Reduction count (%)	-	0.72(80.95%)	0.94(88.52%)
1 h	Mean±S.E	5.54±0.33[a]	4.79±0.12[ab]	4.48±0.15[b]
	Reduction count (%)	-	0.75(82.22%)	1.06(91.29%)
2h	Mean±S.E	5.66±0.28[a]	4.69±0.21[b]	4.45±0.17[b]
	Reduction count (%)	-	0.97(89.28%)	1.21(93.83%)
4 h	Mean±S.E	5.67±0.28[a]	4.69±0.09[b]	4.52±0.18[b]
	Reduction count (%)	-	0.98(89.53%)	1.15(92.92%)
6 h	Mean±S.E	5.68±0.37[a]	4.73±0.12[b]	4.58±0.16[b]
	Reduction count (%)	-	0.95(88.78%)	1.1(92.06%)
24 h	Mean±S.E	5.73±0.30[a]	4.99±0.17[ab]	4.89±0.17[b]
	Reduction count (%)	-	0.74(81.8%)	0.84(85.55%)

CFU/g: Colony forming unit per gram; S.E: Standard error of mean; Min: Minimum; Max: Maximum. Means within the same row with different superscript letters are significantly different (P< 0.05). Reduction count= log mean of control samples- log mean of treated samples. Reduction%: Mean of control samples-Mean of treated samples/Mean of control samples X100.

However, *C. perfringens* was not detected in a study conducted on sausage in Morocco (Malti and Amarouch, 2008). Higher prevalence of *C. perfringens* (30%) was reported in western Massachusetts (Lin and Labbe, 2003), while in Kalyobia Governorate, Egypt, Hassanien (2004) reported that *C. perfringens* was isolated from 40%, 28% and 20% of sausage, beef burger and luncheon samples. In Japan, *C. perfringens* was isolated from 71% of the examined retail raw meat samples (Miki et al., 2008). Meanwhile, in Menoufiea and Gharbia Governorates, Egypt, Atwa and Abou EI-Roos (2011) reported that *C. perfringens* was isolated from ready to cook and ready to eat meat products with the percentages of 48.8% and 21.3%, respectively.

The differences in the prevalence of *C. perfringens* could be attributed to the variation in the unsanitary conditions, poor personal hygiene and cross contamination between raw and cooked meat products (McClane et al., 2006).

Depending on the four major toxins (alpha, beta, epsilon, and iota toxins), *C. perfringens* is classified into different five genotypes from A to E (Fohler et al., 2016). It was found that all the examined *C. perfringens* isolates were positive for alpha toxin, while *C. Perfringens* enterotoxin (*cpe*), not detected in all isolates. Similar findings were reported by Engstrom et al. (2003) who found that all strains of *C. perfringens* were non enterotoxin producers. Moreover, Lin and Labbe (2003) reported that all *C. perfringens* isolates possessed alpha toxin gene, but none of the isolates were identified as carrying the *cpe* gene. Heikinheimo and Korkeala (2005) who found that *C. perfringens* strains possessed alpha encoding genes and were negative for enterotoxin encoding genes. In India, 15.15% of *C. perfringens* isolates were positive for *cpe* (Gurmu et al., 2013). Meanwhile, Yibar et al. (2018) reported that 27.2% of *C. perfringens* isolated from raw, ready to cook and ready to eat meat and meat-based products in Turkey carried the *cpe* gene.

The variations of the results may be attributed to the method of manufacture and contamination level during the processing, packaging and storage (Borch and Arinder, 2002).

The occurrence of multidrug resistant *C. perfringens* in meat and its products raises the public health concerns. In the current study, the tested *C. perfringens* revealed high antimicrobial susceptibility.

C. perfringens isolates showed a relatively lower resistance rate (66% and 56.2%) to tetracycline (Tansuphasiri et al., 2005). Shojadoust et al. (2010) reported the resistance of *C. perfringens* isolates to neomycin (87.5%) and tetracycline (80%). Meanwhile, the percentage of resistance to amoxicillin and ampicillin was less than 7% (Osman and Elhariri, 2013). Silva et al. (2014) reported lower resistance of 22.2% and 27.8% to erythromycin and oxytetracycline respectively. Lower resistance to tetracycline (25.0%) was reported by Chon et al. (2018).

Higher resistance of 100% to gentamycin, erythromycin and streptomycin than the current study as well as 93% to neomycin was reported by Osman and Elhariri (2013). Hamza et al. (2017) reported that *C. perfringens* isolates from processed meat in Cairo, Egypt showed strong resistance (100%) to streptomycin. Mwangi et al. (2019) found the prevalence of *C. perfringens* resistance was 98% and 73% to streptomycin and gentamicin.

The MAR index of 0.2 or more indicates contamination from high risk sources, thus, posing risks to human consumers (Tambekar et al., 2006). More than 50% of *C. perfringens* strains were resistant to more than five antibiotics (Shojadoust et al., 2010). While, in Iran 34.17% of *C. perfringens* strains showed MDR (Akhi et al., 2015). Mehdi and Wannas (2017) reported that all *C. perfringens* isolates showed MDR. High resistance rate of *C. perfringens* may be due to the wide spread of the antimicrobials.

The process of biofilms formation involves different stages including attachment, maturation and dispersion. The obtained results in this study are in accordance with the results obtained by Varga et al. (2008) where *C. perfringens* strains were shown to form biofilms with optical density values between 0.07 and 0.5. On the other hand, Donelli et al. (2012) were able to obtain a higher biofilm formation for *C. perfringens* strain with a mean optical density value of 3.2. Most of the *C. perfringens* were able to form biofilm (230/277) in Canada (Charlebois et al., 2014).

This study showed that the incubation temperature had a significant effect on the ability of *C. perfringens* to produce biofilm. Incubation at 35°C enhanced biofilm formation significantly more than incubation at 4°C and 25°C. Time-temperature abuse in markets could result in the propagation of the bacteria to dangerous levels (Sudha et al., 2012). Temperature has an adverse effect on biofilm formation by affecting flagellar motility, which enhances the movement of bacteria towards the biofilm surface (Bonsaglia et al., 2014). Thus, *C. perfringens* can produce biofilm at markets where temperature abuse occurs, resulting in probable risks to consumers.

Various studies have been conducted on the antimicrobial effects of plant essential oils and their constituents against foodborne pathogens. In a study conducted in Brazil, marjoram essential oil exhibited antibacterial activities against *C. perfringens* (Radaelli et al., 2016). Moreover, Nevas et al. (2004) reported the inhibitory effect of thyme oil against *C. perfringens* and Silva et al. (2013) used the disc diffusion methods to evaluate the antibacterial activity of thyme oil and reported a high antimicrobial activity (inhibition > 95%) against *C. perfringens*.

The best effective lowest concentration dosed of thyme oil was 5% and 2% for marjoram. The results obtained in the current study are comparable to Juneja et al. (2006) who reported that adding 0.1 to 2% thyme to the meat inhibited germination and outgrowth of *C. perfringens* spores at 12 h exponential chill rates. While, Juneja and Friedman (2007) reported that addition of thyme 2% on cooked ground beef and turkey, resulted in 3-5 log CFU/g reduction of spore germination and outgrowth. In addition, Du et al. (2015) reported strong antibacterial effects of thyme oil *in vitro* against a panel of pathogenic bacteria, including *C. perfringens*. In Brazil, a study conducted by Radaelli et al. (2016) revealed that marjoram and thyme with MIC of 5 mg/ ml and 1.25 mg/ ml exhibited antimicrobial activities against *C. perfringens*.

Antimicrobial activities of EOs are related to chemical characteristics such as their hydrophobicity which enables them to interact with the lipids of the bacterial cell membrane thus disturbing bacterial metabolism and cell wall and membrane permeability, leading to extensive leakage of critical molecules and ions from bacterial cells (Diaz Carrasco et al., 2016).

CONCLUSIONS AND RECOMMENDATIONS

Our study revealed a higher prevalence level of *C. perfringens* harbored Alpha toxin gene in raw meat and meat products. These results show that there is a need for applying appropriate food safety practices in meat and meat products production and processing facilities. The inhibition of biofilm formation needs for strict precautions to store food under different conditions. Our results suggest that treatment of meat with thyme oil 5% and marjoram oil 2% can greatly reduce *C. perfringens*, thus enhancing overall food safety. The reduction count was directly proportional.

AUTHOR'S CONTRIBUTION

All authors contributed equally.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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Supplementary Table 1: Supplementary: Concentration and diameter of inhibition zone of antibiotics used for sensitivity test (n=10)

Antibiotic groups	Antimicrobial agent	Conc. (µg)	inhibition zone (R)	(I)	(S)
Penicillins	Amoxicillin (AX)	25	≤22	23-30	≥31
	Ampicillin (AM)	10	≤22	23-30	≥31
Quinolones	Enrofloxacin (ENR)	10	≤ 15	16-20	≥ 21
	Norfloxacin (NOR)	10	≤15	16-20	≥21
Aminoglycosides	Gentamicin (CN)	10	≤12	13-14	≥ 15
	Neomycin (N)	30	≤12	14-16	≥ 17
	Streptomycin (S)	10	≤11	12-14	≥ 15
Aminocoumarin	Novobiocin (NV)	30	≤17	18-21	≥22
Tetracyclines	Oxytetracyclin (T)	30	≤14	15-18	≥ 19
Macrolides	Erythromycin (E)	15	≤13	14-22	≥23

n: Number of tested antibiotics; Conc.: Concentration; R: Resistant; I: Intermediate; S: Sensitive.