Aus dem Institut für Lebensmittelsicherheit und -hygiene
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Investigation of *Vibrio* and ESBL/AmpC-producing *Enterobacteriaceae* in retail seafood and inactivation of *Vibrio* in pure culture and mussel homogenates using high hydrostatic pressure

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Thi Thu Tra Vu
Tierärztin aus Hanoi, Vietnam

Berlin 2018
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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

ace	accessory cholera enterotoxin (gene)
Ace	accessory cholera enterotoxin (protein)
AFLP	amplified restriction fragment length polymorphism
APW	alkaline peptone water
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CC	cellobiose colistin
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
CI	confident interval
CLSI	Clinical and Laboratory Standards Institute
CPS	capsular polysaccharides
CT	cholera enterotoxin
EOs	essential oils
ERIC-PCR	enterobacterial intergenic consensus sequence polymerase chain reaction
ESBL	extended-spectrum β-lactamases
et al.	et alii
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
g	gram
h	hour
HHP	high hydrostatic pressure
ISO	International Organization for Standardization
kDa	kilodalton
kGy	kilogray
KP	Kanagwa phenomenon
LAB	lactic acid bacteria
LB	Luria Bertani
MALDI-TOF	matrix assisted laser desorption ionization – time of flight
mCPC	modified cellobiose polymyxin colistin
mg	milligram
min	minute
ml	milliliter
MFRHA	mannose-fucose resistant hemagglutinin

mm	millimeter
MPa	megapascal
mPCR	multiplex polymerase chain reaction
MPN	most probable number
MSHA	mannose-sensitive hemagglutinin
NSSC	National Shellfish Sanitation Conference
OMPs	outer membrane proteins
PCA	plate count agar
PCR	polymerase chain reaction
pH	potential of hydrogen
ppm	parts per million
qPCR	real-time polymerase chain reaction
RFLP	restriction fragment length polymorphism
rpm	rounds per minute
SD	standard deviation
TCBS	thiosulfate citrate bile salt
TCP	toxin-coregulated pili
tdh	thermostable direct haemolysin (gene)
TDH	thermostable direct haemolysin (protein)
tlh	thermolabile haemolysin (gene)
TLH	thermolabile haemolysin (protein)
trh	heat-labile TDH-related haemolysin (gene)
TRH	heat-labile TDH-related haemolysin (protein)
TSA	tryptic soy agar
US	United States
UV	ultraviolet
VBNC	viable but nonculturable state
zot	zonula occludens toxin (gene)
ZOT	zonula occludens toxin (protein)
δ-VPH	thermolabile haemolysin
µl	microliter
µm	micrometer
®	Trade mark
ºC	degree Celsius
ºF	degree Fahrenheit
%	percent
>	greater than
<	less than

Chapter 1 Introduction

1.1 Seafood production

Seafood, including fish and shellfish, is considered as a valuable food source which contains high nutritional values via e.g. proteins, vitamins and minerals (Oehlenschlager, 2012). Therefore, seafood production and consumption have increased worldwide in recent decades. According to the Food and Agriculture Organization (FAO), global capture and aquaculture production of fish, crustaceans, molluscs and other aquatic animals has grown rapidly and reached 167.2 million tons in 2014, of which aquaculture production was 73.8 million tons (Table 1.1). Aquatic products originate mainly from developing countries, while developed countries for instance the United States (US), European Union countries (EU) and Japan are main importing countries, together accounting for approximately 63% of total world imports (Food and Agriculture Organization of the United Nations (FAO), 2016).

Table 1.1 World caption and aquaculture production of fish, crustaceans, molluscs, etc., by continent

Unit: 1000 tonnes

Continent		2008	2009	2010	2011	2012	2013	2014
World total	Capture	90,192	90,200	89,130	93,683	91,311	92,669	93,445
	Aquaculture	52,914	55,686	58,973	61,809	66,466	70,261	73,784
Africa	Capture	7,311	7,498	7,757	7,752	8,395	8,355	8,636
	Aquaculture	942	989	1,286	1,396	1,484	1,615	1,711
America	Capture	22,025	21,149	17,580	22,545	18,466	18,787	16,930
	Aquaculture	2,470	2,466	2,514	2,774	2,988	3,059	3,351
Asia	Capture	46,504	46,913	48,667	48,810	50,117	50,785	52,795
	Aquaculture	47,001	49,540	52,439	54,783	58,954	62,641	65,602
Europe	Capture	13,085	13,382	13,897	13,386	13,058	13,515	13,748
	Aquaculture	2,327	2,517	2,544	2,659	2,852	2,765	2,930
Oceania	Capture	1,237	1,225	1,210	1,172	1,266	1,205	1,328
	Aquaculture	175	174	190	197	186	181	189
Other	Capture	29	34	19	18	10	22	8
	Aquaculture	-	-	-	-	-	-	-

Source: FAO, 2016

In the EU, the total fishery production in 2015 was approximately 6.4 million live weight tonnes (Eurostat, 2017). The largest fishery countries in Europe in term of production were Norway (3.5 million tonnes of live weight), Iceland (1.3 million tonnes of live weight), Spain (1.2 million tonnes of live weight tonnes), the United Kingdom and Denmark (0.9 million tonnes of live weight each), and France (0.7 million tonnes of live weight). Capture production in the EU declined from 6.5 to 5.1 million tonnes of live weight over the 2000-2015 period, while the aquaculture production was stable around 1.3-1.4 million tonnes of live weight during this period. In 2015, 80.3% of total fisheries production in the EU came from marine capture, while 19.7% originated from aquaculture (Eurostat, 2017). EU capture is taken mainly in the North East Atlantic, the Mediterranean and Black Sea and the Eastern Central Atlantic, while aquaculture takes place in both inland and marine areas. Among more than 130 species cultured in the EU, the ten most common species made up 90% of the production, in terms of both volume and value (Table 1.2). Mussels are the most important bivalve molluscs in Europe and mainly cultured in the North East Atlantic. Among the 10 major species, *Mytilus* (*M.*) *galloprovincialis* (Mediterranean mussels) and *M. edulis* (blue mussels) accounted for more than 25% and over 9% of the overall EU aquaculture production in 2014, respectively (Eurostat, 2017).

Table 1.2 Ten major species by main production method, fishing region and production country, EU-28, 2014 (% of total species production, tonnes of live weight)

Species	Main production method	share (%)	Main fishing region	share (%)	Main production country	share (%)
Mediterranean mussel	Off bottom	99.6	Northeast Atlantic	68.9	Spain	70.3
Atlantic salmon	Cages	99.8	Northeast Atlantic	99.8	United Kingdom	94.8
Rainbow trout	Tanks	64.9	European inland waters	85.0	Italy	17.9
Blue mussel	Off bottom	50.7	Northeast Atlantic	99.6	Netherlands	45.1
Gilthead seabream	Cages	93.1	Mediterranean and Black Sea	93.2	Greece	58.8
Pacific cupped oyster	Off bottom	54.6	Northeast Atlantic	94.2	France	87.4
European seabass	Cages	89.5	Mediterranean and Black Sea	86.1	Greece	51.1
Common carp	Ponds	96.6	European inland waters	100.0	Poland	26.9
Japanese carpet shell	On bottom	100.0	Mediterranean and Black Sea	95.8	Italy	95.6
Atlantic Bluefin tuna	Cages	100.0	Mediterranean and Black Sea	95.3	Malta	50.6

Source: Eurostat, 2017

1.2 The risk associated with seafood consumption

1.2.1 General risks

Despite the nutrient benefits, consumption of seafood containing human pathogenic microorganisms might pose a potential risk of foodborne illness (Butt et al., 2004a). Pathogenic bacteria, viruses, parasites, chemicals, heavy metals and natural toxins have been found in seafood (Butt et al., 2004a, Butt et al., 2004b, Institute of Medicine (US) Committee on Evaluation of the Safety of Fishery Products, 1991, Feldhusen, 2000, Chiocchetti et al., 2017). The threat of illness from seafood consumption depends on seafood species, environmental conditions, harvest location, postharvest processing, and handling during marketing, storage conditions and eating habits such as consumption of raw or minimally processed seafood (Doyle and Buchanan, 2013). Bivalve molluscs are filter feeders, obtaining nutrition and oxygen from the water environment by filtering large volumes of seawater. Therefore, they can accumulate and concentrate heavy metals, chemical agents, viruses and pathogenic bacteria from contaminated water during filtration (Gueguen et al., 2011, De Witte et al., 2014, Strubbia et al., 2016). The microbial pathogens in seafood originate from microorganisms naturally present in the aquatic environment, and could also be a result of contamination with human and/or animal sewage in contaminated growing areas (Feldhusen, 2000, Iwamoto et al., 2010). Seafood could also be contaminated during postharvest handling, processing and preparation. Several factors contributed to the contamination should be considered, such as inappropriate temperatures during storage and transportation, contamination by infected food handles, and cross-contamination via contact with contaminated seawater or seafood (Iwamoto et al., 2010).

A large number of seafood-associated infections is caused by parasites and most of these infections result from consumption of raw or undercooked seafood (Butt et al., 2004b). Parasites causing human infections via seafood consumption include nematodes, trematodes, cestodes and protozoa (Butt et al., 2004b). Viral seafood related infections are usually caused by norovirus and hepatitis A virus. Norovirus has been considered as the leading cause of gastroenteritis and outbreaks worldwide due to the consumption of raw or undercooked shellfish and exposure to contaminated water (Elbashir et al., 2018, Butt et al., 2004a), whereas hepatitis A virus infection is the most serious viral infection related to seafood consumption (Iwamoto et al., 2010). Although viruses have been reported as the most common cause of seafood-borne diseases, most hospitalisations and deaths have been caused by bacteria (Butt et al., 2004a). Pathogenic bacteria in seafood can be classified into three groups: (i) bacteria which are normal components of the marine or estuarine environment, including *Vibrio* (*V.*) *cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Listeria* (*L.*) *monocytogenes*, *Clostridium* (*C.*) *botulinum* and *Aeromonas* (*A.*) *hydrophila* (virulent strains); (ii) enteric bacteria which are present through faecal contamination, such as *Salmonella* (*S.*) spp., pathogenic *Escherichia* (*E.*) *coli*, *Shigella* spp., *Campylobacter* (*C.*) spp. and *Yersinia* (*Y.*) *enterocolitica* (pathogenic serotypes); (iii) bacteria contaminating during processing, e.g. *Bacillus cereus* (toxigenic strains), *L. monocytogenes*, *Staphylococcus* (*S.*) *aureus* and *C. perfringens* (Feldhusen, 2000). Another

public health hazard related to seafood is the rapid increase of antimicrobial resistance among zoonotic pathogens in aquatic populations (Elbashir et al., 2018). The spread of antimicrobial resistance genes to human pathogens via direct transfer or horizontal transfer from aquaculture to humans through the food chain has been reported and became a major concern of public health because of increasing numbers and severity of infections, as well as the frequency of treatment failures (Elbashir et al., 2018).

1.2.2 *Vibrio* spp.

1.2.2.1 Morphology and characteristics

The genus *Vibrio* belongs to the phylum of *Proteobacteria*, the class of *Gammaproteobacteria*, the order of *Vibrionales* and the family *Vibrionaceae*. *Vibrio* spp. are Gram-negative, straight or curved, short rods bacteria with sizes ranging from 1.4 to 2.6 μm in length and from 0.5 to 0.8 μm in width, motile with a single polar flagellum (Bhunia, 2008, Adams and Moss, 2008). *Vibrio* spp. are facultative anaerobic, catalase- and oxidase-positive (except *V. metchnikovii*), ferment glucose and mannitol but unable to ferment lactose (Erkmen and Bozoglu, 2016, Bhunia, 2008). They produce many extracellular enzymes: e.g. amylase, gelatinase, chitinase, and DNase (Bhunia, 2008). Most of *Vibrio* spp. are halophilic and able to grow under conditions of 0.5 to 8% NaCl (Erkmen and Bozoglu, 2016); the optimum level of NaCl for the growth of clinically important species is 1-3% (Adams and Moss, 2008). Although their growth temperature ranges between 5 and 43ºC, the growth of enteropathogenic vibrios occurs optimally at 37ºC (Adams and Moss, 2008). Vibrios are generally acid sensitive and grow well in neutral to alkaline conditions up to pH 9.0, the optimum pH range is 8-8.8 (Bhunia, 2008, Igbinosa and Okoh, 2008).

1.2.2.2 Natural habitats

Vibrio spp. are ubiquitously spread and naturally occur in marine and estuarine environment (Iwamoto et al., 2010, Huehn et al., 2014). Water temperature, concentration of organic materials and salinity have a significant effect on the presence and growth of these organisms in aquatic environments (Butt et al., 2004a). *Vibrio* spp. are commonly isolated from sediment, the water column, plants and vertebrate as well as invertebrate animals in both marine and estuarine environments (Doyle and Buchanan, 2013). There is an association between *Vibrio* and planktonic organisms, since plankton indicates an organically rich microenvironment, where the high nutrient concentrations of the plankton microhabitat can enrich heterotrophic bacteria, including vibrios (Doyle and Buchanan, 2013). Increase in the water temperature and abundance of zooplankton resulted in the increase of *Vibrio* prevalence in seawater (Turner et al., 2014). *Vibrio* spp. are also found in filter-feeding shellfish with concentrations up to 100-fold the amount in the surrounding water (Butt et al., 2004a). The numbers of vibrios in both surface waters and shellfish peak highest during the warm-water months (Butt et al., 2004a, Doyle and Buchanan, 2013).

1.2.2.3 Human pathogenic *Vibrio*

Among different bacterial pathogens detectable in seafood, *Vibrio* spp. are the most commonly associated with human infections. Twelve species have been implicated as potential human pathogen among the members of the genus *Vibrio* (Igbinosa and Okoh, 2008). Infections caused by *Vibrio* are usually associated with the consumption of raw or undercooked seafood particularly shellfish worldwide (by e.g. *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus*), or septicaemia and wound infections via contact with contaminated seawater and seafood (by e.g. *V. vulnificus*, *V. alginolyticus*) (Igbinosa and Okoh, 2008, Huehn et al., 2014). Similar to the occurrence of *Vibrio* in surface seawater and shellfish, cases of V*ibrio* infections have seasonal distribution; mostly occur during summer and early autumn (Iwamoto et al., 2010). *Vibrio* infections are commonly reported in the US (Iwamoto et al., 2010) and in many Asian countries, including Japan, China and Taiwan (Su and Liu, 2007), while *Vibrio* spp. have been implicated to cause sporadic cases of foodborne illness, wound infections and septicaemia in European countries (Huehn et al., 2014). However, currently these pathogens have been paid much more attention in Europe because of an increase in the incidence and clinical burden, owing to climate change and increasing of the global trade as well as seafood consumption (Baker-Austin et al., 2010).

1.2.2.3.1 *V. parahaemolyticus*

V. parahaemolyticus was first identified as the cause of a food associated outbreak with 272 patients in Japan in 1950 (Fujino et al., 1953). Since then, *V. parahaemolyticus* has become one of the leading causes of seafood-borne illness in many Asian countries (Su and Liu, 2007, Yeung and Boor, 2004). Within 10 years from 1998 to 2007, there were a total of 977 foodborne outbreaks of *V. parahaemolyticus* in Japan. The most prevalent serotype was O3:K6, which was involved in 50% of the total outbreak each year. Serotypes O1:K25, O1:K56, O4:K8 and O4:K68 were the major serotypes among more than 60 serotypes isolated (Hara-Kudo et al., 2012). In China, gastroenteritis caused by *V. parahaemolyticus* accounted for 31.1% of foodborne disease outbreaks between 1992-2001 (Han et al., 2015). Additionally, *V. parahaemolyticus* was reported as the leading cause of bacterial diarrhoea in the southern coastal region of China during 2007-2012. Serotype O3:K6 was the most commonly found (66.9%) among 47 serotypes detected, followed by O4:K8 (10.5%) and O3:K29 (3.4%) (Li et al., 2014). Food poisoning by *V. parahaemolyticus* also occurred in other Asian countries such as Taiwan, India, Thailand and Vietnam (Hsiao et al., 2016, Chowdhury et al., 2013, Thongjun et al., 2013, Tuyet et al., 2002). By contrast to Asian countries, *V. parahaemolyticus* infections are scarcely reported in European countries (Su and Liu, 2007). In the US, the Centers for Disease Control and Prevention (CDC) estimates approximately 45,000 cases of foodborne *V. parahaemolyticus* infections each year (Centers for Disease Control and Prevention (CDC), 2017). *V. parahaemolyticus* outbreaks associated with consumption of raw shellfish or cooked seafood have been reported throughout the US coastal areas (Su and Liu, 2007).

V. parahaemolyticus is a halophilic bacterium, which can grow in 1-8% NaCl and the optimum concentrations for growth range from 2 to 4% (Jay et al., 2005). The growth temperatures range from 5 to 44ºC, with the best growth between 30-35ºC (Jay et al., 2005). A pH 4.8-11.0 (with 7.6-8.6 being optimum) are required for the growth (Jay et al., 2005). *V. parahaemolyticus* is serotyped based on both its somatic O and capsular polysaccharide K antigens. Currently, 13 O (LPS) antigens and more than 71 K (acidic polysaccharide) antigens have been recognized (Doyle and Buchanan, 2013).

Gastroenteritis by *V. parahaemolyticus* is commonly associated with consumption of raw, semi-cooked, or cooked but re-contaminated seafood, such as fish, crab, shrimp, lobsters and oysters (Doyle and Buchanan, 2013). The infectious dose required for gastrointestinal illness is 2.10^5-3.10^7 CFU (Doyle and Buchanan, 2013, Bhunia, 2008). *V. parahaemolyticus* is able to generate in a short time (8-9 min) at 37ºC, generation times of 12-18 min have been observed in seafood (Doyle and Buchanan, 2013). The incubation time is 4 to 96 h with a mean of 15 h after ingestion of the bacteria (Doyle and Buchanan, 2013). Symptoms include diarrhoea, abdominal cramps, nausea, vomiting, headache, fever and chills, and the clinical symptoms usually last for 2-3 days (Bhunia, 2008, Doyle and Buchanan, 2013, Su and Liu, 2007, Jay et al., 2005).

V. parahaemolyticus can produce four haemolysins, including the TDH (thermostable direct haemolysin), the TRH (heat-labile TDH-related haemolysin), the TLH (thermolabile haemolysin), and the δ-VPH (thermolabile haemolysin) (Bhunia, 2008, Zhang and Austin, 2005). Both the TLH encoding gene (*tlh*) and the δ-VPH encoding gene have been found in the genomes of all *V. parahaemolyticus* strains; however, the roles of these genes in pathogenesis are still unknown (Zhang and Austin, 2005). Most of the *V. parahaemolyticus* isolates from the environment or seafood are not pathogenic strains (Su and Liu, 2007). Clinical strains have the ability to produce the TDH encoded by *tdh* gene and/or the TRH encoded by *trh* gene, which have been recognized as the major virulence factors of *V. parahaemolyticus* (Thompson et al., 2004, Su and Liu, 2007). TDH causes haemolysis on Wagatsuma blood agar and this phenomenon was called Kanagwa phenomenon (KP). The molecular weight of TDH was determined to be 21 kDa (Zhang and Austin, 2005). This protein is heat-stable (inactivated at 100ºC for 10 min) and produced by KP^+ strains (Bhunia, 2008). The TDH toxin has the ability to damage eukaryotic cells by punching holes in the plasma membrane. Haemolysis by TDH involves three steps: (i) binding to the erythrocyte membrane, (ii) formation of a transmembrane pore, and (iii) disruption of the cell membrane (Honda et al., 1992). TDH can form porin channels and allow the ionic influx, which leads to swelling and death of cells due to ionic imbalance (Bhunia, 2008). The TRH toxin is heat-labile (inactivated at 60ºC for 10 min) and normally produced by KP^- strains (Bhunia, 2008). The role of TRH toxin in inducing diarrhoea has been proven by the ability to stimulate fluid accumulation in the rabbit ileal loop test (Bhunia, 2008, Zhang and Austin, 2005).

1.2.2.3.2 *V. cholerae*

V. cholerae is one of the most important species of the genus *Vibrio*. Cholera caused by ingestion of food or water contaminated with *V. cholerae* remains a major public health concern worldwide. *V. cholerae* causes an estimated 2.9 million cases of cholerae with 95,000 deaths annually (Ali et al., 2015). Seven cholera pandemics have been occurred in the past two centuries (Mandal et al., 2011). Currently, cholera outbreaks are frequently reported in Asia, Africa, South and Central America (Bhunia, 2008). Within 10 years from 2003 to 2012, overall 113 cholera outbreaks were reported in South and Southeast Asia, and *V. cholerae* O1 El Tor (Inaba and Ogawa) as well as *V. cholerae* O139 were identified as the cause of the outbreaks (Mahapatra et al., 2014). In Africa, cholera was the most commonly reported cause of outbreaks during 2003-2007 (Kebede et al., 2010). Between 2001 and 2011, a total of 111 cholera cases caused by *V. cholerae* O1 El Tor (96%) were reported in the US (Loharikar et al., 2015). Among those, 90 (81%) infections were travel-associated cases and 20 (18%) infections were domestically-acquired cases (information for one patient was not available). Most of the domestically-acquired cases (95%) were associated with seafood consumption, such as raw oysters, boiled and/or raw crabs, cooked shrimp and fish (Loharikar et al., 2015).

V. cholerae can grow under temperatures between 10-43ºC, with optimum growth at 37ºC. The growth range of pH is from 5.9 to 9.6, while pH 7.6 is the optimal pH for its growth. *V. cholerae* can grow in concentrations of 0.1-4.0% NaCl and 0.5% NaCl is the optimal concentration (International Commission on Microbiological Specifications for Foods, 1996). *V. cholerae* consists of more than 200 serogroups, which are classified by their somatic O antigen. However, only two toxigenic serogroups O1 and O139 have been identified to cause widespread cholera epidemics (Azman et al., 2013). Based on phenotypic and biochemical characteristics, *V. cholerae* O1 can be divided into the Classical and El Tor biotypes. The two biotypes are further classified as Inaba, Ogawa and Hikojima serotypes based on antigen factors (Azman et al., 2013, Bhunia, 2008). Other *V. cholerae* belonging to non-O1 and non-O139 serogroups can cause gastroenteritis and have been associated with sporadic cases of foodborne outbreaks rather than epidemics (Faruque et al., 1998).

The primary transmission route of cholera is associated with ingestion of faecal contaminated water (Mandal et al., 2011, Rabbani and Greenough, 1999). In addition, food particularly seafood has also been identified as an important vehicle for transmission of *V. cholerae* (Mandal et al., 2011, Rabbani and Greenough, 1999). Seafood, including fish, shellfish, crab, oysters and clams has been implicated in cholera outbreaks in many countries all over the world (Rabbani and Greenough, 1999).

The infectious dose is 10^4-10^{10} CFU/g. The incubation time of cholera can range from 6 h to 5 days and symptoms last for 2-12 days (Bhunia, 2008). The onset of illness may appear suddenly with watery diarrhoea, or with other symptoms such as anorexia, abdominal discomfort and simple diarrhoea. Vomiting normally occurs after the onset of diarrhoea for a few hours (Doyle and Buchanan, 2013). Diarrhoea appears as “rice water” (mucus in the stool)

with a fishy odour (Bhunia, 2008). The watery diarrhoea causes severe dehydration, loss of electrolytes and ions, leading to tachycardia, hypertension and vascular collapse (Doyle and Buchanan, 2013, Bhunia, 2008). The severe dehydration can lead to death within hours of the onset of symptoms, if fluids and electrolytes are not rapidly replaced (Doyle and Buchanan, 2013).

After passing through the acid barrier of the stomach, *V. cholerae* colonizes epithelium of the small intestine via toxin-coregulated pili (TCP) and other colonization factors such as mannose-fucose resistant hemagglutinin (MFRHA), mannose-sensitive hemagglutinin (MSHA) and some outer membrane proteins (OMPs) (Doyle and Buchanan, 2013, Bhunia, 2008, Igbinosa and Okoh, 2008). Invasion into epithelial cells does not occur, production of cholera enterotoxin (CT) produced by adherent vibrios is across the bacterial outer membrane into the extracellular environment and disrupts ion transport by intestinal epithelial cells (Doyle and Buchanan, 2013, Igbinosa and Okoh, 2008). CT is the most important virulence factor of *V. cholerae* O1/O139 and is encoded by virulence genes located in the filamentous cholera toxin phage (CTX phage) (Doyle and Buchanan, 2013, Bhunia, 2008). CT is an A-B subunit toxin, the A subunit is a 27 kDa protein encoded by *ctx*A and the B subunit is a 11.7 kDa protein encoded by *ctx*B (Bhunia, 2008). The B subunit of CT is responsible for binding of the holotoxin to the monosialosyl ganglioside GM_1 receptor on the host intestinal mucosal cell membrane, then the CT is transported inside the cell and the A subunit is detached (Mandal et al., 2011). The A subunit of CT ADP-ribosylates the Gs protein (GTP hydrolysing protein), thereby increases the catalysis of ATP (adenosine triphosphate) to form cAMP (cyclic adenosine monophosphate), this activity causes the increase of Cl^- and HCO_3^- secretion and the decrease of Na^+ and Cl^- absorption by cells, resulting in ion losses (Bhunia, 2008). In consequence, water is drawn from both intravascular and extracellular spaces of the body, and rapidly lost into the intestinal lumen (Mandal et al., 2011).

V. cholerae also can produce zonula occludens toxin (ZOT) and Ace (accessory cholera enterotoxin) toxin, encoded by *zot* and *ace* genes, respectively (Doyle and Buchanan, 2013, Bhunia, 2008). The ZOT toxin increases permeability of the small intestinal mucosa by disrupting the structure of the intercellular tight junction, and also disrupts the ion balance and promotes diarrhoea, while the ace toxin has responsibility in animal, but no role in human diarrhoea (Bhunia, 2008).

Most of *V. cholerae* non-O1/O139 strains do not encode a *ctx* gene, but usually contain genes encoding haemolysin, RtxA and HA/P. The *tdh* gene of *V. parahaemolyticus* and a 17-amino-acid heat-stable enterotoxin (NAG-ST) that shares 50% sequence homology to the STa of enterotoxigenic *E. coli* have been detected in some *V. cholerae* non-O1/O139 strains (Doyle and Buchanan, 2013).

1.2.2.3.3 *V. vulnificus*

V. vulnificus was primarily isolated by the CDC (US) in 1964 (Baker-Austin and Oliver, 2018) and is considered as the most infectious and invasive of all the human pathogenic vibrios

(Bhunia, 2008). Human infections caused by *V. vulnificus* involve gastroenteritis as well as septicaemia via consumption of seafood and wound infections due to exposure to contaminated seawater or seafood (Baker-Austin and Oliver, 2018, Heng et al., 2017). Approximately 85% of *V. vulnificus* infections occur during the warm-water months (Baker-Austin et al., 2010). *V. vulnificus* infections have been reported worldwide such as the US, Belgium, Denmark, Germany, Holland, Italia, Spain, Sweden, Turkey, India, Israel, Japan, Korea, Taiwan, Thailand and Brazil (Heng et al., 2017). In the US, *V. vulnificus* is the leading cause of seafood associated fatalities, responsible for over 95% of all deaths caused by seafood-borne pathogens with 50 cases of *V. vulnificus* infections and 16 deaths every year (Baker-Austin and Oliver, 2018, Heng et al., 2017).

V. vulnificus can grow in the concentration of 1-6% NaCl and forms a capsule (Erkmen and Bozoglu, 2016). *V. vulnificus* is commonly found in warm coastal waters, with salinities between 1.5-2.5% and temperatures between 9-31ºC (Heng et al., 2017). Based on biochemical characteristics, *V. vulnificus* is grouped into three biotypes. Biotype 1 and 3 are indole positive, while biotype 2 is indole negative. In addition, biotype 1 and 2 are able to ferment cellobiose and salicin, while biotype 3 lacks fermentation of these sugars (Doyle and Buchanan, 2013). Biotype 1 strains are found worldwide in sea or brackish waters and are responsible for severe human infections with primary sepsis and fatality rates of over 50% (Heng et al., 2017). Biotype 2 strains are isolated from seawater in Eastern and Western Europe, have been reported as the main cause of fatalities in eel, but rarely cause human infections (Heng et al., 2017). Biotype 3 strains are genetic hybrids of biotypes 1 and 2, and has only been isolated so far in Israel with all cases being wound infections (Doyle and Buchanan, 2013).

Gastroenteritis and primary septicaemia usually result from the consumption of raw contaminated seafood. Gastroenteritis symptoms such as abdominal pain, vomiting and diarrhoea develop within 16 h to 4 days after consumption of contaminated raw seafood particularly oysters with $< 10^3$ viable cells/g (Erkmen and Bozoglu, 2016, Bhunia, 2008). Incubation period of primary septicaemia caused by *V. vulnificus* ranges from 7 h to 10 days (Doyle and Buchanan, 2013). The most significant symptoms include fever, chills, nausea and hypotension (Doyle and Buchanan, 2013). Wound infections by *V. vulnificus* are usually associated with activities in water, including swimming, fishing and handling of seafood (Baker-Austin and Oliver, 2018). Patients with primary wound infections develop rapidly painful cellulitis, local tissue swelling with haemorrhagic bullae; systemic symptoms include fever and chills (Bross et al., 2007).

To cause human infections, *V. vulnificus* attaches to the intestinal epithelial cells, multiplies and produces enterotoxins (lipopolysaccharide). Gastroenteritis results from toxin formation and inflammation of the intestinal cells (Erkmen and Bozoglu, 2016). *V. vulnificus* is highly invasive and produces several virulence factors, including capsular polysaccharides (CPS), siderophores and toxins such as haemolysins, cytolysin and proteases (Bhunia, 2008, Forsythe, 2000). *V. vulnificus* invades epithelial cells by binding to cells with pili, produces haemolysins which induce apoptosis and facilitates bacterial invasion and translocation to the

blood stream (Bhunia, 2008). The CPS protects *V. vulnificus* from phagocytosis by macrophages (Bhunia, 2008). Cytolysin has a role in the tissue destruction on the intestinal epithelial cells and pathogens pass to the blood stream (septicaemia) (Erkmen and Bozoglu, 2016). Bacteria acquire iron from the host transferrin using siderophores causing septicaemia (Bhunia, 2008). Besides that, *V. vulnificus* produces collagenase and metalloproteases which allows colonization of open wounds, and then cause tissue damage leading to haemorrhages (Bhunia, 2008).

1.2.2.3.4 *V. alginolyticus*

V. alginolyticus normally inhabits seawater (Jay et al., 2005), it is often isolated from seawater and seafood such as fish, clams, crabs, oysters, mussels and shrimp (Doyle and Buchanan, 2013). *V. alginolyticus* has been reported to cause disease in finfish and shellfish in many countries (Austin, 2010). In addition, *V. alginolyticus* has been found to cause ear, soft tissue and wound infections in humans (Austin, 2010, Jay et al., 2005). Infections by *V. alginolyticus* mostly occurred after exposure to seawater, or consumption of contaminated fish or shellfish (Doyle and Buchanan, 2013, Penland et al., 2000). *V. alginolyticus* has rarely been implicated as the cause of gastroenteritis, only few cases with acute diarrhoea has been reported (Doyle and Buchanan, 2013).

1.2.2.4 Prevalence of *Vibrio* in seafood

Seafood represents a potential source for foodborne pathogens, including *Vibrio* spp. Prevalence of *Vibrio* spp. have been determined in many types of seafood, for instance fish, oysters, mussels, clams, cockles and shrimp. Table 1.3 presents the prevalence of *Vibrio* spp. in different countries.

Table 1.3 Prevalence of *Vibrio* spp. in seafood

Country	Source	*Vibrio* spp.	Prevalence	Reference
France	Seafood (crustaceans, fish, shellfish)	*Vibrio* spp. *V. parahaemolyticus* *V. vulnificus* *V. cholerae*	34.7% 31.1% 12.6% 0.6%	(Robert-Pillot et al., 2014)
Germany	Blue mussels	*Vibrio* spp. *V. alginolyticus* *V. parahaemolyticus* *V. vulnificus*	74.4% 51.2% 39.5% 3.5%	(Lhafi and Kuhne, 2007)
Italy	Crustaceans	*V. parahaemolyticus*	28%	(Caburlotto et al., 2016)
Poland	Shellfish (mussels, oysters, clams, scallops)	*V. parahaemolyticus*	17.5%	(Lopatek et al., 2015)
Spain	Bivalve molluscs	*V. parahaemolyticus*	14.2%	(Lopez-Joven et al., 2015)
Ecuador	Shrimp	*Vibrio* spp. *V. parahaemolyticus* *V. alginolyticus* *V. cholerae* *V. vulnificus*	95.6% 80.8% 50.2% 11.3% 3.5%	(Sperling et al., 2015)
Mexico	Seafood (raw)	*Vibrio* spp.	44.3%	(Franco Monsreal et al., 2015)
China	Seafood (fish, oysters, shrimp)	*V. parahaemolyticus*	19.4%	(Yang et al., 2017)
India	Seafood (oysters, clams, fish, shrimp)	*V. parahaemolyticus*	89.2%	(Raghunath et al., 2008)
Japan	Seafood (hen-clams, short-neck clams, bloody clams, rock oysters, scallops, other molluscan shellfish, horse mackerel)	*V. parahaemolyticus*	85.2%	(Hara-Kudo et al., 2012)
Malaysia	Shrimp	*V. parahaemolyticus*	57.8%	(Letchumanan et al., 2015)
Taiwan	Oysters Clams	*V. parahaemolyticus* *V. parahaemolyticus*	70.8% 68.8%	(Yu et al., 2013)
Thailand	Seafood (sea bass, shrimp, oysters, cockles)	*Vibrio* spp. *V. parahaemolyticus* *V. cholerae* *V. alginolyticus* *V. vulnificus*	92% 68% 51% 15% 14%	(Woodring et al., 2012)
Vietnam	Shrimp	*Vibrio* spp. *V. parahaemolyticus* *V. alginolyticus* *V. cholerae* *V. vulnificus*	99.5% 96.5% 56.4% 2% 1.5%	(Tra et al., 2016)

1.2.2.5 Detection of *Vibrio*

1.2.2.5.1 Microbiological methods

Conventional cultured-based methods generally involve enrichment of samples, streaking enriched samples on selective solid agars followed by further biochemical tests (Igbinosa and Okoh, 2008). The ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007 standard methods and the US Food and Drug Administration's Bacteriological Analytical Manual were developed for the detection of *Vibrio* spp. The enrichment step is performed in alkaline peptone water (APW) followed by the isolation step on selective thiosulfate citrate bile salt (TCBS) agar and on second selective media for *Vibrio* as optional option, for instance modified cellobiose polymyxin colistin (mCPC) and cellobiose colistin (CC) agar as well as CHROMagar Vibrio (Bonnin-Jusserand et al., 2017, Food and Drug Administration, 2004). Presumptive colonies on selective media need to be confirmed by biochemical tests. Further identification is serotyping for somatic O antigens and capsular K antigens for *V. parahaemolyticus* and O antigens for *V. cholerae* (Food and Drug Administration, 2004). The rapid diagnostic kit API20E can be used alternatively for identification and confirmation of *Vibrio* isolates (Food and Drug Administration, 2004).

However, traditional microbiological techniques for detection of *Vibrio* spp. are laborious and time consuming (Igbinosa and Okoh, 2008). Since the TCBS agar cannot differentiate *Vibrio* spp., confirmation by biochemical tests is required and last for 4-5 days (Su and Liu, 2007). In addition, conventional phenotypic tests with low sensitivity may fail to detect bacterial strains at low concentrations and with unusual phenotypic characteristics (Igbinosa and Okoh, 2008). Therefore, to overcome the disadvantage of conventional microbiological techniques, molecular methods for identification of *Vibrio* spp. and virulent strains have been developed.

1.2.2.5.2 Molecular methods

Molecular methods based on polymerase chain reaction (PCR) have been applied successfully to detect and identify *Vibrio* spp. in clinical, environmental and food samples with high specificity and sensitivity as well as rapid processing time (Di Pinto et al., 2006). The *tlh* gene, the *gyr*B gene which encodes the B subunit of DNA gyrase essential for DNA replication, the *gro*EL gene encoding a heat shock protein and the *tox*R gene which together with *tox*S encodes transmembrane proteins involved in the regulation of virulence associated genes are species-specific genetic markers and have been used for the detection of *V. parahaemolyticus* (Bauer and Rorvik, 2007, Bej et al., 1999, Hossain et al., 2013, Nordstrom et al., 2007, Venkateswaran et al., 1998). Besides, PCR was established to target the *tox*R genes of *V. cholerae* and *V. vulnificus* (Bauer and Rorvik, 2007). Although the primers targeting the *tox*R gene are sensitive and specific for *V. cholerae*, the *omp*W gene encoding an outer membrane protein OmpW of *V. cholerae* can be better targeted for identification of *V. cholerae* (Nandi et al., 2000). In addition, the collagenase genes of *V. alginolyticus*, *V. cholerae* and *V. parahaemolyticus* were used as an alternative genetic marker for species identification of the three *Vibrio* species (Di Pinto et al., 2005).

Multiplex PCR (mPCR) as well as multiplex real-time PCR (qPCR) methods have been applied for the detection of several *Vibrio* species or a species and its virulence factors in the same reaction (Bonnin-Jusserand et al., 2017). For example, Nordstrom et al. (2007) and Hossain et al. (2013) developed a multiplex qPCR and mPCR, respectively, to detect *V. parahaemolyticus* and its virulence genes, including *tdh* and *trh*. The multiplex qPCR is able to detect < 10 CFU/reaction of pathogenic *V. parahaemolyticus* in the presence of > 10^4 CFU/reaction of total *V. parahaemolyticus* bacteria; therefore, it is possible to apply the method for outbreak investigations for the detection of total and pathogenic *V. parahaemolyticus* (Nordstrom et al., 2007). Another multiplex qPCR system was established by Messelhäusser et al. (2010) based on the detection of the *tox*R gene specific for *V. parahaemolyticus*, the *sod*B gene specific for *V. cholerae* and the *vvha* gene specific for *V. vulnificus*. The detection limit for the system was between 1 CFU/ml and 10 CFU/ml in pure culture and in different artificially contaminated sample material. Additionally, two qPCR systems for the detection of different virulence genes of *V. parahaemolyticus* and *V. cholerae* were implemented (Messelhäusser et al., 2010).

In addition to the PCR based techniques, DNA hybridization methods were also developed for specific detection of *V. parahaemolyticus* (Su and Liu, 2007). Other molecular methods for identification and genotyping of *Vibrio* spp. have been used, such as ribotyping, restriction fragment length polymorphism (RFLP), amplified restriction fragment length polymorphism (AFLP), randomly amplified polymorphic DNA and enterobacterial intergenic consensus sequence-PCR (ERIC-PCR) (Bhunia, 2008, Igbinosa and Okoh, 2008).

1.2.3 β-lactamase producing *Enterobacteriaceae*

1.2.3.1 Classification of β-lactamases

The β-lactams are one of the most important groups of antibiotics, which are widely used in human and veterinary medicine (Smet et al., 2010). However, the use of β-lactams might lead to the development of β-lactam-resistance in both Gram-negative and Gram-positive bacteria (Poole, 2004). Among several mechanisms of bacterial resistance to β-lactams, the most common mechanism of β-lactam-resistance in Gram-negative bacteria is the production of β-lactamases, which are hydrolytic enzymes that disrupt the amide bond of the four-membered β-lactam ring (Poole, 2004, Smet et al., 2010).

Table 1.4 Classification scheme of β-lactamases

Bush-Jacoby group	Molecular class	Distinctive substrates	Inhibited by		Characteristics	Representative enzymes
			CA or TZB	EDTA		
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin, hydrolyse cephamycins	ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and other oxyimino-β-lactams	GC1, CMY-10, CMY-19, CMY-37
2a	A	Penicillins	Yes	No	Hydrolyse benzylpenicillin; poor hydrolysis of cephalosporins, carbapenems or monobactams	PC1
2b	A	Penicillins, early cephalosporins	Yes	No	Hydrolyse penicillins and early cephalosporins (cephaloridine and cephalothin)	TEM-1, TEM-2, SHV-1
2be	A	extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1, BEL-1, BES-1, SFO-1, TLA-1, TLA-2
2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, tazobactam	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, tazobactam	TEM-50
2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, cefpirome	RTG-4
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyse cloxacillin or oxacillin and oxyimino-β-lactams but not carbapenems	OXA-11, OXA-15
2df	D	Carbapenems	Variable	No	Hydrolyse cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyse extended-spectrum cephalosporins; inhibited by clavulanic acid or tazobactam but not aztreonam	CepA
2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
	B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1

Note: CA, clavulanic acid; TZB, tazobactam

Source: Bush and Jacoby (2010)

Currently, β-lactamases are classified according to two schemes: the molecular classification scheme and the functional classification system (Table 1.4). The molecular classification is based on the amino acid sequence and divides β-lactamases into four major classes (A to D) (Bush and Jacoby, 2010). Classes A, C and D include serine β-lactamases that hydrolyse their substrates by forming an acyl enzyme through an active site serine, whereas class B enzymes are metallo-β-lactamases that utilize at least one active-site zinc ion to facilitate β-lactam hydrolysis (Bush and Jacoby, 2010). The functional classification divides β-lactamases into three groups (1, 2 and 3) with multiple subgroups according to substrate and inhibitor profiles (Bush and Jacoby, 2010). The β-lactamases can be either plasmid mediated β-lactamases or chromosomal β-lactamases (Shah et al., 2004). To date, more than 400 β-lactamases have been reported and new β-lactamases are emerging worldwide (Smet et al., 2010).

Extended-spectrum β-lactamases (ESBL) belong to molecular class A and functional group 2be (Bush and Jacoby, 2010). ESBL possess the ability to hydrolyse most of the β-lactam antibiotics including penicillins, 1st, 2nd, and 3rd generation cephalosporins and monobactams (e.g. aztreonam) but not cephamycins (e.g. cefoxitin, cefotetan) and carbapenems (e.g. imipenem, meropenem, ertapenem) (Paterson and Bonomo, 2005, Pitout and Laupland, 2008). They can be inactivated by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Paterson and Bonomo, 2005). By contrast, AmpC β-lactamases are not inhibited by β-lactamase inhibitors and are resistant against all β-lactams and cephamycins with the exception of 4th generation cephalosporins (e.g. cefepime, cefpirome) and carbapenems (Jacoby, 2009, Thomson, 2010). In the molecular classification of β-lactamases, AmpC β-lactamases belong to class C, while in the functional classification, they belong to group 1 (Jacoby, 2009).

1.2.3.1.1 ESBL types

TEM β-lactamases

The TEM-type ESBL are derivatives from TEM-1 and TEM-2 (Paterson and Bonomo, 2005). TEM-1 was primarily reported in *E. coli* isolate from a patient, named Temoneira, in Greece in 1965 (Paterson and Bonomo, 2005). TEM-1 hydrolyses penicillins and early cephalosporins such as cephalothin and cephaloridine (Bradford, 2001). TEM-2 has the same hydrolytic profile as TEM-1, but differs from TEM-1 by a single amino acid replacement (Bradford, 2001). Both TEM-1 and TEM-2 are not ESBL. TEM-3 was the first TEM-type ESBL, which is different from TEM-2 by two amino acid substitutions (Bradford, 2001). Currently, more than 90 TEM-type ESBL have been detected in both *Enterobacteriaceae* and non-*Enterobacteriaceae* Gram-negative bacteria (Bradford, 2001, Lahey Clinic, 2017).

SHV β-lactamases

The SHV (sulfhydryl variable)-type ESBL are more commonly found in clinical isolates than other types of ESBL (Paterson and Bonomo, 2005). SHV-1 was first found in *Klebsiella* (*K.*) *pneumonia* and confers resistance to broad-spectrum penicillins but not to the oxyimino-cephalosporins (Shaikh et al., 2015, Smet et al., 2010). SHV-2, an ESBL type was

found from *K. ozaenae* in Germany in 1983, differs from SHV-1 by replacement of glycine by serine at the 238 position, which allows SHV-2 to hydrolyse cefotaxime more than ceftazidime (Paterson and Bonomo, 2005). To date, many SHV-type ESBL derivatives have been found in *Enterobacteriaceae*, and outbreak of SHV-producing *Pseudomonas* (*P.*) *aeruginosa* and *Acinetobacter* spp. have been reported (Paterson and Bonomo, 2005).

CTX-M β-lactamases

The name CTX-M reflects hydrolytic activity of these β-lactamases against cefotaxime (Smet et al., 2010). CTX-M enzymes are related to chromosomal β-lactamases in *Kluyvera* spp. (Bush and Jacoby, 2010). So far, 172 CTX-M have been described (Lahey Clinic, 2017). Additionally, CTX-M have been found in several members of *Enterobacteriaceae*, such as *Citrobacter* (*C.*) *freundii*, *E. coli*, *Enterobacter* spp., *K. pneumoniae* and *Salmonella* spp. (Poole, 2004). Based on the amino acid sequences, they are divided into five groups, including CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Paterson and Bonomo, 2005, Smet et al., 2010).

OXA β-lactamases

The OXA-type β-lactamases are so named based on their oxacillin-hydrolysing abilities (Smet et al., 2010). Most of these β-lactamases do not hydrolyse the extended-spectrum cephalosporins and show the hydrolysis rates for cloxacillin and oxacillin greater than for benzylpenicillin (Paterson and Bonomo, 2005). However, some of these enzymes are ESBL such as OXA-10, OXA-11, OXA-12, OXA-14, OXA-15, OXA-16, OXA-17, OXA-19 and OXA 28 (Lahey Clinic, 2017). The OXA-type ESBL were originally found in *P. aeruginosa* isolates in Turkey (Paterson, 2006).

Other ESBL

Other unusual ESBL have also been discovered, e.g. PER, GES, VEB, BES, BEL, TLA, SFO and IBC (Paterson and Bonomo, 2005).

1.2.3.1.2 AmpC β-lactamases

AmpC β-lactamases, which have been demonstrated or presumed to be chromosomally mediated, have been described in many Gram-negative bacteria, including *Acinetobacter* spp., *Aeromonas* spp., and several *Enterobacteriaceae* such as *Citrobacter* spp., *Enterobacter* spp., *E. coli*, *Hafnia* (*H.*) *alvei*, *Shigella* spp. and *Yersinia* spp. (Jacoby, 2009). When bacteria overproduce the AmpC β-lactamases, they may become resistant to broad-spectrum penicillins, cephalosporins, except the 4th generation cephalosporins, β-lactamase inhibitors and aztreonam (Gutkind et al., 2013). Chromosome-encoded AmpC in many *Enterobacteriaceae* is an inducible enzyme that usually expressed at low levels (Jacoby, 2009). AmpC is inducible by a system involving *ampD*, *ampG*, *ampR* and peptidoglycan recycling (Philippon et al., 2002). However, strains of *E. coli* in which the *ampC* gene is preceded by a strong promoter can express the β-lactamase at high levels (Pfaller and Segreti, 2006). In addition, the expression of chromosomal *ampC* can be at high levels by derepression mechanism (Pfaller and Segreti,

2006). Mutation in *ampD*, which encodes an enzymatic repressor of AmpC synthesis, results in high level production of AmpC in the absence of any inducer (Pfaller and Segreti, 2006).

AmpC β-lactamases can be encoded by *ampC* gene on plasmids of bacteria lacking the chromosomal *ampC* gene such as *Klebsiella* spp. (Smet et al., 2010). Plasmid-mediated AmpC β-lactamases have been found worldwide and named (i) according to the resistance produced to cephamycins (CMY), cefoxitin (FOX), moxalactam (MOX) or latamoxef (LAT), (ii) according to the type of enzyme, such as AmpC type (ACT) or Ambler class C (ACC), and (iii) according to the site of discovery, such as the Miriam Hospital in Providence (MIR) or the Dhahran Hospital in Saudi Arabia (DHA) (Philippon et al., 2002). Besides, BIL-1 was named after the patient (Bilal) who provided the original sample (Philippon et al., 2002). Plasmids carrying *ampC* gene can also carry genes for resistance to aminoglycosides, chloramphenicol, quinolones, sulphonamide, tetracycline, and trimethoprim, as well as genes encoding other β-lactamases (e.g. TEM-1, PSE-1, CTX-M-3, SHV and VIM-1) (Jacoby, 2009).

1.2.3.2 The occurrence of ESBL/AmpC-producing *Enterobacteriaceae* in seafood

ESBL/AmpC-producing *Enterobacteriaceae* have been isolated from humans (Khamsarn et al., 2016, Landers et al., 2016, Rodrigues et al., 2016), companion animals (Baede et al., 2015, Hordijk et al., 2013, Zogg et al., 2018), livestock farms (Abreu et al., 2014, Adler et al., 2015, Fischer et al., 2016, Stefani et al., 2014) and food of animal origin (Belmar Campos et al., 2014, Kola et al., 2012, Ojer-Usoz et al., 2013). Among ESBL/AmpC-producing *Enterobacteriaceae*, *E. coli* and *K. pneumonia* are the most prevalent ESBL/AmpC-producing microorganisms worldwide (Abdallah et al., 2015, Coque et al., 2008, Fernandes et al., 2014). Moreover, among livestock and animal products, the highest prevalence of ESBL/AmpC-producing *Enterobacteriaceae* has been observed in poultry and poultry products with CTX-M-1, TEM-52 and SHV-12 being the most frequently detected ESBL types (Saliu et al., 2017). In addition, these bacteria can also be detected in the surrounding environment of the animal farms (Hering et al., 2014, Laube et al., 2014, Li et al., 2015). Due to increasing treatment difficulties in cases of human and animal infections, and the evidences of transmission of ESBL/AmpC-producing *Enterobacteriaceae* between animals and humans (Dohmen et al., 2015, Fischer et al., 2016, Ljungquist et al., 2016), ESBL/AmpC-producing *Enterobacteriaceae* have become an emerging public health concern.

To date, information on the presence and prevalence of ESBL/AmpC-producing *Enterobacteriaceae* in seafood are still limited. The investigation of Sanjit Singh et al. (2017) on the incidence of ESBL-producing *Enterobacteriaceae* in fresh seafood from retail markets of Mumbai, India showed that 78.6% of the isolates were ESBL phenotype positive with *E. coli*, *K. oxytoca* and *K. pneumonia* being the predominant species. The $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM} genes were found in 76.9%, 63.3% and 44.4% of the phenotype positive isolates, respectively; particularly the New Delhi metallo-β-lactamase gene $bla_{\text{NDM-1}}$ was detected in two ESBL positive isolates (Sanjit Singh et al., 2017). Other previous studies only demonstrated the

occurrence of *E. coli* and *Salmonella* or other bacteria rather than *Enterobacteriaceae* carrying β-lactamase genes in seafood (Bae et al., 2015, Maravic et al., 2013, Maravic et al., 2018, Nguyen do et al., 2016, Ryu et al., 2012, Said et al., 2017). Summary data on the occurrence of bacteria carrying β-lactamase genes in seafood are presented in Table 1.5.

Table 1.5 Occurrence of β-lactamase producing bacteria in seafood

Country	Source	Bacteria	Percentage	β-lactamases	Reference
Croatia	Mussel	*Aeromonas* spp.	14.3% (21/147 isolates)	CTX-M-15, SHV-12, PER-1, FOX-2	(Maravic et al., 2013)
	Mussel	*Pseudomonas aeruginosa*	-	TEM-116	(Maravic et al., 2018)
India	Fresh seafood (fish, shrimp, clams, squid)	*Enterobacteriaceae*	78.6% (169/215 isolates)	CTX-M, SHV, TEM	(Sanjit Singh et al., 2017)
Korea	Seafood (fish, shellfish, molluscs, crustaceans)	*E. coli*	21.4% (15/70 isolates)	TEM	(Ryu et al., 2012)
The US	Imported frozen seafood	*Salmonella*	-	TEM-1, CTX-M-9	(Bae et al., 2015)
Tunisia	Seafood (fish, shellfish)	*E. coli*	5/70 samples	CTX-M-1, TEM-1, OXA-1	(Said et al., 2017)
Vietnam	Fish and shrimp	*E. coli*	29.3% (24/82 samples)	CTX-M-1 group, CTX-M-9 group, TEM, CIT group, DHA	(Nguyen do et al., 2016)

1.2.3.3 Detection of ESBL/AmpC-producing *Enterobacteriaceae*

1.2.3.3.1 Phenotypic detection

The phenotypic methods are commonly used in clinical diagnostic laboratories because of simplicity and effective cost (Pitout and Laupland, 2008). The ESBL phenotypic tests are based on the use of the 3rd generation cephalosporins (cefotaxime and ceftazidime) and a β-lactamase inhibitor (clavulanic acid) (Drieux et al., 2008). The guidelines for ESBL detection in *Enterobacteriaceae* (*E. coli*, *K. pneumonia*, *K. oxytoca* and *Proteus mirabilis*) have been published by the US CLSI, involving both screening and confirmation tests by disk diffusion or broth microdilution methods (Clinical and Laboratory Standards Institute (CLSI), 2014). The CLSI recommends initial screening with cefpodoxime, ceftazidime, aztreonam, cefotaxime or

ceftriaxone, while confirmation test requires the use of both ceftazidime and cefotaxime, alone and in combination with clavulanic acid (Clinical and Laboratory Standards Institute (CLSI), 2014). Phenotypic detection of ESBL can be performed by other methods, such as double-disk diffusion test, three-dimensional test and other commercial tests (e.g. E-test for ESBL, Vitek ESBL test, MicroScan panels, BD Phoenix Automated Microbiology System) (Paterson and Bonomo, 2005, Rawat and Nair, 2010).

Since chromosomal or plasmid-mediated AmpC producers can be distinguished from ESBL by the resistant ability to cephamycins, cefoxitin is recommended to use for screening of AmpC (Al-Bayssari et al., 2015). However, there are presently no CLSI guidelines or other approved criteria for AmpC detection (Jacoby, 2009). Several methods have been developed for phenotypic detection of AmpC, for instance the three-dimensional test, modification of the double-disk test and E-test, which were designed to detect both ESBL and AmpC production (Al-Bayssari et al., 2015, Jacoby, 2009, Pitout et al., 2003)

Although the phenotypic confirmatory tests own high sensitivity and specificity, the phenotypic tests are not able to distinguish between different types of ESBL/AmpC β-lactamases (Jacoby, 2009, Pitout and Laupland, 2008); besides, false-negative as well as false-positive results have been observed with these tests (Paterson and Bonomo, 2005). *K. pneumoniae* or *E. coli* isolates lacking ESBL but hyper-producing SHV-1 may result in false-positive findings of confirmation tests (Paterson and Bonomo, 2005). Some of *K. pneumoniae* have been reported to harbour both ESBL and AmpC β-lactamases; the coexistence of both enzyme types in the same strains may give false-negative results for the detection of ESBL (Paterson and Bonomo, 2005). Additionally, false-negative results can also occur with both screening and confirmatory tests when lower inocula are used (Paterson and Bonomo, 2005).

1.2.3.3.2 Genotypic detection

The molecular detection methods are mainly used in reference or research laboratories (Falagas and Karageorgopoulos, 2009, Pitout and Laupland, 2008). The molecular methods identify specific genes responsible for ESBL/AmpC production even at low-level resistances (Pitout and Laupland, 2008). Moreover, the molecular methods could be done directly from clinical samples without cultivation step and therefore enables reduction of detection time (Al-Bayssari et al., 2015, Pitout and Laupland, 2008). Furthermore, genotypic identification of β-lactamases provides essential information for surveillance systems as well as prevention and control of antimicrobial resistances (Pitout and Laupland, 2008).

Several molecular methods based on amplification of ESBL/AmpC genes have been used, such as multiplex PCR and multiplex real-time PCR (Al-Bayssari et al., 2015, Monstein et al., 2007, Perez-Perez and Hanson, 2002, Roschanski et al., 2014), followed by sequencing. Sequencing is essential to distinguish between the non-ESBL parent enzymes and different variants of ESBL/AmpC (Pitout and Laupland, 2008).

1.3 Control of *Vibrio* spp. and ESBL/AmpC-producing *Enterobacteriaceae* in seafood

1.3.1 Control and decontamination of *Vibrio* spp. in seafood

Since *Vibrio* spp. are natural inhabitants in marine and estuarine environments, it is impossible to obtain seafood free of these bacteria (Su and Liu, 2007). The numbers of *Vibrio* spp. in shellfish normally increase during the warm-water months (Doyle and Buchanan, 2013); therefore, to reduce the risk of *Vibrio* infections in humans, harvest of shellfish for raw consumption should be limited from September to April in the Northern hemisphere (Su and Liu, 2007).

1.3.1.1 Relaying and depuration

Relaying and depuration are one of the common processing practices for reducing bacterial contaminants in shellfish (Su and Liu, 2007). Shellfish is transferred from polluted to unpolluted water in the relaying process for natural biological purification before harvest, followed by the depuration process which allows shellfish to purge sand and grit from the gut into clean seawater (Su and Liu, 2007). These processes usually decrease the levels of microbial contaminants in shellfish; however, several studies revealed that depuration with clean seawater did not significantly reduce the levels of *Vibrio* spp. in shellfish (Eyles and Davey, 1984, Herrfurth et al., 2013, Jones et al., 1992). In order to increase the efficacy in reduction of bacterial contamination in shellfish during the depuration process, seawater used in the depuration process should be treated by ozone, chlorine, iodophors or ultraviolet (UV) light (Doyle and Buchanan, 2013, Su and Liu, 2007). However, only a small reduction in the numbers of *Vibrio* spp. in shellfish (approximately 1-log reduction) was obtained after depuration in treated water (Croci et al., 2002, Ren and Su, 2006).

1.3.1.2 Thermal processes

Thermal processes involve cold storage, freezing, low temperature pasteurization (Su and Liu, 2007). To limit the growth of *Vibrio* in contaminated shellfish, shellfish harvested for raw consumption need to be cooled down to 10ºC within 10, 12 and 36 h of harvest when the average monthly maximum air temperature is ≥ 27ºC, between 19 and 27ºC and < 18ºC, respectively (Su and Liu, 2007). Cook (1994) reported that *V. vulnificus* could not multiply in oysters stored at ≤ 13ºC, but significant growth was observed in oysters stored at 18ºC and under ambient conditions. The study of Muntada-Garriga et al. (1995) revealed that high numbers of *V. parahaemolyticus* can be inactivated at freezing temperatures (-18 and -24ºC). However, *Vibrio* spp. are able to enter into the "viable but nonculturable state" (VBNC) when exposed to temperatures below 10ºC (Doyle and Buchanan, 2013), this might lead to difficulties in *Vibrio* detection. Low temperature pasteurization at 50ºC for 10 to 15 min could decrease the numbers of *V. vulnificus* and *V. parahaemolyticus* in raw shell-stock oysters from > 10^5 MPN/g to non-detectable levels (Andrews et al., 2000). Besides, the US CDC recommends boiling or steaming shell-stock shellfish until the shells open and continue boiling for 3 to 5 min or

steaming for 4 to 9 min, frying shucked oysters for at least 3 min at 375ºF or baking oysters for 10 min at 450ºF (Centers for Disease Control and Prevention (CDC), 2018).

1.3.1.3 High hydrostatic pressure

High hydrostatic pressure (HHP), a non-thermal decontamination process, has been applied to inactivate spoilage and pathogenic microorganisms in a variety of food products (Bajovic et al., 2012, Baker, 2016, Murchie et al., 2005, Torres and Velazquez, 2005). HHP treatment can maintain sensory as well as nutritional values of foods and has additional advantage of shucking or opening shellfish; therefore, HHP technology has been commercially used in the food industry including shellfish (Murchie et al., 2005). Previous studies revealed that HHP could reduce *V. parahaemolyticus* and *V. vulnificus* contaminations in shellfish (Koo et al., 2006, Kural and Chen, 2008, Kural et al., 2008, Mootian et al., 2013, Ye et al., 2012). Mootian et al. (2013) reported that processing conditions of 450 MPa for 4 min and 350 MPa for 6 min reduced the initial concentration of *V. parahaemolyticus* in clams to non-detectable level, achieving > 5-log reductions. Other studies on application of HHP in oysters showed that to achieve a > 5-log reduction of *V. parahaemolyticus*, the pressure treatment needed to be ≥ 350 MPa for 2 min at 1-35ºC or ≥ 300 MPa for 2 min at 40ºC, while pressure levels of ≥ 250 MPa in a short treatment time (≤ 4 min) at -2 or 1ºC were required for the same reductions in *V. vulnificus* (Kural and Chen, 2008, Kural et al., 2008).

1.3.1.4 Irradiation

Irradiation, another non-thermal process, by which the gamma irradiation and X-rays are used to eliminate pathogenic bacteria, has become a popular alternative technology to thermal treatment (Ronholm et al., 2016). The advantage of irradiation is that the food products could be processed frozen to avoid thawing, no residues are left in food, foods can be treated in different stages (liquid, solid and semisolid), and only little changes on seafood sensory at low irradiation dose were observed (Wang et al., 2015). A greater than 6-log reduction of *V. parahaemolyticus* and *V. vulnificus* in whole oysters were achieved with a treatment dose of 5 kGy and 3 kGy X-ray, respectively (Mahmoud, 2009b, Mahmoud and Burrage, 2009). Additionally, a treatment dose of 3 kGy X-ray could also achieve more than 6-log reduction of *V. parahaemolyticus* in ready-to-eat shrimp (Mahmoud, 2009a). Besides, a 6-log reduction in the number of *V. parahaemolyticus* was obtained in oysters exposed to gamma radiation (^{60}Co) at a dose of 1 kGy; moreover, the highest irradiation dose (3 kGy) did not kill the oysters or affect their sensory attributes (Jakabi et al., 2003).

1.3.1.5 Other treatments

Adding essential oils, tea polyphenols and organic acids to seafood products has been suggested to decrease the levels of spoilage and pathogenic bacteria (Ronholm et al., 2016). *V. parahaemolyticus* is highly sensitive to 50 ppm butylated hydroxyanisole and inhibited by

0.1% sorbic acid (Doyle and Buchanan, 2013). Mahmoud (2014) found that treatments with 500, 300 and 150 mg/ml of the grape seed extract containing proanthocyanidins, citric acid and lactic acid, respectively reduced the numbers of *V. vulnificus* to below the detection level (1 log/g) from an initial artificially inoculated concentration of 6 log/g.

Probiotics have been widely used as feed additives in aquaculture to improve the health of aquatic animals and to inhibit pathogenic bacteria (Wang et al., 2015). Probiotic organisms are able to produce inhibitory compounds, such as lytic enzymes, iron-chelating compounds, antibiotics, hydrogen peroxide, organic acids and bacteriocins (Wang et al., 2015). Hwanhlem et al. (2010) reported that probiotic lactic acid bacteria isolated from Kung-Som, a naturally fermented shrimp, completely inhibited the growth of *V. parahaemolyticus* within 24 h of incubation.

Bacteriophages are viruses that are able to infect bacterial host cells, replicate and cause the lysis of host cells (Letchumanan et al., 2016). Application of bacteriophages as a potential biocontrol agent have been increasing, especially after rise of multidrug resistant bacteria (Letchumanan et al., 2016). The combination of the phage Viha10 isolated from oysters and the phage Viha8 isolated from hatchery water were suggested to use as biocontrol agent of luminous vibriosis in aquaculture (Karunasagar et al., 2007). The bacteriophages pVp-1 and VPp1 were applied to reduce the number of *V. parahaemolyticus* in raw oysters (Jun et al., 2014, Rong et al., 2014). After 72 h of the phage pVp-1 application with bath immersion, the count of *V. parahaemolyticus* in oysters decreased from 6.95-log CFU/g to 1.15-log CFU/g (Jun et al., 2014). The phage VPp1 isolated from sewage could reduce *V. parahaemolyticus* in oysters by 1.35-2.76-log CFU/g within 36 h of depuration (Rong et al., 2014).

1.3.2 Control of ESBL/AmpC-producing *Enterobacteriaceae* in seafood

Aquaculture has become a more concentrated industry with large size farms (Romero et al., 2012). However, the intensive farming system has to face growing problems with bacterial diseases, which lead to the intensive use of antimicrobials (Romero et al., 2012). The misuse or abuse of antibiotics in aquaculture results in the development of antimicrobial resistance among bacteria in aquatic animals and environment. In order to control the spread antimicrobial resistant bacteria, including ESBL/AmpC producing *Enterobacteriaceae* in aquaculture, the use of antibiotics in aquaculture should be limited by strict regulations, and the surveillance system for the use of antibiotics and antimicrobial resistance in aquaculture should be developed. In Europe, the prophylactic use of antibiotics was prohibited by the EU Veterinary Medicinal Products Directive (Watts et al., 2017). However, 90% of the world aquaculture production comes from many developing countries, which still lack regulations and enforcement on the use of antibiotics (Watts et al., 2017).

Water used in aquaculture may be contaminated with antimicrobial resistant bacteria from storm-water runoff, agricultural wastes, discharges from sewage treatment plants, or livestock manure; these could be controlled by water disinfection systems with UV application or ozone treatment (Watts et al., 2017).

In addition, alternative treatments to reduce or eliminate pathogenic bacteria in aquaculture have been used, such as vaccination, probiotics, essential oils and phage therapy (Romero et al., 2012). Vaccination, however, is only applied for prevention of infectious diseases, and available commercial products are still limited in the aquaculture field (Romero et al., 2012). Probiotics have been widely used in aquaculture as biocontrol agents, and most of them belong to lactic acid bacteria (LAB) (e.g. *Lactobacillus* and *Carnobacterium* species), *Bacillus* spp., *Aeromonas*, *Enterobacter*, *Pseudomonas*, *Shewanella*, *Lactococcus*, *Leuconostoc*, *Vibrio*, *Enterococcus* and *Saccharomyces* species (Hossain et al., 2017). These probiotics have been successfully used to prevent disease and improve digestion and growth in different aquatic animals, such as teleost fish, prawns, shrimp and bivalve molluscs (Romero et al., 2012). Although essential oils (EOs) in plants have been used as an alternative to antibiotic growth promoters to control the pathogens in the guts of livestock, swine and poultry, the use of EOs in aquaculture systems are still scarce (Romero et al., 2012). Phage therapy has been applied in both terrestrial and marine animals, as well as food and animal products to eliminate bacterial pathogens (Cooper, 2016, Doss et al., 2017). Since recent studies have demonstrated the effectiveness of bacteriophages to stop the growth and destroy multi drug resistant *Salmonella* serovars isolated from broilers (Mahmoud et al., 2018) and ESBL/AmpC-producing *E. coli* isolated from pig and turkey farms (Skaradzinska et al., 2017), there are promising applications on the use of phage therapy against antimicrobial resistant bacteria, particularly ESBL/AmpC-producing bacteria in both aquaculture and seafood products.

1.4 Objectives of the study

Since only limited data on the prevalence of *Vibrio* spp. as well as ESBL/AmpC-producing *Enterobacteriaceae* in seafood are available in Germany; besides, data on the inactivation of *Vibrio* spp. via HHP are still scarce and most of the studies only investigated HHP application in oysters and clams, this study was conducted with the following objectives:

- To determine the prevalence of *Vibrio* spp. in retail seafood in Berlin, Germany
- To determine the prevalence, to investigate the quantitative load, and to characterize ESBL/AmpC-producing *Enterobacteriaceae* in retail seafood in Berlin, Germany
- To investigate the effect of HHP on the inactivation of *Vibrio* spp. in pure culture as well as mussel homogenates

Chapter 2 Prevalence of *Vibrio* spp. in retail seafood in Berlin, Germany

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Chapter 3 Investigation of extended-spectrum and AmpC β-lactamases-producing *Enterobacteriaceae* from retail seafood in Berlin, Germany

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Chapter 4 Inactivation of *Vibrio* spp. in pure cultures and mussel homogenates using high hydrostatic pressure

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Chapter 5 Discussion

Vibrio spp. are normal inhabitants of estuarine and marine environments (Igbinosa and Okoh, 2008); therefore, the presence of these bacteria in seafood could be expected. Notably, contaminated seafood with *Vibrio* spp. might pose a risk to human health, especially since seafood products are widely consumed all over the world. In our survey, four major human pathogenic *Vibrio* spp., *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus* and *V. alginolyticus*, were investigated for the presence in various types of seafood, including shrimp, mussels, venus clams, razor shells and cockles.

The overall prevalence of *Vibrio* spp. in raw seafood in our study (55%) was higher than in studies conducted in France (34.7%) (Robert-Pillot et al., 2014) and in Mexico (44.3%) (Franco Monsreal et al., 2015). Nevertheless, a higher prevalence of *Vibrio* spp. (92%) in uncooked seafood at fresh markets and supermarkets in Bangkok, Thailand was observed (Woodring et al., 2012). Since the presence and density of *Vibrio* spp. in the aquatic environment and shellfish correlates to several parameters such as temperature, salinity, pH and nutrients (Doyle and Buchanan, 2013), the prevalence of *Vibrio* spp. in seafood could vary in different seasons and harvesting areas.

V. parahaemolyticus is well-recognized as a major cause of foodborne illness in many Asian countries and the U.S associated with consumption of raw or undercooked seafood (Su and Liu, 2007). Though only few cases of *V. parahaemolyticus* gastroenteritis have been reported in Europe (Su and Liu, 2007, Baker-Austin et al., 2010), there is a growing concern on non-cholera *Vibrio* in Europe due to global climate change and increase in global consumption of seafood (Baker-Austin et al., 2010). Our results revealed that *V. parahaemolyticus* was the predominant *Vibrio* sp. in shrimp. This result is in accordance with findings in Ecuador and Vietnam (Sperling et al., 2015, Tra et al., 2016). Among the tested bivalves, venus clams were the most commonly contaminated group with *V. parahaemolyticus* (40%, 6/15). Our results are in accordance with previous reports that clams were the main source of *V. parahaemolyticus* (Lopatek et al., 2015, Roque et al., 2009). Other studies of Ripabelli et al. (1999) in Italy as well as Lhafi and Kuhne (2007) in Germany obtained similar results, which demonstrated *V. alginolyticus* as the most frequently detected *Vibrio* sp. in bivalves. Additionally, in agreement with our study, low prevalence of *V. cholerae* and *V. vulnificus* in seafood were reported in various countries (Lhafi and Kuhne, 2007, Sperling et al., 2015, Tra et al., 2016). However, the low frequency of *V. vulnificus* in our study differs from findings of Ripabelli et al. (1999) and Robert-Pillot et al. (2014), who reported *V. vulnificus* was the second most commonly found *Vibrio* sp. in seafood/mussels with a prevalence of 17.7% and 12.6%, respectively.

Regarding the origin of seafood, our results demonstrate that positive samples originated from European, Asian and Latin American countries (Denmark, France, Germany, Ireland, Italy, The Netherlands, Bangladesh, India, Vietnam and Ecuador), where *Vibrio* spp. has already been reported in seafood (Lhafi and Kuhne, 2007, Lopatek et al., 2015, Raghunath et al., 2008, Ripabelli et al., 1999, Robert-Pillot et al., 2014, Sperling et al., 2015, Tra et al., 2016). Due to the increase of seafood consumption as well as the global trade of seafood, contaminated seafood containing *Vibrio* spp. seems to spread worldwide.

It is known that most of the *V. parahaemolyticus* isolates from the environment or seafood are non-pathogenic strains (Su and Liu, 2007). Clinical strains of *V. parahaemolyticus* harbour *tdh* and/or *trh* genes, which are recognized as the major virulence factor of *V. parahaemolyticus* (Su and Liu, 2007, Thompson et al., 2004). Similarly, only *V. cholerae* O1/O139 strains carrying the *ctx* gene can produce cholera toxin (Austin, 2010). Therefore, it is essential to detect virulence genes of both *V. parahaemolyticus* and *V. cholerae* isolates. The virulence gene detection in this study revealed that all of *V. cholerae* isolates were negative for the *ctx*A gene and none of the *V. parahaemolyticus* strains encoded *tdh*/*trh* genes. These findings correspond to other studies, which reported no *tdh*/*trh* genes in *V. parahaemolyticus* isolated from retail and farm shrimp (Sperling et al., 2015, Tra et al., 2016) or from mussels (Lhafi and Kuhne, 2007). By contrast, 8.2% and 12.2% of V. *parahaemolyticus* isolates in seafood from China harboured *tdh* and *trh* genes, respectively (Yang et al., 2017). In addition, Raghunath et al. (2008) reported that *tdh* and *trh* genes were detectable in 8.4% and 25.3% of the seafood samples originating from India, respectively. For risk analysis, only *tdh* and *trh* genes are considered markers of pathogenic strains and used to estimate the load of pathogenic strains in seafood. However, current studies in Chile (Garcia et al., 2009) and Italy (Ottaviani et al., 2012) indicated that *V. parahaemolyticus* lacking *tdh*/*trh* genes can cause acute gastroenteritis in human as well. Therefore, the detection of *tdh*/*trh*-negative *V. parahaemolyticus* isolates should not be neglected because of previous findings on pathogenic strains lacking these virulence markers.

There were several storage conditions for seafood sold at the seafood shops and supermarkets. Packed products were completely enclosed in plastic or cardboard boxes/bags and kept in fridges or freezers, while unpacked products were directly placed in ice or in open-containers then placed in ice with other types of seafood. The obtained data shows that storage conditions seemed to influence the prevalence of *Vibrio* spp. Among chilled samples, the prevalence of *Vibrio* spp. in unpacked samples was significantly higher than in packed samples ($P = 0.006$). Whereas, among packed samples, no significant difference in the prevalence of *Vibrio* spp. between chilled or frozen conditions was observed ($P = 1$). Cross-contamination during packaging process or selling at the supermarkets/seafood shops (via thawing water or handling of seafood by retailers) might occur among unpacked seafood and result in the high prevalence of *Vibrio* spp. in the unpacked samples.

ESBL/AmpC-producing *Enterobacteriaceae* have recently become an emerging public health concern because of the increasing treatment difficulties in cases of human and animal infection as well as the evidence of transmission of ESBL/AmpC-producing *Enterobacteriaceae* between animals and humans. In parallel with detection of *Vibrio* spp., the presence of ESBL/AmpC-producing *Enterobacteriaceae* in retail seafood was also investigated. Up to date, there are several methods could be applied for the detection of ESBL/AmpC production, such as the three-dimensional test, modification of double-disk diffusion test and E-test. In this study, MacConkey agar supplemented with 1 mg/l cefotaxime was used for initial screening of ESBL/AmpC-producing *Enterobacteriaceae*. Confirmation test was done by disk diffusion method with cefotaxime, cefotaxime-clavulanic acid, cetazidime, cetazidime-clavulanic acid and cefoxitin based on guidelines of the CLSI and previous studies (Clinical and Laboratory Standards Institute (CLSI), 2012, Clinical and Laboratory Standards Institute (CLSI), 2014, Sabia et al., 2012). Further analysis included detection and characterization of ESBL/AmpC β-lactamase genes.

Our study demonstrates a high prevalence of ESBL/AmpC-producing *Enterobacteriaceae* in retail seafood (21.3%). ESBL/AmpC-positive samples originated from Bangladesh, Denmark, Ecuador, France, India, Ireland, Italy, Vietnam and other countries. Moreover, the obtained results reveal that ESBL/AmpC-producing *Enterobacteriaceae* can be isolated from different types of seafood, including cockles, shrimp, mussels and venus clams. However, in contrast to *Vibrio* spp., no significant difference between the prevalence of ESBL/AmpC-producing *Enterobacteriacae* among seafood types or under different storage conditions was observed. To the best of our knowledge, this is the first report on the prevalence of ESBL/AmpC-producing *Enterobacteriaceae* in retail seafood. Currently, there is no comparable data on the prevalence of these microorganisms in seafood.

The obtained data show that among different bacterial species isolated, *K. pneumoniae* and *E. coli* were the predominant ESBL/AmpC producers. An investigation of ESBL-producing *Enterobacteriaceae* in fresh seafood in India demonstrated that *E. coli* was the predominant species, followed by *K. oxytoca* and *K. pneumoniae* (Sanjit Singh et al., 2017). Besides, our result agrees with other studies which involved testing of food samples (Abdallah et al., 2015) as well as clinical samples (Mesa et al., 2006, Qin et al., 2008). However, the result differs from other publications which reported *E. coli* and *Serratia fonticola* as the most common ESBL-producing bacteria isolated (Kola et al., 2012, Ojer-Usoz et al., 2013).

The quantitative analysis revealed that most of the positive samples contained an ESBL/AmpC-producing *Enterobacteriaceae* load of < 100 CFU/g (lower detection limit), whereas only three samples contained counts of 100 to 1,000 CFU/g. Our observation on seafood storage conditions indicates that all seafood samples were kept under chilling temperature or frozen. Most members of the *Enterobacteriaceae* grow well at 22-35ºC, while the optimal temperature for the growth of several genera such as *Yersinia*, *Hafnia*, *Xenorhabdus*

and *Photorhabdus* is between 25-28ºC (Garrity et al., 2005). Therefore, low concentrations of ESBL/AmpC-producing *Enterobacteriaceae* in samples would be expected.

It has been known that *bla*$_{\text{SHV-type-ESBL}}$ gene is mainly found in *K. pneumoniae* or *E. coli* strains (Liakopoulos et al., 2016). Additionally, the *bla*$_{\text{CTX-M}}$ gene is widely disseminated in *E. coli* and distributed among healthy and diseased animals as well as humans within the European countries (Coque et al., 2008). However, the *bla*$_{\text{TEM}}$ has been demonstrated as the dominant detected gene in *E. coli* isolated from raw meat and shellfish (Van et al., 2008). Molecular characterization show that four isolates harbouring the *bla*$_{\text{SHV-type-ESBL}}$ gene were *K. pneumoniae* (n = 2) and *E. coli* (n = 2). Furthermore, the *bla*$_{\text{DHA}}$ was the most common ESBL/AmpC gene detected in *K. pneumoniae*, whereas the *bla*$_{\text{CTX-M}}$ gene was frequently found in *E. coli*, and both genes were the most dominant ESBL/AmpC gene types among all isolates investigated. Sanjit Singh et al. (2017) also found that the *bla*$_{\text{CTX-M}}$ was the predominant ESBL gene in *Enterobacteriaceae* from seafood; however, *E. coli* and *K. pneumoniae* were the most common *Enterobacteriaceae* carrying the *bla*$_{\text{TEM}}$ gene.

Interestingly, 8.9% of the isolates harboured a combination of ESBL and AmpC genes, which was also reported in ESBL/AmpC-producing *E. coli* isolated from broiler chicken fattening farms (Laube et al., 2013). In addition, three isolates of *H. alvei* carrying the bla$_{\text{ACC-1/1b}}$ genes did not show the AmpC phenotype. Moreover, 13.3% of the isolates showed the phenotype of ESBL/AmpC but had negative results for the detection of ESBL/AmpC encoding genes. That could be explained by the limited number of tested genes in this study by real-time and multiplex PCR. Other ESBL genes such as *bla*$_{\text{PER}}$, *bla*$_{\text{VEB}}$, *bla*$_{\text{BES}}$, *bla*$_{\text{GES}}$, *bla*$_{\text{TLA}}$, *bla*$_{\text{SFO}}$ and *bla*$_{\text{IBC}}$ have been discovered, but they are rarely found among *Enterobacteriaceae* (Smet et al., 2010). Moreover, there is also the fact that some of the isolates may have possessed intrinsic resistances related to chromosome-encoded genes. Several bacterial species expressing chromosomal AmpC β-lactamases were described previously (e.g. *C. braakii*, *E. aerogenes*, *H. alvei* and *M. morganii*) (Jacoby, 2009). Girlich et al. (2000) found that *H. alvei* clinical isolates encode chromosomal *bla*$_{\text{ACC}}$ gene with low-level inducible expression of cephalosporinase showed susceptibility to extended-spectrum cephalosporins and cefoxitin.

The occurrence of ESBL genes may differ from the geographical areas (Michael et al., 2015). In European countries, ESBL isolated from animals mainly belong to the CTX-M (-1, -2, -3, -8, -9, -13, -14, -15, -24, -28, -32), the SHV (-2, -5, -12) and the TEM (-52, -106, -116) families (Coque et al., 2008). The CTX-M-1 group (CTX-M-1, CTX-M-15 and CTX-M-32), the CTX-M-9 group (CTX-M-9 and CTX-M-14) and the SHV-12 have been reported among *E. coli* isolates from healthy and sick farm animals (including poultry, swine and cattle) in Denmark, France and Italy (Bortolaia et al., 2010, Dahmen et al., 2013, Haenni et al., 2014, Hammerum et al., 2014). Moreover, ESBL production in *Enterobacteriaceae* reported among clinical isolates in these countries frequently belong to the CTX-M-1, CTX-M-9, CTX-M-14, CTX-M-15, CTX-M-32 and SHV-12 (Coque et al., 2008). In Germany, the CTX-M-1 was the

most common spread among ESBL-producing *E. coli* collected from diseased cattle, pigs and poultry, followed by CTX-M-15, CTX-M-14, TEM-52, SHV-12 and CTX-M-3 (Michael et al., 2017).

The seafood samples were collected at retail level, therefore, ESBL/AmpC-producing microorganisms in the samples may originate from other sources during handling, distribution or retail at supermarkets and seafood shops. The release of untreated sewage containing antibiotic residues and antimicrobial resistant bacteria from human and animals might result in the spread of antimicrobial resistant bacteria in the aquatic environment (Sanjit Singh et al., 2017). The spread of ESBL-producing *E. coli* and other *Enterobacteriaceae* harbouring $bla_{\text{CTX-M-1/3/14/15/27}}$ and $bla_{\text{SHV-2/12}}$ from urban households, hospitals, and slaughterhouses into wastewater have been demonstrated in previous studies (Dupouy et al., 2016, Haque et al., 2014). Furthermore, Maravic et al. (2013) found that mussels collected from polluted coasts were potential reservoirs of ESBL/AmpC-producing *Aeromonas* spp. Notably, organic fertilizers, with chicken manure as the most common, were used to increase plankton in pond water and to increase food supply for the cultured shrimp (Graslund et al., 2003). It is possible that, if the organic fertilizers contained antimicrobial resistant microorganisms, using these products would introduce and spread antimicrobial resistant bacteria to shrimp farming. Another important factor, which contributes to the high prevalence of resistant bacteria in seafood, is the misuse and/or abuse of antibiotics in aquaculture. The rapid increase of intensive aquaculture farming caused problems with bacterial diseases, which required intensive use of antibiotics (Romero et al., 2012). Antibiotics used in aquaculture for prevention and treatment of diseases differ between countries (Food and Agriculture Organization of the United Nations, 2005). The wide use of antibiotics in aquatic farms has been reported worldwide (Graslund et al., 2003, Rico and Van den Brink, 2014, Thuy et al., 2011, Tusevljak et al., 2013). In consequence, this might lead to the development and spread of antimicrobial residues and antimicrobial resistant bacteria in aquaculture products and the environment. This is not limited to ESBL/AmpC producers, but also to other antibiotic resistant bacteria, as we recently found carbapenemase producing *Enterobacteriaceae* isolated from the same set of investigated samples (Roschanski et al., 2017).

To sum up, the results from seafood investigations demonstrate high prevalences of both *Vibrio* spp. and ESBL/AmpC-producing *Enterobacteriaceae* in retail seafood in Berlin, Germany. In spite of the low concentration of ESBL/AmpC-producing *Enterobacteriaceae* in seafood, a high prevalence of these bacteria might be of concern to public health due to the potential transmission from seafood to humans via the food chain. Moreover, although the prevalence of *V. parahaemolyticus* was lower than other studies reported and none of the virulence genes were detected among all *V. parahaemolyticus* and *V. cholerae* isolates, *V. parahaemolyticus* lacking *tdh*/*trh* genes can cause acute gastroenteritis (Garcia et al., 2009, Ottaviani et al., 2012). Additionally, in this study, we did not identify the quantitative loads of *Vibrio* spp. in the samples, but the quantitative levels of *Vibrio* spp. should also be of considerable concern because the potential risk of infection rises when seafood contains high

concentrations of *Vibrio* spp. Even though thorough cooking might limit the risk of foodborne illness, potential cross-contamination during preparation or consumption of raw or undercooked seafood might pose a risk of *Vibrio* infections.

In order to reduce the level of pathogenic contamination, including *Vibrio* spp. in seafood, the application of post-harvest processing is necessary to ensure the food safety for human consumption. Among those, HHP has been applied to inactivate spoilage and pathogenic microorganisms in a variety of food products such as fruit juices, meat, meat products and shellfish (Bajovic et al., 2012, Baker, 2016, Murchie et al., 2005, Torres and Velazquez, 2005). HHP disrupts the membrane function of bacterial cells and causes cytoplasmic material leaking (Murchie et al., 2005, Rendueles et al., 2011). HHP can also denature protein, resulting in enzyme inactivation (Bajovic et al., 2012). In addition, HHP induces changes in cell structure and morphology, such as cell lengthening, contraction of the cell wall and pore formation, separation of the cell membrane from the cell wall and the condensation of nucleic material (Murchie et al., 2005, Rendueles et al., 2011). Besides, degradation of bacterial DNA and ribosome destruction in HHP-treated cells has also been observed (Murchie et al., 2005, Rendueles et al., 2011).

The study on the effect of HHP in inactivation of *Vibrio* spp. in pure culture and mussel homogenates was conducted. In this study, four *Vibrio* strains were used, including *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus*. HHP treatment of both pure culture and mussel homogenates contaminated with *Vibrio* spp. were carried out at pressure levels of 250, 350 and 450 MPa for 1 and 3 min at 25ºC. To determine the number of *Vibrio* before and after treatments, the drop plating method was used.

The obtained results showed that significant increases in reductions of *Vibrio* spp. were observed when pressure levels increased in combination with longer processing times. Since the previous findings of Cook (2003) and Ye et al. (2011) revealed that there was no significant difference in inactivation of *V. vulnificus* and *V. parahaemolyticus* in whole oysters and oyster homogenates by HHP, mussel homogenates were used instead of whole mussels in the study. Normally, the density of *Vibrio* in shellfish at harvest is less than 10^3 CFU/g, but the concentration can exceed 10^3 CFU/g during warm season (Froelich et al., 2017). However, *Vibrio* can rapidly multiply to reach the density of 10^5-10^6 cells/g under improper post-harvest conditions (Cook, 1994, Gooch et al., 2002). Therefore, to completely eliminate *Vibrio* spp. in shellfish, it is necessary to apply an adequate treatment with >5 log reduction.

After pressure processing, TCBS agar was used to determine the numbers of survivor cells in pure cultures, whereas both TSCB and TSA agars were used in trials with mussel homogenates. The results showed that the recovery of all *Vibrio* spp. on TSA agar (non-selective medium) was significantly higher than on TCBS agar (*Vibrio* selective medium), particularly at low pressure level of 250 MPa. It is known that high pressure can cause the damage of cell membranes, enzyme denaturation and changes in cell morphology (Murchie et al., 2005), thus injured cells become sensitive and might not have the ability to resuscitate and

grow on selective media within incubation times. Kural and Chen (2008) found that *V. vulnificus* treated at 300 MPa for 1 min at 21ºC recovered better on TSA agar or TSA agar overlay with TCBS agar than on TCBS agar alone. However, in recent studies, selective media were still used for enumeration of other bacteria after HHP treatment in food, e.g. *L. monocytogenes*, *C. jejuni* or *S. enterica* (Bover-Cid et al., 2017, Jackowska-Tracz and Tracz, 2015).

Although pressure ranging from 300 to 600 MPa can inactivate many vegetative bacteria, the pressure susceptibility is widely variable among microorganisms (Murchie et al., 2005). Because of the complexity of the cell membranes, Gram-negative bacteria are more susceptible to high pressure than Gram-positive bacteria (Murchie et al., 2005). As Gram-negative bacteria, *Vibrio* spp. were reported to be sensitive to pressure and can be inactivated by the pressure levels between 200-350 MPa (Cook, 2003, Kural and Chen, 2008, Kural et al., 2008, Ye et al., 2012). In our study, significant differences in the reduction level among four *Vibrio* spp. tested were observed and *V. vulnificus* was the most sensitive species to HHP in both experiments performed in pure culture and mussel homogenates. This is in agreement with the result of Ye et al. (2012) who also found that *V. vulnificus* was more sensitive to HHP than *V. parahaemolyticus*. In addition, differences in pressure resistance were also observed among strains of single *Vibrio* spp., such as *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* (Cook, 2003, Kural and Chen, 2008, Kural et al., 2008). *V. parahaemolyticus* O3:K6 was found to be more resistant to pressure than other *V. parahaemolyticus* serotypes.

Beside pressure level and treatment time, the efficacy of HHP in inactivation of microorganisms in food products can also be influenced by other parameters, such as treatment temperature and product parameters (e.g. pH, water activity and salt content) (Bajovic et al., 2012, Rendueles et al., 2011, Syed et al., 2016). Previous studies have showed that the effectiveness of HHP in inactivation of *Vibrio* can be affected by the processing temperature (Kural and Chen, 2008, Kural et al., 2008, Phuvasate and Su, 2015, Ye et al., 2012). In our study, all trials were performed at 25ºC. Kural et al. (2008) showed that *V. parahaemolyticus* under treatment of 250 MPa was most resistant to pressure at 20ºC, while temperatures above and below 20ºC could enhance pressure inactivation. The authors also found that *V. parahaemolyticus* was most resistant to pressure at 300 MPa between 1 and 20ºC, whereas temperature of ≥ 20ºC could enhance its sensitivity to pressure at 350 MPa. Therefore, the temperature of ≥ 20ºC should be applied to increase the sensitivity of *V. parahaemolyticus* to pressure. However, other studies found that combination of HHP and mild heat treat (40-50ºC) or low temperature (1.5-5ºC) could enhance the reduction of *V. parahaemolyticus* and *V. vulnificus* (Phuvasate and Su, 2015, Ye et al., 2012).

This study demonstrates the efficacy of HHP inactivating *Vibrio* spp. in both pure culture and mussel homogenates. The reduction levels were significant different among four *Vibrio* spp., and *V. vulnificus* was the most susceptible species to HHP. To achieve a > 5-log reduction in mussel homogenates, treatment at 350-450 MPa for ≥ 1 min at 25ºC for *V. alginolyticus*, *V. cholerae* and *V. vulnificus*, or 250 MPa for ≥3 min for *V. vulnificus* are

required, while pressure levels of 350 MPa for ≥ 3 min or 450 MPa for ≥ 1 min should be applied for *V. parahaemolyticus*.

In conclusion, our research highlights the hazard potential of seafood containing both *Vibrio* spp. and ESBL/AmpC-producing *Enterobacteriaceae* in Germany. Further investigations along the seafood chain should be carried out to clarify the contamination route of *Vibrio* spp. and ESBL/AmpC-producing *Enterobacteriaceae* as well as the transmission of resistance genes among these bacteria. Additionally, application of effective post-harvesting methods like HHP is recommended to reduce the contamination level of *Vibrio* spp. in seafood and finally to ensure the food safety for human consumption.

Chapter 6 Summary

Seafood is considered as an important food source which contains high nutritional values via e.g. proteins, vitamins and minerals. Therefore, seafood production and consumption has increased worldwide in recent decades. Despite the nutrient benefits, consumption of seafood containing human pathogens might pose a potential risk of foodborne illness. Pathogenic bacteria, viruses, parasites, chemicals, heavy metals and natural toxins have been found in seafood. Among different bacterial pathogens detectable in seafood, *Vibrio* spp. are the most commonly associated with human infections. Another public health hazard related to seafood is the rapid increase of antimicrobial resistance among zoonotic pathogens in aquatic population. The spread of antimicrobial resistant bacteria, especially ESBL/AmpC-producing *Enterobacteriaceae* to human through the food chain has been reported and become a major concern of public health because of increasing in the number and severity of infections, as well as the frequency of treatment failure.

In this thesis, three studies were conducted with the objectives (i) to determine the prevalence of *Vibrio* spp., (ii) to determine the prevalence, to investigate the quantitative load, and to characterize ESBL/AmpC-producing *Enterobacteriaceae* in retail seafood in Berlin, Germany, and (iii) to investigate the effect of HHP on the inactivation of *Vibrio* spp. in pure culture as well as mussel homogenates.

The seafood investigation results demonstrate a high prevalence of *Vibrio* spp. in retail seafood with 55% (95% CI: 47.2% - 62.8%), and positive samples were detected in all types of seafood investigated. *V. alginolyticus* was the most prevalent species (35.6%), followed by *V. parahaemolyticus* (27.5%), *V. cholerae* (6.3%) and *V. vulnificus* (0.6%). The storage conditions seemed to influence the prevalence of *Vibrio* spp. Among chilled samples, the prevalence of *Vibrio* spp. in unpacked samples was significantly higher than in packed samples ($P = 0.006$). Whereas, among packed samples, no significant difference in the prevalence of *Vibrio* spp. between chilled or frozen conditions was observed ($P = 1$). None of the *V. cholerae* and *V. parahaemolyticus* isolates carried virulence genes. However, this should not be neglected because of previous findings on pathogenic strains lacking these virulence markers.

In parallel with detection of *Vibrio* spp., the presence of ESBL/AmpC-producing *Enterobacteriaceae* in retail seafood was also investigated. The obtained data reveal that ESBL/AmpC-producing *Enterobacteriaceae* were detected in 21.3% of seafood samples (95% CI: 14.8% - 27.7%). Of the positive samples, 91.2% contained an ESBL/AmpC-producing *Enterobacteriaceae* load of < 100 CFU/g (lower detection limit), whereas 8.8% contained counts of 100 to 1,000 CFU/g. *K. pneumoniae* and *E. coli* were the two predominant species among the 45 isolates. β-lactamase genes were detected in 39 isolates, of which 33 isolates

carried ESBL/AmpC β-lactamase genes with the majority of isolates harbouring *bla*$_{\text{CTX-M}}$ (27.3%), *bla*$_{\text{CMY}}$ (21.2%) and *bla*$_{\text{DHA}}$ (21.2%) genes.

The obtained results from HHP experiment indicate the efficacy of HHP inactivating *Vibrio* spp. in both pure culture and mussel homogenates. Significant increases in reductions of *Vibrio* spp. were observed when pressure levels increased in combination with longer processing times. The reduction levels were significant different among the respective *Vibrio* spp., and *V. vulnificus* was the most susceptible species to HHP. To achieve a > 5-log reduction in mussel homogenates, treatment at 350-450 MPa for ≥ 1 min at 25ºC for *V. alginolyticus*, *V. cholerae* and *V. vulnificus*, or 250 MPa for ≥ 3 min for *V. vulnificus* are required, while pressure levels of 350 MPa for ≥ 3 min or 450 MPa for ≥ 1 min should be applied for *V. parahaemolyticus*.

Our research highlights the hazard potential of seafood containing both *Vibrio* spp. and ESBL/AmpC-producing *Enterobacteriaceae* in Germany. Further investigations along the seafood chain should be carried out to clarify the contamination route of *Vibrio* spp. and ESBL/AmpC-producing *Enterobacteriaceae* as well as the transmission of resistance genes among these bacteria. Additionally, application of effective post-harvesting methods like HHP is recommended to reduce the contamination level of *Vibrio* spp. in seafood and finally to ensure the food safety for human consumption.

Chapter 7 Zusammenfassung

Untersuchung von *Vibrio* und ESBL/AmpC-produzierenden *Enterobacteriaceae* in Meeresfrüchten aus dem Einzelhandel und Inaktivierung von *Vibrio* in Flüssigkulturen und Muschelhomogenaten unter Nutzung von hohem hydrostatischem Druck

Meeresfrüchte werden als wichtige Nahrungsquelle angesehen und besitzen einen hohen Gehalt an Nährstoffen, z.B. Proteine, Vitamine und Mineralien. Aus diesem Grund haben die Produktion und der Verzehr von Meeresfrüchten in den letzten Jahrzehnten weltweit zugenommen. Trotz der Nährstoffvorteile kann der Verzehr von Meeresfrüchten, welche humanpathogene Erreger enthalten, ein potenzielles Risiko für lebensmittelassoziierte Erkrankungen darstellen. Pathogene Bakterien, Viren, Parasiten, Chemikalien, Schwermetalle und natürliche Toxine wurden bisher in Meeresfrüchten nachgewiesen. Unter den verschiedenen bakteriellen Pathogenen, die in Meeresfrüchten nachweisbar sind, werden *Vibrio* spp. am häufigsten mit humanen Infektionen assoziiert. Ein weiteres Risiko für die öffentliche Gesundheit in Zusammenhang mit Meeresfrüchten besteht in der schnellen Zunahme antimikrobieller Resistenzen von Zoonoseerregern in der aquatischen Population. Über die Ausbreitung antibiotikaresistenter Bakterien auf den Menschen über die Nahrungskette, insbesondere ESBL/AmpC-produzierende *Enterobacteriaceae*, wurde bereits berichtet und ist zum Hauptanliegen der öffentlichen Gesundheit geworden, da die Anzahl und der Schweregrad von Infektionen sowie die Häufigkeit von Behandlungsversagen zugenommen haben.

In dieser Arbeit wurden drei Studien durchgeführt, mit den Zielen: (i) die Prävalenzen von *Vibrio* spp. (ii) und von ESBL/AmpC-produzierenden *Enterobacteriaceae* in Meeresfrüchten im Einzelhandel in Berlin, Deutschland zu bestimmen, sowie die quantitative Belastung zu untersuchen und die Isolate weiter zu charakterisieren, und (iii) die Wirkung von HHP auf die Inaktivierung von *Vibrio* spp. in Reinkultur sowie in Muschelhomogenisaten zu untersuchen.

Die Ergebnisse der Untersuchung von Meeresfrüchten zeigen eine hohe Prävalenz von *Vibrio* spp. in Meeresfrüchten aus dem Einzelhandel mit 55% (95% CI: 47,2% - 62,8%). Es konnten weiterhin in allen untersuchten Meeresfrüchtesorten positive Proben nachgewiesen werden. *V. alginolyticus* war die am häufigsten vorkommende Art (35,6%), gefolgt von *V. parahaemolyticus* (27,5%), *V. cholerae* (6,3%) und *V. vulnificus* (0,6%). Die Lagerungsbedingungen schienen auf die Prävalenz von *Vibrio* spp. einen Einfluss zu haben. Bei gekühlten Proben war die Prävalenz von *Vibrio* spp. in unverpackten Proben signifikant höher als in verpackten Proben (P = 0,006), wohingegen bei den verpackten Proben kein

signifikanter Unterschied in der Prävalenz von *Vibrio* spp. zwischen gekühlten oder gefrorenen Lagerungsbedingungen beobachtet werden konnte (P = 1). Keines der Isolate von *V. cholerae* und *V. parahaemolyticus* trug Virulenzgene. Dies sollte jedoch aufgrund früherer Befunde zu pathogenen Stämmen, denen diese Virulenzmarker fehlen, nicht unterschätzt werden.

Parallel zum Nachweis von *Vibrio* spp. wurde auch die Präsenz von ESBL/AmpC-produzierenden *Enterobacteriaceae* in Meeresfrüchten aus dem Einzelhandel untersucht. Hierbei konnten ESBL/AmpC-produzierende *Enterobacteriaceae* in 21,3% der Meeresfrüchteproben nachgewiesen (95% CI: 14,8% - 27,7%) werden. Von den positiven Proben enthielten 91,2% eine ESBL/AmpC-produzierende *Enterobacteriaceae*-Belastung von <100 KBE/g (untere Nachweisgrenze), während 8,8% der Proben Belastungen von 100 bis 1000 KBE/g enthielten. *K. pneumoniae* und *E. coli* waren die beiden vorherrschenden Spezies unter den 45 Isolaten. β-Lactamase-Gene wurden in 39 Isolaten nachgewiesen, von denen 33 Isolate ESBL/AmpC-β-Lactamase-Gene trugen, wobei die Mehrzahl der Isolate die Gene $bla_{\text{CTX-M}}$ (27,3%), bla_{CMY} (21,2%) und bla_{DHA} (21,2%) enthielten.

Die Ergebnisse der HHP-Experimente belegen die Wirksamkeit der Inaktivierung von *Vibrio* spp. durch HPP sowohl in Reinkultur als auch in Muschelhomogenisat. Durch Erhöhung des Druckniveaus sowie der Behandlungszeiten ließen sich signifikant höhere Reduktionen von *Vibrio* spp. beobachten. Die Reduktionen der jeweiligen *Vibrio* spp. zeigten signifikante Unterschiede, wobei *V. vulnificus* die empfindlichste Spezies für die HHP-Behandlung war. Um eine > 5-log Reduktion von Muschelhomogenaten zu erreichen, war für *V. alginolyticus*, *V. cholerae* und *V. vulnificus* eine Behandlung bei 350-450 MPa für ≥ 1 min bei 25ºC und für *V. vulnificus* 250 MPa für ≥ 3 min erforderlich. Bei der Behandlung von *V. parahaemolyticus* sollte ein Druck von 350 MPa für ≥ 3 min oder 450 MPa für ≥ 1 min für angewendet werden.

Unsere Untersuchungen unterstreicht das Gefahrenpotenzial von Meeresfrüchten in Deutschland, die sowohl *Vibrio* spp. als auch ESBL/AmpC-produzierende *Enterobacteriaceae* enthalten können. Weitere Untersuchungen entlang der Produktionskette für Meeresfrüchte sollten durchgeführt werden, um den Kontaminationsweg von *Vibrio* spp. und ESBL/AmpC-produzierenden *Enterobacteriaceae* sowie die Übertragung von Resistenzgenen unter diesen Bakterien zu klären. Zusätzlich wird die Anwendung von effektiven Post-harvest-Verfahren wie HHP empfohlen, um den Kontaminationsgrad von *Vibrio* spp. in Meeresfrüchten zu reduzieren und schließlich die Lebensmittelsicherheit zu gewährleisten.

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Vu, T. T. T., Alter, T., Braun, P. G., Dittrich, A. J. & Huehn, S. 2018. Inactivation of *Vibrio* spp. in pure cultures and mussel homogenates using high hydrostatic pressure. *Lett Appl Microbiol*. 67, 220-225.

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Vu, T. T. T., Alter, T., Roesler, U., Roschanski, N. & Huehn, S. 2018. Quantification and characterization of ESBL/AmpC-producing *Enterobacteriaceae* in retail seafood in Germany. SEAOHUN 2018 International Conference "One health academic challenges: Preparing today's workforce to combat tomorrow's infectious diseases", Hanoi, Vietnam, 12-15 Nov. 2018.

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Vu, T. T. T., Alter, T., Braun, P. G., Dittrich, A. J. & Huehn, S. 2018. Conditions for high hydrostatic pressure inactivation of *Vibrio* spp. in mussel homogenates. The 5th Food Safety and Zoonoses Symposium for Asia Pacific, Chiang Mai, Thailand, 6-7 Jul. 2018.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

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