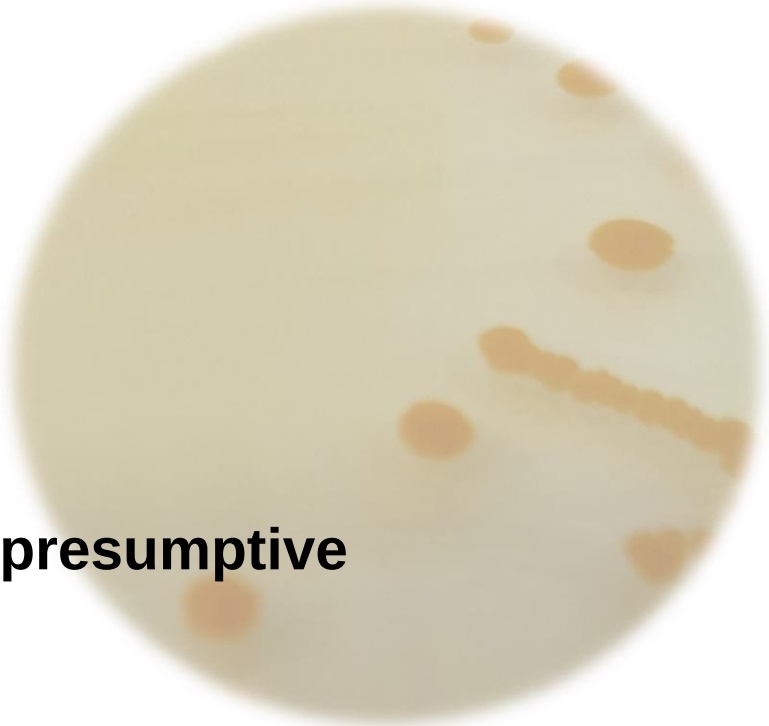

Implementation of a presumptive detection method of enteropathogenic *Vibrio* spp. Detection of *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus.* Method audit according to ISO 21872-1.

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____

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Resumo

As doenças de origem alimentar são causadas por bactérias, vírus, parasitas e substâncias químicas, sendo normalmente infeciosas ou tóxicas. As matrizes alimentares são suscetíveis a contaminação em qualquer parte do seu processamento, até ao seu consumo. O desenvolvimento da indústria alimentar e do comércio global, o envelhecimento da população, bem como o uso excessivo de agentes antimicrobianos, são fatores importantes que influenciam a epidemiologia e os padrões destas doenças. De acordo com a World Health Organization (WHO), perto de 23 milhões de pessoas adoecem e 5000 morrem devido a doenças de origem alimentar na Europa, muitas delas causadas por alimentos contaminados com bactérias. De modo a prevenir e controlar as ameaças causadas por alimentos e rações, foram implementados vários regulamentos, como procedimentos de amostragem e aplicabilidade de critérios microbiológicos, bem como os respetivos valores-guia para a avaliação da qualidade microbiológica.

Este estágio teve como objetivo a implementação de um método de deteção de *Vibrio* spp. potencialmente enteropatogénicos, bem como o cumprimento da rotina de trabalho atribuída, desde a preparação de meio de cultura e amostras até à realização dos procedimentos, de acordo com a norma ISO 21872-1. Vibrios são bactérias comummente encontradas em ambientes de água doce, estuarinos e marinhos, habitando preferencialmente águas quentes e ligeiramente salgadas. Estas bactérias apresentam uma rápida taxa de crescimento e, após a infeção em humanos, podem causar sintomas de ligeiros a graves. Devido às mudanças climáticas e ao aumento da temperatura das águas, estas bactérias são uma preocupação para a saúde pública, uma vez que estão a espalhar-se pelo mundo a um ritmo alarmante.

Foram analisadas um total de 85 amostras e foram realizados 132 ensaios, incluindo Comparações Interlaborariais (ICs), realizadas em anos anteriores. Um total de 91 ensaios (69%) foram contaminados artificialmente, enquanto 41 (31%) não foram contaminados. No total foram obtidos 91 resultados positivos para *Vibrio* spp. e 41 resultados negativos. Foi também detetada *Vibrio cholerae* em 4 das amostras analisadas, possivelmente devido a contaminação natural ou cruzada. Uma auditoria do método foi realizada após a sua implementação. A eficiência dos resultados e da técnica foi avaliada na forma de auditoria vertical e presencial. Não foram registadas não-conformidades nesta auditoria, sendo possível afirmar que o método de deteção de *Vibrio* spp. está a ser realizado de forma correta, segundo a norma ISO 21872-1.

Os resultados obtidos neste estágio enfatizam a importância da atualização constante das metodologias usadas em análise microbiológicas de alimentos e amostras ambientais, a

importância da validação e verificação dos métodos usados nesta indústria, bem como a melhoria constante do Sistema de Gestão de Qualidade.

Palavras-chave: Doenças de origem alimentar; segurança alimentar; qualidade alimentar; análise microbiológica; *Vibrio parahaemolyticus*; *Vibrio cholerae*; *Vibrio vulnificus*; auditoria de método.

Abstract

Foodborne illnesses are caused by bacteria, viruses, parasites, or chemical substances, and are often infectious or toxic. Food matrixes are susceptible to contamination in any part of its processing until its consumption. The development of the food industry and global marketing, the aging population, and the excessive use of antimicrobial agents are important factors that influence their epidemiology and patterns. According to the World Health Organization (WHO), nearly 23 million of people become ill and 5000 die due to foodborne diseases annually in Europe, many of them are caused by food contaminated with bacteria. To prevent and control the threats caused by food or feed, many regulations have been implemented, such as sampling procedures and applicability of microbiological criteria, as well as the respective guide values for the evaluation of microbiological quality.

This internship aimed to implement a detection method of potentially enteropathogenic *Vibrio* spp., as well as following the assigned work routine, from culture media and sample preparation to the performance of the procedure according to the ISO 21872-1. Vibrios are bacteria that are found in freshwater, estuarine, and marine environments, preferentially inhabiting warm and slightly salty waters. These bacteria present a fast growth rate and, upon infection on human, can cause an array of symptoms that go from mild to severe. Due to the ongoing climate changes and rise of sea and ocean water temperatures, these bacteria are a public health concern, since they are spreading across the globe at an alarming rate.

A total of 85 samples were analyzed and 132 assays were performed, including Interlaboratory Comparisons (ICs), performed in previous years. A total of 91 (69%) assays were contaminated artificially and 41 (31%) were not contaminated. In total, 91 assays tested positive for *Vibrio* spp., while 41 tested negative. It was also detected *Vibrio cholerae* in 4 of the analyzed samples, possibly due to natural or cross-contamination. A method audit was performed after its implementation. The results and technique efficiency were evaluated in the form of a vertical and presential audit. In this audit there were no non-conformities registered, it is possible to state that the method of detection of *Vibrio* spp. is being performed correctly, according to the ISO 21872:1.

The results obtained in this internship emphasize the importance of the constant update of the methodologies used in the microbiological analysis of foodstuff and environmental samples, the importance of the validation and verification of the methods used in this industry, as well as the constant improvement of the Quality Management Systems.

Keywords: Foodborne illnesses; food safety; food quality; microbiological analyses; *Vibrio parahaemolyticus*; *Vibrio cholerae*; *Vibrio vulnificus*; method audit.

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Abbreviation List

ADH – Arginine dihydrolase

AP-PCR – Arbitrarily primed polymerase chain reaction

ASAE – Autoridade de Segurança Alimentar e Económica

APW – Alkaline Peptone Water

Aw – Water activity

BHI – Brain-Heart Infusion

CAC – Codex Alimentarius Commision

CCP – Critical Control Points

CDC – Center for Disease and Control Prevention

COVIS – Cholera and Other Vibrio Illness Surveillance System

CPR – Continuous plankton recorder

CPS – Capsular polysaccharide

CT – Cholera enterotoxin

DGERT – Direção Geral do Emprego e das Relações de Trabalho

DPCS – Daily Process Control Samples

EA – European Accreditation

EEA – European Environment Agency

ECDC – European Centre for Disease Prevention and Control

EFSA – European Food Safety Authority

EGI – Sociedade de Engenharia e Gestão de Qualidade, Lda.

ERSAR – Entidade Reguladora de Serviços de Águas e Resíduos

EU – European Union

FAO – Food and Agriculture Organization

FDA – United States Food and Drug Administration

FoodNet – Foodborne Diseases Active Surveillance Network

Fur – Ferric uptake regulator

HACCP – Hazard Analysis and Critical Control Point

HlyA – Hemolysin A

ICs – Interlaboratory Comparisons

IND – Indole tests

INSA – Instituto Nacional Dr. Ricardo Jorge

IPAC – Instituto Português da Acreditação

IPMA – Instituto Português do Mar e Atmosfera

ITS – Intergenic spacer

ISO – International Organization for Standardization

KP – Kanagawa Phenomenon

LB – Luria-Bertani

LDC – Lysine decarboxylase

LPS – Lipopolysaccharides

MLVA – Multilocus variable-number tandem-repeat analysis

MLST – Multilocus sequence typing

MPN – Most-probable-number

NAGs – Non-agglutinating Vibrios

NGS – Next-generation sequencing

NOA – National Organism of Accreditation

ONPG – O-nitrophenyl-β-D-galactopyranoside

PCR – Polymerase chain reaction

PFGE – Pulsed-field gel electrophoresis

PNCA – National Plan for Sample Collection

PNFA – National Plan for Food Inspection

QMS – Quality Management Systems

RAPD-PCR – Randomly amplified polymorphic DNA-polymerase chain reaction

RASFF – Rapid Alert System for Food and Feed

rep-PCR – Repetitive extragenic palindromic polymerase chain reaction

RFLP – PCR-restriction fragment length polymorphism

RTX – Repeat in toxin

SSS – Sea surface salinity

SST – Sea surface temperature

stn – Heat-stable enterotoxin

TCBS – Thiosulfate-Citrate-Bile Salts-sucrose

TCP – Toxin regulated pilus

TDH – Thermolabile Direct Hemolysin

TNF-α – Tumor necrosis factor-alpha

TRH – TDH Related Hemolysin

TTSS – Type 3 secretion systems

USDA-FSIS – United States Department of Agriculture's Food Safety and Inspection Service

vcg – Virulence-correlated gene

VID – chromID *Vibrio*

VBNC – Viable-but-nonculturable

VPI – *Vibrio* Pathogenicity Islands

WHO – World Health Organization

Chapter I: Introduction

1.1 Institution

Silliker Portugal S.A is an independent service provider company linked to the various branches of the agri-food sector. The company is active since July 1992, in the municipality of Vila Nova de Gaia, with the designation of EGI – *Sociedade de Engenharia e Gestão de Qualidade*, Lda. By 2008, the company was named *Silliker* Portugal S.A, after an investment of 86% capital by the multinational North American *Silliker*, which detains one of the largest laboratory groups in the area of food testing. Since 2014, *Silliker* belongs to *Mérieux NutriSciences*, a network of 26 countries with nearly 100 accredited laboratories (Silliker, 2021).

It is an accredited laboratory by *Instituto Português de Acreditação* (IPAC) since 1993, with the accreditation number L0087. It is also recognized by many regulatory bodies, such as *Entidade Reguladora de Serviços de Águas e Resíduos* (ERSAR) and *Instituto Português do Mar e Atmosfera* (IPMA), for the collection and analysis of waters for human consumption, and analysis related to microbiological criteria applied to living bivalve mollusks, respectively. Since 2015, it started to play an important role in consultancy and formation, certified by *Direção Geral do Emprego e das Relações de Trabalho* (DGERT), as a formation entity in the food industry (Silliker, 2021).

Silliker Portugal S.A soon stood out by the way it answered the market needs in the food industry, rapidly becoming the national leader in providing the services it developed. As a company with well-defined objectives, it is committed to the client, by providing results with strict process control. *Silliker* Portugal S.A provides many services, such as analytical services and consultancy in the food industry and nutrition, while providing services to companies of different sectors, namely water and environment, agrochemical, goods, pharmaceutical, and cosmetical areas (Silliker, 2021). The organizational structure is presented in Fig. 1.

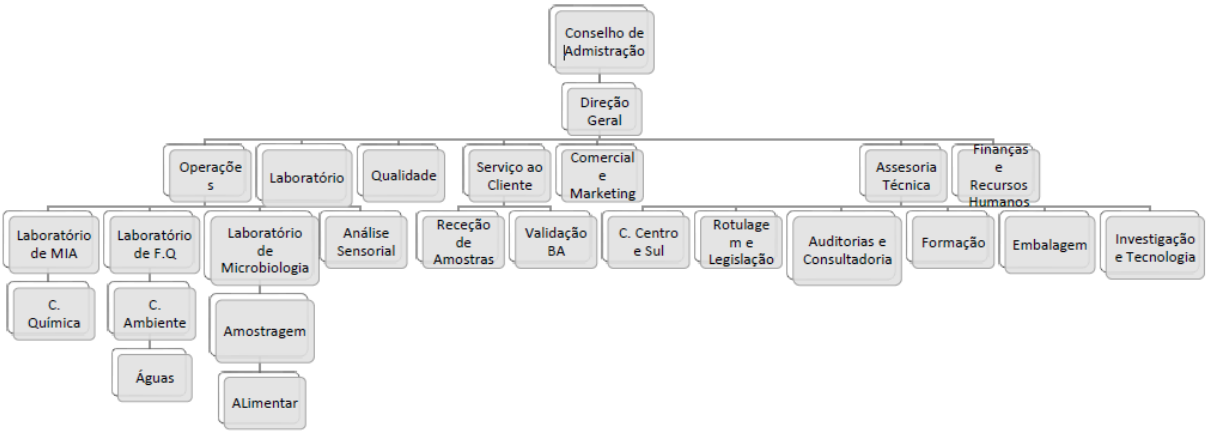


Fig. 1 - Organigram representing the structure of *Silliker* Portugal S.A (Silliker S.A., 2020)

1.2 Food Quality and Safety

The identification of food safe for consumption has always been of huge concern to humanity. Ever since the early humans have been on earth, they developed ways of recognizing and avoiding potentially toxic foods, which were most likely by trial and error, as well as previously acquired knowledge. As humans evolved, from hunter-gatherers to collectors, approximately 15000 years ago, their understanding and capacity of storing and preserving food also improved (Bopp, 2019; Griffith, 2006). The basic forms of food preservation developed included salting, drying, and fermentation. As human habits changed, so did the eating patterns, where the food consumed was different, with an increasing need for more safety in its production and consumption (Griffith, 2006).

Food quality and safety are of extreme importance to society, always trying to improve the control from its processing until its consumption. Despite the efforts to enhance food security by educating the costumers through information campaigns, alongside exploratory microbiology, foodborne illnesses have a major socio-economic impact (Tirado and Schmidt, 2001).

According to the World Health Organization (WHO), “Foodborne illnesses are usually infectious or toxic in nature and caused by bacteria, viruses, parasites or chemical substances entering the body through contaminated food or water” (WHO, 2021a). WHO launched the *Food Surveillance Programme for the Control of Foodborne Diseases* in Europe, in 1980, to provide support to countries that aimed to improve the overall surveillance systems regarding food safety, by developing the appropriate measures to prevent and control foodborne illnesses, whilst identifying the epidemiology of foodborne diseases in those regions, providing relevant information, and aiding the national authorities with the reinforcement of the systems of prevention and disease control (Tirado and Schmidt, 2001).

To improve food safety, the *Codex Alimentarius* was developed in 1963 by the Codex Alimentarius Commission (CAC), established by the Food and Agriculture Organization (FAO) and the WHO and, since then, it has been amended several times. It counts with more than 180 member countries and 200 intergovernmental and international non-governmental organizations (Stankovic, 2016). This document is an international reference for food safety from its primary production to the final consumer, containing a basic structure for other specific codes and norms, applied to specific sectors. The main objectives of this document are to promote, guide, and facilitate the international marketing of foods, by providing information about food handling, preparation, storage, and consumption. Therefore, the main principles of the *Codex Alimentarius* are (FAO and OMS, 2006):

- Protect the consumers against diseases and potential damage caused by food;
- Guarantee that the food is adequate for human consumption;
- Maintain the trust for internationally commercialized food;
- Develop programs that promote the education for food hygiene to the industry and its consumers (FAO and OMS, 2006).

The CAC also established the principles of *Hazard Analysis and Critical Control Point* (HACCP) system. This system allows the identification of dangers and the respective measures for its control, to guarantee food safety, thus, analyzing dangers and preventing them, rather than analyzing the final product (FAO and OMS, 2006). Since 2006, the Regulation (CE) n. 852/2004 of the European Parliament and the Counseling, requires that every operator from the food sector must create, apply and maintain procedures that are based on the seven principles of HACCP, which, according to the CAC, are:

1. Danger identification and preventive measures;
2. Critical Control Points (CCP) identification;
3. Establish critical limits to each CCP;
4. Establish a system that monitors the control of CCP's;
5. Establish which corrective action shall be used when the monitoring indicates that a certain CCP is not under control;
6. Establish the verification procedures to confirm that the system is functioning efficiently;
7. Establish a documentation system of all the procedures and the registration appropriated to those principles and the application of them (ASAE, 2007).

According to the CAC, the implementation of the HACCP system has a set of prerequisite programs, which are basic requirements to produce safe and wholesome food, such as the sanitary conditions of the establishment and equipment used, the good manufacturing practice and food safety programs of the facility suppliers, the requirements for the personal hygiene of employees and other staff, the implementation of pest control programs, the training of employees, as well as the traceability and recall of all raw materials and products (FDA, 1997). One of the benefits of the use of the HACCP system in the food industry is that it can be adapted according to the changes that are applied in the industry, such as equipment upgrades, changes in the procedure, or technological development (FAO and OMS, 2006).

The mandatory implementation of the HACCP system was requested by the *European Food Safety Authority* (EFSA), created by the regulation (CE) n 178/2002. This authority was created based on crises involving food and animal feed, such as the bovine spongiform encephalopathy outbreak (Christian, 2021), to provide independent scientific counseling about the risks associated with food. The EFSA provides advice about the existing and emerging

food risks, by informing European laws, rules, and policymaking, thus protecting consumers from the existing risks in the food chain, while collaborating with external agencies related to the field of health and safety issues for humans, animals and the environment (EFSA, 2021).

According to the EFSA, the European Union (EU) laws and standards aim for health protection in the agriculture, animal husbandry, and food production sectors, with an extensive body of EU-wide law covering not only the entire food production and processing chain inside the EU but also covering the goods that are imported and exported. Therefore, the EU countries have established authorities to implement these harmonized standards, while establishing certain controls to enforce them (EU, 2021). The authority that ensures that these laws and standards are correctly applied in Portugal is the *Autoridade de Segurança Alimentar e Económica* (ASAE), an administrative authority/criminal police agency (ASAE, 2021). The objective of ASAE is to inspect and assure compliance with the regulation legislation in economic activities, as well as communicate the risks regarding the food chain (ASAE, 2020). Therefore, ASAE is responsible for compliance with regulations regarding the food area, such as the regulation regarding the hygiene of food products (Regulation (CE) n.852/2004), and the hygiene regarding food products of animal origin (Regulation (CE) n.852/2004), as well as other regulations in the form of decree-laws. There are also planned controls, such as the *National Plan for Food Inspection* (PNFA) and the *National Plan for Sample Collection* (PNCA), thus based on the food risks, according to the Regulation (CE) n. 882/2004 (ASAE, 2018).

Despite food safety being in the best interest of everyone, food quality must also be assured to the consumers. While food safety refers to all chronic and acute hazards that food might cause to the consumer's health, food quality includes all the attributes that influence the value of the product to the consumer, whether they are negative such as spoilage, or positive such as flavor or texture (FAO, 2003). Therefore, to guarantee that food meets the quality expectation of the consumers, the international norm NP EN ISO 9001 was published. This norm is an international reference for the certification of Quality Management Systems (QMS) (APCER, 2021a).

1.3 Accreditation of Laboratory Procedures and Calibrations

The analysis of food items is of extreme importance and can only be performed by competent laboratories. By detecting problems on the food items, the laboratory can then alert the company, which identifies what measures can be implemented to correct the problem. All the analyses performed in this industry need to comply with the HACCP system, therefore many laboratories of the agro-feeding industry are accredited. Thus, their technical competence is

formally verified to perform conformity assessment tasks, such as assays, calibrations, certifications, and inspections (APCER, 2021b; IAS, 2021; IPAC, 2021). According to the decree-law n.140/2004, accreditation "is the procedure in which the National Organism of Accreditation (NOA) formally recognizes that an entity is technically competent to perform a specific function, according to international, European or national norms, while following the orientation emitted by the international organisms of accreditation that Portugal is inserted in" (DRE, 2004). The national system of accreditation in Portugal is IPAC in compliance with the Regulation (CE) n. 765/2008, therefore:

- There is only one national accreditation organism in each state member;
- It performs the activities as a public authority;
- It is differentiated from other national authorities while being independent and impartial;
- It does not have profit under supervision by the state members;
- It follows the international standard EN ISO/IEC 17011;
- The accreditations performed are recognized by the EU national authorities;
- It is a member of the European Accreditation (EA), and it is subject to the respective evaluation system by its peers.

IPAC also provides several documents to guide laboratories throughout the process of accreditation, such as requirements, regulations, forms, and guides (IPAC, 2021).

The accreditation of the laboratory is performed according to the NP EN ISO/IEC 17025, which is an international standard for testing and calibration of laboratories. The general requirements of competence to perform tests, calibrations, and samplings according to standard and non-standard methods, as well as laboratory-developed methods, are included in the norm. Since this ISO applies to all accredited laboratories throughout the world, the communication between laboratories belonging to different countries is facilitated and the results obtained are similar. The implementation of this norm in a laboratory is very significant, contributing to the laboratory image, thus increasing customers loyalty (ISO, 2017a).

1.4 Validation and Verification in Accredited Laboratories

According to the NP EN ISO/IEC 17025, whenever a new method is implemented; or when an existing method is used for the first time in a laboratory; or when the method has changed, it is necessary to perform its validation, which is the "confirmation of truthfulness through the provision of objective evidence that specified requirements have been fulfilled" (ISO, 2020).

The validation should be as extensive as necessary, while also including procedures related to the sampling, handling, and transportation of calibration and testing items. The techniques used in the validation of a method can be single or combined with other techniques, from testing the robustness of the method by changing controlled parameters, such as temperature, to comparing the obtained results with other validated methods, as well as interlaboratory comparisons (ICs). A method can only be validated if the laboratory has access to the latest release of the protocol, described in a laboratory procedure that includes pieces of equipment, specifications, and additional information that are essential to perform the method (ISO, 2017a). For a microbiological method to be validated, the parameters exhibited in Table 1 must be evaluated (ISO, 2017b).

Table 1 - Parameters evaluated for the validation of a microbiological method according to ISO 13843 (ISO, 2017b).

Parameter	Meaning
Accuracy	Obtained from the results of Interlaboratory comparisons.
Sensitivity	The capability of the method to detect the target organism. The total fraction of positive samples, correctly assigned in the presumptive count. Normal sensitivity: >90%.
Specificity	The total fraction of negatives, correctly assigned in the presumptive count. Normal specificity: >80%.
Rate of false positives	Fraction of positive results (typical colonies) that demonstrated to be non-target organisms.
Rate of false negatives	Fraction of negative results (atypical colonies) that demonstrated to be target organisms
Selectivity	The ratio of the number of colonies/target-assays by the total number of assays.
Efficiency	Fraction of total colonies/assays correctly assigned in the presumptive count

1.5 Internal Audit

To prevent potential problems before they occur, especially in the global food industry, it is important to pinpoint them. This process, known as audit, is a systematic, independent, and documented procedure that allows the objective assessment of the effectiveness of the organization's quality management system and overall performance, according to previously

established criteria (IIA, 2021; Keen, 2021; Mérieux Nutrisciences Australia, 2021). An internal audit can be carried out by an organization to its systems, methods, and facilities, using its qualified staff or contracted auditors. The independence of the staff is exhibited by the non-responsibility for the audited area. As for microbiological methods, the audit is carried out from the sampling procedure to the obtention of the results and its report, while also assessing the traceability of the sample, a component that allows the identification of critical steps in the performance of the method (ISO, 2017a).

1.6 Daily Process Control Sample (DPCS)

The consistency of the results obtained by a laboratory is assured by the control of the performed procedures. This type of control is obtained by evaluating the accuracy of the results to demonstrate that the assays performed in the laboratory are following the criteria established during its validation/verification (Silliker, 2020).

The daily control of the procedures is performed by using *Daily Process Control Samples* (DPCS), as established by internal control procedures of *Silliker* Portugal S.A. DPCS is a sterile food matrix, eliminating the presence of contaminant microflora, with well-defined homogeneity and stability, used for the control verifications and elaboration of control cards. These samples contain the target microorganism, at a known concentration, and are analyzed according to the same method that is used for the samples of the client. By using this control, the laboratory shows that it is capable of executing the analytical method, as well as identifying the target organisms and that the analysts are capable of performing the method (Silliker, 2020).

1.7 Foodborne Illnesses

Foodborne illnesses result from dynamic interactions between hosts, agents, and the surrounding environment where this relationship occurs, known as the epidemiological triad. Different factors influence the epidemiology of foodborne illnesses, such as the development of new foods, the availability of new sources of food, the aging population, and the excessive use of antimicrobial agents or the clinical setting in some production environments. These are some of the examples that can lead to changes in the patterns of illnesses (Doyle, 2020).

This type of illness occurs when food is contaminated at any stage of its production, delivery, or consumption, causing symptoms that range from gastrointestinal to neurological and immunological disturbances (WHO, 2021b). Food contamination can be described as the presence of unwanted organisms, substances, or taint to the packaging, food, or environment.

There are three types of food contamination: biological, physical, and chemical (Manning and Soon, 2016). Biological contamination is considered to be the most relevant form of contamination, in which contamination from bacteria, fungi, virus, and their derivatives (e.g. toxins), as well as other contaminants, are included. Bacterial contamination is one of the most common forms of food contamination, causing the most cases of food poisoning worldwide (Abdolshahi and Yancheshmeh, 2020).

Bacterial growth can be influenced by several factors that fall under four different categories: Food-related factors (intrinsic), including water activity (Aw), nutrient content, pH value, redox potential, mechanical barriers, and the presence of antimicrobial substances; Environment-related factors (extrinsic) regarding food storage, such as temperatures, humidity, and gas composition; Factors related to the microorganisms (implicit), such as food-microorganism interaction, stress tolerations, and their ability to use the sources of nutrition; And processing factors that include the treatments used in the production of the food. The interactions between these four factors affect the growth of bacteria in food items, since the combination of these aspects may have a synergetic or additive effect (Hamad, 2012).

It is estimated that more than 23 million people fall ill annually in Europe, and nearly 5000 people die from eating contaminated food. Amongst all the infectious agents, the most relevant ones are represented in Table 2, along with the number of reported cases occurring in Europe in 2010 (WHO, 2017a).

Table 2 - Leading causes of foodborne illnesses in the WHO European region, in 2010 (WHO, 2017a).

Rank	Pathogen	Number of Cases per year
1	Norovirus	14 850 045
2	*Campylobacter* spp.	4 687 810
3	Non-typhoidal *Salmonella enterica*	1 683 734
4	*Toxoplasma gondii*	1 068 724
5	*Giardia* spp.	485 752
6	*Cryptosporidium* spp.	187 416
7	Shiga toxin-producing *Escherichia coli*	165 250

8	Hepatitis A virus	97 472
9	*Ascaris* spp.	71 884
10	Enteropathogenic *Escherichia coli*	71 395

The most common bacteria that cause foodborne illnesses are *Salmonella* spp., *Campylobacter* spp., and pathogenic strains of *Escherichia coli.* These pathogens are transmitted through the consumption of contaminated food, such as eggs and dairy products for *Salmonella* spp., contaminated broiler meat for *Campylobacter* spp., and, in the case of *E. coli*, handling and consumption of raw/undercooked meat and contaminated dairy products. These pathogens affect the gastrointestinal tract of humans, causing a wide spectrum of symptoms, from diarrhea to nausea and vomiting. *Salmonella* spp. and *Campylobacter* spp. can also cause post-infection complications, such as reactive arthritis as well as respiratory and neurological dysfunctions, respectively. *Listeria* spp. also causes severe foodborne illness through the contamination of food with low moisture and high salt concentration. Although it is less common, this pathogen has a high case-fatality rate. *Vibrio cholerae* is a diarrheal disease agent that caused seven outbreaks throughout the world (WHO, 2017a, 2017b). There are other *Vibrio* species that are known to cause illnesses due to the consumption of seafood. Bacteria from this genus, such as *V. parahaemolyticus*, are a common cause of foodborne illness in Asia, but lately, these bacteria have been spreading to regions previously unreported, causing several outbreaks throughout the world (Food Safety News, 2020).

Even though the understanding and the methods to prevent foodborne illnesses are evolving, the problems associated with food safety are far from getting solved, with cases increasing in both developed and developing countries (Griffith, 2006). Therefore, to prevent and control health threats caused by food or feed, the *Rapid Alert System for Food and Feed* (RASFF) was established by the Regulation (CE) n. 178/2002. This tool provides a safe and fast exchange of information between the Member States, by notifying them about potential dangers towards human health (ASAE, 2016; FAO, 2017). Despite all the dangers mentioned, food and feed do not need to be completely innocuous for them to be marketed. Therefore, the Regulation (CE) n. 1441/2007 established a set of rules for sampling procedures as well as the applicability of microbiological criteria for food and feed, while *Instituto Nacional Dr. Ricardo Jorge* (INSA) provides the guide values for the evaluation of microbiological quality (INSA, 2019).

1.8 *Vibrio* spp.

Vibrio is a genus belonging to the *Vibrionaceae* family, composed of Gram-negative, rod-shaped bacteria commonly found in freshwater, estuarine and marine environments (Baker-Austin et al., 2017, 2018; Doyle, 2020). Species from this genus are genetically diverse, but they also share many biological and genomic features. They inhabit aquatic environments, preferably in warm (above 17ºC), slightly salty waters (5 to 25 ppt), in which the environmental conditions reflect the abundance of organisms (Baker-Austin et al., 2016; Vezzulli et al., 2013). Due to climate changes, the bacteria belonging to this genus are now spreading across the globe at an alarming rate (Baker-Austin et al., 2013, 2017; Doyle, 2020; Vezzulli et al., 2013; Waits et al., 2018).

Many other bacterial pathogens are detected in seawater along with *Vibrio* spp., such as *Escherichia coli*, *Salmonella* spp., *Proteus* spp., *Pseudomonas aeruginosa*, *Aeromonas hydrophila,* and *Staphylococcus aureus*. These bacteria can cause many clinical manifestations, including digestive infections, in which most infections caused by natural aquatic and seafood microbiota are attributed to species of the *Vibrio* genus, especially during warmer months (Baker-Austin et al., 2018, 2017).

Vibrio species can adapt to the environment they live in, assuming a free-living form or attached to biotic or abiotic surfaces in the form of biofilm, increasing their prevalence in the environment (Reen et al., 2006). This phenomenon contributes to their persistence and growth in aquatic environments. These bacteria are commonly found in filter-feeding organisms, such as oysters, in which they can reach a concentration 100 times higher than that found in the surrounding water (Doyle, 2020; Froelich et al., 2017). This group of bacteria includes approximately 130 confirmed species with a dozen of them known to cause infections in humans, with *V. parahaemolyticus* being the most relevant one, followed by *V. cholerae*, *V. vulnificus,* and *V. alginolyticus* (Baker-Austin et al., 2017; Doyle, 2020). The gastrointestinal disturbances can lead to fatal diarrheal diseases, wound infections, and septicemia (Baker-Austin et al., 2017). If the infection is caused by any other type of food, cross-contamination can be the cause of it (Jung, 2018; Liao et al., 2015)

Digestive infections caused by *Vibrio* spp. can be divided into two groups: cholera and non-cholera infections, depending on the species of bacteria that caused the clinical manifestation. Cholera infections are caused by *Vibrio cholerae*, the aetiological agent of cholera, a severe diarrheal disease caused by ingestion of contaminated water and food (Baker-Austin et al., 2017; Howard-Jones, 1984). Non-cholera infections, known as vibriosis, are caused by other *Vibrio* species, such as *V. parahaemolyticus* and *V. vulnificus*. Depending on the route of

infection, host susceptibility, and the species that cause the infection, this group of infections can exhibit different clinical manifestations. They cause mild gastroenteritis or primary septicemia, when raw/undercooked food is ingested followed by a wound infection, or secondary septicemia if skin wounds get exposed to contaminated water (Baker-Austin et al., 2018).

Vibrio spp. cell numbers show a seasonal variation, reaching the highest values in warmer months, during summer and early fall (Iwamoto et al., 2010). This is more noticeable in *V. vulnificus* and *V. parahaemolyticus*, whereas other infections caused, for example, by *V. fluvialis* are observable throughout the year. Although *V. cholerae* infections caused by the serotypes O1 and O139 are recorded by WHO, the same does not apply to other strains or species, where most cases do not get reported worldwide (CDC, 2017; Doyle, 2020; Heng et al., 2017; Vezzulli et al., 2020)

Since the 1970s, in the US, all human infections caused by pathogenic bacteria belonging to the *Vibrionaceae* family are reported by the *Cholera and Other Vibrio Illness Surveillance System* (COVIS). Despite that, only in 2007 did vibriosis become a nationally notifiable disease by the *Centers for Disease and Control Prevention* (CDC). All vibriosis cases in the US are monitored through the *Foodborne Diseases Active Surveillance Network* (FoodNet), a collaborative program of the CDC, 10 state health departments, the *United States Department of Agriculture's Food Safety and Inspection Service* (USDA-FSIS), and the *United States Food and Drug Administration* (FDA) (CDC, 2017). Most seafood-associated outbreaks from 1973 to 2006 were caused by *Vibrio* infections, where a yearly variation was observed in the number of outbreaks (Doyle, 2020; Iwamoto et al., 2010).

The number of vibrioses registered within the U.S. population has been steadily increasing from 1996 to 2010, where *V. parahaemolyticus*, *V. vulnificus,* and *V. alginolyticus* were the most detected bacteria (Newton et al., 2012). According to the CDC, there are nearly 80,000 cases of vibriosis and 100 deaths in the USA every year, mostly recorded during warmer months. The number of *Vibrio* infections and *Vibrio* species is steadily increasing around the world, even in countries that had no previous recorded cases, in contrast to other major foodborne pathogens (Baker-Austin et al., 2017; Paz et al., 2007).

The rise of global sea surface temperature (SST) is a major physical impact of climate change, according to the European Environment Agency (EEA) (le Roux et al., 2015). The coastal European seas have seen their SST increasing 4 to 7 times faster than in global oceans, over the past decades (Reid et al., 2011). The local increase of *Vibrio*-associated human illnesses caused by *V. parahaemolyticus*, *V. cholerae* Non-O1/Non-O139, and *V. vulnificus* is linked to the rising temperature in many European countries. The increase of human infections caused

by these bacteria has been reported in European countries, even in higher latitudes (Baker-Austin et al., 2017, 2013; Martinez-Urtaza et al., 2013). The number of reported infections corresponds temporally and spatially with spikes of domestically-acquired *Vibrio* cases in heatwave years, in the north of Europe (le Roux et al., 2015). This information is corroborated by samples collected for 60 years by the continuous plankton recorder (CPR) survey (Baker-Austin et al., 2017; Vezzulli et al., 2012), which showed that there is a correlation between the increased prevalence of *Vibrio* bacteria in the coastal North Sea and the increase of SST (le Roux et al., 2015).

An accurate estimate of the number of *Vibrio* infections occurring in Europe is not possible, due to the lack of mandatory notification systems for *Vibrio*-associated illnesses (le Roux et al., 2015; Semenza et al., 2017; Vezzulli et al., 2020). Despite that, it is suggested that human activities are directly linked to the increased frequency of mass marine animal mortalities, since these occur particularly in heavily polluted areas, thus favoring disease epidemics. Some of the examples are the devastation of oyster beds in France, the threatened salmonid farming industry, and the mass mortality of benthic corals in the northwest Mediterranean Sea, all caused by *Vibrio* species (le Roux et al., 2015; Munn, 2015; Vezzulli et al., 2010a).

Regarding the lack of tools to monitor *Vibrio* infections throughout Europe, the *European Centre for Disease Prevention and Control* (ECDC) developed a quasi-real-time web platform to monitor marine areas. This tool exhibits coastal waters with suitable growth conditions for *Vibrio* species, based on a real-time model that uses daily updated remotely sensed SST and sea surface salinity (SSS), and compiles the information in a map that shows the risk factor in those areas (Semenza et al., 2017).

Despite the human pandemic strains of *Vibrio* ssp. being well-studied, there is a lack of knowledge about the virulence mechanisms of environmental *Vibrio* species, which may be attributed to the highly diversified virulence mechanisms and the genetic diversity amongst *Vibrio* isolates. Since the virulence factors (toxins and secretions systems) are rarely species-specific and often shared between *Vibrio* strains by horizontal gene transfer, the pathogenic capacity cannot be inferred by taxonomic affiliation (le Roux et al., 2015).

1.8.1 Isolation, identification, and genotyping of *Vibrio* spp.

Vibrio spp. is mostly isolated from sediments, water columns, and animals. To isolate it in the laboratory, it is recommended to use a pre-enrichment medium, such as Alkaline Peptone Water (APW), with plating after 6 to 8 hours to prevent the growth of undesirable bacteria (Doyle, 2020; Kaysner et al., 2004). To improve identification and isolation, streaking in

Thiosulfate-Citrate-Bile Salts-sucrose agar (TCBS) and *Vibrio* chromogenic agar is performed. The differences in the metabolism among the species are used to differentiate them apart. TCBS agar prevents the growth of Gram-positive and *Enterobacteriaceae* species, whilst allowing the differentiation of sucrose-positive species, such as *V. cholerae* and *V. alginolyticus*, from sucrose-negative species, like *V. parahaemolyticus* and *V. vulnificus* (Kaysner et al., 2004). Since several species exhibit the same metabolic profile in TCBS agar, the use of a chromogenic medium is necessary. Therefore, species such as *V. vulnificus* and *V. parahaemolyticus* (both sucrose-negative) are differentiated in chromogenic agar. Thus, the use of these media combined is recommended to obtain the best results (Oliver, 2011).

Presumptive *Vibrio* colonies isolated from TCBS and chromogenic medium are confirmed via a standard series of biochemical media used to identify *Enterobacteriaceae* and *Vibrionaceae*, such as API20E and API20NE, due to the troublesome identification of these species solely based on phenotypic characteristics (Doyle, 2020). Although these tests are widely used throughout the world, they are not 100% accurate, since many of the results obtained can be misleading, whether they do not identify the organism or there is a low chance of it being correctly identified (O'Hara et al., 2003). To ensure the correct identification of the species, a combination of the results obtained on the tests is used (Doyle, 2020; Ramamurthy et al., 2014) as biochemical keys, especially in routine applications, such as environmental and clinical studies with many isolates. These biochemical tests included lysine decarboxylase (LDC), arginine dihydrolase (ADH), o-nitrophenyl-β-D-galactopyranoside (ONPG), and indole tests (IND), obtained in the API20E test (Noguerola and Blanch, 2008).

Due to the problems raised by the identification of the bacteria from this family, several studies using molecular methods are emerging. Methods that utilize polymerase chain reaction (PCR) and sequencing of *Vibrio*-specific DNA markers can be the solution to overcome the limitation of the phenotypic methods. Using molecular methods, the results can be obtained within hours, whereas using culture-based methods, the identification of a bacterium can take up to several days (Silvester et al., 2017). Moreover, molecular methods allow the detection of bacteria that are not viable nor culturable (Doyle, 2020). Newly discovered molecular methods are already used for the discrimination of closely related pathogenic bacteria from the *Vibrio* genus, for example, the use of tetraplex PCR to detect *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus*, and *V. mimicus* simultaneously in cockles (Senachai et al., 2013). Techniques such as pulsed-field gel electrophoresis (PFGE) (Kai et al., 2008), multilocus variable-number tandem-repeat analysis (MLVA) (Harth-Chu et al., 2009), and multilocus sequence typing (MLST) (González-Escalona et al., 2008) are some of the many molecular subtyping methods used to enlighten the scientific community about the genetic diversity and population structure of *V.*

parahaemolyticus strains for epidemiological purposes. These DNA-based techniques allow the establishment of a relation between the *Vibrio* species with the disease epidemics, as well as the discrimination of strains based on pathogenic and epidemic potential, and explore the biology of the population of these bacteria (Doyle, 2020).

The PCR-restriction fragment length polymorphism (RFLP) analysis of the *groEL* gene, a highly conserved gene among *Vibrio* species, can be used to distinguish strains efficiently (Hossain et al., 2014). This gene expresses a heat-shock protein (HSP60) in bacteria, which expression's increase during stress conditions, such as low temperature, pH, and salinity, both inside and outside the host (Chowdhury et al., 1996; Hossain et al., 2013; Kim et al., 2010; Mukhopadhyay et al., 2006). *groEL* also proved to be a better phylogenetic marker than the 16S rRNA, a highly conserved gene, since the *Vibrio* genus shares a high level of sequence similarity with many other closely related species, exhibiting less than 1% difference in the 16S rRNA gene nucleotide sequence with some of those species (Ruimy et al., 1994; Silvester et al., 2017). Therefore, due to all these features, *groEL* is thought to be one of the most reliable conserved genes to distinguish *Vibrio* species with significant differences between the sequences obtained.

Due to the health risks that *Vibrio* infections imply, methods for isolation and enumeration of these bacteria in seafood are also relevant. Different methods are used depending on the pathogen. Most-probable-number (MPN) titration of replicate samples in enrichment broth cultures, followed by direct cultivation in selective agar plates, and posterior incubation and membrane filtration are the most used methods (Kaysner et al., 2004). New protocols are developed constantly for the isolation and enumeration of *Vibrio* species in specific food products (Banerjee and Farber, 2017), such as *V. vulnificus* X-Gal, a selective and differential medium developed for the direct enumeration of *V. vulnificus* organisms from oyster samples, using cellobiose and lactose as carbon sources as well as the antibiotics colistin and polymyxin B as selective agents (Doyle, 2020; Griffitt and Grimes, 2013).

1.8.2 *Vibrio parahaemolyticus*

V. parahaemolyticus was firstly described after a severe foodborne outbreak in Osaka, Japan, in 1950, caused by the consumption of a small, semi-dried sardine known as "*shirasu".* In this outbreak, 20 of the 272 patients who suffered from acute gastroenteritis died (Joseph et al., 1982). Following that incident, *V. parahaemolyticus* has been implicated in many foodborne outbreaks throughout the world. According to the CDC FoodNet data, there is an incidence rate of 0.24 per 100,000 people of *V. parahaemolyticus* in laboratory-confirmed cases, in 2015,

confirming that this bacterium is the most isolated microorganism from clinical cases, from the *Vibrionaceae* family in the US (CDC, 2017; Doyle, 2020).

V. parahaemolyticus is a ubiquitous halophilic, Gram-negative bacterium, inhabiting estuarine and marine environments (Pang et al., 2020). These bacteria can exist planktonically, using flagella for movement, attached or submerged, associated with other organisms/surfaces such as zooplankton, shrimp, fish, and suspended particles (Kaneko and Colwell, 1973; McCarter, 2004, 1999). The detection of *V. parahaemolyticus* is directly associated with the temperature (10 – 44ºC), reaching optimal conditions at 35 to 37 ºC (Odeyemi, 2016). This bacterium prefers slightly salty environments (6% NaCl), using aquatic organisms such as mollusks, shrimp, and fish as hosts (DePaola et al., 1990; Jay et al., 2005).

Infections caused by this microorganism can lead to acute gastroenteritis, where watery/bloody diarrhea, abdominal cramps, nausea, vomiting, fever, and headache are common clinical manifestations (Joseph et al., 1982; Levin, 2006; Odeyemi, 2016; Wagley et al., 2009) that sometimes require hospitalization (Doyle, 2020). In severe cases, the infection can lead to bloody diarrhea and necrotizing fasciitis, causing the death of soft tissues (Pang et al., 2020). If the patient has underlying medical conditions, the illness can evolve to life-threatening septicemia, which requires aggressive antimicrobial therapy. *V. parahaemolyticus* has an incubation period of 4 to 94 hours (Levin, 2006) and the illness caused by this pathogen is self-limiting, lasting from 1 to 12 days, where the symptoms can occur 24 hours after the ingestion of the contaminated food (Doyle, 2020).

Besides the standard identification methods, *V. parahaemolyticus* exhibits a variable expression of urease, a protein coded by the *ure* gene. This gene is associated with the expression of Thermostable Direct Hemolysin (TDH) and TDH Related Hemolysin (TRH), coded by *tdh* and *trh* genes, respectively, both considered important virulence factors (Kaysner et al., 1994; Osawa et al., 1996).

The preference of slightly salty environments by *V. parahaemolyticus* (DePaola et al., 1990; Jay et al., 2005) impacts the reliability of commercial identification systems, such as the API20E system (O'Hara et al., 2003). Therefore, to improve the accuracy of identification of food and environmental isolates of *V. parahaemolyticus*, the media used for testing the biochemical reactions of this bacterium should contain from 2 to 3% of NaCl (Kaysner et al., 2004).

V. parahaemolyticus can be characterized by 3 antigenic components: H (flagellar), O (somatic), and K (capsular). Only the latter two are used for serotyping, despite H being common to all strains. Most of the environmental and clinical isolates can be serotyped by

component O, but some of them cannot be serotyped by component K. Component O consists of 13 lipopolysaccharide antigens, while component K consists of 71 acidic polysaccharide antigens (Li et al., 2021). Until the mid-1990s there was thought to be no correlation between the serotype and virulence. Only in 1996, with the emergence of the pandemic clone O3:K6, new relevance was given to the investigation of outbreaks and epidemiological studies (Doyle, 2020).

1.8.2.1 Pandemic clone O3:K6

In January 1994, an active surveillance of *Vibrio parahaemolyticus* infection amongst hospitalized patients was initiated in Calcutta, India. By February 1996, the incidence cases of infections caused by these pathogens increased and remained high for a while. The frequency of isolation of serovar O3:K6 strains ranged from 50 to 80% between February and August of that year (Nair et al., 2007; Okuda et al., 1997). Eventually, this strain began spreading over to Southeast Asian countries and eventually to Japan. Later, between 1997 and 1998, the pandemic clone O3:K6 was also isolated in several other countries across the world, such as the USA, Taiwan, and Thailand (Matsumoto et al., 2000). The isolates from this serovar had a specific pattern, with nearly identical arbitrarily primed PCR (AP-PCR) profiles, including *tdh*-positive, *trh*-negative, and urease-negative profiles, as well as similar antibiograms (Nair et al., 2007; Okuda et al., 1997).

Based on the results obtained from the AP-PCR, many methods were developed due to the several factors that can influence the outcome of the technique. Therefore, in 2000, Matsumoto et al. performed a PCR on 194 strains of the serovar O3:K6 from eight different countries, based on *toxRS* sequence, concluding that all strains belonged to the same clone (Matsumoto et al., 2000). The sequence identity of *toxRS* between *V. parahaemolyticus* and other *Vibrio* species is lower than the identity for the 16S rRNA, allowing the differentiation of clusters of *V. parahaemolyticus* phylogenetically different (Kim et al., 1999; Matsumoto et al., 2000).

The development of techniques to identify O3:K6 isolates led to the discovery of other serotypes, such as O4:K68, O1:K25 and O1:K untypeable (KUT) with identical *toxRS* sequences, AP-PCR profiles, ribotypes, and PFGE profiles to the O3:K6 serovar (Chowdhury et al., 2000; Matsumoto et al., 2000). The first pandemic of *V. parahaemolyticus* was marked by the isolation of O3:K6 serovariants in the North, Central, and South America, as well as Africa and Europe, by 2006 (Doyle, 2020; Nair et al., 2007).

1.8.2.2 *V. parahaemolyticus* life cycle

The life cycle of *V. parahaemolyticus* is highly dependant on environmental conditions. While it is free in an aquatic medium, it keeps moving with the help of its flagella, but, as soon as it contacts with any solid surface, it enters in a sessile state and begins a distinct differentiation process, colonizing extensive surfaces by “swarming” motility (Fig. 2) (Fraser and Hughes, 1999; McCarter, 2001). During this process cell division is inhibited, resulting in the growth of extremely elongated, filamentous, rod-shaped cells. When this bacterium adheres to a surface, an independent system of secondary flagella (*laf* system) is expressed, resulting in a higher number of lateral flagella throughout the membrane of this organism (Fig. 3). This system stimulates the swarming behavior, leading to swift colonization of the surface (Freitas et al., 2020). Throughout this type of colony, it is possible to observe different cell sizes depending on the position where they grow. In the center, there are several layers of smaller cells, but in the periphery, the number of layers is reduced, while the cells are bigger. This process of cell elongation is extremely important to colonize big surfaces. However, there is no evidence of different gene expressions between the two groups (Freitas et al., 2020).

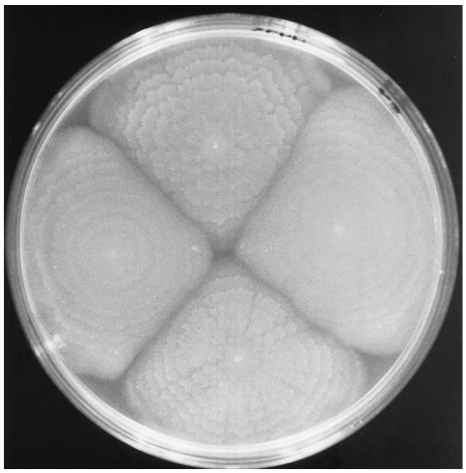


Fig. 2 - Swarm colonies of *Vibrio parahaemolyticus* in solid medium (McCarter, 2001).

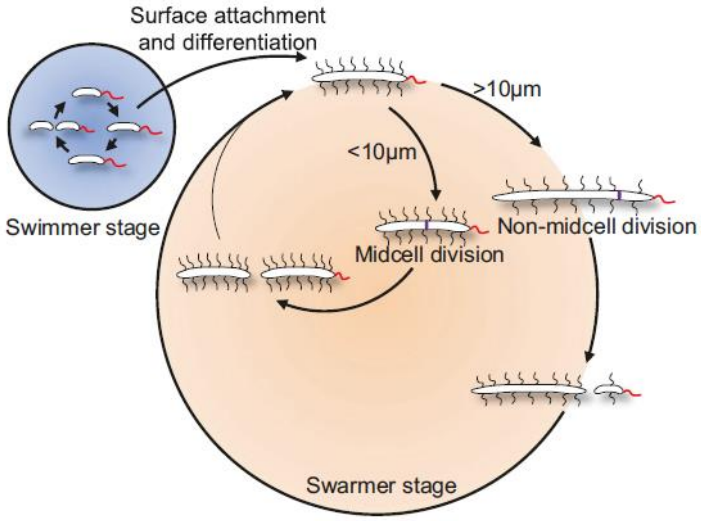


Fig. 3 - Life Cycle of *Vibrio parahaemolyticus* (Freitas et al., 2020).

1.8.2.3 Dissemination

V. parahaemolyticus is characterized by its rapid growth rate (Ulitzur, 1974), exhibiting a generational time of 12 to 18 minutes in seafood (Katoh, 1965). This remarkable characteristic allows this species to reach the estimated infectious dose rapidly in seafood and cross-contaminated food products (10^5 to 10^7 in pathogenic strains) (Doyle, 2020; Joseph et al., 1982).

The dissemination method of this bacterium is based on the size of the cells in the swarm colony. The bigger cells, located in the periphery of the colony, stay attached to the surface, while the smaller ones in the center are released into the liquid medium, contaminating it. The release of these cells not only stimulates the spread of this bacterium but also facilitates the colonization of new surfaces, indicating that they are a different type of cells (Freitas et al., 2020).

1.8.2.4 Virulence

Although bacteria from the *Vibrio* genus are associated with gastrointestinal diseases, some strains are not pathogenic (Croci et al., 2007; Raghunath, 2014). Despite *V. parahaemolyticus* occurring naturally in the marine environment, only a small number of environmental isolates were proven to be pathogenic. The pathogenicity of these microorganisms is characterized by the ability of certain strains to produce hemolysins. In the late 1970s, the production of TDH was detected in clinical strains that exhibited beta-hemolytic activity when inoculated in

Wagatsuma agar, a special blood agar. This process is designated as the Kanagawa Phenomenon (KP) in which positive strains are associated with pathogenicity (Doyle, 2020; Sakazaki et al., 1968). Early studies showed that 96.5% of clinical isolates produced TDH, but only 1% of KP-positive strains prevailed in the environment (Sakazaki et al., 1968). TDH is an amyloid, pore-forming toxin with a wide range of biological activities, such as hemolytic and cytotoxic activities (Raimondi et al., 2000). During infection of *V. parahaemolyticus*, the flux of ions in the intestine is altered, and water absorption is affected, causing diarrhea, the most common clinical manifestation (Doyle, 2020; Raimondi et al., 2000).

Later, in 1988, during the investigation of the Maldives outbreak in 1985, a second hemolysin was discovered that allowed the pathogenicity of *V. parahaemolyticus*, in the absence of the *tdh* gene, the TRH (Honda et al., 1988). Therefore, despite the absence of the *tdh* gene and the presence of the *trh* gene, this strain is characterized as KP-negative, since it does not form hemolytic zones on Wagatsuma agar, although they are observable on other common blood agar (Honda et al., 1988). An example of this type of strain is the *V. parahaemolyticus* strain isolated by Fujino and his research team, in 1950 (strain EB101, WDCM 00037) (Shinoda, 2011).

TRH is immunologically similar to TDH, but it appears to be heat-labile, as it becomes inactive after a 10-minute treatment at 60ºC (Honda et al., 1988). TRH, much like TDH, activates the Cl^- channels, altering the ion flux in the epithelial intestine cells, leading to poor water absorption (Shimohata and Takahashi, 2010). Exhibiting higher sequence variations between strains than *tdh* genes, *trh* genes can be clustered into two main sub-groups (*trh1* and *trh2*), sharing 84% homology (Kishishita et al., 1992) and nearly 68% with *tdh* genes (Nishibuchi et al., 1989).

1.8.3 *Vibrio cholerae*

V. cholerae was first described in 1854 by Pacini in Italy, who reported a “large number of curved bacteria in the intestinal contents of victims”. In 1883, by studying cholera in Egypt, Robert Koch demonstrated that this disease was caused by a comma-shaped microorganism, naming it *Kommabazillen*. This organism was later named *Vibrio comma*, several years before the work of Pacini was recognized, finally changing its name to *Vibrio cholerae* (Kaper et al., 1995).

V. cholerae is a Gram-negative bacterium that does not form spores. This microorganism inhabits brackish and estuarine environments around the world and is classified according to the surface antigen O into nearly 206 serogroups, in which O1 and O139 cause pandemic

cholera (Chowdhury et al., 2017; Safa et al., 2010). *V. cholerae* serogroup O1 caused seven cholera pandemics since its first appearance in 1817, in which the seventh is the currently ongoing pandemic that started in Indonesia in 1961 (Barua, 1992), causing more than 100,000 deaths every year (Albert, 1994; Almagro-Moreno et al., 2015; Kaper et al., 1995).

V. cholerae growth conditions are like other *Vibrio*, a halotolerant organism that has optimal growth at low salinity (0.2% to 3.0% concentration of NaCl), high temperature (ranging from 30ºC to 40ºC), and 8 pH (Vezzulli et al., 2010b). Despite the vast amount of information that there is about cholera and its pathogenic agent, the environmental reservoir for this species is not well defined. Estuarine environments are considered ideal for the survivability and persistence of *V. cholerae*, but these bacteria are also present in arid, central regions, such as in the African continent, far away from coastal waters (Kaper et al., 1995).

Water and food play a critical role in the transmission of cholera (Kaper et al., 1995). While both food and water are considered the major vehicle for the transmission of this pathogen in developing countries, the latter is not as critical as foodborne transmissions in developed countries (Sack et al., 2004). These bacteria are capable of colonizing organisms, from green algae to copepods, and abiotic substrates, such as sediments (Vezzulli et al., 2010b). Due to the capability of producing chitinase, *V. cholerae* can bind chitin, the main component of crustacean shells. This feature allows the colonization of crustaceans, including copepods, reaching from 10^4 to 10^5 *V. cholerae* cells per copepod (Colwell, 1996; Colwell et al., 2003; Doyle, 2020; Pruzzo et al., 2008). *V. cholerae* persists in the environment due to its ability to assume forms that increase their survivability, such as viable-but-nonculturable (VBNC) state, biofilms, and a rugose form (Colwell and Huq, 1994; Huq et al., 2008; Vezzulli et al., 2010b). The ability to form biofilms allows the growth of toxigenic species alongside non-toxigenic species in aquatic environments, increasing their survivability (Watnick and Kolter, 1999).

The clinical manifestations caused by *Vibrio cholerae* can range from asymptomatic colonization to cholera gravis, the most severe form of cholera (Kaper et al., 1995). After the ingestion of contaminated food and/or water, and passing the acid barrier of the stomach, *V. cholerae* starts to colonize the epithelium of the small intestine (Kaper et al., 1995) for 12 to 72 hours before the first symptoms appear (Nelson et al., 2009). The first cholera symptom is usually stomach cramps and vomiting, followed by diarrhea (Nelson et al., 2009). The loss of water and electrolytes can also lead to severe diarrhea. Fatal and explosive, dehydrating diarrhea, characteristic of cholera, is only seen in a small percentage of patients infected with cholera enterotoxin (CT) producing *V. cholerae* (Kaper et al., 1995). Patients suffering from the most severe cholera form have typical rice water stool diarrhea, reaching rates of 500 to 1000 mL/h, harboring between 10^{10} and 10^{12} vibrios per liter (Doyle, 2020; Kaper et al., 1995).

If this disease is not treated, it can lead to severe dehydration, circulatory collapse, and death of the patient (Morris, 2003).

Three important characteristics allow the distinction of microorganisms within this species: serogroup, production of CT, and epidemic spread potential (Kaper et al., 1995). The distinction of *V. cholerae* serogroups O1 through O139 is based on the lipopolysaccharide (LPS) somatic antigen (Kaper et al., 1995) To identify *V. cholerae* serogroup O1 and O139, polyvalent antisera against those antigens can be used to observe bacteria agglutination (Vezzulli et al., 2020). Oxidase-positive bacteria that agglutinate in the antisera can be reported as presumptive pandemic strains *V. cholerae* O1 and O139 and forwarded to a public health reference laboratory for confirmation (CDC, 1999).

Due to the ease and successfulness of the conventional identification methods, nucleic acid probes are not often used for *V. cholerae* identification, despite the accuracy of directly identifying cholera-toxin encoding strains in environmental and complex samples (Doyle, 2020; Kumar et al., 2010; Lyon, 2001; Vezzulli et al., 2015).

Many techniques proved their usefulness when it comes to subtyping *Vibrio cholerae* strains, such as RFLP-PFGE, ribotyping (Bakhshi et al., 2014), and MLST (De et al., 2013; Mohapatra et al., 2009; Vezzulli et al., 2020). The distinction of environmental isolates is of extreme importance since many of the strains lack the production of CT (Mukhopadhyay et al., 1995). Single multiplex PCR is also used to simultaneously amplify 95 regions from *Vibrio* spp., such as toxin-encoding genes and intraspecific genes, hybridizing them to a microarray containing those same genes. By allowing fast and definitive discrimination of *Vibrio* strains, this technique contributes to the environmental, epidemiological, and risk assessment surveillance of species belonging to this genus (Vora et al., 2005). Other techniques involving next-generation sequencing (NGS) have proven to be useful for genotyping and metagenomic analysis of *V. cholerae* strains (Doyle, 2020; Schadt et al., 2010).

1.8.3.1 *V. cholerae* O1 and O139

The survivability of *V. cholerae* O1 in foodstuff is greatly increased by low temperatures, high organic content, high humidity, as well as by the lack of competition with other microorganisms (Morris, 2003). Amongst all the existing food matrixes, seafood plays a critical role in the transmission of this pathogen, although many others can transmit cholera, such as rice, raw fish, raw vegetables, and fruit, all sharing a characteristic near-neutral pH (Morris, 2003). For a long time, CT producing *V. cholerae* O1 was associated with epidemic and pandemic cholera while any other strain was either considered nonpathogenic or occasional pathogens.

However, due to the occurrence of other epidemics of *V. cholerae* O139 (John et al., 1993), as well as the discovery of other pathogenic strains other than O1 and O139, that distinction is no longer valid (Kaper et al., 1995).

There are two biotypes of *V. cholerae* O1: El Tor and classical (Kaper et al., 1995). El Tor strains are considered less virulent, being frequently associated with asymptomatic infections, fewer fatalities, better host-to-host transmission, and better survivability inside the host, as well as in the environment. On the other hand, the classical biotype is linked to more severe clinical manifestations (Sack et al., 2004; Safa et al., 2010). They are differentiated using phenotypic tests and genotypic analysis of specific genes since these types of *V. cholerae* O1 have different phenotypic and genotypic characteristics, as well as survivability, pathogenic potential, and infection patterns in humans (Doyle, 2020; Safa et al., 2010).

Many differences have been identified between the strains for El Tor *V. cholerae* found in the Asian and African continents, known as atypical El Tor variants (Goel et al., 2008; Safa et al., 2010). These atypical variants can produce CT of either biotype and cannot be biotyped based on phenotypic characteristics. Altered El Tor variants are also classified as atypical variants since they can be phenotypically characterized as El Tor strains while producing classical *V. cholerae* cytotoxin (Cho et al., 2010; Grim et al., 2010). These new variants replaced the El Tor *V. cholerae* O1 that caused the seventh pandemic, as reported by the isolation of clinical cases of cholera (Doyle, 2020), being nominated as the most dominant strain in the world (Longini et al., 2002; Nelson et al., 2009; Sack et al., 2003; Villeneuve et al., 1999)

In 1993, a new *V. cholerae* serogroup with similar symptoms and *ctx* sequences to El Tor *V. cholerae* O1 was discovered in Eastern India and Bangladesh, *Vibrio cholerae* O139, or “Bengal” due to its place of origin (Albert, 1994). This new serogroup was indistinguishable from the O1 serogroup, producing CT and toxin regulated pilus (TCP), although it does not produce O1 Lipopolysaccharides (LPS), and possesses a 35-Kbp region encoding the O139 antigen, as well as a polysaccharide capsule (Doyle, 2020; Stroeher et al., 1997; Weintraub et al., 1994). As *V. cholerae* serogroup O1 was replaced by O139 in southeast Asia, a fear of a new pandemic rose. Despite that, only a few cases of the new serogroup were reported outside that region and serogroup O1 soon became dominant again (Albert and Nair, 2005; Doyle, 2020).

1.8.3.2 *V. cholerae* Non-O1/O139

Although *V. cholerae* O1 and O139 are the serogroups that raise more concern, most strains that do not produce CT and TCP are referred to as *V. cholerae* Non-O1/Non-O139 (NOVC) (Vezzulli et al., 2020), or non-agglutinating Vibrios (NAGs) (Doyle, 2020; Dutta et al., 2013). These serogroups rely on other virulence factors to cause infections, such as heat-stable enterotoxin (stn), hemolysin A (HlyA), repeat in toxin (RTX), and type 3 secretion systems (TTSS) (Chatterjee et al., 2009; Dutta et al., 2013).

Some strains from these serogroups are associated with gastroenteritis or mild extraintestinal symptoms, and, although rarely, fatal cases of necrotizing fasciitis and septicemia (Hirk et al., 2016; Khan et al., 2013). Strains from the serogroup O141 were found to produce both CT and TCP, typical from *V. cholerae* O1 and O139, and have been isolated from cases of severe diarrhea (Dalsgaard et al., 2001). Several strains belonging to serogroups other than O1 and O139 have been the origin of many disease outbreaks in Asia, as well as other continents, which is believed to be related to climate change and ocean warming (Doyle, 2020; Hasan et al., 2012; le Roux et al., 2015)

1.8.3.3 *V. cholerae* life cycle

V. cholerae is a natural inhabitant of surface brackish, and estuarine waters, surviving and multiplying in association with zooplankton and phytoplankton (Huq et al., 1983; Islam et al., 1990; Kaper et al., 1979). Since the growth of plankton is highly dependent on environmental factors, climate changes can modify their growth and, consequently, the growth of *Vibrio cholerae* (Sack et al., 2004).

The life cycle of this bacterium consists of two phases, the first one outside the host, and the second one inside the host (Fig. 4). *V. cholerae* can assume a free-living form, as free-swimming cells, or attached to abiotic surfaces, such as sediments (Vezzulli et al., 2010b), or biotic surfaces, including plants, algae, and preferably chitinaceous plankton, such as copepods, due to their chitin-binding interaction using chitinase (Colwell, 1996; Pruzzo et al., 2008; Sack et al., 2004). To persist and survive in the environment, *V. cholerae* can form biofilms and enter a VBNC state (Colwell and Huq, 1994; Huq et al., 2008; Vezzulli et al., 2010b), allowing them to survive between epidemics, in the lack of nutrients (Reidl and Klose, 2002).

Although they are part of normal estuarine flora, toxigenic strains are more commonly isolated from the environment of areas contaminated by infected individuals. On the other hand,

toxigenic strains are not usually detected in regions where there are no *V. cholerae* infections (Faruque et al., 1998b; Sack et al., 2004).

To colonize and survive inside the host, *V. cholerae* must have acquired *Vibrio* pathogenicity islands (VPI), which carry genes for the expression of TCP (Faruque et al., 1998a). These mobile elements are more efficiently expressed in the intestinal milieu of animals. Therefore, based on the life cycle of *V. cholerae*, this bacterium can colonize the human gut and survive during the phase of infection, while living as an autochthonous bacterium of the estuarine environment in interepidemic phases (Sack et al., 2004).

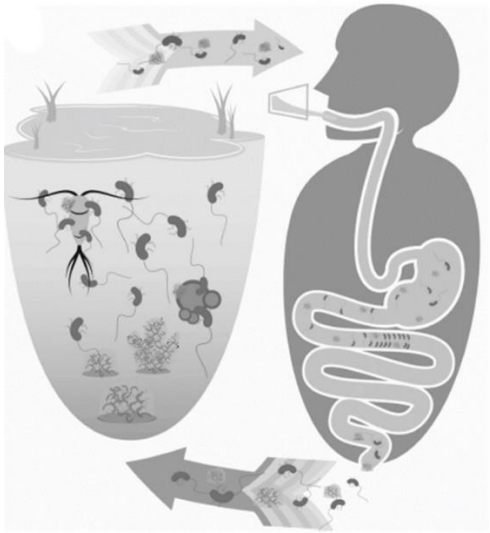


Fig. 4 - Life cycle of *Vibrio cholerae* (Adapted from Conner et al., 2017).

1.8.3.4 Dissemination and Virulence

V. cholerae exhibits a doubling time of 1.1 h in the wild (Gibson et al., 2018). To infect a human host a minimum amount of *V. cholerae* cells must be ingested. The infection dose varies depending not only on the host but also on the strain (Nelson et al., 2009). The successful colonization of a healthy human being requires a dose of 10^8 to 10^{11} cells. This minimum amount decreases to 10^4-10^8 cells if the stomach acid is neutralized beforehand, using, for example, bicarbonate buffer right before the inoculation, raising the infection rate to 90% (Nelson et al., 2009; Nishibuchi and Kaper, 1995). The virulence factors, such as TCP, are expressed in the small intestines after passing the acid barrier of the stomach (Doyle, 2020), and the cells coordinate an exit from the host to make the transmission from host-to-host easier (Schild et al., 2008), shedding nearly 10^{10}-10^{12} cells per liter of rice-water stool (Nelson et al., 2009). Symptomatic patients may shed *Vibrio cholerae* before the illness onset (Cash et al.,

1974b, 1974a), and will continue to shed for 1 to 2 weeks (Dizon et al., 1967; Kaper et al., 1995). This characteristic is important since the symptomatic patients are the main transmission agents of *Vibrio cholerae* (Nelson et al., 2009).

1.8.4 *Vibrio vulnificus*

Vibrio vulnificus was first referred to as "*Benechea vulnificus*", as well as other common names such as "Lactose-positive halophilic *Vibrio*" and "L+ *Vibrio*", until 1979, when it was defined as a new bacterial species of the *Vibrio* genus by Farmer, due to its unusual phenotypical properties, such as being indole and lactose-positive (Farmer, 1979; Hernández-Cabanyero and Amaro, 2020). Due to some clinical manifestations caused by *V. vulnificus*, such as severe wound infections, sometimes media reports this microorganism as flesh-eating bacteria (CDC, 2019; Doyle, 2020).

V. vulnificus is a Gram-negative, halophilic aquatic bacterium that is part of the normal bacterial flora in estuarine and marine environments, in warm regions (Hernández-Cabanyero and Amaro, 2020; Horseman and Surani, 2011; Oliver, 2015). It exhibits a single polar flagellum that uses to move in aquatic media (Strom and Paranjpye, 2000). Due to the great genotype and phenotype variation, this bacteria is divided into three biotypes, all of them able to cause human infection (Froelich and Oliver, 2013), in which the first biotype can be divided into two genotypes, genotype C for clinically isolated strains, and genotype E for environmentally isolated strains, based on the variation in the virulence-correlated gene (*vcg*) (Jones and Oliver, 2009; Warner and Oliver, 2008). The three biotypes are linked to different hosts, biotype 1 is found in salty or brackish water and is responsible for severe human infection (Heng et al., 2017), biotype 2 infects eels (Amaro and Biosca, 1996; Tison et al., 1982), especially in aquaculture, and biotype 3 was only isolated in Israel, linked to the handling of tilapia (Bisharat et al., 1999; Froelich and Oliver, 2013). Even though it is a rare bacterium, this pathogen is the leading cause of death for seafood consumption, in which 1 out of 5 infected individuals sometimes die within one or two days of becoming ill (CDC, 2019). Recent studies have shown that nearly 100 cases are reported every year, with most cases requiring hospitalization, however, most cases are not reported (Heng et al., 2017)

Much like other vibrios, the environment plays an important role in the survivability of this species, with salinity and temperature being the most important factors. The ideal temperature and salinity for its growth range between 10ºC to 30ºC, and 0.2% and 2.5% NaCl, respectively, reaching optimum values at approximately 30ºC, and between 1% to 1.8% NaCl (Oliver, 2015). *V. vulnificus* is an obligate halophilic bacteria, therefore, when presented to environments that

do not fill the salinity requirements of this species, it initiates a stress response, causing their death (Audemard et al., 2011; Jones et al., 2008; Motes and DePaola, 1996; Oliver, 2015). Temperature is also critical not only for the growth of *V. vulnificus* but also for human infection. A 12-year study showed that *V. vulnificus* has a distinct seasonality, much like *V. parahaemolyticus*, with 97% of the cases occurring from April to November, in the Gulf of Mexico, when the water temperature was above 20ºC (Oliver, 2015). Due to climate change, and consequently the rise of sea surface temperature (SST), this microorganism is colonizing different regions around the globe, extending its geographical distribution, causing more infections even in colder regions, such as Denmark, Sweden, Belgium, Germany, and Spain (Heng et al., 2017; Oliver, 2015).

The occurrence of *V. vulnificus* is directly linked with estuarine organisms, sediments/particulates, and plankton. There are two different transmission routes of *V. vulnificus*, via direct contact with skin wounds, and via consumption of raw/undercooked seafood (Heng et al., 2017; Jones and Oliver, 2009). Despite being an aquatic organism, the major vehicle of transmission of this pathogen is seafood, more specifically filter-feeding organisms, such as oysters, that accounts for 93% of the ingestion cases (Oliver, 2015). Even though *V. vulnificus* is part of the natural bacterial flora in estuarine regions, the concentration of this bacterium in the environment is low, reaching values lesser than 10 CFU/g. However, due to the filter-feeding capability of oysters, they can become more than 100 times concentrated inside these organisms, and reach values higher than 10^5/g of tissue (Doyle, 2020; Motes et al., 1998; Oliver, 2015). When the temperature requirements for the survivability of *V. vulnificus* are not met, this microorganism enters a VBNC state to survive (Oliver, 2015).

Cases of human infection from *V. vulnificus* are rare, and mostly affect individuals with underlying illnesses or that are immunocompromised (Feldhusen, 2000). The clinical manifestations vary depending on the route of infection. By consuming contaminated seafood, the patient usually develops gastroenteritis, presenting nausea, vomiting, abdominal pain, and cutaneous manifestations. Eventually, the infection can evolve into septicemia accompanied by chills, fever, and cutaneous manifestations, such as secondary lesions on the extremities within 7 days, and in some patients, up to 14 days (Heng et al., 2017; Jones and Oliver, 2009). Within the first 24 hours of the onset of illness, secondary cutaneous lesions appear on the patient's extremities, such as cellulitis bullae and ecchymoses (Haq and Dayal, 2005). Patients with primary septicemia can also undergo septic shock, changes in the mental status, and thrombocytopenia (Heng et al., 2017), having the worst outcome of all the clinical manifestations, reaching a mortality rate of more than 50% of the patients (Hlady and Klontz,

1996; Horseman and Surani, 2011). It is believed that the infection by *V. vulnificus* starts in the small intestine (Heng et al., 2017), with an incubation period of 26 hours after its ingestion (Oliver, 2013). If the infection is caused due to direct contact with contaminated water, like swimming, fishing, or while handling diseased eels, it can lead to fatal wound infections and septicemia, exhibiting symptoms such as fever, chills, cellulitis, and edema at the infection site. Compared to the infections caused by the ingestion of contaminated food/water, the incubation time of *V. vulnificus* in this type of infection is even faster, averaging 16 hours, with symptoms occurring with 7 to 12 days after exposure (Heng et al., 2017; Horseman and Surani, 2011). While death is not as common with these infections (22%), primary septicemias, tissue debridement, skin grafts, and limb amputation are common (58%) (Oliver, 2015).

The efficiency of the treatment for a *V. vulnificus* infection is directly correlated with the speed and accuracy of the diagnostic (Bross et al., 2007). The use of antibiotics is essential for the treatment of patients with systemic septicemia and wound infection (Heng et al., 2017), and when it is delayed for more than 24 hours in patients with primary septicemia, the mortality rates increase from 33% to 55%, peaking at 100% when the patients are not treated within 72 hours (Hlady and Klontz, 1996; Oliver, 2013). The use of antibiotics to treat *V. vulnificus* infection is generally positive, regardless of the route of infection. However, there is no general first-line antimicrobial agent for the treatment of the infection caused by this pathogen. Since there are different strains scattered throughout the world, they developed different resistances, causing some antibiotics to become ineffective. Therefore, depending on the geographic location of the patient and the resistance of the microorganism, a suitable antibiotic must be chosen to treat the infection (Heng et al., 2017).

Due to the ever-increasing human cases of infection by *V. vulnificus*, there was a need to know more about the differences between the environmental and clinical strains (Oliver, 2015). This distinction is not possible based on traditional methods that use specific media to grow the bacteria followed by the use of biochemical tests to identify them. Therefore, the use of molecular methods is vital to enlighten the scientific community about this matter. Based on the results of a randomly amplified polymorphic DNA-PCR (RAPD-PCR) in 1999, it was observed a unique PCR amplicon of 178 to 200 bp (VV0401) in most human clinical isolates, whereas only 8% of the studied environmental isolates exhibited this region (Rosche et al., 2005; Warner and Oliver, 1999). Further studies around this ~200 bp region allowed the distinction between clinical and environmental strains, based on which pair of primers yielded a 277 bp product. If the first pair generated a PCR product, the strain would have a clinical (C) genotype, but if the second pair generated a PCR product, the strain would have an environmental (E) genotype (Rosche et al., 2005). Based on this distinction between C and E

genotypes, many other molecular techniques have been applied to discriminate them, especially to examine the geographical distribution of potentially pathogenic environmental strains (Jones and Oliver, 2009). One of the techniques that stand out is the DNA sequencing of the 16S rRNA gene, where different sequences were obtained between environmental isolates and clinical isolates (Aznar et al., 1994). The sequencing of the intergenic spacer (ITS) between the 16S rRNA gene and the 23S rRNA gene is also used and it subdivides the strains even further, with clinical strains having more sequence similarity between them and environmental strains being more diverse (González-Escalona et al., 2007). To facilitate the discrimination between the isolates, MLST may also be used, as well as repetitive extragenic palindromic PCR (rep-PCR), which generates a unique “fingerprint” of each strain by targeting conserved repetitive regions of the bacterial genome (Jones and Oliver, 2009). By combining the rep-PCR analysis with the single-locus typing methods to compare *V. vulnificus* strains isolated throughout the world, it was possible to observe that the clinical profile obtained from the infections of this pathogen were rarely observed in environmental oyster isolates (Chatzidaki-Livanis et al., 2006; Jones and Oliver, 2009; Warner and Oliver, 2008).

1.8.4.1 *V. vulnificus* life cycle

The life cycle of *V. vulnificus* has various phases, depending on the host and the surrounding conditions, such as temperature. Iron plays a critical role in the life cycle of *V. vulnificus*, which uses transferrin to sequester iron from the host while being regulated by the ferric uptake regulator (Fur). The life cycle of this pathogen is also dependant on several other genes related to its motility and capsule (Hernández-Cabanyero and Amaro, 2020). As mentioned before, when the conditions are not appropriate for the growth and maintenance of this bacterium, it enters a dormant state of VBNC, which allows it to survive while maintaining its pathogenic potential. In optimal conditions, with an increase of temperature in the presence of nutrients, these cells are resurrected (Marco-Noales et al., 1999). Although they are acapsulated and nonmotile when resurrected, the genes related to those traits are reactivated, and they can move and interact with surfaces and hosts. These vegetative cells can survive in water as a free-swimming organism or attached to biotic and abiotic surfaces (Hernández-Cabanyero and Amaro, 2020; Oliver, 2015). The concentration of iron and temperature influence the behavior of this bacterium. At optimal temperature, when the iron is deficient, the genes related to the pili are activated and the formation of biofilm is favored. On the other hand, when the concentration of iron is high, the genes related to the capsule are activated, interfering with the biofilm formation, and the bacteria begin to disperse. They can also infect other organisms, such as seafood and fish, through chemo-attachment to mucosal tissue or open wounds. Once

V. vulnificus becomes attached to the host, it can start interacting with the iron that is present in hemoglobin and eel-transferrin, due to the presence of iron-uptake-related genes, causing hemorrhages and septicemia (Pajuelo et al., 2016). Diseased fish and contaminated seafood can infect humans via contact and ingestions, respectively, leading to hemorrhages, septicemia, and possible death of the new host, designated as dead-end host (Fig. 5) (Hernández-Cabanyero and Amaro, 2020).

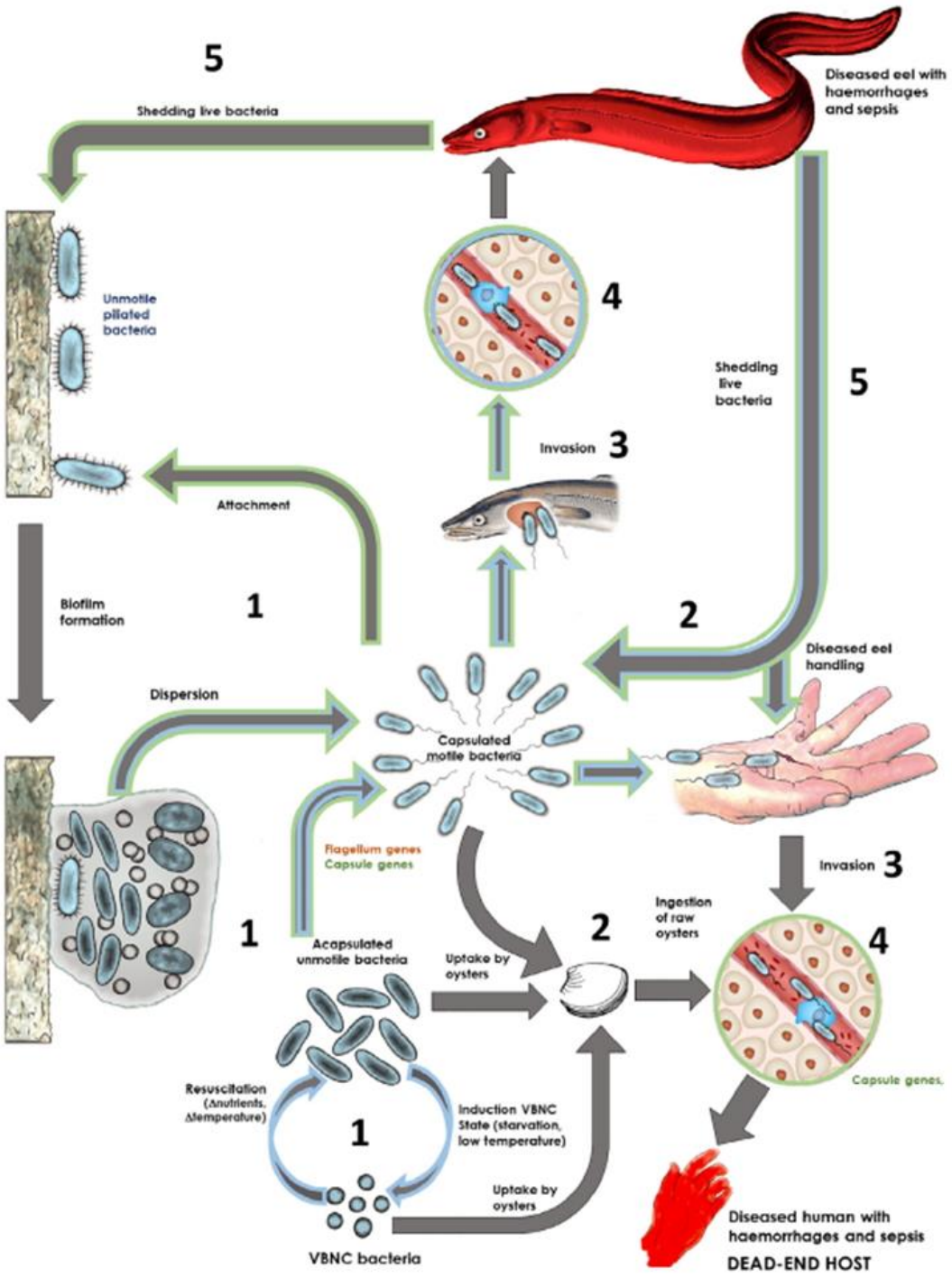


Fig. 5 - Life cycle of *Vibrio vulnificus* (adapted from Hernández-Cabanyero and Amaro, 2020).

1.8.4.2 Dissemination

V. vulnificus infectious dose depends on the variant of the pathogen ingested and the health of the consumer. Oysters are the main vehicle for the biotype 1 *V. vulnificus*, harboring more than 10^7 CFU g^{-1} in warmer months, while eels are the main vehicle for biotype 2 since these organisms shed bacteria when they suffer from sepsis, increasing the risk of infecting humans that handle them. Despite the disease being self-limiting in healthy populations, in susceptible ones, fewer than 100 total bacteria can cause a wide range of clinical manifestations, such as fever, chills, altered psychological status, and hypotension (FDA, 2012; Horseman and Surani, 2011; Oliver, 2015). The generational time depends on the biotype of the strain, in which biotype 1 has the fastest generational time of 3.05 h^{-1} (CFU/mL), followed by the biotype 2 with 1.75 h^{-1}, and biotype 3 with 1.2 h^{-1}, at 30ºC, 2% salinity and neutral pH (Chase and Harwood, 2011).

1.8.4.3 Virulence

V. vulnificus is an extracellular pathogen, therefore this bacterium faces many challenges while colonizing the human host, such as the presence of gastric acid in the stomach. Due to the lack of information regarding this pathogen, the virulence mechanisms are not well characterized, although many factors have been thought to be essential for its virulence, such as capsular polysaccharide (CPS), certain extracellular enzymes, exotoxins, iron acquisition systems, and its acid resistance (Drake et al., 2007; Pettis and Mukerji, 2020; Strom and Paranjpye, 2000).

The best-known virulence factor is the presence of the CPS, which offers protection against phagocytosis by the host defense cells (Pettis and Mukerji, 2020; Strom and Paranjpye, 2000). Depending on the growth phase and the temperature, this bacterium can be capsulated, or nonencapsulated. The genes for the expression of the CPS are activated in the logarithmic growth phase and at the temperature of 30ºC. However, in the stationary growth phase, and at the temperature of 37ºC, these genes are deactivated, and the capsule is absent (Drake et al., 2007; Wright et al., 1990). When streaked on Brain-Heart Infusion (BHI) agar, virulent strains are opaque, while most avirulent are translucid, indicating that the presence of the CPS is essential for virulence (Moreno and Landgraf, 1998).

The presence of LPS is also a known virulent factor, not only of *V. vulnificus* but also of other gram-negative bacteria, such as *Escherichia coli* (Strom and Paranjpye, 2000). LPS is a

mediator of endotoxic shock, which is characteristic of the disease caused by this pathogen, inducing a cytokine response and an increase of tumor necrosis factor-alpha (TNF-α) expression (Jones and Oliver, 2009; Powell et al., 1997). Therefore, there may be a synergetic effect between CPS and LPS, which allows the bacterium to overcome the host immune response, causing inflammation, tissue damage, and septicemia, thus playing an important role in the host cytokine response during systemic *V. vulnificus* infections (Strom and Paranjpye, 2000).

The presence of surface receptors is also generally required to attach and infect a host, in the early phases of most bacterial infections (Jones and Oliver, 2009; Strom and Paranjpye, 2000). The attachment process is often mediated by pili, proteinaceous fibers that protrude from the surface of the cell of some bacteria, that interacts through the polypeptide domains on its structure with specific receptors on the host surface (Strom and Lory, 1993). Since *V. vulnificus* has already been well documented, from which it was concluded that cell to cell contact is required to induce cytotoxicity, the existence of pili is an essential virulence factor for this pathogen (Jones and Oliver, 2009; Kim et al., 2008).

As mentioned before, iron plays an important role in the survivability and life cycle of *V. vulnificus* (Hernández-Cabanyero and Amaro, 2020). Iron is bound to transferrin in human serum, therefore, for *V. vulnificus* to acquire iron from iron-binding compounds, this bacterium developed several iron-acquisition systems (Simpson and Oliver, 1987), in which siderophores are the primary system of this pathogen (Jones and Oliver, 2009). This bacterium is also capable of binding iron through the heme receptor HupA. The expression of these iron-regulated genes is regulated by the Ferric uptake regulator (Fur), an iron-binding repressor protein (Jones and Oliver, 2009; Litwin and Byrne, 1998).

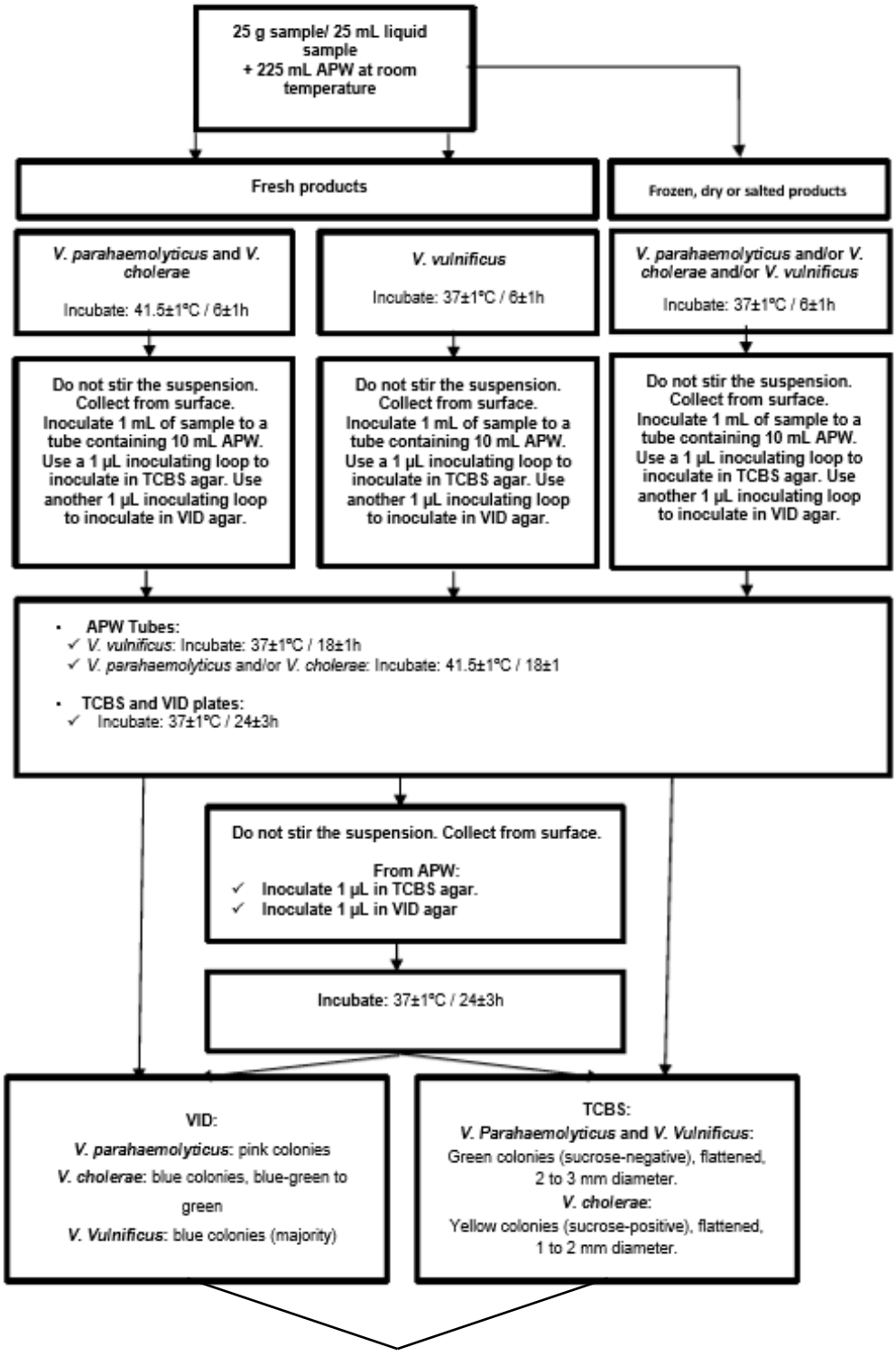
Regarding the enzymes that this pathogen produces, VvhA, VvpE, and RtxA are the ones that stand out as virulence factors (Jones and Oliver, 2009). VvhA is a hemolytic factor that provides iron by releasing it from the hemoglobin of the host, while also being responsible for the cytotoxicity activity of this bacterium (Wright and Morris, 1991). VvpE is an extracellular protease that is thought to be involved in this pathogen virulence, with its broad substrate specificity. When purified, this enzyme contributed to the cause of tissue damage, such as necrosis and cutaneous lesions, along with increased vascular permeability leading to edema, due to the production of bradykinin, a well-known vasodilator (Chang et al., 2005; Jones and Oliver, 2009). RtxA is an enzyme responsible for the development of the systemic disease caused by this pathogen, playing an important role in the spread of the disease to the liver, suggesting that it has an important function in cell injury and potential infection (Jones and Oliver, 2009).

1.9 Aim

This internship had the objective of implementing a method of detection of potentially enteropathogenic *Vibrio* spp. in the microbiology laboratory of *Silliker* Portugal S.A, according to the international standard ISO 21872-1. This included following the assigned work routine, from the preparation of culture media to the preparation of the samples, followed by plating in selective media, and the performance of the tests described in the method for the detection of potentially pathogenic microorganisms. An internal audit was also performed on the method.

Chapter II: Materials and Methods

The detection of potentially enteropathogenic *Vibrio* spp. was performed according to ISO 21872-1. This international standard can be applied to products for human consumption or animal feed and environmental samples in the area of food production and food handling. The flowchart provided by the international standard is presented in Fig. 6 (ISO, 2017c).



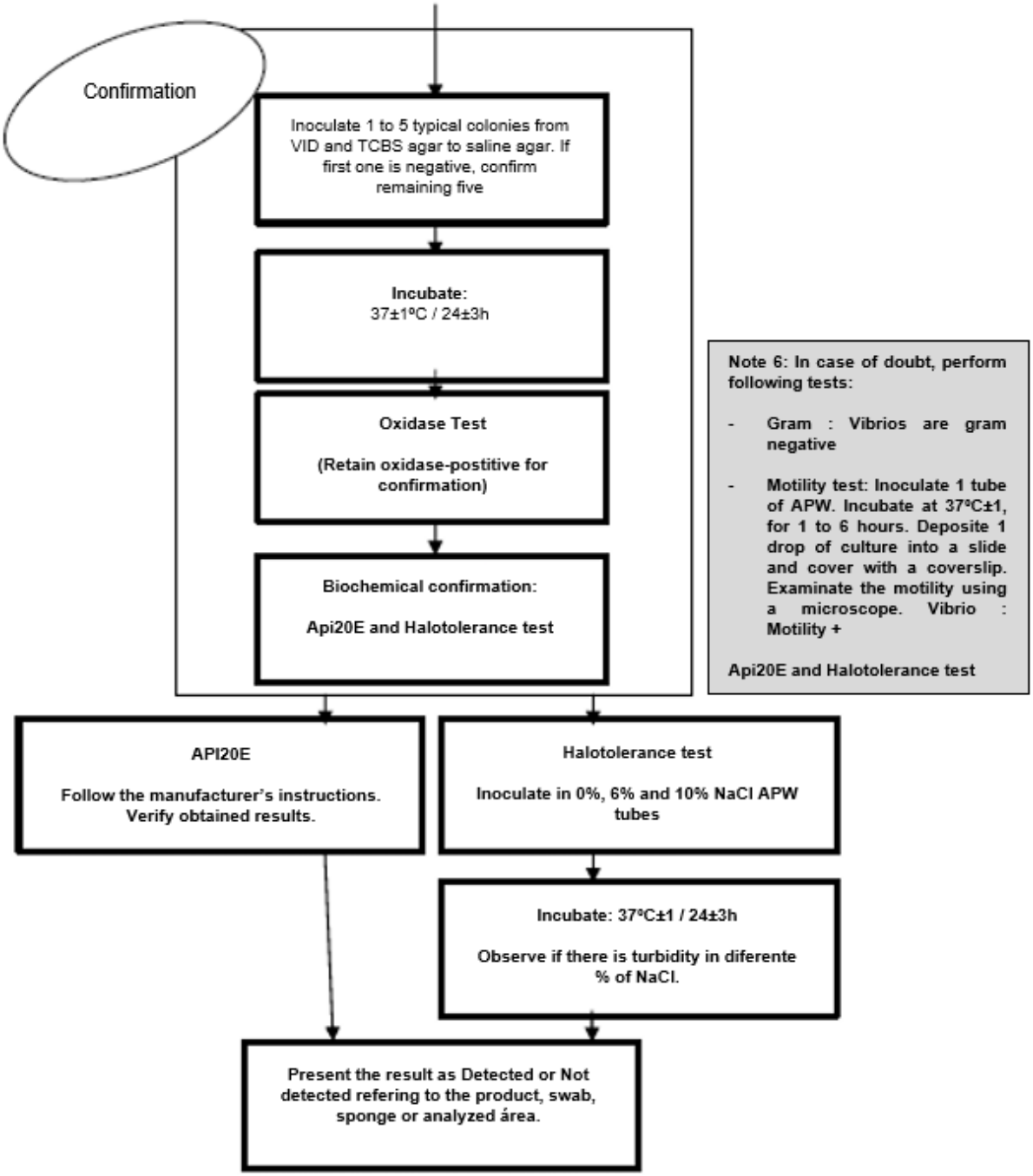


Fig. 6 – Flowchart of the method for detection of *Vibrio* spp., according to the ISO 21782-1 (ISO, 2017c).

2.1 Sample processing

The samples were prepared by suspending 25 g of food sample onto 225 mL of APW (Oxoid), with posterior incubation at (37±1) ºC for frozen, dry, or salty products and (41±1) ºC for fresh products, for (6±1) h, except for *V. vulnificus*, where all the incubations are performed at (37±1) ºC (ISO, 2017c).

2.2 Enrichment and Isolation

The plating of the samples in solid media is performed twice. After the incubation of the sample, 1 µL from the surface of the sample is inoculated in TCBS (Biokar) and chromID *Vibrio* (VID) agar (bioMérieux), using an inoculation loop, and incubated at (37±1) ºC for (24±3) h, to isolate the bacteria. A pre-enrichment process is also performed, where 1 mL from the surface of the sample is transferred to 10 mL of APW and incubated for (18±1) h, at the same temperature as the one used for the sampling procedure. To isolate the bacteria, the same procedure is used as before, inoculating them in TCBS and VID agar. It is recommended to not agitate the suspensions when inoculating. The use of these specific media allows the differentiation of typical *Vibrio* colonies from others (Table 3).

Table 3 - Characteristics of *Vibrio* spp. in VID and TCBS agar. (Adapted from ISO, 2017c).

Bacteria	Characteristics - VID agar	Characteristics - TCBS agar
Vibrio parahaemolyticus	Pink colonies	Blue-green colonies (3-5mm diameter)
Vibrio cholerae	Blue / Blue-green colonies	Yellow, flattened colonies (2-3mm diameter
Vibrio vulnificus	Blue colonies	Green, regular colonies (2-3mm diameter)

2.3 Confirmation step

The confirmation is performed by inoculating one to five colonies in saline nutrient agar (5 g/L meat extract, 3 g/L peptone, 10 g/L sodium chloride 10 g/L agar) and incubating them for (24±3) h, at (37±1) ºC. An oxidase test is performed after the incubation, and the oxidase-positive colonies are kept for posterior biochemical confirmation using API20NE or API20E tests (bioMérieux), according to the manufacturer's instructions, and halotolerance test, using solutions of APW supplemented with 0%, 6%, and 10% NaCl, and posterior incubation for (24±3) h, at (37±1) ºC. A positive result is obtained if turbidity is observed after the incubation period.

Table 4 shows the results of typical *Vibrio parahaemolyticus*, *V. cholerae,* and *V. vulnificus* colonies in all the tests performed, according to the ISO 21872-1.

Table 4 – Positive test results for the *Vibrio* colonies, according to the ISO 21872-1 (Adapted from ISO, 2017c).

Tests	*Vibrio parahaemolyticus*	*Vibrio cholerae*	*Vibrio vulnificus*
Oxidase	+	+	+
LDC	+	+	+
ADH	-	-	-
ONPG	-	+	+
IND	+	+	+
Halotolerance			
0% NaCl	-	+	-
6% NaCl	+	-	+
10% NaCl	-	-	-

2.4 Microbiological results analysis

The method performance for the detection of *Vibrio* spp. was evaluated according to seven parameters presented in the ISO 13843: accuracy, sensitivity, specificity, rate of false negatives, rate of false positives, selectivity, and efficiency (ISO, 2017b), calculated according to the formulas presented in Table 5.

Table 5 – Parameters used for the evaluation of method performance according to the ISO 13843 (Adapted from ISO, 2017b).

		Presumptive Assays		
		+	-	
Confirmed	+	A - True positives	B – False negatives	A+B
	-	C - False positives	D – True negatives	C+D
		A+C	B+D	n

Accuracy	(ICs)
Sensitivity	A/(A+B)
Specificity	D/(C+D)
Rate of False Positives	C/(A+C)
Rate of False Negatives	B/(B+D)
Selectivity	A/n
Efficiency	(A+D)/n

The accuracy is performed according to the results obtained from the ICs (ISO, 2017b).

The sensitivity, also known as the fraction of total positives, evaluated the capability of the method in detecting the target organisms. This parameter must be higher than 90%, and it is calculated through the number of true positives and false negatives (ISO, 2017b).

The specificity is the opposite of sensitivity, therefore evaluating the capability of the method to detect non-target organisms. For a method to be valid, the specificity cannot be any lower than 80%, and it is obtained through the number of false positives and true negatives (ISO, 2017b).

The ratio of false positives and false negatives are two of the most important parameters to evaluate when validating a method. They are related to critical points in the method, such as the correct identification of the colonies, the correct performance, and reading of the biochemical tests and halotolerance tests, errors related to the used technique, or the concentration of the samples. The use of negative and positive controls in each test allows the correction of those errors. Blank samples were included in the assays as negative controls and artificially contaminated samples as positive controls (ISO, 2017b).

Selectivity is a parameter that evaluates the capability of the method to discriminate the target organism. This parameter must be higher than 10%, and it is calculated through the rate of total assays performed and the total positive assays (ISO, 2017b).

The efficiency of a method is obtained through the number of positive and negative results correctly assigned, and the number of total assays performed (ISO, 2017b).

2.5 Cryopreservation

To perform further studies, such as identification by PCR, the positive samples were cultivated in Luria-Bertani (LB) broth and agar (Difco), supplemented with 2,5% NaCl. Three isolated colonies were inoculated in the medium and incubated overnight. After the incubation period, approximately 2 mL were transferred to an Eppendorf tube and centrifugated at 4.4G, for 10 minutes (MicroSpin Plus, Eppendorf). The supernatant is then removed, and the pellet is kept at -20ºC. 700 µL of culture is also transferred to a cryopreservation tube containing 300 µL glycerol. After suspending the culture carefully, the tube is stored at -80 ºC.

Chapter III: Results and Discussion

The microbiological analyses performed on food are of extreme importance to prevent foodborne illnesses and promote food quality and safety (Griffith, 2006). The common practice of these procedures leads to safer commercialization of products, while also protecting the consumer (Stankovic, 2016). The number of illnesses caused by *Vibrio* spp. has been increasing throughout the years, causing many outbreaks throughout the world. Since this genus is mainly present in marine species, especially in seafood, where it is considered natural microbial flora, these are the main food matrixes tested (Baker-Austin et al., 2010; Banerjee and Farber, 2017; Doyle, 2020; Hartnell et al., 2019; Iwamoto et al., 2010; le Roux et al., 2015; Oliver, 2015).

The samples collected from *Silliker* S.A mainly included products for human consumption, such as fish and seafood, but also included animal feed and environmental samples. The sample number used in this study was adapted to protect the customer's identity. The validation of the method of detection of *Vibrio* spp., according to ISO 21872:1, was performed using the samples provided (ISO, 2017c).

3.1 Method Validation

From November 2020 to July 2021, a total of 85 samples were analyzed and 132 assays were performed, including the results obtained from previous Interlaboratory Comparisons (IC) performed in 2018, 2019, and 2020. From those assays, 91 were contaminated artificially (69%) while 41 were not contaminated (31%). It was expected that the positive results were from contaminated assays, while the negative ones were from non-contaminated assays.

The detection of presumptive *Vibrio* spp. was performed using two selective media, TCBS agar and VID, in which the colonies exhibited different colors depending on the metabolism of the species, after incubation at (37±1) ºC for (24±3) h (Fig. 7).

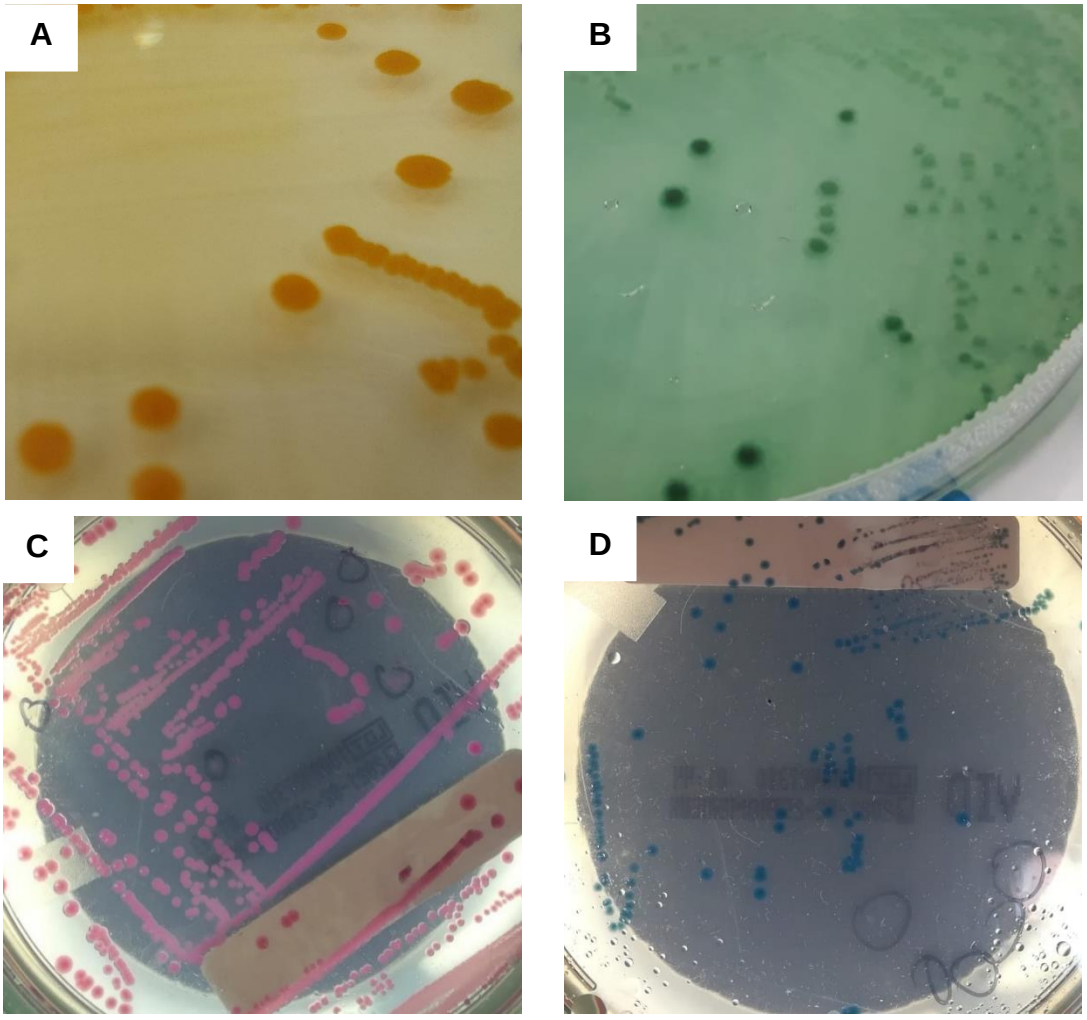


Fig. 7 - Representation of typical colonies of *Vibrio* spp. in TCBS Agar (A – *V. cholerae*; B – *V. parahaemolyticus* and *V. vulnificus*) and in VID Agar (C – *V. parahaemolyticus*; D – *V. cholerae* and *V. vulnificus*).

The presumptively positive *Vibrio* spp. colonies were posteriorly inoculated in saline nutrient agar and incubated at (37±1) ºC for (24±3) h, following up with the identification using biochemical tests (Fig. 8), as well as oxidase and halotolerance tests.

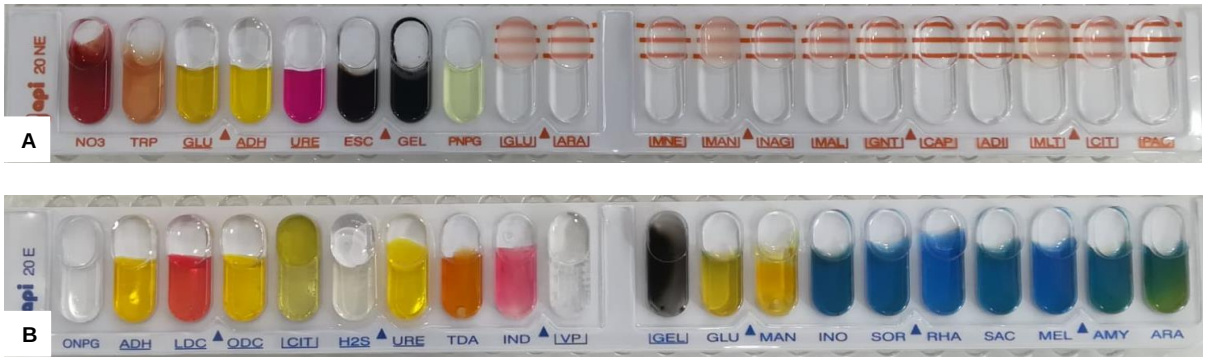


Fig. 8 - Example of a positive result for the detection of *V. parahaemolyticus* using API20NE biochemical test (A) and the API20E biochemical test (B).

According to the ISO 21872:1, both biochemical tests, API20E and API20NE, can be used for the detection of *Vibrio* spp. Even though this bacterium does not belong to the *Enterobacteriaceae* family, it is possible to use the API20E test due to the results being reported according to the result table provided by the ISO. Despite the API20NE test being more suitable for the detection of bacteria belonging to the *Vibrionaceae* family, this test does not provide the essential information for the identification of these organisms, according to this norm.

Therefore, throughout this internship, the use of API20E tests proved to be more reliable, not only providing the necessary information but also minimizing the errors performed by the analysts. By using this test, the correct identification of the organisms is not dependant on getting all the 21 reactions included in the kit correct, since only four of them (ONPG, ADH, LDC, and IND) are essential.

The results of this study are exhibited according to the file provided by *Silliker* S.A. for the determination of the method performance. The results from the ICs performed in the previous years are exhibited in Table 6.

Table 6 - Results obtained from the ICs performed in 2018, 2019, and 2020 by *Silliker* Portugal S.A. Positive results are highlighted. (Adapted from Silliker S.A., 2020).

Date	Sample Number	Product	Observations	Result
5/6/2018	#1	Oat – Interlaboratory	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
2/7/2019	#2	Oat - Interlaboratory	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
17/6/2020	#3	Oat - Interlaboratory	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*

A total of 37 assays were performed on three samples of oats artificially contaminated with *V. parahaemolyticus*, which all of them were detected in the microbiological analyses.

The results obtained during this internship are exhibited in Table 7, which include artificially contaminated samples (reference material), blank samples, and samples analyzed in parallel/double.

Table 7 - Characterization of the assays performed using the method of detection of *Vibrio* spp., according to the ISO 21872:1. Positive results are highlighted. (Adapted from Silliker S.A., 2020).

Date	Sample Number	Product	Observations	Result
29/1/2021	#4	Cod roe	Analyzed in parallel	Not Detected
		Cod roe	Analyzed in parallel	Not Detected
29/1/2021	#5	Cod roe	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
29/1/2021	#6	Sole	Analyzed in parallel	Not Detected
		Sole	Analyzed in parallel	Not Detected
29/1/2021	#7	Sole	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
29/1/2021	#8	Squid	Analyzed in parallel	Not Detected
		Squid	Analyzed in parallel	Not Detected
29/1/2021	#9	Squid	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
29/1/2021	#10	Corvine	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
29/1/2021	#11	Prawn	Contaminated with *V. parahaemolyticus*	Detected *V. parahaemolyticus* and *V. cholerae*
29/1/2021	#12	Squid	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
29/1/2021	#13	Sole	-	Not detected

29/1/2021	#14	Sea bream	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus* and V. cholerae
29/1/2021	#15	Algae	Analyzed in parallel	Not Detected
		Algae	Analyzed in parallel	Not Detected
29/1/2021	#16	Algae	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
29/1/2021	#17	Algae	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
29/1/2021	#18	Algae	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus* and V. cholerae
29/1/2021	#19	Algae	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus* and V. cholerae
29/1/2021	#20	Canned Sardine	-	Not detected
29/1/2021	#21	Canned Sardine	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
29/1/2021	#22	Canned Sardine	-	Not detected
29/1/2021	#23	Cod	-	Not detected
29/1/2021	#24	Frozen Hake	Analyzed in parallel	Not Detected
		Frozen Hake	Analyzed in parallel	Not Detected
9/2/2021	#25	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
10/2/2021	#26	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*

13/2/2021	#27	Hake	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
		Hake	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
13/2/2021	#28	Flounder	-	Not Detected
13/2/2021	#29	Cod	Analyzed in parallel	Not Detected
		Cod	Analyzed in parallel	Not Detected
13/2/2021	#30	Cod	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
13/2/2021	#31	Chile Hake	-	Not Detected
		Chile Hake	-	Not Detected
13/2/2021	#32	Chile Hake	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
13/2/2021	#33	Frozen Squid	Analyzed in parallel	Not Detected
		Frozen Squid	Analyzed in parallel	Not Detected
13/2/2021	#34	Frozen Squid	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
18/2/2021	#35	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
19/2/2021	#36	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
24/2/2021	#37	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
25/2/2021	#38	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*

6/3/2021	#39	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
12/3/2021	#40	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
17/3/2021	#41	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
20/3/2021	#42	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
24/3/2021	#43	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
29/3/2021	#44	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
30/3/2021	#45	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
31/3/2021	#46	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
7/4/2021	#47	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
9/4/2021	#48	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
14/4/2021	#49	DCPS - Milk	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
12/4/2021	#50	Surface	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
12/4/2021	#51	Surface	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
12/4/2021	#52	Surface	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*

12/4/2021	#53	Surface	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
12/4/2021	#54	Surface	-	Not Detected
12/4/2021	#55	Surface	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
12/4/2021	#56	Surface	Analyzed in parallel	Not Detected
		Surface	Analyzed in parallel	Not Detected
12/4/2021	#57	Surface	Contaminated with *V. furnissii*	Not Detected
12/4/2021	#58	Surface	Contaminated with *V. furnissii*	Not Detected
12/4/2021	#59	Surface	Contaminated with *V. furnissii*	Not Detected
12/4/2021	#60	Surface	Contaminated with *V. furnissii*	Not Detected
12/4/2021	#61	Surface	Contaminated with *V. furnissii*	Not Detected
12/4/2021	#62	Surface	Analyzed in parallel	Not Detected
		Surface	Analyzed in parallel	Not Detected
12/4/2021	#63	Surface	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
12/4/2021	#64	Surface	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
12/4/2021	#65	Surface	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*

12/4/2021	#66	Surface	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
12/4/2021	#67	Surface	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
23/4/2021	#68	Animal Feed	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
23/4/2021	#69	Animal Feed	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
23/4/2021	#70	Animal Feed	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
23/4/2021	#71	Animal Feed	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
23/4/2021	#72	Animal Feed	Contaminated with *V. vulnificus*	Detected – *V. vulnificus*
23/4/2021	#73	Animal Feed	Contaminated with *V. furnissii*	Not Detected
23/4/2021	#74	Animal Feed	Contaminated with *V. furnissii*	Not Detected
23/4/2021	#75	Animal Feed	Contaminated with *V. furnissii*	Not Detected
23/4/2021	#76	Animal Feed	Contaminated with *V. furnissii*	Not Detected
23/4/2021	#77	Animal Feed	Contaminated with *V. furnissii*	Not Detected
23/4/2021	#78	Animal Feed	-	Not detected
23/4/2021	#79	Animal Feed	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*

23/4/2021	#80	Animal Feed	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
23/4/2021	#81	Animal Feed	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
23/4/2021	#82	Animal Feed	Analyzed in parallel	Not Detected
		Animal Feed	Analyzed in parallel	Not Detected
23/4/2021	#83	Animal Feed	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
23/4/2021	#84	Animal Feed	Contaminated with *V. parahaemolyticus*	Detected – *V. parahaemolyticus*
23/4/2021	#85	Animal Feed	Analyzed in parallel	Not Detected
		Animal Feed	Analyzed in parallel	Not Detected

According to the results obtained, the outcome of a total of 91 assays was positive for *Vibrio* spp., while 41 were considered negative, which is according to the number of contaminated and non-contaminated samples described before. Although the results came out as expected, there were four samples in which *V. cholerae* was present (#11, #14, #18, and #19) despite also being artificially contaminated with *V. parahaemolyticus*. This indicates that those items were previously contaminated with that bacterium.

The performance of the method for detection of *Vibrio* spp. was then evaluated according to the parameters described in ISO 13843 (ISO, 2017b).

A method accuracy of 100% was achieved since the 37 assays performed in the ICs were all positively confirmed. As for sensitivity, from the 132 assays performed, 91 were presumptively positive, while 41 were negative, resulting in a sensitivity of 100%. A specificity of 100% was also obtained in the performance of this method.

During the validation of this method, no false positive, nor false-negative results were obtained, thus, the ratio of these results while applying this method was 0%. A selectivity of 69% was achieved, whereas efficiency of 100% was obtained in the validation of the method of detection of *Vibrio* spp. since all the correctly assigned results corresponded to the total numbers of assays performed.

All the parameter results are represented in Table 8, along with the assays performed and the result obtained.

Table 8 - Results obtained in the validation of the method of detection of *Vibrio* spp., according to the ISO 21872:1, and the respective formula. (Adapted from Silliker S.A., 2020).

Samples	Assays	Assays of samples contaminated artificially	Assays of non-contaminated samples
85	132	91	41

		Presumptive Assays		
		+	-	
Confirmed	+	91	0	A+B
	-	0	41	C+D
		A+C	B+D	132

		ISO 21872:1
Accuracy	(ICs)	100%
Sensitivity	A/(A+B)	100%
Specificity	D/(C+D)	100%
Rate of False Positives	C/(A+C)	0%
Rate of False Negatives	B/(B+D)	0%
Selectivity	A/n	69%
Efficiency	(A+D)/n	100%

Hartnell et al., (2019) published a study in collaboration with 13 laboratories, where the performance of the method of detection of *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* in seafood, according to the ISO 21872, was evaluated (ISO, 2017c). Moreover, PCR and real-time PCR of *tdh* and *trh* genes were also performed in parallel with the biochemical tests, mentioned in the international standard, to evaluate the pathogenicity of the isolates obtained

in TCBS agar. A total of 800 oysters from the Pacific and five kilograms of prawns were evaluated for the presence of *Vibrio* species. The sensitivity and specificity of the methods used in this study were also calculated and evaluated according to the parameters included in ISO 13843 (ISO, 2017b). Overall good sensitivity and specificity were achieved in the detection and pathogenicity evaluation of *Vibrio* species, except for *V. vulnificus*, where the values obtained were exceptionally low. It was concluded by Hartnell et al., (2019) that the obtained results were difficult to compare, due to sensitivity, specificity, and pathogenicity determination being highly variable. Many authors have reported difficulties in the interpretation of biochemical identifications of *Vibrio* spp. from environmental samples, considering that many atypical tests are reported (Croci et al., 2007; Hartnell et al., 2019).

The presence of naturally contaminated samples (#11, #14, #18, and #19), within the assays performed in the present study, suggest that there might have been issues with the processing of the food matrix, which led to the survival of the bacteria. Despite the excellent results obtained, a method improvement is required (Baker-Austin et al., 2010), since the methods used for the detection of *Vibrio* spp. according to the ISO 21872 are somewhat subjective, from phenotypic to biochemical identification, and require more specialist experience (Croci et al., 2007). The use of molecular methods for the identification of presumptive positive colonies could solve this issue since it is a more objective analysis while being less time-consuming and requiring less experience (Croci et al., 2007; Hartnell et al., 2019; Nordstrom et al., 2007).

3.2 Method Audit

After implementing the method, an audit of the method was performed. The matrixes analyzed were products for human consumption, animal feed, and environmental samples. The efficiency of the technique and truthfulness of the results were evaluated through the performance of a vertical and present audit. The parameters were divided and presented as Conformities (C) and Non-Conformities (NC). If the parameter does not apply to the method, it is presented as Not Applicable (NA) (Silliker, 2020). Several comments were also documented throughout this audit.

3.2.1 Method issues

The questions mentioned in Table 9 were related to potential issues that may compromise the veracity of the results obtained when applying the method. During the audit of the method, no non-conformity was registered and, therefore, the notification of the quality corporative is NA.

Table 9 - Answers obtained regarding potential issues with the method of detection of *Vibrio* spp., according to the ISO 21872:1. Conformities are highlighted. (Adapted from Silliker S.A., 2020).

ITEM	C	NC	NA
Issues/questions were identified during the method audit, that may compromise the precision and trust of the client results, which require the invalidation of the results.	X		
If positive, was the Quality Corporative notified? (It should be notified in less than 24 hours if this issue is observed)			X

3.2.2 Analyst Verification List

The analysts that perform this method need to be qualified to do so. Therefore, the questions mentioned in Table 10 are related to the training of the analysts that participated in this audit. According to the IQ.67, all the analysts can perform this method, in which their training is registered in VM.1037 and confirmed by the supervisor. This method is not verified annually since it was implemented in 2021.

Table 10 - Answers obtained regarding the analysts that performed the method of detection of *Vibrio* spp., according to the ISO 21872:1. Conformities are highlighted. (Adapted from Silliker S.A., 2020).

ITEM	C	NC	NA
The training of the analysts followed the principles of *Silliker*'s SOP: A) The training analyst read and reviewed the method (procedure with a qualified analyst? B) The training analyst observed the performance of the method/procedure of a qualified analyst? C) The training analyst exhibited the ability and conformity with the method/procedure during the direct supervision of a qualified analyst? D) The analyst exhibited competence during the method/procedure?	X		
The registry of the training is complete: tests registers, results of the calculations, and complete traceability.	X		
The training registry is reviewed, verified, and authorized by the Laboratory Manager?	X		
Is the performance of the technique reviewed annually?			X

3.2.3 Method principles

The performance of this method has several critical points that can lead to misleading results. Therefore, to perform this method, the analysts must know which steps are critical, and what can cause false-positive or false-negative results. In this method of detection, according to the ISO 21872:1, it is important to use the correct incubation temperature for each species and food matrix, and the correct identification of the colonies that grow on TCBS and VID agar. The equipment must be sterilized, and the inoculation of the colonies must be performed without agitating the liquid media and collected from the surface. The halotolerance and biochemical test results must be read correctly to avoid misleading identifications. It is shown in Table 11 that there are no non-conformities. Thus, the analysts that performed the method can identify the exiting critical points and in which way they can influence the results.

Table 11 - Answers obtained regarding the principles of the method of detection of *Vibrio* spp., according to the ISO 21872:1. Conformities are highlighted. (Adapted from Silliker S.A., 2020).

ITEM	C	NC	NA
Were the analysts able to identify the critical points of the method? The critical points are those that can compromise the precision of the final results.	X		
The analysts can identify the critical steps that can lead to 1) a high result; 2) a low result; 3) a false-positive; 4) a false-negative.			X

3.2.4 Client complaints – non-conformities

When the methods are not correctly performed, non-conformities and client complaints may occur. When that happens, an investigation is performed, in which the cause analysis is determined, corrective action is implemented, and if the follow-up is efficient. If there is a non-conformity or a client complaint, the general director is responsible for the resume of the work. The questions in Table 12 regarding these issues show that there are only conformities, and since there has not been a complaint or a non-conformity in the past 12 months, the client and regional technical director notification are not applicable.

Table 12 - Answers obtained regarding client complaints and non-conformities of the method of detection of *Vibrio* spp., according to the ISO 21872:1. Conformities are highlighted. (Adapted from Silliker S.A., 2020).

ITEM	C	NC	NA
Was there any non-conformity or complaints from clients related to the audited method in the past 12 months? If positive, was the investigation performed according to the legal requirements? Was the cause analysis determined? Was a correction/corrective action implemented? Was the follow-up efficient?	X		
Were issues identified during the investigation that cast doubt in the validation of the result, including invalidations, efficiently treated, and were the clients notified adequately?			X
In the case of the collection of results, was the regional technical director immediately notified (24h)?			X
In the case of a non-conformity or client complaint, which compromises the precision of the results, did the laboratory define a responsible for the resume of the work?	X		

3.2.5 Norm/Method

The questions mentioned in Table 13 are related to the method of detection. No non-conformities were obtained. This method is performed according to the ISO 21872:1 and updated in 2021. All the analysts have access to an integral copy through the system and all of them follow the method correctly. Since this is an ISO, it is not available in a global format and it is not provided by the client. The validation of the method was performed with samples that are mentioned in the ISO (products for human consumption, animal feed, and environmental samples), therefore, if the matrix is out of the scope of the original application, this method is not applicable. The results of the validation were registered in VM.1037 on 04/05/2021. Since this is a qualitative method, the calculation of uncertainty is not applicable.

Table 13 - Answers obtained regarding the method of detection of *Vibrio* spp., according to the ISO 21872:1. Conformities are highlighted. (Adapted from Silliker S.A., 2020).

ITEM	C	NC	NA
Method/Norm: 1) Does it Exist? 2) Is it properly controlled?	X		
Is the method updated?	X		
Does the analyst have the access to an integral copy of the method?	X		
Is the procedure followed by the analysts?	X		
When applicable, is the test available in global format?			X
Does the analyst have access to the updated version of the procedure and associated documents?	X		
Is the local document consistent with the referenced method?	X		
If the method is provided by the client, does the laboratory have the global requirements of the latest version?			X
Does the scope of the application of validation/verification include the samples to be tested?	X		
If the matrix is out of the scope of the original application, did the laboratory perform the validation/verification of the method for the provided samples?			X
Are the results of the validation/verification registered and the conclusions of the results determined (valid/invalid)?	X		
Was the uncertainty calculation identified and documented for the accredited methods?			X

3.2.6 Reagents, culture media, and method test

All the kits, reagents, culture media, and supplements used by the laboratory must be in mint condition, and within the expiration date. The questions presented in Table 14 are related to that matter, with no registered non-conformities. All the culture media were correctly stored and within the expiration date. The temperature tolerance of the culture media and reagents was also respected. The preparation of the culture media and the conditions of its storage are all updated through the IQ.40, a document that allows the traceability of the culture media used by the laboratory. Regarding the performance of the method, the analyst disinfected the counter and prepared 25g of cooked octopus diluted in 225mL of APW. Due to the properties of the food matrix (cooked dish), the incubation was performed at 37±1ºC for 6±1h. After the incubation, 1mL was transferred to a tube containing 10mL of APW, and 1µL was inoculated in VID and TCBS agar, without stirring and collected from the surface. The tubes were incubated at 37±1ºC for the detection of *V. vulnificus* and 41.5±1ºC for the detection of *V. parahaemolyticus* and *V. cholerae*, for 18±1h. The VID and TCBS agar were incubated at 37±1ºC for 24±3h. After the incubation of the tube, 1µL was again inoculated in TCBS and VID agar, following the same procedure. After the incubations, the plates were analyzed and five typical colonies are transferred to saline nutrient agar and incubated at 37±1ºC for 24±3h. An oxidase test is performed on the colonies. The biochemical test API20E and the halotolerance tests using 10mL APW with 0%, 6%, and 10% NaCl are performed to the oxidase-positive colonies (ISO, 2017c). Finally, all the tests are recorded in the system to allow the traceability of the sample.

Table 14 – Answers obtained regarding the test of the performance of the method of detection of *Vibrio* spp., according to the ISO 21872:1. Conformities are highlighted. (Adapted from Silliker S.A., 2020).

ITEM	C	NC	NA
Was the expiration date of kits/reagents/supplements/culture media exceeded?	X		
Was the temperature tolerance respected?	X		
Were the media/supplements stored correctly?	X		
Was the quality control of the culture media acceptable and with complete traceability?	X		

Did the analyst follow the method? (Cite all the reviewed points and identify if it meets the requirements. Identify the steps if they are critical.)	X		
If it is a quantitative test, were the retests correctly configured? Was the agreement of the results calculated adequately?			X

3.2.7 Traceability

The traceability regarding the method was verified during this audit. The samples are traceable through the Nutria and LIMS system in the laboratory. It is possible to verify the sample number identification, analytical bulletin, list number, when and who prepared the samples, dilutions, and plating. The culture media and reagents used to perform the method are traceable through the document IQ.40, while the readings and subcultures are traceable through the IQ46.1. The registry of the equipment is traceable through IQ199.2A for scales and IQ196 for diluents of 10g and 25g. In Table 15 it is possible to observe that there are no non-conformities regarding this issue.

Table 15 - Answers obtained regarding the traceability in the performance of the method of detection of *Vibrio* spp., according to the ISO 21872:1. Conformities are highlighted. (Adapted from Silliker S.A., 2020).

ITEM	C	NC	NA
Is there complete traceability of the samples in the laboratory program? (Sample number identification, analytical bulletin – registry of the list number, when and who prepared the samples, diluted and plated them.)	X		
Is there complete traceability of the reagents, kits, media, and supplements?	X		
Is there complete traceability of the readings/subcultures?	X		
Is there complete traceability of the equipment registries?	X		
Is there complete traceability in the preparation of culture media?	X		

3.2.8 Equipment and parameters registry

All the laboratory equipment used for the performance of all the methods was verified. The identification and calibration tags were checked, as well as control cards. Regarding the equipment, these were proven to be in ideal conditions until the audit day, in which there were no off-limit points in the control cards. The external calibration tags were updated, and the calibrations and adjustments were correctly performed and identified, exhibiting the date of the next calibration. The internal calibration of the equipment was correctly performed according to the manufacturer. The volumes were registered in the IQ207.3, whereas the masses were recorded in the IQ199.2A document. The questions regarding this matter are presented in Table 16, where there are only conformities.

Table 16 - Answers obtained regarding the equipment, parameters, and calibrations of the laboratory. Conformities are highlighted. (Adapted from Silliker S.A., 2020).

ITEM	C	NC	NA
Are the registries of the used equipment updated? (Check if there are identification, calibration, and verification tags.)	X		
Temperature: Are the calibrations, verifications, and maintenances updated?	X		
Volumes: Are the calibrations, verifications, and maintenances updated?	X		
Masses: Are the calibrations, verifications, and maintenances updated?	X		
Others: Are the calibrations, verifications, and maintenances updated?			X

3.2.9 Registries and documentations – Vertical audit

The questions mentioned in Table 17 are related to the vertical audit of the registries and documentation. Since this method is qualitative, there are no calculation formulas. The results are correctly presented in VM.1037. Regarding the analysts, the qualification is registered in IQ.67 and their roles are registered in IQ.25. Their *Curriculum Vitae* was verified, and it is updated every two years. When there are client samples to detect *Vibrio* spp., a DPCS of *V. parahaemolyticus* is prepared. If there are non-conformities originated by the DPCS prepared,

the NC is registered and corrected. The participation in ICs is registered in VM.1037 and there have not been any non-conformities that originated from ICs since 2014, for *Vibrio* spp.

Table 17 - Answers obtained regarding the registries and documentation of the laboratory. Conformities are highlighted. (Adapted from Silliker S.A., 2020).

ITEM	C	NC	NA
Is the calculation formula according to the norm?			X
Are the results presented according to the ISO 7218 or other norms?	X		
Registries of the qualification of the analysts and description of their roles.	X		
Is the documentation of the analysts in the human resources updated?	X		
Are there Daily Process Control Samples (DPCS)? Are they correctly performed?	X		
Are non-conformities originated from off-limits/tendencies DPCS registered and corrected?	X		
Are there Interlaboratory Comparison (ICs)? Participation twice a year?	X		
Are non-conformities originated from off-limits ICs registered and corrected?	X		

This method audit allowed the verification not only of the performance of the method by the laboratory but also the communication and teamwork between the laboratory analysts. Since it is an extensive method with several steps, some of them critical and with large incubation times, communication is important to trace the sample throughout all the steps. Therefore, traceability is a key factor in the performance of this method, since it eases the communication of which and how were the steps performed.

Based on the results obtained during this audit, in which there were no non-conformities registered, it is possible to state that the method of detection of *Vibrio* spp. is being performed correctly, according to the ISO 21872:1.

Chapter IV: Concluding Remarks

Vibrios are bacteria known to cause illness related to the consumption of seafood. Species from this genus, such as *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*, have been related to foodborne illnesses, especially in Asia. Recently, due to the climate changes and increase of sea surface temperature (SST), these bacteria have been spreading to regions (e.g. Northern Europe) in which they were previously unreported at an alarming rate, causing several outbreaks throughout the world. Due to the health concerns that infections caused by *Vibrio* spp. imply, from severe illnesses to pandemic potential, it is important to study these bacteria, particularly their behavior in foodstuff, animal feed, and environmental samples.

The validation of the method of detection of *V. parahaemolyticus*, *V. cholerae,* and *V. vulnificus*, according to the ISO 21872:1, was performed with the aid of the analysts and under the supervision of the technician responsible for the microbiology laboratory of *Silliker* Portugal, S.A. The implementation of the method was successful, meeting all the microbiological parameters and requirements.

A vertical and presential audit was carried out to evaluate the truthfulness of the results and the efficiency of the technique. The practice of this procedure allows the objective assessment of the effectiveness of the organization's quality management system and overall performance while detecting errors that might lead to non-conformities. During the performance of the audit, no non-conformities were detected, therefore the method of detection of *Vibrio* spp. is being performed correctly, according to the ISO 21872:1.

In the future, molecular approaches should be introduced for the identification of bacteria belonging to the *Vibrio* genus. Due to the complexity of *Vibrio* spp., its detection through culture-based methods and biochemical identification can be challenging and time-consuming since they are somewhat subjective. The interpretation of the results, from the identification of presumptively positive *Vibrio* spp. colonies to the correct interpretation of the biochemical tests performed according to the international standard have revealed to be difficult. The use of molecular identification methods, based on molecular markers, could provide a more efficient and objective solution to this issue. The development of molecular identification techniques of *Vibrio* spp. provided many more options that allow not only the identification of the species but also a pathogenic evaluation by detecting the presence of virulence factors in the isolates. Throughout the years, due to technological development, the performance of these methods has become more accessible. Despite being a large investment, in the beginning, the establishment and performance of these methods might be profitable in the long run, being more objective while providing results faster than phenotypic methods.

Chapter V: Bibliographical References

Abdolshahi, A., Yancheshmeh, B.S., 2020. Food Contamination. Mycotoxins and Food Safety. https://doi.org/10.5772/INTECHOPEN.89802

Albert, M.J., 1994. *Vibrio cholerae* O139 Bengal. Journal of Clinical Microbiology. https://doi.org/10.1128/jcm.32.10.2345-2349.1994

Albert, M.J., Nair, G.B., 2005. *Vibrio cholerae* O139 Bengal - 10 Years on. Reviews in Medical Microbiology. https://doi.org/10.1097/01.revmedmi.0000184743.75679.0a

Almagro-Moreno, S., Pruss, K., Taylor, R.K., 2015. Intestinal Colonization Dynamics of *Vibrio cholerae*. PLoS Pathogens. https://doi.org/10.1371/journal.ppat.1004787

Amaro, C., Biosca, E.G., 1996. *Vibrio vulnificus* biotype 2, pathogenic for eels, is also an opportunistic pathogen for humans. Applied and Environmental Microbiology 62, 1454–1457. https://doi.org/10.1128/aem.62.4.1454-1457.1996

APCER, 2021a. ISO 9001 [WWW Document]. URL https://www.apcergroup.com/pt/certificacao/pesquisa-de-normas/81/iso-9001 (accessed 7.11.21).

APCER, 2021b. O que é a Acreditação? [WWW Document]. URL https://www.apcergroup.com/pt/faqs/68-faqs-pt/179-o-que-e-a-acreditacao (accessed 7.13.21).

ASAE, 2021. A Direção [WWW Document]. URL https://www.asae.gov.pt/asae20/a-direcao.aspx (accessed 7.11.21).

ASAE, 2020. Missão, Visão e Valores [WWW Document]. URL https://www.asae.gov.pt/asae20/missao-visao-e-valores.aspx (accessed 7.11.21).

ASAE, 2018. Área Alimentar [WWW Document]. URL https://www.asae.gov.pt/inspecao-fiscalizacao/plano-de-inspecao-da-asae-pif/area-alimentar.aspx (accessed 7.11.21).

ASAE, 2016. RASFF (Rapid Alert System for Food and Feed) [WWW Document]. URL https://www.asae.gov.pt/seguranca-alimentar/rasff-rapid-alert-system-for-food-and-feed.aspx (accessed 7.18.21).

ASAE, 2007. HACCP [WWW Document]. URL https://www.asae.gov.pt/seguranca-alimentar/haccp.aspx (accessed 6.21.21).

Audemard, C., Kator, H.I., Rhodes, M.W., Gallivan, T., Erskine, A.J., Leggett, A.T., Reece, K.S., 2011. High salinity relay as a postharvest processing strategy to reduce *Vibrio vulnificus* levels in Chesapeake Bay oysters (*Crassostrea virginica*). Journal of Food Protection 74, 1902–1907. https://doi.org/10.4315/0362-028X.JFP-11-152

Aznar, R., Ludwig, W., Amann, R.I., Schleifer, K.H., 1994. Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. International Journal of Systematic Bacteriology 44, 330–337. https://doi.org/10.1099/00207713-44-2-330

Baker-Austin, C., Oliver, J.D., Alam, M., Ali, A., Waldor, M.K., Qadri, F., Martinez-Urtaza, J., 2018. *Vibrio* spp. infections. Nature Reviews Disease Primers 4. https://doi.org/10.1038/s41572-018-0005-8

Baker-Austin, C., Stockley, L., Rangdale, R., Martinez-Urtaza, J., 2010. Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective. Environmental Microbiology Reports 2, 7–18. https://doi.org/10.1111/J.1758-2229.2009.00096.X

Baker-Austin, C., Trinanes, J., Gonzalez-Escalona, N., Martinez-Urtaza, J., 2017. Non-Cholera Vibrios: The Microbial Barometer of Climate Change. Trends in Microbiology 25, 76–84. https://doi.org/https://doi.org/10.1016/j.tim.2016.09.008

Baker-Austin, C., Trinanes, J.A., Salmenlinna, S., Löfdahl, M., Siitonen, A., Taylor, N.G.H., Martinez-Urtaza, J., 2016. Heat Wave–Associated Vibriosis, Sweden and Finland, 2014. Emerging Infectious Diseases 22, 1216–1220. https://doi.org/10.3201/eid2207.151996

Baker-Austin, C., Trinanes, J.A., Taylor, N.G.H., Hartnell, R., Siitonen, A., Martinez-Urtaza, J., 2013. Emerging *Vibrio* risk at high latitudes in response to ocean warming. Nature Climate Change 3, 73–77. https://doi.org/10.1038/nclimate1628

Bakhshi, B., Mohammadi-Barzelighi, H., Hosseini-Aliabad, N., Pourshafie, M.R., 2014. Ribotyping and TCP gene cluster analysis of environmental and clinical *Vibrio cholerae* strains isolated in Iran. Annals of Microbiology 64, 49–53. https://doi.org/10.1007/s13213-013-0631-z

Banerjee, S.K., Farber, J.M., 2017. Detection, enumeration, and isolation of *Vibrio parahaemolyticus* and *V. vulnificus* from seafood: Development of a multidisciplinary protocol. Journal of AOAC International 100, 445–453. https://doi.org/10.5740/jaoacint.16-0290

Barua, D., 1992. History of Cholera, in: Cholera. Springer US, pp. 1–36. https://doi.org/10.1007/978-1-4757-9688-9_1

Bisharat, N., Agmon, V., Finkelstein, R., Raz, R., Ben-Dror, G., Lerner, L., Soboh, S., Colodner, R., Cameron, D.N., Wykstra, D.L., Swerdlow, D.L., Farmer, J.J., 1999. Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing

outbreaks of wound infection and bacteraemia in Israel. Lancet 354, 1421–1424. https://doi.org/10.1016/S0140-6736(99)02471-X

Bopp, A.F., 2019. The Evolution of Food Preservation and Packaging. ACS Symposium Series 1314, 211–228. https://doi.org/10.1021/BK-2019-1314.CH015

Bross, M.H., Soch, K., Morales, R., Mitchell, R.B., 2007. *Vibrio vulnificus* Infection: Diagnosis and Treatment, American Family Physician.

Cash, R.A., Music, S.I., Libonati, J.P., Craig, J.P., Pierce, N.F., Hornick, R.B., 1974a. Response of man to infection with *Vibrio cholerae*. II Protection from illness afforded by previous disease and vaccine. Journal of Infectious Diseases 130, 325–333. https://doi.org/10.1093/infdis/130.4.325

Cash, R.A., Music, S.I., Libonati, J.P., Snyder, M.J., Wenzel, R.P., Hornick, R.B., 1974b. Response of man to infection with *Vibrio cholerae*. I Clinical, serologic, and bacteriologic responses to a known inoculum. Journal of Infectious Diseases 129, 45–52. https://doi.org/10.1093/infdis/129.1.45

CDC, 2019. *Vibrio vulnificus* & Wounds | *Vibrio* Illness (Vibriosis) [WWW Document]. URL https://www.cdc.gov/vibrio/wounds.html (accessed 6.27.21).

CDC, 2017. Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet 2015 Surveillance Report (Final Data) [WWW Document]. Atlanta, Georgia: U.S. Department of Health and Human Services. URL https://www.cdc.gov/foodnet/reports/annual-reports-2015.html (accessed 5.26.21).

CDC, 1999. Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera CDC/NCID Centers For Disease Control and Prevention National Center For Infectious Diseases. Atlanta, Georgia.

Chang, A.K., Kim, H.Y., Park, J.E., Acharya, P., Park, I.S., Yoon, S.M., You, H.J., Hahm, K.S., Park, J.K., Lee, J.S., 2005. *Vibrio vulnificus* secretes a broad-specificity metalloprotease capable of interfering with blood homeostasis through prothrombin activation and fibrinolysis. Journal of Bacteriology 187, 6909–6916. https://doi.org/10.1128/JB.187.20.6909-6916.2005

Chase, E., Harwood, V.J., 2011. Comparison of the effects of environmental parameters on growth rates of *Vibrio vulnificus* biotypes I, II, and III by culture and quantitative PCR analysis. Applied and Environmental Microbiology 77, 4200–4207. https://doi.org/10.1128/AEM.00135-11

Chatterjee, S., Ghosh, K., Raychoudhuri, A., Chowdhury, G., Bhattacharya, M.K., Mukhopadhyay, A.K., Ramamurthy, T., Bhattacharya, S.K., Klose, K.E., Nandy, R.K., 2009. Incidence, virulence factors, and clonality among clinical strains of non-O1, non-O139 *Vibrio cholerae* isolates from hospitalized diarrheal patients in Kolkata, india. Journal of Clinical Microbiology 47, 1087–1095. https://doi.org/10.1128/JCM.02026-08

Chatzidaki-Livanis, M., Hubbard, M.A., Gordon, K., Harwood, V.J., Wright, A.C., 2006. Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. Applied and Environmental Microbiology 72, 6136–6141. https://doi.org/10.1128/AEM.00341-06

Cho, Y.J., Yi, H., Lee, J.H., Kim, D.W., Chun, J., 2010. Genomic evolution of *Vibrio cholerae*. Current Opinion in Microbiology. https://doi.org/10.1016/j.mib.2010.08.007

Chowdhury, F.R., Nur, Z., Hassan, N., Seidlein, L., Dunachie, S., 2017. Pandemics, pathogenicity and changing molecular epidemiology of cholera in the era of global warming. Annals of Clinical Microbiology and Antimicrobials. https://doi.org/10.1186/s12941-017-0185-1

Chowdhury, N.R., Chakraborty, S., Ramamurthy, T., Nishibuchi, M., Yamasaki, S., Takeda, Y., Nair, G.B., 2000. Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. Emerging Infectious Diseases 6, 631–636. https://doi.org/10.3201/eid0606.000612

Chowdhury, R., Sahu, G.K., Das, J., 1996. Stress response in pathogenic bacteria. Journal of Biosciences 21, 149–160. https://doi.org/10.1007/BF02703105

Christian, K., 2021. Food Safety [WWW Document]. URL https://www.europarl.europa.eu/factsheets/pt/sheet/51/food-safety (accessed 7.11.21).

Colwell, R.R., 1996. Global climate and infectious disease: The cholera paradigm, in: Science. American Association for the Advancement of Science, pp. 2025–2031. https://doi.org/10.1126/science.274.5295.2025

Colwell, R.R., Huq, A., 1994. Vibrios in the Environment: Viable but Nonculturable *Vibrio cholerae*, in: *Vibrio cholerae* and Cholera . ASM Press, pp. 117–133. https://doi.org/10.1128/9781555818364.ch9

Colwell, R.R., Huq, A., Islam, M.S., Aziz, K.M.A., Yunus, M., Khan, N.H., Mahmud, A., Sack, R.B., Nair, G.B., Chakraborty, J., Sack, D.A., Russek-Cohen, E., 2003. Reduction of cholera in Bangladeshi villages by simple filtration. Proceedings of the National Academy of Sciences of the United States of America 100, 1051–1055. https://doi.org/10.1073/pnas.0237386100

Croci, L., Suffredini, E., Cozzi, L., Toti, L., Ottaviani, D., Pruzzo, C., Serratore, P., Fischetti, R., Goffredo, E., Loffredo, G., Mioni, R., Paola, P., Raffaello, L., Mara, P., Masini, L., Zampini, M., Bacciaglia, A., Annamaria, P., Stefania, M., Donatella, B., Silvia, Z., Milioni, C., Gasperetti, L., Giovanna, L.S., Carmine, P., Stefano, S., di Giamberardino, F., Maria, G., Claudio, B., 2007. Comparison of different biochemical and molecular methods for the identification of *Vibrio parahaemolyticus*. Journal of Applied Microbiology 102, 229–237. https://doi.org/10.1111/j.1365-2672.2006.03046.x

Dalsgaard, A., Serichantalergs, O., Forslund, A., Lin, W., Mekalanos, J., Mintz, E., Shimada, T., Wells, J.G., 2001. Clinical and environmental isolates of *Vibrio cholerae* serogroup O141 carry the CTX phage and the genes encoding the toxin-coregulated pili. Journal of Clinical Microbiology 39, 4086–4092. https://doi.org/10.1128/JCM.39.11.4086-4092.2001

De, R., Ghosh, J.B., Gupta, S. sen, Takeda, Y., Nair, G.B., 2013. The Role of *Vibrio cholerae* Genotyping in Africa. https://doi.org/10.1093/infdis/jit199

DePaola, A., Hopkins, L.H., Peeler, J.T., Wentz, B., McPhearson, R.M., 1990. Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and oysters. Applied and Environmental Microbiology 56, 2299–2302. https://doi.org/10.1128/aem.56.8.2299-2302.1990

Dizon, J.J., Fukumi, H., Barua, D., Valera, J., Jayme, F., Gomez, F., Yamamoto, S.I., Wake, A., Gomez, C.Z., Takahira, Y., Paraan, A., Rolda, L., Alvero, M., Abou-Gareeb, A.H., Kobari, K., Azurin, J.C., 1967. Studies on cholera carriers. Bulletin of the World Health Organization 37, 737–743.

Doyle, M., 2020. Food Microbiology: Fundamentals and Frontiers, 5th ed.

Drake, S.L., DePaola, A., Jaykus, L.-A., 2007. An Overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Comprehensive Reviews in Food Science and Food Safety 6, 120–144. https://doi.org/10.1111/J.1541-4337.2007.00022.X

DRE, 2004. Decreto-Lei 140/2004 [WWW Document]. URL https://dre.pt/pesquisa/-/search/278973/details/maximized (accessed 7.13.21).

Dutta, D., Chowdhury, G., Pazhani, G.P., Guin, S., Dutta, S., Ghosh, S., Rajendran, K., Nandy, R.K., Mukhopadhyay, A.K., Bhattacharya, M.K., Mitra, U., Takeda, Y., Balakrish Nair, G., Ramamurthy, T., 2013. *Vibrio cholerae* non-O1, non-O139 serogroups and cholera-like Diarrhea, Kolkata, India. Emerging Infectious Diseases 19, 464–467. https://doi.org/10.3201/eid1903.121156

EFSA, 2021. European Food Safety Authority (EFSA) [WWW Document]. URL https://europa.eu/european-union/about-eu/agencies/efsa_en (accessed 7.11.21).

EU, 2021. Food safety in the EU [WWW Document]. URL https://europa.eu/european-union/topics/food-safety_en (accessed 7.11.21).

FAO, 2017. The Rapid Alert System for Food and Feed [WWW Document]. URL http://www.fao.org/family-farming/detail/en/c/1068811/ (accessed 7.18.21).

FAO, 2003. 3. Important Food Issues [WWW Document]. URL http://www.fao.org/3/y8705e/y8705e03.htm#bm03.1 (accessed 7.11.21).

FAO, OMS, 2006. Programa Conjunto da FAO/OMS sobre normas alimentares Comissão do Codex Alimentarius.

Farmer, J.J., 1979. *Vibrio* ("*Beneckea*") *vulnificus*, The Bacterium Associated With Sepsis, Septicaemia, And The Sea. The Lancet. https://doi.org/10.1016/S0140-6736(79)92715-6

Faruque, S.M., Asadulghani, Alim, A.R.M.A., Albert, M.J., Islam, K.M.N., Mekalanos, J.J., 1998a. Induction of the lysogenic phage encoding cholera toxin in naturally occurring strains of toxigenic *Vibrio cholerae* O1 and O139. Infection and Immunity 66, 3752–3757. https://doi.org/10.1128/iai.66.8.3752-3757.1998

Faruque, S.M., Asadulghani, Saha, M.N., Alim, A.R.M.A., Albert, M.J., Islam, K.M.N., Mekalanos, J.J., 1998b. Analysis of clinical and environmental strains of nontoxigenic *Vibrio cholerae* for susceptibility to CTXΦ: Molecular basis for origination of new strains with epidemic potential. Infection and Immunity 66, 5819–5825. https://doi.org/10.1128/iai.66.12.5819-5825.1998

FDA, 2012. Bad Bug Book, Handbook of foodborne pathogenic microorganisms and natural toxins, Second. ed, Food and Drug Administration. Silver Spring, MD, USA,.

FDA, 1997. HACCP Principles & Application Guidelines [WWW Document]. URL https://www.fda.gov/food/hazard-analysis-critical-control-point-haccp/haccp-principles-application-guidelines (accessed 6.22.21).

Feldhusen, F., 2000. The role of seafood in bacterial foodborne diseases. Microbes and Infection. https://doi.org/10.1016/S1286-4579(00)01321-6

Food Safety News, 2020. *Vibrio* increase concerns lead to risk assessment [WWW Document]. URL https://www.foodsafetynews.com/2020/04/vibrio-increase-concerns-lead-to-risk-assessment/ (accessed 7.18.21).

Fraser, G.M., Hughes, C., 1999. Swarming motility. Current Opinion in Microbiology. https://doi.org/10.1016/S1369-5274(99)00033-8

Freitas, C., Glatter, T., Ringgaard, S., 2020. The release of a distinct cell type from swarm colonies facilitates dissemination of *Vibrio parahaemolyticus* in the environment. ISME Journal 14, 230–244. https://doi.org/10.1038/s41396-019-0521-x

Froelich, B., Oliver, J.D., 2013. The Interactions of *Vibrio vulnificus* and the Oyster *Crassostrea virginica*. Microbial Ecology. https://doi.org/10.1007/s00248-012-0162-3

Froelich, B., Phippen, B., Fowler, P., Noble, R.T., Oliver, J.D., 2017. Differences in abundances of total *Vibrio* spp., *V. vulnificus*, and *V. parahaemolyticus* in clams and oysters in North Carolina. Applied and Environmental Microbiology 83. https://doi.org/10.1128/AEM.02265-16

Gibson, B., Wilson, D.J., Feil, E., Eyre-Walker, A., 2018. The distribution of bacterial doubling times in the wild. Proceedings of the Royal Society B: Biological Sciences 285. https://doi.org/10.1098/rspb.2018.0789

Goel, A.K., Jain, M., Kumar, P., Bhadauria, S., Kmboj, D., Singh, L., 2008. A new variant of *Vibrio cholerae* O1 El Tor causing cholera in India. Journal of Infection. https://doi.org/10.1016/j.jinf.2008.06.015

González-Escalona, N., Jaykus, L.A., DePaola, A., 2007. Typing of *Vibrio vulnificus* strains by variability in their 16S-23S rRNA intergenic spacer regions. Foodborne Pathogens and Disease 4, 327–337. https://doi.org/10.1089/fpd.2007.0005

González-Escalona, N., Martinez-Urtaza, J., Romero, J., Espejo, R.T., Jaykus, L.A., DePaola, A., 2008. Determination of molecular phylogenetics of *Vibrio parahaemolyticus* strains by multilocus sequence typing. Journal of Bacteriology 190, 2831–2840. https://doi.org/10.1128/JB.01808-07

Griffith, C.J., 2006. Food safety: where from and where to? https://doi.org/10.1108/00070700610637599

Griffitt, K.J., Grimes, D.J., 2013. A novel agar formulation for isolation and direct enumeration of *Vibrio vulnificus* from oyster tissue. Journal of Microbiological Methods 94, 98–102. https://doi.org/10.1016/j.mimet.2013.04.012

Grim, C.J., Hasan, N.A., Taviani, E., Haley, B., Chun, J., Brettin, T.S., Bruce, D.C., Detter, J.C., Han, C.S., Chertkov, O., Challacombe, J., Huq, A., Nair, G.B., Colwell, R.R., 2010. Genome sequence of hybrid *Vibrio cholerae* O1 MJ-1236, B-33, and CIRS101 and comparative genomics with *V. cholerae*. Journal of Bacteriology 192, 3524–3533. https://doi.org/10.1128/JB.00040-10

Hamad, S.H., 2012. Factors Affecting the Growth of Microorganisms in Food. Progress in Food Preservation 405–427. https://doi.org/10.1002/9781119962045.CH20

Haq, S.M., Dayal, H.H., 2005. Chronic liver disease and consumption of raw oysters: A potentially lethal combination - A review of *Vibrio vulnificus* septicemia. American Journal of Gastroenterology. https://doi.org/10.1111/j.1572-0241.2005.40814.x

Harth-Chu, E., Espejo, R.T., Christen, R., Guzmán, C.A., Höfle, M.G., 2009. Multiple-locus variable-number tandem-repeat analysis for clonal identification of *Vibrio parahaemolyticus* isolates by using capillary electrophoresis. Applied and Environmental Microbiology 75, 4079–4088. https://doi.org/10.1128/AEM.02729-08

Hartnell, R.E., Stockley, L., Keay, W., Rosec, J.P., Hervio-Heath, D., van den Berg, H., Leoni, F., Ottaviani, D., Henigman, U., Denayer, S., Serbruyns, B., Georgsson, F., Krumova-Valcheva, G., Gyurova, E., Blanco, C., Copin, S., Strauch, E., Wieczorek, K., Lopatek, M., Britova, A., Hardouin, G., Lombard, B., in't Veld, P., Leclercq, A., Baker-Austin, C., 2019. A pan-European ring trial to validate an International Standard for detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seafoods. International Journal of Food Microbiology 288, 58–65. https://doi.org/10.1016/J.IJFOODMICRO.2018.02.008

Hasan, N.A., Choi, S.Y., Eppinger, M., Clark, P.W., Chen, A., Alam, M., Haley, B.J., Taviani, E., Hine, E., Su, Q., Tallon, L.J., Prosper, J.B., Furth, K., Hoq, M.M., Li, H., Fraser-Liggett, C.M., Cravioto, A., Huq, A., Ravel, J., Cebula, T.A., Colwell, R.R., 2012. Genomic diversity of 2010 Haitian cholera outbreak strains. Proceedings of the National Academy of Sciences of the United States of America 109. https://doi.org/10.1073/pnas.1207359109

Heng, S.P., Letchumanan, V., Deng, C.Y., Ab Mutalib, N.S., Khan, T.M., Chuah, L.H., Chan, K.G., Goh, B.H., Pusparajah, P., Lee, L.H., 2017. *Vibrio vulnificus*: An environmental and clinical burden. Frontiers in Microbiology. https://doi.org/10.3389/fmicb.2017.00997

Hernández-Cabanyero, C., Amaro, C., 2020. Phylogeny and life cycle of the zoonotic pathogen *Vibrio vulnificus*. Environmental Microbiology. https://doi.org/10.1111/1462-2920.15137

Hirk, S., Huhulescu, S., Allerberger, F., Lepuschitz, S., Rehak, S., Weil, S., Gschwandtner, E., Hermann, M., Neuhold, S., Zoufaly, A., Indra, A., 2016. Necrotizing fasciitis due to *Vibrio cholerae* non-O1/non-O139 after exposure to Austrian bathing sites. Wiener Klinische Wochenschrift 128, 141–145. https://doi.org/10.1007/s00508-015-0944-y

Hlady, W.G., Klontz, K.C., 1996. The epidemiology of *Vibrio* infections in Florida, 1981-1993. Journal of Infectious Diseases 173, 1176–1183. https://doi.org/10.1093/infdis/173.5.1176

Honda, T., Ni, Y., Miwatani, T., 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. Infection and Immunity 56, 961–965. https://doi.org/10.1128/iai.56.4.961-965.1988

Horseman, M.A., Surani, S., 2011. A comprehensive review of *Vibrio vulnificus*: An important cause of severe sepsis and skin and soft-tissue infection. International Journal of Infectious Diseases. https://doi.org/10.1016/j.ijid.2010.11.003

Hossain, M.T., Kim, Y.O., Kong, I.S., 2013. Multiplex PCR for the detection and differentiation of *Vibrio parahaemolyticus* strains using the groEL, tdh and trh genes. Molecular and Cellular Probes 27, 171–175. https://doi.org/10.1016/j.mcp.2013.04.001

Hossain, M.T., Kim, Y.R., Kong, I.S., 2014. PCR-restriction fragment length polymorphism analysis using groEL gene to differentiate pathogenic *Vibrio* species. Diagnostic Microbiology and Infectious Disease 78, 9–11. https://doi.org/10.1016/j.diagmicrobio.2013.10.005

Howard-Jones, N., 1984. Robert Koch and the cholera *Vibrio*: a centenary. British medical journal (Clinical research ed.) 288, 379–381. https://doi.org/10.1136/bmj.288.6414.379

Huq, A., Small, E., West, P., Huq, M., Rahman, R., Colwell, R., 1983. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Applied and environmental microbiology 45. https://doi.org/10.1128/AEM.45.1.275-283.1983

Huq, A., Whitehouse, C.A., Grim, C.J., Alam, M., Colwell, R.R., 2008. Biofilms in water, its role and impact in human disease transmission. Current Opinion in Biotechnology. https://doi.org/10.1016/j.copbio.2008.04.005

IAS, 2021. What Is Accreditation? [WWW Document]. URL https://www.iasonline.org/about-ias/what-is-accreditation/ (accessed 7.13.21).

IIA, 2021. What is internal audit? [WWW Document]. URL https://www.iia.org.uk/about-us/what-is-internal-audit/ (accessed 7.19.21).

INSA, 2019. Interpretação de resultados de ensaios microbiológicos em alimentos prontos para consumo e em superfícies do ambiente de preparação e distribuição alimentar Valores-guia.

IPAC, 2021. IPAC [WWW Document]. URL http://www.ipac.pt/ipac/funcao.asp (accessed 7.13.21).

Islam, S., Drasar, B.S., Bradley, D.J., 1990. Long-term persistence of toxigenic *Vibrio cholerae* 01 in the mucilaginous sheath of a blue-green alga, *Anabaena variabilis*. Journal of Tropical Medicine and Hygiene 93, 133–139.

ISO, 2020. ISO/IEC 17000:2020(en), Conformity assessment — Vocabulary and general principles [WWW Document]. URL https://www.iso.org/obp/ui#iso:std:iso-iec:17000:ed-2:v2:en (accessed 7.17.21).

ISO, 2017a. ISO - ISO/IEC 17025:2017 - General requirements for the competence of testing and calibration laboratories [WWW Document]. URL https://www.iso.org/standard/66912.html (accessed 7.13.21).

ISO, 2017b. ISO - ISO 13843:2017 - Water quality — Requirements for establishing performance characteristics of quantitative microbiological methods [WWW Document]. URL https://www.iso.org/standard/62979.html (accessed 9.20.21).

ISO, 2017c. ISO - ISO 21872-1:2017 - Microbiology of the food chain — Horizontal method for the determination of *Vibrio* spp. — Part 1: Detection of potentially enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*.

Iwamoto, M., Ayers, T., Mahon, B.E., Swerdlow, D.L., 2010. Epidemiology of seafood-associated infections in the United States. Clinical Microbiology Reviews. https://doi.org/10.1128/CMR.00059-09

Jay, J.M., Loessner, M.J., Golden, D.A., 2005. Modern food microbiology, 7th edn, Springer, New York.

John, M., Siddique, A.K., Islam, M.S., Faruque, A.S.G., Ansaruzzaman, M., Faruque, S.M., Sack, R.B., 1993. Large outbreak of clinical cholera due to *Vibrio cholerae* non-01 in Bangladesh. The Lancet. https://doi.org/10.1016/0140-6736(93)90481-U

Jones, M.K., Oliver, J.D., 2009. *Vibrio vulnificus*: Disease and pathogenesis. Infection and Immunity. https://doi.org/10.1128/IAI.01046-08

Jones, M.K., Warner, E., Oliver, J.D., 2008. Survival of and in situ gene expression by *Vibrio vulnificus* at varying salinities in estuarine environments. Applied and Environmental Microbiology 74, 182–187. https://doi.org/10.1128/AEM.02436-07

Joseph, S.W., Colwell, R.R., Kaper, J.B., 1982. *Vibrio parahaemolyticus* and related halophilic vibrios. Critical Reviews in Microbiology 10, 77–124. https://doi.org/10.3109/10408418209113506

Jung, S.W., 2018. A foodborne outbreak of gastroenteritis caused by *Vibrio parahaemolyticus* associated with cross-contamination from squid in Korea. Epidemiology and health 40, e2018056. https://doi.org/10.4178/epih.e2018056

Kai, M.K., Luey, C.K.Y., Parsons, M.B., Cooper, K.L.F., Nair, G.B., Alam, M., Atiqul Islam, M., Cheung, D.T.L., Chu, Y.W., Ramamurthy, T., Pazhani, G.P., Bhattacharya, S.K., Watanabe, H., Terajima, J., Arakawa, E., Ratchtrachenchai, O.A., Huttayananont, S., Ribot, E.M., Gerner-Smidt, P., Swaminathan, B., 2008. Evaluation and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping *Vibrio parahaemolyticus*: An International Multicenter Collaborative study. Journal of Clinical Microbiology 46, 2766–2773. https://doi.org/10.1128/JCM.00424-08

Kaneko, T., Colwell, R.R., 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. Journal of Bacteriology 113, 24–32. https://doi.org/10.1128/jb.113.1.24-32.1973

Kaper, J., Lockman, H., Colwell, R.R., Joseph, S.W., 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. Applied and environmental microbiology 37, 91–103. https://doi.org/10.1128/aem.37.1.91-103.1979

Kaper, J.B., Morris, J.G., Levine, M.M., 1995. Cholera. Clinical Microbiology Reviews. https://doi.org/10.1128/cmr.8.1.48-86.1995

Katoh, H., 1965. Studies on the growth rate of various food bacteria. 3. The growth of *V. parahaemolyticus* in raw fish meat. Nippon saikingaku zasshi. Japanese journal of bacteriology 20, 541–544. https://doi.org/10.3412/jsb.20.541

Kaysner, C., Abeyta, C., Trost, P.A., Wetherington, J.H., Jinneman, K.C., Hill, W.E., Wekell, M.M., 1994. Urea hydrolysis can predict the potential pathogenicity of *Vibrio parahaemolyticus* strains isolated in the Pacific Northwest. Applied and Environmental Microbiology. https://doi.org/10.1128/aem.60.8.3020-3022.1994

Kaysner, C., DePaola, A., Jones, J., 2004. BAM Chapter 9: Vibrio [WWW Document]. URL https://www.fda.gov/food/laboratory-methods-food/bam-chapter-9-vibrio (accessed 5.20.21).

Keen, R., 2021. Internal audit explained [ISO 9001:2015] [WWW Document]. URL https://www.iso-9001-checklist.co.uk/internal-audit-explained-eur.htm (accessed 7.19.21).

Khan, S., Kumar, A., Meparambu, D., Thomas, S., Harichandran, D., Karim, S., 2013. Fatal non-O1, non-O139 *Vibrio cholerae* septicaemia in a patient with chronic liver disease. Journal of Medical Microbiology 62, 917–921. https://doi.org/10.1099/jmm.0.049296-0

Kim, D.G., Kim, Y.R., Kim, E.Y., Cho, H.M., Ahn, S.H., Kong, I.S., 2010. Isolation of the *groESL* cluster from *Vibrio anguillarum* and PCR detection targeting *groEL* gene. Fisheries Science 76, 803–810. https://doi.org/10.1007/s12562-010-0266-y

Kim, Y.B., Okuda, J., Matsumoto, C., Takahashi, N., Hashimoto, S., Nishibuchi, M., 1999. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. Journal of Clinical Microbiology 37, 1173–1177. https://doi.org/10.1128/jcm.37.4.1173-1177.1999

Kim, Y.R., Lee, S.E., Kook, H., Yeom, J.A., Na, H.S., Kim, S.Y., Chung, S.S., Choy, H.E., Rhee, J.H., 2008. *Vibrio vulnificus* RTX toxin kills host cells only after contact of the bacteria with host cells. Cellular Microbiology 10, 848–862. https://doi.org/10.1111/J.1462-5822.2007.01088.X

Kishishita, M., Matsuoka, N., Kumagai, K., Yamasaki, S., Takeda, Y., Nishibuchi, M., 1992. Sequence variation in the thermostable direct hemolysin-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*. Applied and Environmental Microbiology 58, 2449–2457. https://doi.org/10.1128/aem.58.8.2449-2457.1992

Kumar, P., Peter, W.A., Thomas, S., 2010. Rapid detection of virulence-associated genes in environmental strains of *Vibrio cholerae* by multiplex PCR. Current Microbiology 60, 199–202. https://doi.org/10.1007/s00284-009-9524-6

le Roux, F., Wegner, K.M., Baker-Austin, C., Vezzulli, L., Osorio, C.R., Amaro, C., Ritchie, J.M., Defoirdt, T., Destoumieux-Garzón, D., Blokesch, M., Mazel, D., Jacq, A., Cava, F., Gram, L., Wendling, C.C., Strauch, E., Kirschner, A., Huehn, S., 2015. The emergence of *Vibrio* pathogens in Europe: Ecology, evolution and pathogenesis (Paris, 11-12 March 2015). Frontiers in Microbiology 6. https://doi.org/10.3389/fmicb.2015.00830

Levin, R.E., 2006. *Vibrio parahaemolyticus*, a notably lethal human pathogen derived from seafood: A review of its pathogenicity, characteristics, subspecies characterization, and molecular methods of detection. Food Biotechnology. https://doi.org/10.1080/08905430500524275

Li, R., Wang, J., Wang, L., Hu, B., Liu, Z., Sun, N., Yang, Y., Miao, S., Guo, X., 2021. Genetic and serological characterization of capsular antigen untypeable *Vibrio parahaemolyticus* strains reveal novel K serotypes and epidemiological characteristics in Shandong, China. International Journal of Food Microbiology 347, 109188. https://doi.org/10.1016/j.ijfoodmicro.2021.109188

Liao, Y., Li, Y., Wu, S., Mou, J., Xu, Z., Cui, R., Klena, J.D., Shi, X., Lu, Y., Qiu, Y., Lin, Y., Xie, X., Ma, H., Li, Z., Yu, H., Varma, J.K., Ran, L., Hu, Q., Cheng, J., 2015. Risk Factors for *Vibrio parahaemolyticus* Infection in a Southern Coastal Region of China. Foodborne Pathogens and Disease 12, 881–886. https://doi.org/10.1089/fpd.2015.1988

Litwin, C.M., Byrne, B.L., 1998. Cloning and characterization of an outer membrane protein of *Vibrio vulnificus* required for heme utilization: regulation of expression and determination of the gene sequence. Infection and immunity 66, 3134–3141. https://doi.org/10.1128/IAI.66.7.3134-3141.1998

Longini, I.M., Yunus, M., Zaman, K., Siddique, A.K., Sack, R.B., Nizam, A., 2002. Epidemic and endemic cholera trends over a 33-year period in Bangladesh. Journal of Infectious Diseases 186, 246–251. https://doi.org/10.1086/341206

Lyon, W.J., 2001. TaqMan PCR for Detection of *Vibrio cholerae* Ö1, O139, Non-O1, and Non-O139 in Pure Cultures, Raw Oysters, and Synthetic Seawater†. Applied and Environmental Microbiology 67, 4685–4693. https://doi.org/10.1128/AEM.67.10.4685-4693.2001

Manning, L., Soon, J.M., 2016. Food Safety, Food Fraud, and Food Defense: A Fast Evolving Literature. Journal of Food Science 81, R823–R834. https://doi.org/10.1111/1750-3841.13256

Marco-Noales, E., Biosca, E.G., Amaro, C., 1999. Effects of salinity and temperature on long-term survival of the eel pathogen *Vibrio vulnificus* biotype 2 (serovar E). Applied and Environmental Microbiology 65, 1117–1126. https://doi.org/10.1128/aem.65.3.1117-1126.1999

Martinez-Urtaza, J., Baker-Austin, C., Jones, J.L., Newton, A.E., Gonzalez-Aviles, G.D., DePaola, A., 2013. Spread of Pacific Northwest *Vibrio parahaemolyticus* Strain . New England Journal of Medicine 369, 1573–1574. https://doi.org/10.1056/nejmc1305535

Matsumoto, C., Okuda, J., Ishibashi, M., Iwanaga, M., Garg, P., Rammamurthy, T., Wong, H.C., Depaola, A., Kim, Y.B., Albert, M.J., Nishibuchi, M., 2000. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. Journal of Clinical Microbiology 38, 578–585. https://doi.org/10.1128/jcm.38.2.578-585.2000

McCarter, L.L., 2004. Dual flagellar systems enable motility under different circumstances. Journal of Molecular Microbiology and Biotechnology. https://doi.org/10.1159/000077866

McCarter, L.L., 2001. Polar Flagellar Motility of the *Vibrionaceae*. Microbiology and Molecular Biology Reviews 65, 445–462. https://doi.org/10.1128/mmbr.65.3.445-462.2001

McCarter, L.L., 1999. The multiple identities of *Vibrio parahaemolyticus*, in: Journal of Molecular Microbiology and Biotechnology. pp. 51–57.

Mérieux Nutrisciences Australia, 2021. Food Safety Audit - Audit & Certification [WWW Document]. URL https://www.merieuxnutrisciences.com/au/services/food-safety-and-quality/audit-and-certification (accessed 7.19.21).

Mohapatra, S.S., Ramachandran, D., Mantri, C.K., Colwell, R.R., Singh, D. v., 2009. Determination of relationships among non-toxigenic *Vibrio cholerae* O1 biotype El Tor strains from housekeeping gene sequences and ribotype patterns. Research in Microbiology 160, 57–62. https://doi.org/10.1016/j.resmic.2008.10.008

Moreno, M.L., Landgraf, M., 1998. Virulence factors and pathogenicity of *Vibrio vulnificus* strains isolated from seafood. Journal of applied microbiology 84, 747–751. https://doi.org/10.1046/J.1365-2672.1998.00404.X

Morris, J.G., 2003. Cholera and other types of vibriosis: A story of human pandemics and oysters on the half shell. Clinical Infectious Diseases 37, 272–280. https://doi.org/10.1086/375600

Motes, M.L., DePaola, A., 1996. Offshore suspension relaying to reduce levels of *Vibrio vulnificus* in oysters (*Crassostrea virginica*). Applied and Environmental Microbiology 62, 3875–3877. https://doi.org/10.1128/aem.62.10.3875-3877.1996

Motes, M.L., DePaola, A., Cook, D.W., Veazey, J.E., Hunsucker, J.C., Garthright, W.E., Blodgett, R.J., Chirtel, S.J., 1998. Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). Applied and Environmental Microbiology 64, 1459–1465. https://doi.org/10.1128/aem.64.4.1459-1465.1998

Mukhopadhyay, A.K., Saha, P.K., Garg, S., Bhattacharya, S.K., Shimada, T., Takeda, T., Takeda, Y., Nair, G.B., 1995. Distribution and virulence of *Vibrio cholerae* belonging to serogroups other than O1 and O139: A nationwide survey. Epidemiology and Infection 114, 65–70. https://doi.org/10.1017/S0950268800051918

Mukhopadhyay, S., Miller, R.D., Sullivan, E.D., Theodoropoulos, C., Mathews, S.A., Timms, P., Summersgill, J.T., 2006. Protein expression profiles of *Chlamydia pneumoniae* in models of persistence versus those of heat shock stress response. Infection and Immunity 74, 3853–3863. https://doi.org/10.1128/IAI.02104-05

Munn, C.B., 2015. The Role of Vibrios in Diseases of Corals. Microbiology Spectrum 3. https://doi.org/10.1128/microbiolspec.ve-0006-2014

Nair, G.B., Ramamurthy, T., Bhattacharya, S.K., Dutta, B., Takeda, Y., Sack, D.A., 2007. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clinical Microbiology Reviews. https://doi.org/10.1128/CMR.00025-06

Nelson, E.J., Harris, J.B., Morris, J.G., Calderwood, S.B., Camilli, A., 2009. Cholera transmission: The host, pathogen and bacteriophage dynamic. Nature Reviews Microbiology. https://doi.org/10.1038/nrmicro2204

Newton, A., Kendall, M., Vugia, D.J., Henao, O.L., Mahon, B.E., 2012. Increasing rates of vibriosis in the United States, 1996-2010: Review of surveillance data from 2 systems. Clinical Infectious Diseases. https://doi.org/10.1093/cid/cis243

Nishibuchi, M., Kaper, J.B., 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: A virulence gene acquired by a marine bacterium. Infection and Immunity. https://doi.org/10.1128/iai.63.6.2093-2099.1995

Nishibuchi, M., Taniguchi, T., Misawa, T., Khaeomanee-Iam, V., Honda, T., Miwatani, T., 1989. Cloning and nucleotide sequence of the gene (*trh*) encoding the hemolysin related to the thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infection and Immunity 57, 2691–2697. https://doi.org/10.1128/iai.57.9.2691-2697.1989

Noguerola, I., Blanch, A.R., 2008. Identification of *Vibrio* spp. with a set of dichotomous keys. Journal of applied microbiology 105, 175–185. https://doi.org/10.1111/J.1365-2672.2008.03730.X

Nordstrom, J.L., Vickery, M.C.L., Blackstone, G.M., Murray, S.L., DePaola, A., 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Applied and Environmental Microbiology 73, 5840–5847. https://doi.org/10.1128/AEM.00460-07

Odeyemi, O.A., 2016. Incidence and prevalence of *Vibrio parahaemolyticus* in seafood: a systematic review and meta-analysis. SpringerPlus 5. https://doi.org/10.1186/s40064-016-2115-7

O'Hara, C.M., Sowers, E.G., Bopp, C.A., Duda, S.B., Strockbine, N.A., 2003. Accuracy of Six Commercially Available Systems for Identification of Members of the Family *Vibrionaceae*. Journal of Clinical Microbiology 41, 5654–5659. https://doi.org/10.1128/JCM.41.12.5654-5659.2003

Okuda, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay, A.K., Garg, S., Bhattacharya, S.K., Nair, G.B., Nishibuchi, M., 1997. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. Journal of Clinical Microbiology 35, 3150–3155. https://doi.org/10.1128/jcm.35.12.3150-3155.1997

Oliver, J.D., 2015. The Biology of *Vibrio vulnificus*. Microbiology Spectrum 3. https://doi.org/10.1128/microbiolspec.ve-0001-2014

Oliver, J.D., 2013. *Vibrio vulnificus*: Death on the Half Shell. A Personal Journey with the Pathogen and its Ecology. Microbial Ecology. https://doi.org/10.1007/s00248-012-0140-9

Oliver, J.D., 2011. Chapter 18. Culture Media for the Isolation and Enumeration of Pathogenic *Vibrio* Species in Foods and Environmental Samples, in: Handbook of Culture Media for Food and Water Microbiology. Royal Society of Chemistry, pp. 377–402. https://doi.org/10.1039/9781847551450-00377

Osawa, R., Okitsu, T., Morozumi, H., Yamai, S., 1996. Occurrence of urease-positive *Vibrio parahaemolyticus* in Kanagawa, Japan, with specific reference to presence of thermostable direct hemolysin (TDH) and the TDH-related-hemolysin genes. Applied and Environmental Microbiology 62, 725–727. https://doi.org/10.1128/aem.62.2.725-727.1996

Pajuelo, D., Hernández-Cabanyero, C., Sanjuan, E., Lee, C. te, Silva-Hernández, F.X., Hor, L.I., Mackenzie, S., Amaro, C., 2016. Iron and fur in the life cycle of the zoonotic pathogen *Vibrio vulnificus*. Environmental Microbiology 18, 4005–4022. https://doi.org/10.1111/1462-2920.13424

Pang, R., Li, Y., Chen, M., Zeng, H., Lei, T., Zhang, Junhui, Ding, Y., Wang, J., Wu, S., Ye, Q., Zhang, Jumei, Wu, Q., 2020. A database for risk assessment and comparative genomic analysis of foodborne *Vibrio parahaemolyticus* in China. Scientific Data 7, 1–8. https://doi.org/10.1038/s41597-020-00671-3

Paz, S., Bisharat, N., Paz, E., Kidar, O., Cohen, D., 2007. Climate change and the emergence of *Vibrio vulnificus* disease in Israel. Environmental Research 103, 390–396. https://doi.org/https://doi.org/10.1016/j.envres.2006.07.002

Pettis, G.S., Mukerji, A.S., 2020. Structure, Function, and Regulation of the Essential Virulence Factor Capsular Polysaccharide of *Vibrio vulnificus*. International Journal of Molecular Sciences 21. https://doi.org/10.3390/IJMS21093259

Powell, J.L., Wright, A.C., Wasserman, S.S., Hone, D.M., Morris, J.G., 1997. Release of tumor necrosis factor alpha in response to *Vibrio vulnificus* capsular polysaccharide in in vivo and in vitro models. Infection and immunity 65, 3713–3718. https://doi.org/10.1128/IAI.65.9.3713-3718.1997

Pruzzo, C., Vezzulli, L., Colwell, R.R., 2008. Global impact of *Vibrio cholerae* interactions with chitin. Environmental Microbiology. https://doi.org/10.1111/j.1462-2920.2007.01559.x

Raghunath, P., 2014. Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. Frontiers in Microbiology. https://doi.org/10.3389/fmicb.2014.00805

Raimondi, F., Kao, J.P.Y., Fiorentini, C., Fabbri, A., Donelli, G., Gasparini, N., Rubino, A., Fasano, A., 2000. Enterotoxicity and cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin in in vitro systems. Infection and Immunity 68, 3180–3185. https://doi.org/10.1128/IAI.68.6.3180-3185.2000

Ramamurthy, T., Chowdhury, G., Pazhani, G.P., Shinoda, S., 2014. *Vibrio fluvialis*: An emerging human pathogen. Frontiers in Microbiology. https://doi.org/10.3389/fmicb.2014.00091

Reen, F.J., Almagro-Moreno, S., Ussery, D., Boyd, E.F., 2006. The genomic code: Inferring *Vibrionaceae* niche specialization. Nature Reviews Microbiology 4, 697–704. https://doi.org/10.1038/nrmicro1476

Reid, P.C., Gorick, G., Edwards, M., 2011. Climate Change and Marine Ecosystem Research. Sir Alister Hardy Foundation for Ocean Science (SAHFOS), Plymouth.

Reidl, J., Klose, K.E., 2002. *Vibrio cholerae* and cholera: out of the water and into the host . FEMS Microbiology Reviews 26, 125–139. https://doi.org/10.1111/j.1574-6976.2002.tb00605.x

Rosche, T.M., Yano, Y., Oliver, J.D., 2005. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. Microbiology and Immunology 49, 381–389. https://doi.org/10.1111/j.1348-0421.2005.tb03731.x

Ruimy, R., Breittmayer, V., Elbase, P., Lafay, B., Boussemart, O., Gauthier, M., Christen, R., 1994. Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. International Journal of Systematic Bacteriology 44, 416–426. https://doi.org/10.1099/00207713-44-3-416

Sack, D.A., Sack, R.B., Nair, G.B., Siddique, A.K., 2004. Cholera, in: Lancet. Elsevier B.V., pp. 223–233. https://doi.org/10.1016/S0140-6736(03)15328-7

Sack, R.B., Siddique, A.K., Longini, I.M., Nizam, A., Yunus, M., Islam, M.S., Morris, J.G., Ali, A., Huq, A., Nair, G.B., Qadri, F., Faruque, S.M., Sack, D.A., Colwell, R.R., 2003. A 4-year study of the epidemiology of *Vibrio cholerae* in four rural areas of Bangladesh. Journal of Infectious Diseases 187, 96–101. https://doi.org/10.1086/345865

Safa, A., Nair, G.B., Kong, R.Y.C., 2010. Evolution of new variants of *Vibrio cholerae* O1. Trends in Microbiology. https://doi.org/10.1016/j.tim.2009.10.003

Sakazaki, R., Tamura, K., Kato, T., Obara, Y., Yamai, S., Hobo, K., 1968. Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus*. Japanese Journal of Medical Science and Biology 21, 325–331. https://doi.org/10.7883/yoken1952.21.325

Schadt, E.E., Linderman, M.D., Sorenson, J., Lee, L., Nolan, G.P., 2010. Computational solutions to large-scale data management and analysis. Nature Reviews Genetics. https://doi.org/10.1038/nrg2857

Schild, S., Bishop, A.L., Camilli, A., 2008. Ins and Outs of *Vibrio cholerae* transitions between the human gut and the aquatic environment are aided by specific shifts in gene expression.

Semenza, J.C., Trinanes, J., Lohr, W., Sudre, B., Löfdahl, M., Martinez-Urtaza, J., Nichols, G.L., Rocklöv, J., 2017. Environmental suitability of *Vibrio* infections in a warming climate: An early warning system. Environmental Health Perspectives 125. https://doi.org/10.1289/EHP2198

Senachai, P., Chomvarin, C., Namwat, W., Wongboot, W., Wongwajana, S., Tongkanakul, W., 2013. Application of tetraplex PCR for detection of *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. mimicus* in cockle. The Southeast Asian journal of tropical medicine and public health 44(2), 249–258.

Shimohata, T., Takahashi, A., 2010. Diarrhea induced by infection of *Vibrio parahaemolyticus*. Journal of Medical Investigation. https://doi.org/10.2152/jmi.57.179

Shinoda, S., 2011. Sixty years from the discovery of *Vibrio parahaemolyticus* and some recollections. Biocontrol Science. https://doi.org/10.4265/bio.16.129

Silliker, 2021. Silliker Portugal, S. A. | Mérieux NutriSciences Portugal [WWW Document]. URL https://www.merieuxnutrisciences.com/pt/silliker-portugal (accessed 6.18.21).

Silliker, 2020. Silliker Portugal, S.A. - Internal procedures.

Silvester, R., Alexander, D., Antony, A.C., Hatha, M., 2017. GroEL PCR- RFLP – An efficient tool to discriminate closely related pathogenic *Vibrio* species. Microbial Pathogenesis 105, 196–200. https://doi.org/10.1016/j.micpath.2017.02.029

Simpson, L.M., Oliver, J.D., 1987. Ability of *Vibrio vulnificus* to obtain iron from transferrin and other iron-binding proteins. Current Microbiology 1987 15:3 15, 155–157. https://doi.org/10.1007/BF01577265

Stankovic, I., 2016. Codex Alimentarius. Encyclopedia of Food and Health 191–196. https://doi.org/10.1016/B978-0-12-384947-2.00180-X

Stroeher, U.H., Parasivam, G., Dredge, B.K., Manning, P.A., 1997. Novel *Vibrio cholerae* O139 Genes Involved in Lipopolysaccharide Biosynthesis. Journal of Bacteriology 179, 209–216.

Strom, M.S., Lory, S., 1993. Structure-function and Biogenesis of the Type IV Pili. Annual Reviews in Microbiology 47, 565–596. https://doi.org/10.1146/ANNUREV.MI.47.100193.003025

Strom, M.S., Paranjpye, R.N., 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microbes and Infection. https://doi.org/10.1016/S1286-4579(00)00270-7

Tirado, C., Schmidt, K., 2001. WHO surveillance programme for control of foodborne infections and intoxications: Preliminary results and trends across greater Europe, in: Journal of Infection. W.B. Saunders Ltd, pp. 80–84. https://doi.org/10.1053/jinf.2001.0861

Tison, D.L., Nishibuchi, M., Greenwood, J.D., Seidler, R.J., 1982. *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. Applied and environmental microbiology 44, 640–646. https://doi.org/10.1128/aem.44.3.640-646.1982

Ulitzur, S., 1974. *Vibrio parahaemolyticus* and *Vibrio alginolyticus*: Short generation-time marine bacteria. Microbial Ecology 1, 127–135. https://doi.org/10.1007/BF02512384

Vezzulli, L., Baker-Austin, C., Kirschner, A., Pruzzo, C., Martinez-Urtaza, J., 2020. Global emergence of environmental non-O1/O139 *Vibrio cholerae* infections linked with climate change: a neglected research field? Environmental Microbiology 22, 4342–4355. https://doi.org/10.1111/1462-2920.15040

Vezzulli, L., Brettar, I., Pezzati, E., Reid, P.C., Colwell, R.R., Höfle, M.G., Pruzzo, C., 2012. Long-term effects of ocean warming on the prokaryotic community: Evidence from the vibrios. ISME Journal. https://doi.org/10.1038/ismej.2011.89

Vezzulli, L., Colwell, R.R., Pruzzo, C., 2013. Ocean Warming and Spread of Pathogenic Vibrios in the Aquatic Environment. Microbial Ecology. https://doi.org/10.1007/s00248-012-0163-2

Vezzulli, L., Previati, M., Pruzzo, C., Marchese, A., Bourne, D.G., Cerrano, C., 2010a. *Vibrio* infections triggering mass mortality events in a warming Mediterranean Sea. Environmental Microbiology 12, 2007–2019. https://doi.org/10.1111/j.1462-2920.2010.02209.x

Vezzulli, L., Pruzzo, C., Huq, A., Colwell, R.R., 2010b. Environmental reservoirs of *Vibrio cholerae* and their role in cholera. Environmental Microbiology Reports. https://doi.org/10.1111/j.1758-2229.2009.00128.x

Vezzulli, L., Stauder, M., Grande, C., Pezzati, E., Verheye, H.M., Owens, N.J.P., Pruzzo, C., 2015. GbpA as a novel qPCR target for the species-specific detection of *Vibrio cholerae* O1, O139, Non-O1/Non-O139 in environmental, stool, and historical continuous plankton recorder samples. PLoS ONE 10, e0123983. https://doi.org/10.1371/journal.pone.0123983

Villeneuve, S., Boutonnier, A., Mulard, L.A., Fournier, J.M., 1999. Immunochemical characterization of an Ogawa-Inaba common antigenic determinant of *Vibrio cholerae* O1. Microbiology 145, 2477–2484. https://doi.org/10.1099/00221287-145-9-2477

Vora, G.J., Meador, C.E., Bird, M.M., Bopp, C.A., Andreadis, J.D., Stenger, D.A., 2005. Microarray-based detection of genetic heterogeneity, antimicrobial resistance, and the viable but nonculturable state in human pathogenic *Vibrio* spp. Proceedings of the National Academy of Sciences of the United States of America 102, 19109–19114. https://doi.org/10.1073/pnas.0505033102

Wagley, S., Koofhethile, K., Rangdale, R., 2009. Prevalence and potential pathogenicity of *Vibrio parahaemolyticus* in Chinese mitten crabs (*Eriocheir sinensis*) harvested from the River Thames estuary, England. Journal of Food Protection 72, 60–66. https://doi.org/10.4315/0362-028X-72.1.60

Waits, A., Emelyanova, A., Oksanen, A., Abass, K., Rautio, A., 2018. Human infectious diseases and the changing climate in the Arctic. Environment International. https://doi.org/10.1016/j.envint.2018.09.042

Warner, E., Oliver, J.D., 2008. Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. Applied and Environmental Microbiology 74, 80–85. https://doi.org/10.1128/AEM.01434-07

Warner, J.M., Oliver, J.D., 1999. Randomly amplified polymorphic DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other *Vibrio* species. Applied and Environmental Microbiology 65, 1141–1144. https://doi.org/10.1128/aem.65.3.1141-1144.1999

Watnick, P.I., Kolter, R., 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. Molecular Microbiology 34, 586–595. https://doi.org/10.1046/j.1365-2958.1999.01624.x

Weintraub, A., Widmalm, G., Jansson, P.E., Jansson, M., Hultenby, K., Albert, M.J., 1994. *Vibrio cholerae* O139 Bengal possesses a capsular polysaccharide which may confer increased virulence. Microbial Pathogenesis 16, 235–241. https://doi.org/10.1006/MPAT.1994.1024

WHO, 2021a. Food safety [WWW Document]. URL https://www.who.int/news-room/fact-sheets/detail/food-safety (accessed 6.19.21).

WHO, 2021b. Foodborne diseases [WWW Document]. URL https://www.who.int/health-topics/foodborne-diseases#tab=tab_1 (accessed 7.17.21).

WHO, 2017a. The Burden of Foodborne Diseases In the WHO European Region.

WHO, 2017b. Cholera [WWW Document]. URL https://www.who.int/news-room/fact-sheets/detail/cholera (accessed 7.18.21).

Wright, A., Morris, J., 1991. The extracellular cytolysin of *Vibrio vulnificus*: inactivation and relationship to virulence in mice. Infection and immunity 59, 192–198. https://doi.org/10.1128/IAI.59.1.192-197.1991

Wright, A., Simpson, L., Oliver, J., Morris, J., 1990. Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. Infection and immunity 58, 1769–1773. https://doi.org/10.1128/IAI.58.6.1769-1773.1990