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Bacteriological evaluation of imported canned fish with special reference to Clostridium perfringens .

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ABSTRACT

Anaerobic and facultative anaerobic microorganisms are important group of microorganisms responsible for many health hazards to consumer of canned food. This study was conducted to bacteriologically evaluating 105 sample of imported canned fish from different markets at kaluobia governorate . Bacteriological examination indicated that the mean values of the examined samples of canned mackerel, sardine and tuna were 1.7 x104±1.1 x104, 1.4 x103±5 x102&1.7 x103±9.2 x102/ g for total anaerobic counts and 1.1 x103±5.7 x102, 7.5 x102±2.7 x102&1.9 x103±1.5 x103/g for total *C. perfringens* counts, respectively. Moreover, C. perfringens was detected in 25.7%, 14.3% and 11.4% of the examined canned mackerel, sardine and tuna samples, respectively. However, *C. bifermentans, C. subterminal, C. sporogenes, C. sordelli* were isolated from such examined samples of canned fishes at different percentages .The current study classified the presence of unacceptable canned imported fish for human consumption so recommended future studies must using advanced methods to control this problems in The future and save people health.

1. INTRODUCTION

The Clostridia species produce the highest number of toxins of any type of food poisoning bacteria. Among Clostridium sp., *C. perfringens* is the largest toxin producer and also the most widespread, being found as part of the microbiota of animals and humans and in the soil. In most cases, *C. perfringens* food poisoning results by eating improperly cooked and stored foods. C. perfringens food poisoning is quite common, and it is an important cause of outbreaks worldwide Hailegebreal (2017). Anaerobic and facultative anaerobic micro-organisms are important group of microorganisms responsible for many health hazards to consumer of canned food. Clostridium botulinum and clostridium poisoning, isolation of clostridium botulinum from food is general considered to be less significant than the detection of its toxins (Hobbs, 1987).

Canned tuna as product from tuna or bonito fish flesh while canned sardines are the product produced from sardine fish. and canned mackerel as the product produced from mackerel. This fish flesh is preserved in sealed cans with edible oils, brine, or both of them and is exposed to commercial sterilization. The same standards stated that canned tuna, sardine and mackerel should not have clostridium or anaerobic spore forming bacteria EOS (2005) and GSO (2012, 2013 and 2016). The total viable count (TVC) ranged from 0.1 to $2.8x10^8$ CFU/g for canned sardine in vegetable oil under cold storage condition. while under ambient temperature, TVC ranged from 0.1 to $4.4x10^8$ CFU/g. All counts were above the ICMSF (maximum limit of 1.0x106 CFU/g, for acceptability) (Agwa, et al., 2018). Clostridium perfringens type A is a common cause of food poisoning because of its ability to sporulation, rapid multiplication and production of an enterotoxin. Early investigations about its incidence in foods revealed it's widespread in food animals and foods; while recent data showed that the presence of the enterotoxin gene (cpe) is rare in non-outbreak isolates. Necrotic enteritis, a more serious hazard, is caused by *C. perfringens* type C strains, but this illness is rare in industrialized countries. Improper cooling remains the single most important contributing factor in cases of foodborne illness by this organism (Labbe and Juneja, 2016). Therefore, this study was conducted to record the bacteriological evaluation of imported canned fish to detect anaerobic bacterial count and isolation.

2. MATERIAL AND METHODS

Total of 105 sample of imported canned fish collected from different markets at kaluobia governorate and handled under complete aseptic conditions by surface sterilization with alcohol.

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2.1.Bacteriological examination:

2.1.1. Preparation of samples (ICMSF, 1996):

The different cans (tuna -sardine- mackerel) 35 samples of each type were handled under complete aseptic conditions by surface sterilization with alcohol and flame. Further, the cans were opened by using sterile can opener to induce small opening To 10 g of each sample, 90 ml of sterile peptone water were added and thoroughly mixed by using sterile blender for 1.5 minutes to give a final dilution of 1/10. Accordingly, tenfold serial dilutions were prepared by transferring 1 ml of the original homogenate into sterile test tube containing 9m·l of sterile peptone water (0.1%) from which further dilutions were obtained until the dilution of 10·5 .

2.1.2. Determination of total anaerobic bacterial count (Roberts et al., 1995):

One ml from each previously prepared serial dilutions were spread into clostridia agar plates (Roberts et al., 1995). The plates were then incubated in upright position in anaerobic gar (Mackintosh jar) at 37°C/24 h. The suspected plates were selected and counted, and the results were interpreted as colony forming units (cfu) per gram of the samples.

2.1.3. Enumeration of viable C. perfringens (ISO, 2004):

One ml from each previously prepared serial dilution was spread into Tryptose Sulphate Cycloserine agar plates (ISO, 2004). The plates were then incubated in upright position in anaerobic gar (Mackintosh jar) at 37°C/48 h. Plates showed black colonies were selected and counted, and the results were interpreted as colony forming units (cfu) per gram of the samples.

2.1.4. Enumeration of C. perfringens spores (Weiss and Strong, 1967):

Alternatively, a portion of the previously prepared serial dilution was heated at 80°C/15 min. to destroy vegetative cells and activate *C. perfringens* spores then one ml was spread into *C. perfringens* agar plates (Weiss and Strong, 1967). The plates were then incubated in upright position in anaerobic gar (Mackintosh jar) at 37 OC for 48h. The suspected plates showing black colonies were selected and counted, and the results were interpreted as colony forming units (cfu) per gram of the samples.

2.1.5. Isolation of C. perfringens (Carter and Cole, 1990):

Two grams of each sample were aseptically inoculated into sterile previously boiled and cooked meat media. The inoculated tubes were incubated anaerobically at 37°C for 24 hours. Positive tubes showing turbidity and gas production were subcultured on neomycin sheep blood agar plates then incubated anaerobically at 37 °C for 24 hours. The suspected colonies were selected and purified in a pure culture for further identification.

2.1.6. Identification of C. perfringens (MacFaddine, 1980 and Cato et al. 1986):

2.1.6.1. Staining (Cruickshank et al., 1975):

Films from suspected cultures were, stained with Gram's stain and examined microscopically to investigate their shape, arrangement, size, Gram's stain reaction, presence or absence of spores and the shape of the spores. Clostrdium perfringens appears as strongly Gram positive straight rods with parallel sides and rounded ends.

2.1.6.2. Motility tests (Quinn et al., 2002):

The growth culture was inoculated by stabbing the center of the semi-solid agar tubes and incubated at 25ºC for 48 hours. Positive result: Motile organisms migrate from the stab line and diffuse into medium. Negative results: Nomigration from the stab line observed.

2.1.6.3. Cultural characteristics (Cruickshank et al., 1975):

Suspected isolated cultures of anaerobic microorganisms were inoculated and subcultured onto the following different kinds of media:

2.1.6.3.1. Cooked meat media:

The inoculated cooked meat broth were examined for pink coloration of meat particles due to growth of saccharolytic species, while dark red, gray and black color (sludge) in the bottom of the tubes accompanied by putrid odor as a result of digestion of meat particles due to growth of proteolytic species.

2.1.6.3.2. Sheep blood agar media:

Suspected colonies were anaerobically subcultured onto 10% sheep blood agar for 24-48 hours at 37°C to study the morphological character of the colonies and the type of hemolysis.

2.1.6.3.3. Egg yolk agar media (Nagler's reaction):

One half of egg yolk agar plates were inoculated with C. perfringens type (A) alpha antitoxin and allowed to dry in the incubator for 30 min.. The suspected isolated organisms were streaked across the plate starting from the half without antitoxin to the other side. The plates were incubated anaerobically at 37°C for 24 hours. The plates were then examined for the appearance of opalescence and formation of pearly layers on the half of the plate without antitoxin.

2.1.6.3.4. Nutrient gelatin media:

Nutrient gelatin tubes were inoculated with the isolated strains, covered with paraffin wax seals to attain anaerobic conditions and incubated anaerobically at 37 °C for up to 14 days. The tubes were examined every two days for gelatin liquefaction after being cooled at 4 °C for 30 min.

2.1.7. Biochemical reactions:

2.1.7.1. Nitrate reduction test (Willis, 1977):

The purified culture were inoculated into 5 ml of trypticase nitrate broth tubes, then covered with sterile paraffin wax and incubated at 37°C for 3-4 days, to each tube, 2 ml of reagent A (Reagent Greiss 1: Alpha-naphthol amine + N/5 acetic acid) followed by 2 ml of reagent B (Reagent Greiss 2: Sulphanilic acid + N/10 acetic acid) were added. The tubes were shacked and allowed to stand for 10 min.. Developing of a pink color in the culture was considered as a positive result.

2.1.7.2. Indol production test (MacFaddine, 1980):

Sterile peptone water 1% tubes were inoculated with suspected culture and anaerobically incubated at 37°C for 48hours, and then 0.5 ml of Kovac's reagent was

trickled down on the side of the tubes. Developing of red colored ring indicated positive result.

2.1.7.3. Hydrogen sulphide test (MacFaddine, 1980):

Tubes of triple sugar iron agar were inoculated by suspected culture through stabbing into the butt and the slant then covered with sterile melted paraffin wax and incubated anaerobically for 48h at 37°C, appearance of blackening in the butt indicates H2S production.

2.1.7.4. Sugar fermentation test (Willis, 1977):

Suspected culture was inoculated to 1% peptone water tubes containing 2% bromocresol purple and 1% quantities of the following sugar: mannose, glucose, lactose, maltose, mannitol, sucrose and xylose were added. The tubes covered with sterile layer of paraffin wax and incubated anaerobically at 37°C for 7 days.

3. RESULTS

Table (1) that the total anaerobic counts ranged from $1.2x10^2$ to$1.6 x10^5$ with an average of $1.7 x10^4 \pm 1.1 x10^2$/g for canned mackerel, $2.2 x10^2$ to $3.5 x10^3$ with an average of $1.4 x10^3 \pm 5 x10$ g for canned sardine and $1.6x10^2$ to $4.5 x10^3$ with an average of $1.7 x10^3 \pm 9.2 x10$/g for canned tuna. Moreover 40%, 22.9% and 14.3% of the examined samples of canned mackerel, sardine and tuna were contaminated with anaerobic organisms, respectively.

Table (2) showed that the mean values of C. *perfringens* counts were $1.1 x10^3 \pm 5.7 x10$,$7.5 x10^2 \pm 2.7 x10$ and $1.9 x10^3 \pm 1.5 x10$ /g for the examined samples of mackerels sardine and tuna, respectively.

Differences associated with the examined samples of various canned fishes were highly significant ($P \geq 0.05$) as result of total C. *perfringens* count (Table,4).

Results given in Table (3) indicated that C. *sporogenes* organisms were isolated at the highest level in examined samples of canned mackerel (42.6%) followed by C. *subterminal* (34.2%),C. *bifermentans* (14.3%), and *C.sordelli* (13.4%), Concerning canned sardine, *C. sporogenes*(28.6%), *subterminal* (20%), and *C. bifermentans (2.9%)*where isolated and identified. While, C.*sporogenes*(28.6%), *subterminal* (17.1%) and *C. sordelli*(2.9%) were isolated from the examined samples of canned tuna.

Table (1) statistically analytical results of total anaerobic plate count of examined canned fish for each samples (n =35)

Canned fish samples	Positive samples		Count C.F.U./g		
	No.	%*	Min.	Max.	Mean ± SE
Tuna	5	14.3	$1.6x10^2$	$4.5 x10^3$	$1.7 x10^3 \pm 9.2 x10$
Mackerel	14	40	$1.2 x10^2$	$1.6 x10^5$	$1.7 x10^4 \pm 1.1 x10^2$
Sardine	8	22.9	$2.2 x10^2$	$3.5 x10^3$	$1.4 x10^3 \pm 5 x10$

*% calculated according to the number of each sample (n=35)

Table (2) Results of total Clostridium perfringens count in different examined canned fish products (n =35)

Canned fish samples	Positive samples		Count C.F.U./g		
	No.	%	Min.	Max.	Mean ± SE
Tuna	4	11.4	1.3 x10	$6.5 x10^3$	$1.9 x10^3 \pm 1.5 x10$
Mackerel	9	25.7	1.1 x10	$5.3 x10^3$	$1.1 x10^3 \pm 5.7 x10$
Sardine	5	14.3	7.5 x10	$1.7 x10^3$	$7.5 x10^2 \pm 2.7 x10$

*% calculated according to the number of each sample

Table (3) Incidence of Clostridial species other than clostridium perfringens in the examined canned fish for each samples (n =35)

Canned fish samples	C.bifermentans		C. sporogens		C. subterminal		C. Sordellii	
	No.	%	No.	%	No.	%	No.	%
Tuna	-	-	10	28.6	6	17.1	1	2.9
Mackerel	5	14.3	15	42.6	12	34.2	11	13.4
Sardine	1	2.9	10	28.6	7	20	-	-

*% calculated according to the number of each sample

Table (4) Incidence *of Clostridium perfringens* in the examined canned fish for each samples (n=35)

Canned fish samples	*C. perfringens*	
	No.	%
Tuna	3	8.6
Mackerel	7	20
Sardine	4	11.4

*% calculated according to the number of each sample

Table (5) Incidence of lecithinase activity (toxigenicity) of *Clostridium perifringens* isolated from examined canned fish samples (n =35)

Canned fish samples	Isolates		*Clostridium perfringens*			
			Lecithinase +ve		Lecithinase –ve	
	No.	%	No.	%	No.	%
Tuna	3	8.6	1	2.9	2	5.7
Mackerel	7	20	4	11.4	3	8.6
Sardine	4	8.6	2	5.7	2	5.7

*% calculated according to the number of each sample (n=35)

Table (6) typing of lecithinase +ve (toxigenic) *Clostridium perifringens* isolated from examined canned fish for each samples (n =35) by intradermal inoculation of guinea pigs

Canned fish samples	Typing							
	A		B		C		D	
	No.	%	No.	%	No.	%	No.	%
Tuna	1	100	-	-	-	-	-	-
Mackerel	4	100	-	-	-	-	-	-
Sardine	2	100	-	-	-	-	-	-

*% calculated according to the number of lecithinase +ve sample.

4. DISCUSSION

It is evident from the results recorded in Table (1) that the total anaerobic counts ranged from $1.2x10^2$to$1.6\ x10^5$ with an average of $1.7\ x10^4 \pm 1.1\ x10^2$/g for canned mackerel, $2.2\ x10^2$ to $3.5\ x10^3$ with an average of $1.4\ x10^3 \pm 5$ x10 g for canned sardine and $1.6x10^2$ to $4.5\ x10^3$ with an average of $1.7\ x10^3 \pm 9.2$ x10/g for canned tuna. more over 40%, 22.9% and 14.3% of the examined samples of canned mackerel, sardine and tuna were contaminated with anaerobic organisms, respectively. Fish is subjected to many risks of contamination from different sources during fishing, marketing, manufacturing and processing till reaching to consumer. The chief sources of fish contamination are water, soil, sewage, worker hands and equipment which may render the product unfit for human consumption resulting in economic losses or public health hazard to consumer (National Academy of Science,1985).

The lack of stiff standardization and monitoring system towards smoked dried fish had invariably permitted poor handling practices. This resulted to gross exposure of fish and fish products to a wide range of microbiological and chemical contaminations (Jop ,2016) .

The total viable count (TVC) ranged from 0.1 to $2.8\ x10^8$ CFU/g for canned sardine in vegetable oil under cold storage condition. While under ambient temperature , TVC ranged from 0.1 to $4.4\ x10^8$ CFU/g. All counts were above the ICMSF (maximum limit of $1.0x10^6$ CFU/g, for acceptability) (Agwa *et al* , 2018)

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Differences associated with the examined samples of various canned fishes were highly significant ($P \geq 0.05$) as result of total C. *perfringens* count(Table,4)

Results given in Table (3) indicated that C.*sporogenes* organisms were isolated at the highest level in examined samples of canned mackerel (42.6%) followed by C.*subterminal*(34.2%),C. *bifermentans* (14.3%) , and *C.sordelli*(13.4%), Concerning canned sardine, *C.sporogenes*(28.6%), *subterminal*(20%), and *C.bifermentans (2.9%)*where isolated and identified. While, C.*sporogenes*(28.6%), *subterminal*(17.1%) and *C.sordelli*(2.9%) were isolated from the examined samples of canned tuna. Statistically estimated the incidence of *C. perfringens* food poisoning outbreaks in UK and Wales between 1992 and 2008; they reported that out of 1000 persons, 24 personswere presented to *C. perfringens* illness per year. *C. perfringens* was identified as the cause of 10% of food-borne outbreaks Gormley*et al.* (2011) and Tam *et al.* (2012) .

Canned tuna ,sardine and mackerel should not have *clostridium* or anaerobic spore forming bacteria *EOS (2005)* .

Results achieved in Table (5) indicated that the incidence of lecithinase +ve C. *perfringens* was 11.4%, 5.4% and 2.9% of the examined samples of canned mackerel, sardine and tuna, respectively. While, the incidence of C. *perfringens* was 20%, 11.4% and 8.6% of the examined samples of canned mackerel, sardine and tuna, respectively.

Multiplication of C. *Perfringens* occurs during the long period of storage between boiling and consumption causing food poisoning (Hewitt *et al*,1986). Furthermore, C. *perfringens* is able to grow at concentration of 3-5% NaCl and can gain access during processing or food service operations (ICMSF,1986).

The presence of clostridia in canned fish products indicates improper processing or contamination during handling and storage of such products. Also, the quality of used raw fish has a major role in the presence of clostridia in examined canned fish .On the other band ,proper handling of fishes after landing can reduce the chance of any public health hazard by C. *perfringens* (Lalitha and Lyer,1986).

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