Clostridium perfringens in foods and fish

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Foodborne diseases are considered an important public health problem at a global level due to their levels of incidence and mortality as well as their negative consequences in economic and social aspects. Foodborne diseases are defined as those that are generated by the ingestion of food and water contaminated by chemical or biological agents commonly affecting health at the level of the gastrointestinal system. Among the risks and dangers to health from food are the diseases caused by *Clostridium perfringens*, a common bacterium which inhabits water, soil, vegetables, fish, the gastrointestinal system of human and animals and of course foods. The importance of this bacterium in health and food lies both in its cosmopolitan distribution, ability to generate heat-resistant spores and food poisoning, which makes control and prevention actions indispensable along the food chain. This article presents a general description of foodborne diseases, including those caused by consumption of food, such as fish, derived from contamination by *C. perfringens*; likewise, the actions and recommendations undertaken around the world for the prevention and control of these diseases are shown, including aspects related to the antimicrobial resistance phenomenon and its impact on public health.

Keywords: foodborne diseases; food safety; microorganisms; gastrointestinal symptoms

Foodborne diseases generalities

Foodborne diseases (FD) are those caused by the ingestion of food and water contaminated by microorganisms or chemical substances, the most common clinical manifestation being the appearance of gastrointestinal symptoms (WHO, 2018). These diseases are considered an important public health problem at a global level due to their incidence and mortality together with the negative economic and social repercussions (Palomino & Gonzalez, 2014; Lorenzo & Gálvez, 2015).

Estimates from the World Health Organization (WHO) indicate that annually around the world FD cause 420,000 deaths, of which one third are of children; while for the Americas region they indicate that around 77 million people fall ill and more than 9,000 die from these diseases (WHO, 2018a). In the United States alone, it is estimated that around 76 million people suffer from FD, 325,000 are hospitalized and 5,000 die each year, implying high health services costs (Olea et al., 2012). Factors such as the mobility of populations, growing urbanization, new forms and industries of production and sale of food, changes in eating habits, the globalization of the market and the lack of knowledge or perception of risk to health by all those involved in the chain of food production and service have contributed to a higher rate of contamination and incidence of diseases in consumers (Olea et al., 2012; Palomino & Gonzalez, 2014; Lorenzo & Gálvez, 2015).

Food due to its composition in water and nutrients is a favorable environment for microbial growth and in turn constitutes a vehicle for the transport of different diseases. Furthermore, if warm environmental conditions are present, microbial growth can be further favored by unhygienic practices during the handling of products; pathogenic microorganisms can be transferred to the surface of food from many sources (soil, water, insects, food handlers), which generates health risks for the consumer (Barbosa & Bermudez, 2010). Food contamination can be widespread and varied, with approximately 250 causal agents of diseases described; these contaminants can be of physical, chemical and biological origin, among which are included bacteria, viruses, fungi, parasites, prions, toxins and metals that can compromise the consumer's health; where contamination by biological agents (bacteria, fungi, viruses and parasites) represents the highest incidence and risk to health (Barreto et al., 2010; Olea et al., 2012; PAHO, 2016). Among the bacteria generally involved in the production of diseases through food are *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Clostridium perfringens*, *Campylobacter jejuni*, *Salmonella* spp., *Shigella* spp., among others (USDA, 2013).

This article presents a general description of foodborne diseases, including those caused by food consumption, such as fish deriving from contamination by microbial agents such as *C. perfringens*; likewise, the actions and recommendations for the prevention and control of these diseases are shown; in addition to showing the incidence of the phenomenon of antimicrobial resistance by this pathogenic bacterium.

The genus *Clostridium*

The genus *Clostridium* is formed by around 150 species, which are both phylogenetically and phenotypically heterogeneous. Some species are of importance in various industrial areas, such as *C. acetobutylicum*, *C. beijerinckii* and *C. butirycum*, since they produce organic solvents (Castaño, 2008), *C. pasteurianum* are involved in deterioration in storage stability of food and juices that undergo inadequate thermal processes (Feng et al., 2010) while species such as *C. botulinum*, *C. perfringens*, *C. tetani* and *C. difficile* are of clinical importance and must be considered pathogenic to food, giving rise to diseases by action of extracellular toxins (Rhodehamel & Harmon, 2001; Morris & Fernandez, 2008; El-Shorbagy et al., 2012; García et al., 2016). The bacteria of

the genus *Clostridium*, are characterized by having a vegetative form in the form of bacilli. They are gram positive, anaerobic, do not form pili and fimbriae, have peritrichous flagella except for *C. perfringens* and form spores and toxins. They are located generally in nature and commonly as a constituent part in the gastrointestinal system of animals, spreading through food, thus generating food intoxication (Romero, 2007; Elika, 2013). The species associated with diverse pathologies in humans, including the alimentary toxi infections (*C. difficile*, *C. novyi*, *C. septicum*, *C. histolyticum*, *C. sordelli*, *C. fallax*, *C. botulinum* and *C. perfringens*); the toxins of the latter affect the nervous and gastrointestinal system respectively (Romero, 2007; Elika, 2013; OPS, 2016).

Clostridium perfringens

C. perfringens is a bacterium belonging to the family Bacillaceae in the form of a bacillus measuring: 4–8 µm x 0.3–1.5 µm, Gram positive, capped, non-mobile, sporulated, it is relatively aerotolerant, catalase negative, fermented carbohydrates (glucose, lactose, sucrose, fructose, maltose and galactose) with production of acid and carbon dioxide, produce H_2S and hydrogen, its optimal growth temperature is 37–45 °C and it tolerates NaCl concentrations of 2%, is anaerobic but able to grow at values of Eh of +350 mV and reduce its environment to less than –400 mV, it has a cosmopolitan distribution in the environment mainly in soil, wastewater, spices, vegetables, raw foods, processed foods and in the gastrointestinal system of man and animals (Pascual & Calderón, 2000; Morris & Fernández, 2008; Massoc, 2008; Santos & Heredia, 2011; Gamboa et al., 2011; El-Shorbagy et al., 2012; CDC, 2017). Being a microorganism sporulated under unfavorable conditions or growth stress in its vegetative forms, it generates heat-resistant spores as forms of survival, whose process is linked to the production of toxins (Pascual & Calderón, 2000; Morris & Fernandez, 2008; Massoc, 2008; El-Shorbagy et al., 2012). A high correlation has been established between the ability of *C. perfringens* strains to produce toxins and generate food poisoning and conditions such as necrotic enteritis and gas gangrene (Rhodehamel & Harmon, 2001; El-Shorbagy et al., 2012). The ability of *C. perfringens* to generate spores by sulfite-reducing clostridia, being inhabitants of the gastrointestinal system of mammals and resistant to different conditions of environmental stress, makes them useful as indicators of fecal contamination of water for human use and consumption which can put health at risk (Rios et al., 2017).

Food poisoning by *C. perfringens* tends to be self-limiting, symptoms appearing suddenly such as watery diarrhea, severe abdominal pain and cramping 8 to 16 hours after ingestion lasting approximately 24 hours. The vast majority of outbreaks of disease are related to inadequate handling, preparation and preservation of food, such as temperature control, especially in meat and meat products (Perdomo & Melendez, 2004; Massoc, 2008; CDC, 2017). It is estimated that *C. perfringens* is capable of generating toxinfection through ingestion in foods with inocula above 10^5 to 10^8 CFU/g (Pascual & Calderón, 2000; Santos & Heredia, 2011; PAHO, 2016). The population in general is susceptible to poisoning by this pathogen but children under 5 years of age, older adults, pregnant women and people with weakened immune systems are at higher risk of *C. perfringens* infection and experience more severe symptoms (CDC, 2017).

The virulence factors attributable to *C. perfringens* include the capacity to generate different toxins. Among those one usually finds the toxin alpha (α), beta (β), epsilon (ε) and iota (ι) that are used for the bacterial classification of the species into five toxinotypes from A to E according to the toxins produced where the type A produces (α), B produces (α), (β) and (ε), C produces (α) and (β), D produces (α) and (ε) and E produces (α) e (ι) (Morris & Fernandez, 2008; Gamboa et al., 2011; El-Shorbagy et al., 2012). This microorganism also generates different enzymes hydrolytics: lecithinases, hemolysins, hyaluronidases, collagenases, DNAase and amylases (Santos & Heredia, 2011). It also presents the synthesis of other protein toxins that also contribute to virulence but are not considered for their classification, such as CPE enterotoxin encoded by the cpe gene responsible for diarrhea in humans and animals, the NetB associated with necrotic enteritis in birds and β_2 toxin, related to enteritis cases (Morris & Fernandez, 2008; Gamboa

et al., 2011). Foodborne diseases that have *C. perfringens* as a causative agent are commonly related to type A strains, while necrotic enteritis is caused by type C and some type A strains (Santos & Heredia, 2011). According to estimates from the Centers for Disease Control and Prevention of the United States of America (CDC), approximately one million cases of foodborne diseases caused by *C. perfringens* are recorded annually, it being one of the most common causative agents of food poisoning (CDC, 2017). Reporting food poisoning by *C. perfringens* is not mandatory in this country. However, the centers for disease control and prevention estimate 250,000 cases per year due to food poisoning due to *C. perfringens* type A (Santos & Heredia, 2011).

C. perfringens and fish

Fish is considered an important source of food due to its easy digestibility, high nutritional value due to its composition of water, proteins, lipids, vitamins, minerals and carbohydrates, besides representing a source of economic and living income for millions of people around of the world (FAO, 1998, 2016; Sheyin & Solomon, 2017). However, these same characteristics make this food a highly susceptible source of deterioration and decomposition by enzymes of the fish itself (autolysis) and microorganisms, converting them into a vehicle of diseases when consumed (Avdalov, 2009). The microbiote present in fish is related to various factors such as: the nature of the water, temperature, food, season of year from which they are extracted, among others. Human activities have had a detrimental effect on water, so there may be a risk of contamination with pathogenic microorganisms from it, as well as by those activities involved in capture, cultivation (aquaculture), processing and conservation actions, such as for example the use of poor quality water, poor hygiene practices in handling, while fish processing facilities which are contaminated can be contaminated by chemical or biological agents (García & Calvario, 2008; Avdalov, 2009; Sheyin & Solomon, 2017). *C. perfringens* being a cosmopolitan microorganism can be located in diverse environments such as the soil, aquatic ecosystems, gastrointestinal system of animals and fish, as well as raw and processed foods (Matches et al., 1974; Kimura, 1996; Santos & Heredia, 2011; Sheyin & Solomon, 2017). This microorganism has been implicated in outbreaks of food-borne diseases that are related to the consumption of meat and derivatives, including fish; in the United States of North America in the period from 1998 to 2008, 2468 cases of foodborne diseases were reported, in which there were 461 cases of this pathogen being identified as the causative agent (Kimura, 1996; El-Shorbagy et al., 2012; Painter et al., 2013; CDC, 2017). The cases of foodborne diseases that have *C. perfringens* as a producing agent are related to inadequate storage, processing and food service operations, preferably in places that concentrate large groups of people such as hospitals, school canteens, prisons and old people's homes, or in events with food service (Kimura, 1996; El-Shorbagy et al., 2012; CDC, 2017). The ubiquitous nature of this microorganism and its spores make it a frequent problem and challenge for the food industry and establishments that produce large amounts of food (Santos & Heredia, 2009).

Isolation of *C. perfringens* in foods and fish

For the isolation and detection of *C. perfringens* in food, there are several standardized methods in the scientific collection, such as the one developed by the International Organization for Standardization (ISO) ISO 7937:2004 for the colony count of *C. perfringens* (Fig. 1), the method reported by Rhodehamel & Stanley (2001), presented in the Bacteriological Analytical Manual (BAM) of the Food and Drug Administration (FDA) of the United States of America (Fig. 2) is also the method reported by McNamara & Lattuada, (1998) presented in the microbiology laboratory guide of the United States Department of Agriculture (USDA) and the Food Safety and Inspection Service (FSIS) (Fig. 3). All these methods present some similarities in conditions and culture media used for isolation, quantification, and biochemical confirmation for the emission of results to *C. perfringens*. It should be noted that prior to the microbiological analysis, a core point of the method to be developed is actions that involve the collection and transport of the

sample, so it is recommended that the sample once collected for analysis should be transported immediately to the laboratory at temperatures 0–10 °C. The samples collected for analysis that cannot be analyzed immediately require special treatment; being sensitive, *C. perfringens*

may lose viability in prolonged periods of refrigeration and freezing, so they should be treated with a buffered glycerin saline solution, stored or sent frozen to the laboratory (McNamara & Lattuada, 1998; Rhodehamel & Harmon, 2001).
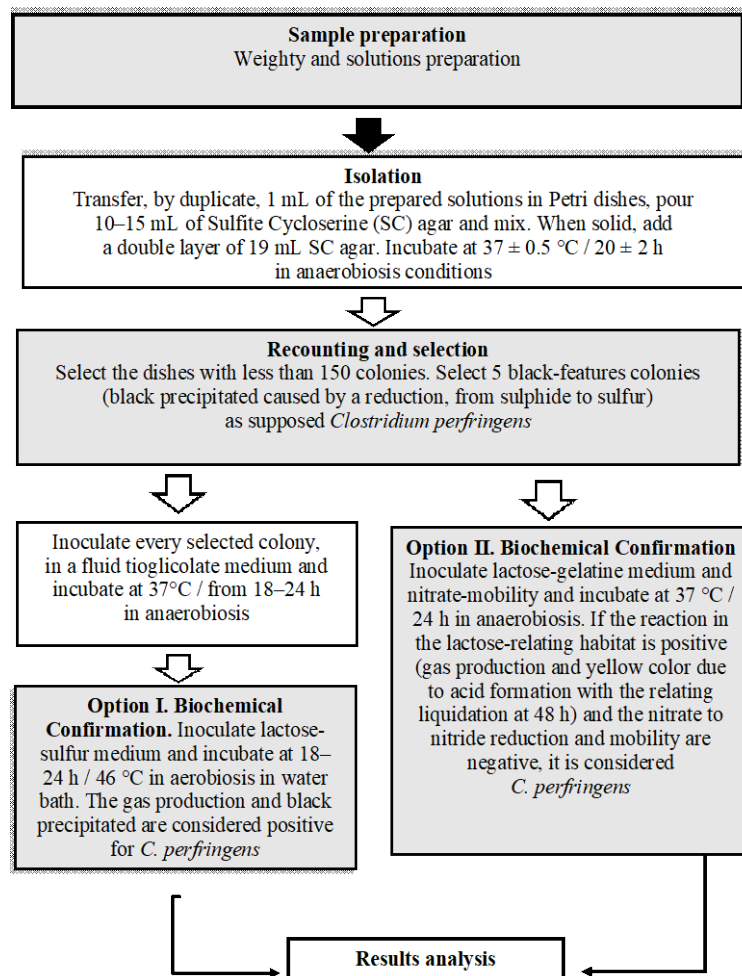
Sample preparation
Weighty and solutions preparation

Isolation
Transfer, by duplicate, 1 mL of the prepared solutions in Petri dishes, pour 10–15 mL of Sulfite Cycloserine (SC) agar and mix. When solid, add a double layer of 19 mL SC agar. Incubate at 37 ± 0.5 °C / 20 ± 2 h in anaerobiosis conditions

Recounting and selection
Select the dishes with less than 150 colonies. Select 5 black-features colonies (black precipitated caused by a reduction, from sulphide to sulfur) as supposed *Clostridium perfringens*

Inoculate every selected colony, in a fluid tioglicolate medium and incubate at 37°C / from 18–24 h in anaerobiosis

Option II. Biochemical Confirmation
Inoculate lactose-gelatine medium and nitrate-mobility and incubate at 37 °C / 24 h in anaerobiosis. If the reaction in the lactose-relating habitat is positive (gas production and yellow color due to acid formation with the relating liquidation at 48 h) and the nitrate to nitride reduction and mobility are negative, it is considered *C. perfringens*

Option I. Biochemical Confirmation. Inoculate lactose-sulfur medium and incubate at 18–24 h / 46 °C in aerobiosis in water bath. The gas production and black precipitated are considered positive for *C. perfringens*

Results analysis

Fig. 1. Method for plate count of *Clostridium perfringens* in food samples (ISO 7937: 2004; ANMAT, 2013)

Control and prevention of foodborne diseases

Food poisoning by *C. perfringens* is commonly due to inadequate handling of the temperature in the processing, maintenance or reheating of food, as well as its preparation in advance of consumption (Santos & Heredia, 2011). Low levels of *C. perfringens* occur in raw meats, poultry, soups, dehydrated sauces, raw vegetables or spices during food preparation; this coupled with the heat resistance and oxygen that can be decreased during cooking processes favors the growth of bacteria; thus spores that survive cooking can germinate and grow rapidly in foods that do not undergo adequate refrigeration processes after the cooking treatment (Rhodehamel & Harmon, 2001; Santos & Heredia, 2011). On the other hand, *C. perfringens* generally loses its viability when food is frozen or kept in prolonged refrigeration; for analytical issues in the laboratory this characteristic can hinder isolation and detection in food as well as establish the causes of an outbreak of food poisoning (Rhodehamel & Harmon, 2001). The actions focused on the control and prevention of diseases by this pathogen in food are involved in the stages of production and storage of food in which the cooling of food must be below 10 °C, for two or three hours, and the preservation of hot foods above 60 °C prior to consumption. In the case of reheating cold or refrigerated food, these should reach a minimum internal temperature of 75 °C. Likewise, cross contamination with utensils and contaminated surfaces must be prevented (OPS, 2016).

Throughout the food chain, from primary production through industry to household manipulation prior to consumption, strategies and procedures have been developed, recommended and implemented in a

global manner that contribute to minimize the health risks of the consumer, some of these are: good agricultural practices (GAP), good livestock or livestock practices, good fishing practices and aquaculture that contribute to reducing the contamination of food with soil and fecal animal matter, thus minimizing the bacterial load on the subject premium, as well as the application of good manufacturing practices (GMP) appropriate hygienic practices in the handling and processing, development and implementation of control systems based on Hazard Analysis and Critical Points (HACCP), compliance with the microbiological criteria of raw materials in order to reduce or prevent pollution by diverse microorganisms including strains of the genus *Clostridium* and finally, for home consumption, the World Health Organization (WHO) has developed informative handbooks aimed at the general population on hygiene conditions in the storage, handling and preparation of food in a healthy and safe way (WHO, 2007; García & Calvario, 2008; Ramírez & Ishihara, 2008; Avdalov, 2009; FAO, 2009; Elika, 2013; ANMAT, 2013; Gómez et al., 2015). On the other hand, other measures of control and prevention of contamination and deterioration of food include the use of chemical preservatives; in the food industry this is considered an alternative and common practice to reduce the pathogenic and deteriorating microbial population thus avoiding risks for the health and prolonging the life of the products; In different investigations around the world, effective results have been reported when combined with other barrier technologies against *C. prefringens* (Santos & Heredia, 2011).

At the international level, there are different limits or microbiological specifications established for the sanitary quality of various pro-

cesssed foods which are susceptible to contamination with *Clostridium* spp., Mexico has the official Mexican standard NOM-130-SSA1-1995 focused on packaged foods in hermetically sealed containers and subjected to heat treatment; indicating the sanitary dispositions and specifications in the microbiological character, such as the presence of anaerobic mesophiles that consist of bacteria of the genus *Clostridium*, among which are *C. sporogenes*, *C. putrificans*, *C. hystoliticum*, *C. bifermentans*, *C. perfringens* and *C. botulinum* of sanitary interest. For specific products such as formulas for infants, foods and non-alcoholic beverages for infants and young children, the Official Mexican Standard NOM-131-SSA1-2012, mentions the provisions, sanitary and nutritio-

nal specifications, as well as the labeling and testing methods including in the microbiological specifications the anaerobic mesophilic microorganisms. With respect to sanitary quality and safe consumption of food, products and fishing services, the Official Mexican Standard NOM-242-SSA1-2009 establishes sanitary specifications and test methods for fresh, chilled, frozen and processed fishery products, being the microbiological specification acceptable for *C. botulinum*, in fresh, chilled and frozen products as "absent" while the specification corresponding to the presence of sporulated anaerobic thermophiles and sporulated anaerobic mesophiles in commercially acceptable sterilized fishery products is "negative".
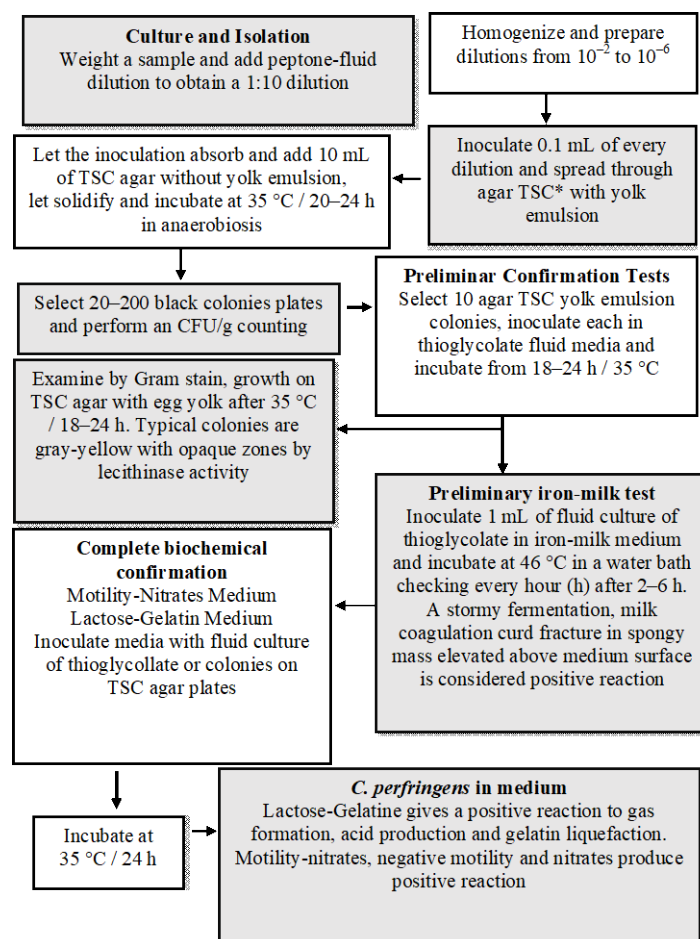
Culture and Isolation
Weight a sample and add peptone-fluid dilution to obtain a 1:10 dilution

Homogenize and prepare dilutions from 10^{-2} to 10^{-6}

Let the inoculation absorb and add 10 mL of TSC agar without yolk emulsion, let solidify and incubate at 35 °C / 20–24 h in anaerobiosis

Inoculate 0.1 mL of every dilution and spread through agar TSC* with yolk emulsion

Select 20–200 black colonies plates and perform an CFU/g counting

Preliminar Confirmation Tests
Select 10 agar TSC yolk emulsion colonies, inoculate each in thioglycolate fluid media and incubate from 18–24 h / 35 °C

Examine by Gram stain, growth on TSC agar with egg yolk after 35 °C / 18–24 h. Typical colonies are gray-yellow with opaque zones by lecithinase activity

Preliminary iron-milk test
Inoculate 1 mL of fluid culture of thioglycolate in iron-milk medium and incubate at 46 °C in a water bath checking every hour (h) after 2–6 h. A stormy fermentation, milk coagulation curd fracture in spongy mass elevated above medium surface is considered positive reaction

Complete biochemical confirmation
Motility-Nitrates Medium
Lactose-Gelatin Medium
Inoculate media with fluid culture of thioglycollate or colonies on TSC agar plates

Incubate at 35 °C / 24 h

***C. perfringens* in medium**
Lactose-Gelatine gives a positive reaction to gas formation, acid production and gelatin liquefaction. Motility-nitrates, negative motility and nitrates produce positive reaction

Fig. 2. Culture and isolation of *C. perfringens* from food (Rhodehamel & Harmon, 2001): * – Tryptose-Sulphite-Cycloserine Agar (TSC)

On the other hand, the Official Mexican Standard NOM-251-SSA1-2009, indicates the guidelines for hygiene practices for the process of food, beverages or food supplements; involving facilities, areas, equipment and utensils, services, storage, control of operations and raw materials, it also refers to the good practices of health and hygiene of the personnel, which also recommends the establishment of a system of hazard analysis and critical control points (HACCP) in order to prevent the generation of food that carries a risk to the health of the consumer. In Europe, on the part of the European Parliament and the Council of the European Union, Regulation (EC) No 178/2002 establishing the principles and general requirements of food legislation was established. European Food Safety Authority and procedures relating to safety to food safety are set; likewise by Commission Recommendation 2004/24/EC (DOCE 19/12/03) on the official control program for food products that recommends taking representative samples of spices at the level of import, production, packaging establishments, wholesale trade, establishments that use spices in food preparation and retail trade, in order to count *C. perfringens* with a microbiological limit of 100–1000 CFU/g (Elika, 2013).

Several studies have been carried out around the world that have reported the isolation and profile of resistance to different antimicrobials

by pathogenic microorganisms (*C. perfringens*, *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*), among others which come from different natural environments such as soil, food, animals and human beings. These studies generate information for a more rational use of antibiotics in the areas of clinical, agriculture, livestock, fisheries and aquaculture, in addition to the detection of strains with antimicrobial resistance, which contributes to alert us about possible genetic transfer mechanisms in nature and generate strategies in control (Abraham et al., 2011; Gamboa et al., 2011; Granier et al., 2011; Puig et al., 2011; Slavic et al., 2011; Elhadi et al., 2014; Gharaibeh et al., 2014).

At global level, different actions have been developed and recommended in order to reduce the transmission of resistance to antimicrobials in the food chain and thus achieve food safety, among these actions are good manufacturing practices, sanitary control through of HACCP systems, good agricultural, livestock and aquaculture hygiene practices, in addition the Codex Alimentarius has issued guidelines such as CAC/GL 77-2011 for the risk analysis of antimicrobial resistance transmitted by food and the code of practice to minimize and contain antimicrobial resistance CAC/RCP 61-2005 (PAHO, 2015; FAO, 2017; FAO, 2018). The control of resistance to antimicrobials is a common task and, therefore, it is necessary that the participation of govern-

ments around the world with the support of the scientific community generate actions focused on control and surveillance in the agricultural and clinical field by implementing surveillance and control policies and systems for the use of antimicrobials and the phenomenon of resistance, all in favor of the availability to the world population of food of good in nutritional quality and safety (Padgett et al., 2011; Gestal et al., 2014).
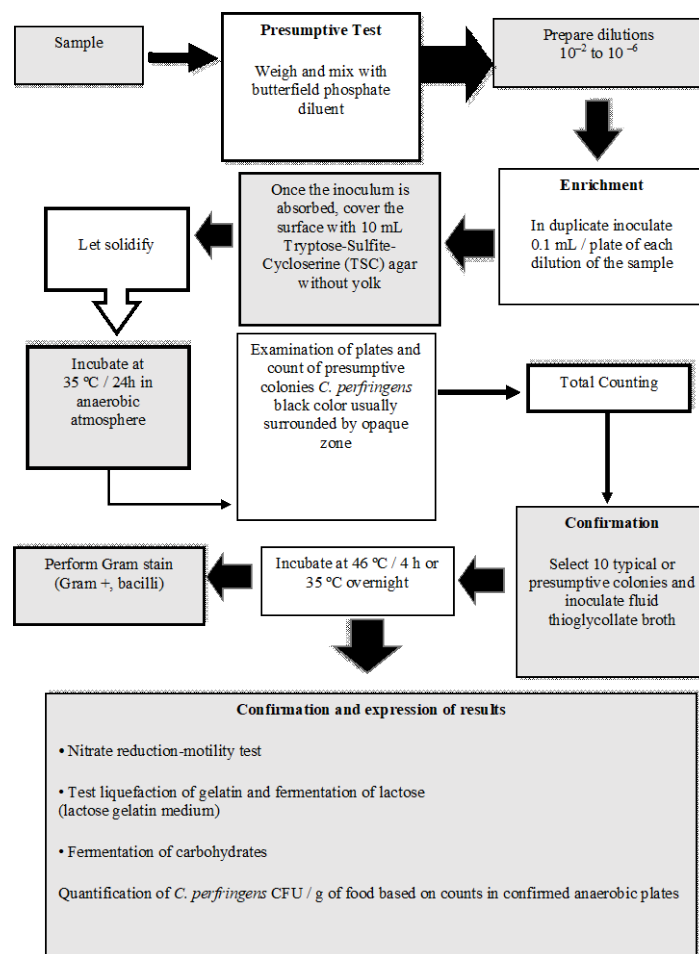

Fig. 3. Method of quantification of *C. perfringens* in foods (McNamara & Lattuada, 1998)

Conclusions

Food is a priority need for human beings to achieve survival, growth, development and for fulfillment of their daily activities. However, during the production of food and throughout the food chain there can be microbial contaminations and these can be vehicles of biological hazards to health, giving rise to different diseases. Foodborne diseases are considered to be of great relevance worldwide in public health aspects due to their incidence and mortality rate, together with the fact that several years ago the appearance and increase of resistance to antimicrobials was reported. These diseases have different causal agents thus potentiating their importance, given the negative repercussions on health, economy and society.

C. perfringens is considered a danger of biological origin along the food chain and is described as a threat to human and animal health, since through the contamination of food and subsequent consumption it generates negative effects on health. This is complicated by reports worldwide on the isolation of strains resistant to different antimicrobials. Different strategies, procedures and actions have been developed and implemented around the world through international organizations, the food industry, government and academia to control and prevent food contamination by food pathogens such as *C. perfringens*, in an effort to guarantee the supply of safe food to the general population in all stages that go from its primary production through its processing and transformation until its conservation, manipulation and preparation prior to its consumption.

Satisfying the growing demand for safe and nutritious food globally still requires a great common effort from the food industry, governments, and academia, including the general population, through their awareness of the different risks and dangers to health from food, handling and storage conditions for consumption in order to minimize the incidence of foodborne diseases and their impact on health.

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