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Occurrence of anaerobic spore formers including *Clostridium perfringens* and its virulence genes in some vacuum-packed chilled meat products

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ABSTRACT

Ninety samples of various vacuum-packed meat products, including sausage, frankfurter and salami, were selected randomly from several Menofyia governorate supermarkets in order to be examined for the presence of *Clostridium perfringens* and its virulence genes, as well as anaerobic spore formers. The results showed that the prevalence of anaerobic spore forming bacteria was 18 (60%), 25(83.33%) and 26 (86.67%) in salami, frankfurter and sausage, respectively. The mean values of anaerobic spore forming bacteria count in vacuum packed salami, frankfurter and sausage were $1.09\times10^3\pm 0.17\times10^3$, $3.22\times10^3\pm 0.58\times10^3$ and $5.61\times10^3\pm 1.20\times10^3$, respectively. Also, the prevalence of *C. perfringens* in same products was 46.67%, 63.33% and 70%, respectively**.** Furthermore, the mean values of *C. perfringens* counts for salami, frankfurter and sausage were $9.95\times10^2\pm 2.14\times10^2$, $2.18\times10^3\pm 0.37\times10^3$ and $3.87\times10^3\pm 0.65\times10^3$CFU/g, respectively. The acceptability of same products based on their contamination with *C. perfringens*, was 53.33%, 36.67% and 30 % was accepted, while 46.67%, 63.33% and 70% were unaccepted, respectively. Moreover, *cpa*(402 bp), *cpb* (236 bp), *cpx* (541bp) and *iap* (317 bp) *C. perfringens* virulence enterotoxin genes was detected from meat products by using multiplex PCR and their incidence were 50%, 50% and 25% for *cpa gene* while it was25%,25% and 25 % for *cpa* and *cpb* genes and 25%, 25% and25% for *cpa* and *cpx* genes and 0%,0% and 25% for *cpa*, *cpb* and *iap* genes isolated from the salami, frankfurter and sausage meat products respectively. Sausage samples were the most contaminated products. So, periodical examination and HACCP system application considered very important in meat products plants.

1. INTRODUCTION

Meat processing sector frequently encounters numerous significant issues pertaining to the safety and hygiene of diverse products (Ruiz-Capillas and Herrero, 2019).Pathogen transmission into the product may occur from the carcass surface or during handling procedures (Palmer et al., 2007). Anaerobic spore-forming foodborne pathogen as *Clostridium perfringens* is found in a vast range of foods, including meat and fish products. Because *C. perfringens* spores are heat resistant, they may withstand the heating process, germinate, and spread throughout food (Chang et al., 2020).This pathogen produces diarrheal illness (Limbo et al., 2010) or even mortality (Grass, et al. 2013) by affecting the permeability of the cell membrane in the intestine when the contaminated food is consumed. Foodborne infections have been linked to multiple outbreaks and have been linked to a number of serious illnesses and public health issues (Okeke et al., 2005). Foodborne epidemics caused by *C. perfringens* were recently discovered during the Panhellenic Handball Championship for kids (Mellou et al., 2019) consequently, techniques for managing *C. perfringens*, particularly its spores in diverse meat products, are required (Juneja et al., 2013). When *C. perfringens* spores germinate in food and survive cooking, they can cause an outbreak of food poisoning. These germinated spores eventually cause enterotoxins to be produced in the digestive tract following ingestion. Thus, the current study sheds insight on the number of anaerobic spore formers, particularly *C. perfringens* bacteria; found in sausage, frankfurters and salami as well as the multiplex PCR method used to identify their virulence genes.

2. MATERIAL AND METHODS

2.1. Collection of samples

Randomly selected ninety samples of vacuum-packed meat products, sausage, frankfurter, and salami 30 of each were gathered from various supermarkets in the Menofyia governorate, Egypt, in order to look for anaerobic spore formers, specifically *C. perfringens* and its genes virulence

2.2. Preparation of samples

The collected samples were prepared according to APHA (2001).

2.3. Enumeration of anaerobic spore formers (ISO 15213:2003)

2.4. Enumeration and isolation of C. perfringens

The volume of 0.1 ml was pipetted into the center of tryptose sulphate cycloserine agar plates (TSC).The plates were incubated upright and anaerobically at 37°C for 48 hrs. The plates containing characteristic black colonies (1-2 mm in

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diameter surrounded by district wide zone of opaque precipitate, 3-4 mm in diameter) were selected; the black colonies were counted (Collee et al., 1996).

*2.5. Identification of C. perfringens (*Koneman et al., 1992*)*

2.6. Detection of Virulence genes of Clostridium perfringens by PCR (Meer and Songer, 1997)

2.7. Statistical Analysis

ANOVA test was used to statistically examine the results (Feldman *et al.,* 2003)

3. RESULTS

The results showed that the prevalence of anaerobic spore forming bacteria was 18(60%), 25(83.33%) and 26 (86.67%) in salami, frankfurter and sausage, respectively (table 1). Also, the mean counts of anaerobic spore forming bacteria of vacuum packed salami, Frankfurter and Sausage were $1.09\times10^3\pm 0.17\times10^3$, $3.22\times10^3\pm 0.58\times10^3$and $5.61\times10^3\pm 1.20\times10^3$, respectively (table 2).

Table (3) reported that the prevalence of *C. perfringens* in the vacuum packed Salami, Frankfurter and Sausage were46.67%, 63.33% and 70%, respectively. Further, the mean values of *C. perfringens* count for Salami, frankfurter and sausage were $9.95\times10^2\pm 2.14\times10^2$, $2.18\times10^3\pm 0.37\times10^3$and $3.87\times10^3\pm 0.65\times10^3$CFU/g, respectively (table 4).

In table (5) the acceptability of vacuum packed Salami, Frankfurter and Sausage meat products based on their contamination with *C. perfringens* was 53.33%, 36.67% and 30% were accepted, while 46.67%, 63.33% and 70% were unaccepted, respectively. Moreover, in table (6) the incidence of virulence genes of *C. perfringens* were50%, 50% and 25% for *cpa*, 25%, 25% and 25 % for cpa and cpb and 25%, 25% and 25% for cpa and cpx and 0%, 0% and 25% *cpa*, *cpb* and *iap* isolated for Salami, Frankfurter and Sausage meat products respectively.

Figure (1) proved the presence of*cpa*(402 bp), *cpb* (236 bp), *cpx* (541bp) and *iap* (317 bp) virulence enterotoxin genes of *C. perfringens* that isolated from meat products by using Agarose gel electrophoresis of multiplex PCR

Table (1): Prevalence of anaerobic spore formers in the examined samples of vacuum packed meat products (n=30)

Meat products	No of positive samples	%
Salami	18	60
Frankfurter	25	83.33
Sausage	26	86.67
Total	69	76.67

Table (2): Statistics of anaerobic spore formers counts in the examined samples of vacuum packed meat products (n=30)

Meat products	Min	Min	Mean ± S.E*
Salami	1.0×10^2	4.7×10^3	$1.09\times10^3\pm 0.17\times10^{3a}$
Frankfurter	1.0×10^2	9.2×10^3	$3.22\times10^3\pm 0.58\times10^{3b}$
Sausage	1.0×10^2	1.5×10^4	$5.61\times10^3\pm 1.20\times10^{3c}$

S.E* = standard error of mean, the different superscripted small letters represent a the significance difference at level $p \leq 0.05$

Table (3): Acceptability of vacuum packed meat products based on their contamination with anaerobic spore formers (n=30)

Meat products	Anaerobic count /g*	Accepted		Unaccepted	
		No	%	No	%
Salami	≤ 100	21	70	9	30
Frankfurter	≤ 100	13	43.3	17	56.7
Sausage	≤ 100	12	40	18	60
Total (90)		46	51.1	44	48.9

*Egyptian Organization for Standardization "EOS" (2005)

Table (4): Prevalence of *Clostridium perfringens* in the examined samples of vacuum packed meat products (n=30)

Meat products	No of positive samples	%
Salami	14	46.7
Frankfurter	19	63.3
Sausage	21	70
Total	54	60

Table (5): Statistics of *Clostridium perfringens* counts in the examined samples of vacuum packed meat products (n=30)

Meat products	Min	Min	Mean ± S.E*
Salami	1.0×10^2	3.1×10^3	$9.95\times10^2\pm 2.14\times10^{2a}$
Frankfurter	1.0×10^2	6.0×10^3	$2.18\times10^3\pm 0.37\times10^{3b}$
Sausage	1.0×10^2	8.9×10^4	$3.87\times10^3\pm 0.65\times10^{3c}$

S.E* = standard error of mean, the different superscripted small letters represent a the significance difference at level $p \leq 0.05$

Table (6): Acceptability of vacuum packed meat products based on their contamination with *Clostridium perfringens* (n=30)

Meat products	*Clostridium perfringens* count /g*	Accepted		Unaccepted	
		No	%	No	%
Salami	Free	16	53.3	14	46.7
Frankfurter	Free	11	36.7	19	63.3
Sausage	Free	9	30	21	70
Total (90)		36	40	54	60

*Egyptian Organization for Standardization "EOS" (2005)

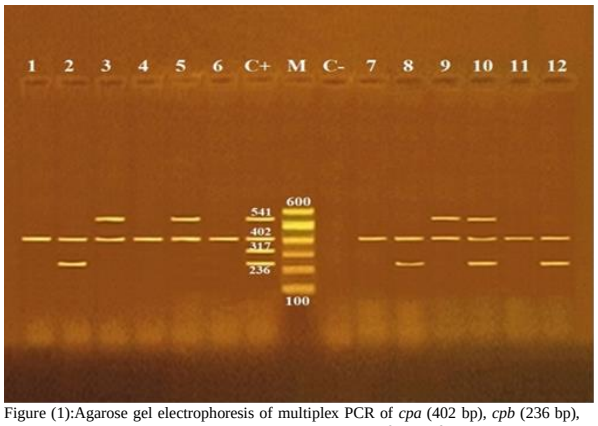

Figure (1):Agarose gel electrophoresis of multiplex PCR of *cpa* (402 bp), *cpb* (236 bp), *cpx* (541bp) and *iap* (317 bp) virulence enterotoxin genes of *C. perfringens*. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive for*cpa*,*cpb*, *cpx* and *iap*genes. Lane C-: Control negative. Lanes 1, 4, 6, 7 and 11: Positive *C. perfringens* strains for *cpa* gene. Lanes 2, 8 and12: Positive *C. perfringens* strains for *cpa* and *cpb* genes. Lanes 3, 5 and 9: Positive *C. perfringens* strains for *cpa* and *cpx* genes. Lane 10: Positive *C. perfringens* strain for *cpa*, *cpb* and *cpx* genes

4. DISCUSSION

Many meals can be spoiled by anaerobic spore-forming, *Clostridium* species, and meat typically produces gas and/or foul odors (Kyrylenko et al., 2023). The development of *C. perfringens* outbreaks is known to be mostly influenced by improper chilling and holding temperatures, unsatisfactory food processing, and insufficient reheating and temperature management of meat (Leung et al., 2017). As can be observed in table (1), revealed more total anaerobic spore forming bacteria recovered from vacuum-packed salami were found by El-Rays (2014), who found that 28% of such microorganisms were associated from vacuum-packed salami, also anaerobic spore forming were more common in the vacuum-packed sausage samples than were examined in the samples reported by (Abd El- Rahman and WH 1996), who found that anaerobic bacteria were present in 80% of the examined samples. On the other hand, Ibrahim et al. (2015) found that the prevalence of anaerobic spore-forming bacteria isolated from meat products was 84%, 56%, and 0% for sausage, salami, and frankfurter, respectively. Shaltout (1999), Mohammed (2002), El-Mossalami (2003), and Torky-Amal (2004) found that the mean values of the total anaerobic count of the sausage samples under examination were 2.62, 3.1, 5.59 and 5.22 log cfu/g, respectively. These results were differ from otheras by Zakaria (2009) failed to detect anaerobic spore forming microorganisms in the examined vacuum-packed sausage samples andAhmed et al. (2016)who isolated anaerobes from sausages with mean value $1.5\times10^7\pm3.5\times10^6$ and according to the permissible limit stipulated by E.O.S (2005) for the total anaerobic count in salami (not more than 2 log CUF /g), 44%of the examined samples were accepted and 56% of the examined samples were unaccepted. Regarding to results of the bacteriological analysis of *Cl. perferinges* in the examined samples under

study table (4), The outcomes of Abd El Salam (2015) isolation of *Clostridium perfringens* from animal products were almost same, while these results were higher than that of El-Rays (2014) who detected anaerobic spore forming in salami and frankfurter with prevalence of 28% and 48%, respectively and Zakaria (2009) who failed to isolated anaerobic bacteria from sausage while the prevalence of anaerobic bacteria isolated from frankfurter was 13.3%. Similar results reported by Ibrahim et al. (2015) who reported that the prevalence of *Clostridium perfringens* in salami and sausage were 44% and 80%, respectively, while they failed to detect them in frankfurter. *Clostridum* spp. are frequently found in food and the environment so, they are a major cause of food poisoning and spoilage (Gribble et al. 2014). Since meat is not a sterile product, surface contamination with bacteria during processing is what causes post processing spoilage (Boerema et al., 2007).In addition, 16% and 28% of the examined samples of salami and frankfurter were contaminated with *C.perfringens*, respectively (El-Rays, 2014)and the count of *C.perfringens* were $9.65\times10^2\pm 2.08\times10^2$ and $2.81\times10^3\pm 0.44\times10^3$ for salami and frankfurter, respectively. Hamoda(2011) recorded that *C.perfringens* counts (cfu/g) from sausage were $2.5x10^3$.Shalabyet al. (2011) examined 30 samples of sausage, the incidence of C.*perfringens* in the examined samples was 40% also ,Ahmed et al. (2016)isolated *C.perfringens* from 33.3% of examined sausage. More poisons are produced by clostridial spp than by any other kinds of bacteria, *Clostridium perfringens* is the most common and greatest producer of toxins among the *Clostridium* species; it can be found in soil, animals' microbiota and human bodies. Due to their involvement in the etiology of *C. perfringens*-associated illness (CPAD) in both humans and animals, enterotoxin and beta toxin, two of the virulence components of *C. perfringens*, have drawn more attention than others (Monma et al.,2015).*C. perfringens* can be serotyped into five groups (A to E) based on the production of specific exotoxins [alpha (α), beta (β), epsilon (ε), and ι (iota)](Moustafa et al., 2022). The α-toxin, encoded by a plasmid mediated cpa gene, is associated with serotype A, as well as all other serotypes of *C. perfringens* (Cooper and Songer 2010). Meanwhile, the serotype B harbors *cpb* and *etx* plasmid-mediated genes that encode β- and ε-toxins, respectively. The β- and ε-toxins are associated with serotypes C and D, respectively (Chenetal., 2011), but serotype E has the plasmid-mediated *iap* gene, which produces ι-toxin(Park et al. 2015).Christoph et al. (2004) recorded the toxin genes *cpa, cpb,* and *iap* using multiplex PCR; similar results were obtained by Bendary et al. (2022) and Ibrahim et al. (2015) as the authors found *cpa* from the analyzed materials. However, Deng et al. (2006) were unable to find *C.* P*erfringens cpb* gene. *Clostridium perfringens* is now the second most common bacterium linked to foodborne illness in the United States, accounting for one million cases annually, according to the Centers for Disease Control and Prevention (Grass et al. 2013).

5. CONCLUSIONS

From the obtained results it could be concluded that sausage samples were the most contaminated products. So, periodical examination and HACCP system application considered very important in meat product plants.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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