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Review

Foods, fish and salmonellosis

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Foodborne diseases are those caused by the consumption of water and food contaminated by different causal agents such as: viruses, bacteria, parasites, toxins, among others, being considered an important public health problem global due to its incidence and mortality and for several years for the isolation of microorganisms that cause these diseases resistant to antimicrobials. *Salmonella* species is considered a food pathogen frequently responsible for infectious outbreaks through the consumption of contaminated food, also presenting resistance to different antimicrobials. Fishery products are recognized as an important source of food, nutrition, income and a source of livelihood for a large part of the world's population. However, fish is also considered to be a vehicle that transmits different pathogens (*Salmonella* spp*., Shigella* species*, Escherichia coli, Listeria monocytogenes* among others) mainly due to inadequate hygiene practices along the food chain. The purpose of this article is to show in a general way a perspective of foodborne diseases, specifically those caused by bacteria of the genus *Salmonella* spp., through fish such as tilapia, the control and prevention measures of these pathogens in food, the phenomenon of resistance to antimicrobials by these bacteria isolated in food and fish around the world that exacerbates the problem in food safety and public health.

Key words: *Salmonella*, food, fish, processing, antimicrobial.

INTRODUCTION

Foodborne diseases (FD) are considered an important public health issue at the international level due to their incidence and mortality rates as well as the negative economic-productive repercussions associated with health services and the implementation and monitoring of health policies and food safety (Zamudio et al., 2011; Olea et al., 2012; Puig et al., 2013). These diseases are generated due to the consumption of food or water contaminated by physical, chemical or microbiological agents during any phase of the food chain (primary production, processing, handling, conservation, transport, distribution or commercialization) distinguishing themselves in infections or food poisoning (De Fuente and Barboza; 2010; Zamudio et al., 2011; Badui, 2015; Jorquera et al., 2015).

Foodborne diseases are characterized by different

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symptoms that generally include nausea, vomiting, diarrhea, abdominal pain, and fever; in some cases, severe complications may occur, such as sepsis, meningitis, Reiter syndrome, Guillan Barré syndrome or death, with population groups such as children, pregnant women and the elderly being more severely affected (Soto et al., 2016). According to estimates of the World Health Organization (WHO) annually around the world, 1500 million cases of diarrhea are generated, passing away three million children under five years of age is a high proportion due to the consumption of food and water contaminated (López et al., 2013). Only in countries like the United States of America (USA), it is estimated that around 76 million people suffer from a foodborne illness, of which 325,000 require hospital care and 5,000 dies every year and involving high health costs (Olea et al., 2012).

Approximately, more 250 disease-causing agents derivate by consumption of food has been identified, which includes bacteria, viruses, fungi, parasites, prions, toxins and metals (Olea et al., 2012). Most infections are generated by bacteria, viruses, and parasites; being between these the bacterial the mostly reported among which are*: Salmonella, Vibrio, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens, Escherichia coli, Listeria monocytogenes, Campylobacter,* and *Shigella* species among others (Puig et al., 2013; Jorquera et al., 2015; Soto et al., 2016).

Some of the factors that have caused a higher rate of food contamination and incidence of these diseases are market globalization, new manufacturing technologies and eating habits (Olea et al., 2012; Jorquera et al., 2015). The incidence of these diseases is a direct indicator of the hygienic-sanitary quality of food, demonstrating that the contamination of these can occur at any stage of the food chain either in the processing or use of contaminated raw materials (Flores and Herrera, 2005).

In the analysis of foodborne diseases and specifically related to fish and its relationship with biological agents contaminating foods such as *Salmonella* spp., Alerte et al. (2012) reported that in the Metropolitan Region of Chile between January 2005 and June 2010, there were 2806 outbreaks of which 2472 were investigated finding that 15.1% of the outbreaks are related to food such as fish, being *Salmonella* spp., the causal agent is 20.9% of the total outbreaks, also discovering that the main causes of the loss of food safety was the commercial and domestic manipulation, raw material, inadequate storage and processing.

On the other hand in Europe, Espinosa et al. (2014) through the data issued by the national network of epidemiological surveillance (RENAVE) of Spain in the period of the year 2008 to 2011 reported a total of 30219 cases of which 1763 were hospitalizations and 24 deaths of which *Salmonella* spp. was involved in 33.9% of the cases and the food involved was fish and products in 6.5% and among the contributing factors to such diseases were mainly the poor hygiene practices in handling, processing, storage, etc.

The purpose of this document is to show a general perspective of foodborne diseases, especially those caused by bacteria of the genus *Salmonella* spp*.*, through fish such as tilapia, as well as the control and prevention measures of these pathogens in food. In addition, an outline of resistance to antimicrobials by *Salmonella* spp*.*, in foods and fish is presented, which exacerbates the problem of loss of food safety and public health.

THE GENUS *SALMONELLA* SPP*.*

Bacteria of the genus *Salmonella* spp*.*, belong to the family *Enterobacteriaceae*, are bacillus-shaped, Gram negative, non-sporulated, mobile, catalase positive, oxidase negative, aerobic and facultative anaerobes, grow in a temperature range from 5 to 47°C, with an optimum temperature of 35 to 37°C, the pH of growth is between 4 and 9 with an optimum between 6.5 and 7.5, and an activity water (Aw) of 0.99 to 0.94; two species are currently recognized: *Salmonella enterica* and *Salmonella bongori*, among which there are 2500 serotypes, classified according to flagellar antigen "H", somatic "O" and virulence "Vi". *S. enterica* is divided into six subspecies such as: *enterica, salamae, arizonae, diarizonae, houtenae* and *indica*, where *S. enterica*, subspecies *enterica*, presents 99% of serotypes isolated and related to infections in animals and humans (González et al., 2014; Soto et al., 2016; Cortes-Sánchez et al., 2017). The main reservoir of *Salmonella* spp. is the gastrointestinal tract of mammals, reptiles, fish, birds, and insects (Nwiyi and Onyeabor, 2012; Juno et al., 2013; González et al., 2014; Bibi et al., 2015).

The genus *Salmonella* spp*.,* is of great importance in aspects of public health worldwide due to its incidence, virulence, adaptability and resistance to antimicrobials, causing diseases such as salmonellosis through the consumption of contaminated food and water such as meat, fish, vegetables, etc., and the most vulnerable population groups are children, the elderly, pregnant women and weakened immune system (Rivera et al., 2012; Nwiyi and Onyeabor, 2012; Junod, 2013; Soto et al., 2016; Cortes-Sánchez et al., 2017; Sheyin and Solomon, 2017). Once the salmonellosis is contracted, the symptoms manifest between 6 and 72 h after the ingestion of contaminated food, lasting between 2 and 7 days (WHO, 2018c). Annually, an estimated incidence of 1.3 billion cases of salmonellosis is estimated worldwide, with 3.0 million deaths and its common symptoms being vomiting, abdominal cramps, fever, headache, enterocolitis, diarrhea, blood in stools in severe cases can cause sepsis, endotoxemia, disseminated intravascular coagulation, multiple organ failure, and death (Rivera et al., 2012; Bibi et al., 2015).

FISH AS FOOD AND HEALTH

Fish is considered a globally important food, as it represents a source of nutrition, income, and livelihoods for hundreds of millions of people. In 2014, the world production of fish by capture and aquaculture was 167.2 million tons, of which 146.3 million were for human consumption (FAO, 2016). For the aquaculture sector, global trade in these products has increased considerably in recent decades and the expansion of aquaculture production, particularly in Asia, has the potential to satisfy a considerable part of the growing global demand for fish and fishery products; according to data from the Food and Agriculture Organization of the United Nations (FAO), aquaculture contributes around 50% of the world demand for fish and fishery products, with approximately 90% of aquaculture products coming from the Asian continent (Elhadi, 2014).

Fish is considered a basic nutritious food that is part of a healthy diet due to its composition as a source of water (66-81%), high-quality protein and digestibility due to being a source of essential amino acids (16-21%), carbohydrates (<0.5%), fat-soluble and water-soluble vitamins, minerals (1.2-1.5%) mainly calcium, potassium and phosphorus and lipids (0.2-25%) that include polyunsaturated fatty acids omega-3 such as: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that provide benefits to cardiovascular and nervous system with hypo-triglyceridemic, hypo cholemic, antithrombotic, anti-inflammatory, anti-arrhythmic effects, among others. It should be noted that the chemical and nutritional composition of fish varies considerably between different species and also between individuals of the same species, according to age, sex, diet, environment and season (FAO, 1998; Bakr et al., 2011; De Oliveira and Amancio, 2012; Valenzuela et al., 2014, 2016; FAO, 2016; Sheyin and Solomon, 2017).

However, these same nutritional and chemical composition qualities mentioned earlier convert fish from the capture or culture through aquaculture into a highly perishable food product susceptible to deterioration, as well as to contamination of chemical origin (metals, pesticides, antibiotics) or biological (biotoxins, parasites, viruses and bacteria) that label it as a food of considerable risk to consumer health (Ferre, 2001; Martínez et al., 2008; Uresti et al., 2008; Chalen et al., 2010; Romero and Negrete, 2011; Martinez et al., 2012; Quintero et al., 2012; Ortega, 2014; Sheyin and Solomon, 2017; FAO, 2018).

FISH AS A SOURCE OF FOOD: THE CASE OF TILAPIA

It is generically called tilapia a group of teleost fishes belonging to the Cichlidae family originating from the African continent. The family includes the genera *Tilapia* and *Oreochromis*, which have gained relevance around the world in recent years at the level of cultivation and trade as a source of food for humans. Species of the genus *Oreochromis* species such as *Oreochromis niloticus*, called "Nile tilapia", *Oreochromis aureus*, "blue tilapia" and *Oreochromis* spp. or "red tilapia" are the most accepted in cultivation, because they have a period of short growth, easy handling (sowing, harvesting, transfers, etc.), tolerance to extreme conditions (low oxygen concentrations, high planting densities), high ammonium levels, low and high pH values), adaptability in food habits and good production parameters (feed conversion, weight gain, survival) (Wicki and Gromenida, 1998; Vega et al., 2009; Garcia et al., 2012).

Tilapia is considered a fish of good appearance, quick acceptance and nutritional value by be source of proteins with a content of 23.34 g/100 g, ashes of 1.94 g/100 g, water 72.36 g/100 g and total lipids of 2.26/100 g, which has a proportion of omega-3 fatty acids of 33 g/100 g of lipids and omega-6 of 47.7g/100 g of lipids which has been reported to be beneficial effects for consumer health and that make it part of a balanced diet and healthy (Izquierdo et al., 2000).

Tilapia comprising its seven species constitutes the second most important group of farmed fish, only behind carp species; this has spread more among all the farmed fish (Aquaculture). Worldwide, Asian countries such as China, the Philippines, Thailand, Indonesia, Taiwan, India, Malaysia followed by countries of the American continent such as Brazil, Honduras, Costa Rica, Ecuador, and United States of America, are listed as the main producers of tilapia (*O. niloticus*) (FAO, 2018a).

In Mexico, tilapia farming and fishing due to its volume is positioned in the fifth place of the fishing production in Mexico and its commercial value is positioned in the third place. The average annual growth rate of production in the last 10 years is 3.28% where aquaculture through extensive, semi-intensive and intensive systems (according to the fish culture density, food supply and cultivation technification) performs the main contribution of this product; cultivating eight types of tilapia, mainly: herbivorous tilapia, Nile tilapia, Stirling tilapia, white tilapia, Mozambique tilapia, orange tilapia, tilapia mojarra and Florida red tilapia (SAGARPA, 2013, 2016) being the states of the Mexican Republic as Jalisco, Chiapas, Veracruz and Michoacán the largest producers (SAGARPA, 2013).

DETERIORATION AND CONTAMINATION OF FISH AND TILAPIA

The deterioration of the fish starts from the death of the animal and is considered as all those stages that involve internal and external changes through which the fish passes since die until it reaches a decomposition or putrefaction degree, which results in a product not

suitable for consumption (Avdalov, 2009; Gentile, 2010).

The most notorious change is the attack of rigor mortis where immediately after death, the muscle of the fish is relaxed, flexible and elastic texture commonly maintained for few hours and then the muscle becomes hard and rigid; the whole body then becomes inflexible so the fish is in rigor mortis; this condition is maintained for one or more days, subsequently the muscle relaxes again and regains flexibility, but not the elasticity prior to rigor. The ratio between the beginning and the resolution of the rigor varies according to the species and is affected by the temperature, handling, size and physical conditions of the fish; for example, *Tilapia mossambica* in a state of non-exhaustion prior to its death of 2 to 9 h, which is the beginning of rigor mortis after death with a duration of 26.5 h at a temperature of 0 to 2°C (FAO, 1998). In general, within the activities that the fish experience in the deterioration are: autolysis (self-digestion, endogenous enzymes), lipid oxidation and microbiological activity; changes in fish during spoilage can be perceptible at the sensory level and through chemical, physical and microbiological methods (FAO, 1998; Avdalov, 2009; Gentile, 2010).

The speed with which the fish deteriorates is related to the initial microbial load and the temperature at which it is handled and preserved, the higher the maintenance temperatures and the microbial count, the faster the deterioration is generated (Gentile, 2010; Sheyin and Solomon, 2017). A characteristic of fish such as tilapia is that they have a large number of nitrogen compounds which makes them even more prone to deterioration, which is why they are commonly kept alive even before consumption (Sheyin and Solomon, 2017).

The fish's microbiota is related to the environmental conditions and food availability of their habitat. Water from rivers and lakes has a complex population that includes aquatic species, as well as terrestrial, animal and plant sources. On the other hand, anthropogenic activities have had a detrimental effect on both coastal and continental waters being contaminated with wastewater, giving rise to the risk that enteric microorganisms may be present and contaminate aquatic sources and therefore fish products. This does not further exempt that, depending on the type of fishing, aquaculture and hygiene practices during inappropriate handling or processing to which the different products are subjected, they may contribute to contamination with harmful biological or chemical agents, generating a risk to the health of the consumer (Avdalov, 2009; Gentile; 2010; Quintero et al., 2012; Sheyin and Solomon, 2017).

Pathogenic microorganisms in fresh fishery products may be related to fecal contamination of the water from where they were captured or cultivated. Fish function as a passive carrier of *Salmonella* spp. which can excrete it without apparent symptoms and clinical disease in fish species such as tilapia (*O. niloticus*) where the isolation of *Salmonella* spp. has been reported, mainly in organs such as intestines, skin, and galls (Bibi et al., 2015). Studies have also been carried out on the prevalence of this pathogen around the world in fish such as tilapia, which is extracted from various aquatic sources, showing biological contamination such as *Salmonella* spp. which is reflected in the possible risk of acquiring diseases when extracted and consume these products; such is the case of Nile tilapia (*O. niloticus*) from Lake Victoria Beaches in Western Kenya where contamination with enteric bacteria such as *S. Typhimurium* was reported, followed by *typhi* and *enteritidis*, estimating that the source of contamination is of human or animal origin (Awuor et al., 2011).

Likewise, in microbiological studies of fresh tilapia sold to the general public in three markets of Nigeria, it was reported that samples of fresh tilapia in a period of the year 2010 to 2012 presented in body, gills, and guts the presence of *Salmonella* spp. suggesting a source of cross-contamination in the preparation of these products for consumption as well as focusing and promoting further studies of pathogen transmission along the food chain (Nwiyi and Onyeabor, 2012). On the other hand, Ismail et al. (2016) conducted a study among associations on water quality and the presence of bacteria in tilapia culture in floating cages in lakes and rivers in Peninsular Malaysia (Pedu Lake in Kedah and Kenyir Lake in Terengganu and Terengganu River) over a period of 24 months indicating the presence of various bacteria including those of a pathogenic nature for both humans (*Salmonella* spp.) and fish (*Vibrio* spp., *Aeromonas hydrophila,* and *Streptococcus* spp.) where water quality is an important aspect of aquaculture. Concluding that non-optimal physicochemical parameters of water (dissolved oxygen, pH, salinity, ammonia, temperature, etc.) and poor management practices (overfeeding, inadequate nutrition, overcrowding, etc.) can cause stress in intensively farmed fish and make them susceptible to outbreaks of diseases with harmful effects on water quality, so it is necessary to understand the microbiological-fish-environment association for an adequate and timely management of food production and safety through aquaculture.

CONTROL AND PREVENTION OF FOODBORNE DISEASES

Food safety is considered as a guarantee that food will not cause harm to the consumer when prepared and consumed according to the intended use, and is considered one of the four basic groups of characteristics that, in combination with nutritional, organoleptic and commercial, make up the total quality of food (De la Fuente and Barboza, 2010; Jorquera et al., 2015).

Fishery products are recognized as an important transmitter of microorganisms thus compromising their safety as food. The pathogenic microorganisms related to

fish and products can be grouped into three general groups: (a) Autochthonous bacteria that belong to the natural microflora of fish (*Clostridium botulinum, Vibrio* spp*.,* and *A. hydrophila*); (b) Enteric bacteria whose presence is due to fecal contamination (*Salmonella* spp., *Shigella* spp., *Escherichia coli, Staphylococcus aureus*); and (c) Bacterial contamination during processing, storage or preparation for consumption (*Bacillus cereus, L. monocytogenes, Staphylococcus aureus, C. perfringens, Salmonella* spp.) (Elhadi, 2014).

The knowledge of the microbiota of fishery products through the microbial load of organs such as the skin, gills, and intestine allows to determine the microorganisms that are indicators of hygienic-sanitary quality and which ones can be activated during deterioration; the relevance of these organs lies in that they are in direct contact with the muscle and constitute the origin of the bacterial invasion, so if the number of bacteria increases in the said organs, proportionally it will increase in the muscle (Fuentes et al., 2011). The presence of various pathogenic bacteria is important for fish processing where process design and handling are involved in order to eliminate or inhibit them (Sheyin and Solomon, 2017).

The actions that have been developed around the world in order to generate safe food, avoiding and controlling the presence of bacterial pathogenic as *Salmonella* spp., including fish and products is through the application of different hygiene procedures along the food chain such as good agricultural practices, good livestock practices, good aquaculture and fishing practices and good manufacturing practices as well as the implementation of systems for Hazard Analysis and Critical Control Point (HACCP) and the development of microbiological analysis methods in fast, sensitive, efficient and reliable food for timely decision making in protection of the health of the population (Flores and Rojas, 2005; Quintero et al., 2012; Ledezma et al., 2013; Balbuena, 2014; Jorquera et al., 2015; FAO, 2018b).

ANALYSIS IN THE LABORATORY FOR THE DETECTION OF *SALMONELLA* SPP., AS PART OF CONTROL AND PREVENTION IN FISH AND OTHER FOODS

The analysis of food in the search for pathogenic microorganisms is considered an essential phase for the control of the safety and quality of food (Zadernowska and Chajęcka, 2012). Among the traditional methodologies of isolation and detection of *Salmonella* spp*.,* in foods which are based on the culture and identification through differential and selective means, biochemical and serological tests (Figure 1 and Table 1). Several methods have been reported around the world, some of them standardized and commonly used for this purpose, such as the US Food and Drug Administration (US FDA) in the manual of analytical bacteriology (BAM) by Andrews et al. (2017), the method developed by International Organization for Standardization (ISO) ISO 6579: 2002 or the MLG 4.09 method in its version of 2017 for the detection of *Salmonella* spp. in foods of the Department of Agriculture of the United States of America (USDA, 2017); and finally, the official method of national regulation in the Mexican Republic contemplated in the official Mexican standard NOM-210-SSA1-2014, for the detection of pathogens such as *Salmonella* spp., in food. Likewise, complementary or alternative molecular methods for the detection of pathogens have been developed, such as the polymerase chain reaction (PCR) in its multiple variants, immune enzymatic or proteomics such as electrophoresis and MALDI-TOF mass spectrometry (matrix-assisted laser desorption/ionization-time of flight) in order to reduce the analysis times presented by the phenotypic methods and to have an early detection; although its implementation is not universal due to the high cost and degree of specialization required for its application (Bou et al., 2011; Zadernowska and Chajęcka, 2012; Sparbier et al., 2012; Pavlovic et al., 2013; Soto et al., 2016; Cortes et al., 2017).

Foodborne diseases are a complex issue that impacts several social sectors, so detection and prevention are considered a joint effort between several social actors ranging from consumers, regulatory authorities, health, food industry and academia, whose actions lead to a decrease in the risks of contamination of food. To guarantee consumers a safe and hygienic food, it is necessary to control pathogenic microorganisms in all stages of the food chain and although the actions have focused on the production of healthy and safe food, using good hygiene practices such as good manufacturing practices, and quality and safety control measures, such as the implementation of hazard analysis and critical control point (HACCP), there is still much to be done as foodborne diseases continue to be responsible for high morbidity rates and in some cases of mortality, around the world, generating large losses for public health, animal health and the food industry (González and Rojas, 2005; Jorquera et al., 2015; WHO, 2018a).

REGULATION OF FOOD SAFETY AROUND THE WORLD OF FOOD AND FISH PRODUCTS FOR CONTROL AND PREVENTION OF SALMONELLOSIS AND OTHER FOODBORNE DISEASES

In food, and specifically fishery and aquaculture products, globalization and commercialization efforts have been directed towards guaranteeing food safety and quality, as well as environmental sustainability through different standards, codes, guidelines, and/or certifications to the global level, for example: general principles of food hygiene, microbiological criteria, risk assessment,

Table 1. Biochemical and serological identification tests for the isolation of *Salmonella* spp., in the laboratory (Perilla et al., 2004; Andrews et al., 2017; Cortes-Sánchez et al., 2017).

Test	The reaction of species of *Salmonella* spp.*
Production of glucose acid (TSI)	+
Lysine decarboxylase (LIA)	+
H_2S in TSI and LIA	+
Hydrolysis of urea	-
Lysine decarboxylase broth	+
Methyl red	+
Voges-Proskauer	-
Malonate broth	-c
Indole production	-
Urease	-
Citrate metabolism	v
Mobility	+
Somatic polyvalent test	Agglutination+
Flagellar multi-purpose test	Agglutination+

LIA: Iron lysine agar; TSI: triple sugar iron agar. * +: 90% or more positive in 1 or 2 days; -: 90% or more negative in 1 or 2 days. v: variable. b mostly cultures of *S. arizonae* cultures are negative. The majority of *S. arizonae* cultures are positive.

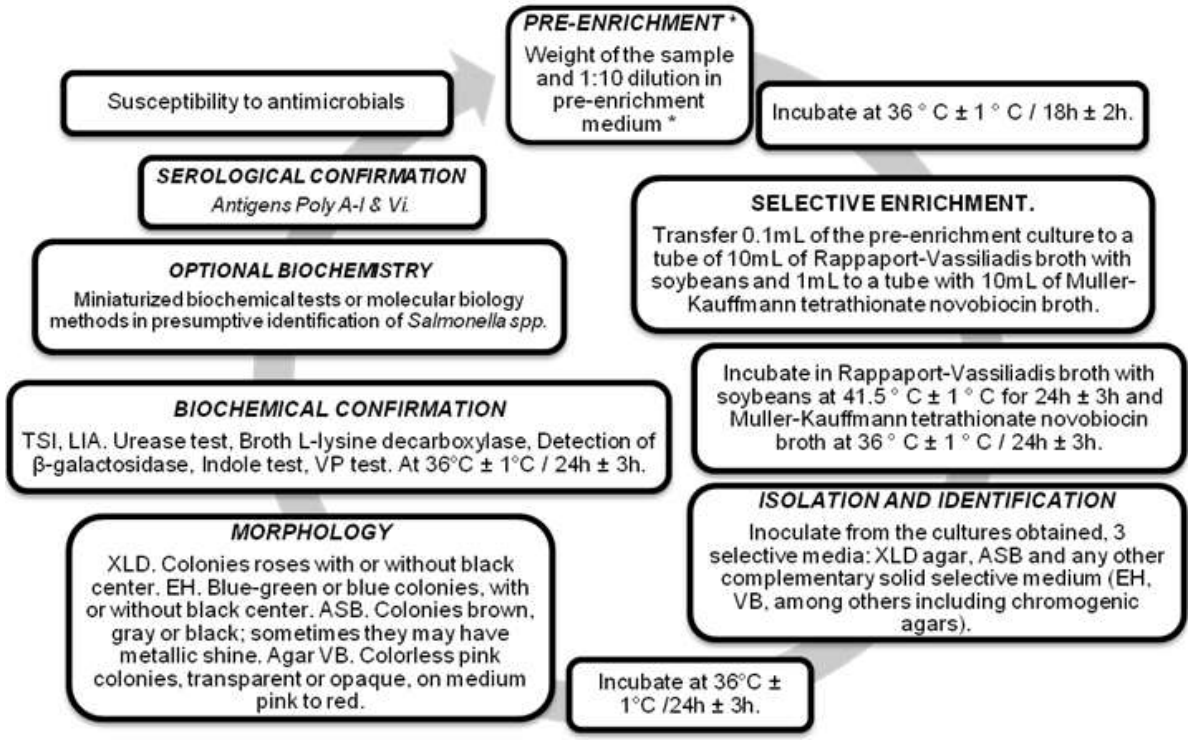

Figure 1. Method for the isolation and identification of *Salmonella* spp., in food. *The pre-enrichment medium will be according to the food under analysis; generally buffered peptone water is used. XLD: Xylose Deoxycholate Agar; ASB: Bismuth Sulfite Agar; VB: Bright Green Agar; EH: Hecktoen Agar; TSI: Triple Sugar Iron Agar, LIA: Iron Lysine Agar; VP: Vogues-Proskauer.
Source: Perilla et al. (2004); NOM-210-SSA1-2014; ISO 6579: 2002; Cortes-Sánchez et al. (2017).

HACCP system, ISO 22000, Safe Quality Food (SQF), British Retail Consortium (BRC), GlobalGAP, etc., generated and validated by different organizations like WHO, United Nations Organization for Food and Agriculture (FAO) through the Codex Alimentarius, World Organization for Animal Health (OIE), International

Organization for Standardization (ISO) and Global Food Safety Initiative (GFSI) (RECUA, 2018).

On the other hand, sanitary regulation and safety of food and fishery products in Europe has established different food safety legislation, such as the case of the European community that issued Regulation (EC) No 178/January 28, 2002 of the European Parliament establishing the principles and general requirements of food legislation, creating the European Food Safety Authority (EFSA) and establishing procedures related to food safety. Subsequently, through Regulation (EC) No 853/2004 of the European Parliament of April 29, 2004, the specific rules of hygiene of foods of animal origin including fish directed at company operators are instituted.

Likewise, on November 15, 2005, Commission Regulation (EC) No. 2073/2005 was issued, which is related to the microbiological criteria applicable to food products to serve as guidance on the acceptability of food products and their products manufacturing, handling and distribution processes; where for *Salmonella* spp., it indicates a criterion of "Absence in 25 g of product" using the method of analysis of the International Organization for Standardization (ISO) ISO 6579 likewise makes the application of procedures based on the principles of Hazard Analysis and Critical Control Point (HACCP) and other hygiene control measures.

In countries of the American continent such as Mexico, sanitary regulation through the Official Mexican Standard NOM-242-SSA1-2009, contemplates for fresh, refrigerated, frozen and processed fishery products, the physical, chemical and microbiological sanitary specifications, as well as test methods for your laboratory analysis. Indicating that for *Salmonella* spp., in all fishery products it must be "absent in 25 g of the product". While the Official Mexican Standard NOM-251-SSA1-2009, which refers to hygiene practices for the process of food, beverages or food supplements involve the minimum requirements of good hygiene practices that must be observed in the process of food, beverages or food supplements and their raw materials in order to avoid their contamination throughout their process, also including the HACCP system and guidelines for their application.

RESISTANCE TO ANTIMICROBIALS

Resistance is the mechanism obtained either naturally or acquired by which microorganisms decrease the action of antimicrobial agents on them when exposed to antimicrobials (antibiotics, antifungals, antivirals, antimalarials or anthelmintic). The resistant microorganisms are of cosmopolitan location and can be transmitted from person to person or between people and animals, even by food being the most common attributable causes to its spread of excessive, insufficient, indiscriminate and inappropriate use of these antimicrobial agents in the human clinic, agricultural and aquaculture production as prophylactic, therapeutic and growth promoters, in addition to poor sanitary conditions and inadequate handling in food production (FAO, 2002; Puig et al., 2011; Rivera et al., 2012; Cabello, 2004; WHO, 2018e).

There is a great concern around the world regarding public health due to the incidence of infections by microorganisms with this characteristic due to the increase in disability, death, prolongation of the disease, cost of health care due to the longer duration of hospitalizations and the need for more intensive care. It is estimated that approximately 500,000 people die each year globally due to causes related to antimicrobial resistance. In addition, this phenomenon affects food safety, food security and economic well-being as food is involved in the development and spread of antimicrobial resistance, and the presence of antimicrobial-resistant microorganisms in the food chain is a potential means of exposure (WHO, 2018a; FAO, 2018c).

Antimicrobial resistance in microorganisms can be intrinsic or acquire resistance by Novo mutations or by the transfer of genetic material (plasmids, transposons, and integrons) from other organisms. The ability to resist the action of antimicrobials is carried out through various mechanisms such as modification of the permeability of the membrane, expulsion of the compound by pumping excretion, enzymatic inhibition and modification of the attack target or alteration of the composition and the content of cell wall glycoproteins (Tafur et al., 2008; Becerra et al., 2009; Puig et al., 2011; Pérez and Robles, 2013).

Antimicrobial resistance is a growing threat to global public health and requires action by international organizations, the government sector, and society to reduce the incidence of this phenomenon (WHO, 2018a). Global action plans have been established by the World Health Organization (WHO) against antimicrobial resistance, where five strategic objectives are established: (1) Improve awareness and understanding of antimicrobial resistance; (2) Reinforce knowledge through surveillance and research; (3) Reduce the incidence of infections; (4) Optimally use antimicrobial agents and prepare economic arguments in favor of a sustainable investment that takes into account the needs of all countries; and (5) Increase investment in new medicines, diagnostic tools, vaccines and other interventions. Likewise, guidelines have been developed for the use of antimicrobials in animals intended for food production and thus avoid abuse and indiscriminate use (WHO, 2018b, FAO, 2018c). The Codex Alimentarius has developed scientifically based on guidelines and codes to manage antimicrobial resistance and its transmission along the food chain, such as risk analysis of foodborne antimicrobial resistance: CAC/GL 77-2011 and code of practice to minimize and contain antimicrobial resistance:

CAC/RCP 61-2005 (FAO, 2018c).

Salmonella spp. is one of the different pathogenic microorganisms isolated in foods that have shown resistance to different antimicrobials, affecting a large extent the food chain (Puig et al., 2011; Hur et al., 2012; WHO, 2018d). Resistance to antimicrobials by *Salmonella* spp., can be observed and transmitted mainly by consuming food contaminated with antibiotics or eating food contaminated with feces of animals or human carriers, who continue to suffer from the disease after various incomplete or failed treatments (Rivera et al., 2012). In the search for strains resistant to antimicrobials, several studies on animals and food have been carried out globally in order to improve the analysis, control, and prevention of infections by this bacterium, such as the case of food from fisheries where Rahimi et al. (2013) analyzed the prevalence and susceptibility to antimicrobials by different serotypes of *Salmonella* spp., such as *Salmonella Typhimurium, Salmonella enteritidis, Salmonella* Typhi*, Salmonella paratyphi* B, and *Salmonella newport* isolated in fishery products from three provinces (Bushehr, Hormozgan and Khuzestan) from the Persian Gulf on the southern coast of Iran, reporting the susceptibility of 19 isolates to different antimicrobial drugs using the disk diffusion method; the resistance to nalidixic acid was 47.4%, followed by resistance to tetracycline in 36.8%, streptomycin 15.8%, trimethoprim 15.8% and ciprofloxacin 5.3%. On the other hand, Budiati et al. (2013) conducted a study to determine the prevalence and resistance to antibiotics by *Salmonella* spp., in fish isolates such as: catfish (*Clarias gariepinus*) and tilapia (*T. mossambica*) obtained from wet markets and ponds fed chicken offal, eggs and commercial fish feed during the period of 2008 to 2009 in Malaysia; from a total of 172 samples (32 catfish and 32 catfish intestines, 32 tilapia carcass and 32 tilapia intestines and 44 water samples), the isolation of seven *Salmonella* serovars in catfish 9/32 (28.1%), tilapia 14/32 (43.8%) and water samples 11/44 (25%) were reported. Isolated serotypes include *Salmonella albany, Salmonella agona, Salmonella corvallis, Salmonella stanley, S. Typhimurium, Salmonella mikawashima* and *Salmonella bovis-mobificans*. The sensitivity of these isolates to different antibiotics was for chloramphenicol (37.2%), clindamycin (100%), rifampicin (90.7%), spectinomycin (27.9%) and tetracycline (67.4%).

Finally, Elhadi (2014) carried out the analysis of the proportion of imported frozen fish contaminated with *Salmonella* spp. between retail food stores and supermarkets in the Eastern Province of Saudi Arabia wherein a total of 225 fish samples analyzed which included 75 of tilapia from India and Thailand; the results showed that 30 of these tilapia samples were positive for *Salmonella* spp. with 20 and 22 isolates, respectively. The isolates also showed resistance to antibiotics such as tetracycline, ampicillin and clavulanic acid-amoxicillin. In all the previous studies, it is concluded that the increase and spread of this antimicrobial-resistant food pathogen is related to the wide use of antimicrobial agents, in human, veterinary medicine, livestock production and aquaculture as prophylactic, therapeutic or growth promoters; in addition to the fact that the increase of the antimicrobial resistance phenomenon by *Salmonella* spp., could restrict the therapeutic options for clinical cases that appear to be derived from the transmission of food, so it is recommended that food control authorities generate and implement in a robust manner regular surveillance systems for this pathogen transmitted through fish and aquaculture products destined as food for human consumption and sharing the information globally to improve the effectiveness of the control programs.

The monitoring of antimicrobial resistance by *Salmonella* spp., through the development of monitoring networks between countries for the detection of resistant bacteria, can improve epidemiological studies for the control of outbreaks or epidemics in humans around the world; likewise, the control of this phenomenon involves different actions such as sanitary and hygienic regulation in agricultural, fishing and aquaculture production, the generation and implementation of educational plans for human, veterinarian, and personnel working in the different phases of the food chain, the rationalized use and rotation of drugs are useful for safeguarding food safety and for combating antimicrobial resistance (Puig et al., 2011; Hur et al., 2012; Rivera et al., 2012; FAO, 2018c; WHO, 2018d).

CONCLUSION

Foodborne diseases are a major global public health problem due to their incidence, mortality and global threat due to resistance to antimicrobials by causal agents of biological origin that put at risk the human and animal health and food safety.

Salmonella spp. is considered a food pathogen frequently related to outbreaks of foodborne diseases together with the fact that different serotypes of clinical isolates and foods have been shown to be resistant to antimicrobials commonly used in human therapeutics.

Reports around the world continue to indicate the isolation of resistant pathogens, so that international health and food organizations, government authorities, the food industry and academia must continue actions in surveillance, regulation and promote to avoid the indiscriminate and inappropriate use of antimicrobials for therapeutic purposes, prophylactic in human and animal health as well as in the production of food.

Fish is considered a highly nutritious food by their contents: water, proteins, lipids, vitamins and minerals, but also too perishable and susceptible to deterioration and contamination by pathogenic microorganisms such as *Salmonella* spp., during any phase of the food chain

(primary production, processing, conservation, distribution, commercialization, among others), improper hygiene practices were mainly derived.

Tilapia is a group of fish widely known, produced and intended for human consumption for its adaptation and tolerance to environmental and food conditions of growth in culture, as well as being a source of proteins, vitamins, lipids and minerals.

Tilapia is marketed in different presentations (fresh whole, gutted, fillets, refrigerated, frozen, among others) according to the preference of the consumer; this fish is obtained through capture fisheries and aquaculture throughout the world, with the Asian countries being the main producers. And like other fish in their production, capture is susceptible to contamination and deterioration by various chemical and biological agents (among these bacteria of the genus *Salmonella* spp.) derived mainly to poor hygiene practices in fishing activities, aquaculture, processing and manipulation prior to consumption, turning them into high-risk food products for the health of the consumer.

For several years, different international organizations such as the WHO, Codex Alimentarius, International Organization for Standardization and others around the world have generated recommendations, guidelines, certifications concerning fish and products in order to produce, distribute and market food of nutritional quality and protect public health.

Contamination of food can occur in any link of the food chain turning them into carriers of diseases where the causative microorganisms have the ability to resist different antimicrobials, some of them commonly used in therapeutic treatments. World reports indicate that some of the factors involved in the emergence and increase of resistance to antimicrobials is the indiscriminate use of antimicrobials in human and animal health as well as in food production; the isolation of microorganisms resistant to antimicrobials from clinical cases and food continues to increase, generating a priority concern and challenge at the global level in developing guidelines and programs for action focused on control and prevention jointly between different social actors such as different international organizations in the field of health and nutrition, government agencies, academics, primary producers of food, food industry and general population in order to generate awareness of regular surveillance of the use of antimicrobials, which are a factor involved in the safety of food, including products from fisheries and aquaculture.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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Review

Response of microbial communities to oil spill in the Gulf of Mexico: A review

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Crude oil has become a part of the marine ecosystem through natural seeps and oil spills. Microbial communities have adopted various response mechanisms to adjust to oil spills that contaminate the marine environment and help restore the ecosystem to its original state. These response mechanisms ranges from change in indigenous microbial community composition, change in microbial diversity to gene diversity and modification. An instance for review was the deep-water horizon (DWH) oil spill in the Gulf of Mexico. The DWH oil spill was distinctive from other spills in terms of profundity, extent as well as its scale hence fears and enquiries about the state and outcome of the hydrocarbons at large is required. The main inquiry was about the metabolism ability of microbial communities; the ways, and to what degree the hydrocarbons can be metabolized. Various researches after the spill revealed that change in the successional patterns from the water column, shallow water and bottomless sea sediments to the coastline sediments saw the dominance of *Roseobacter* cluster within the Alphaproteobacteria on the surface, the Deltaproteobacteria closest to the wellhead, *Cycloclasticus* in shallow oil slicks, Colwellia and Alteromonas in the deep-sea hydrocarbon plume and sediment. These microbial communities help in bioremediation of oil during oil spills through their response mechanism. Factors such as nutrient limitation, hydrocarbon availability, ocean mixing and circulation among others also limit the rate at which microbial communities degrade hydrocarbons. This review outlines the response mechanism of microorganisms and how they help in hydrocarbon degradation.

Key words: Oil spills, microbial communities, microbial response, microbial degradation, hydrocarbons, deepwater horizon, Gulf of Mexico.

INTRODUCTION

Crude oil has become a vital part of the world's economy and industrial growth and is currently in a period of industrial development, alteration and increase (Bao et al., 2014). An increase in this industry involved in

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production, transportation and storage of oil has exposed most shores along oil mining areas and marine environment to oil spill accident (McGenity et al., 2012) from cargo/wholesale ocean carriers which are the major source carriers of oil (Bao et al., 2014). These oil spills affect human life, the environment and ecosystem as a whole. For example, the 2002 Prestige oil spill affected shores of Spain with about 66% of species abundance lost (Huz et al., 2005); the Deepwater Horizon spill (2010) conceded biodiversity of vertebrates and metazoan meiofauna (Baguley et al., 2015). Some other intense oil spill examples are the tanker collision in the Mumbai coast and the oil spill in Montora (Sakthipriya et al., 2015)

Microbial communities are known to control most major processes that occur in the marine environment (Karl, 2007). One important function and process is biodegradation of contaminants and nutrient recycling that helps to ensure an effective and efficient ecosystem (Wu et al., 2014). Hydrocarbon degradation by microorganisms has received much consideration because of its un-hazardous, inflammability, extensive and environmentally friendly state when likened to other orthodox methods (Sakthipriya et al., 2015). Microorganism from all the three domains of life are known to utilize the ~600 000 tons of oil which moves into the marine ecosystem from natural leaks yearly (NRC, 2003). A total of 175 prokaryotic genera of microorganisms from seven phyla of Bacteria, Archaea, and an equivalent amount of fungal genera, can utilize hydrocarbons as the only or main source of carbon (Hazen et al., 2015). Most of these microorganisms are found in the water column, sea bed sediments and on the shorelines of the marine environment through the tropics to the Polar Regions eg. Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Pelagibacter, Actinobacteria, Planctomycetes and Bacteroidetes (Yang et al., 2016).

Upon the introduction of crude oil in the oceanic ecosystem, there is a change in microbial community consisting of numerous mutually surviving species which is described via resource sharing (Yakimov et al., 2005). The native oil utilizing bacteria becomes enriched, with organisms of the order *Oceanospirillales* comprising up to 90% of bacterial community, when related with 5% of the pure sample (Hazen et al., 2015). McNutt et al. (2012) after the DWH oil spill in the Gulf of Mexico projected ~5.0 M drums to ~210 M tons of oil and gas released. Other research from these oil spill in April 2010 discovered impacts of the oil on marine organisms populations involving corals (Goodbody-Gringley et al., 2013), meio-, macro- and mega-fauna (Fisher et al., 2014) coupled with its influence on microorganisms assemblies (Hazen et al., 2010; Edwards et al., 2011; Mason et al., 2012; Redmond and Valentine, 2012; Gutierrez et al., 2013).

This study reviews the responses of microbial communities from the introduction of oil from oil spills to the marine environment, from the water column, shallow water, and bottomless sea sediments to the coastline sediments, in particular, with reference to the Deepwater Horizon oil spill.

Constituents of crude oil and biodegradation

Crude oil is a composite combination of organic compounds principally consisting of hydrocarbons (HC), averagely ~30% linear and branched alkanes, ~30% cyclic alkanes, ~30% aromatics and ~10% molecules with heteroatoms such as sulphur, oxygen and nitrogen (Tissot and Welte, 1984) with the latter giving oils most of their color. The aromatics consist of molecules that contain at least one single or condensed rings and aromatic ring, coupled by recurring and linear alkane substituents like the monocyclic (Tissot and Welte, 1984). The aromatics also comprise of polycyclic aromatic hydrocarbons (PAHs). Low molecular weight compounds (benzene, naphthalene and anthracene) are favorably degraded and high molecular weight (pyrene and fluoranthenes) being also broken down (Bacosa and Inoue, 2015). The polar fractions of crude oil comprise mainly of Asphaltenes and resins; however, the characterization of this polar molecule has not yet been widely done in the marine ecosystem. Microbes are able to take up some, and others if constantly exposed to the sun are generated by photochemistry (Aeppli et al., 2012), making their gross fate in the environment unclear. Hydrocarbons with about 40 carbon atoms are near total biodegradability in a one or two months when presented with a conducive atmosphere, except for certain hopanes and steranes (Prince et al., 2013); however bigger molecules degrade much more slowly, example are the constituents intended for roads.

The introduction of oil into a marine ecosystem makes considerable excess of carbon available in the development of microorganisms (Prince et al., 2003). However, unavailability of nutrients (nitrogen and phosphorus) is able to lessen the development of microbes and hence oil degradation (Liu et al., 2017). In an attempt to promote oil degradation on diverse coastlines (including the Arctic), fertilizers containing available nitrogen and phosphorus were added (Prince et al., 2003). Wide research and field-testing discovered that the development of hydrocarbon degrading micro-organisms was stimulated by adding nutrients and consequently raises the detected speed of oil decomposition (Atlas and Bragg, 2009).

Microbial degradation eliminates the hydrocarbons in marine environs and regenerates the oil polluted biota (Al-Hadhrami, 1995). Significant progress has been made within previous era in understanding hydrocarbons degradation by microorganisms (Atlas et al., 2011). The major microbes that help in degradation of oil have been identified from the 3 domains of life: bacteria, archaea and eukaryotes (Bacosa et al., 2015a) via different degradation pathways (Table 1). Most of the active oil degrading

Table 1. The major oil degrading microbes and the hydrocarbons degraded.

Taxa	Phyla	Degradation pathway/metabolism	HC degraded
Bacteria	Proteobacteria (Alpha, Beta ,Gamma and Delta)	Anaerobic, aerobic/sulfate and nitrate reducer	Aromatic HC, alkaliphilic and halophilic HC, phenanthrene, PAHs, carbazole, heterocyclic aromatics, naphthalene, BTEX, mono-aromatic HC, fluoranthene, linear chain alkanes, benzo[a]pyrene, propane, ethane, butane
Bacteria	Actinobacteria	Anaerobic	Aromatic hydrocarbons (phenanthrene), PAHs
Bacteria	Cyanobacteria	Aerobic pathway	Naphthalene, n-alkanes and isoalkanes, other aromatic HC
Bacteria	Firmicutes	Anaerobic/fermentative, sulfate reducing, saprophytes	Long chain alkanes, naphthalene, pyrene, other aromatics
Bacteria	Bacteroides	Anaerobic/ fermentative, nitrogen reducing	Carbazole, PAHs, benzo[a]pyrene
Bacteria	Deinococcus-thermus	Aerobic	Hexadecane and a broad spectrum of PAHs, toluene
Archaea	Halobacteria	Aerobic/ nitrate reducers	Phenanthrene, benzene, toluene, n-Alkane, octadecane, heptadecane
Archaea	Euryarchaeota	Anaerobic/ Methanogen, Fermentative	Methane
Eukaryote	Fungi	Aerobic	Alkanes

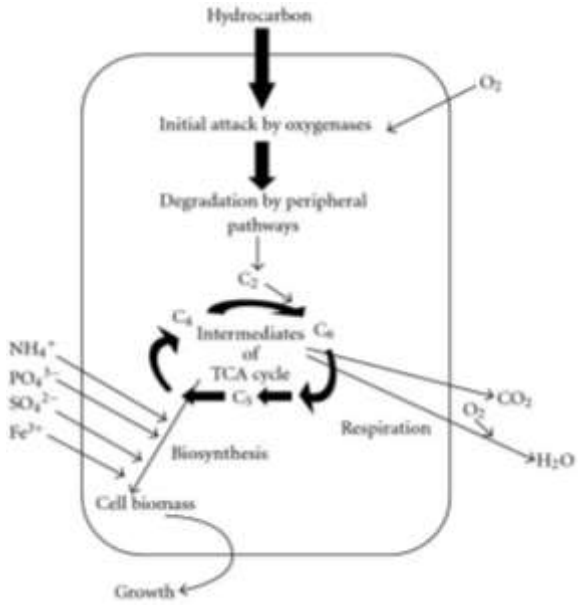

Figure 1. Principle for hydrocarbon breakdown (Das and Chandran, 2011).

microbes are bacteria and over 79 genera of bacteria have been known to be able to use hydrocarbons as the main and exclusive origin of energy among those with the ability to breakdown or convert hydrocarbons (Head et al., 2006). The oceanic ecosystem only shelters a minimal of 25 genera of bacteria that decompose hydrocarbons and are known primarily for pollutants attenuation (Das and Chandran, 2011). Due to the diversified nature of hydrocarbon compounds related to oil (iso-, cyclo, and linear alkanes, monoaromatic compounds and polycyclic aromatic hydrocarbons), the breakdown of different groups of hydrocarbon compounds needs different microorganisms with distinct biochemical mechanisms (Timmis et al., 2010). Distinctively, microbial communities have the ability to biologically breakdown a large array of hydrocarbons collectively as compared to an individual microorganism. Succeeding the DWH spill, there was a fast improvement in the quantity of hydrocarbon-degrading microorganisms in the water column, shallow and subsurface column water and ruled by recognized hydrocarbon-decomposing bacteria (King et al., 2015).

The degradation of hydrocarbons by microorganisms mostly involves oxygen combined with hydrocarbons to produce water and carbon dioxide achieved by the enzymes oxygenase and peroxidase (Figure 1). Hence, free oxygen is required for degradation to occur through these pathways. Shallow waters hardly lack oxygen; however, the concentration of oxygen can reduce as it travels through the water column and sometimes lacking

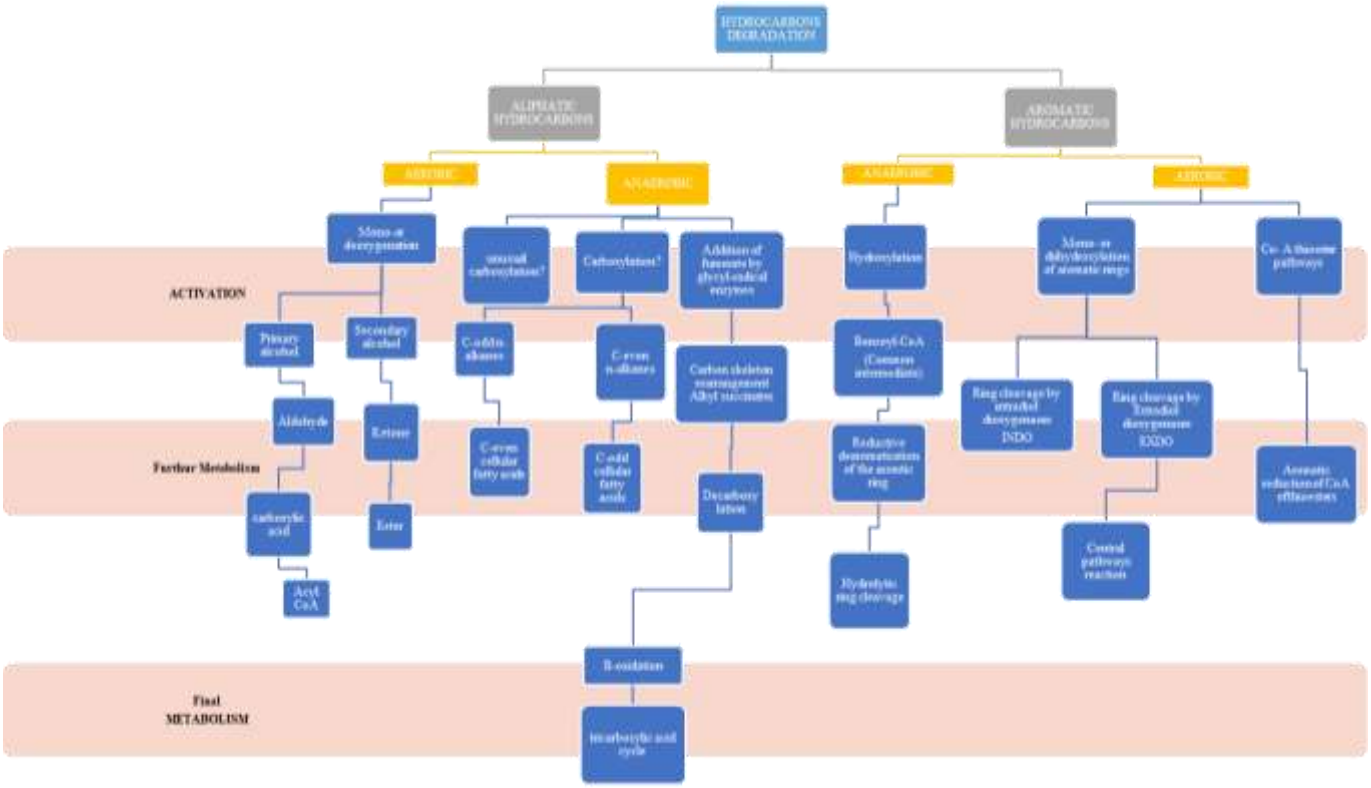

Figure 2. Pathway for microbial degradation of hydrocarbon.

Table 2. The enzymes involved in specific hydrocarbon degradation (Nilanjana and Preethy, 2011).

Enzymes	Substrates
Soluble-methane monooxygenases	C1–C8 alkanes alkenes and cycloalkanes
Particulate-methane monooxygenases	C1–C5 (halogenated) alkanes and cycloalkanes
AlkB-related, hydroxylases, alkane	C5–C16 alkanes, fatty acids, alkyl benzenes, cycloalkanes and so forth
Eukaryotic P450	C10–C16 alkanes, fatty acids
Bacterial P450 oxygenase system	C5–C16 alkanes, cycloalkanes
Dioxygenases	C10–C30 alkanes

in bottom waters. Most often, sediments usually lack oxygen (anoxic condition) below the surface; however, in some cases, oxygen is able to infiltrate below to the seabed (Roy et al., 2012). It is unlikely for anoxic condition on shallow slicks of the water plume to prevent or reduce biodegradation due to the ability of oil droplets to dilute quickly. Anoxic conditions can however occur in lower levels or bottom depths depending on the oil concentration and degree of oxygen renewal by waves and currents. The degradation of hydrocarbons by microorganisms via aerobic pathways (Figure 2) is known as aerobic biodegradation of hydrocarbons. The Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria are the major oil-degrading bacteria that utilize oxygen and are mostly outlined (Head et al., 2006; Van Beilen and Funhoff, 2007).

Biodegradation by aerobic microorganisms is activated by monooxygenase and dioxygenase enzymes (Austin and Callaghan, 2013). *Alcanivorax borkumensis* an oil-degrading microorganism and a marine gammaproteobacterium is widely studied and defined to consume wide array of aliphatic hydrocarbons (Dos Santos et al., 2010) through various pathways using terminal oxidation process through numerous enzymes (Table 2) introducing hydroxyl groups to the organic compounds (alkB1, P450 and cytochrome monooxygenase) (Sabirova et al., 2006).

Despite the fact that hydrocarbon degradation by aerobes symbolizes a fast and acknowledged method (Fritsche and Hofrichter, 2008), much consideration is

given to degradation by anaerobes (Heider and Schühle, 2013) for its role in marine ecosystems associated with oil spills (Head et al., 2006).

The scale of anaerobic hydrocarbon degradation is slow as compared to aerobic hydrocarbon degradation; however, it is mostly needed in marine sediments (Roy et al., 2012). When oxygen is limiting, Fe^{3+}, SO_4^{2-} and NO_3^- are known to occur for biodegradation of hydrocarbon (Foght, 2008). Marine harbor sediments are known to degrade petroleum hydrocarbons when oxygen is limiting and sulfate and iron-reducing conditions are present (Coates et al., 1996). For example, the Caspian Sea permanently lacks oxygen in bottom waters, so it is possible that any hydrocarbon degradation is under oxygen limitation. Microorganisms adapted to oxygen limiting conditions comprises of sulfate reducers, denitrifying and nitrate ammonifying bacteria, phototrophs, and metal ion reducers, with the ability of breaking down several hydrocarbons, ranging from n-alkanes and n-alkenes to the more stubborn aromatic compounds (Heider and Schühle, 2013).

The often discussed method for initiating and breakdown of hydrocarbon under anaerobic conditions is the addition of fumarate (Heider and Schühle, 2013). Other substitute methods defined include intra-aerobic hydroxylation, oxygen-independent hydroxylation and carboxylation (Callaghan, 2013a; Heider and Schühle, 2013) (Figure 2).

Activation for further metabolism in aerobic pathway requires terminal and sub-terminal reaction for aliphatic HCs and peripherical hydroxylation reactions for aromatic HCs. Anaerobic pathways require double reaction for this process (Garcia and Oliveira, 2013).

Post spill microbial response mechanisms

Research over time has proven that after an oil spill incident, microbial communities mostly make use of 3 major response mechanism from the water column, surface water and bottomless sea sediments to the shoreline sediments mainly change in succession of native microbial community structure, change in microbial diversity and gene diversity and modification (Engel and Gupta, 2014; Rodriguezr et al., 2015).

For instance, with respect to DWH oil spill, discharge was a Louisiana lighter crude oil (contains little sulfur and great gasoline/kerosene fractions as compared to denser oils); dominated by alkanes (saturated hydrocarbons), 16% aromatic hydrocarbons and 10% polar compounds (Reddy et al., 2012). Shortly succeeding the blast, shallow waters were covered with huge quantities of oil. However assumptions were undiscovered but then possibly bulky quantities of oil was confined in deep waters due to the great depth. In the first month of the DWH accident, a bottomless water oil column interrelated with dispersed MC252 oil was discovered at ~1100 m depth (Hazen et al., 2010). The column was composed of a composite combination of hydrocarbons comprising of alkanes, monoaromatic hydrocarbons (BTEX) and polycyclic aromatic hydrocarbons (PAHs) with concentrations up to189 µg/L (ppb) and extending principally in a west-south west direction from the still gushing wellhead (Reddy et al., 2012). These hydrocarbons are more often than not differentiated by known biomarkers and their corresponding fractions. Additionally, constituents of natural gas; methane, ethane and propane too was discovered at significant, then again doubtful levels (Joye et al., 2011; Reddy et al., 2012). This composite combination of hydrocarbons discharged at bottomless waters and in cold waters (4 to 6°C) lead to microbial biomass bloom in the column (Hazen et al., 2010).

In the water column, it was noticed that there was a substantial difference and variety deficit in deep and shallow oil slick populations when compared with populations at the non-oil impacted water column. This was due to the phylogenetic shift undergone by the indigenous populations (Redmond and Valentine, 2012; Yang et al., 2016). Findings according to Liu et al. (2017) stated the role of environmental factors especially temperature on microbial succession and shift. Further discoveries stated that succession patterns at every specific location were determined by the availability of specific hydrocarbon compounds (Dubinsky et al., 2013). The carbon branded hydrocarbons and segregation methods gave extra proof of fluctuating populations of impacted water column with ability of disintegrating several groups of hydrocarbons (Gutierrez et al., 2013). The 16S rRNA studies of microorganisms sample within the first and second month after the DWH spill saw the dominance of an uncultivated gammaproteobacterium from order *Oceanospirillales* dominating (Hazen et al., 2010; Redmond and Valentine, 2012; Mason et al., 2012). Even though GeoChip 4.0 submitted proof of enriched genes included in aerobic and anaerobic hydrocarbon degradation in the column, metatranscriptomic data obtained from the same cruise samples suggested aerobic degradation of hydrocarbons as most dominant on site procedure during that time (Mason et al., 2012). Genes that help in aerobic alkane degradation (e.g., alkane monooxygenase, cyclohexanol dehydrogenase and cyclohexanone monooxygenase) remained highly at significant peaks in the column metatranscriptomes. These genes were also found in single-cell genomes of *Oceanospirillales*, however aromatic hydrocarbon degradation were discovered in low levels or remained undiscovered (Mason et al., 2012). Around mid- to late June 2010, there was change in succession of microbial community in the water column, dominantly by two different gammaproteobacteria groups, *Cycloclasticus* and *Colwellia* (Redmond and Valentine, 2012). With regards to microcosm experiments, the crude oil enriched areas (methane, ethane, propane and benzene) were dominated by *Colwellia* spp., hence the anticipation of its

essential role of degrading ethane and propane *in situ* within this period (Redmond and Valentine, 2012). Transcriptomic data further revealed that the *Colwellia* spp. was active and succeeded preceding Oceanospirillales because of their gaseous and aromatic hydrocarbon degradation ability (Mason et al., 2014a). The *Colwellia* spp. experienced a bloom in June 2010 even though they were scarce in the column during late May 2010 (Mason et al., 2012). By September 2010, initially discovered microorganisms (*Oceanospirillales Cycloclasticus and Colwellia*) were substituted by initially undiscovered methylotrophic bacteria (*Methylococcaceae, Methylophaga* and *Methylophilaceae*), *Flavobacteria* and *Rhodobacterales* due to the presence of methane and methane oxidation observed in minute quantities in the September plume, in addition to the enrichments achieved by the September plume samples (Redmond and Valentine, 2012). Later in October 2010 and July 2011, the analysis of microbial community composition (clone libraries and pyrosequencing) from post-column samples showed likeness for the pre-spill pelagic community (800 m, March 2010) (Yang et al., 2016).

The surface water of the Gulf of Mexico in May 2010 showed dominating population of gammaproteobacteria and *cycloclasticus* (known to degrade aromatic hydrocarbons), similar to that of the deep water plume (Yang et al., 2016). However, pyrosequencing of oil mousses and clone library studies from other distinct sites within this same period showed a dominance (above 65%) of alpha and gammaproteobacteria and cyanobacteria (Redmond and Valentine, 2012), hence highlighting the distinction of surface slick communities from the bottomless water column. There is a possibility for both shallow slick and bottomless water column undergoing the same succession changes of microbial community composition dependent on type and availability of hydrocarbons at each definite time and location.

The deep-sea sediments are a home of various microbial communities (Bacosa et al., 2018, 2015b) similar in genera with the water column. After the DWH spill, large quantity of PAH compounds (>24,000 µg/kg) were observed in deep-sea sediments near the oil platform as compared to distant cores (~50 µg/kg); hence, the uncovering of the indigenous microflora to aromatic hydrocarbons near the oil platform (OSAT-I, 2010). Metagenomic analysis and targeted functional gene assays of subsurface (1.5 to 3 cm) deep sea sediment cores from September to October 2010 was highly dominated by Deltaproteobacteria and anaerobic degrading genes for aliphatic and aromatic hydrocarbons (For example, bssA, benzoyl-CoA reductase genes and assA) in the sediments located near the well (1-3 km) in espect to the distant (128 km) control samples (Kimes et al., 2013). Alphaproteobacteria was second-most abundant and was in line with alphaproteobacterial blooms observed in metagenomic analyses of seafloor sediments collected in September 2010 (Mason et al., 2014). Other discovered groups according to Yang et al. (2016) are the Deltaproteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes and Verrucomicrobia. The abundance of the groups increased or decreased over time depending on specific hydrocarbon availability and successional changes of microbial communities observed at the order, family or genus level of the various groups (Figure 3). The presence of Oceanospirillales, even though in low quantities with no variation at contaminated and non-contaminated sites, presented the genomic proof of aerobic hydrocarbon-degradation in sediments (Kimes et al., 2013).

Metagenomic analysis of samples taken 3 years after the spill (June 2013) still revealed genes related to the initial oxidation of aromatic rings, alkane monooxygenase and those involved in metabolizing hydrocarbon degradation by-products (alcohol and cyclohexanol dehydrogenases), and fatty acid metabolism was the most abundant (Bacosa et al. In Press). Bacterial communities of *Alteromonadales, Oceanospirillales*, *Methylococcales, Rhodobacterales* and *Bdellovibrionales* increased in oil treatments.

In coastal sediments/sands, oils discovered after an oil spill were extensively weathered; mostly reducing C9-C16 n-alkanes (0.3 to 1.6% of total composition as compared to 54% in MC252 oil) and BTEX/C3-benzenes (an order of magnitude lower levels) (Liu et al., 2012). The concentrations of PAH are also altered losing the dominant MC252 PAH and naphthalene, reduced to 3-9% of total PAHs in relation to 64% in unaltered MC252 oil. However, according to Engel and Gupta (2014), coastal sandy beach had shifting regime of microbial communities with respect to depth, location and time. Some of the dominant groups found on the open beach and swash zones were Gammaproteobacteria, Alphaproteobacteria, Firmicutes, Bacteroidetes, Enterobacteriales, Xanthomonadales and Chromatiales. Successional changes occurred in the various species of these groups over time. Aeppli et al. (2012) discovered high levels (10 times less in MC252) of oxyhydrocarbons (major component of tar balls) in beach sand after a long term study (18 months after spill). Kiruri et al. (2013) discovered obstinate free radicals on coastal beach Tar balls providing evidence of the oxidation of aromatic hydrocarbons with transition metals and weathering of hydrocarbon through multiple processes. However, it should be noted that these processes vary based on the site deposited.

Factors affecting microbial biodegradation

Microbial degradation of oil spills is a composite procedure influenced by several features of the oil and the surroundings. For example, the scope of oil droplets

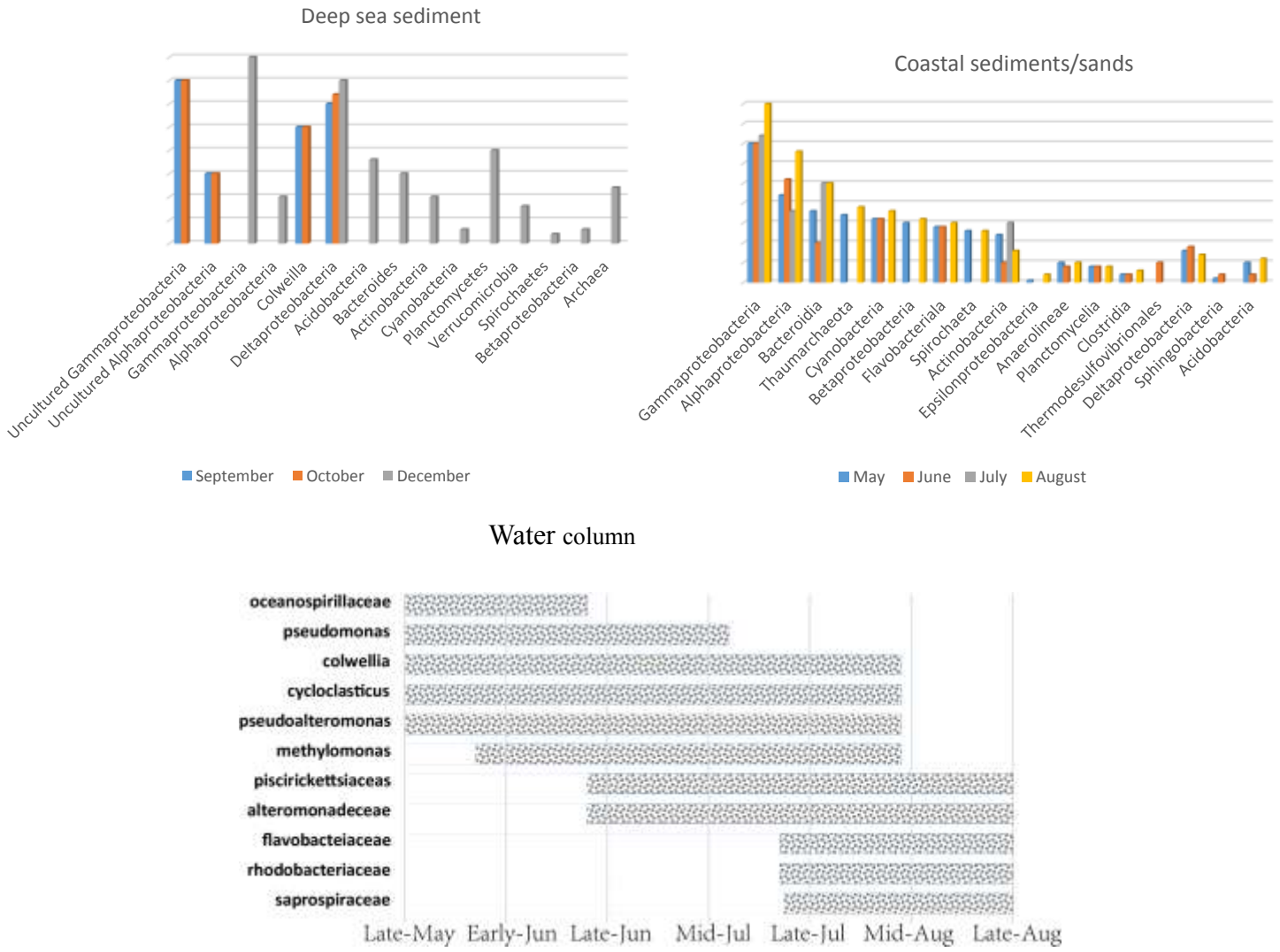

Figure 3. Overview of the relative abundance and successional change of microbial community at various sites in response to oil spills (Mason et al., 2014; Kimes et al., 2014; Rodriquez-R et al., 2015).

dissemination determines the prospects of the oil in the subsequent time frame (Yapa et al., 2012). Physical and microbial processes degrade tiny oil droplets easily (Hazen et al., 2015).

Dubinsky et al. (2013) established that the simultaneous operation of multiple hydrocarbon-degrading bacteria throughout the spill was regulated by change of hydrocarbon availability. For example, *Oceanospirillales* and *Pseudomonas* were the major genera in the initial stages of the oil spill with very high n-alkanes and cycloalkanes levels. Kleindienst et al. (2015) observed a change in deep plume community with respect to a change in quantity of hydrocarbon available (increased aromatic hydrocarbons and decreased petroleum hydrocarbons), that is, *Colwellia, Cycloclasticus* and *Pseudoalteromonas* dominating.

The process of biodegradation also involves degrading transformational byproducts and exopolymeric substances yielded by microbial communities to combine hydrocarbons and enable oil availability. The measurement of a higher peptidase activity and enzymatic hydrolysis of carbohydrates in the water samples within the oil spill plume when likened to the outside predicted that, the deep-water column microbial communities were structured to degrade singled out array of explicit high molecular weight substrates (Ziervogel and Arnosti, 2014). Gutierrez et al. (2013) researched on the function of amphiphilic exopolysaccharides produced by indigenous Halomonas bacteria for the period of the DWH oil spill. They discovered that strain TG39 produced Halomonas exopolysaccharides which efficiently amplified the solubility of aromatic hydrocarbons and improved their degradation, hence influencing oil removal and oil aggregates formation.

Nutrient deficiency is a normal limiting factor for microbial growth. Nitrogen and phosphorous are known as the key restrictive elements for oil degradation in the sea (Beyer et al., 2016). In oil-contaminated shallow waters of the Northern Gulf of Mexico (NGOM), it has been proposed that deficiency of dissolved nutrients mainly phosphate, reduces microbial growth (Liu et al., 2017). This is because when incubation mixtures were altered with nutrients, bacterial cell numbers and biomass improved speedily (Edwards et al., 2011). The addition of nitrogen fertilizers mainly on shorelines and uric acid at sea help stimulate and enhance oil bioremediation (Ron and Rosenberg, 2014).

Oceanic mixing and circulation processes are essential for microbial oil degradation (Beyer et al., 2016). The assessment of the impact of physical mixing processes on marine bacterial communities showcased biodegradation patterns of a faster growth rate for bacteria from seed populations that are scarce in nature. There is possibility of major participation of oceanic mixing process in allocating hydrocarbons, accompanied by bacterial blooms, and accelerated hydrocarbon degradation across an autoinoculation effect inside the northeast Gulf waters. The impacts of mixing and circulation processes on degradation of oil spills was shown by the higher abundance of HC-degrading bacteria in past bloomed waters that later re-circulated to the spill site, feeding successive bacterial blooms (Valentine et al., 2012).

Conclusion

In an era where crude oil production and consumption is on the rise, oil spills are inevitable in the marine environment. Even though the frequency and quantity of oil spills have reduced over the years, the marine environment still remains a reservoir for oil from natural seeps. Microbial communities have developed the ability to degrade these crude oil products. Bacteria from the phylum Proteobacteria has been known as the major crude oil degrading microbe. Other microbial groups: Firmicutes, Bacteroidetes and some arhaeal groups also have the ability to breakdown hydrocarbons. They have developed mechanisms such as acquisition of oil degrading functional genes, change in biodiversity where species with the ability to degrade specific hydrocarbons dominate and change in succession patterns. How microbial communities respond to crude oil has been far researched over the years; however, the amount and quantity of hydrocarbons degraded is still lacking. Other researches have also focused on the large fractions of oil introduced into the system leaving the residues, weathered components and the biodegraded byproducts which have detrimental effects on other marine organisms. More researches should focus on the concentration and quantity of hydrocarbons degraded by specific oil degrading microbial species and using oil residues from past spills to assess the impacts and possible ways for total ecosystem recovery.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Detection of alpha toxin and enterotoxins of *Clostridium perfringens* isolated from minced meat by real time polymerase chain reaction (PCR)

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Clostridium perfringens **is one of the most widespread pathogen producing toxins related to variable pathogenic conditions, particularly food poisoning in humans. Thus, this study described the prevalence, enumeration, toxigenic types and antibiotic susceptibility of *C. perfringens* strains isolated from minced meat in Egypt as well as the validation of a real-time polymerase chain reaction (PCR) test for the identification of *C. perfringens* toxin genes. A high prevalence of *C. perfringens* (98/105, 93.3%) was detected in minced meat samples. The total count of viable *C. perfringens* in 23 samples was 2.0 × 10^2 to 4.5 × 10^2 with a mean value 3.7 × 10^2 ± 1.07 × 10^2 CFU/g. The toxin typing of *C. perfringens* based on lecithinase activity and dermonecrotic reactions in albino guinea pig exhibited 33 (33.7%) as toxigenic strains of *C. perfringens* type A and 65 (66.3%) as non-toxigenic strains. Antibiotic susceptibility testing of isolates against 15 different antimicrobial agents indicated that *C. perfringens* was extremely sensitive to penicillin, followed by erythromycin, tetracycline, doxycycline and amoxicillin. All the other drugs were relatively less effective against the isolates. The real time PCR (RT-PCR) was performed for screening of alpha (*cpa*), beta, epsilon, iota toxins and enterotoxin (*cpe*) genes in toxigenic isolates of *C. perfringens* type A. All toxigenic strains of *C. perfringens* type A (33, 33.7%) were positive for alpha toxin (*cpa*) and enterotoxin (*cpe*) genes, while none of these isolates carried beta, epsilon and iota toxin genes. To the best of the authors' knowledge, this is one of the studies that used RT-PCR for the determination of toxigenic strains of *C. perfringens* in Egypt. It is suggested that RT-PCR could be used instead of the conventional culture procedures for identification of *C. perfringens* in minced meat in Egypt.**

Key words: *Clostridium perfringens*, minced meat, alpha toxin (*cpa*) gene, enterotoxin (*cpe*) gene, real time-polymerase chain reaction (RT-PCR).

INTRODUCTION

Meat and meat products as a source of vital nutrients represent an essential part of the human food because

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humans cannot easily get these nutrients through vegetables and their derived products (Byers et al., 2002; Basyoni, 2003).The microbiological quality and safety of commercially processed meat and poultry products have a special concern for producers, consumers and public health officials all over the world (Okolocha and Ellerbroek, 2005). *Clostridium perfringens,* as a Gram-positive spore producing and non-motile bacilli, inhabits the environment (soil) and intestinal tract of humans and animals (Hayes, 1992; Labbe and Juneja, 2006).

C. *perfringens* is one of the most common clostridia genus isolated from minced meat and related to food poisoning in humans. The pathogenicity of *C*. *perfringens* organisms is connected to numerous toxins that are also evaluated for bacterial toxin typing, within them, all toxigenic isolates of the bacteria yield alpha (α) toxin coded by *cpa* gene. The other major lethal toxin formed by the organisms are beta (β), epsilon (ε) and iota (ι) toxins which are thoroughly associated with the virulence of bacterium (Hatheway, 1990; Titball et al., 1999). Besides these major lethal toxins, some isolates with a percentage of 0 to 5% have an ability of forming enterotoxin coded by *cpe* gene which is the major reason for public *C*. *perfringens* type A food poisoning (Mcclane, 2007; Juneja et al., 2010).

The exposure of meat dishes or meat products to insufficient cooking with the presence of high counts of *C*. *perfringens* in them is responsible for food outbreaks. The meat and meat products can be contaminated with *C*. *perfringens* through variuos sources, mostly internally from animals after slaughtering as post mortem invasion or externally from polluted hands, animals skin, soil, water and processing equipments (Satio, 1990). The toxin neutralization test is classically utilized in mice or guinea pigs for typing of *C*. *perfringens* (Stern and Batty, 1975; Mcdonel, 1986). This method is time consuming and costly; thus, it has mainly been substituted by molecular techniques for example, polymerase chain reaction (PCR), especially the real time PCR for typing of *C*. *perfringens* in the last years (Baums et al., 2004; Chon et al., 2012). Therefore, the present study was undertaken to throw light on the occurrence, enumeration, typing, chemotherapeutic agents susceptibility and determination of the toxin profile (alpha, beta,epsilon, iota and enterotoxin) of *Clostridium perfringens* strains in minced meat via real time-PCR (RT-PCR) technique in Egypt.

MATERIALS AND METHODS

Samples collection

In total, 105 samples from minced meat were obtained from large supermarket, butcher shops and retail meat shops distributed in different geographic areas in Mansoura province, Egypt during September to December, 2016. The samples were immediately transferred to the laboratory in sterile polyethylene bags placed inside an icebox and subjected to required investigation without delay.

Isolation and identification of *C. perfringens*

The samples were enriched in freshly prepared cooked meat media (CMM), and then incubated anaerobically using anaerobic jar at 37°C for 24 h. A loopful from the inoculated medium was subcultured onto 10% sheep blood agar supplemented with neomycin sulphate (200 µg/ml) and incubated anaerobically at 37°C for 48 h. The presumptive colonies were picked and subjected to standard morphological and biochemical identification (nitrate reductive, sugar fermentation, gelatin liquefaction, indole, methyl red and Vogus Proskauer tests) (Koneman et al., 1992).

Enumeration of viable *C. perfringens* in minced meat

The counting of *C. perfringens* was performed based on FAO (1992) Briefly, twenty-five grams of each samples were aspectically taken and homogenated in stomacher 400 (Seward, UK) with 25 ml of 0.1% peptone water to provide original dilution 1/10, followed by serial two fold dilutions. The pour plate method was performed using tryptose sulphite cycloserine (TSC) agar followed by incubation of the plates anaerobically at 37°C for 20 h. Next, the number of suspected (black) colonies was calculated for the plate having an optimal counting more than 20 colonies. Not regarding the count, a maximum of ten colonies were picked up for verification from each sample. The interpretation of results occurred as colony forming units (CFU) per gram of the sample.

Test of Nagler's reaction (lecithinase activity)

This test was applied on the positive *C. perfringens* isolates as described by Smith and Holdeman (1986).

Typing of *C. perfringens* isolates

The typing of *C. perfringens* isolates was done for toxigenic and non-toxigenic strains by dermonecrotic test in albino guinea pigs as recommended by Stern and Batty (1975).

Sensitivity of *C. perfringens* isolates to chemotherapeutic agents

The disc diffusion assay was employed on a pure subculture from isolates of *C. perfringens* based on the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC, 2011). The 15 most effective antibiotics (Oxoid) frequently utilized for treatment of *C. perfringens* infections were examined. In brief, the antibiotic discs were placed on the surface of seeded Muller Hinton agar (Oxoid) plates, followed by their incubation anaerobically at 37°C for 24 h. *C. perfringens* ATCC 13124 was used as a control strain. The sensitivity was judged as stated by BSAC approaches for antimicrobial susceptibility testing (2011). The isolates categorized as intermediate were regarded as sensitive to simplify the data analysis.

Real-time polymerase chain reaction (RT-PCR) for *C. perfringens* toxin genes determination

The real-time PCR was applied for screening of alpha (*cpa*), beta (*cpb*), epsilon, iota (*cpi*) toxin genes and enterotoxin (*cpe*) gene in toxigenic isolates of *C. perfringens* type A. The bacterial DNA was extracted from isolates by the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with some changes of the manufacturer's recommendation. Specific primers and cycling were used in this

Table 1. Oligonucleotide primers, sequences, target genes, amplicon sizes and cycling conditions for SYBR Green RT-PCR.

Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	References
Alpha toxin (cpa)	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402	Yoo et al. (1997)
Beta toxin (cpb)	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAGAC	236	
Epsilon toxin	ACTGCAACTACTACTCATACTGTG CTGGTGCCTTAATAGAAAGACTCC	541	
Iota toxin (*cpi*)	GCGATGAAAAGCCTACACCACTAC GGTATATCCTCCACGCATATAGTC	317	
Enterotoxin (*cpe*)	ACATCTGCAGATAGCTTAGGAAAT CCAGTAGCTGTAATTGTTAAGTGT	247	Kaneko et al. (2011)

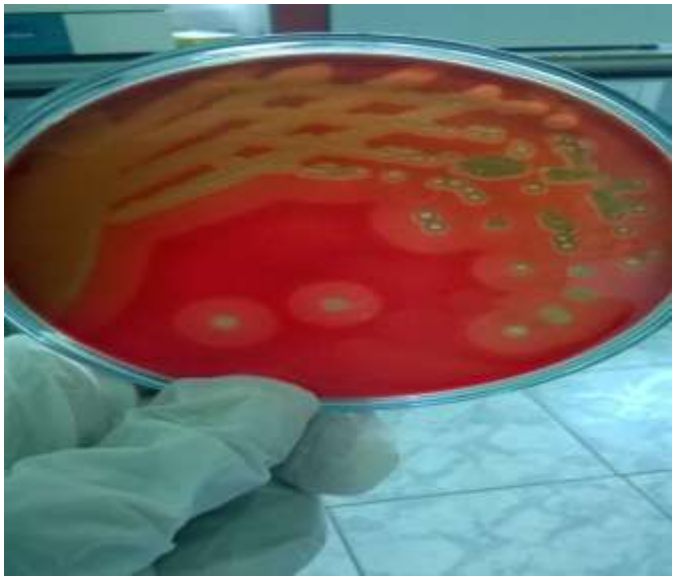

Figure 1. *C. perfringens* isolates showing double zone of haemolysis on sheep blood agar.

assay as described by Yoo et al. (1997) and Kaneko et al. (2011) (Table 1). Primers were utilized in 25 ml reaction, comprising of 12.5 ml of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.5 ml of each primer of 20 pmol concentration, 6.5 ml of water and 5 ml of DNA template. The reaction was achieved in a stratagene MX3005P real time PCR machine with the following program: one cycle for 5 min at 94°C, after that, 40 cycles consisting of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and then one cycle for 1 min at 95°C, 1 min at 55°C and 30 s at 95°C as the dissociation curve assay.

Statistical analysis

The data obtained were evaluated using statistical package for social science, version 17 (SPSS Software, SPSS Inc., Chicago, USA) and stated as means ± standard deviation (SD).

RESULTS

The prevalence of *C. perfringens* in minced meat

C. perfringens was isolated from 98 (93.3%) out of 105 different minced meat samples with regards to traditional methods (Table 2). *C. perfringens* isolates were Gram positive short plumb rarely sporulated and non motile bacilli. *C. perfringens* isolates revealed double zone of haemolysis on sheep blood agar (Figure 1) and black zone on TSC (Figure 2).

Table 2. Prevalence and typing of *C. perfringens* isolated from minced meat samples.

Samples	Positive samples	Lecithinase positive isolates	Type of toxigenic isolates							
			A		B		C		D	
			No.	%	No.	%	No.	%	No.	%
Minced meat (105)	98 (93.3%)	33 (33.7%)	33	33.7	0	0	0	0	0	0

Figure 2. *C. perfringens* isolates showing black zone on tryptose sulphite cycloserine agar.

Enumeration of *C. perfringens* isolates

Total count of viable *C. perfringens* in the examined minced meat (98) were less than 10 CFU/g in 75 samples and more than 20 colonies in the remaining (23) samples. These 23 samples showed total count of 2.0 $\times 10^2$ to 4.5 $\times 10^2$ with a mean value $3.7 \times 10^2 \pm 1.07 \times 10^2$ CFU/g (Table 3).

Toxin typing of *C. perfringens* isolated from minced meat

Nagler's test (Lecithinase activity) represented the action of *C. perfringens* alpha toxin on Lecithin of egg yolk which appeared as pearly opalescence zone surrounding the colonies, while this reaction was inhibited by *C. perfringens* alpha toxin antiserum (Figure 3). The lecithinase activity was detected in 33 (33.7%) strains of *C. perfringens* in the examined minced meat.

Moreover, toxin typing of lecithinase positive *C. perfringens* based on dermonecrotic reactions in albino guinea pig showed that 33 (33.7%) strains were determined as *C. perfringens* type A, while other strains were regarded as non-toxigenic strains (65, 66.3%) (Table 2 and Figure 4).

Antibiotic susceptibility of *C. perfringens* isolates

The sensitivity of *C. perfringens* isolates derived from minced meat to 15 different antibiotics was determined. The C. *perfringens* isolates were highly sensitive to penicillin (88, 89.8%), followed by erythromycin, tetracycline (65, 66.3%, each), then doxycycline and amoxicillin (64, 65.3 % each). In contrast, *C. perfringens* isolates were resistant to ofloxacin (96, 97.95%), streptomycin, cloxacillin (94, 95.9% each), amikacin (90, 91.8%), trimethoprime sulphamethazole (85, 86.7%), oxytetracycline (83, 84.7%), cephalothin, cefeprime (80,

Table 3. Total viable count of *C. perfringens* isolated from minced meat (n=98).

Count of *C. Perfringens* (CFU/g)	No. of samples
4.5×10^2	6
4.1×10^2	4
2.4×10^2	5
2.3×10^2	3
2.2×10^2	2
2.0×10^2	3
Total	23
*Mean ± SD	3.2×10^2 ± 1.07×10^2
Less than10 CFU/g	75
Overall total	98

*The mean of bacterial count ± standard deviation (SD).

Figure 3. Lecithinase activity of *C.perfringens* alpha toxin on lecithin of egg yolk agar appearing as pearly opalescence zone surrounding the colonies

81.6% each) and kanamycin (78,79.6%) (Table 4).

RT-PCR for toxin produced by *C. perfringens* determination

The RT-PCR showed that all tested *C. perfringens* type A (33, 100%) harbored alpha toxin gene (*cpa*) (Figure 5) and enterotoxin gene (*cpe)* (Figure 6). On the other hand, they were negative to beta, epsilon and iota toxin genes.

DISCUSSION

Food illness related to *C. perfringens* is one of the major diseases associated with the ingestion of contaminated food, particularly meat and poultry products. It has been severely developed that the production of enterotoxins in the intestine from ingested vegetative cells is related to these diseases (Duncan, 1973). In the last years, many investigations were established on the prevalence of *C. perfringens* in raw and processed meat as well as poultry.

Table 4. Antimicrobial sensitivity test of C. *perfringens* isolates (n=98).

Antimicrobial agents	Code	R		S	
		No.	%	No.	%
Ofloxacin	OFX5	96	97.95	2	2.1
Streptomycin	S10	94	95.9	4	4.1
Cloxacillin	Cx1	94	95.9	4	4.1
Amikacin	AK30	90	91.8	8	8.2
Ampicillin	Amp10	87	88.8	11	11.2
Trimethoprime-sulphamethazole	SXT25	85	86.7	13	13.3
Oxytetracycline	T30	83	84.7	15	15.3
Cephalothin	V1f30	80	81.6	18	18.4
Cefeprime	Fep30	80	81.6	18	18.4
Kanamycin	K30	78	79.6	20	20.4
Doxycycline	DO30	34	34.7	64	65.3
Amoxicillin	Ax25	34	34.7	64	65.3
Erythromycin	E15	33	33.7	65	66.3
Tetracycline	TE30	33	33.7	65	66.3
Penicillin	P10	10	10.2	88	89.8

S: Sensitive; R: resistant; %: percentage of sensitive isolates.

Figure 4. The dermonecrotic reactions in albino guinea pig displayed by *C. perfringens* type A.

These documents indicated wide spread of *C. perfringens* in raw and processed meat and poultry (Labbe and Doyle, 1989; Labbe et al., 2000). Therefore, the high prevalence of *C. perfringens* (93.3%) in minced meat in the present study is not surprising. These results confirmed the findings of the previous studies obtained by Miwa et al. (1998), Mcclane (2007) and Grass et al. (2013) who noted the high prevalence *of C. perfringens* in market meats. Similarly, Guran et al. (2014) found that 96 and 88% of the ground beef and sheep meat samples were contaminated with *C. perfringens* in Turkey, respectively. Also, Wen and Mcclane (2004) isolated *C. perfringens* from 66% ground meat samples. Additionally, Kamber et al. (2007) isolated *C. perfringens* from 55% of minced meat samples in Turkey. On the other hand, a lower prevalence of *C. perfringens* in minced meat was

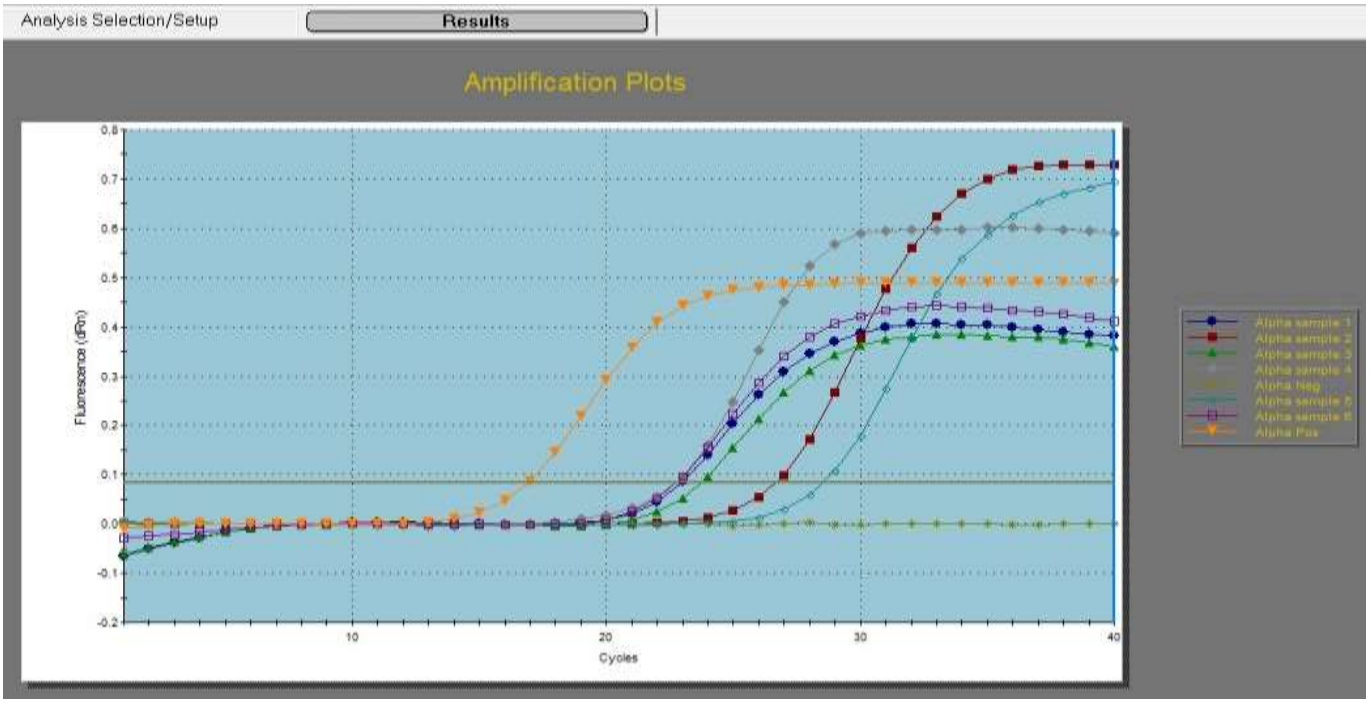

Figure 5. Representative real-time PCR amplification plots for alpha toxin (cpa) gene in toxigenic strains of *C. perfringens* isolated from minced meat samples that show threshold amplification cycle at 14 to 28 cycles.

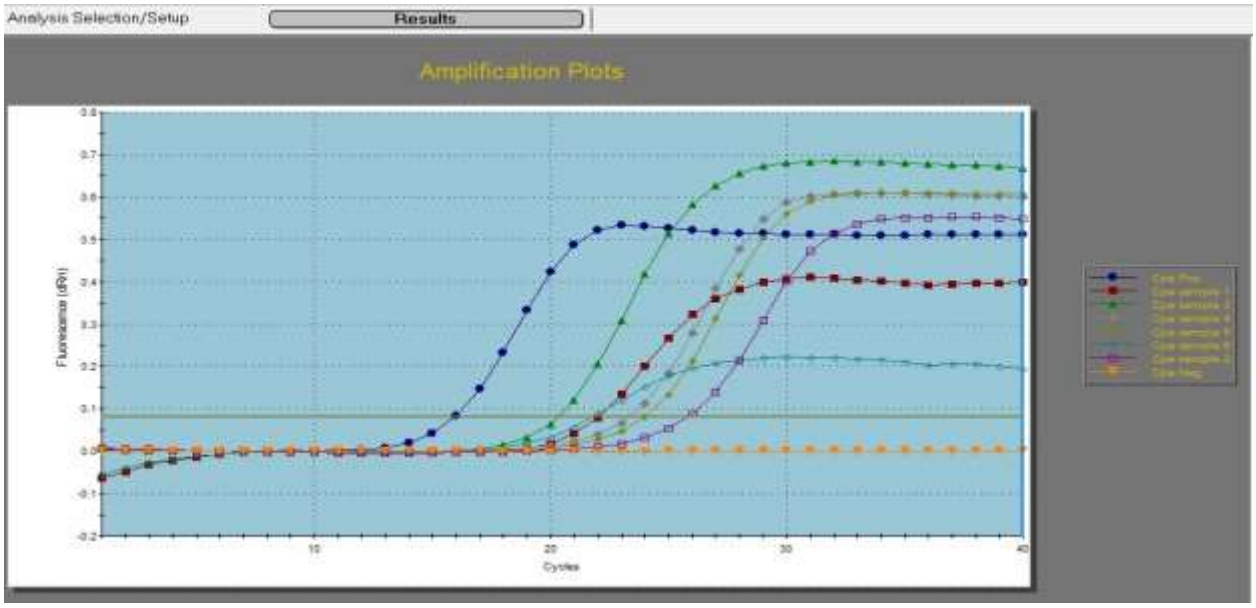

Figure 6. Representative real-time PCR amplification plots for enterotoxin (*cpe)* gene in toxigenic strains of *C. perfringens* isolated from minced meat samples that show threshold amplification cycle at 13 to 26 cycles.

recorded as 12.5% by Afshari et al. (2015), 16.67% by Abd El-Tawab et al. (2015), 20% by Hamoda (2012) and 40% by Alkheraije (2013). The lowest prevalence was found by Herrer (1995) as 7.1%. The variation in the incidence of *C. perfringens* in minced meat may be related to the original contamination of minced meat and poor hygienic measures in processing factors. The cutting, handling and wrapping procedures could

separately be associated with the adding of *C. perfringens* spores and vegetative cells.

Food poisoning occurs due to ingestion of foods containing large populations of viable vegetative cells of *C. perfringens* and the subsequent production of toxins in the intestine. The presence of about 1 million organisms/gram of food was necessary to produce food poisoning after ingestion of such food (Johnson et al., 2007). Thus, enumeration of *C. perfringens* in food is usually done to investigate the suspected involvement of this bacterium in food poisoning. The current study revealed that the total count of viable *C. perfringens* in 75 examined minced meat samples were less than 10 CFU/g. Thus, the count of such samples was neglected because the anaerobic counts of the examined samples were within the permissible limits requested by the Egytian Standard Specification and not enough to create food poisoning in humans, and millions of viable *C. perfringens*/g may be needed for induction of food poisoning in humans. However, the current study showed total count of viable *C. perfringens* in the remaining 23 samples with a mean value of $3.7 \times 10^2 \pm 1.07 \times 10^2$ CFU/g that might pose hazards. These results were consistent with Kamber et al. (2007) who stated that the average value of *C. perfringens* recovered from minced meat were 2.75×10^2 and 6.82×10^2 CFU/g from local markets and butcher's shops, respectively. Also, Ali (2009) documented $1.7 \times 10^2 \pm 3.5 \times 10$ and $2.1 \times 10^3 \pm 1.1 \times 10^3$ CFU/g as the average of *C. perfringens* count of fore and hind quarters of raw cattle meat, respectively. In addition, El-Atwa and Abou El-Roos (2011) recorded a lower mean count of 1.2×10 of C. *perfringens* in minced meat. However, other studies by Abo Zaied (1998) and El-Melegy (2015) showed a higher mean count of *C. perfringens* with an average of $2.2 \times 10^4 \pm 3.8 \times 10^3$ CFU/g in meat samples. Shaltout et al. (2017) enumerated the total count of vegetative form of *C. perfringens* in the tested raw beef samples as 1.7×10^2 to 2.50×10^3 with an average of $6.22 \times 10^2 \pm 2.35 \times 10^2$ CFU/g. It is likely that a high prevalence of *C. perfringens* in minced meat is indicative of neglect of sanitary measures during production and handling of this product. Furthermore, presence of this bacterium in large numbers could constitute a public health hazard.

Toxin typing of *C. perfringens* strains revealed that the prevalence of *C. perfringens* type A was 33.7% in minced meat samples, whereas *C. perfringens* type B, C and D were not identified. Similarly, Shaltout et al. (2017) detected the high incidence of *C. perfringens* type A (50%) amongst strains isolated from raw beef samples with the absence of other toxin types in Egypt. El-Jakee et al. (2013) demonstrated that *C. perfringens* belonging to type A was the most dominant ones in poultry. On the other hand, this result was higher than that obtained by Emara (2014) who documented the occurrence of *C. perfringens* type A in meat samples as 16.73% in Egypt. Additionally, this result was lower than literature reports that indicated the prevalence of *C. perfringens* type A in 77.4% of the ground beef and sheep meat samples in Turkey (Guran et al., 2014) and 81% of minced meat in Iran (Afshari et al., 2015). Also, Kamber et al. (2007) determined 12, 1, 4 and 2% of minced meat samples contaminated with *C. perfringens* types A, B, C and D in Turkey, respectively.

In the last decades, the development of antimicrobial resistance among pathogenic bacteria is widespread. Hence, the *C. perfringens* isolates were tested for their antibiotic sensitivity to 15 frequently used antibiotics belonging to different antimicrobial classes to assess the most appropriate antibiotic for *C. perfringens* infection. This study reveals the high resistance of C. *perfringens* isolates from minced meat to the most examined antibiotics. Ofloxacin, streptomycin, cloxacillin, amikacin and trimethoprime sulphamethazole were the least effective antibiotic as most of the strains were resistant to these agents followed by oxytetracycline, cephalothin, cefeprime and kanamycin. These results are compatible to previous studies documented by Johansson et al. (2004) and Silva et al. (2009). This resistance of *C. perfringens* to these antibiotics is due to the excessive use of these agents either as therapeutic agent or growth promotor in the food of farm animals. However, a higher sensitivity of *C. perfringens* isolates to penicillin was noticed, followed by erythromycin, tetracycline, doxycycline and amoxicillin. The observations were similarly detected by Skariyachan et al. (2010), Abd El-rhman (2015) and Khan et al. (2015) where *C. perfringens* recovered from meat exhibiting susceptibility to penicillin, ampicillin and tetracycline while the organisms were moderately sensitive to erythromycin and vancomycin. Consequently, these antibiotic agents were proved to be most effective drugs against these isolates based on their high rate of sensitivity.

C. perfringens is still a common cause of food borne diseases through its ability to produce toxins particularly alpha toxin (*cpa*) and enterotoxins (*cpe*) which are responsible for food poisoning (Schalch et al., 1999). The present investigation showed that all tested *C. perfringens* type A (33, 100%) harbored alpha toxin (*cpa*) gene and *cpe* gene by RT-PCR. In contrast, these isolates were negative to beta, epsilon and iota toxin genes. The application of RT-PCR showed specificity of the oligonucleotide primers that was verified by positive amplification of 402 bp fragments for *C. perfringens* alpha toxin genes (*cpa*) and 247 bp fragments for *C. perfringens* enterotoxin genes (*cpe*) from DNAs extracts of all tested isolates from minced meat. These results were compatible with Guran et al. (2014) and Abd Eltwab et al. (2016) who found alpha toxin (*cpa)* gene in all *C. perfringens* type A isolates. Hence, it is clarified that the results obtained by RT-PCR provided a good compatibility with the results obtained by conventional culture means. Also, in contrast to conventional culture approach, the RT-PCR assay is a rapid and specific tool

and has probable practice as an identifying method for enterotoxigenic C. *perfringens* in food samples, considering its detection ability and time-saving efficiency (Singh, 2005; Albini et al., 2008; Yang et al., 2010; Chon et al., 2012). A lot of studies have used RT-PCR for detection of *C. perfringens* and their toxin genes in different samples (Wu et al., 2011; Chon et al., 2012). Albini et al. (2008) identified toxigenic strains of *C. perfringens* in animal isolates using RT-PCR. Mizher et al. (2016) revealed alpha (cpa) toxin genes of *C. perfringens* in 40 and 70% of cattle and sheep, respectively using real time PCR. In previous studies, the *C*. *perfringens* enterotoxin genes (*cpe*) were identified in 2.2 and 28.57% of examined isolates using multiplex PCR (Guran et al., 2014; Abd Eltwab et al., 2016), while, Razmyar et al. (2014) found that all isolates of *C. perfringens* obtained from ostrich flocks carried alpha toxin gene (*cpa*) and absence of enterotoxin gene (*cpe*) by multiplex PCR. Also, Afshari et al. (2015) detected 81% of alpha toxin (*cpa*) gene and absence of *cpe* gene in minced meat isolates by multiplex PCR. However, few studies are available on the use of such technique (RT-PCR) for estimation of *C. perfringens* and their toxin genes in minced meat in Egypt.

Conclusion

This investigation was concluded on the high prevalence of *C. perfringens,* particularly type A in minced meat, which is regarded as a public health hazard to consumers in Egypt. In this respect, strict hygienic measures and suitable regulations should be imposed for production, handling and distribution of minced meat to safeguard consumers. Moreover, the real time PCR is a promising molecular method for the rapid determination of toxigenic strains of *C. perfringens* instead of conventional microbiological techniques as it is much faster and more accurate.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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