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Incidence, antibiotic resistance, and virulence profile of *Clostridium perfringens* in traditional salted sardine, feseikh and renga

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ABSTRACT

Salted fish products are one of the most desirable, traditional, and commercial seafood in Egypt. However, some sulfite-reducing anaerobes do not potentiate health hazards. *C. perfringens* is one of vigorously causing food poisoning globally. This study investigated the drug susceptibility profile and virulence factors of toxigenic *C. perfringens*, which was isolated from ninety samples of salted *Mugil cephalus* (feseikh), sardine, and smoked herring (renga) (thirty samples of each), that were randomly collected from different retailers at Qalyubia governorate. The results revealed the isolation of eighteen *C. perfringens* strains from the examined samples (20%); it was detected in 30, 20, and 10% of the examined feseikh, renga, and sardine samples, respectively. The eighteen detected isolates appeared to be resistant to 41.7% of the tested antibiotics; where, the major resistance was noted against clindamycin (72.2%), while the lowest resistance level was noted against metronidazole (11.1%). For the determination of the detected isolates' virulence profile, *plc*, *pro*A, and *cpe* genes were examined; where *plc* and *pro*A were positively detected in all of the examined five isolates; whereas, *cpe* was positively detected in 80% out of the examined isolates. In addition, *bla* and *erm*B resistance genes were examined; where they were detected in 80% and 100% of the examined isolates, respectively. Based on the results obtained, multi-drug resistance toxigenic *C. perfringens* was found at a substantial rate in one of Egypt's most popular seafood products, indicating possible harm and health hazards for users of such fish products.

1. INTRODUCTION

Clostridium perfringens (*C. perfringens*) is one of the most significant pathogens found in the gastrointestinal tracts of both humans and animals and widely dispersed in the environment (Beres et al., 2023). It is an anaerobic, Gram-positive rod that is encapsulated, non-motile, and spore-forming bacterium upon microscopic examination that appears as spherical, glossy, smooth, and encircled by two zones of hemolysis colonies on blood agar. Additionally, it has a biochemical catalase-negative and saccharolytic state (Dar et al., 2017).

Clostridium perfringens is ranked as one of the most often reported causes of food poisoning in the industrialized world each year and is thought to be the third most frequent cause of foodborne disease worldwide, especially type A, which is linked to human food poisoning. It has often been noted that the most well-known food sources for *C. perfringens* foodborne infections include various types of meat, particularly shellfish (El Bayomi et al., 2020; Saliman et al., 2024).

Initially, *C. perfringens* was categorized into five types from (A to E) according to secretion of significant lethal toxins; alpha (α) (*cpa*), beta (β) (*cpb*), epsilon (Ɛ) (*etx*), and iota (l) (*itx*) toxins. While, in a recently revised toxin typing scheme, *C. perfringens* are classified into seven toxico-types, A to G. *Clostridium perfringens* virulence is attributed to its ability to produce various potent protein exotoxins and extracellular enzymes. *Clostridium perfringens* type A produces α toxin only, type B produces α, β, and Ɛ toxins, while type C produces α and β toxins, type D produces α and Ɛ toxins, and type E yields α and l toxins. Enterotoxin (*cpe*), in addition to the alpha toxin, is produced by type F, while type G produces α and *net*B (necrotic enteritis responsible toxin) which has a critical role in the pathogenesis of necrotic enteritis disease in the infected host (Mamsin et al., 2024).

Seafood has been considered a necessary source of animal protein for a wide scale of people. Fish is more susceptible to be loaded with pathogenic bacteria, which may come from the contaminated water depending on fishing practices and further contamination sources during handling, processing, and packaging. Although fish flesh is normally sterile, microorganisms can penetrate from the skin and the gut to the flesh, and this penetration and contamination increases when fish are caught from polluted areas where there is a high density of bacteria (Okyere et al., 2018).

Salting fish is among the oldest ways of fish preservation. The major objective of this strategy is to increase the product's shelf life by allowing the muscle to absorb salt leading to a reduction in the fish's moisture content to a degree where bacterial and enzyme activity is inhibited, accompanied by the toxic action of chloride ions on the microbial growth. Moreover, the influence that sodium chloride has on different biochemical pathways—such as those that result in the enzymatic activity of certain enzymes that are responsible for the development of particular

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organoleptic characteristics—also contributes to the enhancement of flavor (Gassem, 2019; FAO, 2021).

Products made from salted fish are a common dish in many countries around the world. Unfortunately, improper hygienic measures, bad quality salt, insufficient time and amount of salting, improper storage, and handling conditions have been sorted as the main reasons for threatening consumer's health through consumption of salted fish products (Talab and Ghanem, 2021).

Despite that the Centers for Disease Control and Prevention (CDC) has not mentioned fish as a typical source of *C. perfringens* infections (CDC, 2023), Saliman et al. (2024) discovered toxigenic *C. perfringens* in salted fish samples that included food poisoning potential.

Recent studies have highlighted a concerning trend: the increasing antibiotic resistance among strains of *C. perfringens.* Tansuphasiri et al. (2005) found that 56.2% of *C. perfringens* strains were resistant to tetracycline, followed by 24.9% to imipenem, and lower rates to metronidazole (9.5%) and penicillin G (9%); Another study revealed that 34.17% of the *C. perfringens* isolates exhibited multiple drug resistance to antibiotics such as imipenem, metronidazole, ceftriaxone, clindamycin, chloramphenicol, and penicillin (Akhi et al., 2015).

Therefore, the current study focused on the incidence, antibiotic resistance, and virulence profile of *C. perfringens* in traditional salted sardine, feseikh, and renga samples collected from different retailers in Benha City, Qalyubia governorate during the autumn season of 2023.

2. MATERIAL AND METHODS

The research was performed after approval of the ethical committee of the Faculty of Veterinary Medicine, Benha University (BUFVTM 44-11-23).

2.1. Sampling

Ninety random samples of salted fish-feseikh, sardine, and smoked renga, thirty of each were gathered from various retail vendors in Benha, Qalyubia governorate. Before being examined, each sample was individually wrapped in a sterile polyethylene bag and stored in the refrigerator.

2.2. Isolation and identification of C. perfringens

According to ISO 15213-1 (2021), nearly 10g of each sample's pectoral muscle were mixed and incubated in thioglycolate broth (OXOID: CM0391) for 24h in 37ºC in anaerobic condition as an enrichment process of the anaerobic bacteria, followed by streaking a loopful, from the previously enrichment broth, on Tryptose Sulfite Cycloserine (TSC) agar (OXOID; CM0587), and incubated at 37oC for 24h. The suspected black colonies were isolated, purified, and re-enriched on thioglycolate broth, as previously mentioned, for further microscopic and biochemical identification (including investigation of its lecithinase activity, hemolysis on blood agar (OXOID: CM0271), H2S productivity in lactose sulfite broth (Conda Lab: C1009), indole and sugar fermentation.

2.3. Antimicrobial susceptibility test

The in Vitro susceptibility test was conducted on each *C. perfringens* isolate to study their sensitivity against twelve different antimicrobial agents, that were obtained from OXOID®, represented by amoxicillin-clavulanic acid (AMC – 30 µ/g), ampicillin/sulbactam (SAM – 10/10 µ/g), chloramphenicol (C – 30 µ/g), clindamycin (CD – 2 µ/g), colistin (CT – 10 µ/g), doxycycline (DO – 30 µ/g), erythromycin (E – 30 µ/g), gentamicin (GN – 10 µ/g), metronidazole (MTZ - 5 µ/g), tetracycline (TE – 30 µ/g), trimethoprim – sulfamethoxazole (SXT - 1.25/23.75 µ/g), and vancomycin (VA – 30 µ/g), using the disc diffusion method on Mueller-Hinton agar (OXOID: CM0337) and incubation for 24h in 37^{O}C according to CLSI (2020) and EUCAST (2024).

2.4. Molecular identification of toxigenic C. perfringens virulence and resistance genes

Five random isolates of *C. perfringens* (2 from feseikh (1st and 2nd sample), 2 from renga (3rd and 4th sample), and 1 from sardine (5th sample)) were screened for three virulence genes (*cpe*, *plc*, *pro*A), and two resistant genes (*bla* and *erm*B) represented β lactam and erythromycin resistance genes, respectively.

Using the QIAamp DNA Mini Kit (Catalogue No. 51304) and the primers listed in Table (1), genomic DNA was extracted from isolated strains and amplified using Emerald Amp GT PCR master mix (Takara) Code No. RR310A. The PCR program was carried out according to the amplification kit manufacturer's instructions, and the PCR products were separated by electrophoresis in 1.5% agarose gel with bands visualized by ethidium bromide staining (Sambrook et al., 1989).

Table (1) Oligonucleotide primer sequences used for amplification of *C. perfringens* genes.

Gene		Sequence (3' - 5')	Amplified product	Reference
plc (Alpha toxin)	F	GTTGATAGCGCAGGACATGTTAAG	402 bp	Yoo et al., 1997
	R	CATGTAGTCATCTGTTCCAGCATC		
*pro*A	F	AAGAGGAGGAAGTGAGGCTAT	97 bp	Charlebois et al., 2016
	R	TTGAACTGATCCCTCTGGAA		
cpe	F	ACATCTGCAGATAGCTTAGGAAAT	247 bp	Kaneko et al., 2011
	R	CCAGTAGCTGTAATTGTTAAGTGT		
*erm*B	F	GAA AAG GTA CTC AAC CAA ATA	638 bp	Soge et al., 2009
	R	AGT AAC GGT ACT TAA ATT GTT TAC		
bla	F	ATGAAAGAAGTTCAAAAATATTTAGAG	780 bp	Catalán et al., 2010
	R	TTAGTGCCAATTGTTCATGATGG		

2.5. Statistical analysis

Simple descriptive analytics was used to analyze the data. The incidence of the isolated strains was calculated in relation to the total number of the examined samples according to Feldman et al. (2003).

3. RESULTS

3.1. Isolation and identification

The incidence of *C. perfringens* in the salted fish samples under examination (Fig. 1) was found to be greater in the feseikh samples as compared to the renga and sardine samples, where 9/30, 6/30, and 3/30 samples of feseikh, renga, and sardine were positive for *C. perfringens* representing 30, 20, and 10%, respectively. On the other hand, *C. perfringens* was isolated from 20% of all samples that were analyzed.

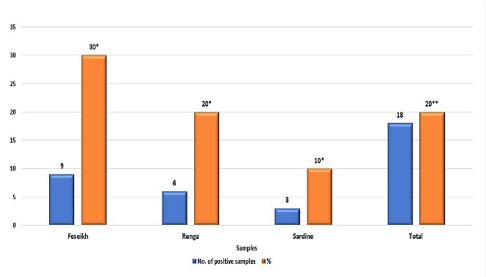

Fig. (1). Incidence of *Clostridium perfringens* in the examined salted fish samples (n=30). *: incidence (%) in relation to number of each fish product (30). **: incidence (%) in relation to the total number of the examined fish samples (90).

Grossly, *C. perfringens* isolates appeared black colonies in TSC agar, which may be surrounded by a hollow zone in egg yolk-supplemented media representing lecithinase positive strain (Fig. 2 A & B). On blood agar, it characterized by a

double zone of hemolysis (Fig. 3). It gave black precipitation (H2S positive) with gas formation in lactose sulfite broth (Fig. 4). Moreover, it showed positive fermentation of mannose, maltose, sucrose, lactose and glucose, and indole negative reaction. In addition, it appears Gram-positive, blunt-ended bacilli, encapsulated with or without central oval spore formation (Fig. 5).

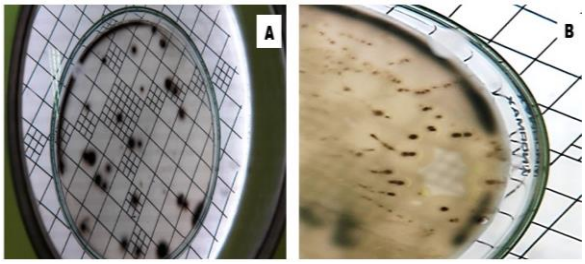

Fig. (2). Black colonies of *C. perfringens* on TSC agar without egg yolk (A), and with egg yolk "lecithinase active" (B)

Fig. (3). Characteristic double zone of hemolysis of *C. perfringens* on blood agar

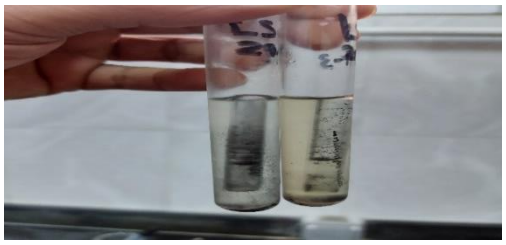

Fig. (4). Characteristic H2S and gas formation of *C. perfringens* on lactose sulfite broth

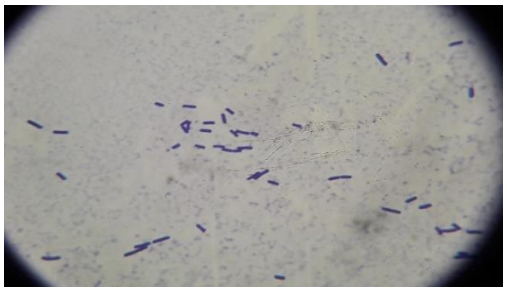

Fig. (5). Gram-positive *C. perfringens* bacilli

3.2. Antimicrobial susceptibility of *C. perfringens* isolates

Table (2) and Fig. (6) showed that the examined *C. perfringens* isolates were resistant to over 41.7% of the used antibiotics revealing them as multidrug-resistant strains. Variable degrees of resistance were recorded; where they showed high resistance levels against clindamycin (72.2%), followed by colistin (61.1%), and ampicillin/sulbactam, tetracycline, trimethoprim-sulfamethoxazole with ratio of 55.5% for each; while the highest sensitivity was recorded for metronidazole (88.9%)

Table (2) Antibiotic sensitivity test results (n=18).

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		AA
		No	%	No	%	No	%	
Amoxicillin-Clavulanic acid (AMC)	30	9	50.0	2	11.1	7	38.9	S
Ampicillin/sulbactam (SAM)	10/10	6	33.4	2	11.1	10	55.5	R
Chloramphenicol (C)	30	9	50.0	1	5.6	8	44.4	S
Clindamycin (CD)	2	3	16.7	2	11.1	13	72.2	R
Colistin (CT)	10	6	33.4	1	5.5	11	61.1	R
Doxycycline (DO)	30	12	66.6	2	11.1	4	22.2	S
Erythromycin (E)	30	10	55.6	0	0	8	44.4	S
Gentamicin (GN)	10	13	72.3	1	5.5	4	22.2	S
Metronidazole (MTZ)	5	16	88.9	0	0	2	11.1	S
Tetracycline (TE)	30	6	33.4	2	11.1	10	55.5	R
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	6	33.4	2	11.1	10	55.5	R
Vancomycin (VA)	30	13	72.3	1	5.5	4	22.2	S

%. Susceptibility was calculated according to the ratio between sensitive and resistant isolates in relation to the total number of isolates (18).

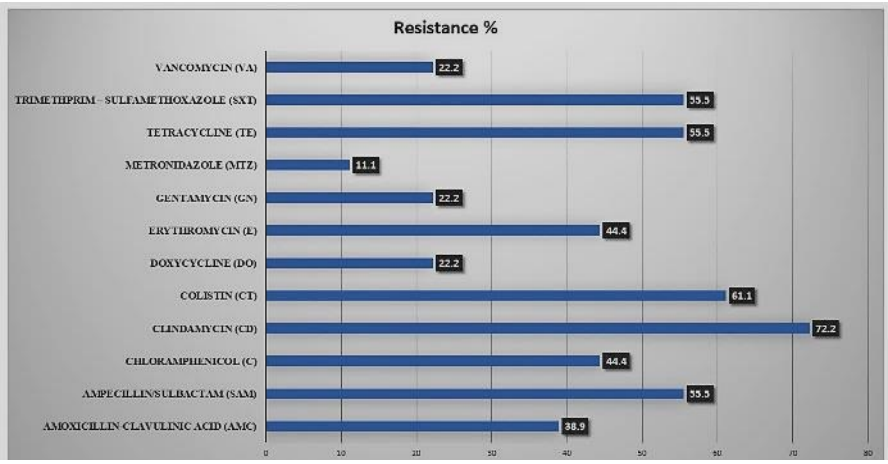

Fig. (6). Resistance (%) of *C. perfringens* isolates to the selected antibiotics (n=18).

3.3. Molecular characterization of virulent, drug-resistant genes in isolated *C. perfringens* isolates

The *plc* and *pro*A were detected in all of the examined isolates, while *cpe* was detected in 80% out of the isolates. In addition, *bla* and *erm*B resistance genes were examined; where they were detected in 80% and 100% of the examined isolates, respectively (Figures 7-11).

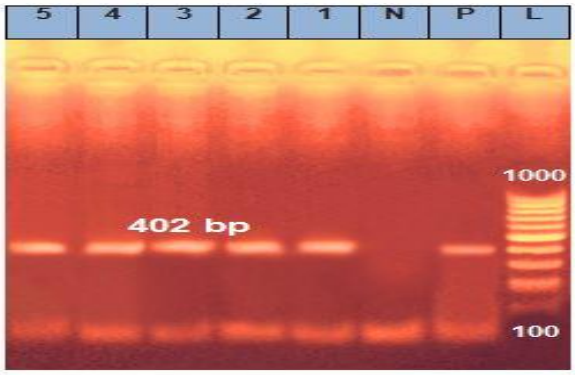

Fig. (7). Molecular characterization of the *plc* virulence gene in *Clostridium perfringens* isolates using agarose gel electrophoresis. 100 bps DNA StepLadder, positive control (*C. perfringens*, ATCC 04502303), and negative control (*E. coli* ATCC 20192507) were inoculated in Lane (L), Lane (P), and Lane (N), respectively. Lanes 1 through 5 provide positive results for the *plc* gene, which is amplified at 402 bps.

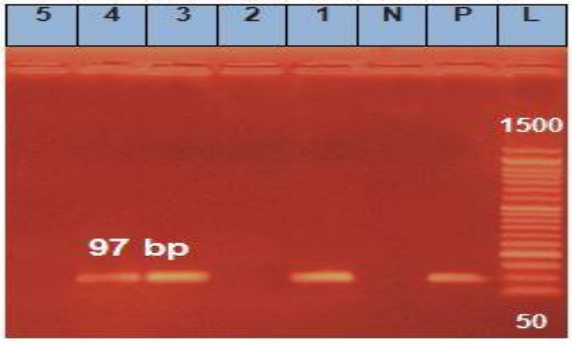

Fig. (8). Molecular characterization of the *proA* virulence gene in *Clostridium perfringens* isolates using agarose gel electrophoresis. 50 bps DNA StepLadder, positive control (*C. perfringens*, ATCC 04502303), and negative control (*E. coli* ATCC 20192507) were inoculated in Lane (L), Lane (P), and Lane (N), respectively. proA gene amplified at 97 bp in lanes 1, 3, and 4. While, negative isolates were found in lanes (2, 5).

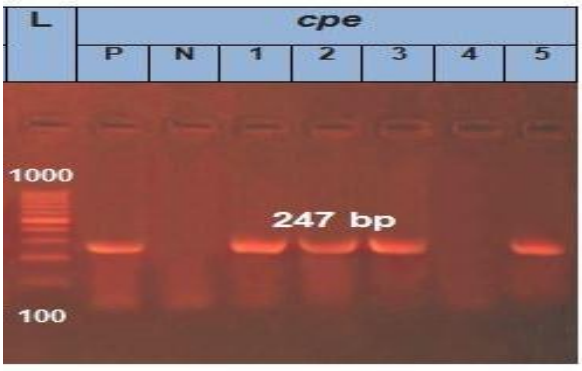

Fig. (9). Molecular characterization of the *cpe* virulence gene in *Clostridium perfringens* isolates using agarose gel electrophoresis. 100 bps DNA StepLadder, positive control (*C. perfringens*, ATCC 04502303), and negative control (*E. coli* ATCC 20192507) were inoculated in Lane (L), Lane (P), and Lane (N), respectively. *cpe* gene amplified at 247 bp in lanes 1, 2, 3, and 5. While negative isolate was found in lane 4.

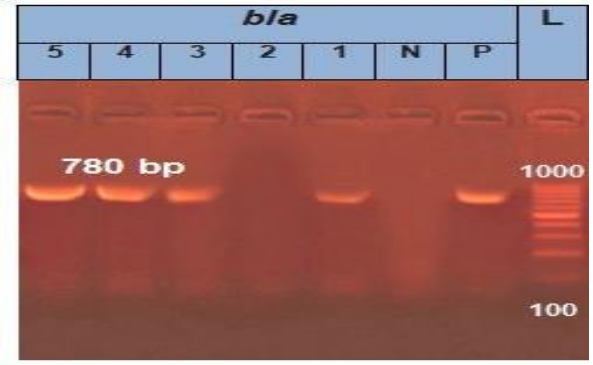

Fig. (10). Molecular characterization of the *bla* resistance gene in *Clostridium perfringens* isolates using agarose gel electrophoresis. 100 bps DNA StepLadder, positive control (*C. perfringens*, ATCC 04502303), and negative control (*E. coli* ATCC 20192507) were inoculated in Lane (L), Lane (P), and Lane (N), respectively. *bla* gene amplified at 780 bp in lanes 1, 3, 4 and 5. While, negative isolate was found in lane 2.

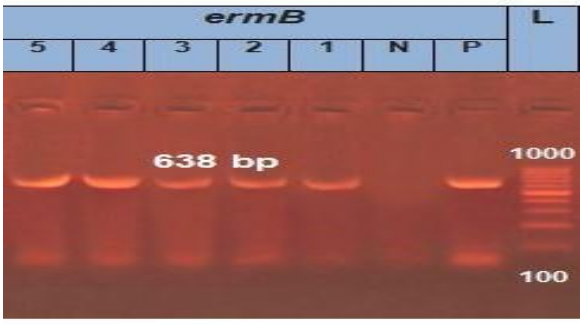

Fig. (11). Molecular characterization of the *ermB* resistance gene in *Clostridium perfringens* isolates using agarose gel electrophoresis. 100 bps DNA StepLadder, positive control (*C. perfringens*, ATCC 04502303), and negative control (*E. coli* ATCC 20192507) were inoculated in Lane (L), Lane (P), and Lane (N), respectively. Lanes 1 through 5 provide positive results for the *ermB* gene, which is amplified at 638 bps.

4- DISCUSSION

Clostridium perfringens is one of the most important foodborne bacteria because of its extensive distribution more than some other harmful bacteria. It is typically found in the soil and the digestive tracts of both humans and animals (Camargo et al., 2024). There is strong evidence that fish and other seafood are most likely contaminated by such anaerobic bacteria (Bradley et al., 2013). Since *C. perfringens* is widely present in processed and raw fish, several surveys conducted in the last few decades have documented this fact (Saad et al., 2015; Ghanem et al., 2019); therefore, the current study was conducted to investigate the prevalence of *C. perfringens* in some salted fishes than were commercially marketed in Qalyubia governorate markets.

Fish are very sensitive to bacteria, especially aerobic Gram-negative Bacillus species, which can cause a wide range of microbial infections. It is challenging to ascertain these bacteria's possible involvement as fish pathogens because there aren't many known anaerobic bacteria that might impair fish products (Pękala-Safińska, 2018).

Clostridium perfringens spores, sulfite-reducing anaerobes, are found in large quantities in environments such as soil, wastewater, and animal and human waste; where their spores can withstand long submersion in water because they are more resistant to the impacts of chemicals and physical factors than vegetative forms; so, its presence might be an indicator of polluted groundwater and drinking water, especially with fecal contamination (García-Prieto et al., 2022).

Regarding the present results, feseikh samples showed a higher incidence of *C. perfringens* contamination than the examined Renga and salted sardine samples, respectively; which came in agreement with the recorded results of Saad et al. (2015) (3.33% and 10.0% in sardine and feseikh samples, respectively) and Ghanem (2019) (20.0%, 46.0% and 38.0% of the examined renga, feseikh and sardine samples, respectively); whereas, El-Sheshnagui (2006) (85.0% and 55.0% for feseikh and sardine samples, respectively), and Amer (2018) (18, 28 and 62% for renga, feseikh, and sardine samples, respectively), found higher anaerobic bacterial contamination in the examined salted sardine samples than feseikh samples. While, Lela (2012) failed to detect *C. perfringens* from all the examined salted fish samples.

Higher initial bacterial loads of the mugil fish used in feseikh production, their culture environment, and their additional anaerobic fermentation process other than the salted sardine and renga may be the cause of higher feseikh level contamination (Ibrahim et al., 2021).

Variations between different authors may refer to the difference in the initial bacteriological quality of raw fish, the season of collection, and the hygienic procedures followed during processing and storage (Saliman et al., 2024).

There are several virulence factors produced by *C. perfringens*. Phospholipase C, or alpha-toxin, which is encoded as plc gene, is one of the main virulence agents that *C. perfringens* produces. The phospholipase C enzyme, breaks down sphingomyelin and phosphatidylcholine in cell membranes, causing necrosis, tissue damage, and cell lysis (Monturiol-Gross et al., 2014). The enterotoxin responsible for food poisoning is encoded by the cpe gene (Cornillot et al., 1995). According to Freedman et al. (2016), the *C. perfringens* enterotoxins (CPE) is known to cause diarrhea and abdominal disturbances by holes' formation in the host's intestinal cell membranes leading to intestinal epithelial barrier dysfunction. The phosphoglycerate dehydrogenase enzyme, which is involved in the production of the amino acid proline, is also encoded by the *proA* gene. Although not directly a virulence factor, *proA* plays a role in bacterial metabolism and can contribute to the bacterium's ability to thrive in various environments (Grant, 2018).

For the determination of the detected isolates' virulence profile, the presence of *plc*, *proA*, and *cpe* genes were investigated; where *plc* and *proA* were positively detected in all of the examined isolates (100%), while cpe was detected in 80% out of the examined isolates. The proficiency of the PCR technique as a useful diagnostic technique of virulence factors of *C. perfringens* has been previously recorded by Omer et al. (2020) who recorded the detection of virulence and resistance genes in the isolated *C. perfringens* isolates; which was confirmed by advanced genotyping.

The uncontrolled use of antibiotics as treatments or growth promoters in fish production has led to an expansion of antibiotic resistance in the normal intestinal flora and an increase in the occurrence of drug-resistant *C. perfringens*; which may be attributed to the microbial adaptation process to repeated un optimum antibiotic application that has led to establishment of MDR and high virulent *C. perfringens* strains (Ibrahim et al., 2020 and Ammar et al., 2021).

The present study revealed that *C. perfringens* isolates are multidrug-resistant strains, in addition to the presence of resistance genes against β lactams and macrolide antibiotic generations. These results came in line with those recorded by García-Vela et al. (2023) and Duc et al. (2024) who recorded the isolation of MDR *C. perfringens* from different meat products confirming the relationship between the rearing environment and MDR foodborne bacteria.

5. CONCLUSIONS

In the present study, feseikh samples showed higher incidence of *C. perfringens* than those of renga and sardine, respectively. The detected isolates revealed high prevalence of drug resistance possessing a potential hazard for human being. Positive detection of such virulence factors in the detected isolates proved that *C. perfringens* isolated from salted and smoked fish samples can be a threaten factor for the consumers.

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