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Review

Food Safety in the Production and Consumption of Fish: The Case of *Vibrio Vulnificus*

[1]Alejandro De Jesús Cortés-Sánchez, [2]Luis Daniel Espinosa-Chaurand, [3]Mayra Diaz-Ramirez, [4]Ma. de la Paz Salgado-Cruz and [5]Erika Torres-Ochoa

[1]*Consejo Nacional de Ciencia y Tecnología (CONACYT), Departamento de Ciencias de la Alimentación, Division de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana, Unidad Lerma, Lerma de Villada, Estado de Mexico, Mexico*
[2]*Consejo Nacional de Ciencia y Tecnología (CONACYT), Unidad Nayarit del Centro de Investigaciones Biológicas del Noroeste (UNCIBNOR+), Tepic, Nayarit, México*
[3]*Departamento de Ciencias de la Alimentación, Division de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana, Unidad Lerma, Lerma de Villada, Estado de Mexico, Mexico*
[4]*Departamento de Ingeniería Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Ciudad de Mexico, Mexico*
[5]*Departamento Académico de Ingeniería en Pesquerıas, Universidad Autónoma de Baja California Sur, La Paz, Baja California Sur, Mexico*

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Corresponding Author
Alejandro De Jesús Cortés-Sánchez
Consejo Nacional de Ciencia y Tecnología (CONACYT), Departamento de Ciencias de la Alimentación, Division de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana, Unidad Lerma, Lerma de Villada, Estado de Mexico, Mexico
Email: alecortes_1@hotmail.com

Abstract: Foodborne Diseases (FD) constitute a major global public health challenge due to their incidence and related mortality. Bacteria are frequently related to the generation of FD outbreaks. Fish is a highly nutritious food that is marketed and consumed globally. However, they are also susceptible to deterioration and contamination due to biological hazards related to unsanitary conditions and practices occurring throughout the food chain. *Vibrio vulnificus* is considered a human pathogen related to diseases and is transmitted by manipulation and consumption of aquatic foods, mainly in the raw state. This microorganism is also a fish pathogen that generates epizootics in culture with consequent economic losses and risks to human health due to transfer in the food chain. Aquaculture is a source of food such as fish that has grown over the years, becoming an important form of subsistence worldwide. Tilapia is an aquaculture species that, depending on culture conditions, may be at risk of *V. vulnificus* infections that would generate negative effects on animal health and compromise food safety. Therefore, the purpose of this document is to provide a general perspective on diseases transmitted by the food of aquatic origin derived from the handling and consumption of fish, specifically tilapia and whose causal agent is *V. vulnificus*. The metabolic, ecological, isolation, and identification characteristics of this pathogen are pointed out, in addition to the various actions to control and prevent negative impacts on public health due to this biological hazard in foods of aquatic origin.

Keywords: Aquaculture, Fishing, Food Pathogens, Gastroenteritis, Zoonoses, Fish-Borne, Tilapia

Introduction

Food and Health

Food is an essential factor in biological processes and supplies organisms with the necessary elements for subsistence (Chasquibol *et al*., 2003; Izquierdo *et al*., 2004; FAO, 2016a; Ortega and Hernandez, 2017). The ingestion of food (natural or processed) needs to be conducted safely to fulfill its function of providing nutritional content; however, food is likely to become a vehicle for disease when it does not meet optimal conditions for consumption (Izquierdo *et al*., 2004; FAO, 2016a; Ortega and Hernandez, 2017). Food safety guarantees serve to ensure that food will not generate consumer illnesses (Fuertes *et al*., 2014; Palomino-Camargo *et al*., 2018). Food safety is an important global public health issue and is one of the basic characteristics that, in addition to nutritional, sensory, and commercial characteristics, constitute the total quality of food (De la Fuente *et al*., 2010; Fuertes *et al*., 2014). This document focuses on providing general information regarding foodborne diseases, specifical information on human and fish pathogenic *Vibrio vulnificus* and its effects on tilapia, characteristics of the pathogen and disease generated, as well as actions to control and prevent hazards to human and animal health, are described, with a focus on the production of safe food and the protection of public health.

Foodborne Diseases

Foodborne Diseases (FDs) are considered an important public health issue due to the high rates of morbidity and mortality and negative socioeconomic burdens resulting from them around the world, mainly in developing countries (Soto *et al*., 2016; Weiler *et al*., 2018). It is estimated that approximately 600 million people fall ill every year from eating contaminated food and 420,000 die from the same cause (WHO, 2020b).

The appearance of FD around the world is related to different causes, such as industrialization and globalization of the food supply, microbial adaptation, new forms of transmission, resistance to antimicrobials, changes in food production systems, climate change, changes in demographic behavior, lifestyle changes among the human population and the appearance of vulnerable groups (Jorquera *et al*., 2015; Centeno and Rodríguez, 2005; Palomino-Camargo *et al*., 2018; Thorstenson and Ullrich, 2021).

Approximately 250 foodborne disease-causing agents have been described, including physical, chemical, and biological agents (bacteria, viruses, parasites, and fungi), with bacteria being the most frequently related to disease cases and outbreaks (Jorquera *et al*., 2015; Weiler *et al*., 2018; WHO, 2020) (Table 1).

Two types of diseases are caused by bacteria that are taken in through food: (I) Food infections: Occur when a pathogen is present in the food and establishes and multiplies in the consumer. These infections present two variants: (a) Invasive infections are characterized by bacterial colonization of tissues and organs of the affected person; examples of these bacteria are *Salmonella spp*., *Aeromonas* spp., *Campylobacter* ssp., *Shigella* spp., *Vibrio parahaemolyticus, Yersinia* spp. and enteroinvasive *Escherichia coli*. (b) Toxi infections are caused by noninvasive bacteria capable of colonizing and multiplying in the intestinal tract of the host, where they excrete their toxins; some examples are *Vibrio cholerae, Bacillus cereus* (enterotoxin-producing strains), *Clostridium botulinum*, and *Clostridium perfringens*. (II) Food poisoning: This is caused by the intake of toxins produced by bacteria that have developed to a certain concentration in food. Poisonings, in general, present a more rapid clinical manifestation than foodborne infections. The type of microorganisms that cause food poisoning is *Clostridium botulinum*, *Bacillus cereus* (strains that produce emetic toxin), and *Staphylococcus aureus* (Torrens *et al*., 2015).

FDs are syndromes derived from ingesting food or water that contain etiological agents in amounts that affect consumer health (Soto *et al*., 2016; WHO, 2020). These diseases can reach populations with different susceptibility and consumption patterns with consequent health risks, mainly affecting children, pregnant women, the elderly, and people with compromised immune systems (López *et al*., 2014; Soto *et al*., 2016).

An FD is characterized by a variety of gastrointestinal symptoms, such as nausea, vomiting, diarrhea, abdominal pain, and fever and there may also be severe complications, such as sepsis, meningitis, spontaneous abortions, Reiter's syndrome, Guillan Barré syndrome, and/or death (Soto *et al*., 2016; WHO, 2020a). Food contamination with various agents harmful to health can occur at any stage of the food chain, that is, from primary production to the consumption of food (from farm to table) (De la Fuente *et al*., 2010; WHO, 2020a). For a food to be considered suitable for consumption, it must undergo sanitation procedures at all stages of the food chain; it must have adequate organoleptic characteristics (taste, smell, texture, and color) and no pathogenic microorganisms or toxins, foreign chemical substances or substances that are not allowed (FAO, 2016a).

For some years, food safety has grown in its global relevance for consumers and the food industry, and the population is estimated to increase in the coming years with a parallel increase in the rate of urbanization and expectations of a simple lifestyle. These factors will lead to the demand for natural resources and the production and supply of quality food, which will be a great challenge for producers, government organizations, academia, and a planet affected by environmental disasters, the transformation of the environment, and scarce natural resources (Wajid *et al*., 2020).

Fish

Fish are defined as all the food extracted from continental or ocean waters used for human or animal consumption, encompassing all portions and products (Soares and Goncalves, 2012; Silva *et al*., 2017). The sources of fish for human use and consumption are capture fisheries and aquaculture. Estimates by the Food and Agriculture Organization (FAO) of the United Nations report a world production of 178.5 million tons of fish in live weight for 2018 and a per capita consumption of 20.5 kg for both activities (FAO, 2020a). Fish are considered a healthy and nutritious food source as well as an important means of subsistence for humans around the world due to their biological value and highly digestible protein content (15-25%) (greater than 95% depending on the species), polyunsaturated fatty acids, vitamins and minerals (FAO, 2009; Soares and Goncalves, 2012; Fuertes *et al*., 2014; Silva *et al*., 2017).

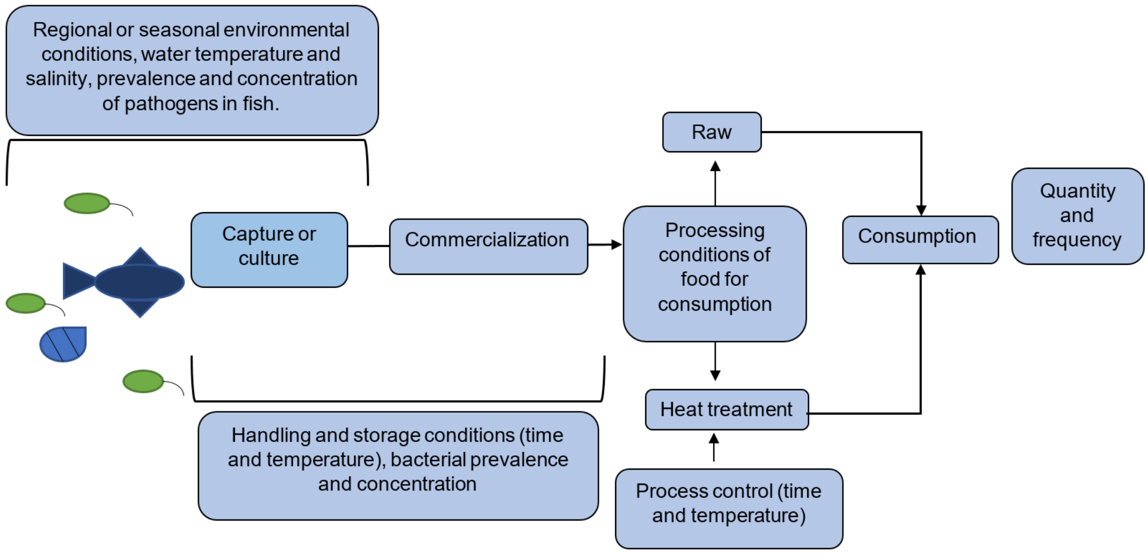

Fig. 1: Schematic representation of fish and seafood contamination by *V. vulnificus* and health risk (Adapted from FAO, 2003)

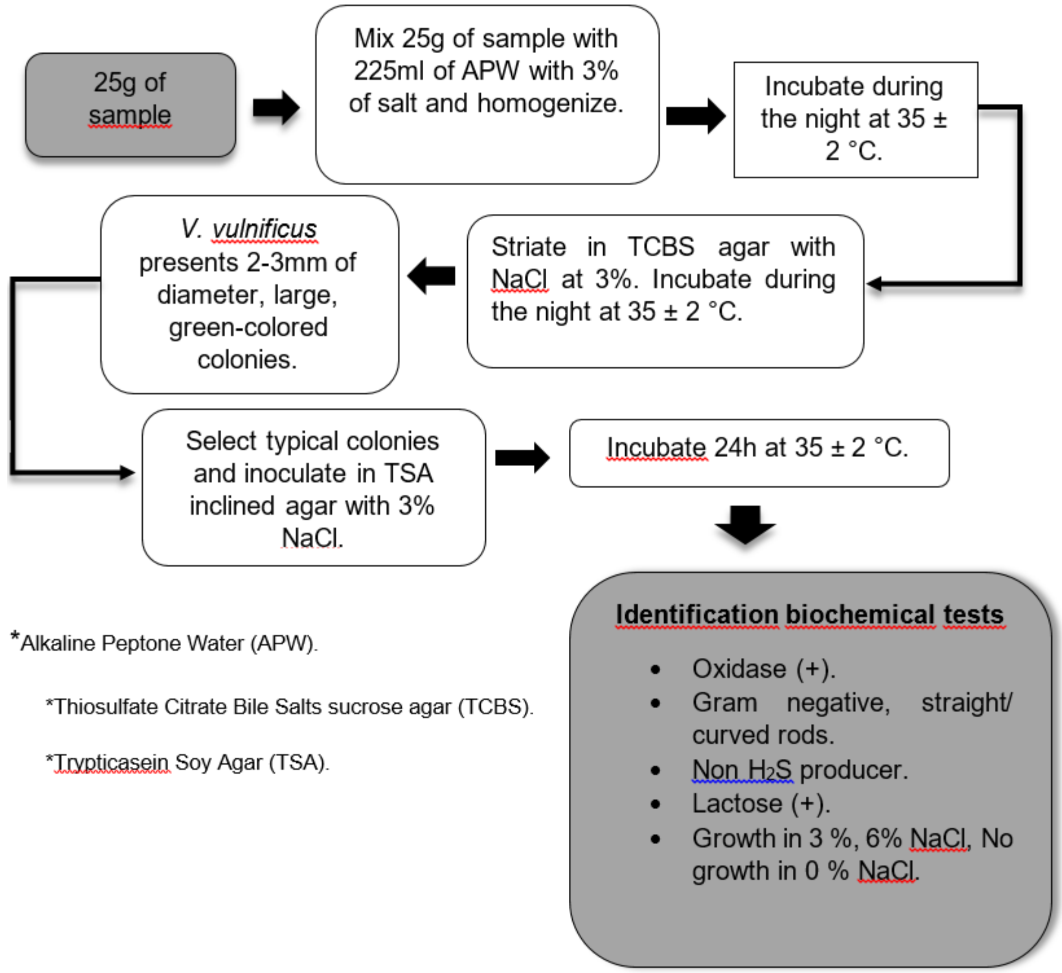

Fig. 2: Isolation of *Vibrio vulnificus* in fish and shellfish protocol (Narasimha *et al*., 2018)

Table 1: Different bacterial agents causing foodborne illnesses of importance in public health (Soto *et al*., 2016; FDA, 2018; CDC, 2021; Elika, 2021; Minsalud, 2022)

Microorganism	*Food/Source*
Escherichia coli (pathogenic)	Meat, dairy, fish, shellfish, water, ice and legumes not subjected to adequate heat treatment or raw, unpasteurized juice and contaminated water
Listeria monocytogenes	Refrigerated and ready-to-eat foods such as beef, poultry, seafood, and dairy (unpasteurized milk and dairy products), melons, sausage, pâtés, deli meats, smoked fish and shellfish
Vibrio spp.,	Products of aquatic origin, such as raw or inadequately cooked fish, oysters, mussels, shrimp and clams
Aeromonas spp.	Water, vegetables, meat and fish
Salmonella spp.	Raw or undercooked eggs, raw beef, pork, poultry, seafood, raw milk, dairy and fresh produce, vegetables, fruits, processed foods including nut butters, frozen pies, chicken nuggets and stuffed chicken.
Staphylococcus aureus	Dairy products, salads, cream-filled pastries and other desserts, high-protein foods (cooked ham, raw beef, and chicken), and those foods that are not cooked after handling, such as sliced meats, desserts, puddings, cakes, and sandwiches
Shigella spp.	Salads, dairy products, raw oysters, ground beef, chicken, and water, foods of animal origin contaminated or with contact with infected people
Campylobacter spp.	Poultry: Chicken, turkey, duck, goose and other birds, raw or undercooked fish, fruits, vegetables, water and dairy products.
Clostridium spp.	Canned and home-prepared foods, vacuum-packed and hermetically-wrapped foods, beef and seafood products, herb-infused cooking oils and beef and derivatives
Brucella spp.	Raw milk and unpasteurized derivatives. Contaminated water
Bacillus cereus	Cereals, custards and sauces, meatballs, sausages, vegetables subjected to inadequate heat treatment.
Yersinia enterocolitica	Often beef, pork, fish and fish products, dairy, and water

Table 2: Biochemical characteristics used for the identification of species of the genus Vibrio from foods of aquatic origin (Oliver, 2005; Cortés-Sánchez *et al*., 2016; Kaysner *et al*., 2019)

Test	*V. cholerae*	*V. alginolyticus*	*V. fluvialis*	*V. furnissii*	*V. metschnikovii*	*V. hollisae*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*
TCBS agar	GY	GY	GY	GY	GY	NG	G	G	G
mCPC agar	P	NG	NG	NG	NG	NG	NG	NG	GY
Oxidase	+	+	+	+	-	+	+	+	+
Arginine dihydrolase	-	-	+	+	+	-	-	-	-
Ornithine decarboxylase	+	+	-	-	-	-	+	+	+
Lysine decarboxylase	+	+	-	-	+	-	+	+	+
Gelatinase	+	+	+	+	+	-	+	+	+
Urease	-	-	-	-	-	-	-	V	-
Lipase (corn oil)	+	85	+	89	+	-	17	+	+
ONPG	+	0	40	35	50	0	+	5	75
String	+	+	+	+	+	+	+	64	+
Growth in NaCl (w/v)									
0%	+	-	-	-	-	-	+	-	-
3%	+	+	+	+	+	+	+	+	+
6%	-	+	+	+	+	-	+	+	+
8%	-	+	V	+	V	-	-	+	-
10%	-	+	-	-	-	-	-	-	-
Growth at 42°C	+	+	V	-	V	ND	+	+	+
Acid production of:									
Sucrose	+	+	+	+	+	-	-	-	-
D-cellobiose	-	-	+	-	-	-	-	V	+
Lactose	-	-	-	-	-	-	-	-	+
Arabinose	-	-	+	+	-	+	-	+	-
D-mannose	+	+	+	+	+	+	+	+	+
VP	V	+	-	-	+	-	-	-	-

+, (generally, approximately 90–100% positive); −, strains negative (generally approximately 0–10% positive). ONPG: ortho-nitrophenol for β-galactosidase production. VP: Voges-Proskauer. TCBS, thiosulfate-citrate-bile salts-sucrose. mCPC, modified cellobiose polymyxin colistin. GY: growth of yellow colonies. G: growth of green colonies. P: growth of purple colonies. NG: no growth. V: variable reaction. ND: not determined.

Table 3: Different biochemical characteristics of *Vibrio vulnificus* biotypes have been identified (Colodner *et al*., 2004; Oliver, 2005; Silveira *et al*., 2016)

	Vibrio vulnificus		
Test	Biotype 1	Biotype 2	Biotype 3
Indole	+	-	+
Ornithine decarboxylase	+	-	+
Fermentation:			
Mannitol	+	-	-
Salicin	ND	ND	-
Cellobiose	-	+	-
Lactose	-	-	-
Citrate	-	+	-
ONPG	-	+	-
Lysine decarboxylase	+	+	+

ONPG: O-nitrophenyl-β-D-galactopyranoside. ND: Not determined. Mannitol fermentation and growth at 42°C. *V. vulnificus* presents great phenotypic variation since approximately 15% of the strains are lactose negative despite being considered lactose-positive *Vibrio*

However, due to the composition of fish, they are also highly susceptible food to deterioration and contamination (physical, chemical, or biological) due to their pH being close to neutral, high-water activity, nutrient content, microbial activity, autolysis, and oxidation (Centeno and Rodriguez, 2005; Soares and Goncalves, 2012; Fuertes *et al*., 2014; Rabiela, 2015; Silva *et al*., 2017). It should be noted that the freshness, nutritional quality, and safety of fish depend on factors such as species, age, habitat, diet, catch conditions, harvest, processing, conservation, transport, distribution, and commercialization (Huss, 1998; Centeno and Rodriguez, 2005; Soares and Goncalves, 2012; Fuertes *et al*., 2014; Rabiela, 2015; Silva *et al*., 2017).

The safety challenges for foods such as fish are the diseases transmitted by these foods that are derived from contamination by bacteria, viruses, parasites, biogenic amines, biotoxins, heavy metals, polychlorinated biphenyls, pesticides, and residues of veterinary drugs (FAO, 2009).

The presence of bacteria is mainly related to the deterioration and shelf life of fish and bacteria also generate diseases due to the consumption of this food (Corrales *et al*., 2011; Alerte *et al*., 2012; Espinosa *et al*., 2014). In terms of qualitative and quantitative aspects, the bacterial content of fish is influenced by factors such as the time of year, diet, geographic area, species, capture system, handling, and storage conditions (Romero and Negrete, 2011; Corrales *et al*., 2011).

Fish are vehicles for bacteria that are pathogenic to humans and these bacteria can be classified into two groups: (1) One group contains the bacteria that naturally inhabit aquatic environments, where water temperature has a selective effect (*C. botulinum, Vibrio* spp., *Aeromonas* spp. and *Plesiomonas* sp., among others). These bacteria are found in the skin and gastrointestinal contents of fish and when the fish are alive, these bacteria do not invade the muscular package and are sterile due to the protection of their natural defenses. However, when fish die, these bacteria penetrate inside the tissues fish´s and contribute to contamination and deterioration. (2) The second group contains bacteria that are present in water due to fecal matter contamination and are associated with handling processes during capture, harvesting, and processing (*Escherichia coli, Shigella* spp., *Salmonella* spp., *Listeria monocytogenes, Staphylococcus aureus, Streptococcus faecalis,* among other) (Huss, 1997; Romero and Negrete, 2011; Corrales *et al*., 2011).

Vibrio

Members of the *Vibrio* genus belong to the *Vibrionaceae* family; they are Gram-negative bacilli with a straight and curved morphology and mobile with a single polar flagellum; they do not generate spores; they are thermolabile, facultative anaerobes; they produce catalase and oxidase (Table 2) and ferment glucose without gas; their growth temperature range is 8°C to 43°C; they can survive refrigeration temperatures; they can grow at a pH of 5 to 10; and some are halophiles (Davalos *et al*., 2005; Zúñiga and Caro, 2014; Cortés-Sánchez *et al*., 2016; Kaysner *et al*., 2019). They live in aquatic environments, such as in water or in association with fish, crustaceans, and zooplankton (Cortés-Sánchez *et al*., 2016; Brumfield *et al*., 2021).

Species such as *V. cholerae, V. parahaemolyticus, V. vulnificus, V. mimicus, V. alginolyticus, V. fluvialis, V. furnissii, V. metschnikovii,* and *V. hollisae* are considered human pathogens (Ramamurthy *et al*., 2014; Cortés-Sánchez *et al*., 2016; Silveira *et al*., 2016; Kaysner *et al*., 2019; Brumfield *et al*., 2021). Similarly, species of the genus *Vibrio* can be pathogens of freshwater and saltwater fish and are responsible for outbreaks of diseases in animals called epizootics (Mustapha *et al*., 2013; Rehulka *et al*., 2015).

In countries such as the United States of America, the (CDC, 2019) reported 2014 a total of 1,252 cases of diseases caused by *Vibrio* species called "Vibriosis" (excluding *V. cholerae* O1 and toxigenic O139), where from the total number of cases, *Vibrio parahaemolyticus* was associated in most cases (605 cases), followed by *Vibrio alginolytic us* (239 cases), *Vibrio vulnificus* (124 cases), *Vibrio cholerae* (80 cases) and *Vibrio fluvial is* (71 cases) (CDC, 2019). The high incidence of vibriosis in the United States occurs frequently during the warmest years, estimating that if the global warming phenomenon continues, it will contribute to the increase in the appearance of Vibrio infections (Brumfield *et al*., 2021). The transmission and infections by pathogenic *Vibrio* sp. have been reported, it derives from various factors such as the consumption of raw or undercooked contaminated food, handling of fish and shellfish, and skin exposure to water (CDC, 2019; Brumfield *et al*., 2021). The mortality rate from *V. vulnificus* infections in humans is estimated to be one of the highest for a waterborne pathogen, over 50% for primary septicemia, and is responsible for approximately 95% of all deaths related to water and seafood in the United States (Brumfield *et al*., 2021).

Vibrio Vulnificus

V. vulnificus is a Gram-negative halophilic bacillus found naturally in coastal, marine, and freshwater environments around the world at tropical temperatures, especially between 22 and 30°C (at temperatures <13°C it becomes viable but not culturable), and salinities from 1% to 34%, as free-living cells or symbionts (Yano *et al*., 2004; Oliver, 2005; Jones and Oliver, 2009; Horseman and Surani, 2011; Castillo *et al*., 2013; Zúñiga and Caro, 2014; Lin *et al*., 2021; Thorstenson and Ullrich, 2021). It can be isolated from water, sediments, plankton, fish, and shellfish (Yano *et al*., 2004; Oliver, 2005; Jones and Oliver, 2009; Horseman and Surani, 2011; Castillo *et al*., 2013; Zúñiga and Caro, 2014; Lin *et al*., 2021).

V. vulnificus has been classified based on biotypes, Lipopolysaccharide (LPS) antigens, capsules, and genetic sequences (Gulig *et al*., 2005; Oliver, 2005; Horseman and Surani, 2011; Silveira *et al*., 2016). Three biotypes of *V. vulnificus* have been reported and they are identified by different biochemical characteristics, such as indole and ornithine decarboxylation and growth at 42°C (Table 3) (Colodner *et al*., 2004; Oliver, 2005; Fouz *et al*., 2010; Horseman and Surani, 2011; Silveira *et al*., 2016; Cho *et al*., 2018).

All biotypes are pathogenic in humans, biotype 1 occurs worldwide and is associated mostly with human diseases, biotype 2 is mainly associated with infections in fish (eels and tilapia) and biotype 3 has been reported as a hybrid of biotypes 1 and 2 and has been identified in freshwater aquaculture, associated with cases of foodborne bacteremia and tissue infections that in some cases have required amputation (Oliver, 2005; Fouz *et al*., 2010; Horseman and Surani, 2011; Silveira *et al*., 2016; Cho *et al*., 2018; Brumfield *et al*., 2021; López-Pérez *et al*., 2021).

The pathogenicity of the genus *Vibrio* is related to the ability of *Vibrio* microorganisms to cause diseases (through the entry, colonization, and multiplication in the human body). Clinical symptoms occur due to certain pathogenic factors (hydrolases, hemolysins, hemagglutinins, and metalloproteases, among others), in addition to epidemiological factors such as the carrier vehicle (food of aquatic origin), aquatic activities (bathing, diving, and fishing), bites or stings by fish rays or fins (Mira and Garcia, 1997; Silveira *et al*., 2016). *V. vulnificus* has various pathogenic factors, such as the ability to tolerate stomach acidity; a polysaccharide capsule that resists phagocytosis; the production of lipopolysaccharides, enzymes for vascular permeability (proteases, phospholipases, lecithinases, and chitinases), cytotoxins and proteins that contribute to tissue adhesion and invasion (fimbriae or pili); and the ability to acquire iron (Davalos *et al*., 2005; Gulig *et al*., 2005; Oliver, 2005; Horseman and Surani, 2011; Silveira *et al*., 2016; Brumfield *et al*., 2021). Furthermore, *V. vulnificus* can develop biofilms regulated by a quorum-sensing system (dependent on cell density) through the synthesis of various extracellular polysaccharides; these biofilms are responsible for survival, pathogenicity, and resistance to stress conditions. Additionally, the development of these biofilms is considered a preferable trait that improves growth and survival by allowing the entry of nutrients and avoiding antimicrobials (Lee *et al*., 2013; Packiavathy *et al*., 2013).

This bacterium is related to various diseases, such as gastroenteritis, fever, sepsis, and necrotizing fasciitis; in sporadic cases, it can cause bacterial peritonitis, pneumonia, endometritis, meningitis, septic arthritis, osteomyelitis, endophthalmitis and keratitis (Gulig *et al*., 2005; Jones and Oliver, 2009; Horseman and Surani, 2011). It is considered a pathogen of importance in public health due to its resulting morbidity and lethality, which has been reported to occur at a rate of over 50% in septicemia, edema, and ulcers in humans; gastroenteritis and sepsis usually occur due to the consumption of undercooked or raw fish and shellfish and this pathogen also cause wound infections when the skin comes in contact with water and when contaminated fish are handled (Yano *et al*., 2004; Oliver, 2005; Davalos *et al*., 2005; Chen *et al*., 2006; Jones and Oliver, 2009; Horseman and Surani, 2011; Cortés-Sánchez *et al*., 2016; Kaysner *et al*., 2019; Brumfield *et al*., 2021; López-Pérez *et al*.,2021). No cases of person-to-person transmission have been reported yet; *V. vulnificus* also has an incubation period of 16 h to 1.6 days, an infection causes diarrhea and progresses after consumption of raw or undercooked fish and shellfish, primarily oysters in quantities of 10^5 UFC/g o more (Davalos *et al*., 2005; Zúñiga and Caro, 2014; Brumfield *et al*., 2021). *Vibrio vulnificus* infections are usually rapidly acute, with symptoms presenting within 24 h or less of exposure, where even with therapy the case fatality rate is approximately 3–40% (Brumfield *et al*., 2021). In addition, the most vulnerable population groups are people with chronic diseases, liver damage, immunodeficiency, iron storage disorders, hypertension, kidney disease, and diabetes mellitus (Yano *et al*., 2004; Horseman and Surani, 2011; Zúñiga and Caro, 2014; Dickerson *et al*., 2021). Antimicrobial treatment against *V. vulnificus* infections consists of treatments that involve cephalosporins, betalactam–beta-lactamase inhibitors, carbapenems, tetracyclines, aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole or chloramphenicol; surgery is recommended in severe cases of tissue infection and necrotizing fasciitis (Horseman and Surani, 2011; Zúñiga and Caro, 2014).

Tilapia

Tilapia are fish of African origin that belong to the *Cichlidae* family. Tilapia can be categorized into three genera: *Tilapia* sp., *Oreochromis* sp., and *Sarotherodon* sp., according to standards of parental care (Wicki and Gromedia, 1998; Jácome *et al*., 2019). These fish are found in many tropical, subtropical, and temperate regions of the world, with the genus *Oreochromis* (*O. niloticus*, *O. aureus*, and *O. mossambicus*) and hybrids being considered some of the main organisms in freshwater or saltwater aquaculture activities due to their tolerance to high densities, fast growth, resistance to diseases, adaptability to captivity, acceptance of balanced diets and good quality and affordable meat; these characteristics make tilapia one of the most commercialized foods in the global market as whole fish or as fillets (Younes *et al*., 2016; Jácome *et al*., 2019).

Aquaculture activities in 2018 accounted for 46% of global fish production; finfish were one of the groups with the highest production and among them, Tilapia (*Oreochromis* spp. and *Oreochromis niloticus*) accounted for 10.2% of the total species produced through aquaculture globally (FAO, 2020b). Countries on the Asian continent are the main producer of tilapia (Martínez-Cordero *et al*., 2021).

In Latin America, Mexico has a tropical climate conducive to tilapia fishing and aquaculture production, with the latter producing 52,748 tons in 2018; thus, tilapia has become the species with the second highest production amount and importance in terms of animal protein after whiteleg shrimp (*Litopenaeus vannamei*) in Mexico (Martínez-Cordero *et al*., 2021). Mexico is considered the fifth largest producer of tilapia in controlled systems globally (SIAP, 2018). The main states of the Mexican Republic that produce tilapia through aquaculture and fishing are Chiapas, Jalisco, Tabasco, Campeche, Nayarit, Veracruz, Michoacán, Sinaloa, State of Mexico, Puebla, and Guerrero (INP, 2018; SIAP, 2018; Martínez-Cordero *et al*., 2021).

The tilapia market is local, regional, and national; the average presentation weight of a tilapia is 250-300 g and it can be whole, fresh, and gutted; whole, frozen, and gutted; a fresh fillet; or a frozen fillet (INP, 2018). The annual national per capita consumption of mojarra tilapia is 2 kg (SIAP, 2018). On the other hand, the intensification of aquaculture activities has led to an increase in disease outbreaks, generating a significant limit on the freshwater and saltwater aquaculture industry and significant economic losses worldwide (Caipang *et al*., 2014). Examples of the different causative agents of fish diseases are *Edwardsiella tarda, Aeromonas hydrophila, Streptococcus iniae, Vibrio vulnificus,* and *Mycobacterium* spp. These diseases are considered zoonoses, which are relevant to not only animal health but also food safety and public health (Lehane and Rawlln, 2000; Quijada *et al*., 2005; Gauthier, 2015).

V. vulnificus has been identified as part of the microbiota of healthy fish such as tilapia (Al-Harbi and Uddin, 2005). However, *V. vulnificus*, specifically biotype 2, which is divided into two main serotypes, A and E, has also been associated with epizootics (under stress conditions) called "vibriosis", mainly in eels (*Anguilla* and *Anguilla japonica*), tilapia (*Oreochromis* spp.) and pompano (*Trachinotus ovatus*) in aquaculture (Al-Harbi and Uddin, 2005; Chen *et al*., 2006; Fouz *et al*., 2010; Packiavathy *et al*., 2013; Lo *et al*., 2014; LaFrentz and Shoemaker, 2015).

The bacteria are transmitted through water and infection is generated by their adherence to and penetration of external mucous, with or without existing wounds or injuries, followed by multiplication in the blood and spread to different organs of the fish; this process causes episodes of septicemia, hemorrhages and histological changes in the gastrointestinal tract, gill, heart, liver, and kidney leading to death (Chen *et al*., 2006; Fouz *et al*., 2010). As a result, economic losses for the aquaculture sector can occur from these potential zoonoses (Amaro *et al*., 2015).

Sanitary Control of Food for Human Consumption

Among the measures to avoid contracting *V. vulnificus* diseases through fish, it is recommended that consuming raw fish and shellfish should be avoided, and consuming them should occur only if they are cooked completely to avoid cross contamination of cooked fish and shellfish with those that are raw; in addition, fish and shellfish should be refrigerated once cooked (Fig. 1) (Davalos *et al*., 2005; Horseman and Surani, 2011; Zúñiga and Caro, 2014). In addition, to prevent infections from routes other than consumption, brackish water should be avoided when one has open wounds or broken skin, protective clothing (gloves) should be worn when handling raw seafood and protective footwear should be worn when in contact with salt water (Horseman and Surani, 2011; Zúñiga and Caro, 2014).

The development of the food industry and international trade have generated the distribution of food globally. Thus, the chemical, physical and biological hazards present in food can reach populations with different degrees of vulnerability and consumption patterns with the consequent health risk. Since food-borne risks to human health may be due to biological, chemical, or physical causes, risks from microbiological hazards constitute a serious health problem. Due to the above, different policies, regulations, and tools (risk analysis) have been developed and implemented by governments around the world and international organizations for food safety management (López *et al*., 2014).

The regulation of fish quality and safety around the world is varied and extensive. Several international organizations contribute to the quality and safety of food, such as the World Organization for Animal Health (OIE), World Trade Organization (WTO), Food and Agriculture Organization (FAO), and World Health Organization of the United Nations (WHO). The Codex Alimentarius contains standards of sanitation practices for food production based on the general principles of food sanitation safety (CAC/RCP 1-1969) and the Code of Practice for Fish and Fishery Products (CAC/RCP 52-2003) contains standards for the stages of breeding, harvesting, handling, producing, processing, storing, transporting and selling of fish destined for human consumption. In both cases, the application of Hazard Analysis and Critical Control Point (HACCP) systems is included. Similarly, in 2009, the FAO developed guidelines for risk-based fish inspections to ensure that consumers have access to high-quality, safe, and nutritious fish and products, regardless of the source of the food and competent authorities, public and private organizations, producers, industrial entities, academia and civil society as users of these regulations with an interest in the safety of fish and products (FAO, 2009).

In the United States of America, the Food and Drug Administration (FDA) and the Department of Agriculture (USDA) regulate the safety of processed foods and animal health, respectively. Title 21 of the Federal Code of Regulations (21 CFR), section 117 (21 CFR 117), includes good manufacturing practices, hazard analysis, and risk-based preventive controls for food intended for human consumption (FDA, 2019a). In addition, for fish and seafood, section 123 of title 21 of the CFR (21 CFR 123) outlines the HACCP system for the safety management of these foods (FDA, 2019b).

On the other hand, the regulation of fish safety in the European Union occurs through Commission Regulation (EC) N° 2073/2005, which establishes microbiological criteria and analytical reference methods for guidance on the acceptability of food products, including manufacturing, handling, and distributing processes. However, for *Vibrio vulnificus*, this regulation does not establish any microbiological criteria, only the application of good hygiene practices and procedures based on HACCP at different stages of the food chain.

Similarly, European regulation N° 852/2004 establishes general rules for food business operators in terms of sanitation for food products at all stages of the production, transformation, and distribution of food; exports; and the application of HACCP principles. In addition to regulation N° 853/2004, where sanitation rules for food of animal origin are specifically established, including fish; all the aforementioned regulations are in addition to regulation N° 854/2004, where specific rules are established for the organization of official controls for products of animal origin intended for human consumption.

In Latin American countries such as Mexico, regulations for the safety of fresh, refrigerated, frozen, and processed fishery products are based on the official Mexican standard NOM-242-SSA1-2009, where the sanitary specifications for *V. cholerae* and *V. vulnificus* (absent in 50 g of product) are given. The NOM-251-SSA1-2009 standard establishes the minimum requirements for good sanitation practices in food processing to avoid contamination, as well as the implementation guidelines for a HACCP system. The standard NOM-128-SSA1-1994 establishes the application of a risk analysis system and control of critical points in the industrial fishery product processing plant. In addition, the NMX-F-605-NORMEX-2016 standard promotes sanitation management of prepared food service in fixed establishments (restaurants, hotels, hospitals, and industrial canteens, among others), and an establishment in compliance with this standard is granted an "H", which shows it is implementing good sanitation practices in all its phases: Reception, storage, preparation, and service.

For all the foregoing processes, it is generally considered that the control and reduction of food contamination risks that may cause illness in the consumer occur through the application of various sanitation procedures, such as Sanitation Standard Operating Procedures (SSOP), good aquaculture and fisheries practices, Good Manufacturing Practices (GMP) and the Hazard Analysis and Critical Control Point (HACCP) system, at all stages of the food chain (from the farm to a consumer's table) and through education on sanitation procedures for the final food handler in a home or the population in general, thus the entire food chain (WHO, 2007; FAO, 2009; Corrales *et al*., 2011; Quintero *et al*., 2012; Espinosa *et al*., 2014; Jorquera *et al*., 2015; Fuertes *et al*., 2014; Rabiela, 2015; Palomino-Camargo *et al*., 2018).

Sanitation Control in Aquaculture Production

It has been established that in terms of animal health and aquaculture safety, some of the basic measures for preventing animal diseases are the application of good aquaculture sanitation practices that incorporate the following actions: Focusing on animal health and stress reduction when handled, implementing personnel sanitation measures and training and monitoring the microbiological and physicochemical quality of water (Al-Harbi and Uddin, 2005; FAO, 2009; Balbuena, 2011; Balbuena *et al*., 2011; Amaro *et al*., 2015; FAO, 2016b; FAO, 2020b).

To minimize the risk of diseases in fish, there are some fundamental European regulations, such as Directive 2006/88/EC, that outline zoo sanitation requirements for aquatic animals and aquaculture products and the prevention and control of certain diseases. In Latin America, countries such as Mexico regulate aquaculture production in terms of food safety and animal health through the implementation of sanitation procedures such as good aquaculture practices developed and promoted by the National Service of Health, Safety and Agro-food Quality (SENASICA, 2019).

The use of antimicrobials in livestock and aquaculture production activities to prevent and treat diseases is a relevant topic. Using these compounds under proper management and administration is possible; however, preventive actions are recommended, prioritizing the implementation of good aquaculture practices to address the current health challenges related to mitigating the spread of antimicrobial resistance due to the inappropriate use of these compounds in food production activities, which can affect food safety and security (FAO, 2020a; 2020b;

FAO/WHO, 2020). On the other hand, in addition to the application of good aquaculture practices such as fish disease prevention measures, vaccination may be a promising possibility for ensuring animal health where the experimental development of various vaccines has produced good results in different fish, such as in tilapia related to resisting various bacterial pathogens, including *V. vulnificus* (Caipang *et al*., 2014; LaFrentz and Shoemaker, 2015).

Animal health linked to food safety has been a relevant issue for consumers and the food industry worldwide for several years. The increase in food production, including food from aquaculture and fishing, to meet demand has led to the trend in focusing on and guaranteeing food safety, for which various food certification schemes have been developed, involving different phases of production and aspects of sustainability, social responsibility, animal health, and product safety for commercialization. Some of these certification schemes are the Global GAP, International Federation of Organic Agriculture Movements (IFOAM), Accredited Fish Farm Scheme, Safe Quality Food (SQF), British Retail Consortium (BRC), Sanitation Standard Operating Procedures, and HACCP systems (CECOPESCA, 2012; Badui, 2015).

Microbiological Analysis

Microbiological analysis is part of controlling and preventing diseases. Although it does not guarantee 100% food safety, microbiological analysis is a requirement and part of food quality and safety assurance systems in addition to HACCP systems, good aquaculture and fishing practices, good manufacturing practices, sanitation practices, traceability and recall management (Narasimha *et al*., 2018).

The microbiological analysis of food is based mainly on aspects that involve the collection of samples, selection of analytical methods, and interpretation of results (Pascual and Calderon, 2000; González Flores and Rojas, 2005). The results of a food microbiological analysis provide essential information on sanitation levels in food production and handling processes, the food processing environment, a specific product batch, product shelf life, and sample collection procedures; all this information is used to verify whether an entity has complied with the corresponding health regulations as well as the control and prevention of foodborne diseases (Narasimha *et al*., 2018; Cruces *et al*., 2021).

In terms of microbiological analyses of *Vibrio* spp., various methods have been used for the detection and isolation of species based on conventional methodologies through phenotypic features using gelatin phosphate salt broth; alkaline enrichment culture media such as Alkaline Peptone Water (APW); or differential and selective culture media such as Thiosulfate Citrate Bile Salt Sucrose agar (TCBS), modified Cellobiose Polymyxin Colistin (mCPC) agar, Cellobiose Colistin (CC) agar, chromogenic agar, and Gelatin Phosphate Salt (GPS) agar and through biochemical identification tests (Fig. 2) using samples of food, whole fish specimens, or internal organs for the analysis (Graü *et al*., 2004; Fouz *et al*., 2010; FSSAI, 2012; Oliver *et al*., 2012; Castillo *et al*., 2013; Narasimha *et al*., 2018; Kaysner *et al*., 2019).

There are standard protocols for the analysis of *V. vulnificus* in foods; one of which is that of Kaysner *et al*. (2019) in the US FDA's Bacteriological Analytical Manual (BAM). This protocol establishes conditions for the collection and storage of samples for analysis, specifying that once a sample is collected, it must be cooled immediately (approximately between 7 and 10°C) and analyzed as soon as possible. The analysis involves microbial quantification based on determining the Most Probable Number (MPN), enrichment phases for growth with Alkaline Peptone Water (APW), and isolation in selective and differential media (CC and mCPC agar), with subsequent biochemical identification (Table 2).

The N° 21872-1:2017 method, developed by the International Standard Organization (ISO), is another standard method that focuses on the detection of potentially enteropathogenic *Vibrio spp*. (*Vibrio parahaemolyticus*, *Vibrio cholerae*, and *Vibrio vulnificus*). This method includes enrichment phases with alkaline saline peptone water, isolation in selective and differential culture media (TCBS agar and chromogenic agar), and confirmation through biochemical tests or the inclusion of molecular techniques such as Polymerase Chain Reaction (PCR) (Jean-Philippe *et al*., 2012).

In Mexico, the method used to detect *Vibrio* species in fishery products is part of the official Mexican standard NOM-242-SSA1-2009 and this method uses APW as the enrichment medium and TCBS and mCPC agar for the isolation and selection of *V. vulnificus*, producing flattened yellow colonies with opaque centers and translucent edges. On the mCPC agar, most other *Vibrio* species do not grow easily; therefore, the use of different biochemical tests for the differentiation and confirmation of the isolates is involved (Table 2).

On the other hand, some disadvantages are associated with conventional or traditional methods of microbiological analysis, as these methods can require substantial effort and a long time to obtain results; in addition, transporting samples to a laboratory can affect the conservation of viable microorganisms in the sample, thus resulting in the sample having a low sensitivity level (González Flores and Rojas, 2005; Lopez-Hernandez *et al*., 2017). Furthermore, pathogenic species such as *Vibrio alginolyticus, Vibrio cholerae, Vibrio harveyi, Vibrio parahaemolyticus*, and *Vibrio vulnificus* have been reported to occur in the form of Viable but Uncultivatable Cells (VBNCs), which is a dormancy state derived from exposure to adverse environmental conditions. Therefore, they are bacteria that do not grow in culture media but are metabolically active and capable of causing infections in animals (Brauns *et al*., 1991; Fakruddin *et al*., 2013; Lopez-Hernandez *et al*., 2017). This behavior makes traditional microbiological analysis and control and disease prevention measures difficult, so in comparison to conventional methods, molecular techniques are useful as they are more efficient and sensitive, allow a greater number of sample analyses, and decrease the time it takes to detect microorganisms in samples of food (Brauns *et al*., 1991; González Flores and Rojas, 2005; Fakruddin *et al*., 2013; Palomino-Camargo and Gonzalez, 2014; Lopez-Hernandez *et al*., 2017). Although they are not widely incorporated into standardized methods and require a comparison with cultural methods, the rapid identification of biological hazards in food is important in terms of processing food and determining quality, as well as in terms of identifying causative agents of disease outbreaks to decrease morbidity, mortality and economic costs related to foodborne diseases (Pavlovic *et al*., 2013; Palomino-Camargo and Gonzalez, 2014; Lopez-Hernandez *et al*., 2017).

The molecular techniques used to detect *V. vulnificus* are based on PCR from housekeeping gene sequences (16S rRNA, *rpoD, gyrB* and *glp*); genes encoding various virulence factors, such as virulence correlated gene (*vcg)*, Capsular Polysaccharide (CPS), hemolysine (*vvhA*) responsible for hemolytic and cytolytic activity; and the coding gene for pilus proteins (*pilF*); in addition, other molecular techniques include Pulsed Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphism (RFLP), Multilocus Sequence Typing (MLST), Multiple-Locus Variable-number tandem repeat analysis (MLVA), Random Amplified Polymorphic DNA (RAPD) and complete genome analysis, which are used to identify and type strains in ecology research and epidemiological surveillance (Brasher *et al*., 1998; Oliver, 2005; Neogi *et al*., 2010; Pan *et al*., 2013; Reynaud *et al*., 2013; Bier *et al*., 2013; Yokochi *et al*., 2013; Cho *et al*., 2018; 2017; Kaysner *et al*., 2019; Lydon *et al*., 2021).

Other rapid techniques for the analysis of food pathogen isolates, useful over conventional phenotypic methods and sometimes even molecular methods due to their reported cost-benefit ratios related to implementation, are Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Pavlovic *et al*., 2013; Böhme *et al*., 2013). MALDI-TOF MS is a chemotaxonomic method that detects different biomolecules (nucleic acids, sugars, peptides, and proteins) and producer microorganisms through the generation of highly abundant protein fingerprints, followed by correlation with reference spectra in databases (Pavlovic *et al*., 2013).

Some studies have been conducted with good results related to identifying various *Vibrio* species through the use of MALDI-TOF MS, which can be useful for the rapid analysis of isolates in environmental studies, food quality and safety, epidemiological surveillance, and evaluation of health risks (Dieckmann *et al*., 2010; Bier *et al*., 2013; Böhme *et al*., 2013; Erler *et al*., 2015; Cho *et al*., 2017).

Antimicrobial Resistance and Vibrio Vulnificus

Antimicrobial resistance occurs when microorganisms undergo changes that make the antimicrobials used to cure infections caused by these bacteria resistant (Serra, 2017). Antimicrobial resistance affects all microbial species, especially bacteria, including those that are transferred to humans through the food chain (Puig *et al*., 2011; Verraes *et al*., 2013; Serra, 2017).

The increase in antimicrobial resistance is considered one of the largest problems for public and animal health globally due to the health risk of infections, increased morbidity and mortality rates of diseases caused by resistant causal agents, prolongation of disease, increased health costs, and impacts on production, food safety and food security (Puig *et al*., 2011; 2019; FAO/WHO, 2020; Kumar *et al*., 2020). It has been reported that in the United States and the European Union, antibiotic resistance is the cause of more than 23,000 and 25,000 deaths per year, respectively (Kumar *et al*., 2020) and according to WHO estimates alone, 500,000 people die each year from causes related to antimicrobial resistance (FAO/WHO, 2020).

Various studies around the world have already reported resistance to different food pathogens, such as *Salmonella* spp., *Campylobacter* spp., *Escherichia coli, Clostridium difficile, Listeria monocytogenes, Staphylococcus aureus, Vibrio cholerae, Vibrio vulnificus, and Vibrio parahaemolyticus*, to various antimicrobials (Lungu *et al*., 2011; Kim *et al*., 2011; Puig *et al*., 2011; Kitaoka *et al*., 2011; Elmahdi *et al*., 2016; Lammie and Hughes, 2016; Bennani *et al*., 2020). Antimicrobial resistance in bacteria has been mainly considered due to factors such as the inappropriate and excessive use of antimicrobials in humans and animals, with antimicrobials in animals being used with a therapeutic, prophylactic, or growth-promoting approach in primary food production (Puig *et al*., 2011; Kumar *et al*., 2020).

Antimicrobial resistance can be naturally transmitted vertically (daughter cells) or acquired by mutations in chromosomes and the exchange of genetic material (plasmids, integrons, and transposons), which can be transferred horizontally through conjugation processes, transduction, and transformation (Verraes *et al*., 2013; Quiñones, 2017). The resistance mechanisms of microorganisms to antimicrobials are varied; mechanisms can involve a modification to the permeability of the membrane, an extraction of the compound by pumping, enzyme inhibition, alternative metabolic pathways, a modification of the attack target, or alteration of its composition and generation of biofilms (Tafur *et al*., 2008; Puig *et al*., 2011; Verraes *et al*., 2013; Quiñones, 2017).

Food is involved in the development and spread of antimicrobial resistance (Verraes *et al*., 2013; FAO/WHO, 2020). The presence of antimicrobial-resistant microorganisms in production systems (agricultural, livestock, and aquaculture) and the food chain is considered a potential route of exposure for a population and a health risk (Verraes *et al*., 2013; FAO/WHO, 2020).

Food contamination by antimicrobial-resistant bacteria or resistant genetic material can occur through different routes: 1. Presence of antimicrobial-resistant bacteria in food due to the use of antibiotics during primary production, 2. Presence of resistant genes in bacteria that are intentionally added during food processing (starter cultures, probiotics, bio preservative microorganisms, and bacteriophages) and 3. Cross-contamination with antimicrobial-resistant bacteria during food processing and handling (Verraes *et al*., 2013).

In food processing, raw food that has not been previously processed or preserved or food subjected to minimum conditions of processing or preservation (being frozen, acidified, or modified atmosphere packaging) can maintain cells that are sub fatally damaged or stressed and these cells present a high risk of transferring antimicrobial resistance since the resistant bacteria present were not eliminated during processing or preservation; thus, in the food matrix or intestines after being ingested, these bacteria can transfer resistance between the existing microbiota mainly through conjugation mechanisms (Verraes *et al*., 2013).

Bacteria, including pathogens, in aquatic environments, can transfer and exchange genetic material that confers antimicrobial resistance, generating the spread of this phenomenon (Puig *et al*., 2019). The isolation of *V. vulnificus* from different foods and aquatic environments, including aquaculture, and its resistance to various antimicrobials (mainly ampicillin) have been reported by various studies around the world (Donkeng *et al*., 2011; Kim *et al*., 2011; Elmahdi *et al*., 2016; Igbinosa, 2016; Heng *et al*., 2017; Lin *et al*., 2021). Thus, *V. vulnificus* constitutes a risk to public health due to its transmission to humans through the food chain and monitoring the sanitation quality procedures for fish and shellfish, and implementation of different strategies related to the use of antimicrobials to control infections in humans and animals in the aquaculture environment (probiotics or phage therapy) are necessary to ensure these bacteria are not a source of resistant bacteria (Elmahdi *et al*., 2016; Igbinosa, 2016).

The FAO and WHO, through the Codex Alimentarius, have established codes and guidelines to address antimicrobial resistance and public health risks and to achieve food safety and these codes include the code of practice to minimize and contain antimicrobial resistance (CAC/RCP 61-2005) and guidelines for risk analysis of foodborne antimicrobial resistance (CAC/GL 77-2011). In addition to codes related to veterinary drugs and their residues (CX/MRL 2-2018), codes focused on animal nutrition (CAC/RCP 54-2004) as well as food sanitation codes of practice (CAC/RCP 1-1969 and CAC/RCP 52-2003) (FAO/WHO, 2020) establish good sanitizing and processing practices (time/temperature combinations in heat treatments) where processes lethal to bacteria in food reduce the risk of transmission of antimicrobial resistance (Verraes *et al*., 2013; FAO/WHO, 2020). Similarly, in the intentional use of microorganisms for food production, it is necessary to ensure that they are free of genetic material that transfers resistance. In countries such as the United States of America, microorganisms added to food processes, such as fermentation, are evaluated based on their food quality and are Generally Recognized As Safe (GRAS), while in Europe, microorganisms safe for use in food are subject to evaluations based on a Qualified Presumption of Safety (QPS) developed by the scientific committee of the European Food Safety Authority (EFSA); thus, microorganisms deemed safe to acquire a QPS status (Verraes *et al*., 2013; EFSA, 2020).

Conclusion

Currently, foodborne diseases are important health topics worldwide due to their incidence, mortality, and negative consequences on health, the economy, and the productivity of the affected society.

Fish are a widely consumed food around the world, with a high nutritional value, but they are highly susceptible to deterioration and contamination by various hazards that are mainly biological, such as bacteria; originate from the environment where the fish are caught or cultivated and occur due to the sanitation procedures used for the fish until their consumption.

The sources of fish for human consumption are fishing and aquaculture activities. For aquaculture, tilapia is one of the fish species with the highest production and commercialization worldwide due to its adaptability to farming conditions and its meat is of good quality and nutritional value (mainly protein) at affordable prices.

Vibrio vulnificus is a human pathogen that is contracted when consuming fish and shellfish, mainly in the raw state, as well as when coming into contact with and manipulating aquatic species. Similarly, this species is considered an animal pathogen that causes outbreaks of diseases in fish such as tilapia (epizootics) with consequent economic losses in the aquaculture sector. Therefore, *V. vulnificus* is considered a hazard with a high risk to human health since its incorporation into the food chain can lead to zoonosis.

Vibrio vulnificus, like other food pathogens, has been reported to be resistant to antimicrobials based on isolates from food and aquatic environments; this resistance is a significant risk to human and animal health, and the spread of this phenomenon can affect the production and safety of food.

Actions to prevent and control the diseases caused by *Vibrio vulnificus* and the spread of antimicrobial resistance must be carried out considering two factors that address the entire food chain (from farm to consumer table); first, these actions need to be implemented concerning animal production and health, where they mainly focus on implementing good aquaculture practices that include sanitation, water quality, fish density, feeding, and use of veterinary drugs. At the same time, postharvest food handling must be considered and the implementation of sanitation procedures such as good manufacturing practices, sanitation standard operating procedures, and HACCP systems must be involved. In addition, knowledge on food sanitation must be provided to the general population, focusing on the biological hazards present in aquatic foods, the sanitation management of these foods until their consumption, and the prevention of food being eaten in a raw state.

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Author's Contributions

Alejandro De Jesús Cortés-Sánchez: Conceptualization and study designed, data analysis, manuscript writing.

Luis Daniel Espinosa-Chaurand, Mayra Diaz-Ramirez, Ma. de la Paz Salgado-Cruz and Erika Torres-Ochoa: Data acquisition and analysis manuscript writing.

Ethics

The corresponding author confirms that all of the other authors have read and approved the manuscript and that are not ethical issues and conflict of interest involved before and after the publication of this manuscript.

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