Towards a better overview of virulence and risk management of Vibrio parahaemolyticus, a marine bacterium potentially pathogenic for humans

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Quand rien n'est certain, tout est possible

When nothing is sure, everything is possible

Margareth Drabble

Summary

Abbreviations

Amino Acid ..... aa
Buffered-Physiological Water ..... BPW
Brain Heart Infusion Salted ..... BHIS
Cytochalasin B ..... CB
Centre for Disease Control and Prevention ..... CDC
Colony Forming Unit ..... CFU
Chitin-Regulated Pilus ..... ChiRP
Cluster of Orthologous Gene ..... COG
Coding sequences ..... CDS
Diploid 2N
Direction Générale de l'Alimentation ..... DGAl
Electrolyzed Oxidizing Water ..... EOW
Filtered and sterile seawater ..... FSSW
Flow cytometry ..... FCM
Fluid Accumulation Ratio ..... FAR
Food & Drug Administration - Bacteriological Analytical Manual ..... FDA-BAM
Green Fluorescent Protein ..... GFP
Heart Infusion Salter ..... HIS
Hour Post Infection ..... HPI
Intergovernmental Panel on Climate Change ..... IPCC
Internal Positive Control ..... IPC
International Organization Standardization ..... ISO
Kanagawa Phenomenon ..... KP
Lactic Acid Bacteria ..... LAB
Lethal Dose 50 ..... LD50/LD_{50}
Luria Bertoni Salted ..... LBS
Matrix Assisted Laser Desorption Ionization - Time of Flight ..... MALDI-TOF
Man Rogosa Sharpe ..... MRS
Mannose-Sensitive Hemagglutinin ..... MSH
Most Probable Number ..... MPN
National Shellfish Sanitation Program ..... NSSP
Pacific Northwest ..... PNW
Peptone Buffered water ..... PBW
Plate enumeration ..... PE
Post-Harvest Processes ..... PHP
Seawater Surface Temperature ..... SST

Sequence Type ..... ST
Système de secretion de type III SST3
Thiosulfate Citrate Bile Saccharose..... TCBS
Thermostable-Direct Haemolysin ..... TDH
Thermostable-Direct Haemolysin (TDH)-Related haemolysin ..... TRH
Triploid..... 3N
Trypticase Soy Agar or Trypticase Soy Broth..... TSA or TSB
Type III Secretion System..... T3SS
Type VI Secretion System T6SS
Ultraviolet..... UV
Viable But Nonculturable..... VBNC
Vibrio parahaemolyticus..... *Vp*
Vibrio Pathogenicity Island number 7 ..... VPaI-7
Whole Genome Sequencing..... WGS
World Health Organization ..... WHO
Zonula Occludens Toxin ..... ZOT

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Scientific context

I. Global changes and diseases

It is increasingly accepted that global changes, characterized by the increase of greenhouse gases emission, result in numerous climatic hazards such as warming, droughts or floods, among others. According to the World Health Organization (WHO), climate changes are the greatest challenge of the 21st century, thus increase the threat to human lives and health (WHO, 2015). Indeed, climatic hazards can impact respiratory diseases (asthma associated to air pollutant exposure), allergies (increased production of pollen), cardiovascular disease (air pollutant and heatwaves), heat strokes (heatwaves), but also infectious diseases (WHO, 2015).

1. Climatic hazards and infectious diseases

Climate hazards can result in emergence of new infectious diseases, and in resurgence and expansion of existing infectious diseases (**Figure 1**). Those hazards can affect simultaneously the wide taxonomic diversity of human pathogens (viruses, bacteria, parasites…) and the transmission types (vector-, food-, waterborne…) (Mora et al., 2022). Moreover, those hazards can also increase human and pathogens proximity, strengthen pathogens (reproduction, life cycle, virulence…) and also decrease human capacity to fight these pathogens (stress, malnutrition…) (Mora et al., 2022).

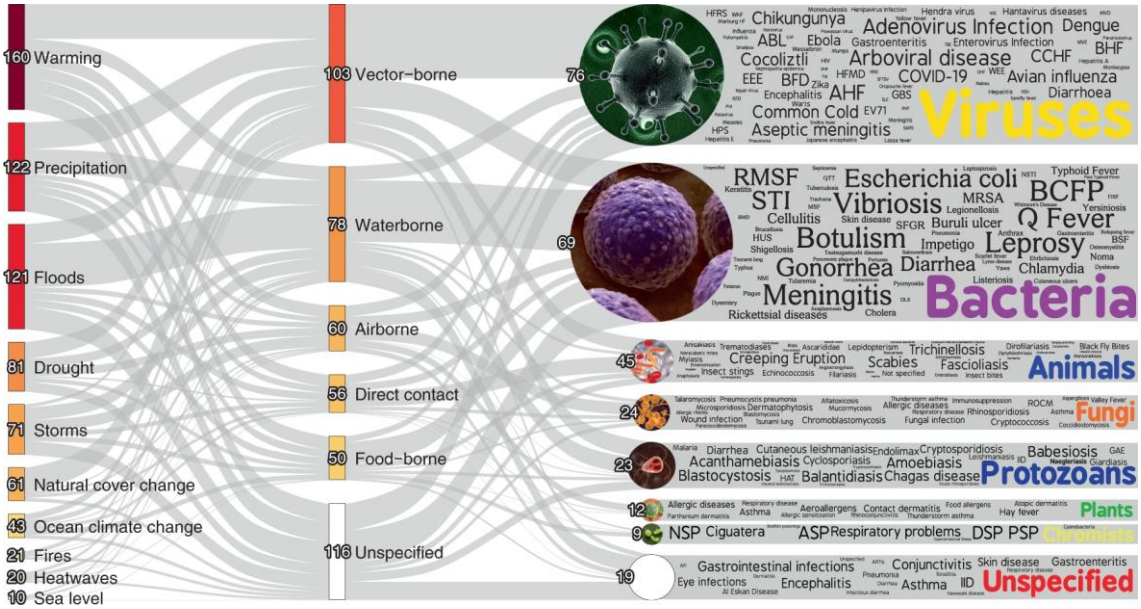


Figure 1. Pathogenic diseases aggravated by climatic hazards (Mora et al., 2022).

2. Vibriosis cases

Among those pathogenic diseases, the increase of vibriosis cases was shown to be associated to global change, in particular to ocean warming (Baker-Austin et al., 2013;

Vezzulli et al., 2015). Vibriosis are caused by gram negative bacteria of *Vibrio* genus, composed of 137 species (https://lpsn.dsmz.de/genus/vibrio) which can infect marine organisms (corals, molluscs, fishes…) but also human (de Souza Valente and Wan, 2021; Ina-Salwany et al., 2019; Thompson et al., 2004). *Vibrio* spp. are halophilic bacteria commonly found in estuarine and marine environment, and whose the proliferation is affected by the surface seawater temperature (SST) (Froelich et al., 2019). Human pathogenic *Vibrio* are principally represented by three species: *Vibrio cholerae*, *Vibrio vulnificus* and *Vibrio parahaemolyticus*. Data from USA (**Figure 2**) and France (Centre National de Référence des Vibrions et du Choléra, 2021) showed that the increase in vibriosis cases was principally due to an increase in *V. parahaemolyticus*-associated cases.

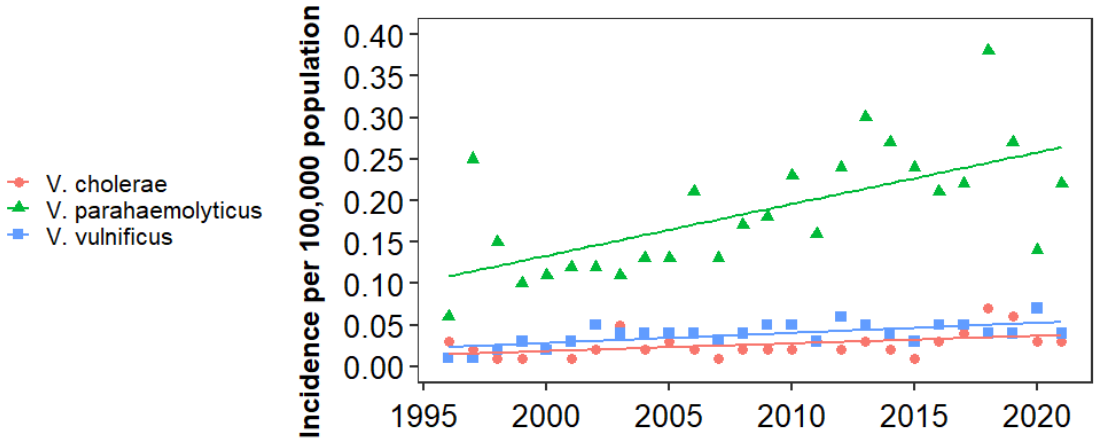


Figure 2. Incidence per 100,000 population of *V. cholerae* (pink circle), *V. parahaemolyticus* (green triangle) and *V. vulnificus* (blue square) cases (FoodNet Fast | CDC).

II. *Vibrio parahaemolyticus*: biology and ecology

Vibrio parahaemolyticus is a curved or straight bacillus of 0.5 - 1 µm wide and 1.2 - 3.5 µm long with a polar flagellum (**Figure 1B**) that allows motility. In the environment, *V. parahaemolyticus* can be found in two forms: swimmers (*i.e.* free-living form) and swarmers (*i.e.* form adapted for motility on surface or in very viscous environments) characterized by different morphologies (McCarter, 1999). The swimmers are characterized by small and opaque colonies while swarmers are larger and translucent colonies on agar plates. At the phenotypic level, the difference between the two forms is the presence of lateral flagella on surface of swarmers allowing their attachment to solid surface (**Figure 1A**). Swarmers are the principal morphologies found in biofilms (McCarter, 1999).

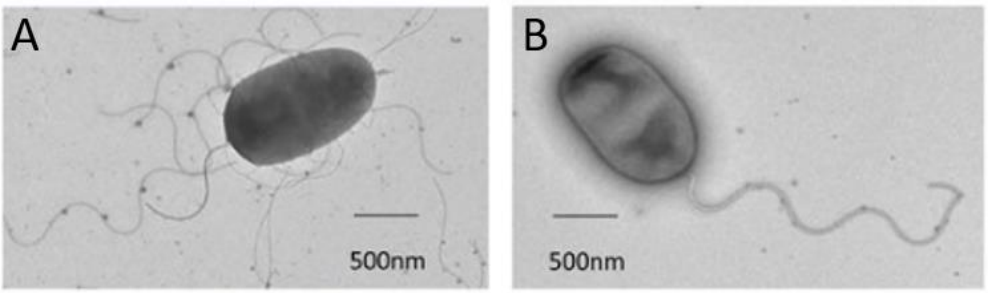


Figure 3. Swarmer (A) and swimmer (B) cells of *V. parahaemolyticus*. Expression of lateral flagella in swarmer conformation whereas only the polar flagella in swimmer conformation. Figure from (Zhang et al., 2016)

Vibrio parahaemolyticus is aero-anaerobic, able to live in both marine environment (aerobic) and human gut (anaerobic) and is heterotroph, needs exogenous organic matter to produce their constitutive elements (Joseph et al., 1982). *V. parahaemolyticus* is a mesophilic organism because of the duality marine bacteria – human pathogens, thus can grow in medium with temperatures ranging from 5°C to 43°C, salinity from 0.5% to 30% and pH from 4.8 to 11 (WHO/FAO, 2011). However, *V. parahaemolyticus* optimal growth is obtained at 35-37°C, 1.5-3% of salinity and pH of 7.8 – 8.6 (Joseph et al., 1982).

3. Ecology of *Vibrio parahaemolyticus* in environment

Vibrio parahaemolyticus is considered a common inhabitant of environmental compartment which constitute its ecological niches: seawater column (Deter et al., 2010; Johnson et al., 2012), shellfishes (Deter et al., 2010; Johnson et al., 2010; Johnson et al., 2012), sediments (Caburlotto et al., 2010; Deter et al., 2010; Johnson et al., 2012) and is associated to zooplankton (Caburlotto et al., 2010; Rehnstam-Holm et al., 2014). It appears that *V. parahaemolyticus* abundance in these different niches varies according to diverse factors.

a. Factors influencing *V. parahaemolyticus* ecology

Seawater temperature

Seawater temperature was shown to be the main environmental determinant of *V. parahaemolyticus* abundance worldwide. Total *V. parahaemolyticus* was positively correlated with temperature in seawater (Caburlotto et al., 2010; Deter et al., 2010; Johnson et al., 2010; Johnson et al., 2012; Rehnstam-Holm et al., 2014), in oysters (Johnson et al., 2010; Johnson et al., 2012) and in sediments (Johnson et al., 2012). Thus, *V. parahaemolyticus* proliferation follow a seasonal dynamics with an increase from May to July-August and decrease until October in northern hemisphere (Caburlotto et al., 2010; Johnson et al., 2010).

Salinity

Salinity was shown to be an environmental determinant of *V. parahaemolyticus* abundance and distribution, thus was positively (Johnson et al., 2010; Rehnstam-Holm et al., 2014) or negatively (Caburlotto et al., 2010) correlated with total *V. parahaemolyticus* in seawater. Same conflicted results were observed in oysters (Johnson et al., 2010) but only positive correlations were observed in sediments (Deter et al., 2010; Johnson et al., 2010). Indeed, higher salinity lead to higher sedimentation allowing *V. parahaemolyticus* to resist to low temperatures in particular during winter (Deter et al., 2010).

Other abiotic factors

Turbidity was positively correlated with total *V. parahaemolyticus* in seawater (Johnson et al., 2012; Rehnstam-Holm et al., 2014), in oysters (Deter et al., 2010; Johnson et al., 2012) and in sediments (Deter et al., 2010; Johnson et al., 2012). However, pH showed more contrasted results about its impact on total *V. parahaemolyticus* concentrations. Indeed, seawater is well-buffered, thus, its pH does not vary drastically. Though, most studies have found little to no correlation between pH and total *V. parahaemolyticus* concentration (Caburlotto et al., 2010). Dissolved organic carbon and phosphate were positively correlated with total *V. parahaemolyticus* in seawater (Rehnstam-Holm et al., 2014) and in oysters (Johnson et al., 2012) while nitrate and nitrite (Rehnstam-Holm et al., 2014) were negatively correlated with total *V. parahaemolyticus* in seawater.

Planktons

Studies of phytoplankton were often investigated by the proxy of chlorophyll a in seawater. Chlorophyll a was positively correlated with total *V. parahaemolyticus* concentrations in seawater (Caburlotto et al., 2010; Rehnstam-Holm et al., 2014), in mussels (Deter et al., 2010) and in sediments (Johnson et al., 2012). Moreover, presence of diatom was positively correlated with total *V. parahaemolyticus* concentrations in seawater (Rehnstam-Holm et al., 2014).

b. Response to stress: viable but nonculturable state

Variations of these factors could lead to stress on bacterial population, thus, resulting in viable but nonculturable (VBNC) bacteria. This state is characterized by rounded cells, incapacity to growth on agar medium and decrease of metabolic activity. VBNC *V. parahaemolyticus* can be induced at low temperatures (~ 3.5°C) under starvation conditions (Jiang and Chai, 1996) followed by regrowth, showed by cell division and recovery of culturability (Coutard et al., 2007). In the VBNC state, *V. parahaemolyticus* can

resist to thermal stress (47°C), low salinity (0% NaCl) and acidification (pH 4) (Wong and Wang, 2004).

4. Context of global changes

Various studies showed an increase in *Vibrio* spp. infection cases in years in USA (Froelich et al., 2019; Newton et al., 2012; Tack et al., 2019), around Baltic sea (Baker-Austin et al., 2013), in Australia (Harlock et al., 2022) and in *V. parahaemolyticus* infection cases in Canada (Galanis et al., 2020). These increased incidences have been associated to global changes characterized principally by an increase of SST. According to the most recent projection of the Intergovernmental Panel on Climate Change (IPCC), the different scenarios predicted an increase of SST of 1.0-1.8°C and of 3.3-5.7°C by 2081-2100 in the best and worst cases, respectively (IPCC, 2021). As indicated previously, higher temperatures promote proliferation of *Vibrio* spp. and of *V. parahaemolyticus* (Johnson et al., 2010; Johnson et al., 2012). In the epidemiological history of *V. parahaemolyticus* infection, outbreaks and epidemic expansion were often correlated with anomalies in seawater temperatures such as an unusual warm coastal seawater temperatures detected parallelly with new outbreaks or high *Vibrio* cases (Baker-Austin et al., 2010; González-Escalona et al., 2008; McLaughlin et al., 2005). Projections of future effects of climate change on temporal and spatial concentrations of *V. parahaemolyticus* are necessary to predict future microbial risks for public health. In particular, Muhling *et al.* (2017) showed that the concentration of *V. parahaemolyticus* in oysters from Chesapeake Bay could increase from 1.5 to 3 times by 2071-2100 (Muhling et al., 2017) and by 1.2 on Mexico coasts by 2100 (Ortiz-Jiménez, 2018). Moreover, they suggested that future climate change could also increase season length, spatial habitat and infection rates of *V. parahaemolyticus*. A recent study showed that *V. parahaemolyticus* concentration in oysters in Taiwan could increase up to 39-86% by 2081-2100 (Ndraha and Hsiao, 2022). All of these predictions allow a good forecasting in future public health issues concerning *V. parahaemolyticus* infections, emphasizing the need to further characterize *V. parahaemolyticus* and to develop methods to protect consumers from infection.

III. *Vibrio parahaemolyticus*: public health issues

1. Human pathogen *Vibrio*

Vibrio spp. can be characterized by their serotype according to combination of antigenic structures found on their surfaces: 13 variants of O (lipopolysaccharide) and 71 variants of K (polysaccharidic acid) antigens (ANSES, 2011). Ten *Vibrio* species are pathogens for human (**Table 1**) and are divided in two groups: choleric or non-choleric. Choleric vibriosis are caused by *Vibrio cholerae* O1 or O139 while non-choleric vibriosis are principally caused by *V. cholerae* non O1/O139, *V. parahaemolyticus* and *Vibrio vulnificus*, and in rarer cases by six other *Vibrio* species (**Table 1**) (Quilici and Robert-Pillot, 2011).

Table 1. Human pathogenic species of *Vibrio* genus

Species frequently isolated	*Vibrio cholerae* *Vibrio parahaemolyticus* *Vibrio vulnificus* *Vibrio alginolyticus*
Species rarely isolated	*Vibrio fluvialis* *Vibrio mimicus*
Species with non-defined pathogenicity	*Vibrio metschnikovii* *Vibrio cincimatiensis* *Vibrio furnissii*

1. Transmission and symptoms

To this day, *V. parahaemolyticus* is classified as the first causal bacterial agent in seafood-gastroenteritis worldwide, in particular in Asia and USA (Chen et al., 2022; Scallan et al., 2011). Infections are in most cases the result of consumption and manipulation of raw or undercooked contaminated seafood but also wound infection. Symptoms appear between 4 and 24 h after ingestion of the contaminated food and can last up to 2 weeks. Most frequents symptoms are diarrhoea, abdominal cramps, nausea, vomiting, headache, fever and chills, and in rarer cases, sepsis (Baker-Austin et al., 2018).

2. Epidemiology and pandemic clones

a. *Vibrio parahaemolyticus* infection epidemiology

Vibrio parahaemolyticus was first identified in food poisoning outbreak in 1951 in Japan causing 272 cases and 20 deaths (Fujino et al., 1953). Starting in 1969, cases of *V. parahaemolyticus* food poisoning were recorded in USA (Molenda et al., 1972), in Alaska (McLaughlin et al., 2005) and in Canada (CDC, 2006). In Europe, only sporadic

outbreaks were caused by *V. parahaemolyticus* in Spain (Lozano-León et al., 2003) and in France (Lemoine et al., 1999). Nowadays, *V. parahaemolyticus* is a common cause of foodborne poisoning in Asia, in particular in China (Chen et al., 2022), Japan (Alam et al., 2002) and USA (Scallan et al., 2011). In France, the National Reference Laboratory at the Institut Pasteur, Paris (Centre National de Référence des Vibrions et du Choléra, CNRVC) is confirming identification of *Vibrio* species isolated from clinical cases. Their annual report showed that the number of non-cholera infections was between seven and 12 between 1995 and 2014 and increase to 69 cases in 2019 (Centre National de Référence des Vibrions et du Choléra, 2021), but this is probably underestimated due to the lack of mandatory notification.

b. O3:K6 pandemic clone *Vibrio parahaemolyticus*

In 1996, a sudden outbreak occurred in Calcutta (India) and a unique clone of *V. parahaemolyticus*, never isolated previously, was identified as serotype O3:K6 representing 50 to 80% of infections during this outbreak (Okuda et al., 1997). This serovar[1] rapidly spread to other Asiatic countries (Matsumoto et al., 2000), American continent (Velazquez-Roman et al., 2014), Africa (Ansaruzzaman et al., 2005) and Europe (Martinez-Urtaza et al., 2005), thus, becoming pandemic (**Figure 2**). Various serovariants[2] *i.e.* O4:K68, O1:K25 or O1:KUT emerged worldwide (Nair et al., 2007). In Asiatic countries, percentage of pandemic clones implicated in outbreaks rose with time (Chiou et al., 2000; Yamazaki et al., 2003), thus representing 67% of *V. parahaemolyticus* clinical isolates in China between 2007 and 2012 (Li et al., 2014). In France, only a few cases caused by O3:K6 were identified (Quilici et al., 2005).

c. Pacific northwest *Vibrio parahaemolyticus* sequence type 36

Prior to 1997, a specific serovar of *V. parahaemolyticus*, O4:K12, was associated to gastroenteritis cases recorded on USA and Mexican Atlantic coasts (Abbott et al., 1989). In 1997-1998 an important outbreak occurred on the USA and Canadian Pacific coasts associated to the serovar O4:K12 (Daniels et al., 2000). In 2012, two outbreaks occurred simultaneously on the USA and Spanish Atlantic coasts associated to the serovar O4:K12 (Martinez-Urtaza et al., 2013; Turner et al., 2013). This serovar was associated to the sequence type 36 (ST36) and was confirmed as endemic from USA and Mexican Pacific coasts (Turner et al., 2013) (**Figure 2**).

[1] Bacterial population characterized by one serotype of O and K antigens
[2] Bacterial populations with similar genotype and molecular profiles of O3:K6

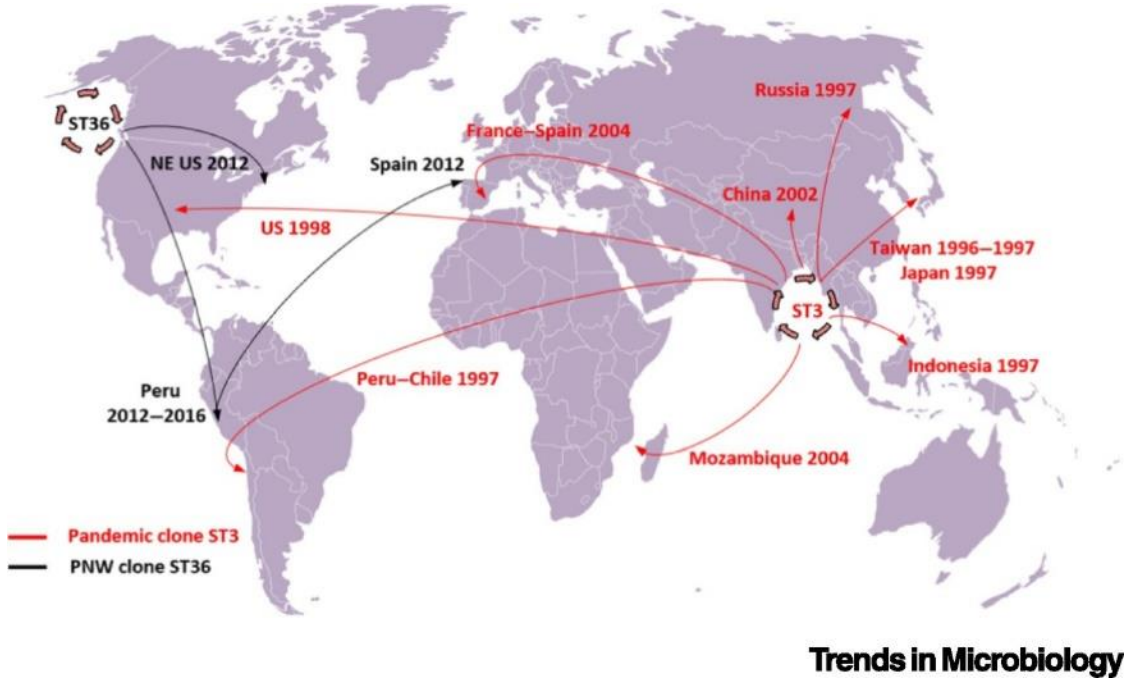


Figure 4. Geographical expansion of the Pandemic (red) and the Pacific Northwest (PNW, black) clones. Figure from Martinez-Urtaza and Baker-Austin (2020).

In France, prevalence of *V. parahaemolyticus* in environment is high (Cantet et al., 2013; Deter et al., 2010; Esteves et al., 2015) but low number of cases/outbreaks are recorded each year (Centre National de Référence des Vibrions et du Choléra, 2021). Indeed, pathogeny of *V. parahaemolyticus* in humans depend on virulence factors, thus, it is important to distinguish non-pathogenic (also qualified as non-toxigenic) from potentially pathogenic *V. parahaemolyticus* strains isolated from environment. In order to manage infection issue, anticipation and characterization of virulence and proliferation in environment are crucial research axis.

IV. *Vibrio parahaemolyticus* genome and virulence

1. Circular genome

The physical card of genome of clinical strain AQ4673 of *V. parahaemolyticus* was determined in 1999 allowing to highlight that the genome was constituted to two circular chromosomes (Yamaichi et al., 1999), a characteristic shared within the *Vibrio* genus. Whole genome sequencing (WGS) of the strain AQ4673 showed that chromosomes have sizes around 3.3 Mbp and 1.9 Mbp for chromosome 1 and 2, respectively (Yamaichi et al., 1999). WGS of the strain RIMD2210633, selected as reference strain in most studies, revealed that the number of coding sequences are around 3,000 and 1,800 for chromosome 1 and 2, respectively (Makino et al., 2003). This study allowed to confirm that chromosome 1 harbours coding sequences for essential functions such as growth,

replication and motility whereas chromosome 2 harbours mainly genes for environmental adaptation and virulence.

2. Virulence and markers

a. Pathogenicity

The bacterial pathogenicity is the capacity of a microorganism to produce disease (qualitative notion) and virulence is the degree of pathogenicity (quantitative notion). Virulence factors, molecules or mechanisms involved in virulence, can be of different natures and functions such as protease, transporters, toxins or secretion systems (Falkow, 2008).

Pathogenic and non-pathogenic (non-toxigenic) strains of *V. parahaemolyticus* were classified according to the result of the "Kanagawa Phenomenon", consisting in a culture on Wagatsuma agar [3]. Pathogenic strains are characterized as KP^+ "Kanagawa phenomenon-positive", able to induce haemolysis, while non-pathogenic as KP^- "Kanagawa phenomenon-negative", enable to induce haemolysis (Miyamoto et al., 1969). The main drivers of virulence in *V. parahaemolyticus* was identified as two haemolysins and two type III secretion systems.

b. Thermostable Direct Haemolysin (TDH) and TDH-Related Haemolysin

Sakurai *et al.* (1973) purified a haemolysin, called the Thermostable Direct Haemolysin (TDH), starting from filtrate of KP^+ *V. parahaemolyticus* culture (Sakurai et al., 1973). In 1985, strains isolated from clinical cases were identified as KP^-. The TDH-Related Haemolysin (TRH) was purified from these *V. parahaemolyticus* strains (Honda et al., 1988).

Gene and protein characteristics

The TDH protein is encoding by *tdh* gene in the form of five variants, named *tdh1* to *tdh5*, located on chromosome 2 (Baba et al., 1991; Nishibuchi and Kaper, 1990). The *tdh* gene encodes a protein composed of the mature TDH of 165 amine acids (aa) and a signal peptide in N-terminal of 24 aa. The C-terminal is important for the oligomerization of the four monomers of TDH forming the active protein (Nishibuchi and Kaper, 1985). Origin of the haemolysin thermostability comes from a phenomenon known as Arrhenius effect characterized by structural modifications of the dimer resulting in haemolysin inactivation when temperature ranges from 60 to 80°C. In this temperature interval, homodimer formed β-strands called fibrils (Fukui et al., 2005).

[3] Culture medium composed of rabbit erythrocytes allowing haemolysis

The *trh* gene in the form of two variants, *trh1* and *trh2* (Kishishita et al., 1992) which are located on chromosome 2, are similar at 54% and 68% to the *tdh* gene, respectively, and encodes a protein composed of the mature TRH (165 aa) and a signal peptide (24 aa) like the *tdh* gene. The two toxins are immunologically similar but not identical (Nishibuchi et al., 1989).

Modes of action

TDH and TRH haemolysins form pores (pore-forming toxins) of approximately 2 nm in the lipidic bilayer of erythrocytes through which water and ions (K^+, Na^+, Cs^+ or Li^+) enter in the cytoplasm. These ionic and watery imbalances are responsible for the lysis of human and other mammal cells but not horse erythrocytes (Honda et al., 1988; Zen-Yoji et al., 1971). Absence of ganglioside receptor GT1 in horse and inactivation of haemolysis activity of TDH by inhibition of GT1 suggested that GT1 is a good candidate for potential TDH receptor on erythrocytes, although haemolysin receptor is still unknown (Verma and Chattopadhyay, 2021).

TDH and TRH exhibit numerous activities: modifications of cytoplasm (Sakurai et al., 1976), changes within cytoskeleton (Fabbri et al., 1999), apoptosis induction (Naim et al., 2001), cardio-cytotoxicity (Honda et al., 1976a), cytotoxicity (Honda and Iida, 1993). Moreover, it was shown that both TDH and TRH induced a fast increase of free calcium (Ca^{2+}) in target cells (Fabbri et al., 1999; Takahashi et al., 2000). This Ca^{2+}, secondary messenger in phosphorylation pathway of kinase protein, play a role in various cellular processes such as induction of cellular death in presence of different toxins (Schanne et al., 1979).

c. Type III secretion system

The type III secretion system (T3SS) is a secretion mechanism often identified in gram negative bacteria and is composed of more than 20 proteins (Coburn et al., 2007). This system allows injection of proteins, called effectors, in a target cell. According to translocated effectors, symptoms could vary. Makino *et al.* (2003) highlighted the presence of two clusters encoding T3SS in the genome of the *V. parahaemolyticus* clinical strain RIMD2210633, *i.e.* T3SS1 and T3SS2. Proteins composing the T3SS are classified in three groups in function of their localization: structural proteins (cytoplasmic and intramembrane), translocon proteins (plasma membrane of target cell) and effector proteins (secreted) (**Appendix 1**).

Gene and protein characteristics

The first T3SS, located on chromosome 1 (VP1656-VP1702)[4], encodes the T3SS1 and possesses a gene organization like those observed in other bacterial species such as *Yersinia* spp. (Coburn et al., 2007). It seems that this cluster, with a GC content similar to the remaining genome (*i.e.* approximatively 45%), was acquired by a common ancestor of these species and was conserved across bacterial multiplication. However, it was shown recently that this cluster could vary between strains of *V. parahaemolyticus* with three profiles of T3SS1 in *V. parahaemolyticus*, called *VP*-I to *VP*-III according to genes present in variable region previously identified (**Figure 3**) (Wu et al., 2020). Presence of T3SS1 in all *V. parahaemolyticus* strains (Makino et al., 2003) suggests its involvement in avirulent interactions with marine organisms (Zhang and Orth, 2013). This mechanism, although non-required for colonization, could influence severity of infection (Ritchie et al., 2012).

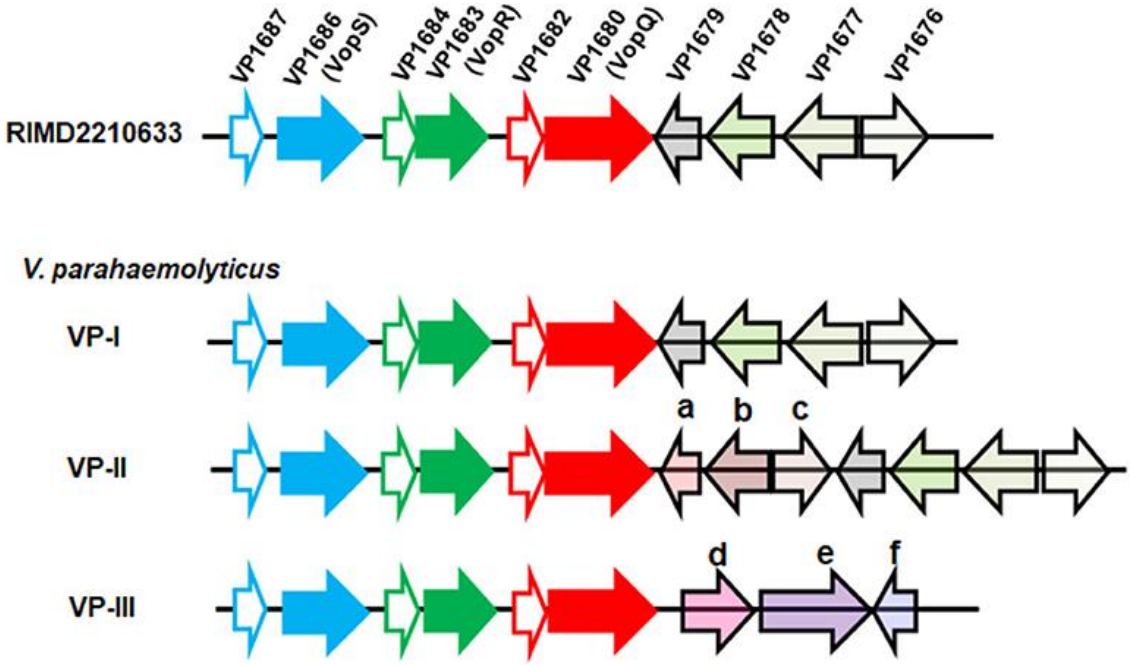


Figure 5. Genetic organisation of region encoding effectors of T3SS-1 in *V. parahaemolyticus*. Genes (arrow) are in the same colour when they are an orthologous in RIMD2210633. In contrary, genes are in another colour and indicated by a letter as followed: a: GNAT-family N-acetyltransferase, b: EamA-family transporter, c: LysR-family transcriptional regulator, d: HlyD-family secretion protein, e: MFS transporter, f: TetR/AcrR-family transcriptional regulator. Figure and legend from Wu *et al.* (2020).

The second T3SS, located on chromosome 2 (VPA1321-VPA1370), encodes the T3SS2 and possesses a different gene organization than all the T3SS described in other bacterial species (Makino et al., 2003). Moreover, study of a clinical strain *tdh*$^{-}$ *trh*$^{+}$ (TH3996)

[4] Nomenclature of genes according their accession number in the strain RIMD2210633

reveals differences in genes encoding T3SS2 in comparison to the one initially identified in RIMD2210633 (Okada et al., 2009). Distinction was made between the first cluster identified in RIMD2210633 called thereafter T3SS2α and this newly identified cluster, called T3SS2β. Studies showed that T3SS2β variant was present in all *trh*$^+$ strains, even in *tdh*$^+$ *trh*$^+$ strains (Jones et al., 2012), suggesting that *trh* gene was concomitant with the T3SS2β variant.

Effector proteins and action modes

Effectors ARNm possess in 5' a fragment that is a potential translocation signal resulting in a coupling of translation with secretion (Coburn et al., 2007). Chaperon proteins, often encoding near respective effector genes, protect effectors against early interaction with other component of the secretion system or their degradation by proteases (Coburn et al., 2007). The **Table 2** list effectors of the two T3SS systems (Broberg et al., 2011). T3SS1 is involved in general cytotoxicity (cellular lysis) while T3SS2 is involved in enterotoxicity and pathogenicity resulting in symptoms such as fluidic accumulation, inflammation and diarrhoea (Ham and Orth, 2012). Absence of one or more effectors could explain absence of virulence of some strains (Park et al., 2004b).

Table 2. Effectors of T3SS1 and T3SS2 identified in RIMD2210633. *ND*: Not determined.

	Effector	Gene	Activity	Biological activity
T3SS-1	VopQ	VP1680	Pore-forming	Autophagy, cellular lysis
	VopS	VP1686	Inhibition of Rho GTPases	Actin cytoskeleton disorganization
	ND	VPA0450	Inositol polyphosphate 5-phosphatase	Cellular lysis
	VopR	VP1683	Binds PIP2 in membrane	Chaperone effectors
T3SS-2	VopC	VPA1321	Activation of Rac1 and Cdc42	Bacterial invasion
	VopT	VPA1327	ADP-ribosylation of Ras	Cytotoxicity induction
	VopZ	VPA1336	Inhibition of TAK1 and downstream MAPK and NF-κB	Intestinal colonization and diarrhoea induction
	VopA/VopP	VPA1346	Inhibition of MAPK by acetylation of MKK	Immune response and cellular growth inhibitions
	VopV	VPA1357	Actin binding and bundling	Enterotoxicity
	VopL	VPA1370	Actin nucleation	Fibrillar stress
	ND	VPA1380	Cysteine protease	Pore-forming in host cells
	VopO	VPA1329	Polymerise actin	Actin stress fiber, remodel tight junction
	VopW	VPA1345	Translocate T3SS2 effectors	Colonization and fluid accumulation in rabbit intestine
	VgpA	VPA1360	Gate way of T3SS2 secretion	Switches secretion of T3SS2 translocon and effector
	VgpB	VPA1359		

d. Pathogenicity Island number 7

In numerous bacteria, regions called pathogenicity island regroup genes encoding potential virulence factors. These regions have often a different GC percentage and harbour a different codon utilization in comparison to the rest of the genome (Hacker and Kaper, 2000). In pandemic O3:K6 *V. parahaemolyticus*, seven pathogenicity islands called VPaI-1 to VPaI-7 (for "*Vibrio*-pathogenicity island") were characterized (Hurley et al., 2006). VPaI-1 to VPaI-6 were present in only few strains while the most studied was the VPaI-7 (39% GC) located on chromosome 2 and harbouring virulence factors previously described: TDH, TRH and T3SS2 cluster (Makino et al., 2003). VPaI-7 seemed to be integrated by homologous recombination by means of a translocator from the Tn7 superfamily (Sugiyama et al., 2008). In actual *V. parahaemolyticus* strains, Tn7 is non-functional due to lack of genes encoding biosynthesis proteins (Sugiyama et al., 2008).

e. Other virulence factors

For the past fifteen years, studies showed that between 6.6 and 28% of *V. parahaemolyticus* clinical isolates do not harbour TDH, TRH and/or T3SS2 genes (Chen et al., 2019; Jones et al., 2012). Other factors were investigated to further characterize mechanisms potentially involved in virulence of these strains.

Type VI secretion system

The Type VI secretion system (T6SS) was described for the first time in 2006, with structural and genetic similarities suggesting a parallel evolution with bacteriophages such as T4 (Records, 2011). Locus of T6SS share 13 conserved essential genes (**Appendix 2**) with a total of 15 to 20 genes (Records, 2011). In *V. parahaemolyticus*, it was shown that clinical strain RIMD2210633 harbour two clusters of T6SS: the T6SS1 (VP1386-VP1414) composed of 29 genes located on chromosome 1 and the T6SS2 (VPA1024-VPA1046) composed of 23 genes on chromosome 2 (**Appendix 2**) (Makino et al., 2003). The T6SS2, present in all *V. parahaemolyticus* strains, is involved in adhesion and host cell invasion whereas the T6SS1, present only in few *V. parahaemolyticus* strains, take part in antibacterial activities (Salomon et al., 2013).

Motility: flagella role

Motility is an important virulence factor allowing access to the target niche in the host. *Vibrio parahaemolyticus* have a polar flagella constitutively expressed and lateral flagella expressed only in stress condition such as environmental changes (McCarter, 1999). Polar flagella allow swimming in liquid media whereas lateral flagella allow swarming on solid surfaces. Flagella is composed of three structural entities: a filament, a hook and

a basal body, which are assembled in hierarchical manner starting from intracellular to extracellular structures (Chaban et al., 2015). Genes encoding the two structures were identified in RIMD2210633: 60 genes organised in five clusters on chromosome 1 for the polar flagellum constitutively expressed and essential for swimming motility (Kim and McCarter, 2000) and 38 genes in two clusters on chromosome 2 for lateral flagella whose expression is induced in viscous media or on surface, allowing swarming motility (Stewart and McCarter, 2003) (**Figure 5**).

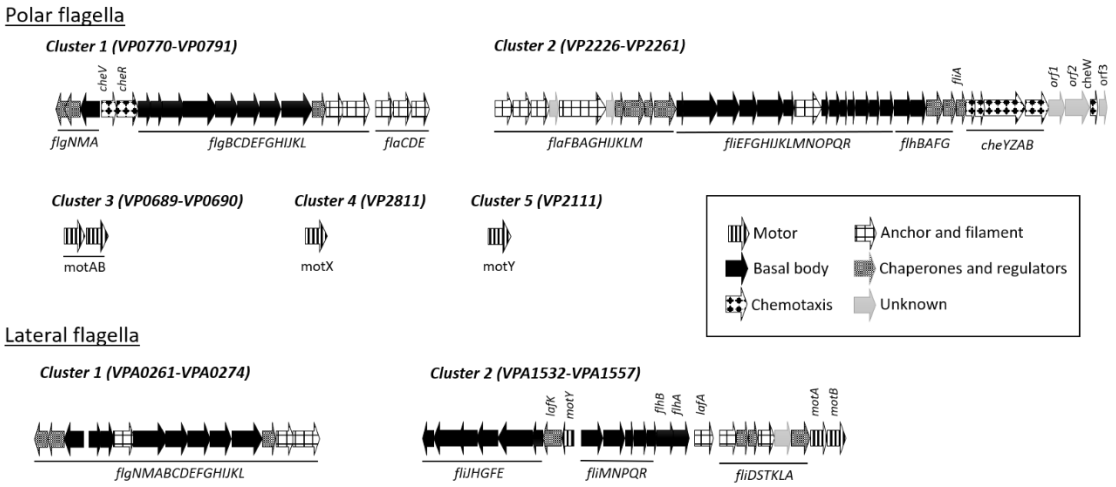


Figure 6. Genetic organization of polar and lateral flagella clusters in RIMD2210633. Figure based on (Kim and McCarter, 2000; Stewart and McCarter, 2003)

Adhesion and colonization of host cells

In order to colonize host tissues, bacteria need to adhere to cells. Pili such as type IV pilus allow adhesion via proteins present on cell surfaces. In *V. parahaemolyticus*, two type IV pili were identified: the chitin-regulated pilus (ChiRP, VP2523-VP2526) and the Mannose-sensitive hemagglutinin (MSH, VP2693-VP2707) represented in **Annexe 3** (Marsh and Taylor, 1999). Both are involved in attachment to chitin and biofilm formation (Shime-Hattori et al., 2006).

Polysaccharides

Extracellular surface-polysaccharides are essential components of biofilm matrix. In *V. parahaemolyticus*, two polysaccharides are known: the capsular polysaccharide (CPS) encoding by *cps* locus (VP0190-VP0237) (Chen et al., 2010) and the lipopolysaccharide (LPS) presumably encoding by *lps* locus (VPA1402-VPA1412) (Okura et al., 2008). These two polysaccharides are used for serotyping with the K-typing with CPS and O-typing with LPS. K antigen CPS may play an important role in adherence of *V. parahaemolyticus* to enteric cells (Hsieh et al., 2003) while the O antigen LPS was poorly studied. Recently, the polysaccharide biosynthesis clusters *scv* (VPA1458-VPA1469 and VPA1473-

VPA1476) locus, implicated in biofilm formation, was identified in *V. parahaemolyticus* (Liu et al., 2022).

Iron Acquisition

Iron is essential in cellular composition, intermediary and secondary metabolisms, and in enzymatic activity as a cofactor. Microorganisms secrete proteins called siderophores able to capture and transport iron from surrounding environment to bacteria. Two types of siderophores are distinguished: endogenous siderophores that are recognized by bacteria which produced them, and xenosiderophores that are recognized by bacteria which are not able to produce them but that possess the corresponding receptor. *Vibrio parahaemolyticus* encoded one endogenous siderophore (vibrioferrine) and receptors for three exogenous siderophores (aerobactin, ferrichrome and enterobactine) (**Table 3**) (León-Sicairos et al., 2015). Iron has a signalling function on gene expression involved in colonization and virulence (Gode-Potratz et al., 2010).

Table 3. List of iron acquisition mechanisms identified in *V. parahaemolyticus*.

Receptor	Ligand	Transporter	TonB
PvuA1	Fe^{3+}-Vibrioferrine	PvuBCDE	TonB2
PvuA2			TonB1-B2
IutA	Fe^{3+}-Aerobactin	*ND*	TonB2
FhuA	Ferrichrome	FhuBCD	TonB-B2
VctA/IrgA	Fe^{3+}-Enterobactine	VctPGCD	TonB1-B2
IrgA			TonB1-B2
PeuA			TonB2

ND: not determined

Factors involved in virulence are more or less known but their presence do not necessarily mean expression. Use of *in vitro* and *in vivo* models is essential to evaluate virulence expression and determine virulence phenotypes. These models are also useful for confirming the role of newly identified genes in virulence. Each model can bring different information about virulence phenotype of a strain.

V. Virulence expression of *Vibrio parahaemolyticus*

Study of human pathogens and understanding of mechanisms involved in pathogenicity need use of models. These models, *in vitro* or *in vivo*, allow an accurate characterization of virulence phenotype of bacterial strains.

1. *In vitro* cell lines

Cell lines are cultures of animal cells than can propagate repeatedly and can be useful for examination of alterations in cell structure and biology resulting from infection by a bacterial pathogen. Various cell lines were used to investigate roles of haemolysins and T3SS. The majority of cell lines used with *V. parahaemolyticus* derived from human intestinal epithelium: HCT 116 (Bhattacharjee et al., 2005), Intestine 407 (Tang et al., 1995), Caco-2 (Kodama et al., 2007; Raimondi et al., 2000) and HCT-8 (Kodama et al., 2007). Cell lines derived from rat intestine (IEC-6) (Fabbri et al., 1999; Raimondi et al., 2000) and human uterus epithelium (HeLa) (Ono et al., 2006) were also used. This method is reduced to the interaction bacteria-cell does not inform on global bacteria phenotype, in particular enteritis in *V. parahaemolyticus* case. In this purpose, *in vivo* models are needed. Several *in vivo* models have been used to study *V. parahaemolyticus* infection.

2. *In vivo* mice model

The laboratory mouse is frequently used as first instance for studying bacterial infection *in vivo*. Indeed, this model is low cost and easy to housing, and characterized by rapid reproduction and large litter numbers. Moreover, existence of genetically identical mouse strains allowed to study specific immune response (Sarkar and Heise, 2019). Mouse was the first *in vivo* model used for the study of *V. parahaemolyticus* virulence. This model allowed, among others, to characterize the cytotoxicity of TDH (Honda et al., 1976b) and TRH (Honda et al., 1988) using intra-peritoneally infected mice. Moreover, intraperitoneal systemic infection of mice revealed roles for T3SS1 in mice lethality (Hiyoshi et al., 2010; Piñeyro et al., 2010). More recently, VopC role in invasion of intestinal mucosa was shown using oro-gastrically infected mice (Yang et al., 2019). Intraperitoneal-infected mice were previously used to characterize virulence phenotypes of strains isolated in the Laboratoire Santé Environnement Microbiologie (Ifremer – Centre Bretagne, France) and showed that some *trh*$^{+}$ strains were avirulent in this model (Coutard, 2007). Although showing interesting results, this model is not the most accurate in *V. parahaemolyticus* virulence characterization.

3. *In vivo* infant rabbit model

Development of an *in vivo* model that allowed investigation of colonization, host response, histopathology and inflammation appeared to be essential to decipher the entire pathogenicity of *V. parahaemolyticus*. This model uses infant rabbits that are small animals that allowed non-surgical infection. Ritchie *et al.* (2012) showed that the

V. parahaemolyticus strain RIMD2210633 colonized the small intestine of infant rabbits (Ritchie et al., 2012). Decrease in permeability of epithelial barrier and tissues inflammation were observed in infected rabbits. Bacteria, suggested to be in « swarmer » form (McCarter, 1999), created micro-colonies that adhered to epithelial cells. *Vibrio parahaemolyticus* disrupted intestinal villi resulting in cavities that protected and increased nutriment access to *V. parahaemolyticus*. Tight junctions were also redistributed, thus, resulting in permeability of paracellular integrity. Finally, epithelial cells were dropped in intestinal lumen resulting in diarrhoea. This study confirmed the role of T3SS-2 but not of T3SS-1 in enterotoxicity, fluid accumulation and colonization of the small intestine. Results invalidated the role of TDH in fluid accumulation (Ritchie et al., 2012) in comparison to what was shown with ligated ileal loops (Hiyoshi et al., 2010; Park et al., 2004a). Although this model reproduced the inflammatory enteritis and watery diarrhoea, no blood in the stools was detected in infected rabbits. This model is the more accurate regarding the infection way fitting the natural infection by ingestion.

4. *In vivo Caenorhabditis elegans* nematode model

The use of non-mammal models for the study of bacterial pathogens allows a broad screening at low coasts. Nematode *Caenorhabditis elegans* appeared to be a suitable model to study the mechanisms of microbial pathogenicity and innate immunity (Sifri et al., 2005). In laboratory, experiments with *C. elegans* to study bacterial pathogenicity are simplified by the fact that infection is performed by replacement of the food source, *Escherichia coli* OP50, by the pathogenic bacteria. Colonization of *C. elegans* by a non-toxigenic strains of *V. parahaemolyticus* was performed (Durai et al., 2011). In this study, it was shown that *V. parahaemolyticus* colonized nematode intestine resulting in the death after 12 h. However, precise mechanisms involved in this phenotype need to be further described to expect the use of this *in vivo* model to assess *V. parahaemolyticus* pathogenicity.

5. *In vivo Galleria mellonella* larvae model

Another non-mammal *in vivo* model, the larvae of *Galleria mellonella*, a wax moth, was shown to be sensitive to fungi and bacteria (Tsai et al., 2016). *Galleria mellonella* was used to investigate virulence of pathogenic (*tdh*$^+$ and/or *trh*$^+$ and T3SS2$^+$) and non-toxigenic strains (*trh*$^-$, *tdh*$^-$, *T3SS2*$^-$) of *V. parahaemolyticus* (Wagley et al., 2018). These authors showed that *G. mellonella* was sensitive to pathogenic and to non-toxigenic strains of *V. parahaemolyticus*. Moreover, *V. parahaemolyticus* strains deleted of *mutT* gene, encoding

a Nudix hydrolase[5], were not able to kill larvae, suggesting involvement of this gene in pathogenicity of *V. parahaemolyticus* in this model (Wagley et al., 2018).

Characterization of factors involved in virulence of *V. parahaemolyticus* could be useful for a more accurate detection of potentially pathogenic strains in environment. Indeed, the French Food General Administration bases the compliance control of seafood and shellfishes for the presence of *V. parahaemolyticus* on detection of *tdh* and/or *trh* genes (absence in 25 g of tissues and shell fluid). When batches of seafood or shellfishes are not in compliance (*i.e.* detection of *tdh* and/or *trh*), they are removed from the market (DGAl, 2019), resulting in economical loss for farmers/fishermen. Although prevalence of *V. parahaemolyticus* differs among countries and marine organisms, according to the meta-analysis of Odeyemi (2016)[6], *V. parahaemolyticus* was isolated in 63% of oysters followed by 53% and 28% of clam and mussel/scallop, respectively (Odeyemi, 2016).

VI. Pacific oyster *Crassostrea gigas*

Cupped oysters, *Crassostrea* spp., are the marine organism identified as most frequently involved in *V. parahaemolyticus* infection cases in humans. The two species the most studied are *Crassostrea gigas* and *Crassostrea virginica*. *Crassostrea gigas* was introduced in France in 70's after the collapse of *Crassostrea angulata* and *Ostrea edulis* production due to viral and parasitic infections.

1. Oyster production in numbers

Due to their flavour and nutritional value, oysters are one of the most popular seafood products in the world. In 2019, the global production of oysters exceeded 6 million metrics (FAO, 2021), represented at 85% by China, thus making it the top oyster-producing country. The Republic of Korea, US, Japan, France, Philippines, Thailand, Taiwan, Canada and Ireland were the other countries in the top 10 of oyster production in 2019 (**Table 4**). French production represented 77% of the European production of oysters, with more than 85.000 tonnes (5th country worldwide) for more than 4 million USD (2nd country worldwide).

[5] Superfamily of hydrolytic enzymes able to cleave nucleoside diphosphates (NDP) linked to any moiety into nucleoside monophosphate (NMP).
[6] This meta-analysis included 48 studies published between 2003 and 2015 from 24 countries

Table 4. Top 10 countries producing oysters. Statistics of oyster production in quantity (tonnes, t) and values (USD 1000). (FAO, 2021)

COUNTRY	TONNES (t)	VALUES (USD 1000)
China	5,225,595 (85.5%)	5,627,966 (78,6%)
Republic of Korea	326,190 (5.3%)	196,326 (2.7%)
US	174,570 (2.9%)	215,949 (3%)
Japan	162,100 (2.7%)	286,007 (4%)
France	85,947 (1.4%)	445,838 (6.2%)
Philippines	36,194 (0.6%)	13,626 (0.2%)
Thailand	27,300 (0.4%)	69,889 (1.0%)
Taiwan	19,332 (0.3%)	117,091 (1.0%)
Canada	14,903 (0.2%)	40,879 (0.6%)
Ireland	10,716 (0.2%)	51,975 (0.7%)

2. Oyster anatomy and biology

a. Anatomy

Cupped oyster, *Crassostrea* spp., is composed of a soft body inside a mineral shell (**Figure 7**). This shell is constituted of two asymmetrical valves linked together by a hinge ligament that can be opened. Gills play a role in nutrient intake by creating water flow and transport particles to the mouth. An initial sorting was performed along gills with discarding of large particles as pseudo-faeces, followed by a second sorting at the mouth level. Remaining particles are transported to the stomach where they are shredded by a mechanical (crystalline style) and an enzymatic processes (diastases). This nutritive shred is transported to the digestive gland, and digested intracellularly. Debris of this digestion are transported to the intestine and are discarded as faeces. Gills are also responsible for capture of dissolved O_2 that is transported in circulatory apparatus allowing oxygenation of all the oyster tissues.

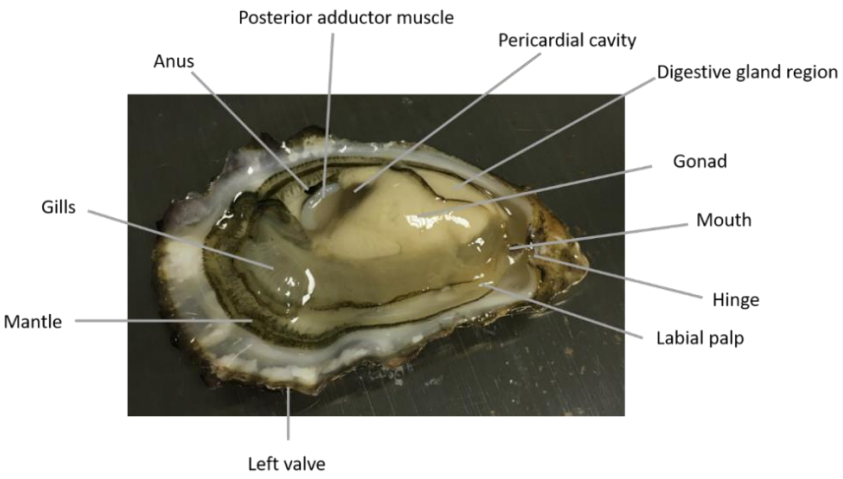


Figure 7. Graphic of left valve of diploid *Crassostrea gigas* oysters

b. Immune system

Oysters exhibit an efficient innate immune system mainly ensured by circulating cells called haemocytes. These cells circulate through an open system, called haemolymph, allowing haemocyte circulation in all the tissues. Haemocytes are able to phagocytosis and to cytotoxic reactions such as respiratory burst (Goedken and De Guise, 2004). Recognition of microorganisms can be performed by a direct (binding of a receptor at the haemocytes surface to surface components) or by an indirect (serum component as bridge between microorganism and haemocytes) manner.

Whereas adaptative immune system does not exist in oysters, an innate immune memory was described and called "immune priming". This phenomenon is explained by a primary stimulation to a specific pathogen leading to an increased immune response during a second exposure to this pathogen. In case of bacterial pathogen, such as *Vibrio splendidus*, the priming was characterized by an increase of total haemocytes count and an enhanced phagocytic rate (Zhang et al., 2014). For viral pathogen, such as Ostreid herpes virus (OsHV-1), the priming was characterized by an increase in immune gene expression (Lafont et al., 2020).

c. Oyster-associated microbiota

Host-associated microbiota play key role in host health and physiology. It was shown that seagrass microbiota can detoxify sediments to improve the plant growth (Crump et al., 2018). Moreover, microbiota was shown to be involved in regulation of intestinal absorption and fatty acids metabolism in the zebrafish (Semova et al., 2012). Concerning oyster-associated microbiota, whereas their roles in bivalve health just starting to be investigated (Paillard et al., 2022), it was shown that communities can differ in function of tissues (Lokmer et al., 2016a), environment (Dupont et al., 2020; Lokmer and Wegner, 2015) or pathogen presence (Lokmer and Wegner, 2015).

Some phyla were frequently identified in oyster microbiota such as Firmicutes (in particular *Mollicutes* class) in *Crassostrea gasar* and *Crassostrea arhizophorae* (Horodesky et al., 2020), in *C. virginica* (King et al., 2012; Pimentel et al., 2021) and in *C. gigas* (Wegner et al., 2013; Yang et al., 2022). Proteobacteria were also frequently identified in oyster microbiota of *C. virginica* (King et al., 2012; Pimentel et al., 2021) and *C. gigas* (Fernandez-Piquer et al., 2012; Wegner et al., 2013; Yang et al., 2022).

d. Reproduction

Sex-determination of cupped oysters induced a lot of contradictory studies with two paradigms: either oysters are sequential hermaphrodites[7] or oysters are protandrous hermaphrodites[8]. A recent study monitored individual *C. gigas* oysters from two cohorts over the six first-years of their lifespan and sexed them each year (Broquard et al., 2020). From this study, it appeared that *C. gigas* reproduction could only involve sequential hermaphrodites and some rare simultaneous hermaphrodites[9]. According to this study, 59 to 67% of oysters are females during the first year of their lifespan, and that 53 to 66% of oysters did not change sex between the first and the second year. This percentage increased with years and reached approx. 90% after the fifth year, suggesting a decrease of sex change with the oyster age (Broquard et al., 2020). During the reproduction, gametes are expulsed in environment and fecundation happen immediately when an ovule meet a spermatozoid. The embryo follows a classical development cycle (morula, blastula and gastrula), then form trochophore larva and pelagic veliger larva. These larval forms allow dissemination of the species in environment. After this stage, the pediveliger larva develops a foot that allow adhesion to a substrate, signal for metamorphosis into spat followed by adult stage.

Mature oyster life cycle is composed of three stages (in northern hemisphere): an inactive stage from September to March, a gametogenesis stage between March and August, and a spawning stage from August to September. During gametogenesis, commercialization of diploid oysters decrease because consumers do not appreciate gravid oysters (Allen and Downing, 1991), that is why work was performed to produce triploid oysters.

3. Triploid oysters: economical and biological progress

To overcome the decrease in diploid oyster commercialization during summer, triploid oysters are commonly raised in several countries such as USA, France, Australia and New Zealand. To this day, almost all the French production from hatcheries is triploid *C. gigas* oysters.

a. Triploidy induction

Two approaches were developed to induce triploidy in oysters: inhibition of polar body release during meiosis I or II after fertilization, or crossing diploid and tetraploid oysters.

[7] Oysters sex change at some point during their lifespan
[8] Oysters born males and become female
[9] One oyster has the two sexual organs and can produce both gamete types

Triploid oysters were originally developed in USA using cytochalasin B (CB) (Stanley et al., 1981), which is a chemical product potentially toxic for the operator. Other methods were investigated with relative low triploidy yield (Gérard et al., 1999; Nell, 2002). The only method allowing 100% triploidy is the crossing of tetraploid males with diploid females, or tetraploid females with diploid males, which only depend on availability of tetraploid breeding stocks (Guo et al., 1996). Nowadays, in France, triploid oysters are only produced using this method (Dégremont et al., 2019).

b. Triploidy advantages and disadvantages

Gonadal development in triploid oyster is limited and delayed but not absent in comparison to diploid oysters (Allen and Downing, 1990). This limited gametogenesis allows commercialization of triploid oysters during summer when diploid oysters are gravid (Allen and Downing, 1991). Moreover, it was shown that triploid oysters can reallocate the energy, initially allocated to gametogenesis, to somatic processes such as cellular process and growth, resulting in faster growth of triploid oysters (Allen and Downing, 1986). Production time of triploid oysters (*i.e.* reaching the commercial size) is shorter, thus limiting the time during which oysters might be exposed to disease, compared to diploid oysters. Moreover, it was suggested that triploidy could confer higher resistance for *Perkinsus marinus* in *C. virginica* (Dégremont et al., 2012) and for *Bonamia roughleyi* in *Saccostrea glomerata* (Hand et al., 1998). However, studies showed lower survival rates of triploid over diploid oysters to experimental challenges with *V. aestuarianus* (Azéma et al., 2016), and to summer mortalities in the Puget Sound (Cheney et al., 2000), the Chesapeake Bay (Guévélou et al., 2019) and the northern Gulf of Mexico (Wadsworth, 2018), possibly triggered by environmental stressors such as high temperatures and low salinity. Moreover, triploid oyster seeds increase culture cost for farmers, dependent to commercial hatcheries. However very few studies were performed concerning human pathogens in diploid and triploid oysters (Jones et al., 2020; Walton et al., 2013)

In order to reduce *V. parahaemolyticus* infection risk for oyster consumers, standard protocols were developed to detect *V. parahaemolyticus* in seafood before commercialization using cultural, biochemical and molecular methods.

VII. Detection of *Vibrio parahaemolyticus* in shellfish

1. Detection methods

Presence and concentrations of *V. parahaemolyticus* in shellfish depend on detection methods, sampling time and localization. Variation in prevalence and abundance of *V. parahaemolyticus* was shown to depend on environmental factors such as seawater temperature and salinity as presented above. Prevalence and abundance of *V. parahaemolyticus* also vary according to the detection methods (Parveen et al., 2008; Parveen et al., 2020). Regulatory standard methods were developed in order to detect and/or enumerate bacteria with the Food & Drug Administration - Bacteriological Analytical Manual (FDA-BAM) (FDA-BAM, 2004) and the ISO/TS 21872-1:2017 (ISO stand for International Organization of Standardization) (ISO, 2017). Both methods require a preliminary enrichment from seafood homogenates and are culture-based detection methods associated to biochemical confirmations. Identification of *V. parahaemolyticus* can also be performed using molecular methods.

a. Most probable Number

The most probable number (MPN) is used to estimate the viable bacteria in a sample. This method is a statistical approach according to the principle of extinction dilution consisting of serial dilutions of samples until no more viable bacteria is present. In this purpose, multiple serial dilutions are inoculated into a suitable growth medium and the turbidity is used as the growth indicator. The pattern of positive replicates for growth and statistical probability tables (available here: https://standards.iso.org/iso/7218/) are used to estimate the concentration of bacteria in the original sample ($MPN.g^{-1}$ or $MPN.mL^{-1}$ according the sample type). The more replicate tubes are used, the greater and the more precise the estimation of bacterial concentration is. According to the FDA-BAM, a three-tube MPN method is accepted for enumeration of *V. parahaemolyticus* potentially pathogenic. This method is also based on specificity and accuracy of agar medium used to determine positive replicates.

b. Cultural methods

Detection and enumeration of *V. parahaemolyticus* can be performed on selective Thiosulfate Citrate Bile Saccharose (TCBS) agar plates as recommended in the standard methods FDA and ISO. This medium allows *Vibrio* growth and differentiation between *Vibrio* species according to the colour of the colony. Indeed, *V. parahaemolyticus* and *V. vulnificus* colonies are blue-green while *V. cholerae* and *V. alginolyticus* colonies are

yellow. If selective for *Vibrio* spp., this medium does not allow a reliable differentiation of *Vibrio* species (Hara-Kudo et al., 2001; Oliver, 2011). Various medium were investigated and chromogenic media such as CHROMagar *Vibrio* (Di Pinto et al., 2011) or Bio-Chrome *Vibrio* medium (Su et al., 2005) were shown to be more accurate than TCBS agar for *Vibrio* species identification. The ISO standard method (2017) advise to use TCBS and a second medium such as chromogenic media for *V. parahaemolyticus* identification.

c. Colony hybridization

The colony hybridization method involves fluorescent-labelled probes to detect and identify microbial organisms at the species or genus levels, followed by an analysis with a fluorescence microscope. Colony material was transferred onto a membrane and was then hybridized with labelled-DNA probes. For *V. parahaemolyticus* identification, probes labelled with alkaline phosphatase and digoxigenin were developed for *tlh*, *tdh* and *trh* (FDA-BAM, 2004; McCarthy et al., 2000; Nordstrom et al., 2006). However, this method lack sensibility, that is why molecular methods were developed for identification of total and potential pathogenic *V. parahaemolyticus*.

d. Molecular methods

Both the FDA-BAM and ISO standard methods recommend polymerization chain reaction (PCR) method as a final step for the identification procedure (FDA-BAM, 2004; ISO, 2017). Detection of *tlh*, *toxR* or R72H DNA sequence allow *V. parahaemolyticus* identification, while detection of *tdh* and *trh* allows identification of potentially pathogenic *V. parahaemolyticus*. Real-time PCR was developed to detect R72H DNA sequence (Robert-Pillot et al., 2010) and *toxR* gene (Messelhäusser et al., 2010). Multiplex PCR (Bej et al., 1999) as well as multiplex real-time PCR (Messelhäusser et al., 2010) were developed to detect *tlh*, *tdh* and *trh* in seafood samples at the same time. To this day, these methods are more accurate, and detection and identification of *V. parahaemolyticus* are faster.

These standard methods are used by Institutions to set regulation about *V. parahaemolyticus* contamination in shellfish.

2. Regulations for *Vibrio parahaemolyticus* contamination in oysters

Using these standard methods, regulations were implemented in Japan, Peru, Brazil and US while only recommendations were suggested in Australia, New Zealand and the United Kingdom concerning *V. parahaemolyticus* contamination in ready-to-eat seafood

(**Table 5**). Neither regulation nor recommendation are suggested by the European commission concerning *V. parahaemolyticus* contamination in ready-to-eat seafood regarding current available scientific data (European Commission, 2005). With the increase of *V. parahaemolyticus* infection cases in countries already concerned (Froelich et al., 2019) and with the appearance of *V. parahaemolyticus* infection in new countries associated to global changes (Baker-Austin et al., 2013), control of seafood contamination and surveillance of *V. parahaemolyticus* infection cases could prevent serious outbreaks in the coming years. Especially since the absence of mandatory notification could result in an underestimation of *V. parahaemolyticus* infection cases.

Table 5. Contamination limits for total *V. parahaemolyticus* level in ready-to-eat raw shellfishes in some countries.

Country	Regulation level	Reference
Canada	100/g	(CFIA, 2011)
Japan	100/g	(JETRO, 2010)
USA	30/g	(US FDA, 2022)
Peru	Undetectable for 25 g	(MINSA, 2008)
Brazil	10^3/g	(ANVISA, 2001)
Country	**Recommendation level**	**Reference**
United Kingdom	10^3/g	(UKHPA, 2009)
Australia	10^2/g	(FSANZ, 2022)
New Zealand	10^2/g	(FSANZ, 2022)

In order to satisfy these recommendations, physical, chemical and biological methods to reduce *V. parahaemolyticus* in oysters were developed.

VIII. Processes to reduce *Vibrio parahaemolyticus*

The particularity of bivalves such as oysters, which directly impact consumer security, is that they are filter-feeder organisms. By this process, contaminants present in seawater such as bacteria, virus, toxins or chemical pollutants are accumulated in oyster tissues resulting in a risk for consumer health. Thus, to protect consumers, aquaculture areas are under strict surveillance (Lee et al., 2008). Various physical, chemical or biological methods were investigated to reduce contaminants before commercialization, called post-harvest processes (PHP). In order to validate a PHP, a 3.52 log reduction of viable

bacteria and a *V. parahaemolyticus* contamination reduction to undetectable level (< 30 MPN.g^{-1}) is recommended (NSSP, 2017).

1. Traditional processes for depuration

Depuration exploits the natural capacity of bivalve filtration in order to eliminate contaminants from the tissues. This process consists to place bivalves in a recirculated system allowing them to filtrate "clean" seawater to purify their tissues. It showed good results for enterobacteria (*Escherichia coli*, *Enterococcus faecalis*) and moderate efficacies for other contaminants and even some bacteria (Love et al., 2010). This method has to be coupled to inactivation methods such as chlorination, ultraviolet (UV) light and ozone (Lee et al., 2008). However, these methods are rather inefficient concerning *V. parahaemolyticus* elimination (Croci et al., 2002; Vasconcelos and Lee, 1972), and chlorine and ozone produce components that can be harmful to oysters and/or humans (Lee et al., 2008). For these reasons, various alternative methods were investigated.

2. Alternative methods for depuration

a. Physical methods

Various physical methods were investigated for a more efficient elimination of *V. parahaemolyticus* in oysters before commercialization such as fast freezing (Shen et al., 2009), radiations (Mahmoud and Burrage, 2009), low temperature pasteurization (Andrews et al., 2000) and high hydrostatic pressure (Ye et al., 2012). However, these methods can be detrimental for oysters (Campus, 2010) or modify sensory qualities of oysters (Mudoh et al., 2014; Ye et al., 2012). Moreover, although efficient to reduce *V. parahaemolyticus* to undetectable levels, some of these methods can be difficult to accept for consumers (irradiated oysters) or provide oysters not to their taste (frozen oysters in France). Finally, these methods can be very expensive for oyster farmers.

b. Chemical methods

Some chemical methods, such as electrolyzed oxidizing water (Ren and Su, 2006) or use of antimicrobial agents such as lactic acid, chitosan and tea extract (Shirazinejad et al., 2010; Terzi and Gucukoglu, 2010; Xi et al., 2012) were investigated with promising results. Electrolyzed oxidizing water can result in oyster death and can only be applied on oysters with low *V. parahaemolyticus* levels.

c. Biological methods

Biological methods were recently investigated for oyster depuration. The first is the use of bacteriophages that are viruses which infect specific bacteria without affecting

surrounding cells. Since the approval of the FDA in 2006 for use of bacteriophage for the control of *Listeria monocytogenes* in food, studies of bacteriophages as biocontrol agent for food pathogens increased. Although the use of bacteriophages for *V. parahaemolyticus* elimination from oysters showed interesting results (Jun et al., 2014; Rong et al., 2014), it is difficult for consumers to accept seafood treated with virus. The second method, is the use of probiotics. A general definition of probiotic is "a live microbial feed supplement that is beneficial to health" (Salminen et al., 1998). Probiotics are widely accepted by people as "beneficial bacteria", thus resulting in an easy food application (FAO and WHO, 2006). Their use is appreciated in aquaculture to prevent appearance of multidrug resistance bacteria.

3. Probiotics in aquaculture

An aquatic organism has a particular relationship with its environment in contrast to terrestrial organisms. Indeed, aquatic organism microbiota is subject to environmental factors and to presence of potential pathogens while living or feeding. The general definition of probiotics was slightly modified for aquatic environment as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community" (Verschuere et al., 2000). The potential probiotics used in aquaculture include gram-positive lactic acid bacteria (LAB) such as *Lactococcus* and *Latilactobacillus*, other gram-positive bacteria such as *Bacillus*, and gram-negative bacteria such as *Aeromonas*, *Pseudomonas* and *Vibrio* (Hoseinifar et al., 2018). Probiotic benefits are improvement of growth, disease resistance, stress response and health status, as shown for the Pacific white shrimp *Litopenaeus vannamei* using *Latilactobacillus* sp. resulting in an increase of body weight, immune and digestive enzyme activities, and decrease mortalities (Zuo et al., 2019).

a. Probiotic in shellfish aquaculture

Until today, most of the studies investigating the use of probiotics in shellfish aquaculture have focused on white shrimps production (Ringø, 2020). In oyster farming, probiotics were mainly investigated for their implication in survival and growth of larvae (Campa-Córdova et al., 2011; Douillet and Langdon, 1994; Gibson et al., 1998; Karim et al., 2013; Kesarcodi-Watson et al., 2012) and less as PHP to reduce bacteria levels in oysters after depuration (Kang et al., 2018; Khouadja et al., 2017; Xi et al., 2014). Mechanisms explaining the decrease of bacterial loads are diverse and appeared to depend on probiotic species.

b. Mechanisms of action

Firstly, exclusive competition was suggested to prevent pathogens colonization by adhering to epithelium or mucus cells in the host. Moreover, some of the probiotic microorganisms are able to produce bactericidal or bacteriostatic molecules such as bacteriocins, siderophores, lysozymes, proteases or even hydrogen peroxide which target surrounding microorganisms (Teplitski et al., 2009; Verschuere et al., 2000). Finally, it was also shown that probiotics could improve immune response of host in order to increase resistance against pathogens (Zuo et al., 2019)

c. Probiotics and cupped oysters

The use of probiotics in cupped oyster production mainly focused on resistance to oyster pathogens *Vibrio* such as *V. harveyi*, *V. tubiashii* , *V. coralliilyticus* or *V. alginolyticus* (**Table 6**). Only few studies investigated the impact of bioprotective agents such as *Enterococcus faecium* or *Latilactobacillus* spp. on oyster depuration capacities to eliminate *V. parahaemolyticus* (Kang et al., 2018; Khouadja et al., 2017; Xi et al., 2014). However, these studies were not conclusive because reduction of *V. parahaemolyticus* loads were limited (less than 0.8-log reduction) compared to control conditions (without the bioprotective agent). Thus, further assays are needed to determine the parameters allowing an optimal depuration, including bioprotective agent species, bacterial concentrations, time of exposure, seawater temperature among others.

Table 6. Non-exhaustive list of probiotics used with *Crassostrea* spp. oysters. ↑: increase, ↓: decrease

Probiotic	Species	Observations	References
LAB strain NS61 *Pseudomonas aeruginosa* YC58 *Burkholderia cepacian* Y021	*Crassostrea corteziensis* (larvae)	↑ survival	(Campa-Córdova et al., 2011)
Bacillus licheniformis MAt32 *Bacillus subtilis* Mat43 *B. subtilis* GAtB1	*Crassostrea sikamea* (spat)	↑ survival ↑ growth	(Sánchez-Ortiz et al., 2020)
Pseudoalteromonas sp. D41 *Phaeobacter gallaeciensis* S4	*Crassostrea gigas* (larvae)	↑ resistance against *V. coralliilyticus*	(Kesarcodi-Watson et al., 2012)
Bacillus sp.		↓ haemocytes mortality ↑ resistance against *V. harveyi*	(Fdhila et al., 2017)
Aeromonas media A199		↑ resistance against *V. tubiashii*	(Gibson et al., 1998)
Unintentified P02-45 and P02-1			(Douillet and Langdon, 1994)
Unidentified S21		↑ resistance against *V. alginolyticus*	(Elston et al., 2008)
Phaeobacter sp. S4 *B. pumilus* RI06-95	*Crassostrea virginica* (larvae)	↑ resistance against *V. tubiashii* and *Roseovarius crassostreae*	(Karim et al., 2013)
Phaeobacter sp. S4 *B. pumilus* RI06-95		↑ resistance against *V. tubiashii*	(Karim et al., 2013)
Enterococcus faecium HL7	*C. virginica* (juvenile)	↓ establishment of *V. parahaemolyticus*	(Kang et al., 2018)
L. plantarum ATCC 8014	*C. gigas* (adult)		(Xi et al., 2014)
Latilactobacillus spp. L5, L9, L10, L11			(Khouadja et al., 2017)

IX. Thesis objectives and general methodology

In most cases and studies, virulence of *V. parahaemolyticus* was explained by haemolysins and type III secretion systems, although some clinical strains were found to lack these virulence factors. Extended characterization of factors involved in *V. parahaemolyticus* virulence is crucial in order to better identify and improve detection of potentially pathogenic *V. parahaemolyticus* in environmental samples. Moreover, *V. parahaemolyticus* proliferation and concentrations in seawater, sediment and oysters were shown to vary according to environmental abiotic parameters, while biotic parameters of oysters were rarely studied. These observations need to be further investigated in order to decrease any risk for human health. The principal objective of the thesis « Towards a better overview of virulence and risk management of *V. parahaemolyticus*, a marine bacterium potentially pathogenic for humans » was to investigate *Vibrio parahaemolyticus* virulence genes and factors influencing risk of *V. parahaemolyticus* infections associated to oyster consumption. My thesis work was organized as followed:

What are the genetic traits and virulence phenotypes of environmental and clinical *Vibrio parahaemolyticus* strains selected and sequenced for this study?

Characterization of *V. parahaemolyticus* clinical isolates lacking haemolysins and type III secretion systems (Jones et al., 2012) highlighted the necessity 1) to further identify factors involved in virulence and especially since the pathogenic potential of *V. parahaemolyticus* is currently based on detection of haemolysin genes, and 2) to evaluate virulence expression of *V. parahaemolyticus* strains presenting different gene profiles.

Thus, for this chapter, 11 *V. parahaemolyticus* strains collected by the laboratory were selected and sequenced, to determine their whole genome, and annotated to identify genes present. Genomic analyses were performed in order to further characterize the selected strains considering the general genomic organization and the presence of virulence genes, principally. Two *in vivo* models, infant rabbits and wax moth *Galleria mellonella* larvae, were used to further characterize virulence phenotypes of the selected *V. parahaemolyticus* strains.

Does life history and/or ploidy level of *Crassostrea gigas* oysters impact *Vibrio parahaemolyticus* accumulation and depuration?

Accumulation refers to the process that allows increase of contaminant concentrations in oysters, whereas depuration is a purification process during which oysters are placed in fresh seawater resulting in decrease of contaminant concentrations. Previous studies mostly investigated the influence of environmental factors on accumulation and/or depuration of oysters concerning *V. parahaemolyticus*. Here, we proposed to investigate the impact of life history and/or ploidy level of oysters on *V. parahaemolyticus* experimental accumulation and depuration.

In order to perform oyster experimentations and be able to distinguish experimental *V. parahaemolyticus* strain from indigenous *V. parahaemolyticus* in oysters, we electroporated *V. parahaemolyticus* strains with a plasmid harbouring Green Fluorescent Protein (GFP) gene (*Vp-gfp*$^+$). Experimental design for oyster experimentations and methodological developments for *Vp-gfp*$^+$ quantification and sample collections were undertaken. Quantification of *Vp-gfp*$^+$ in oysters was investigated in haemolymph and in oyster tissues by flow cytometry and plate enumeration. The *V. parahaemolyticus* IFVp201-*gfp*$^+$ strain (environmental, *tdh*$^+$ *trh*$^+$) was selected to perform experimental contamination of oysters.

To study the impact of oyster life history on *V. parahaemolyticus* accumulation and depuration in *C. gigas*, we used two groups of oysters: oysters grown in inland controlled facilities at Ifremer and oysters grown in an intertidal area (three batches in each group). Each batch was individually exposed to IFVp201-*gfp*$^+$ strain for one night and depurated for 24 h. Concentrations of IFVp201-*gfp*$^+$ in oyster haemolymph were determined by flow cytometry after the overnight accumulation and 24 h of depuration.

To study the impact of ploidy level on *V. parahaemolyticus* accumulation and depuration in *C. gigas*, three batches of diploid and three batches of triploid oysters were brought from a commercial hatchery and maintained in the intertidal environment (La Floride, Charente Maritime – France) for a few months before the experimentations. Oysters were collected once a month between May and November 2021 and were analysed for their i/ contamination in indigenous *V. parahaemolyticus* and ii/ experimental accumulation and depuration of IFVp201-*gfp*$^+$. Oyster contamination with indigenous *V. parahaemolyticus* was analysed using MPN-qPCR. Concentrations of experimental IFVp201-*gfp*$^+$ in haemolymph of oysters were determined by flow cytometry after the overnight accumulation, and 24 h and 48 h of depuration.

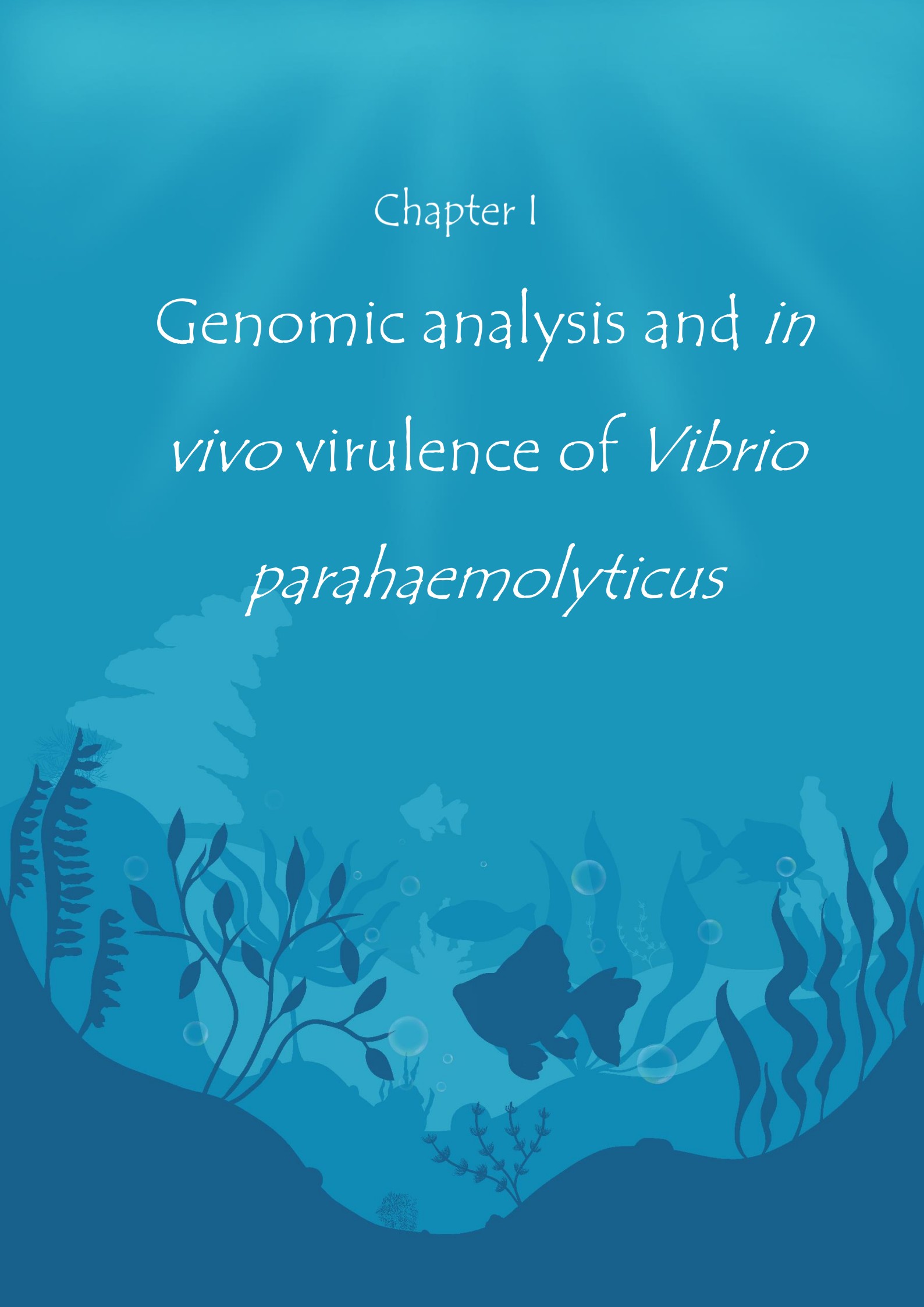
Could marine lactic acid bacteria be used to reduce *Vibrio parahaemolyticus* levels in *C. gigas* oysters?

Reduction of *V. parahaemolyticus* levels in oysters is crucial to ensure consumer health and shellfish safety but it is often limited using classical methods of depuration. Different depuration processes such as fast freezing, high hydrostatic pressures, irradiation were investigated (Ndraha et al., 2020). So far, very few studies propose biological treatments to reduce *V. parahaemolyticus* levels in oysters. Here, we proposed to investigate the impact of lactic acid bacteria (LAB) on *V. parahaemolyticus* experimental depuration in oysters.

A collection of salt and cold tolerant LAB strains isolated from seafood products was tested for their capacity to inhibit growth of the *V. parahaemolyticus* strain LMG2850^{T} *in vitro* (ViLAB project, Ifremer). Three of these LAB strains were tested for their inhibition activities against the 11 *V. parahaemolyticus* strains previously selected for the genomic and virulence analyses, and were then evaluated for their capacities to accelerate depuration of *C. gigas* experimentally-exposed to four *Vp-gfp*$^{+}$ strains. For these assays, juvenile oysters were exposed overnight to each *Vp-gfp*$^{+}$ strain individually and then exposed to one of the three LAB strains tested for 24 h.

Chapter I

Genomic analysis and *in vivo* virulence of *Vibrio parahaemolyticus*



Contexte

Vibrio parahaemolyticus est une bactérie marine responsable de gastroentérites liées à la consommation de fruits de mer crus ou insuffisamment cuits. Des facteurs tels que la TDH, la TRH et le système de sécrétion de type III (SST3), ont été identifiés comme étant impliqués dans la virulence de *V. parahaemolyticus* par l'utilisation de mutants délétés pour ces gènes chez modèles d'études *in vitro* et *in vivo* (Hiyoshi et al., 2010). Cependant, l'isolement de souches de *V. parahaemolyticus* ne possédant ni les hémolysines ni le SST3 dans des cas cliniques (Jones et al., 2012) questionne quant à la pertinence de ne prendre en compte que ces gènes en tant que prédicteurs de la virulence de *V. parahaemolyticus*.

La recherche de nouveaux facteurs de virulence de *V. parahaemolyticus* est d'intérêt afin de distinguer les souches de *V. parahaemolyticus* potentiellement pathogènes de celles non pathogènes et ainsi prévenir le risque d'infection de façon plus fiable. L'analyse de génomes ne permet pas à elle seule d'identifier des facteurs de virulence, la présence d'un gène ne signifie pas forcément qu'il est exprimé. L'infection de modèles *in vitro* et *in vivo* par une souche bactérienne permet de définir un phénotype (pathogène ou non pathogène) de cette souche. Ces modèles peuvent être étudiés afin de suivre l'apparition des symptômes, la colonisation bactérienne au sein des tissus ou même la mort des individus. Les données issues de ces modèles, couplées à des données de génomiques, peuvent permettre de mettre en évidence des gènes uniquement présents chez les souches pathogènes. Le recours à des mutants délétés pour ces gènes permet de vérifier leur implication dans la virulence de la souche.

Dans ce chapitre, le séquençage des génomes complets de 11 souches de *V. parahaemolyticus* (10 environnementales et une clinique) a permis d'étudier la présence de facteurs de virulence connus chez cette espèce (hémolysines et système de sécrétion de type III) ainsi que des facteurs plus généraux. Ce séquençage a également permis de prédire la présence de prophages et d'ilots de pathogénicité décrits précédemment. Deux souches, dont l'une (IFVp201) présentant des caractéristiques génomiques particulières, ont été testées sur le modèle lapin. Toutes les souches (11) ont été testées sur un modèle chenille *Galleria mellonella* qui a pour objectif de déterminer s'il pouvait être utilisé en première attention comme modèle simple et à bas coût permettant le criblage d'un large spectre de souches de *V. parahaemolyticus*. Le protocole utilisé pour le modèle lapin permet une voie d'infection par ingestion comme chez l'Homme à la différence de la chenille (injection).

Publication I

Titre de l'article	**"Comparative genomics and *in vivo* virulence analysis of environmental *Vibrio parahaemolyticus*"**
Statut de l'article	Draft en cours
Objectif de l'étude	Comparaison génomique de souches de *V. parahaemolyticus* et étude *in vivo* de l'expression de la virulence chez deux modèles animaux : les larves de chenille *Galleria mellonella* et le lapin
Résultats principaux	Le séquençage et l'analyse comparative des 11 génomes de *V. parahaemolyticus* révèlent des combinaisons différentes de gènes associés à la virulence. Une souche *tdh*$^+$ *trh*$^+$ a induit rapidement les symptômes chez les lapins alors qu'une souche *tdh*$^+$ était non virulente. Bien que le modèle des larves *G. mellonella* a permis de bien discriminer les souches de *V. parahaemolyticus* en fonction de leur potentiel de virulence (Kéomurdjian, 2015), les phénotypes observés chez ce modèle étaient l'opposé de ceux observés chez les lapins (Hervio Heath et al., 2016). Les infections en utilisant les mutants ont suggéré que le système de sécrétion de type III numéro 1 participait aux mortalités larvaires.
Conclusions	Les souches environnementales de *V. parahaemolyticus* sont génétiquement différentes et sont capables de provoquer la maladie. La présence des hémolysines ne signifie pas forcément que la souche est virulente.

TITLE: Comparative genomics and *in vivo* virulence analysis of environmental *Vibrio parahaemolyticus*

RUNNING TITLE: Genomic content and virulence of environmental *V. parahaemolyticus*

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Abstract:

Vibrio parahaemolyticus is a leading cause of gastroenteritis associated with seafood comsumption worldwide. Characterization of virulence mechanisms is essential in order to establish a reliable detection method of pathogenic strains in environment and prevent infections in humans. Although virulence factors were identified (TDH, TRH and type III secretion system 2), strains lacking these factors are isolated in clinical cases, suggesting existence of other virulence mechanisms. This study aimed to investigate genomic and genetic particularities of 11 new *V. parahaemolyticus* genomes and characterize their phenotypes using two *in vivo* models: infant rabbits and *Galleria mellonella* larvae. Comparative genetic analyses of the isolates revealed different combinations of virulence-associated genes, with the majority encoding *tdh* and/or *trh* genes. A *tdh/trh* positive isolate induced rapid disease onset in infant rabbits while a *tdh* positive isolate was avirulent. Although *G. mellonella* larvae was a good model to discriminate *Vp* strains according to their virulence potential, phenotypes observed in this model were the opposite of those observed in rabbits. Infection using defined mutants suggests that *Vp*'s T3SS1 take part in larvae mortality. When taken together, our findings highlight the diversity of *Vp* isolates found in the environment and their potential to cause disease.

1. Introduction

Vibrio parahaemolyticus (*Vp*), a common halophilic bacterium that inhabits estuarine and marine environments, is the leading cause of infection associated with the consumption of raw or undercooked seafood worldwide (Su and Liu, 2007). Historically most cases of human infection have been reported in tropical and sub-tropical regions (Nair et al., 2007; Pan et al., 1997). However, climate-driven increases in seawater temperatures support *Vp* proliferation in the environment and have contributed to the global expansion of *Vibrio* illness in temperate regions (Baker-Austin et al., 2013; Deter et al., 2010; Vezzulli et al., 2013). The prevailing dogma asserts that not all *Vp* strains are pathogenic; however, correlations between genetic markers for virulence and clinical infections are not straightforward (Froelich and Noble 2016).

Early studies identified an association between human infection and strains that exhibit hemolytic activity on Wagatsuma's media (Kanagawa phenomenon), a phenotype attributed to the production of thermostable direct hemolysin (TDH) encoded by *tdh* gene in the form of five variants named *tdh1* to *tdh5* sharing 96 to 99% of homologies (Baba et al., 1991; Nishibuchi and Kaper, 1990; Sakurai et al., 1973). Subsequently, TDH-

related haemolysin (TRH) producing strains (encoding by *trh* gene in the form of two variants *trh1* and *trh2* sharing 84% of homologies) were recognised, and, despite being Kanagawa negative, these were also linked to disease cases (Honda et al., 1988; Kishishita et al., 1992). Conversely, TDH and/or TRH producing strains were rarely found in the environment (Nair et al., 2007), providing evidence for their role as major virulence factors. However, recent years has seen a number of studies documenting the isolation of TDH and/or TRH-producing isolates from shellfish, seawater or sediments (Bacian et al., 2021; Hazen et al., 2015; Hervio Heath et al., 2002; Jones et al., 2012; Wagley et al., 2008) as well as the recovery of non-TDH/TRH producing isolates from patients (Bhoopong et al., 2007; Jones et al., 2012; Ottaviani et al., 2012). While it is recognised that there are several possible explanations for the latter including colony selection bias (Bhoopong et al., 2007), polystrain infections and loss of genes during laboratory isolation, the pathogenic risk of such strains remains unclear. Moreover, whole genome sequencing (WGS) has revealed that *Vp* harbours an extensive arsenal of pathogenic attributes including two type six secretion systems (T6SS), various adhesins and toxins, and two type three secretion systems (T3SS) that are located on chromosome 1 (T3SS1) and 2 (T3SS2), respectively (Makino et al., 2003). Notably, while T3SS1 has been detected in all *Vp*, T3SS2 of which there are two types, T3SS2α and T3SS2β, were found in *trh*$^{-}$ and *trh*$^{+}$ isolates respectively, has not been detected in all *Vp* (Park et al., 2000).

Most research on T3SS1 and T3SS2 activities has occurred in the pandemic reference isolate RIMD2210633 (Makino et al., 2003) but less is known about the pathogenic impact of *trh*$^{+}$ isolates. Epidemiological data suggest such strains have higher attack rates and cause more severe diseases (Martinez-Urtaza et al., 2016; McLaughlin et al., 2005). Progress in understanding *Vp* pathogenesis has been further complicated by the emergence of novel genetic variants of clinical relevance (e.g. ST36, ST88), and a paucity of data from mammalian models which recapitulate the disease.

Several *in vivo* models have been used to study *Vp* infection. Rabbit illeal loops have been used to demonstrate the enterotoxic nature of *Vp* infection and uncover roles for *tdh* and T3SS2 (Hiyoshi et al., 2010; Park et al., 2004a). Conversely, intraperitoneal systemic infection of mice revealed role for T3SS1 in mice lethality (Hiyoshi et al., 2010; Piñeyro et al., 2010). Microbiota-based colonisation resistance limits murine oral infection studies although models based on antibiotic pre-treatment or germ-free mice have recently been developed (Santos et al., 2021; Yang et al., 2019). However, microbiota loss could create a bias in deciphering *Vp* pathogenicity as it was shown that microbiota could play a potentially role in *Vp* infection (Wang et al., 2020). Infant rabbits are

susceptible to oral infection of *Vp* and use of this host has revealed insights into the pathological changes that occur during the course of infection for *tdh*$^+$ pandemic strain RIMD2210633 (Ritchie et al., 2012) and identified critical factors involved in intestinal colonisation (Zhou et al., 2012; Zhou et al., 2013). The use of non-mammalian model hosts such as those utilising wax moth (*Galleria mellonella*) larvae is increasing in bacterial infection studies for deciphering virulence and antimicrobial efficacy (Champion et al., 2016; Ménard et al., 2021) and is promising in particular with enteric bacteria due to similarities between intestinal epithelial cells from larvae and mammalian digestive tracts (Mukherjee et al., 2013). Moreover, insects harbour an innate immune system similar to that of mammals, comprising of haemocytes and soluble effector molecules (Wojda, 2017). Injection of larvae with a range of clinical and environmental *Vp* strains revealed a role for *mutT*, a gene coding for a nudix hydrolase, in TDH/TRH-negative strains (Wagley et al., 2018). However, the impact of haemolysin or the T3SSs was not assessed.

The purpose of this study was to examine the genetic diversity of *Vp* isolates collected from shellfish production areas in France and Portugal and assess their pathogenic potential using two models of *Vp* infection. Comparative genetic analyses of the isolates revealed different combinations of virulence-associated genes, with the majority encoding *tdh* and/or *trh* genes. Surprisingly, a *tdh/trh* positive isolate induced more rapid disease onset in infant rabbits than the pandemic *tdh*+ isolate, but failed to cause mortality in wax moth larvae. Infection using defined mutants suggests that *Vp*'s T3SS1 take part in larvae mortality. When taken together, our findings highlight the diversity of *Vp* isolates found in the environment and their potential to cause disease.

2. Results

a. General features of *V. parahaemolyticus* genomes

Eleven isolates (hereafter called IFVp) were sequenced to further characterize the genomic features of environmental *Vp* (**Table S1**). IFVp genomes were 4.9 to 5.4 Mbp in size, exhibited GC contents between 45.12 and 45.59% and contained on average 3,223 and 1,888 kbp in chromosomes 1 and 2, respectively. A total of 4,715 to 5,420 coding sequences (CDS) were predicted. One strain, IFVp182, contained a 40.45 kbp plasmid with 45 CDS, including a recognised type IV secretion system (**Table S1**). Genomic comparisons revealed that some previously described virulence clusters such as the mannose-sensitive hemagglutinin (MSH), chitin-regulated pilus (ChiRP), polar and lateral flagella, T6SS2 and T3SS1 were present in all IFVp strains (**Fig 1**). Additionally, it

revealed that strain IFVp5 was highly similar to that of the reference strain RIMD2210633, both clinical O3:K6 sequence type three (ST3) strains. *Vp* island-7 (VPaI-7) of RIMD2210633 which contains the organism's second T3SS was absent in IFVp18 and IFVp69 but present with varying levels of homology in the other IFVp strains. Cluster of Orthologous Genes (COG) analysis of the 11 strains revealed similar proportions of genes were involved in cellular processes and signalling (24%), information storage and processing (15%), metabolism (33%) or poorly characterized (19%) (**Fig S1**).

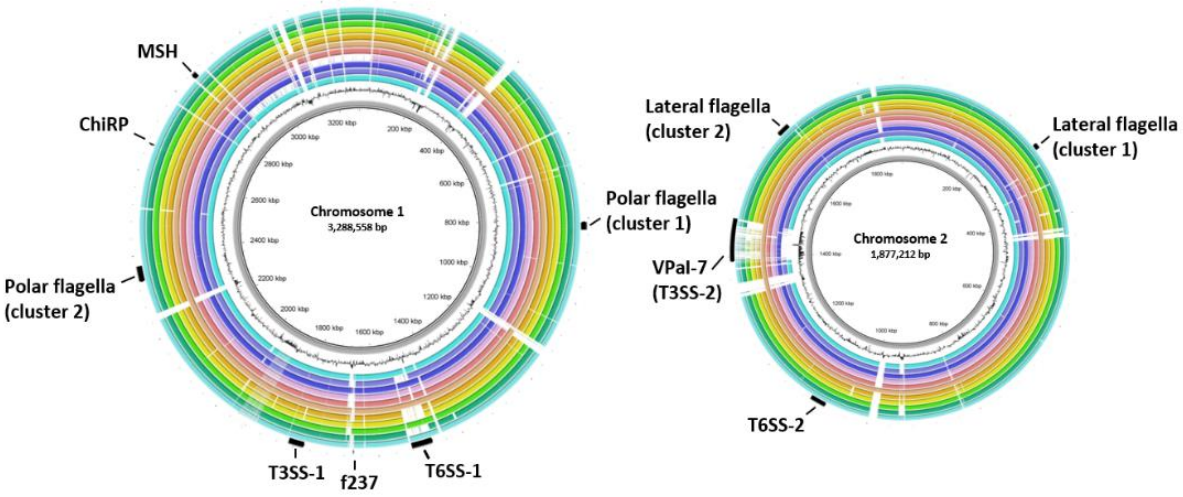

Fig 1. Comparison of environmental *Vp* genomes with RIMD2210633 as reference. Analysis performed by BLASTn using BRIG software. Left: chromosome 1, right: chromosome 2. From the inside out: RIMD2210633 (grey), GC content (black), IFVp18 (light blue), IFVp69 (purple), IFVp182 (dark blue), IFVp195 (light pink), IFVp408 (pink), IFVp5 (light orange), IFVp201 (orange), IFVp203 (yellow), IFVp136 (green), IFVp177 (turquoise) and IFVp22 (light turquoise). Names of previously described virulence clusters are indicated on the external ring ; MSH: mannose-sensitive hemagglutinin, ChiRP: chitin-regulated pilus, T3SS: type III secretion system, T6SS: type VI secretion system, VPaI-7: Pathogenicity Island of *Vp* number 7.

b. Haemolysin contents

To enable comparisons, the genomes of eight previously published complete *Vp* genomes (hereafter called NCBI genomes) containing varying *tdh* and/or *trh* gene profiles were included in subsequent analysis (see **Table S2**). A minimum evolution tree was performed with *tdh* and *trh* sequences from IFVp and NCBI genomes, determined using BLAST+ (see **Fig S2**). Determination of haemolysin variants was performed by adding sequences from NCBI database of four variants of *tdh* (*tdh1* to *tdh4*) and the two variants of *trh* (*trh1* and *trh2*) in the analyses (see **Fig S2**). In our analysis, we observed five profiles of haemolysin variant combinations: *tdh1, tdh1 tdh2, tdh3 trh1, trh1* and *trh2*

(**Table 1**), thus suggesting that *tdh2* and *tdh3* could not be present alone and that *trh2* could be only present alone. This analysis showed also that FDA_R31 harboured a TDH haemolysin (M634_24535) which shared 89%, 91%, 92% and 92% of homologies with *tdh1*, *tdh2*, *tdh3* and *tdh4*, respectively, thus did not allow a clear classification of this *tdh* gene.

c. Genomic features and phylogenetic analyses

The pan genome of all 19 genomes (11 IFVp and eight NCBI genomes) comprised of 9,161 coding genes including 3,616 (39%) core genes and 5,545 (61%) accessory genes (**Fig 2A**). The number of accessory genes ranged from 915 to 1,800 depending on the strain (**Table S2**). As evident in **Fig 2A**, *Vp* has an open pan-genome structure as the number of total coding genes increased with the addition of each new genome, while the number of new genes decreased (**Fig 2B**). Scoary analysis indicated that no genes were significantly over- or under-represented in accessory genome of clinical versus environmental strains, or of *tdh*$^+$ versus *tdh*$^-$ strains. However, 51 genes were over-represented in accessory genome of *trh*$^+$ strains compared to *trh*$^-$ strains including those coding for the urease operon and some for the T3SS2 cluster (**Table S3**).

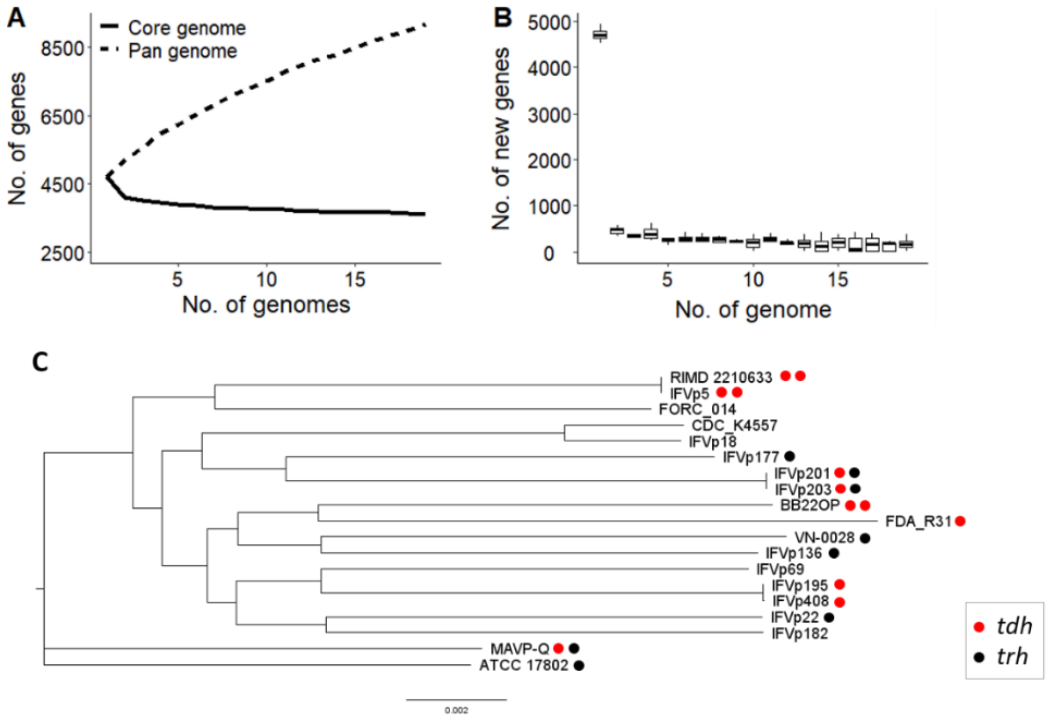

Fig 2. Pan-genome analysis and core-genome phylogenetic tree of the 19 *Vp* strains. (A) Estimated number of the core (3,616 genes) and pan-genomes (9,161 genes), and (B) number of new genes. (C) Maximum-likelihood tree obtained from concatenated nucleotide sequence alignment of core genes using RaxML with 100 bootstraps.

Phylogenetic analysis of the core genomes showed that the clinical strain IFVp5 (*tdh1*$^+$ *tdh2*$^+$) clustered with RIMD2210633 (*tdh1*$^+$ *tdh2*$^+$) but not with BB22OP (*tdh1*$^+$ *tdh2*$^+$) (**Fig**

2C). Additionally, IFVp18 (*tdh*$^-$ *trh*$^-$) clustered together with CDC_K4557 (*tdh*$^-$ *trh*$^-$), but not with IFVp69 (*tdh*$^-$ *trh*$^-$). Conversely, MAVP-Q (*tdh*$^+$ *trh*$^+$) did not cluster with IFVp201 and IFVp203 (*tdh*$^+$ *trh*$^+$) and FDA_R31 (*tdh*$^+$ *trh*$^-$) did not cluster with IFVp195 and IFVp408 *(tdh*$^+$ *trh*$^-$). Finally, IFVp182 (*tdh*$^-$ *trh*$^-$ T3SS2$^+$) did not cluster with FORC_014 (*tdh*$^-$ *trh*$^-$ T3SS2$^+$), and IFVp136 (*tdh*$^-$ *trh*$^+$) and IFVp177 (*tdh*$^-$ *trh*$^+$) did not cluster with VN-008 (*tdh*$^-$ *trh*$^+$) (**Fig 2C**). These observations suggest that core gene phylogeny was not associated with haemolysin gene content; instead, strains with the same sequence type (ST) (**Table S1**) were found to cluster more closely *i.e.* IFVp5 and RIMD2210633, IFVp201 and IFVp203, and IFVp195 and IFVp408.

d. Potentially virulent gene content

The presence of known virulence genes, determined using BLAST+, are shown in **Table 2**. T3SS2 was present in 16 strains and T6SS1 was present in 12 strains. Heatmap of T3SS2 genes showed that the presence of the T3SS2β was concomitant with the presence of the *trh* gene even in *tdh*$^+$ *trh*$^+$ strains, except for FDA_R31 (*tdh*$^+$ *trh*$^-$) which harboured the T3SS2β (**Fig 3**). Among the 19 strains, *zot* and *hly* genes were detected in five and four strains, respectively. A cellulose operon (*bcs*) was identified in six strains that lacked the T6SS1 gene cluster (**Table 1**). Although T3SS1 was present in all the *Vp* strains, its organization differed between strains. Indeed, gene region VP1676-VP1679 was absent in IFVp201, IFVp203 and FDA_R31 strains (**Fig 3**) but was replaced by three new genes in IFVp201 and IFVp203: a secretion protein, a MFS transporter and a TetR/AcrR family transcriptional regulator gene. Moreover, the ATCC 17802 harboured three new genes between VP1676-VP1679 and *vopQ* gene: a N-acetyltransferase, a transporter protein and a LysR family transcriptional regulator genes. Concerning the T3SS1 effectors, the four (VopQ, VopS, VopR and VPA0450) were present in all the strains. T3SS2 effectors VopC, VopZ, VopP, VopL and VPA1380 were present in all the strains while VopT and VopV were absent in *trh*$^+$ and *tdh*$^+$ *trh*$^+$ strains.

Table 1. Distribution of known virulence genes in *Vp* strains by *in sillico* detection

Strains	Haemolysins		T3SS		T6SS		Toxins			Type IV pili		Polar flagella	Lateral flagella	Colonization			Iron-acquisition genes			Biofilm
	tdh	*trh*	T3SS-2	T3SS-1	T6SS1	T6SS2	ZOT	*hly*	RTX	MSH	ChiRP	*fla*	*laf*	*VPA1701*	*acfD*	*acfC*	*pvs-pvu*	*IutA*	*irgA-vctA*	*bcs*
Vp18				+	+	+	+		+	+	+	+	+		+		+	+	+	
Vp69				+		+			+	+	+	+	+		+		+	+	+	
Vp182			α	+	+	+				+	+	+	+		+		+	+	+	
Vp195	1		α	+	+	+			+	+	+	+	+		+	+	+	+	+	
Vp408	1		α	+	+	+			+	+	+	+	+		+	+	+	+	+	
Vp5	1+2		α	+	+	+				+	+	+	+	+	+		+	+	+	
Vp201	3	1	β	+		+			+	+	+	+	+		+		+	+	+	+
Vp203	3	1	β	+		+	+		+	+	+	+	+		+		+	+	+	+
Vp136		1	β	+	+	+	+	+		+	+	+	+	+	+		+	+	+	
Vp177		1	β	+		+			+	+	+	+	+		+		+	+	+	+
Vp22		2	β	+	+	+		+	+	+	+	+	+		+		+	+	+	
CDC_K4557				+	+	+				+	+	+	+		+		+	+	+	
FORC_014			α	+		+	+			+	+	+	+	+	+	+	+	+	+	+
FDA_R31	*		β	+		+	+	+		+	+	+	+		+		+	+	+	+
RIMD2210633	1+2		α	+	+	+	+			+	+	+	+		+		+	+	+	
BB22OP	1+2		α	+	+	+			+	+	+	+	+		+		+	+	+	
MAVP-Q	3	1	β	+		+				+	+	+	+		+		+	+	+	+
VN-0028		1	β	+	+	+				+	+	+	+		+		+	+	+	
ATCC_17802		2	β	+	+	+		+	+	+	+	+	+		+		+	+	+	

*: novel *tdh* variant

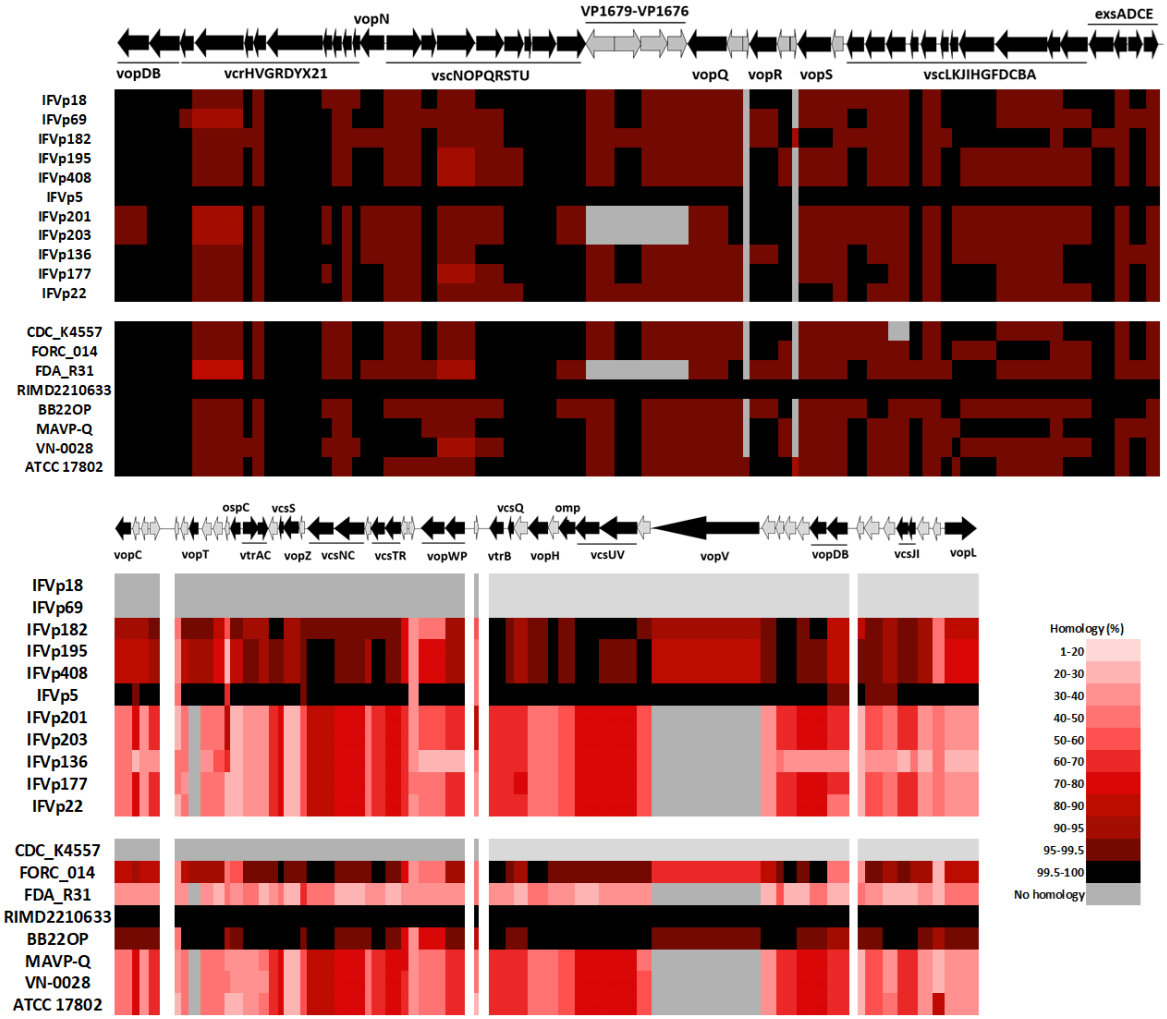

Fig 3. Heatmaps of the two T3SS clusters in the 19 *Vp* strains.
(A) T3SS1 cluster. (B) T3SS2 cluster. Results are based on BLASTp analyses with RIMD2210633 sequences as reference: T3SS1 (VP1656-VP1702) and T3SS2 (VPA1321-VPA1370). Black arrow: genes with known function. Grey arrow: genes with unknown function

e. Prediction of prophages and genomic islands

Intact prophages were predicted by PHASTER in 13 *Vp* genomes including eight IFVp and five NCBI genomes (**Table S4**). The analysis revealed the presence of intact VfO3K6 prophages in IFVp18, IFVp136, IFVp203, FDA_R31, FORC_014 and RIMD2210633. This prophage was shown to harbour a *zot* gene (Castillo et al., 2018a) which is in accordance with the presence of *zot* gene in these strains as shown previously (**Table 1**). Moreover, intact VFJ prophages were detected in IFVp195, IFVp408, IFVp201, IFVp203 and CDC_K4557. Vibrio 12A4 prophages, mainly composed of hypothetical proteins, were identified in IFVp201, IFVp203, IFVp177 and FORC_014. Moreover, prophage identified in *Aeromonas media* called phiO18P was predicted in FDA_R31 and BB22OP while the Vibrio phage VPSUM 8 was predicted in FORC_014. Vibrio phage Vf12, which harbours a *zot*-like gene, was detected in IFVp182.

Genomic islands defined in RIMD2210633 (Hurley et al., 2006) were investigated in the 19 *Vp* genomes and those present in each strain are indicated in **Table S5**. BLASTp analysis revealed that VPaI-1 was partially present in IFVp18, IFVp5, CDC_K4557 and VN-0028 while it was absent in all other strains, except for the presence of a single gene (VP0394) in IFVp177 and FDA_R31. VPaI-2 and VPaI-3 were partially present in all strains, whereas VPaI-4 was partially present only in IFVp18. VPaI-5 was also partially present in IFVp18 and BB22OP, while VPaI-6 could be found in IFVp5, IFVp22, FDA_R31, BB22Op and MAVP-Q. A schematic illustration of VPaI-7 identified in the 19 *Vp* genomes is shown in **Fig S3A**. These results showed that the VPaI-7 was absent from IFVp18, IFVp69 and CDC_K4557 (**Fig S3A**) and that the island organization mainly depended upon presence/absence and variants of *tdh* and/or *trh* genes, except for FDA_R31. This analysis showed that a gene encoding the accessory colonization factor D (*acfD*) was present near the T3SS-2 cluster. These results confirmed that the urease operon was present in all the trh^+ strains. Moreover, we observed the presence of toxin B gene only in the $trh2^+$ strains while the *hly* operon (*hlyCABD*) was present in some trh^+ strains (IFVp136, IFVp22 and ATCC 17802). The COG analysis of the VPaI-7 showed variation in proportion according to haemolysins presence/absence (**Fig S3A**). Indeed, excluding FDA_R31, the trh^+ strains had higher proportion of metabolism (28 ± 4%) and lower unknown function (11 ± 4%) than in trh^- strains (7 ± 2% and 31 ± 3%, respectively).

f. Infant rabbit infections

To begin to assess the pathogenic potential of the IFVp isolates, 2-3 days old infant rabbits were orogastrically inoculated with either IFVp201 or IFVp195; strains which

harboured contrasting virulence features (IFVp201: *tdh3/trh1*, T3SS2β, T6SS1$^-$ *vs* IFVp195: *tdh1*, T3SS2α, T6SS1$^+$; see **Table 1**). IFVp201 caused diarrhoea with most rabbits contaminated by liquid faeces from about 12 hours post infection (HPI). By 15 HPI, significant amounts of fluid had accumulated in their small intestines and between 10^8 to 10^9 CFU.g^{-1} could be recovered from this region (**Fig 4**). In marked contrast, IFVp195 failed to cause observable diarrhoea or fluid accumulation in the cecum of rabbits even by 120 HPI. Concentrations of IFVp195 were nearly 5 logs lower than those of IFVp201 in all regions of the small intestine at 15HPI, the only timepoint where direct comparisons were possible ($p \leq 0.001$). IFVp195 concentrations increased to approximatively 6 log CFU.g^{-1} by 38 HPI, then appeared to stabilise. A few animals infected with IFVp201 were found to contain ~10^3 CFU.g^{-1} in bile and spleen but cross-contamination from the intestine at the time of organ collection could not be ruled out. Overall, our findings suggest that IFVp201 induced more severe disease and appeared more able to colonise the mammalian intestine than the *tdh*$^+$ IFVp195 strain.

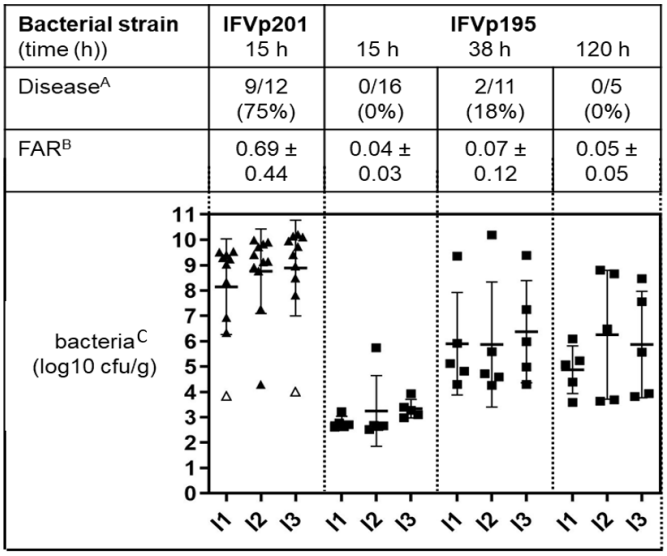

Fig 4. Kinetics of disease, fluid accumulation and intestinal colonisation of infant rabbits infected with *V. parahaemolyticus* IFVp201 and IFVp195.
Infant rabbits were oro-gastrically inoculated with ~5 x 10^8 CFU of IFVp201 or IFVp195 and disease state, intestinal fluid accumulation ratios (FAR) and recovery of *Vp* determined at 15, 38 and 120 hours post infection (HPI). [A] Disease was recorded for individual animals. [B]Fluid accumulation ratios (FAR) were represented as mean ± standard deviation of all rabbits within the group. C Concentrations of *Vp* recovered in intestinal homogenates taken from the proximal (I1), mid (I2) and distal regions (I3) of the small intestine.

g. *Galleria mellonella* larvae infections

While infant rabbits offer a useful model to dissect *Vp* pathogenesis, they are not suited to high-throughput studies. Thus, in order to explore the pathogenic potential of all the

sequenced *Vp* strains, *Galleria mellonella* larvae were trialled as an alternative infection model (**Fig 5A**). First, we sought to determine the impact of *tdh* and the two T3SS on *Vp*-induced killing of the larvae. Using the same mutants as examined in infant rabbits (Ritchie et al., 2012), larvae were infected with approx. 7 log CFU.mL^{-1} and *G. mellonella* survival was evaluated after 24 h. While wild type, Δ*tdh*, and ΔT3SS2 mutants were highly virulent killing 100% of larvae, 38 and 20% of those infected with ΔT3SS1 or the triple mutant survived (**Fig 5B**).

Next, larvae survival was compared following injection of the IFVp strains sequenced in this study. Slighter lower concentrations of IFVp strains (range from 6.52 to 6.84 log CFU.mL^{-1}) were used due to minor differences in growth conditions in the different laboratories. Larvae survival ranged widely, revealing that four strains (IFVp182, IFVp201, IFVp203 and IFVp22) were less virulent (larvae survival > 75%), five strains (IFVp18, IFVp69, IFVp408, IFVp4 and IFVp177) were highly virulent (larvae survival < 15%) with the remaining two strains (IFVp195 and IFVp136) induced an intermediate phenotype (**Fig 5C**) (Kéomurdjian, 2015). Differences in virulence corresponded to the experimentally-determined lethal dose $(LD)_{50}$ of the strains, with those exhibiting greater virulence having LD_{50} values close to the injected concentrations (**Table S6**). Notably, non-toxigenic strains could be found in both high (IFVp18, IFVp69) and low (IFVp182) virulence groups, respectively. Further studies uncovering the pathogenic mechanisms and threat to human health posed by these and other *Vp* strains are urgently needed.

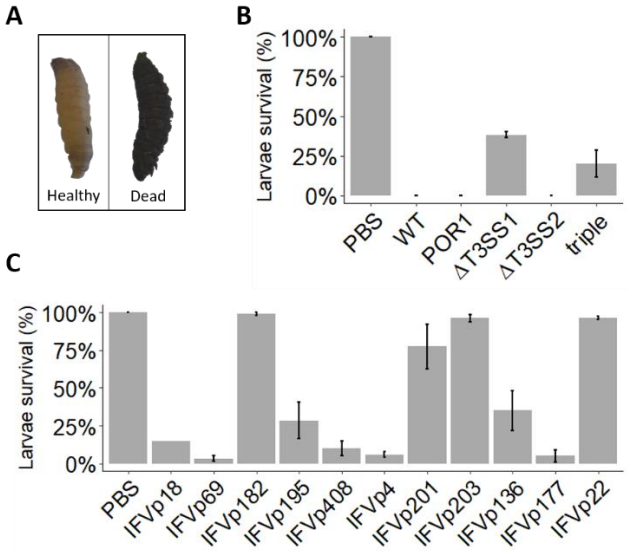

Fig 5. *Galleria mellonella* infections.
(A) Images of healthy and dead larvae. (B) Percentage of survival of larvae at 24 h post-injection with approx. 1 x 10^{7} CFU.mL^{-1} of RIMD2210633 and mutants. Data represented

mean ± standard error. (C) Percentage of survival of larvae at 24 h post-injection with approx. 5 x 10^6 CFU.mL^{-1} of IFVp strains. Data are represented as mean ± standard error.

3. Discussion/perspectives

Identification of human pathogenic *Vp* is necessary to reduce the risk of infection for seafood consumers. Yet, the presence of classical genetic markers (*i.e. tdh, trh*) do not seem to accurately reflect pathogenic potential as non-toxigenic strains lacking the aforementioned genes, have been associated with clinical cases in some countries (Jones et al., 2012; Ottaviani et al., 2012). Here, we sequenced 11 new *Vp* genomes and compared their features to 8 previously published strains. Two infection models were used to explore their pathogenic potential.

The core genome of all 19 strains used in this study consisted of 3,616 genes, slightly smaller than that of some non-toxigenic strains (Castillo et al., 2018b; Ronholm et al., 2016) and slightly larger than one of O3:K6 strains (Chen et al., 2011) and of *Vp* strains isolated from ready-to-eat food (Pang et al., 2019). In our study, the open pan genome suggested constant gene gain allowed by high rates of horizontal gene transfers, frequent in environmental strains, thus increasing bacterial fitness in the environment (McInerney et al., 2017). Core genome phylogeny of these strains showed that the clustering was not related to haemolysin content. However, we observed that strains with the same ST clustered together (RIMD2210633 and IFVp5, and IFVp195 and IFVp408) as found previously for clinical and environmental *Vp* strains (Jesser et al., 2019; Ronholm et al., 2016).

As a measure of virulence potential of *Vp* strains, we determined *in silico* presence of known virulence factors, specific or not to *Vp*. All the strains harboured a T3SS1 cluster as previously shown (Hazen et al., 2015). It was also shown that organisation of the T3SS1 around VP1676-VP1679 genes can vary between *Vp* strains, thus, distinguishing three groups called VPI to VPIII (Wu et al., 2020). Analyses of gene syntheny of this cluster showed that among the 19 strains, two were VPIII (IFVp201 and IFVp203), one was VPII (ATCC 17802), one presented a unique organization not observed previously (FDA_R31) and the remaining strains were VPI. Deletion of VP1676-VP1679 has previously been shown to have no cytotoxic effect on mammalian (Ono et al., 2006) or fish cells (Wu et al., 2020), and thus remained with an unknown function. As shown previously by Hazen *et al.* (2015) and Jones *et al.* (2012), the relation between variants of T3SS2 and the presence of *trh* was confirmed in our study, *i.e.* the T3SS2β variant was present in *trh*$^+$ strains (even in *tdh*$^+$ *trh*$^+$ strains) and the T3SS2α variant in *trh*$^-$ strains, with the exception of FDA_R31 which harboured a T3SS2β variant

while this strain is *trh*-. All the *Vp* strains carried the T6SS2 cluster (Yu et al., 2012) while only 12 of the 19 strains harboured the T6SS1. T6SS1 may enhance *Vp* fitness in marine environments via conferring anti-bacterial activity that outcompetes other bacteria (Salomon et al., 2013). Interestingly, a cellulose operon (*bcs*) was identified in strains lacking T6SS1. This operon was suggested to be involved in survival in aquatic environment and to replace T6SS1 (Meparambu Prabhakaran et al., 2021). Some strains harboured a *zot* gene, encoding a secreted toxin which increases intestinal permeability that was firstly identified in *Vibrio cholerae* (Fasano et al., 1991). It was shown that the *zot* gene was encoded in the filamentous phage f237 associated to pandemic O3:K6 *Vp* strains (Nasu et al., 2000), but *zot* gene was identified in non-toxigenic strains as well (Castillo et al., 2018b). Analyses of prophages identified in our strains showed that the prophage VfO3K6 (**Table S4**) harboured a *zot* gene and that *zot*+ strains in our study all possessed the prophage VfO3K6. BLASTp of VfO3K6 proteins showed high homologies (> 90%) with f237 proteins from RIMD2210633, except for ORF10 (> 59%) and ORF8 (ranging from 22 to 26%) (not shown). Interestingly, the pandemic O3:K6 strain IFVp5 isolated in 2004 did not harbour ORF8 and ORF10. Previous studies of VPaI identified in RIMD2210633 showed that VPaI-1, VPaI-4, VPaI-5 and VPaI-6 were restricted to pandemic post-1995 O3:K6 strains (Hazen et al., 2015; Hurley et al., 2006). In our study, we observed contradictory, yet interesting results. Indeed, IFVp18 strain (*tdh*- *trh*- T3SS-2-) was shown to harbour the VPaI-1 almost intact, and the entire VPaI-4 and VPaI-5 while these three VPaI were absent from IFVp5 (pandemic post-1995 O3:K6). Otherwise, VPaI-2 and VPaI-3 were partially present in all strains, as shown previously (Ronholm et al., 2016). Global organization of VPaI-7 was suggested to depend on haemolysins content. Difference in COG proportions (metabolism group) could be explained by presence of urease operon in *trh*+ strains but not in *tdh*+. Collectively, virulence genes, prophages and pathogenicity islands are important for *Vp* fitness in the environment, providing genetic adaptability in response to changing conditions. Evaluation of pathogenicity of a bacterial strain need *in vivo* experiments using animal models.

Two environmental *Vp* strains, IFVp201 and IFVp195, were selected for phenotypic characterization of virulence using the infant rabbit model of *Vp* infection (Ritchie et al., 2012). These two strains exhibited two distinct virulence phenotypes. Indeed, IFVp201 (*tdh3*+ *trh1*+ T3SS2β+) showed a highly virulent phenotype in this model, even more virulent than the one of the reference strain RIMD2210633 concerning FAR (0.69 ± 0.44 and 0.29 ± 0.13, respectively) (Ritchie et al., 2012), but similar to the disease kinetics seen in rabbits infected with non-O1, non-O139 *V. cholerae* (Shin et al., 2011). It was shown

that most of the disease caused by *Vp* RIMD2210633 could be attributed to the activity of T3SS2α, and in particular VopZ (VPA1336) with *tdh* and T3SS1 playing more minor roles (Ritchie et al., 2012; Zhou et al., 2013). In our study, presence of T3SS2β, *tdh3* variant and a *trh* gene could explain the high virulence phenotype of IFVp201. Interestingly, the *tdh3* variant showed lower haemolytic activity than *tdh1* and *tdh2* variant (Nishibuchi and Kaper, 1990). Moreover, IFVp201 harbours the same haemolysin variants (*tdh3*$^+$ *trh1*$^+$) as ST36 strains that caused severe disease in Spain and Pacific northwest coast of USA (Martinez-Urtaza et al., 2013; McLaughlin et al., 2005). Conversely, IFVp195 (*tdh1*$^+$ T3SS2α$^+$) appeared less virulent, with the amount of fluid present in the distal small intestine of infected rabbits (0.04 ± 0.03) similar to that seen for historic mock infected rabbits (0.05 ± 0.02) (Ritchie et al., 2012). These results suggested that T3SS2 does not explain all the pathogenicity in this model and that other virulence mechanisms are involved. In order to decipher *Vp* pathogenicity and its mechanisms, it appears essential to perform phenotypic characterization of *Vp* strains presenting a wide range of genetic content.

Use of *G. mellonella* as a model to study *Vp* infection was evaluated. Infection with RIMD2210633 mutants showed that the deletion of T3SS1 resulted in less lethality among the larvae. These findings suggest that contrary to infant rabbits, *G. mellonella* larvae were more sensitive to the activities of T3SS1, and possibly lend support to a role for TDH in dampening the immune response as noticed previously (Ritchie et al., 2012). Moreover, these results correlate with results observed in intraperitoneally infection mice (Hiyoshi et al., 2010; Piñeyro et al., 2010), suggesting a role of T3SS1 in systemic infections. In the present and in previous studies, infection with IFVp strains showed varying virulence phenotypes. Interestingly, IFVp195 was virulent (28% of larvae survival) whereas IFVp201 was less virulent (78 % of larvae survival) in *G. mellonella* larvae (Kéomurdjian, 2015). Thus, disease outcomes associated with the two *Vp* strains did not correlated in the two model hosts, this may provide insights into different aspects of *Vp* infection. Difference in virulence phenotype between the two models tested in our study could be explained by the difference in immune response associated to infection way (injected in larvae and oro-gastrically in rabbits). Indeed, using murine model, it was shown that virulence mechanisms differed using intra-peritoneally infected mice (T3SS1) (Hiyoshi et al., 2010) and oro-gastrically infected mice (T3SS2) (Yang et al., 2019), respectively. A protocol of bacterial infection using a forced feeding of *G. mellonella* larvae was established and could allow to avoid this injection bias (Ramarao et al., 2012). Intriguingly, reduced virulence in both RIMD2210633 ΔT3SS1 and

IFVp182, IFVp201, IFVp203 and IFVp22 strains, appears to correlate with loss of, or reduced homology of VP1678 in T3SS1. VP1678 encodes a putative dienelactone hydrolase (Nydam et al., 2014) that could be involved in *Vp* virulence in this model. Indeed, a dienelactone hydrolase was shown to be involved in virulence of the fungi *Fusarium pseudograminearum* in cereal host wheat (Gardiner et al., 2012). Moreover, *Vp* need to compete with microbiota to successfully colonize and survive in the host. Thus, the T6SS, known to play a role in antimicrobial competition, could be involved in *Vp* virulence. Indeed, T6SS was identified as a potential virulence factor involved in virulence of *Francisella tularensis* (Brodmann et al., 2021) and *Campylobacter jejuni* (Liaw et al., 2019) in *G. mellonella* larvae model. However, the mutT gene encoding a nudix family protein identified as a potential virulence factor of *Vp* in *G. mellonella* (Wagley et al., 2018) was present in all our strains, thus it did not corroborate with virulence phenotype in this model. Further genomic analyses are needed to identify mechanisms involved in *Vp* virulence in larvae.

To conclude, we sequenced genetically different strains of *Vp* which could be useful to further identify potential virulence mechanisms, in association to phenotypic characterization in *in vivo* models. Use of infant rabbit model in this study showed that understanding of mechanisms involved in *Vp* virulence was not a simple task and needed more accurate characterization of *Vp* strains. The phenotypic characterization of a wide range of *Vp* strains using *G. mellonella* larvae can reproduce phenotype readout of some disease aspects, thus, could be useful for high throughput screening, even if mechanisms involved *Vp* virulence in this model need to be further identified. Criteria used by national institutions to characterize the potential pathogenicity of *Vp* strains are *tdh* and *trh*, thus when detected in seafood/shellfish result in their removal from market or farms closure (DGAl, 2019; NSSP, 2017). According to our study, presence of these genes does not necessarily mean virulence, thus made these criteria not enough accurate to identify potentially pathogenic strains. Further studies using more diverse *Vp* strains, *in vivo* models and genomic analyses are needed to identify more relevant criteria.

4. Materials and methods

a. Bacterial strains

Eleven *Vp* strains (IFVp), mainly isolated from the environment and selected from a larger collection to represent a range of isolation date, sample origins and serotype (**Table 1**), were sequenced in this study. Data from eight additional previously sequenced *Vp* genomes were collected from NCBI (NCBI genomes) for genomics

analyses: five clinical isolates (RIMD2210633 (Makino et al., 2003), ATCC 17802 (Yang et al., 2015), CDC_K4557 (Lüdeke et al., 2015), MAVP-Q (Xu et al., 2017) and VN-0028 (Accession no. MVKK00000000.1)) and three environmental isolates (FORC_014 (Ahn et al., 2016), FDA_R31 (Lüdeke et al., 2015), and BB22OP (Jensen et al., 2013)). The reference strains were selected based on their complete genomes (except for VN-0028) and *tdh/trh* gene profiles. Previously described genetically defined deletion mutants of RIMD2210633 were used for *Galleria mellonella* infection experiments: Δ*tdh*, ΔT3SS-1, ΔT3SS-2 and deletions in of all three virulence factors (*aka* triple mutant) (Hiyoshi et al., 2010).

Table 2. Characteristics of *V. parahaemolyticus* strains used in this study

Strain	Isolation year	Country	Source	Serotype
IFVp18	1999	France	E (mussel, *Mytilus edulis*)	O2:KUT
IFVp69	2002	France	E (mussel, *M. edulis*)	O3:KUT
IFVp182	2005	France	E (seawater)	O11:KUT
IFVp195	2010	France	E (seawater)	O4:KUT
IFVp408	2014	France	E (oyster, *Crassostrea gigas*)	O4:K37
IFVp5	2004	France	Clinical	O3:K6
IFVp201	2009	France	E (mussel, *M. edulis*)	O5:KUT
IFVp203	2009	France	E (mussel, *M. edulis*)	O5:KUT
IFVp136	2004	Portugal	E (mussel, *Mytilus galloprovincialis*)	O11:KUT
IFVp177	2006	Portugal	E (oyster, *C. gigas*)	O11:KUT
IFVp22	1999	France	E (mussel, *M. edulis*)	O2:KUT

E: environmental, KUT: K-untypeable

b. Whole-Genome Sequencing (WGS), assembly and annotation

IFVp strains for WGS were initially grown on Luria-Bertani agar containing 3% NaCl (LBS) at 37°C for approx. 18 h, prior to the aseptical transfer of a single colony into 1.5 mL of peptone buffered-water (PBW: 20 $g.L^{-1}$ of peptone, 20 $g.L^{-1}$ of sodium chloride) and subsequent incubation with shaking for 18 h at 37°C. DNA was extracted from the bacterial cells using a GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, UK) following manufacturer's recommendations. The purity and concentration of extracted DNA was assessed from 260/280 nm readings taken using the Epoch ™ Microplate Spectrophotometer (BioTek, USA). DNA extracts were sent to the GATC platform (Eurogentec, France) for sequencing (Illumina HiSeq 4000 type paired-end (2 x 150 bp) 450 bases, approximately 5 million read pairs and 100X average coverage). DNA integrity was verified by electrophoresis with a 2% agarose gel.

Quality of reads was verified using FastQC v0.11.5 and reads were mapped using Bowtie2 (Langmead et al., 2019). The sequences were assembled using Velvet *de novo*

v1.2.10 (Zerbino and Birney, 2008). The two chromosome sequences were determined by alignment against the *Vp* RIMD2210633 reference strain using Mauve Aligner (Rissman et al., 2009) and visualized using BRIG v0.95 (Alikhan et al., 2011). Genome annotation was performed with the interface Magnifying Genomes (MaGE) of the MicroScope web-based service from GenoScope (Vallenet et al., 2009). The Sequence Type (ST) of each genome was determined using PubMLST (https://pubmlst.org/organisms/vibrio-parahaemolyticus) (Jolley et al., 2018).

c. Core and accessory genome analyses

Prokka v1.14.6 (Seemann, 2014) was used to collect GFF format using Fasta files of the 19 *Vp* genomes. Roary v3.13.0 (Page et al., 2015) was used to analyse the total core and accessory genomes of *Vp* strains. Core genes were defined to be present in all the strains and accessory genes to be absent in at least one strain (sum of shell and cloud genes). Software packages were used under default settings. "Pan/Core-Genome" tool from MaGe platform was used to determine accessory gene numbers (sum of variable and strain-specific genes) present in each strain. The core-alignment, generated by Roary, was used to construct a maximum-likelihood phylogeny using RaxML v8.2.4 (Stamatakis, 2014) with a general time reversible gamma model and 100 bootstrap values.

The relationships between accessory gene presence-absence and (i) origin (*i.e.* clinical and environmental), (ii) *tdh* presence-absence and (iii) *trh* presence-absence were investigated using Scoary v1.6.16 (Brynildsrud et al., 2016). For these analyses, the gene presence-absence matrix, generated by Roary, and a binary matrix indicating the traits of each strain were used. Pairwise comparisons were deemed significant when the Benjamini-Hochberd adjusted *p*-values were less than 0.05.

d. Characterization of genome

Cluster of orthologous genes

Distribution of protein coding genes was determined within the Cluster of orthologous genes (COG) functional categories using the COG automatic classification tool on MaGe platform in each chromosome. These values are computed using the automatic results obtained with the COGNiTOR software (http://www.ncbi.nlm.nih.gov/COG/).

In silico identification of known virulence genes

In sillico identification of known virulence genes was performed using BLAST+ v2.11.0 (Camacho et al., 2009). Genes identified in this study were *tdh1-4* (Nishibuchi and Kaper, 1985; Nishibuchi and Kaper, 1990), *trh1-2* (Kishishita et al., 1992; Nishibuchi et al., 1989),

T3SS-1 and T3SS-2 (Makino et al., 2003; Okada et al., 2009), T6SS-1 and T6SS-2 (Salomon et al., 2013), polar (Kim and McCarter, 2000) and lateral (Stewart and McCarter, 2003) flagella, type IV pilus (Marsh and Taylor, 1999), iron-acquisition genes (León-Sicairos et al., 2015), toxins such as ZOT (VP1558), HlyA (VCA0219) and RtxA (WP_010895441), and colonization factors such as VPA1701 (Gu et al., 2019) and AcfCD (Peterson and Mekalanos, 1988).

Sequences of *tdh* and *trh* from 14 *Vp* genomes (eight IFVp and six NCBI genomes) and six NCBI haemolysin reference sequences (two *trh* and four *tdh* sequences) were aligned using MUSCLE and a minimum evolution (ME) phylogeny with 1000 bootstrap was constructed using MEGA X (Kumar et al., 2018). This analysis involved 26 amino acid sequences. Tree was annotated using Interactive Tree of Life (iTOL v6) (http://itol.embl.de).

Pathogenicity islands and prophages

Pathogenicity islands VPaI-1 to VPaI-6, characterized in RIMD2210633 (Hurley et al., 2006), were determined using BLASTp. VPaI-7, which was composed of the T3SS2 cluster and *tdh/trh* haemolysin genes, was identified using the MaGe platform "Regions of Genome Plasticity" (RGP) tool. COG analyses were performed on VPaI-7 regions as described above.

Integrated prophage regions were identified and annotated using PHASTER (Arndt et al., 2016). PHASTER provided the best hit prophage as well as the position, the length and the GC content of the region. Determination of completeness scores are described in (Zhou et al., 2011).

e. Infant rabbit experiments

All experimental protocols were approved by the local Animal Welfare and Ethical review Body, the Home Office and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Time-mated adult New Zealand White females were obtained from a commercial breeder (Harlan Laboratories, UK). Experiments were performed on individual litters of 2-3 days old infant rabbits that were housed as a group in a nest box with the lactating doe for the duration of the study. The number of animals per litter varied but for the two test *Vp* strains (IFVp201 and IFVp195) and for each timepoint (15, 38 and 120 h post infection (HPI)), infections were performed on animals derived from at least two independent litters to counter reporting of litter-specific effects. Low birth weight rabbits ('runts') were removed from the litters prior to the experiment commencing as these animals tend to exhibit different disease kinetics.

Otherwise, all animals within a litter were infected. Experiments were performed generally as described (Ritchie et al., 2012), except animals were pre-treated with ranitidine (5 $mg.kg^{-1}$ bodyweight via intraperitoneal injection; GlaxoSmithKline) 3 hr prior to oro-gastric inoculation. *Vp* inoculum was prepared from stationary phase cultures grown with shaking in tryptone soy broth (TSB) at 37°C for 18 h. For IFVp201, TSB or tryptone soy agar (TSA) was supplemented with carbenicillin (50 $\mu g.mL^{-1}$). Cells were collected by centrifugation (5 min at 5,000 g) and the cell pellet resuspended in sodium bicarbonate solution (2.5g in 100ml; pH 9) to give a final concentration of ~ 1.10^9 $CFU.mL^{-1}$. Rabbits were oro-gastrically inoculated using a size 4 French catheter at a dose equivalent to 0.5 mL per 90 g rabbit bodyweight and then after monitored frequently for signs of disease. Disease was recorded when visible signs of solid or liquid faecal contamination were observed on the anus or ventral surface of the rabbits; healthy rabbits remained clean with no signs of contamination. At the indicated time points, rabbits were humanely killed and the entire intestinal tract from the duodenum to the rectum was removed and processed to determine levels of fluid accumulation and *Vp* colonisation. Fluid accumulation ratios (FAR) were obtained by determining the weight of fluid present in a section of the distal small intestine divided by the entire weight (fluid and tissue) of the section. Concentrations of *Vp* (in log $CFU.g^{-1}$) were determined in tissue samples taken from the proximal (I1), mid (I2) and distal (I3) regions of the small intestine. Tissue samples were homogenised between two glass slides using 2 mL sterile PBS prior to serial dilution and plating on tryptone soy agar. Colonies were confirmed as *Vp* following growth on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. For some rabbits, the internal organs including the gall bladder, spleen and liver were also collected, homogenized and plated on selective media to check for systemic spread of *Vp*.

f. *Galleria mellonella* larvae experiments

Pathogenicity of RIMD2210633 mutants and IFVp strains were evaluated using the *Galleria mellonella* model, with minor differences in inoculum preparation due to the work being performed in different laboratories. RIMD2210633 derived mutants were grown on TSA and incubated at 37°C for 24 h. Following growth, 1-2 discrete colonies were inoculated into 5 mL TSB and incubated with shaking at 37°C for 18 ± 2 h. The resulting cell suspensions were centrifuged to remove spent media, washed once in an equivalent volume of PBS and then adjusted with PBS to yield a final concentration of ~1.10^7 $CFU.mL^{-1}$. When testing the IFVp strains, due to issues with its growth, IFVp5 was replaced with IFVp4, which was also O3:K6 and harboured the same haemolysin

and T3SS profile (unpublished data). IFVp strains were initially grown on Heart Infusion (DIFCO ™, USA) agar containing 0.5% NaCl (HIS) at 37°C for 24 h, prior to aseptically transfer of a single colony into 20 mL of HIS broth and subsequent incubation with shaking for 8 h at 37°C. After incubation, cultures were washed twice and diluted with buffered-physiological water (BPW; 0.4 $g.L^{-1}$ of $K_2H_3PO_4$; 4.5 $g.L^{-1}$ of Na_2HPO_4, $12H_2O$; 7.2 $g.L^{-1}$ of NaCl) to obtain a *Vp* inoculum with final concentration of 5.10^6 $CFU.mL^{-1}$ (confirmed by plate enumeration onto HIS at 37°C for 24 h).

In both laboratories, larvae of *G. mellonella* measuring 2.0 to 3.0 cm in body length (body weight of approx. 200 to 300 mg) were used in infection experiments. For each experiment, 10 µL of *Vp* inoculum (~ 5.10^4 CFU of bacteria) was injected into the right foremost proleg of larvae (n = 20) using a syringe pump (KD Scientific, USA). Control groups (n = 20) were inoculated with PBS or BPW in the same conditions. Larvae were maintained in petri dishes at 37°C in the dark. All larvae were monitored for 24 h, and melanisation and survival were recorded. The percentage of survival was determined for all the strains at 24 HPI. Larvae were scored as dead when they did not show any response to touch. Three independent infection experiments were performed for each *Vp* strain. Data represent the mean survival (± standard error) of three independent experiments each consisting of 20 larvae per group.

Determination of LD_{50} was performed as described above by larvae injection with concentrations of *Vp* inoculum ranging from 5.10^2 to 5.10^6 CFU of bacteria. Mortality was recorded at 24 HPI as described above. LD_{50} was calculated as the concentration inducing 50% of mortality and was expressed in $CFU.mL^{-1}$.

g. Statistical analyses

All statistical analyses of the data were performed using RStudio 2021.09.0+351 "Ghost Orchid" Release (2021-09-20) for Windows (Core R Team, 2019) or in GraphPad Prism (version 8.4.3). Data are presented as means ± standard error and the significance level was set to $p < 0.05$. The proportion of infant rabbits infected with IFVp201 and IFVp195 at 15 HPI were compared using Fisher's exact test. Fluid accumulation ratios between IFVp201 and IFVp195 at 15 HPI in the three regions of small intestine were analysed using unpaired Student t-test. Concentrations of IFVp201 and IFVp195 at 15 HPI in the three regions of small intestine were log transformed prior to analyses using unpaired Student t-test. Percentages of larvae survival during bacterial infections between RIMD22210633 and its mutants, and between IFVp strains were evaluated using

ANOVA with strain as factor, followed by the post hoc Tukey test to determine where differences occur.

h. Genome accession numbers

Draft genomes of *Vp* strains are available from the European Nucleotide Archive (Project ID: PRJEB53525) under accession number shown in **Table S1**.

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SUPPLEMENTARY FIGURES AND TABLES

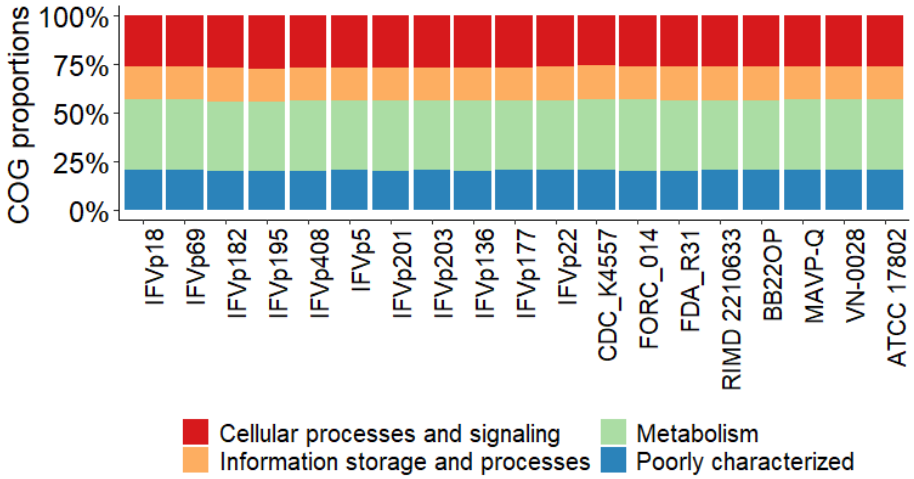

Fig S1. Cluster of Orthologous Genes of *Vibrio parahaemolyticus* genomes.

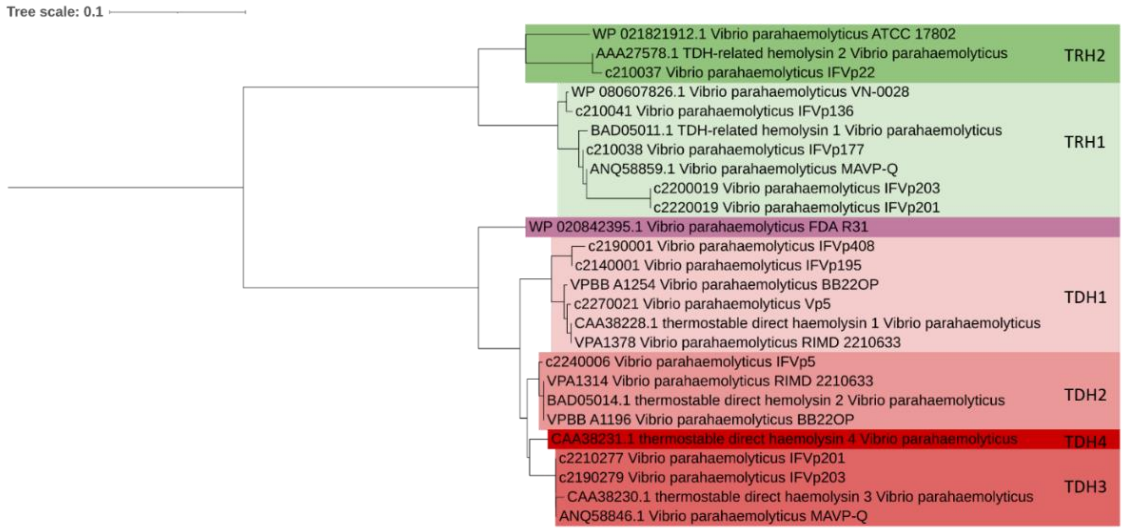

Fig S2. Minimum evolution tree (ME) constructed from TDH and TRH sequences
Tree was constructed using MEGA X and annotated using iTOL v6. Bold: reference sequences

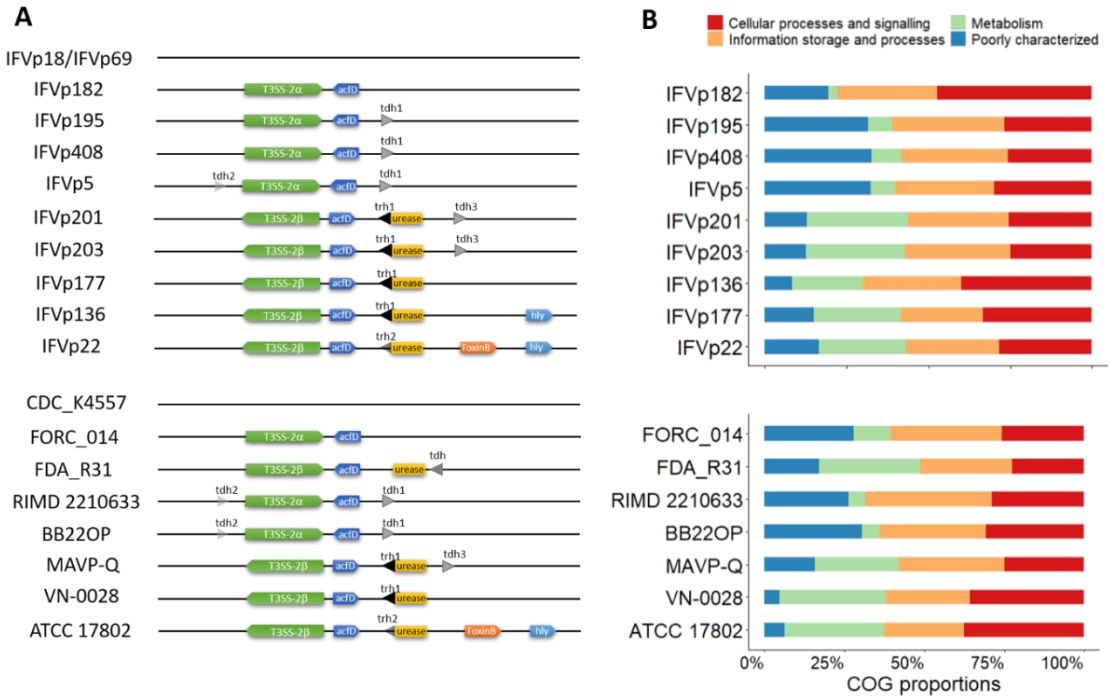

Fig S3. Pathogenicity island number 7 of *Vibrio parahaemolyticus* (VPaI-7).
(A) Schematic representation of the VPaI-7 from the 19 strains used in this study. (B) Cluster of Orthologous Genes (COG) analysis of the VPaI-7. Blue: poorly characterized, light green: metabolism, light orange: information storage and processes, red: cellular processes and signalling.

Table S1. General features of *V. parahaemolyticus* genomes sequenced in this study

Strain	Replicon	Size (kbp)	%GC	Genes	CDS	rRNA	tRNA	ST	Biosample accession No.
IFVp18	Ch. 1	3,300	45.19	3,281	3,145	5	93	2581	ERS12231012
	Ch. 2	1,805	45.43	1,685	1,657	2	10		
IFVp69	Ch. 1	3,004	45.17	2,949	2,833	4	77	2935	ERS12231019
	Ch. 2	1,965	45.59	1,847	1,802	1	25		
IFVp182	Ch. 1	3,329	45.21	3,308	3,172	3	88	1294	ERS12231013
	Ch. 2	2,076	45.2	2,112	2,060	1	29		
	Plasmide	40.18	41.45	46	45	0	0		
IFVp195	Ch. 1	3,000	45.19	2,891	2,787	2	68	1140	ERS12231014
	Ch. 2	1,851	45.26	1,824	1,796	2	9		
IFVp408	Ch. 1	3,203	45.26	3,110	2,999	2	71	1140	ERS12231018
	Ch. 2	1,853	45.27	1,826	1,794	2	13		
IFVp5	Ch. 1	3,217	45.28	3,107	2,988	5	77	3	ERS12231020
	Ch. 2	1,868	45.32	1,795	1,763	2	11		
IFVp201	Ch. 1	3,266	45.32	3,214	3,085	4	84	2942	ERS12231015
	Ch. 2	1,864	45.39	1,853	1,818	3	15		
IFVp203	Ch. 1	3,262	45.31	3,226	3,097	3	86	2942	ERS12231016
	Ch. 2	1,862	45.38	1,847	1,814	3	13		
IFVp136	Ch. 1	3,306	45.12	3,238	3,107	3	90	73	ERS12231010
	Ch. 2	1,850	45.3	1,772	1,736	3	15		
IFVp177	Ch. 1	3,281	45.26	3,283	3,149	5	90	1584	ERS12231011
	Ch. 2	1,877	45.22	1,772	1,742	3	10		
IFVp22	Ch. 1	3,284	45.26	3,267	3,138	4	86	987	ERS12231017
	Ch. 2	1,898	45.2	1,868	1,831	3	15		

Ch.: Chromosome, ST: Sequence Type, *: new ST

Tables S2. Gene count for each genome of *V. parahaemolyticus*.

Strains	No. of CDS			% of total CDS	
	Total	Core	Accessory	Core	Accessory
IFVp18	4,802	3,616	1,228	75	26
IFVp69	4,635	3,616	1,132	78	24
IFVp182	5,275	3,616	1,698	69	32
IFVp195	4,579	3,616	915	79	20
IFVp408	4,79	3,616	1,149	75	24
IFVp5	4,748	3,616	1,118	76	24
IFVp201	4,903	3,616	1,245	74	25
IFVp203	4,911	3,616	1,263	74	26
IFVp136	4,843	3,616	1,310	75	27
IFVp177	4,890	3,616	1,386	74	28
IFVp22	4,966	3,616	1,481	73	30
CDC_K4557	4,844	3,616	1,278	75	35
FORC_014	5,064	3,616	1,704	71	47
FDA_R31	5,117	3,616	1,800	71	50
RIMD 2210633	5,131	3,616	1,687	70	47
BB22OP	4,818	3,616	1,397	75	39
MAVP-Q	5,178	3,616	1,739	70	48
VN-0028	4,891	3,616	1,447	74	40
ATCC 17802	5,112	3,616	1,586	71	44

Tables S3. Genes over or under-represented in the accessory genome of *trh*⁺ and *trh*⁻ *V. parahaemolyticus* strains. Results from Scoary analysis

		Number of strains				
Gene	**Annotation**	**gene present in *trh+***	**gene present in *trh-***	**gene absent in *trh+***	**gene absent in *trh-***	***p*-value**
appA	Oligopeptide-binding protein AppA	8	1	0	10	0.013
ddpC	putative DD-dipeptide transport system permease protein DdpC	8	1	0	10	0.013
group_1068	hypothetical protein	8	1	0	10	0.013
group_121	hypothetical protein	8	0	0	11	0.013
group_1522	hypothetical protein	8	1	0	10	0.013
group_1524	hypothetical protein	8	1	0	10	0.013
group_1758	hypothetical protein	8	1	0	10	0.013
group_1759	hypothetical protein	8	1	0	10	0.013
group_1760	hypothetical protein	8	1	0	10	0.013
group_1761	hypothetical protein	8	1	0	10	0.013
group_196	hypothetical protein	8	1	0	10	0.013
group_2486	hypothetical protein	8	1	0	10	0.013
group_2488	hypothetical protein	8	1	0	10	0.013
group_2492	hypothetical protein	7	0	1	11	0.013
group_2494	hypothetical protein	8	1	0	10	0.013
group_2497	hypothetical protein	8	1	0	10	0.013
group_2502	hypothetical protein	8	1	0	10	0.013
group_2504	hypothetical protein	8	1	0	10	0.013
group_2647	hypothetical protein	8	1	0	10	0.013
group_2648	hypothetical protein	8	0	0	11	0.013
group_2650	hypothetical protein	8	1	0	10	0.013
group_2651	hypothetical protein	8	1	0	10	0.013
group_3647	hypothetical protein	8	1	0	10	0.013
group_3649	hypothetical protein	8	1	0	10	0.013
group_416	hypothetical protein	8	1	0	10	0.013
group_472	hypothetical protein	8	0	0	11	0.013
group_473	hypothetical protein	8	0	0	11	0.013
group_476	hypothetical protein	8	1	0	10	0.013
group_602	hypothetical protein	8	1	0	10	0.013
group_666	hypothetical protein	8	1	0	10	0.013
group_913	hypothetical protein	8	1	0	10	0.013
group_915	hypothetical protein	8	1	0	10	0.013
hns_2	DNA-binding protein H-NS	8	1	0	10	0.013
hrcN	Type III secretion ATP synthase HrcN	8	1	0	10	0.013
nikE	Nickel import system ATP-binding protein NikE	8	1	0	10	0.013
ompA_8	Outer membrane protein A	8	1	0	10	0.013
oppB_4	Oligopeptide transport system permease protein OppB	8	1	0	10	0.013
oppD_6	Oligopeptide transport ATP-binding protein OppD	8	1	0	10	0.013
pdeG_2	putative cyclic di-GMP phosphodiesterase PdeG	8	1	0	10	0.013
sctC_4	Type 3 secretion system secretin	8	1	0	10	0.013
spaP_2	Surface presentation of antigens protein SpaP	8	1	0	10	0.013
ureA	Urease subunit gamma	8	1	0	10	0.013
ureB	Urease subunit beta	8	1	0	10	0.013
ureC	Urease subunit alpha	8	1	0	10	0.013
ureD	Urease accessory protein UreD	8	1	0	10	0.013
ureE	Urease accessory protein UreE	8	1	0	10	0.013
ureF	Urease accessory protein UreF	8	1	0	10	0.013
ureG	Urease accessory protein UreG	8	1	0	10	0.013
ureR	Urease operon transcriptional activator	8	1	0	10	0.013
yedA	putative inner membrane transporter YedA	8	1	0	10	0.013
yscU_2	Yop proteins translocation protein U	8	1	0	10	0.013

Table S4. Prophages regions in the 19 V. parahaemolyticus genome.

Results from PHASTER analysis. Green: intact prophages, blue: questionable prophages and red: incomplete prophages.

strain	Chromosome	REGION_LENGTH	COMPLETENESS(score)	MOST_COMMON_PHAGE_NAME(hit_genes_cour	_PERCENTAG
IFVp18	1	20.4Kb	incomplete(40)	PHAGE_Entero_mEp390_NC_019721(2),PHAGE_E	41.84%
	1	8.5Kb	intact(131)	PHAGE_Vibrio_VfO3K6_NC_002362(8),PHAGE_Vi	44.95%
	2	No phage were found in this sequence!			
IFVp69	1	39.1Kb	incomplete(50)	PHAGE_Escher_500465_1_NC_049342(7),PHAGE_	41.25%
	2	No phage were found in this sequence!			
IFVp182	1	9Kb	intact(150)	PHAGE_Vibrio_Vf12_NC_005949(7),PHAGE_Vibri	46.86%
	1	56.8Kb	questionable(80)	PHAGE_Vibrio_12A4_NC_021068(22),PHAGE_Esch	43.31%
	2	No phage were found in this sequence!			
IFVp195	1	14Kb	incomplete(20)	PHAGE_Klebsi_ST147_VIM1phi7.1_NC_049451(4)	43.45%
	1	23.7Kb	incomplete(30)	PHAGE_Escher_500465_1_NC_049342(7),PHAGE_	43.61%
	2	14.7Kb	intact(112)	PHAGE_Vibrio_VFJ_NC_021562(9),PHAGE_Vibrio	44.36%
IFVp408	1	13.9Kb	incomplete(20)	PHAGE_Klebsi_ST147_VIM1phi7.1_NC_049451(4)	43.43%
	1	23.7Kb	incomplete(30)	PHAGE_Escher_500465_1_NC_049342(7),PHAGE_	43.61%
	2	14.7Kb	intact(112)	PHAGE_Vibrio_VFJ_NC_021562(9),PHAGE_Vibrio	44.36%
IFVp5	1	14.7Kb	incomplete(20)	PHAGE_Klebsi_ST13_OXA48phi12.1_NC_049453(4	44.63%
	1	24.3Kb	incomplete(40)	PHAGE_Escher_500465_1_NC_049342(7),PHAGE_	42.33%
	2	No phage were found in this sequence!			
IFVp201	2	13.7Kb	intact(100)	PHAGE_Vibrio_VFJ_NC_021562(8),PHAGE_Vibrio	47.38%
	2	8.4Kb	incomplete(40)	PHAGE_Mannhe_vB_MhM_1127AP1_NC_047750(	39.82%
	1	15.3Kb	incomplete(50)	PHAGE_Escher_500465_1_NC_049342(8),PHAGE_	44.32%
	1	71.1Kb	intact(100)	PHAGE_Vibrio_12A4_NC_021068(22),PHAGE_Shig	43.66%
IFVp203	1	15.3Kb	incomplete(50)	PHAGE_Escher_500465_1_NC_049342(8),PHAGE_	44.32%
	1	6.6Kb	intact(131)	PHAGE_Vibrio_VfO3K6_NC_002362(8),PHAGE_Vi	46.88%
	1	71.1Kb	intact(100)	PHAGE_Vibrio_12A4_NC_021068(22),PHAGE_Esch	43.66%
	2	12Kb	intact(100)	PHAGE_Vibrio_VFJ_NC_021562(8),PHAGE_Vibrio	47.05%
	2	8.4Kb	incomplete(40)	PHAGE_Mannhe_vB_MhM_1127AP1_NC_047750(	39.82%
IFVp136	1	31.8Kb	incomplete(60)	PHAGE_Escher_500465_1_NC_049342(7),PHAGE_	40.21%
	1	16.2Kb	incomplete(40)	PHAGE_Edward_GF_2_NC_026611(2),PHAGE_Pec	40.86%
	1	12.2Kb	intact(135)	PHAGE_Vibrio_VfO3K6_NC_002362(9),PHAGE_Vi	43.71%
	2	No phage were found in this sequence!			
IFVp177	1	22.2Kb	incomplete(40)	PHAGE_Escher_500465_1_NC_049342(8),PHAGE_	45.02%
	1	57.5Kb	intact(100)	PHAGE_Vibrio_12A4_NC_021068(22),PHAGE_Esch	43.39%
	1	16Kb	incomplete(40)	PHAGE_Pectob_ZF40_NC_019522(2),PHAGE_Edw	40.83%
	2	No phage were found in this sequence!			
Vp22	1	20Kb	incomplete(40)	PHAGE_Escher_500465_1_NC_049342(8),PHAGE_	43.40%
	1	56.2Kb	questionable(90)	PHAGE_Vibrio_12A4_NC_021068(23),PHAGE_Esch	42.88%
	1	16.2Kb	incomplete(40)	PHAGE_Edward_GF_2_NC_026611(2),PHAGE_Pec	40.88%
	2	6.7Kb	incomplete(30)	PHAGE_Shigel_SfII_NC_021857(2),PHAGE_Shigel	40.57%
CDC_K4557	1	8.5Kb	intact(116)	PHAGE_Vibrio_VFJ_NC_021562(8),PHAGE_Vibrio	45.82%
	2	No phage were found in this sequence!			
FORC_014	1	38.9Kb	intact(150)	PHAGE_Vibrio_8_NC_022747(21),PHAGE_Vibrio_	45.57%
	1	9.6Kb	intact(150)	PHAGE_Vibrio_VEJphi_NC_012757(9),PHAGE_Vib	43.79%
	1	66.7Kb	intact(100)	PHAGE_Vibrio_12A4_NC_021068(21),PHAGE_Shig	43.68%
	2	8Kb	intact(95)	PHAGE_Vibrio_VCY_phi_NC_016162(7),PHAGE_V	43.19%
FDA_R31	1	8Kb	intact(135)	PHAGE_Vibrio_VfO3K6_NC_002362(9),PHAGE_Vi	45.69%
	1	57.7Kb	incomplete(60)	PHAGE_Vibrio_12A4_NC_021068(23),PHAGE_Shig	43.68%
	1	48.8Kb	intact(150)	PHAGE_Aeromo_phiO18P_NC_009542(21),PHAGE	45.28%
	2	27.3Kb	intact(150)	PHAGE_Vibrio_VEJphi_NC_012757(9),PHAGE_Vib	43.18%
RIMD 2210633	1	10.6Kb	intact(150)	PHAGE_Vibrio_VfO3K6_NC_002362(10),PHAGE_V	43.98%
	2	No phage were found in this sequence!			
BB22OP	1	32.9Kb	intact(140)	PHAGE_Aeromo_phiO18P_NC_009542(19),PHAGE	49.85%
	2	7.8Kb	incomplete(20)	PHAGE_Gordon_GMA6_NC_030906(2),PHAGE_Ba	43.05%
MAVP-Q	1	3.7Kb	incomplete(10)	PHAGE_Gordon_Schwabeltier_NC_031255(1),PHA	42.22%
	1	16.3Kb	incomplete(20)	PHAGE_Stx2_vB_EcoP_24B_NC_027984(2),PHAGE	40.80%
	1	10.1Kb	incomplete(30)	PHAGE_Acinet_vB_AbaS_TRS1_NC_031098(1),PHA	45.87%
	2	No phage were found in this sequence!			
VN-0028	1	24.8Kb	incomplete(40)	PHAGE_Escher_500465_1_NC_049342(7),PHAGE_	42.59%
	2	No phage were found in this sequence!			
ATCC 17802	1	No phage were found in this sequence!			
	2	No phage were found in this sequence!			

Tables S5. Distribution of pathogenicity island in *V. parahaemolyticus* strains. Results from BLASTp.

Strains	VPal-1	VPal-2	VPal-3	VPal-4	VPal-5	VPal-6
	VP0380-VP0403	**VP0635-VP0643**	**VP1071-VP1094**	**VP2131-VP2144**	**VP2900-VP2910**	**VPA1253-VPA1270**
IFVp18	VP0380 + VP0382-VP0384 + VP0386-VP0388 + VP0394-VP0396 + VP0398 + VP0400 + VP0402-VP0403	VP0635-VP0636 + VP0643	VP1088 + VP1091-VP1094	VP3131-VP3139 + VP3142-VP3144	VP2900-VP2903 + VP2905-VP2910	-
IFVp69	-	VP0635-VP0636 + VP0638	VP1088 + VP1091-VP1094	-	-	-
IFVp182	-	VP0635-VP0636 + VP0643	VP1088 + VP1091-VP1094	-	-	VPA1258
IFVp195	-	VP0635-VP0636 + VP0643	VP1088 + VP1091-VP1094	-	-	VPA1258
IFvp408	-	VP0635-VP0636 + VP0643	VP1088 + VP1091-VP1094	-	-	VPA1258
IFvp5	VP0380 + VP0382-VP0384 + VP0386-VP0389 + VP0391-VP0392 + VP0394-VP0395 +	VP0635-VP0639 + VP0641-VP0643	VP1071 + VP1079-VP1088 + VP1091-VP1094	-	-	VPA1254-VPA1263 + VPA1266-VPA1270
IFVp201	-	VP0635-VP0636 + VP0638-VP0639 + VP0641-VP0643	VP1088 + VP1091-VP1094	-	-	-
IFVp203	-	VP0635-VP0636 + VP0638-VP0639 + VP0641-VP0643	VP1088 + VP1091-VP1094	-	-	-
IFvp136	-	VP0635-VP0636 + VP0643	VP1088 + VP1091-VP1094	-	-	-
IFVp177	VP0394	VP0635-VP0636 + VP0638-VP0639 + VP0641-VP0643	VP1088 + VP1091-VP1094	-	-	VPA1258
IFvp22	-	VP0635-VP0636 + VP0638-VP0639 + VP0643	VP1088 + VP1091-VP1094	-	-	VPA1258-VPA1259
CDC_K4557	VP0380 + VP0394 + Vp0398-VP0400 + VP0402-VP0403	VP0635-VP0639 + VP0641-VP0643	VP1090 + VP1093-VP1094	-	-	-
FORC_014	-	VP0635-VP0636 + VP0643	VP1088 + VP1091-VP1094	-	-	-
FDA_R31	VP0394	VP0635-VP0636 + VP0643	VP1088 + VP1091-VP1094	-	-	VPA1258-VPA1259 + VPA1266-VPA1270
RIMD 2210633	+	+	+	+	+	+
BB22OP	-	VP0635-VP0636 + VP0638-VP0639 + VP0643	VP1088 + VP1091-VP1094	-	VP2901-VP2903	VPA1257-VPA1258
MAVP-Q	-	VP0635-VP0643	VP1088 + VP1091-VP1094	-	-	VPA1258-VPA1259
VN-0028	VP0386 + VP0395 VP0388-VP0389 + VP0391-VP0392	VP0635-VP0636 + VP0638-VP0639 + VP0641-VP0643	VP1088 + VP1091-VP1094	-	-	-
ATCC 17802	-	VP0635-VP0636 + VP0643	VP1088-VP0089 + VP1091-VP1094	-	-	VPA1253

Table S6. Lethal dose 50 (LD50) of IFVp strains in *Galleria mellonella* larvae (Kéomurdjian, 2015)

Strain	DL50 (CFU/mL)
IFVp182	*ND*
IFVp69	$2{,}26 \times 10^{1}$
IFVp182	$1{,}70 \times 10^{6}$
IFVp195	$3{,}67 \times 10^{4}$
IFVp408	$6{,}02 \times 10^{3}$
IFVp5	$7{,}08 \times 10^{3}$
IFVp201	$4{,}27 \times 10^{5}$
IFVp203	$1{,}11 \times 10^{6}$
IFVp136	$7{,}81 \times 10^{4}$
IFVp177	$5{,}72 \times 10^{5}$
IFVp22	*ND*

ND: not determined

Conclusion

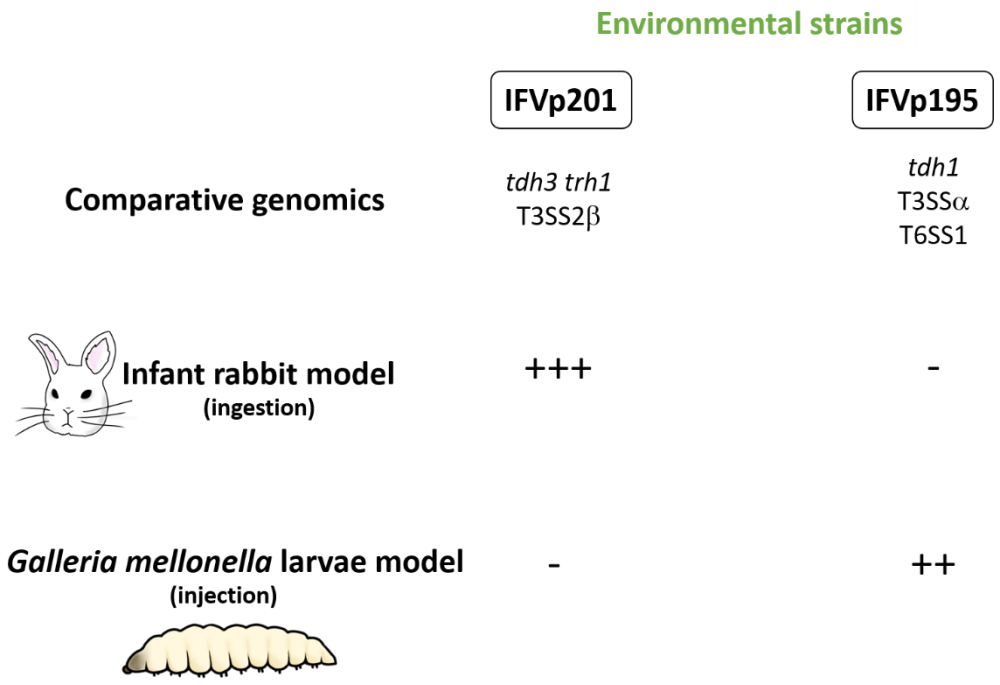

Figure 8. Résumé graphique de l'étude de genomes complets de *V. parahaemolyticus* et de l'expression *in vivo* de la virulence chez le modèle lapin et le modèle *Galleria mellonella* pour IFVp201 et IFVp195. +++ : très virulence, ++ : moyennement virulente, - : non virulente.

Cette étude comparative de génomes de *V. parahaemolyticus* et de l'expression *in vivo* de leur virulence chez deux modèles animaux soulignent la nécessité et l'importance de bien caractériser les marqueurs et mécanismes impliqués dans la virulence – d'autant

plus que ces marqueurs sont utilisés pour détecter les *V. parahaemolyticus* potentiellement pathogènes chez les produits issus de la mer. Afin de mieux gérer le potentiel risque d'infection à *V. parahaemolyticus*, il est aussi important d'identifier les facteurs influençant l'accumulation et la dépuration de *V. parahaemolyticus* chez les organismes marins, et plus particulièrement chez l'huître creuse, *C. gigas*.

Chapter II

Vibrio parahaemolyticus contamination in *Crassostrea gigas* oysters

Contexte

Les huîtres, sentinelles de l'environnement, sont des bivalves filtreurs capables d'accumuler des contaminants chimiques et biologiques présent dans l'eau. L'accumulation est le processus induisant l'augmentation de la concentration d'un contaminant dans l'huître. A contrario, la dépuration représente la diminution de cette concentration. La compréhension des facteurs influençant ces processus est cruciale pour évaluer les risques pour les consommateurs concernant les pathogènes humains tels que *V. parahaemolyticus* (Ndraha et al., 2020). De nombreuses études ont montré que les paramètres abiotiques tels que la température (Johnson et al., 2010) ou la salinité (Caburlotto et al., 2010; Rehnstam-Holm et al., 2014) influençaient la contamination des huîtres par *V. parahaemolyticus*. Cependant, des paramètres biotiques liés à l'huître peuvent être considérés comme des facteurs influençant cette contamination. Il a été montré que l'origine parentale, la génétique (Azéma et al., 2017; Dégremont et al., 2019), le microbiote (Petton et al., 2015) ou le système immunitaire (Lafont et al., 2020) des huîtres pouvaient être associés à une résistance ou sensibilité à une infection par OsHV-1 et/ou *Vibrio* spp.. A notre connaissance, l'implication de tels facteurs dans la contamination par *V. parahaemolyticus* chez l'huître n'a pas encore été étudiée.

Dans ce premier chapitre, nous avons cherché à savoir si l'historique de vie de l'huître d'une part et le niveau de ploïdie de l'huître d'autre part pouvaient impacter l'accumulation et la dépuration de *V. parahaemolyticus*. En effet, il a été montré que ces deux paramètres pouvaient influer sur la survie des huîtres lors d'épisodes de mortalités (Azéma et al., 2016; Fallet et al., 2022).

Dans un premier temps, nous avons étudié l'accumulation et la dépuration expérimentales d'une souche de *V. parahaemolyticus* chez des huîtres *C. gigas* élevées dans des installations contrôlées et des huîtres élevées dans la zone intertidale afin d'évaluer l'impact de l'exposition des huîtres à un environnement naturel sur la contamination en *V. parahaemolyticus*. Afin de différencier les *V. parahaemolyticus* indigènes et expérimentaux, les souches ont été génétiquement modifiées pour exprimer la GFP (Publication II).

Dans un second temps, nous avons cherché à savoir si le niveau de ploïdie impactait la contamination par *V. parahaemolyticus*. Nous avons utilisé trois lots d'huîtres 3N et d'huîtres 2N que nous avons collectées sur l'estran une fois par mois entre mai et novembre 2021 pour estimer la contamination naturelle de *V. parahaemolyticus* et l'accumulation et la dépuration expérimentale de *V. parahaemolyticus* (Publication III).

Publication II

Titre de l'article	**"Life history of oysters influences *Vibrio parahaemolyticus* accumulation in Pacific oysters (*Crassostrea gigas*)"**
Statut de l'article	Publié dans *Environmental Microbiology* DOI: 10.1111/1462-2920.15996
Objectif de l'étude	Caractériser l'effet de l'historique de vie de l'huître *C. gigas* dans l'accumulation et la dépuration de *V. parahaemolyticus*
Résultats principaux	Nous avons développé une méthodologie simple basée sur de l'électroporation, afin de différencier les *V. parahaemolyticus* indigènes et expérimentaux par l'expression de la GFP, et de la cytométrie en flux, afin de quantifier les *V. parahaemolyticus* GFP^+ dans des huîtres collectées en environnement. En utilisant cette méthodologie, nous avons montré que des huîtres élevées dans la zone intertidale accumulaient moins de *V. parahaemolyticus* que des huîtres élevées dans des structures sécurisées lors de contaminations expérimentales.
Conclusions	Moins de contamination à *V. parahaemolyticus* chez des huîtres élevées dans la zone intertidale que dans des structures sécurisées.

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Life history of oysters influences *Vibrio parahaemolyticus* accumulation in Pacific oysters (*Crassostrea gigas*)

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Summary

Vibrio parahaemolyticus **infection in humans is associated with raw oyster consumption. Evaluation of *V. parahaemolyticus* presence in oysters is of most interest because of the economic and public health issues that it represents. To explore *V. parahaemolyticus* accumulation and depuration in adult *Crassostrea gigas*, we developed a GFP-tagged *V. parahaemolyticus* strain (IFVp201-*gfp*$^{+}$), as well as a rapid and efficient quantification method in *C. gigas* oysters haemolymph by flow cytometry. Impact of the life history of *C. gigas* on accumulation and depuration of *V. parahaemolyticus* IFVp201 was subsequently investigated. We found that naive oysters, i.e. grown in controlled facilities with UV treated seawater, accumulated significantly more IFVp201 than environmental oysters, i.e. grown in intertidal environment. We hypothesized that environmental oysters could have been immune primed, thus could limit *V. parahaemolyticus* accumulation. Meanwhile, both naive and environmental oysters had similar depuration rates.**

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Introduction

Vibrio parahaemolyticus is a halophilic bacterium widely inhabiting estuarine and marine environments. *Vibrio parahaemolyticus* is the first causative bacterial agent of acute gastroenteritis in Human worldwide and has been extensively studied to distinguish pathogenic from non-pathogenic strains. To date, three major virulence factors have been characterized: two haemolysins named Thermostable Direct Haemolysin (TDH) and TDH-Related Haemolysin, and effectors of the type III secretion system number II (Sakurai *et al*., 1973; Honda *et al*., 1988; Park *et al*., 2004). *Vibrio parahaemolyticus* strains have been isolated in seawater and in association with other marine organisms in Europe, Asia and North and South America (World Health Organization, 1999; Baker-Austin *et al*., 2010; Luo *et al*., 2017). Review of 48 scientific publications conducted from 2003 to 2015 highlighted that oysters showed the highest percentage of contamination by *V. parahaemolyticus* (63.4%) in comparison to other marine organisms such as mussels, clams, fish or shrimp (Odeyemi, 2016). Thus, analysis of the accumulation and persistence of potentially pathogenic *V. parahaemolyticus* in oysters is important to evaluate the subsequent risk for humans consuming these oysters especially since factors associated with efficiency of accumulation and depuration of Human-pathogenic *Vibrio* are not well known (Froelich and Noble, 2014). Thus, identification of conditions and factors that limit/promote *V. parahaemolyticus* accumulation and depuration in oysters could help to improve risk management practices. Furthermore, the Pacific oyster, *Crassostrea gigas*, is a real economic issue worldwide. Most of the oyster production in France (98%) is based on *C. gigas* representing 437 million of US$ for 84 760 tons in 2019 (FAO, 2021).

Bivalve molluscs, in particular oysters, are considered sentinels of the environment because of their sessile characteristic and filtration capacity. Hence, oyster microbiota reflects the diversity of microbial environment that changes

in function of biotic and abiotic factors (Lokmer *et al*., 2016; Scanes *et al*., 2021). *Vibrio* spp. are an integral part of the marine bacterial diversity, explaining why they are frequently isolated from the microbiome of *Crassostrea* spp. oysters (Clerissi *et al*., 2020). Jones *et al*. (2014) showed that all oysters collected from environment during the proliferation period of *Vibrio* spp. (from May to September) were contaminated up to 4 log of most probable number per gram of oyster tissues of *V. parahaemolyticus* (Jones *et al*., 2014). Oysters can therefore represent an important ecological niche for *V. parahaemolyticus* and an issue for public health. This characteristic complexifies the study of particular *V. parahaemolyticus* strains in laboratory.

Numerous studies rely on the use of Green Fluorescent Protein (GFP) as genetic marker to track a specific *Vibrio* spp. strain in bivalve molluscs (Cabello *et al*., 2005; Travers *et al*., 2008; Aboubaker *et al*., 2013; Dubert *et al*., 2016). Flow cytometry (FCM) can subsequently be used for bacterial quantification, thus, simplifying the study of *Vibrio* spp. in marine organisms (Travers *et al*., 2008; Aboubaker *et al*., 2013) in comparison with standard microbiology procedures such as plate enumeration (PE). Introduction of exogenous DNA in *Vibrio* strain is generally performed by conjugation. This strategy, although efficient (but not in all strains), is time consuming, with a bi- or triparental mating between *E. coli* donor cells harbouring the plasmid of interest and the *Vibrio* transformants, sometimes with the use of an *E. coli* helper strain containing the conjugative machinery (Christensen *et al*., 2020). Recently, a rapid electroporation protocol was developed for *Vibrio harveyi* (Delavat *et al*., 2018). This transformation protocol allowed obtention of transformants after only 1–2 days, without the need of counter-selecting the *E. coli* strain(s).

In this study, we used the IFVp201 strain (environmental, *tdh*$^+$ *trh*$^+$), preliminary electroporated with a GFP-plasmid (IFVp201-*gfp*$^+$) to investigate accumulation and depuration in haemolymph of *C. gigas* oysters using FCM. The main objective of this study was to investigate the impact of life history of oysters on the accumulation and depuration of this *V. parahaemolyticus* strain. This was evaluated for naive oysters, i.e. grown in controlled facilities with filtered and UV treated seawater, and environmental oysters, i.e. grown in the intertidal environment.

Experimental procedures

Bacterial strains and culture conditions

The *V. parahaemolyticus* IFVp201 strain used in this study was isolated in 2009 from a sample of mussels (*Mytilus edulis*) in Poitou Charentes (France). This environmental strain was characterized as *tdh*$^+$ *trh*$^+$ (Lozach *et al*., in preparation) by qPCR, which are virulence genes widely present in clinical cases but rarely in environmental strains.

Wild type IFVp201 strain was grown overnight at 37°C on agar plates of Luria–Bertani containing 3% NaCl (LBS) and on LBS supplemented with 50 μg ml^{-1} of trimethoprim (LBS50) for IFVp201-*gfp*$^+$ (see 'Electroporation protocol'). A single colony formed on LBS or LBS50 agar plate was transferred into the corresponding liquid medium (LBS or LBS50) and was incubated at 37°C overnight at 50–70 rpm. Optical density of overnight culture was measured at 600 nm (OD600) using a Spectrophotometer UV/visible Evolution™ (Thermo Scientific™) and culture was plated on LBS or LBS50 (incubated overnight at 37°C) to estimate the concentration of each cultured isolate.

Before experimentation in filtered and sterile seawater (FSSW, natural seawater filtered with 1 μm polypropylene mesh and treated with UV), IFVp201-*gfp*$^+$ overnight cultures were washed twice using buffered-physiological water (BPW, 0.4 g L^{-1} of $K_2H_3PO_4$; 4.5 g L^{-1} of Na_2HPO_4, $12H_2O$; 7.2 g L^{-1} of NaCl) and analysed by FCM to determine bacterial concentration (log bacteria ml^{-1}).

Electroporation protocol

The plasmid used in this study was pFD086 harbouring *gfp* gene and trimethoprim-resistance expression cassettes (Morot *et al*., 2021). IFVp201 strain was electroporated as described previously (Delavat *et al*., 2018). The positive clones were confirmed by PCR amplification on colony with the primers 181101 and 181102 (Morot *et al*., 2021), and with VP32 and VP33 (Lee *et al*., 1995) which target the pFD086 plasmid and the pR72H region for identification at the species level respectively. PCR amplifications were performed using a Taq DNA Polymerase Good Manufacturing Product grade (Roche Diagnostics) with the following thermal cycle: 5 min of initial denaturation at 95°C followed by 35 cycles at 95°C for 30 s, 56°C for 1 min and 72°C for 1 min 30 s. To verify GFP expression, colonies were placed under UV light to visualize fluorescence emission.

Estimation of plasmid stability in filtered and sterile seawater

The ability of pFD086 to persist in IFVp201-*gfp*$^+$ was determined in FSSW. For this purpose, overnight cultures washed twice in BPW were diluted to obtain a final concentration of 10^8 CFU ml^{-1}. This bacterial culture was suspended in FSSW up to a final concentration of 10^5 CFU ml^{-1}. At 0, 24 and 48 h FSSW samples were plated onto LBS agar and incubated overnight at 37°C. Fifty isolated colonies were plated onto LBS50 and then onto LBS (overnight at 37°C). The stability percentage

was estimated as the number of colonies grown onto LBS50 divided by number of colonies grown onto LBS and multiplied by 100.

Growth kinetics of wild type and Vp-gfp$^+$ *strains*

Overnight cultures of wild type IFVp201 and IFVp201-*gfp*$^+$ were carried out in LBS and in LBS50 respectively, at 37°C. The OD600 of the overnight cultures were measured and cultures of IFVp201 and IFVp201-*gfp*$^+$ were 100-fold diluted in LBS and in LBS50 respectively, then incubated at 37°C. Concentrations of IFVp201 and IFVp201-*gfp*$^+$ in the 100-fold diluted cultures were determined in triplicates by PE on LBS and LBS50 respectively and incubated at 37°C overnight. The OD600 of these diluted cultures were measured every hour for 8 h. The experiment was performed in triplicate.

The doubling time (*G*) corresponds to the time allowing the doubling of bacterial population during the exponential growth phase. Graphically, *G* was calculated as the time between $OD600_t$ at a *t* time and $2 \times OD600_t$.

*Quantification of IFVp201-*gfp$^+$ *by FCM*

Overnight cultures of IFVp201-*gfp*$^+$ were used to establish the settings of quantification by FCM. Bacteria were identified and counted using a Cyflow Space (Sysmex-Partec, Munster, Germany). The bacterial counting was performed according to the number of cells detected in a volume of 200 µl of sample. They were detected by their green fluorescence (FL1 detector of the flow cytometer) and their relative size and granularity [Forward SCatter (FSC); Side SCatter (SSC)]. Concentrations were expressed in log bacteria ml^{-1}.

*Preliminary assays of oyster contamination with IFVp201-*gfp$^+$

The first assay was performed to select the most appropriate matrix for IFVp201-*gfp*$^+$ quantification, i.e. haemolymph or oyster tissues, and to estimate the appropriate concentration of IFVp201-*gfp*$^+$ that could be used for the following experimentations of this study. Adult diploid *C. gigas* (approx. 30–40 g of total weight and 7 cm long) were bought from an oyster farmer located in the Bay of Brest (Pointe du Chateau, Finistère, France, 4°19′3″ W, 48°20′2″ N) in February 2020. Experimentation occurred less than 10 h after collection from their culture site. IFVp201-*gfp*$^+$ was inoculated at initial concentrations of 10^5 CFU ml^{-1} (5 log) and 10^7 CFU ml^{-1} (7 log) in 2 L of FSSW into two separate tanks. Four oysters ($n = 4$) were then placed into each tank and maintained overnight at room temperature (20°C–22°C). Control oysters ($n = 4$) not exposed to IFVp201-*gfp*$^+$ were kept in the same conditions. After the overnight accumulation, and using sterile tools and workstation, each oyster was shucked, and abductor muscles were cautiously cut with a scalpel to allow opening of the shell and avoid damaging of the pericardial cavity. Haemolymphs were collected into the pericardial cavity using a 25G needle and 1 ml insulin syringe. Collected haemolymphs (approx. 1–2 ml) were filtered on a 30 µm nylon mesh to remove aggregates and/or debris. All oyster tissues were shredded during 3×30 s at low speed using a Laboratory blender (Waring Blender). Analyses were performed at the individual level, and were carried out by FCM and by PE (on LBS50 and incubated overnight at 37°C) for both the haemolymph and oyster tissues.

This second assay was performed to validate experimental design. Wild diploid *C. gigas* (named E1), caught on the Atlantic coast of Charente Maritime (France), were maintained in mesh bags at La Floride (Ronce les Bains, Charente Maritime, France, 1°09′15″ W, 45°48′12″ N). These oysters were collected in February 2021 and transferred within 24 h at the Ifremer experimental platform at Bouin (Vendée, France). IFVp201-*gfp*$^+$ was inoculated in one 20 L tank of FSSW with initial concentration of 10^5 CFU ml^{-1}. Initial concentrations of IFVp201-*gfp*$^+$ in FSSW were verified in triplicates by FCM in the tank. E1 oysters were placed in the contaminated tank ($n = 15$) and in a control 20 L tank of FSSW not exposed to IFVp201-*gfp*$^+$ ($n = 9$), and maintained at 19°C for an overnight balneation (17–19 h). Aeration was provided to all tanks. After the overnight balneation, IFVp201-*gfp*$^+$ concentration in seawater was determined in triplicates by FCM and oysters were transferred 1 h into 'fresh' FSSW to remove potential IFVp201-*gfp*$^+$ present on shells and in shell fluid. Haemolymphs were collected as described previously from one pool of three oysters from each batch and IFVp201-*gfp*$^+$ was quantified by FCM in each pool (two analytical replicates for each pool). Remaining oysters from the batch exposed to IFVp201-*gfp*$^+$ ($n = 12$) and from the control batch ($n = 6$) were transferred in 6 L tanks of FSSW and maintained at 19°C for 24 h of depuration. After this depuration, haemolymphs were collected from three pools and one pool of three oysters from the batch exposed to IFVp201-*gfp*$^+$ and from the control batch respectively. IFVp201-*gfp*$^+$ was quantified by FCM in each pool (two analytical replicates for each pool).

*Challenge tests of naive and environmental oysters with IFVp201-*gfp$^+$

The following experiment was performed in order to evaluate the impact of the life history of oysters on *V. parahaemolyticus* accumulation and depuration. Experimental protocol is represented in Fig. S1.

For this experiment, two groups of adult oysters (approx. 30–40 g of total weight and 7 cm long) with three batches for each group were tested: naive oysters, i.e. grown in controlled inland facilities using sand-filtered and UV-treated seawater, and environmental oysters, i.e. grown in the intertidal environment. For the naive group, the three selected batches were hatchery-produced diploid oysters and were always maintained in controlled inland facilities at Ifremer. Two batches, called A1 and A2, were maintained in raceways at the experimental platform at Bouin (Vendée, France), and the third, called A3, was maintained in raceway at the experimental platform at La Tremblade (Charente Maritime, France). For the environmental group, two wild diploid stocks, E1 (as in the second assay) and E2 from Chaucre (Saint-Georges-d'Oléron, Charente Maritime, France, 1°23′44″ W, 45°58′56″ N), as well as one hatchery-produced diploid batch (E3) were used. E3 was bought to an oyster farmer that raised them near Bouin coasts (Port des Champs, Vendée, France, 2°2′54″ W, 46°57′54″ N). All six oyster batches were transferred at the experimental platform at Bouin within 24 h after collection and experimentations were carried out upon arrival at the platform in October 2021.

IFVp201-*gfp*$^+$ was inoculated in six 10 L tanks of FSSW with initial concentration of 10^5 CFU ml^{-1}. Initial concentrations of IFVp201-*gfp*$^+$ were verified in triplicates by FCM in each tank. Each batch of oysters was individually placed in the contaminated tank ($n = 30$) and in six control 4 L tanks of FSSW not exposed to IFVp201-*gfp*$^+$ ($n = 15$), and maintained at 19°C for an overnight balneation (17–19 h). Aeration was provided to all tanks. After the overnight balneation, IFVp201-*gfp*$^+$ concentration in seawater was determined in triplicates by FCM and oysters were transferred 1 h into 'fresh' FSSW. Haemolymphs were collected as described previously from two pools and one pool of five oysters from each batch exposed to IFVp201-*gfp*$^+$ and each control batch respectively, and IFVp201-*gfp*$^+$ was quantified by FCM in each pool (two analytical replicates for each pool). Remaining oysters from batches exposed to IFVp201-*gfp*$^+$ ($n = 20$) and from the control batch ($n = 10$) were transferred in 10 and 4 L tanks of FSSW respectively and maintained at 19°C for 24 h of depuration. After this depuration, haemolymphs were collected from three pools and one pool of five oysters from each batch exposed to IFVp201-*gfp*$^+$ and each control batch respectively, and IFVp201-*gfp*$^+$ was quantified by FCM in each pool.

Statistical tests

Data used to investigate reliability and agreement between FCM and PE represented a wide range of *Vp*-*gfp*$^+$ enumeration by FCM (in log bacteria ml^{-1}) and PE (in log CFU ml^{-1}) in different mediums and for different strains. Bland–Altman plot was used to explore the agreement between the two methods (Bland and Altman, 1986). The graphical representation shows the difference between enumeration by FCM and by PE for one sample in function of average enumeration between FCM and PE for one sample. The intraclass correlation coefficient (ICC) was used to explore the reliability that reflects both the degree of correlation and of agreement between the FCM and the PE methods. ICC values were calculated using a two-way mixed model with absolute agreement for single measurements. We rated ICC values as poor if 0–0.5, fair if 0.5–0.75, good if 0.75–0.9 and excellent if 0.9–1 (Koo and Li, 2016; R Core Team, 2019).

All statistical analyses were performed using RStudio 2021.09.0 + 351 'Ghost Orchid' Release (2021-09-20) for Windows. Significance level was set to $p < 0.05$. Prior further analyses, normality and variance homogeneity of the data were tested using Shapiro test and Levene test respectively. For the following analyses, quantification of IFVp201-*gfp*$^+$ in seawater (SW) and, in haemolymph after the overnight accumulation (ACCU) and after 24 h of depuration (DEPU) were analysed between naive and environmental oysters (CONDITION), and between each batch of naive and environmental oysters (BATCH). Comparisons of SW were carried out using non-parametric Kruskal–Wallis test and *post hoc* Dunn test in function of CONDITION and BATCH. Comparisons of ACCU were carried out using parametric one-way ANOVA and *post hoc* Tukey test in function of CONDITION and BATCH. Comparisons of DEPU were carried out using an ANCOVA with ACCU as covariable and *post hoc* Tukey test in function of CONDITION and BATCH. Comparisons of ACCU with DEPU were carried out using a parametric one-way ANOVA and *post hoc* Tukey test for each CONDITION and BATCH. This last analysis had for purpose to determine significance of depuration rates.

Results

Construction and validation of IFVp201-gfp$^+$ *transformants*

IFVp201 was electroporated with the pFD086 plasmid harbouring the *gfp* gene (Morot *et al*., 2021). Transformation efficiency was 1.2×10^3 CFU µg^{-1} of DNA. Plasmid presence in the transformants was confirmed by PCR and by visualization of fluorescent colonies under UV light (not shown). Assays of plasmid stability in FSSW showed that pFD086 plasmid persisted in 100% of IFVp201-*gfp*$^+$ strain after an inoculation of 10^5 CFU ml^{-1}

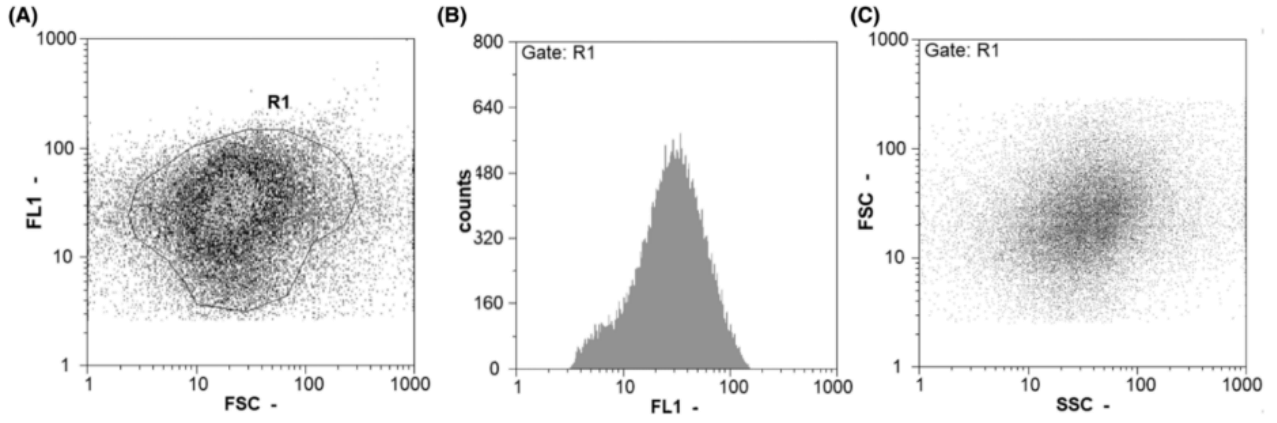

Fig. 1. Detection of IFVp201-*gfp*$^+$ by FCM. Results of overnight culture of IFVp201-*gfp*$^+$ in LBS diluted up to 10^5 CFU m^{-1} in buffered-physiological water based on OD600. Properties of green fluorescence (FL1, A and B), Forward Scatter (FSC, A and C) and Side Scatter (SSC, C) are shown.

in FSSW for 48 h. This result showed that the construction was stable in experimental conditions.

To determine the impact of pFD086 plasmid, growths of IFVp201-*gfp*$^+$ and IFVp201 strains were compared. Interestingly, OD600 of IFVp201 and IFVp201-*gfp*$^+$ of the overnight cultures were 1.41 $\pm$ 0.09 and 1.74 $\pm$ 0.04 respectively. However, concentrations in the 100-fold cultures before growth monitoring were 7.00 $\pm$ 0.13 log CFU ml^{-1} and 7.00 $\pm$ 0.11 log CFU ml^{-1} for IFVp201 and IFVp201-*gfp*$^+$ respectively. Only a minor difference was observed between the growths after 8 h of culture. The difference of doubling time between wild type IFVp201 and IFVp201-*gfp*$^+$ strains was 3 min (30 and 27 min respectively) thus showing that the pFD086 plasmid did not impact growth of IFVp201. Moreover, colonies size and aspect were similar for wild type and IFVp201-*gfp*$^+$ strains.

Quantification of IFVp201-gfp$^+$ *culture by FCM*

Quantification by FCM was first set from a pure culture of IFVp201-*gfp*$^+$. A dot plot of FL1 against FSC was used to select the gate in which particles were identified as IFVp201-*gfp*$^+$, named R1 population (Fig. 1A). Dot plot of FSC against SSC, and histogram of count of particles against FL1 were used to confirm the identification of R1 population by gating results on R1 population (Fig. 1B and C). Importantly, no event was detected in the R1 gate for control LBS, control FSSW and for wild type IFVp201 strains (not shown).

Selection of oyster matrix (haemolymph or tissues) and quantification method (FCM or PE)

Haemolymphs and oyster tissues were analysed for IFVp201-*gfp*$^+$ quantification by FCM and PE. For the control oysters not exposed to IFVp201-*gfp*$^+$ strain, 4.60 log event ml^{-1} (in case of control oysters, we could not express the concentration in log bacteria ml^{-1}) were detected by FCM in R1 gate in oyster tissues, whereas no event was detected in haemolymph. The events detected in control oysters were associated to autofluorescence of oyster tissues, and if considered, they would impair bacterial quantification by fluorescence measurement in contaminated conditions. Consequently, only haemolymph was considered for IFVp201-*gfp*$^+$ enumeration by FCM. After an oyster exposition to 5 log of IFVp201-*gfp*$^+$, 3.32 log bacteria ml^{-1} and 3.01 log CFU ml^{-1} were enumerated in haemolymph by FCM and PE respectively. A similar slight overestimation was found for oysters exposed to 7 log of IFVp201-*gfp*$^+$ with 7.04 log bacteria ml^{-1} and 6.93 log CFU ml^{-1} by FCM and PE respectively. As a concentration of 5 log allowed a sufficient accumulation for further analysis, this concentration was used for the subsequent experiments using haemolymph as sample.

To assess the concordance of IFVp201-*gfp*$^+$ quantification by FCM in comparison to PE in several media (LBS, seawater, haemolymph and artificial seawater), a Bland and Altman graphical representation was carried out (Bland and Altman, 1986) (Fig. 2). The bias between FCM and PE was 0.44 log bacteria ml^{-1} and the standard deviation was 0.27 log bacteria ml^{-1}. The 95% confidence interval (limits of agreement, in red dashed line in Fig. 2) for the bias was between 0.96 and $-$0.09 log bacteria ml^{-1} that contained 20/22 (91%) of the values. The standard error of the limit was 0.10 log bacteria ml^{-1}. This analysis suggests that this overestimation was not significant because the 95% confidence interval included the '$y = 0$' axis. The ICC for the enumeration method was 0.975 with a 95%-confidence interval ranging between 0.387 and 0.994.

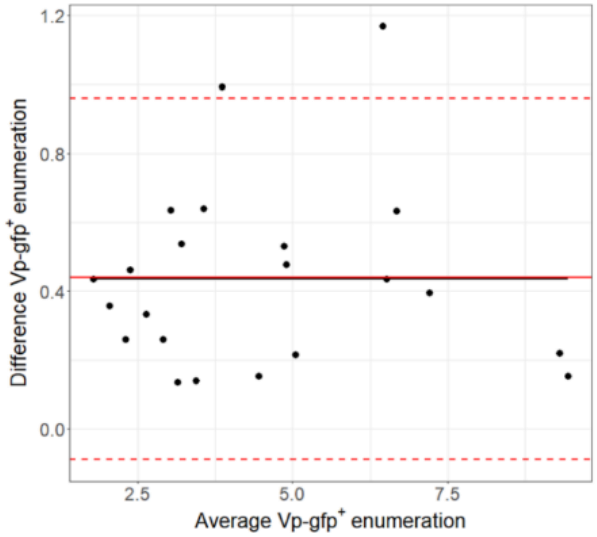

Fig. 2. Bland and Altman representation for IFVp201-*gfp*$^+$ enumeration in different media by FCM and PE. *X*-axis: average enumeration by FCM and by PE for one sample. *Y*-axis: difference between the enumeration by FCM and by PE for one sample. Red solid line: bias; red dashed line: limits of agreement/95% confidence interval.

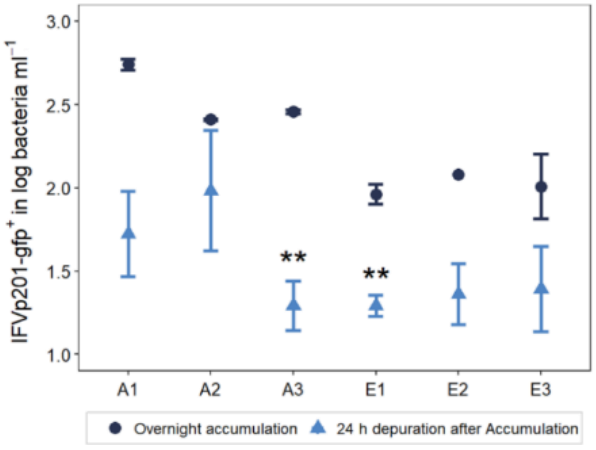

Fig. 3. IFVp201-*gfp*$^+$ quantification by FCM in haemolymphs of oysters after an overnight accumulation (black circles) and after 24 h of depuration (blue triangles). Oysters exposed overnight to 10^5 CFU mL^{-1} of IFVp201-*gfp*$^+$ then depurated for 24 h, A1-A2-A3: naive oysters, E1-E2-E3: environmental oysters. Data represent the mean of two pools of five oysters (two analytical replicates per pool) for overnight accumulation and the mean of three pools of five oysters (two analytical replicates per pool) for 24 h depuration ± standard error. Statistical analysis: one-way ANOVA and *post hoc* Tukey tests ($p < 0.05$).

In order to validate the experimental design and IFVp201-*gfp*$^+$ quantification by FCM, an assay was performed in February 2021 with E1 oysters. After the overnight accumulation, 3.22 log bacteria ml^{-1} of IFVp201-*gfp*$^+$ were quantified in haemolymph. Concentration of IFVp201-*gfp*$^+$ after 24 h of depuration was 0.81 log bacteria ml^{-1} thus the depuration rate of E1 was significant ($p < 0.001$). Those results confirmed the experimental design previously established.

Impact of the life history of oysters on accumulation and depuration of IFVp201-gfp$^+$

In order to evaluate the impact of life history of naive and environmental oysters on accumulation (during 17–19 h) and depuration (during 24 h) of IFVp201-*gfp*$^+$, strains were determined in haemolymphs. Initial concentrations of IFVp201-*gfp*$^+$ in tanks (before oyster exposure) were 5.26 ± 0.03 log bacteria ml^{-1}, whereas no bacteria was detected in control tanks. After the overnight accumulation in oysters, concentrations of IFVp201-*gfp*$^+$ in seawater were 4.70 ± 0.26 and 4.58 ± 0.13 log bacteria ml^{-1} for environmental and naive oysters respectively and no bacteria was detected in control tanks. No significant difference ($p = 0.749$) of concentration was observed between each tank before and after accumulation. Figure 3 shows IFVp201-*gfp*$^+$ concentration in haemolymph of oysters directly after the overnight accumulation and followed by 24 h of depuration.

Control oysters were all negative for IFVp201-*gfp*$^+$ by FCM. Batches of naive oysters (A-batches) accumulate significantly more IFVp201-*gfp*$^+$ than batches of environmental oysters (E-batches) ($p < 0.001$). Batches comparison showed that the difference of accumulation was only significant between A1 and E3 ($p = 0.0169$), and between A1 and E1 ($p = 0.013$), and on other fronts, between A1 and A2 ($p = 0.00265$) and between A1 and A3 ($p = 0.00419$). In contrast, IFVp201-*gfp*$^+$ concentrations after 24 h of depuration were not significantly different between A-batches and E-batches ($p = 0.55$). Finally, depuration rate (between the overnight accumulation and 24 h of depuration) was significant for E-batches ($p < 0.001$) and A-batches ($p = 0.00142$). Batches comparison showed that depuration rate was only significant for A3 ($p = 0.00878$) and E1 ($p = 0.00565$). Environmental oysters accumulated more but depurated equally to naive oysters.

Discussion

Study of human-pathogenic bacteria in filter feeding bivalves is a major challenge for public health worldwide. *Vibrio parahaemolyticus*, first causal bacterial pathogen agent of gastroenteritis by seafood consumption, is a bacterium naturally present in marine and estuarine environments. The first shellfish implicated in these foodborne diseases are the cupped oyster (Odeyemi, 2016), *Crassostrea gigas* (Asia and Europa) and *Crassostrea virginica* (United States). Analysis of *V. parahaemolyticus*

accumulation and persistence in oysters and investigation of the conditions that favour or not accumulation and persistence are important to evaluate the risk for oyster consumers (Froelich and Noble, 2014).

The study of particular bacterial strains in laboratory requires the use of new methodologies in order to differentiate indigenous *Vibrio* spp. from *Vibrio* spp. experimentally accumulated in bivalve. The use of fluorescent molecules such as GFP was an interesting way to visualize and quantify *V. parahaemolyticus* accumulated in oysters in experimental conditions. In this study, an electroporation approach (Delavat *et al*., 2018) was used to produce the strain IFVp201-*gfp*$^+$ (Morot *et al*., 2021). To our knowledge, it is the first time that transformants of *V. parahaemolyticus* are obtained by an electroporation method. This method allowed fast production of transformants (2 working days) in contrast to conjugation (1 week), commonly used for marine bacteria (Cabello *et al*., 2005;Travers *et al*., 2008; Aboubaker *et al*., 2013). Electroporation efficiency for the IFVp201-*gfp*$^+$ was comparable to what was obtained with *Vibrio harveyi* in Delavat *et al*. (2018), i.e. around 10^3 CFU μg^{-1} DNA. Moreover, it is known that an exogenous plasmid could have an effect on bacterial physiology like bacterial growth. For example, Muturi *et al*. (2019) showed that GFP-tagged bacterial strains had lower growths than the wild type strains (Muturi *et al*., 2019). In our study, GFP-plasmid presence showed a minor difference between IFVp201 and IFVp201-*gfp*$^+$ growth, suggesting that GFP-plasmid did not have any noticeable impact on the growth of IFVp201-*gfp*$^+$.

The scope of application of FCM expands, allowing characterization of cell types, verification of cell viability and cell quantification. FCM was used in previous studies for the quantification of bivalve pathogens *Vibrio* spp. (Travers *et al*., 2008; Aboubaker *et al*., 2013). In our study, production of IFVp201-*gfp*$^+$ allowed us to develop a protocol for immediate quantification of bacterial culture or biological samples by FCM (GFP detection). FCM represented a real advantage in terms of time gained for experimentation. In our study, the reliability and agreement between FCM and PE were checked. On the one hand, Bland and Altman representation (Bland and Altman, 1986) showed that bacterial concentration calculated by FCM was slightly overestimated in comparison to PE. However, this overestimation was minimal and not significant at a concentration of 10^5 CFU ml^{-1}. This difference could be explained by the fact that the quantification by FCM did not depend on bacterial culturability in contrast to PE. This was also considered by Gao *et al*. (2018) who showed that quantification of *Bifidobacterium longum* by FCM was four times higher than by PE (Gao *et al*., 2018). On the other hand, determination of the ICC (Koo and Li, 2016) showed that the reliability between the two methods was 'excellent' (ICC > 0.90). Thus, we showed that FCM was a good alternative to PE for *Vp-gfp*$^+$ quantification. In contrast to molecular approaches (e.g. qPCR), FCM quantified viable cells only. Indeed, the dead fluorescence-tagged bacteria not expressing the fluorescence (Lowder *et al*., 2000). On this basis, qPCR paired with propidium monoazide was developed to quantify only viable cells and proved to be a reliable for quantification of viable *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* in biofilm (Sánchez *et al*., 2014). Unlike molecular approaches, which need DNA extraction and could result in biological material loss, FCM allows immediate quantification of individual cells. The prerequisite of FCM use is the preliminary tagging of bacteria with fluorescent tag. As a result, FCM appears to be the simplest method for bacterial quantification in oysters exposed to fluorescent bacteria in an experimental setting.

Enumeration of *V. parahaemolyticus* from shellfish samples is commonly carried out with oyster tissues homogenates using cultural methods (NSSP, 2017). The use of FCM in our study was shown to be reliable and a promising method to replace cultural methods for experiments using fluorescence-tagged bacteria. In our study, auto-fluorescence of oyster tissues complicated the interpretation of FCM results, and haemolymph was an interesting alternative for quantification of IFVp201-*gfp*$^+$ by FCM during experimental infections of oysters. Indeed, Cabello *et al*. (2005) used a GFP-tagged *V. parahaemolyticus* strain (ATCC 17802) to study its fate and its capacity of proliferation in oysters (*Ostrea chilensis*). They showed that concentration of *Vp-gfp*$^+$ did not differ significantly between different individual tissues, including pericardium (Cabello *et al*., 2005). Furthermore, the presence of fluorescent particles in R1 population in tissues of control oysters showed that haemolymph was a better matrix for FCM analyses than oyster tissues. Concerning the IFVp201-*gfp*$^+$ initial concentration, although an exposure of 7 log showed a higher accumulation, 5 log provided a sufficient accumulation in haemolymph and was commonly used in literature for bacterial balneation with bivalves (Travers *et al*., 2008; Aagesen *et al*., 2013, 2018). This explained why we used the IFVp201-*gfp*$^+$ initial concentration of 5 log for bacterial quantification in haemolymph by FCM. Thus, we developed a reliable protocol to challenge oysters considering *V. parahaemolyticus* contamination, which could be adapted to other fluorescence-tagged bacteria infecting oysters or any other bivalve molluscs.

In order to evaluate the impact of life history of oysters on the accumulation and depuration of *V. parahaemolyticus*, environmental oysters and naive oysters maintained in secured structures were used. We

observed that naive oysters accumulated significantly more IFVp201 than environmental oysters. First, these results could be explained by the fact that filtration capacity of naive oysters could be less effective than the one of environmental oysters. However, no significant difference in IFVp201 concentration was observed in seawater after the overnight accumulation between naive and environmental oysters and each oyster batch. This result indicated that filtration capacities of the six batches of oysters were quite similar and thus, would not explain the accumulation differences between naïve and environmental oysters. Moreover, it was shown that the activity times of oysters linked to clearance rate and oxygen consumption (feeding time activity and respiratory activity) did not permit to distinguishing the oysters, when tested at 20°C, as a function of their life history trait (naive oysters vs. environmental oysters), their ploidy level (diploid and triploid) and their tolerance for OsHV-1 (Haure *et al*., 2021). Second, these results could be explained by the fact that naive oysters have never been exposed to environmental microflora, including *Vibrio* spp., contrary to environmental oysters. Indeed, Zhang *et al*. (2014) and Lafont *et al*. (2020) showed that a first exposure to agents (*Vibrio vulnificus* and OsHV-1 respectively) could lead to a development of a stronger immune response of the oyster for future reinfection (Zhang *et al*., 2014; Lafont *et al*., 2020). This phenomenon is called the innate immune priming. Furthermore, our results could also be explained by the oyster microbiota composition and diversity. Indeed, Lokmer *et al*. (2016) showed that bacterial community of oyster haemolymph varied between laboratory conditions and after field deployment (Lokmer *et al*., 2016). Thus, oyster haemolymphs from field condition were characterized by lower relative abundances of ε-, γ-proteobacteria (especially *Arcobacter* and *Vibrionaceae*) and Flavobacteria, and by higher relative abundances of α-proteobacteria, Mollicutes and bacterium related to Spirochaetes in comparison to oyster haemolymphs from laboratory condition. To our knowledge, characterization of haemolymph microbiota was never studied as a condition to favour or not bacterial accumulation or persistence unlike digestive gland microbiota which was shown to be linked to resistance or sensitivity to Pacific Oyster Mortality Syndrome (POMS). Indeed, previous studies showed that *Mycoplasmatales* (Clerissi *et al*., 2020) as well as *Vibrionaceae* (*Vibrio* and *Pseudomonas*) in digestive gland microbiota (King *et al*., 2019; Clerissi *et al*., 2020) were linked to higher sensitivity of oysters to POMS. Moreover, Offret *et al*. (2020) showed that oysters grown in controlled inland facilities at Ifremer (like naive oyster from our study) were characterized by high levels of *Mycoplasmatales* (Offret *et al*., 2020). Those results could explain why naive oysters accumulate more IFVp201 than environmental oysters from our study. Moreover, it was shown that oyster genetic take part in resistance or sensitivity of oysters against pathogens (Dégremont *et al*., 2015). In contrast, this was not studied for human pathogenic *Vibrio*. Indeed, it could be interesting to be taken into account for further analyses. Moreover, the presence of this microbiota could lead to niche competition with exogenous bacteria, i.e. bacteria experimentally accumulated. Srivastava *et al*. (2009) showed that treatment of *C. virginica* oysters, collected from environment, with tetracycline resulted in the decrease of more than 3 log CFU g^{-1} of indigenous *V. vulnificus*. That treatment allowed the accumulation of *V. vulnificus* strain during experimental infection at approximately 4.5 log CFU g^{-1} after a 24 h exposure at 10^6 CFU ml^{-1} which was comparable to experimental *V. vulnificus* accumulation in oysters collected during the winter months (Srivastava *et al*., 2009). In our study, oysters from intertidal environment could have indigenous *V. parahaemolyticus* that can prevent the accumulation of *V. parahaemolyticus* during experimental infection. Indeed, 60% of oyster samples ($n = 10$) were positive for the detection of *V. parahaemolyticus* by PCR in October 2019 in France (Copin *et al*., 2021). To verify this hypothesis, depuration of oysters before experimentation in water at low temperature and high salinity with variable efficiencies of depuration (Phuvasate *et al*., 2012; Phuvasate and Su, 2013) could be considered for further experimentations. Another way is the pre-treatment of oysters with antibiotics to eliminate indigenous bacteria (Srivastava *et al*., 2009).

In conclusion, we developed and validated the use of FCM as an alternative to PE for *V. parahaemolyticus* quantification in oysters using IFVp201-*gfp*$^+$ transformant. This strategy contributed to the investigation of the accumulation and depuration of *V. parahaemolyticus* in oysters, with a link with immune priming and microbiota of oysters. Further investigations are required on oyster microbiota using metagenomic to compare microbiota of naive and environmental and tentatively evaluate if there will be any correlations between higher/lower accumulation and diversities of microbiota. Moreover, it could be interesting to include season and physiological status of oysters in further studies of oyster microbiota, considering that it was shown that microbiota structure varied according to seasons (Lokmer *et al*., 2016). Finally, broader spectrum of *V. parahaemolyticus* strains, and combination of *in vivo* experimentation and genomic analyses of those strains could help to identify mechanisms implied or possibly implied in accumulation and colonization of oysters.

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Author Contributions

Biological resources: B.P., F.D., L.D. Conception and design: M.S., D.H.H. Acquisition of data: M.S., D.H.H., C.L., M.P., S.L. Analysis and interpretation of data: M.S., D.H.H., L.D., M.P., D.P. Manuscript: M.S., D.H.H., L.D., D.P., F.D., C.L.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Experimental protocols with IFVp201-*gfp*$^{+}$ for the preliminary assay in February 2021 (A) and the challenge test with naive and environmental oysters in October 2021 (B).

Supplementary figures

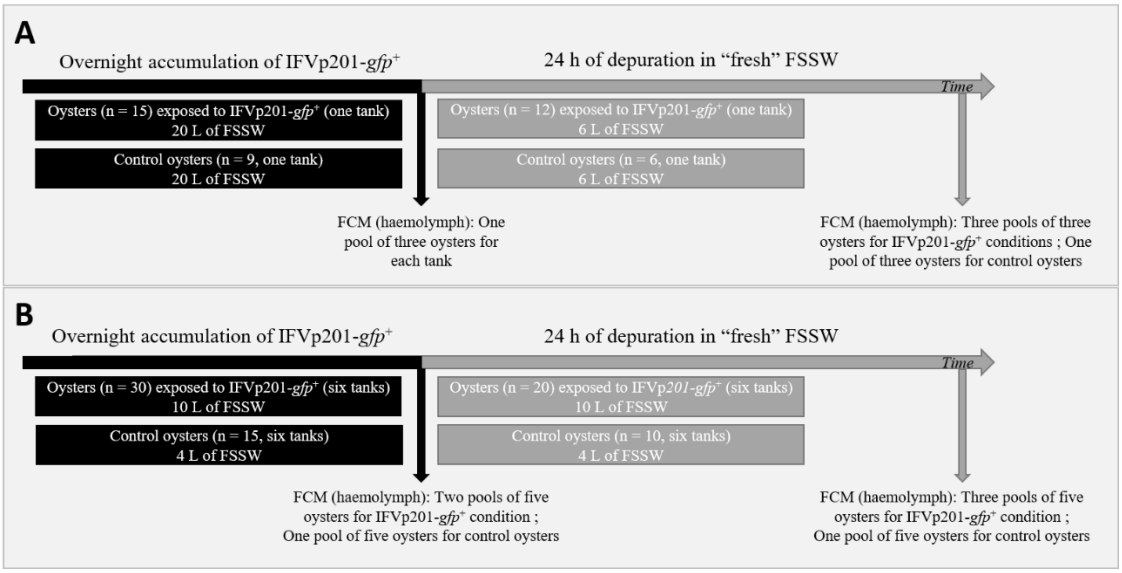

Figure S1. Experimental protocols with IFVp201-*gfp*⁺ for the preliminary assay in February 2021 (A) and the challenge test with naive and environmental oysters in October 2021 (B).

Conclusion

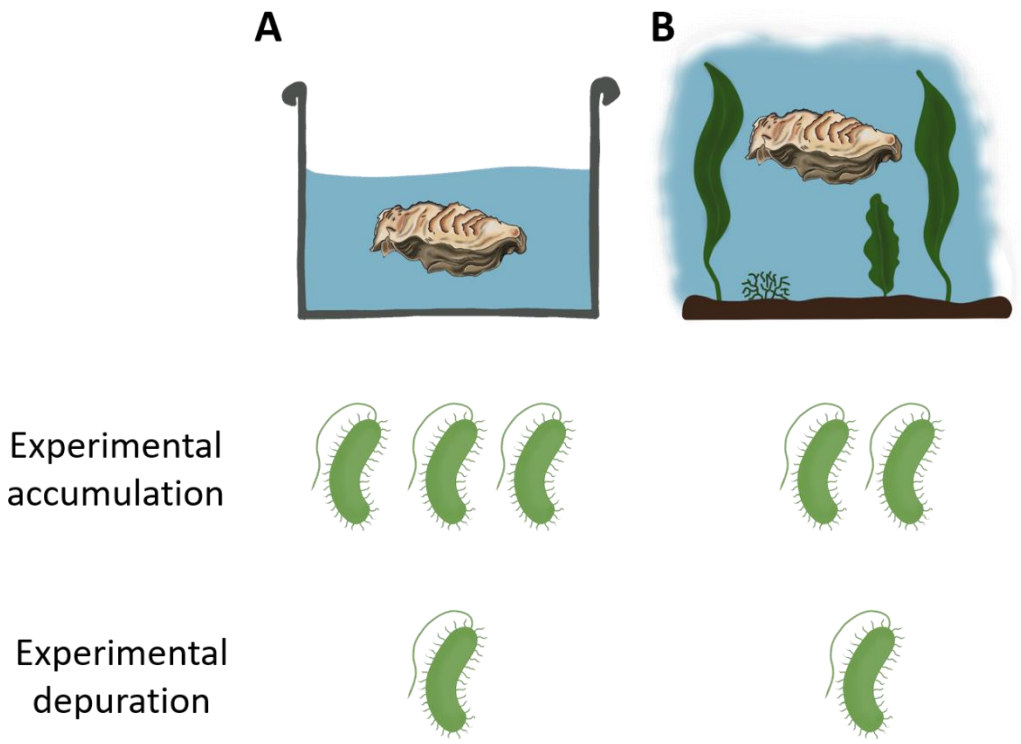

Figure 9. Résumé graphique de l'impact de l'historique de vie de *C. gigas* sur l'accumulation de *V. parahaemolyticus* lors de contaminations expérimentales avec la souche IFVp201-*gfp*$^{+}$. (A) huîtres élevées dans des structures sécurisées (huîtres naïves). (B) huîtres élevées dans la zone intertidale (huîtres environnementales). Bactérie : niveau de contamination en *V. parahaemolyticus*.

Publication III

Titre de l'article	**"Impact of ploidy level of *Crassostrea gigas* oysters on *Vibrio parahaemolyticus* accumulation and depuration"**
Statut de l'article	Publié dans *Aquaculture* DOI : 10.1016/j.aquaculture.2022.738992
Objectif de l'étude	Evaluer l'impact du niveau de ploïdie de l'huître *C. gigas* sur la contamination naturelle par *V. parahaemolyticus*, et l'accumulation et la dépuration expérimentales de *V. parahaemolyticus*.
Résultats principaux	Nous n'avons pas observé de différence en termes de contamination ou de profils géniques de virulence (*tdh* et/ou *trh*) des *V. parahaemolyticus* indigènes entre des huîtres 2N et 3N. Une tendance est cependant visible en Novembre 2021 avec moins de *V. parahaemolyticus* indigènes chez les 3N que chez les 2N. Il n'y a pas de différence d'accumulation et de dépuration expérimentales de *V. parahaemolyticus* entre les 2N et 3N. Nous avons cependant observé la variation des profils géniques de virulence des *V. parahaemolyticus* indigènes ainsi que des profils de dépuration expérimentale en fonction des saisons ploïdies confondues.
Conclusions	Le niveau de contamination de *V. parahaemolyticus* chez *C. gigas* n'est pas influencé par le niveau de ploïdie. Des variations saisonnières des *V. parahaemolyticus* indigènes (totaux et potentiellement pathogènes) et de la dépuration expérimentale de *V. parahaemolyticus* sont observées.

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Effect of ploidy level on accumulation and depuration of *Vibrio parahaemolyticus* in Pacific oyster *Crassostrea gigas*

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ABSTRACT

Triploid cupped oysters represent an advantage over diploid oysters due to their faster growth and their whole year marketable span, especially during the spawning season occurring in the summer. Thus, their commercialization during this warm season could present a risk for human health due to the proliferation of human-pathogenic *Vibrio* spp. such as *Vibrio parahaemolyticus (Vp)*. The purpose of this study was to evaluate the impact of *Crassostrea gigas* oyster ploidy on contamination with indigenous *Vp* in an oyster farm, and on the accumulation and depuration of *Vp* in laboratory experiments. The study was conducted from May through November 2021 using three batches of diploid oysters and three batches of triploid oysters. We observed that ploidy did not significantly influence contamination by indigenous *Vp* although the contamination with indigenous *Vp* trended to be lower in triploids (0.93 log MPN.g^{-1}) in comparison with diploids (3.08 log MPN.g^{-1}) in November. These results could suggest a safer consumption of triploid oysters over diploid oysters in autumn concerning *Vp* infection risk in the site of this study. Moreover, pathogenic profiles of indigenous *Vp* varied significantly between July (8% of *tdh*$^+$ and 100 of *trh2*$^+$) and November (98% of *tdh*$^+$ and 0% of *trh2*$^+$) with no significant difference between ploidies. Ploidy did not significantly influence experimental accumulation or depuration at any time. However, depuration was higher during summer (June to August) than during the other months. In conclusion, our study suggests that *Vp* levels in *C. gigas* are not influenced by oyster ploidy. In addition, seasonal variations of indigenous *Vp* pathogenic profiles and *Vp* experimental depuration were observed.

1. Introduction

Vibrio parahaemolyticus (*Vp*) is a natural inhabitant of coastal and estuarine waters worldwide. This bacterium is the principal pathogenic agent implicated in foodborne infections by the consumption of raw or insufficient cooked seafood causing acute gastroenteritis in humans (Center for Disease Control and Prevention, 2019). These foodborne cases of vibriosis peak during the summer (Baker, 2016), following the seasonal cycle of *Vp* observed in waters and oysters with proliferation occurring when temperatures exceed 15 °C (Parveen et al., 2008). Pathogenicity has first been characterized by the Thermostable-Direct haemolysin (TDH) (Sakurai et al., 1973), the TDH-Related Haemolysin (TRH) (Honda et al., 1988) and more recently by the type III secretion system (Makino et al., 2003). Both pathogenic and non-pathogenic strains can be isolated from seawater and bivalves with seasonal dynamics (Deter et al., 2010; Zimmerman et al., 2007).

The highest prevalence of *Vp* was found in oysters (63%), followed by clams (53%), fish (51%) and shrimps (48%) (Odeyemi, 2016). Oyster aquaculture is growing worldwide and is widely dominated by China which represented 85% of global production by weight and 79% by value in 2019 (FAO, 2021). Within Europe, 77% of the oyster production occurs in France representing 85,947 t and $445 million in 2019, thus

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positioning this country as the 5th producing country by weight and the 2nd country by value worldwide (FAO, 2021). The main oyster species cultivated in France is the Pacific oyster *Crassostrea gigas,* present and cultivated worldwide (FAO, 2009). Oysters cultured are either diploid or triploid. Triploid oysters were originally developed in the United States in the 80's (Stanley et al., 1981), and their production increased over the years, especially in France (Boudry et al., 1997; Gérard et al., 1999). The amount of triploid spat produced by French commercial hatcheries has increased regularly each year since 2000, reaching nearly 3 billion units in 2012 (Dégremont and Benabdelmouna, 2014). Triploid oysters are associated with faster growth than diploids, and are of particular interest during the summer as a result of their partial sterility (Allen and Downing, 1986). Indeed, the partial sterility results in better meat quality compared to diploid oysters, enabling to expand the market for oysters to the warmer months. However, the fact that triploid oysters are marketable during summer raise the public health issues associated with human pathogenic bacteria that proliferate in summer such as *Vibrio* spp. (Di et al., 2017).

Investigations about the effect of ploidy level and disease resistance have been broadly investigated in oyster species, as one indirect mechanism for triploids to avoid disease. Indeed, the faster growth limits the span of time when oysters might be exposed to disease (Dégremont et al., 2015). Previous study showed that triploidy confers neither advantage nor disadvantage over diploidy for OsHV-1 infections in *C. gigas* (Dégremont et al., 2016), while other studies showed higher disease resistance of either triploids, as observed for *Perkinsus marinus* in *C. virginica* (Dégremont et al., 2012) and for *Bonamia roughleyi* in *Saccostrea glomerata* (Hand et al., 1998), or diploids, as found for *Vibrio aestuarianus* in *C. gigas* (Azéma et al., 2016). Moreover, diploid and triploid oysters were shown to be physiologically different throughout the seasons according to their reproductive patterns (Goulletquer et al., 1996; Jeung et al., 2016; Normand et al., 2009). Previous studies showed that ripe *C. gigas* oysters or at the postspawning stage were more susceptible to the summer mortality phenomenon (Huvet et al., 2010; Samain et al., 2007), to *Vibrio splendidus* and *Vibrio aestuarianus* (De Decker et al., 2011), and OsHV-1 (Dégremont et al., 2013) infections. Altogether, these observations highlighted the importance of the physiological status of oysters on *Vibrio* spp. infections for diploid and triploid oyster comparisons. Concerning human pathogenic *Vibrio*, recent studies did not show any differences in *Vp* contamination between diploid and triploid *C. virginica* oysters (Grodeska et al., 2019; Jones et al., 2020; Walton et al., 2013). To our knowledge, this has not been studied in *C. gigas*.

The aim of this study was to investigate the impact of ploidy level of *C. gigas* oysters on natural contamination of *Vp* in field condition, and on experimental accumulation and depuration of *Vp* in laboratory condition throughout seasons. For each ploidy, three replicated spawns were used to disentangle the ploidy and batch effects, as significant variation for disease resistance exists among spawns in oysters as demonstrated by Dégremont et al. (2012). Oysters were sampled once a month from May to November 2021, and were directly analysed for indigenous *Vp* contamination (May, July, September and November) and exposed to the *Vp* strain IFVp201-*gfp*$^+$ (environmental strain, *tdh*$^+$ *trh*$^+$) to investigate experimental accumulation and depuration (May, June, July, August, September and November).

2. Materials and methods

2.1. Bacterial strains and culture conditions

For this study, the *Vp* IFVp201 strain isolated from mussels (*Mytilus edulis*) in December 2009 in Poitou Charentes (France) was used. This environmental strain was characterized as *tdh*$^+$ *trh*$^+$ by qPCR (Lozach et al., in preparation)(Bej et al., 1999), which are virulence genes widely present in clinical isolates but rarely in environmental strains. IFVp201 was previously electroporated with pFD086 plasmid harbouring *gfp* gene and trimethoprim-resistance expression cassettes named thereafter IFVp201-*gfp*$^+$ (Sorée et al., 2022).

IFVp201-*gfp*$^+$ was grown overnight at 37 °C on agar plates of Luria Bertani containing 3% NaCl supplemented with 50 µg.mL^{-1} of trimethoprim (LBS50). A single colony formed on LBS50 agar plate was transferred into LBS50 liquid medium and incubated at 37 °C overnight at 50–70 rpm. Overnight cultures were washed twice with buffered-physiological water (BPW, 0.4 g.L^{-1} of $K_2H_3PO_4$; 4.5 g.L^{-1} of Na_2HPO_4, $12H_2O$; 7.2 g.L^{-1} of NaCl) before inoculation in filtered and sterile seawater (FSSW, natural seawater filtered with 1 µm polypropylene mesh and treated with UV). This washed-culture of IFVp201-*gfp*$^+$ was analysed using Cyflow Space flow cytometer (Sysmex-Partec, Munster, Germany) to determine the bacterial concentration (log bacteria.mL^{-1}).

2.2. Oyster batches and sampling site

Three batches of diploid *C. gigas* oysters (called thereafter D1, D2 and D3) and three batches of triploids (called thereafter T1, T2 and T3), were bought at a commercial hatchery in November 2020. Each batch was produced using common practices developed by commercial hatcheries, by mating several dozens of females with a few males from broodstocks developed by the commercial hatchery. For diploid batches, all parents were diploids, while triploids batches were produced by mating diploid females with tetraploid males, as usually done by French commercial hatcheries (Dégremont et al., 2019). Batches used in this study are representative of hatchery-produced oysters used by oyster farmers in France. For each batch, oysters weighted around 30 g in November 2020, which is the beginning of the market-size in France. For each batch, oysters were deployed in six bags fixed on racks in the experimental farm at Agnas on November 16th 2020 (1°10′35″ W, 45°52′14″ N), only accessible by boat. Due to COVID issues, the boat operation was restricted, and so, oysters were transferred to La Floride (1°09′15″ W, 45°48′12″ N) on April 12th 2021, accessible from the laboratory by walking. Both sites are located in the Marennes-Oléron Bay which is the most important area of shellfish culture in Europe, mainly based on cupped oysters and mussels, and both sites are in the intertidal area as most of the oyster farms (Goulletquer and Le Moine, 2002). For each batch and sampling date, 75 to 85 oysters were collected and then transferred to Ifremer mollusc experimental platform at Bouin (France) on May 17th, June 21st, July 26th, August 23rd, September 20th and November 22nd 2021. Experimentations were performed <24 h after oyster collection except in June when a delivery issue led to a 24 h delay. Indigenous *Vp* presence in oysters was investigated every two months, in May, July, September and November 2021. Experimental accumulation and depuration were performed in May, June, July, August, September and November 2021. For each sampling date, shell length and wet tissue weight were recorded from 30 oysters for each diploid and triploid batch to estimate their growth kinetics. One of the triploid batches (T2) underwent severe mortalities in the environment to the point that this batch was missing in September and November 2021 experimentations.

Environmental parameters were measured regularly by IFREMER LER/PC for the "Reseau d'Observatoires de Microbiologie Environnementale intégrée" at the sampling point (1° 09′ 17" O, 45° 47′ 53" N). Seawater temperature (in °C) and salinity (ppt) were measured every 15 min by STPS sensors (NKE, France) placed in proximity of the sampling site during the entire period of experimentation. In addition, a Thermobutton sensor measured the temperature (°C) every 30 min inside the oyster bags between 22nd July and 30th November 2021.

2.3. Ploidy verification

Due to a risk of contamination among ploidy before purchasing the oysters, the ploidy of each batch was checked. For each ploidy, six pools of five individuals were analysed, using small pieces of gills (1 mm^2) transferred into 2 mL of Cystain UV Ploidy solution (Sysmex, Japan).

Gills were disrupted by pipetting and the mix was filtered on 30 μm Celltricks (Sysmex, Japan) into a new tube and 1 mL of Cystain UV Ploidy solution was added before analyses using Cyflow Space flow cytometer (Sysmex-Partec, Munster, Germany) with UV LED (450/50 nm). A diploid oyster was used as standard before analyses.

2.4. Quantification and characterization of indigenous Vp contamination

2.4.1. Preparation of samples, culture enrichment and DNA extraction

For each batch and each sampling date (in May, July, September and November 2021), one pool of 10 oysters was analysed for indigenous *Vp*. The Most Probable Number (MPN)-qPCR protocol of (Luan et al., 2008), used to estimate the viable number of bacteria in a sample, was adapted to 96-deep well plates. Briefly, the pool of 10 oysters was blended three times for 30 s with Laboratory Waring Blender. 25 g of oyster homogenate were transferred into 225 mL of alkaline peptone water 2% NaCl (APWS: 20 $g.L^{-1}$ of bacteriological peptone, 20 $g.L^{-1}$ of sodium chloride). This suspension, corresponding to the $1/10^{e}$ dilution of the homogenates, was serially-diluted until the $1/10{,}000^{e}$ dilution and 1.5 mL of each dilution was transferred into a 96-deep well plate (six replicates per dilution) and incubated at 36 ± 2 °C for 20 h. Three wells of APWS (1.5 mL) were used as negative controls. After incubation, 1 mL of the bacterial suspension from each well was transferred into a new 96-deep well plate. These suspensions were washed twice with BPW followed by centrifugation. Pellets were re-suspended in 150 μL of DNAse-free water (Merck Millipore™, Germany) and transferred into 96-well microplate. Nucleic acids were extracted by thermal lysis (15 min at 95 °C) using a TC5000 96 × 0.2ML thermocycler (Techne, UK). Plates were centrifuged and supernatants were transferred into new 96-well microplate and maintained at −20 °C. DNA concentrations in each well were determined using Epoch Microplate Spectrophotometer (BioTek Instrumentals, Inc., Winooski, VT, USA) and concentrations were adjusted if necessary at approximatively 50 $ng.\mu L^{-1}$ with DNAse free-water (Merck Millipore™, Germany).

2.4.2. Quantification and characterization of indigenous Vp

Firstly, affiliation to the species level (*Vp*) was determined by qPCR targeting *toxR* gene using the Platinum™ Quantitative PCR SuperMix-UDG Kit (Invitrogen™, CA, USA) and the TaqMan™ Exogenous Internal Positive Control (IPC) Reagents (Applied Biosystems ™, CA, USA) to detect qPCR inhibition (Lozach et al., in preparation). The PCR reaction mixture (25 μL) consisted of 2 μL of DNA, 12.5 μL of the reaction buffer (2× SuperMix-UDG PCR quantitative Platinum ™), 0.5 μL of IPC DNA (50× Exo IPC DNA), 1 μL of $MgCl_2$ (50 mM), 2.5 μL of IPC mix (10× Exo IPC mix), 5.625 μL of DNAse water, 0.375 μL of probe (20 mM), 0.125 and 0.375 μL of reverse and forward primers (20 mM), respectively. DNA was replaced by 2 μL of block IPC (10× block IPC) for non-amplification control wells and by DNAse free-water for non-template control. A standard was performed by dilution of a reference plasmid in DNAse free-water from 10^{5} to 10^{0} copies per well and was used to validate positivity of wells when the concentration was superior to 10 copies. The amplification was carried out with a Mx3000P QPCR System (Agilent™ Technologies, CA, USA). Most probable number (MPN) enumeration (in log $MPN.g^{-1}$ of oyster tissues) was performed for *toxR* detection following https://standards.iso.org/iso/7218/. *toxR*$^{+}$ wells were further characterized for the genetic markers *tdh*, *trh1* and *trh2* genes. Previous studies performed on French coasts (Cantet et al., 2013; Deter et al., 2010; Esteves et al., 2015) showed a high prevalence of *trh*$^{+}$ strains that were further characterized according to the presence of *trh1* and *trh2* genes. The qPCR were performed using the Platinum™ Quantitative PCR SuperMix-UDG Kit (Invitrogen™, CA, USA) (Lozach et al., in preparation) and with a PCR reaction mixture (25 μL) consisted of 2 μL of DNA, 12.5 μL of the reaction buffer (2× SuperMix-UDG PCR quantitative Platinum ™), 0.5 to 1 μL of $MgCl_2$ (50 mM), 8 to 8.375 μL of ultrapure water, 0.375 to 0.625 μL of each primer (20 mM). Results were expressed for each batch as a ratio of the number of positive wells for the gene (*tdh, trh1* and *trh2*) over the total number of *toxR*$^{+}$ wells.

2.5. Oyster bacterial challenge with IFVp201-*gfp*$^{+}$

2.5.1. Accumulation of IFVp201-*gfp*$^{+}$

Oyster bacterial challenge was performed as previously described in (Sorée et al., 2022) with slight modifications. Briefly, IFVp201-*gfp*$^{+}$ was inoculated in six 20 L tanks of FSSW (33.5 ± 0.8 ppt) with initial concentration of 10^{6} $CFU.mL^{-1}$. For each diploid and triploid batch, 50 oysters were placed into one contaminated tank and 25 oysters were held as controls in a 6 L tank of FSSW without IFVp201-*gfp*$^{+}$. The 12 tanks were maintained at 19 °C for 24 h and aeration was provided to all tanks. After 24 h, IFVp201-*gfp*$^{+}$ concentration was assessed by flow cytometry in triplicate samples of contaminated and control seawater. Oysters were then transferred one hour into "fresh" FSSW. Haemolymph was collected as described in 2.5.3 from two pools and one pool of five oysters from each contaminated and control tank, respectively. IFVp201-*gfp*$^{+}$ was quantified by flow cytometry from two analytical replicates per haemolymph pool. These data represented the 24 h-accumulated concentration of IFVp201-*gfp*$^{+}$ in oysters.

2.5.2. Depuration of Vp201-*gfp*$^{+}$

Remaining oysters exposed to IFVp201-*gfp*$^{+}$ ($n = 40$) and control oysters ($n = 20$) were transferred into new separate tanks with 20 L and 6 L of FSSW, respectively, and maintained in the same conditions as for accumulation. After 24 h and 48 h, haemolymphs were collected from three haemolymph pools of five oysters previously exposed to IFVp201-*gfp*$^{+}$, and from one haemolymph pool of five oysters for the control. IFVp201-*gfp*$^{+}$ was quantified by flow cytometry in two analytical replicates per pool. In June 2021, data could not be collected at the 48 h of time point due to a delay in the oyster delivery to the laboratory processing the samples. Log reduction was calculated as the difference between the accumulated concentration and concentrations after 24 h (24 h log reduction) and 48 h of depuration (48 h log reduction).

2.5.3. Sample preparation and analysis

At each step (accumulation and depuration), oysters were shucked using a sterile shucking knife on a sterile surface. Abductor muscles were cautiously cut with sterile scalpel blades to enable shell opening. Haemolymphs were collected using a sterile 25G needle and sterile 1 mL insulin syringe from the pericardial cavity. Collected haemolymphs were filtered on a 30 μm-sterile nylon mesh to remove aggregates. Samples were then analysed by flow cytometry using a Cyflow Space (Sysmex-Partec, Munster, Germany) by detection of their green fluorescence (FL1 detector of the flow cytometer) and their relative size and granularity (Forward SCatter [FSC]; Side SCatter [SSC]). The bacterial counting was performed according to the number of cells detected by the flow cytometer in a volume of 200 μL of sample and was expressed in log $bacteria.mL^{-1}$ (Sorée et al., 2022).

2.6. Statistical analyses

All statistical analyses were performed using RStudio 2021.09.0 + 351 "Ghost Orchid" Release (2021-09-20) for Windows. Data are presented as means ± standard error and the significance level was set to $p < 0.05$.

2.6.1. Growth of oysters

For each sampling date, wet tissue weight and shell length were analysed using non-parametrical Kruskal-Wallis test and post hoc Dunn test between ploidies and batches nested within ploidy.

2.6.2. Quantification and characterization of indigenous Vp in oysters

Contamination with indigenous *Vp* and pathogenicity characterization of indigenous *Vp* in oysters were analysed using an ANCOVA with wet tissue weight as covariables, and with ploidy (diploid vs triploid),

sampling date (July and November 2021, when *Vp* was detected) and their interaction as factors. Absence of replicates for each batch nested within ploidy did not allow to include this factor in the analysis. Differences among factors were estimated using a post hoc Emmeans test.

2.6.3. Experimental IFVp201-*gfp*$^+$ concentrations in seawater

For each sampling date, concentrations of IFVp201-*gfp*$^+$ (in log bacteria.mL^{-1}) in seawater before accumulation by oysters were analysed using an ANOVA with tank as factor. For each sampling date, log reductions of IFVp201-*gfp*$^+$ concentrations in seawater before and after accumulation by oysters were analysed using an ANCOVA with wet tissue weight as covariables and ploidy as factor. Absence of replicates for each batch nested within ploidy did not allow to include this factor in the analysis. To evaluate differences of log reduction in seawater among months of experimentation, an ANCOVA and a post hoc Emmeans tests were performed with wet tissue weight as covariable and sampling date as factor.

2.6.4. IFVp201-*gfp*$^+$ accumulation and depuration in haemolymph

For each sampling date, IFVp201-*gfp*$^+$ accumulation in haemolymph (in log bacteria.mL^{-1}) was analysed using an ANCOVA with wet tissue weight as covariable, and ploidy and batch nested within ploidy as factors. For each sampling date, log reductions of IFVp201-*gfp*$^+$ concentrations after 24 h and 48 h of depuration were analysed using an ANCOVA with wet tissue weight as covariable and ploidy as factor. Absence of replicates of log reduction for each batch did not allow analysis with batches nested within ploidy as factor.

To evaluate differences in accumulation and log reduction in haemolymph among the months of experimentation, an ANCOVA and post hoc Emmeans test were performed with wet tissue weight as covariable and sampling date as factor.

3. Results

3.1. Ploidy validation and environmental condition

Analyses of oyster ploidy level by flow cytometry confirmed the ploidy of each batch of oysters used for this study.

Seawater temperature measured at the sampling site ranged from 11 °C in November 2021 to 23 °C in July 2021 (Fig. 1A). Temperatures were higher than 15 °C between May 17th and October 30th, and a mean of 19 °C was observed during the sampling period. Salinity ranged from 30 ppt in July 2021 to 34 ppt in September 2021 with a mean of 33 ppt during the sampling period (Fig. 1B).

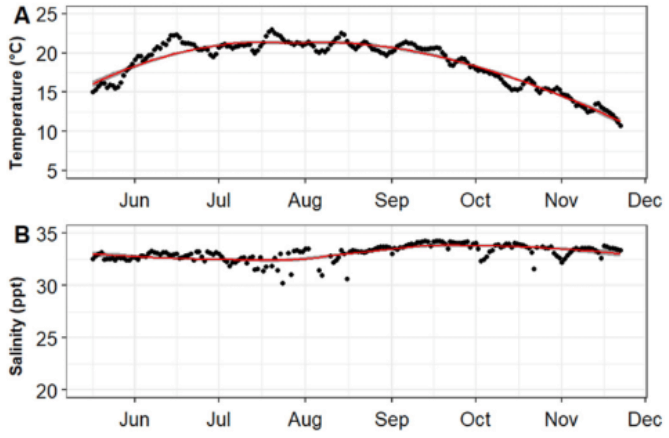

Fig. 1. Kinetic of seawater temperature (°C, A) and salinity (ppt, B) from 17th May through 22nd November 2021. Regressions line were represented in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Growth of diploid and triploid oysters

Wet tissue weights and shell lengths for each ploidy from May to November 2021 are shown in Fig. 2A and B, respectively. Details of each growth trait for each batch are given in Table S1.

In May 2021, similar wet tissue weights were observed for diploid (5.33 ± 0.21 g) and triploid (5.32 ± 0.15 g) oysters (Fig. 2A). The wet tissue weight increased until July for both diploid (7.70 ± 0.22 g) and triploid (7.84 ± 0.26 g) oysters with no significant differences from May to July but with significant difference among batches in May (p = 0.013), June (p = 0.003) and July ($p < 0.001$). For these three months, one diploid batch was lighter than the two others batches. Moreover, in July, one triploid batch was lighter than the two other batches (Table S1). Triploid wet tissue weights continued to increase until November (10.04 ± 0.29 g) while diploid wet tissue weights decreased until September (6.44 ± 0.24 g) followed by a new increase until November (7.24 ± 0.26) (Fig. 2A). The difference of wet tissue weights between diploid and triploid oysters was significant in August (p = 0.015), September ($p < 0.001$) and November ($p < 0.001$) (Fig. 2A) and among batches in August (p = 0.002) with one diploid batch heavier than the two others (Table S1). The shell lengths were not significantly different between diploid and triploid oysters in May (6.71 and 6.67, respectively) and in July (7.6 and 7.9 cm, respectively) while triploids exhibited a significantly longer shell length than diploids in June (7.4 cm vs 7.1 cm, $p < 0.01$), August (8.3 cm vs 7.6 cm, $p < 0.001$), September (8.4 cm vs 7.5 cm, $p < 0.001$) and November (8.1 cm vs 7.3 cm, $p < 0.001$) (Fig. 2B). Differences among batches were significant in June (p = 0.027) and in July (p = 0.049). Thus, one batch of diploid oysters was shorter than the two other batches in June, and one batch of diploids and one batch of triploids were shorter than the two others in July (Table S1).

3.3. Indigenous *Vp* contamination

Vp natural concentration (*tox*R$^+$, in log MPN.g^{-1}) in tissues of diploid and triploid oysters deployed at this study site are reported in Fig. 3A, whereas Fig. 3B shows the prevalence (in %) of *tdh*, *trh1* and *trh2* genes among the *toxR*$^+$ samples collected in July and November 2021.

Indigenous *Vp* were detected in oysters in July and November but not in May and September 2021 (Fig. 3A). The interaction between ploidy and dates was not significant (p = 0.135). Concentrations of indigenous *Vp* tended to be higher in diploid than in triploid oysters (p = 0.0531) especially in November with 0.93 ± 0.93 log MPN.g^{-1} in triploid and 3.08 ± 0.70 log MPN.g^{-1} in diploid oysters (Fig. 3A). No significant difference in the indigenous *Vp* contamination was observed between sampling dates (p = 0.225).

Concerning the prevalence of virulence genes, the interaction between ploidy and dates was not significant ($p > 0.05$). No significant difference was observed between diploid and triploid oysters for the prevalence of *tdh* (p = 0.37), *trh1* (p = 0.82) and *trh2* (p = 0.79) genes (Fig. 3B) while it was significant between sampling dates for *tdh* ($p < 0.001$) and *trh2* ($p < 0.001$) genes but not for *trh1* (p = 0.416). Thus, prevalence of pathogenic genes from July to November decreased for *trh2* (100% to 0%, respectively, $p < 0.001$) and increased for *tdh* (9% to 98%, respectively, $p < 0.001$) genes (Fig. 3B).

3.4. Experimental IFVp201-*gfp*$^+$ accumulation and depuration in diploid and triploid oysters

3.4.1. Experimental IFVp201-*gfp*$^+$ concentrations in seawater

Details of concentrations of IFVp201-*gfp*$^+$ in seawater before and after accumulation with oysters. and log reductions for each batch are given in Table S2.

For each sampling date, IFVp201-*gfp*$^+$ concentrations in seawater before accumulation ranged from 4.89 to 6.10 log bacteria.mL^{-1} (mean: 5.80 ± 0.44 log bacteria.mL^{-1}) with no significant difference among

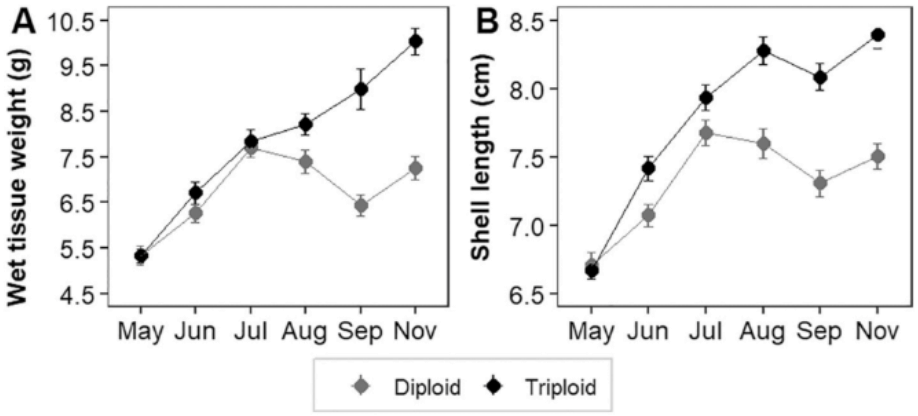

Fig. 2. Weights (g) of wet tissues (A) and lengths (cm) of shells (B) from May to November 2021 of diploid (grey) and triploid (black) *C. gigas* oysters. For each batch at each sampling date $n = 30$. Data are represented as mean of the three batches per ploidy ± standard error.

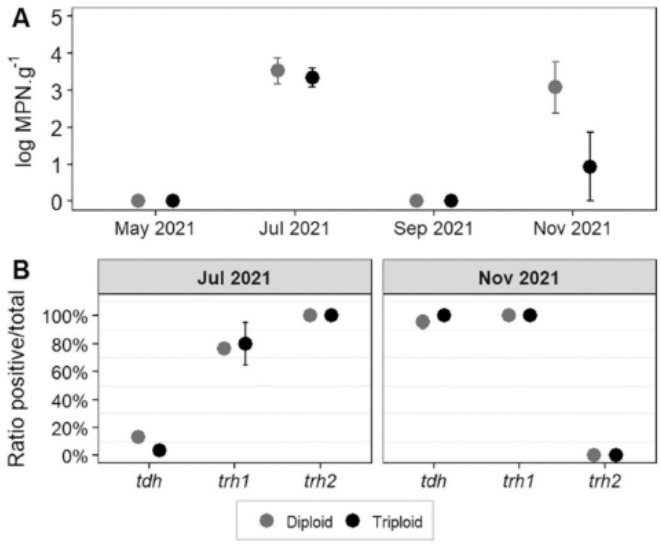

Fig. 3. Mean *Vp* levels (log MPN.g^{-1}, n = 3) (A) and prevalence (%) of virulence markers (*tdh. trh1* and *trh2*) among *toxR*$^+$ samples (n = 3) (B) in diploid (grey) and triploid (black) oysters deployed at La Floride. Prevalence data represent the ratio of number of positive samples for each of the genes (*tdh. trh1* and *trh2*) among the samples in which *toxR* was detected (*toxR*$^+$ samples). Data are represented as means of the three batches per ploidy ± standard error.

tanks ($p > 0.05$) (Table S2). For each sampling date, the log reduction of IFVp201-*gfp*$^+$ concentrations in seawater after accumulation by oysters ranged from 0.34 to 1.71 log with no significant difference between diploid and triploid oysters ($p > 0.05$) (Table S2). The log reduction of IFVp201-*gfp*$^+$ concentrations in seawater was significantly different among sampling dates ($p < 0.001$) with the highest value in July (1.19 log), intermediate in May (0.71 log), August (0.85 log) and September (0.71 log), and the lowest in June (0.58 log) and November (0.46 log) (Fig. 4).

3.4.2. *Experimental IFVp201-gfp*$^+$ *accumulation and depuration in haemolymph*

Fig. 5 represents the quantification of IFVp201-*gfp*$^+$ in haemolymph by flow cytometry after 24 h of accumulation (0 h), and after 24 h and 48 h of depuration for diploid and triploid oysters for each month. Details of accumulation and depuration at 24 h and 48 h for each batch are given in Table S3.

Accumulation of IFVp201-*gfp*$^+$ in haemolymph varied between 2.10 and 3.70 log bacteria.mL^{-1} for diploid oysters, and between 2.17 and

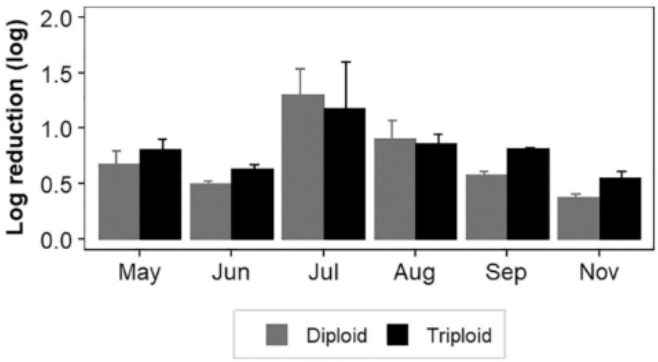

Fig. 4. Log reduction of IFVp201-*gfp*$^+$ concentrations in seawater after experimental accumulation by the diploid (grey) and triploid (black) oysters (n = 3). Data are represented as the mean between the three batches for each ploidy ± standard error.

3.97 log bacteria.mL^{-1} for triploids for all the sampling dates (Fig. 5). For each sampling date, no significant difference in accumulation of IFVp201-*gfp*$^+$ was observed between diploid and triploid oysters ($p > 0.05$), whereas significant differences were observed among batches only in July ($p = 0.021$) and August ($p < 0.01$). Thus, accumulation for one batch of diploids and one batch of triploids was lower than in the two other batches in July, while it only concerned one batch of triploids in August (Table S3). Moreover, concentration of IFVp201-*gfp*$^+$ in haemolymph decreased for both ploidies at 24 h of depuration for each sampling date, and in a lesser extent at 48 h of depuration except in September, where it remains stable, and in November, where it increased from 1.44 log bacteria.mL^{-1} at 24 h to 2.41 log bacteria.mL^{-1} at 48 h (Fig. 5). Reduction of IFVp201-*gfp*$^+$ concentrations in haemolymph after 24 h of depuration (24 h log reduction) varied between 0.12 and 2.34 log for diploid oysters, and between −0.05 and 2.48 log for triploid oysters for all the sampling dates (Fig. 5). For each sampling date, no significant difference in 24 h log reduction was observed between diploid and triploid oysters ($p > 0.05$). Similar finding was observed for the reduction of IFVp201-*gfp*$^+$ concentrations in haemolymph after 48 h of depuration ranging from −0.13 to 2.62 log for diploid oysters, and 0 to 3.07 log for triploids ($p > 0.05$) (Fig. 5).

The date of sampling had a significant impact on accumulation ($p < 0.01$), and on 24 h ($p < 0.001$) and 48 h log reduction ($p < 0.001$) of *Vp* in oysters. Thus, accumulation of IFVp201-*gfp*$^+$ was the highest in June (3.20 log bacteria.mL^{-1}), intermediate in May (2.98 log) and July (3.02 log), and the lowest in August (2.76 log), September (2.54 log) and November (2.56 log) (Fig. 5 and Table S4). The 24 h log reduction was the higher in June (1.72 log) and July (1.64 log), intermediate in August

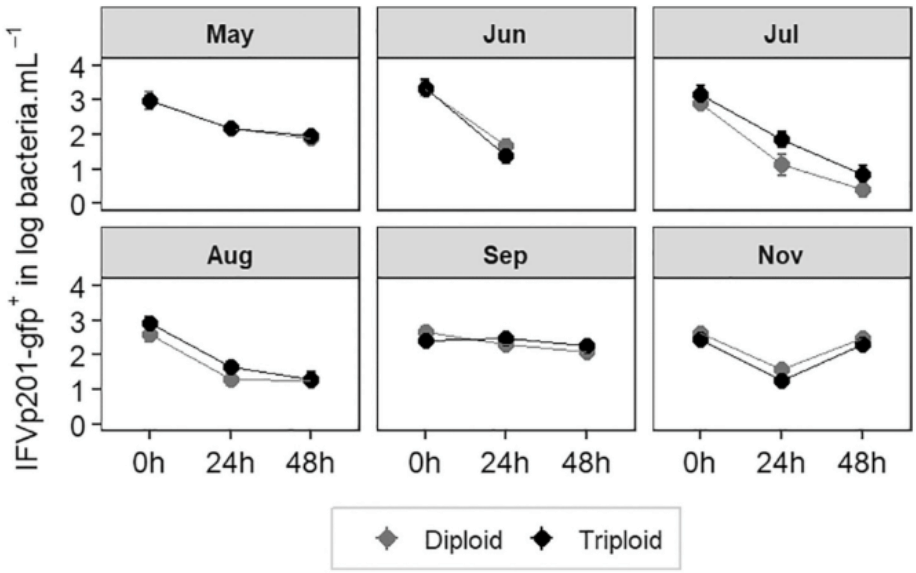

Fig. 5. IFVp201-*gfp*$^+$ quantification (log bacteria.mL^{-1}) in haemolymph of diploid (grey) and triploid (black) oysters by flow cytometry after 24 h of accumulation (0 h), and at 24 h and 48 h of depuration. Oysters were exposed to 10^6 CFU.mL^{-1} of IFVp201-*gfp*$^+$. 0 h: mean of two pools of five oysters per batch ± standard error (n = 3); 24 h and 48 h data: mean of three pools of five oysters per batch ± standard error (n = 3).

(1.31 log), and the lower in May (0.91 log), September (0.27 log) and November (1.13 log) (Fig. 5 and Table S4). The 48 h log reduction was the higher in July (2.42 log), intermediate in August (1.57 log) and May (1.07 log), and the lower in September (0.46 log) and November (0.12 log) (Fig. 5 and Table S4).

4. Discussion

Vibrio parahaemolyticus is the primary bacterial cause of gastroenteritis associated with seafood consumption, and is naturally present in coastal and estuarine marine environments worldwide. *Vp* is isolated more frequently in oysters (63%) than in other marine organisms (Odeyemi, 2016). In France, consumers prefer triploid *C. gigas* during the summer due to their higher meat quality compared to diploids which are gravid. Since the summer is also the season during which *Vp* are more likely to proliferate (Baker, 2016), defining the potential human health risks associated with consumption of triploid oysters during this season is critical. In our study, we assessed contamination by indigenous *Vp* naturally present in the environment in diploid and triploid oysters. Additionally, oysters were experimentally exposed to a GFP-tagged *Vp* strain to investigate the impact of oyster ploidy level on accumulation and depuration of the bacteria. Finally, *Vp* accumulation, both natural and experimental, and depuration were investigated temporally throughout the study to determine if a higher risk for human consumption would be observed.

Occurrence and concentrations of indigenous *Vp* in *C. gigas* oysters varied according to the seasons with the exception of the September time point. In our experimental farm, there was no detection of *Vp* in May or September 2021, while it was detected in July and November 2021 for both diploid and triploid oysters. Absence of indigenous *Vp* in May can be explained by the seawater temperature just reaching 15 °C, which is the threshold temperature for *Vp* proliferation as shown in previous studies (Deter et al., 2010; Kaneko and Colwell, 1973). In September, however, the seawater temperature was 20 ± 1 °C (Fig. 1), thus it did not explain the absence of indigenous *Vp* in oysters. This was particularly surprising since *Vp* had been isolated two months earlier (Fig. 2A), and also from the same geographical area in September in a previous study (Deter et al., 2010). Although human error cannot be ruled out, weather conditions might have played a role in our study. Indeed, extremely dry conditions in August were recorded, together with unusually high air temperatures (air temperature > 35 °C), high temperatures inside oyster bags (seawater temperature > 30 °C, Fig. S1) and high salinity levels (> 34 ppt, Fig. 1B) in September. High salinity might have affected oyster physiology resulting for example in a decrease of the oxygen consumption rate and enzyme activities, and an increase in glycogen decomposition and lactic acid concentrations (Chen et al., 2022), or in decrease in haemocyte locomotion (Fisher and Nowell, 1986) as it was shown in C. gigas and C. virginica, respectively. Moreover, it was shown that postspawning oysters were at higher risk during heat shock compared to prespawning oysters resulting in higher mortality, lower energy for metabolic activities, and reduced haemocyte phagocytosis and haemolymph antimicrobial activity (Li et al., 2007). Furthermore, it was shown that Vp proliferation was reduced in high salinity conditions (Parveen et al., 2017). During a drought with particularly high salinity levels in North Carolina from 2007 to 2009, *V. vulnificus* was shown to be undetectable in oysters (Froelich et al., 2012). Taken all together, those results could explain the absence of *Vp* in oysters in September in our study. Our study showed also that ploidy did not influence indigenous *Vp* contamination, suggesting a similar *Vp* infection risk for human consumption, although in November 2021 we observed a tendency for lower indigenous *Vp* contamination to occur in triploid compared to diploid oysters. These results confirmed previous studies showing no significant difference in *Vp* contamination between diploid and triploid *C. virginica* oysters (Grodeska et al., 2019; Jones et al., 2020; Walton et al., 2013). It is interesting to note that profiles of pathogenicity of indigenous *Vp* in oysters did not differed between diploid and triploid oysters even if they differ between months. Thus, we observed less *trh2*$^+$ profile strains during autumn in comparison to summer which is in agreement with previous studies (Cantet et al., 2013; Esteves et al., 2015).

Experimental accumulation and depuration of *Vp* by oysters did not differ between diploid and triploid oysters, which is consistent with the log reductions of IFVp201-*gfp*$^+$ concentration in seawater. Similar results were observed previously (Phuvasate and Su, 2013). This could be explained by similar clearance rate and oxygen consumption for both ploidies (Haure et al., 2021). In addition, we showed that, for both diploid and triploid oysters, *Vp* depuration was higher during summer (June to August) than during the other months. This can be explained by higher filtration rates of oysters during the summer months (Ehrich and Harris, 2015). Higher depuration in our study was consistent with the

reductions of IFVp201-*gfp*$^+$ concentration in seawater for both diploid and triploid oysters significantly higher in July than in other months. Another hypothesis explaining our results relies on the reproductive cycle of oysters. A previous study showed that oysters in gametogenesis (May–June) displayed lower haemocyte activities than oysters in ripe gametes and post-spawning periods (Gagnaire et al., 2006). In our study, both diploid and triploid oysters could exhibit low haemocyte activities during gametogenesis (May–June), resulting in less depuration of oysters than during the following months. These differences of depuration could also be explained by niche competition between indigenous *Vp* and the Vp201-*gfp*$^+$ strain. Indeed, higher concentrations of indigenous *Vp* in July was correlated with higher experimental depuration in comparison to May and September when the low experimental depuration was associated with the absence of indigenous *Vp* in oyster tissues. Similar results were observed by Froelich et al. (2012) after experimental contamination of *V. vulnificus* with oysters free of indigenous *V. vulnificus*. Furthermore, the natural exposition of oysters to *Vp* in July could result in an innate immune priming. Indeed, Zhang et al. (2014) showed that a first infection with *V. splendidus* induced a significant increase of the total count of haemocytes and of the immune processes after a second infection with *V. splendidus* (Zhang et al., 2014). Based on these studies, the influence of season and physiological status of oysters on *Vp* accumulation and depuration warrants further investigations.

In our study, triploid oysters displayed faster wet tissue weight gain and shell length increase than diploid oysters which is consistent with previous studies (Dégremont et al., 2012; Wadsworth et al., 2019). The slight decrease in wet tissue weight for diploid oysters (−1 g) between July and September can be explained by the release of gametes at this period. Moreover, the decrease observed in shell lengths for both diploid (0.36 cm) and triploid (0.19 cm) oysters between July and September can be explained by the vulnerability of peripheral part of shells during growth which could have been damaged during collection or transportation. Our results suggested that the growth advantage of triploid oysters over diploid oysters would not have an effect on human health risks associated with *Vp*.

5. Conclusions

In conclusion, our study suggests that *Vp* levels in *C. gigas* are not influenced by oyster ploidy similarly to the results obtained on *C. virginica* (Grodeska et al., 2019; Walton et al., 2013). Together, our study and these previous studies suggest that *Vp* infection risk for consumers is not affected by oyster ploidy. However, in November, the lower contamination by indigenous *Vp* of triploid oysters than diploid oysters would suggests a reduced risk of *Vp* infection for consumers, although complementary studies are needed to confirm these observations. In addition, seasonal variations of indigenous pathogenic profiles and *Vp* experimental depuration were observed. This study provides meaningful comparison of diploid and triploid *C. gigas* regarding *Vp* contamination. Accumulation and depuration.

CRediT authorship contribution statement

Marion Sorée: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Anna Le Meleder:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Elise Maurouard:** Resources, Writing – review & editing. **Mathias Papin:** Methodology, Writing – review & editing. **Christophe Stavrakakis:** Resources, Funding acquisition, Writing – review & editing. **Corinne Audemard:** Writing – review & editing. **Dominique Hervio Heath:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Lionel Dégremont:** Conceptualization, Resources, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738992.

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SUPPLEMENTARY FIGURE AND TABLES

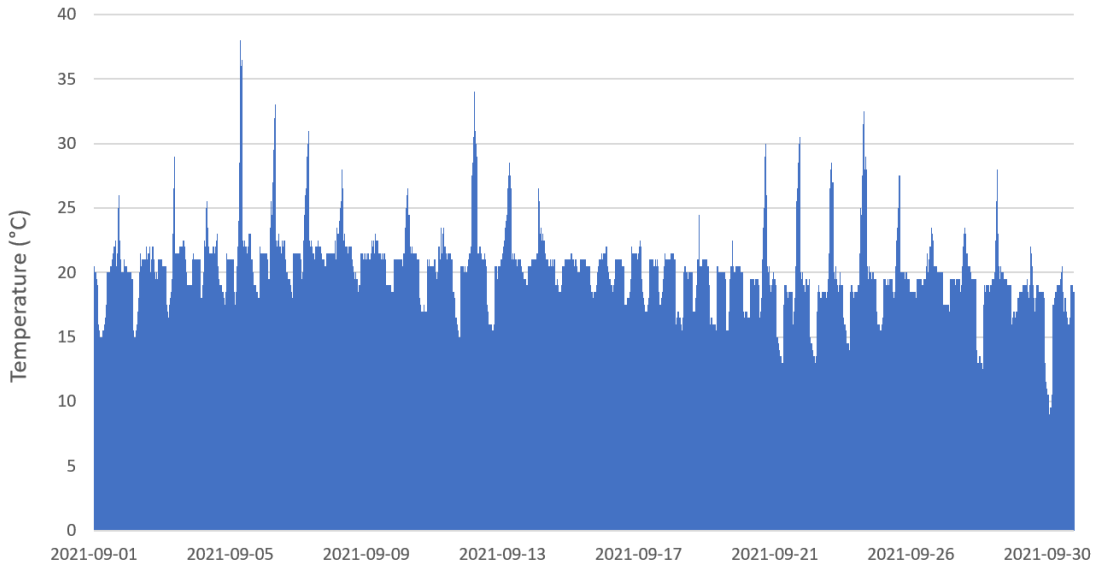

Fig S1. Kinetic of seawater temperature (°C) from 1st through 30th September 2021 within oyster bags.

Table S1. Wet tissues weights (A) and shell lengths (B) from May to November 2021 of diploid and triploid oyster batches (n = 30). Data are represented as the mean of 30 oysters per batch ± standard error

	Wet Tissues Weight (g)					
	Diploid			Triploid		
A	**D1**	**D2**	**D3**	**T1**	**T2**	**T3**
May	5.62 ± 0.33	4.48 ± 0.25	5.88 ± 0.45	5.12 ± 0.22	5.23 ± 0.24	5.62 ± 0.31
June	6.63 ± 0.28	5.41 ± 0.29	6.71 ± 0.37	6.36 ± 0.38	6.15 ± 0.41	7.63 ± 0.47
July	7.64 ± 0.28	6.57 ± 0.27	8.67 ± 0.45	8.23 ± 0.49	6.62 ± 0.32	8.63 ± 0.45
August	6.7 ± 0.42	6.66 ± 0.38	8.73 ± 0.41	8.11 ± 0.35	8.01 ± 0.41	8.52 ± 0.48
September	6.41 ± 0.43	6.16 ± 0.41	6.77 ± 0.38	8.39 ± 0.46	*ND*	9.63 ± 0.76
November	6.67 ± 0.37	7.15 ± 0.45	7.93 ± 0.50	9.76 ± 0.36	*ND*	10.32 ± 0.46

	Shell Length (cm)					
	Diploid			Triploid		
B	**D1**	**D2**	**D3**	**T1**	**T2**	**T3**
May	6.85 ± 0.16	6.38 ± 0.14	6.89 ± 0.19	6.62 ± 0.13	6.73 ± 0.11	6.67 ± 0.12
June	7.20 ± 0.13	6.88 ± 0.15	7.13 ± 0.15	7.04 ± 0.15	7.63 ± 0.16	7.60 ± 0.15
July	7.68 ± 0.17	7.41 ± 0.17	7.66 ± 0.16	8.26 ± 0.18	7.68 ± 0.14	7.87 ± 0.16
August	7.53 ± 0.21	7.82 ± 0.19	7.45 ± 0.18	8.38 ± 0.16	7.94 ± 0.15	8.51 ± 0.20
September	7.54 ± 0.15	7.42 ± 0.16	7.55 ± 0.17	8.13 ± 0.15	*ND*	8.65 ± 0.14
November	7.43 ± 0.16	7.34 ± 0.20	7.15 ± 0.14	7.92 ± 0.13	*ND*	8.25 ± 0.14

ND: Not determined

Table S2. Concentrations of IFVp201- *gfp*+ in seawater before (n = 3) and after accumulation (n = 3), and the log reduction (n = 1) of oysters. D1, D2, D3: batches of diploid oysters, T1, T2, T3: batches of triploid oysters. Data are represented as the mean of three replicates per batch ± standard error

		Concentration of IFVp201-gfp+ (log bacteria,mL-1)		
Sampling Date	**Batch**	**Before accumulation**	**After accumulation**	**log reduction**
May	D1	4.91 ± 0.01	4.43 ± 0.01	0.48
	D2	4.90 ± 0.01	4.26 ± 0.01	0.64
	D3	4.85 ± 0.01	3.96 ± 0.00	0.9
	T1	4.88 ± 0.00	3.90 ± 0.02	0.98
	T2	4.85 ± 0.01	4.07 ± 0.01	0.78
	T3	4.88 ± 0.01	4.20 ± 0.01	0.67
June	D1	5.93 ± 0.00	5.41 ± 0.00	0.53
	D2	5.92 ± 0.01	5.45 ± 0.00	0.47
	D3	5.93 ± 0.00	5.42 ± 0.01	0.5
	T1	5.92 ± 0.00	5.24 ± 0.00	0.67
	T2	5.91 ± 0.00	5.33 ± 0.01	0.58
	T3	5.92 ± 0.00	5.26 ± 0.00	0.66
July	D1	5.85 ± 0.01	4.30 ± 0.00	1.56
	D2	5.88 ± 0.01	5.01 ± 0.00	0.86
	D3	5.88 ± 0.00	4.38 ± 0.01	1.5
	T1	5.87 ± 0.04	4.16 ± 0.01	1.71
	T2	5.90 ± 0.02	5.54 ± 0.00	0.36
	T3	5.92 ± 0.01	4.47 ± 0.01	1.46
August	D1	6.07 ± 0.00	4.90 ± 0.01	1.16
	D2	6.06 ± 0.00	5.43 ± 0.01	0.62
	D3	6.03 ± 0.00	5.08 ± 0.01	0.95
	T1	6.06 ± 0.00	5.19 ± 0.00	0.87
	T2	6.01 ± 0.04	5.01 ± 0.00	1,00
	T3	6.03 ± 0.01	5.32 ± 0.00	0.71
September	D1	6.06 ± 0.03	5.45 ± 0.00	0.61
	D2	6.07 ± 0.01	5.47 ± 0.01	0.61
	D3	6.04 ± 0.06	5.52 ± 0.03	0.51
	T1	6.07 ± 0.03	5.26 ± 0.00	0.81
	T2	ND	ND	ND
	T3	6.10 ± 0.01	5.27 ± 0.01	0.82
November	D1	6.10 ± 0.01	5.71 ± 0.01	0.39
	D2	6.10 ± 0.05	5.77 ± 0.01	0.34
	D3	6.11 ± 0.06	5.70 ± 0.00	0.41
	T1	6.05 ± 0.03	5.55 ± 0.00	0.5
	T2	ND	ND	ND
	T3	6.16 ± 0.06	5.55 ± 0.01	0.61

ND: Not determined

Table S3. Concentration of IFVp201-*gfp*+ in haemolymph of oysters after 24 h of accumulation, after 24 h and 48 h of depuration (n = 3) and log reductions after 24 h and 48 h of depuration for each batch of oysters. D1, D2, D3: batches of diploid oysters. T1, T2, T3: batches of triploid oysters. 24 h of accumulation: mean of two pools of five oysters ± standard error. 24 h and 48 h of depuration: mean of three pools of five oysters ± standard error

		Concentration of IFVp201-*gfp*+				
		24 h of accumulation	24 h of depuration		48 h of depuration	
Sampling Date	Batch	log bacteria.mL^{-1}	log bacteria.mL^{-1}	log reduction	log bacteria.mL^{-1}	log reduction
May	D1	3.48 ± 0.14	2.37 ± 0.14	1.12	2.34 ± 0.16	1.15
	D2	3.09 ± 0.35	2.18 ± 0.17	0.91	1.78 ± 0.12	1.31
	D3	2.35 ± 0.45	1.78 ± 0.21	0.57	1.51 ± 0.06	0.83
	T1	3.00 ± 0.23	2.22 ± 0.12	0.78	2.11 ± 0.04	0.88
	T2	3.03 ± 0.32	2.06 ± 0.10	0.98	1.69 ± 0.07	1.35
	T3	2.93 ± 0.25	2.19 ± 0.11	0.74	2.04 ± 0.06	0.89
June	D1	3.42 ± 0.34	1.17 ± 0.46	2.25	*ND*	*ND*
	D2	3.70 ± 0.56	1.68 ± 0.28	2.02	*ND*	*ND*
	D3	3.01 ± 0.36	1.79 ± 0.13	1.22	*ND*	*ND*
	T1	3.48 ± 0.44	1.00 ± 0.53	2.48	*ND*	*ND*
	T2	2.49 ± 0.59	1.71 ± 0.18	0.79	*ND*	*ND*
	T3	3.10 ± 0.39	1.53 ± 0.13	1.57	*ND*	*ND*
July	D1	2.93 ± 0.07	1.59 ± 0.38	1.33	0.39 ± 0.39	2.53
	D2	3.11 ± 0.02	0.80 ± 0.42	2.31	0.49 ± 0.49	2.62
	D3	2.67 ± 0.19	0.33 ± 0.33	2.34	0.33 ± 0.33	2.34
	T1	2.77 ± 0.18	2.15 ± 0.32	0.62	0.97 ± 0.55	1.8
	T2	3.97 ± 0.04	2.20 ± 0.26	1.77	0.91 ± 0.47	3.07
	T3	2.68 ± 0.12	1.20 ± 0.27	1.48	0.50 ± 0.50	2.18
August	D1	3.06 ± 0.08	1.34 ± 0.16	1.72	1.42 ± 0.15	1.63
	D2	2.62 ± 0.39	1.69 ± 0.07	0.93	1.46 ± 0.25	1.15
	D3	2.10 ± 0.17	0.81 ± 0.41	1.29	0.47 ± 0.00	1.63
	T1	2.87 ± 0.17	1.34 ± 0.17	1.52	0.47 ± 0.23	2.4
	T2	2.61 ± 0.11	2.04 ± 0.17	0.57	1.73 ± 0.13	0.88
	T3	3.30 ± 0.22	1.48 ± 0.18	1.82	1.60 ± 0.34	1.70
September	D1	2.15 ± 0.12	2.03 ± 0.30	0.12	2.23 ± 0.15	-0.08
	D2	2.97 ± 0.20	2.51 ± 0.03	0.46	2.41 ± 0.04	0.55
	D3	2.72 ± 0.18	2.49 ± 0.08	0.23	1.74 ± 0.15	0.98
	T1	2.30 ± 0.14	2.34 ± 0.06	-0.05	2.17 ± 0.08	0.12
	T2	*ND*	*ND*	*ND*	*ND*	*ND*
	T3	2.55 ± 0.08	2.58 ± 0.08	-0.03	2.37 ± 0.14	0.18
November	D1	2.73 ± 0.11	1.40 ± 0.18	1.34	2.41 ± 0.23	0.33
	D2	2.62 ± 0.06	1.79 ± 0.11	0.84	2.31 ± 0.12	0.31
	D3	2.56 ± 0.14	1.50 ± 0.09	1.06	2.69 ± 0.11	-0.13
	T1	2.17 ± 0.15	0.98 ± 0.10	1.19	2.17 ± 0.16	0
	T2	*ND*	*ND*	*ND*	*ND*	*ND*
	T3	2.72 ± 0.19	1.51 ± 0.10	1.21	2.45 ± 0.13	0.28

ND: Not determined

Table S4. p-values of ANCOVA with wet tissue weight as covariable and sampling date as factor for accumulation and log reduction in haemolymph of diploid and triploid oysters. In red, significant *p*-value

	p-values		
	Accumulation	Log reduction 24 h	Log reduction 48 h
May *vs* June	0.01	< 0.001	*ND*
May *vs* July	0.145	0.005	< 0.001
May *vs* August	0.888	0.037	0.027
May *vs* September	0.261	0.186	0.153
May *vs* November	0.583	0.135	0.073
June *vs* July	0.297	0.549	*ND*
June *vs* August	0.014	0.134	*ND*
June *vs* September	< 0.001	< 0.001	*ND*
June *vs* November	0.583	0.07	*ND*
July *vs* August	0.126	0.32	0.002
July *vs* September	0.002	< 0.001	< 0.001
July *vs* November	0.011	0.143	< 0.001
August *vs* September	0.138	< 0.001	< 0.001
August *vs* November	0.376	0.58	< 0.001
September *vs* November	0.5	0.002	0.468

ND: Not determined

Conclusion

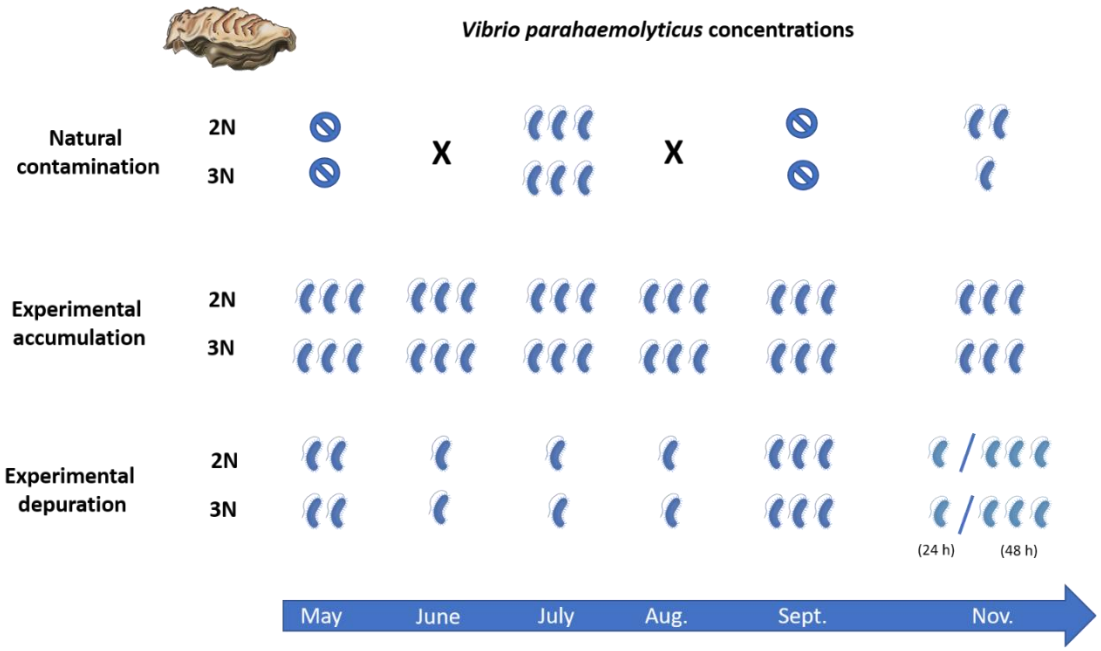

Figure 10. Résumé graphique de l'effet du niveau de ploïdie de l'huître *C. gigas* sur la contamination naturelle, et l'accumulation et la dépuration expérimentales en *V. parahaemolyticus* au cours de l'étude. Le niveau de contamination en *V. parahaemolyticus* des huîtres est imagée par le nombre de bactérie(s) pour chaque analyse. Les chromosomes représentent la ploïdie de l'huître (deux pour diploïde et trois pour triploïde). Les croix noires représentent l'absence de donnée pour ces dates. Les ronds bleus barrés représentent l'absence de *V. parahaemolyticus* pour ces dates. La dépuration expérimentale particulière en Novembre est représentée par une couleur différente des bactéries.

Les études sur l'effet de l'historique de vie et de la ploïdie de l'huître dans l'accumulation et la dépuration de *V. parahaemolyticus* apportent d'importants résultats quant au risque associé pour l'Homme. Malgré la dépuration, le risque d'infection à *V. parahaemolyticus* est présent car les critères sanitaires de contrôle des huîtres avant commercialisation (coliformes et *Escherichia coli*) ne permettent pas d'assurer l'absence totale de *V. parahaemolyticus*. De nombreuses techniques ont été développées pour éliminer *V. parahaemolyticus*, *e.g.* la congélation ultra rapide ou l'irradiation. Cependant ces techniques peuvent avoir des impacts négatifs pour l'ostréiculteur (coût d'installation et de maintenance), pour l'huître (pessimisme des consommateurs face à certaines méthodes comme l'irradiation, préservation du goût). Le développement de nouvelles méthodes afin d'assurer l'élimination de *V. parahaemolyticus* des huîtres est aujourd'hui nécessaire, ce qui fait l'objet du travail suivant.

Chapter III

Investigation of lactic acid bacteria as oyster post-harvest treatment

Contexte

L'utilisation de probiotiques a été suggérée dès le début du 20e siècle, bien que le terme « probiotique » en lui-même date des années 60. En effet, les travaux de Tissier et de Metchnikoff sont considérés comme les pierres fondatrices de la notion de bactéries bénéfiques pour l'Homme. Metchnikoff a suggéré de modifier la flore de nos corps en remplaçant les bactéries intestinales néfastes par des bactéries utiles. Tissier, quant à lui, a montré que les selles d'un enfant sain et d'un enfant diarrhéique se distinguent par rapport à leur contenu bactérien, notamment des bactéries en « Y » uniquement présentes chez l'enfant sain. Leurs travaux ont été mis de côté jusque-là fin du 20e siècle, où la recherche sur la sélection et la caractérisation de probiotiques a considérablement augmenté. De nos jours, de nombreux probiotiques sont disponibles à la consommation parmi lesquels, *Latilactobacillus* et *Bifidobacterium* sont les genres bactériens les plus représentés. Les bienfaits sur la santé humaine sont principalement orientés sur l'amélioration du transit et de la digestion ainsi que sur le traitement d'infections bactériennes causant des diarrhées (FAO and WHO, 2006).

Dès 1980, il a été suggéré que les bactéries pouvaient être utiles pas uniquement pour l'alimentation humaine, mais également comme contrôleurs biologiques pour les maladies des poissons et comme activateurs de la régénération des nutriments (Yasuda and Taga, 1980). Le nombre d'études portant sur l'utilisation de contrôles biologiques en aquaculture s'intensifie à partir des années 90 (Gatesoupe, 1999) et continue d'augmenter (Hoseinifar et al., 2018). L'utilisation des probiotiques, tels que des bactéries lactiques (lactic acid bacteria en anglais, LAB) des genres *Latilactobacillus* et *Lactococcus*, est largement étudiée en aquaculture en bassins fermés. Cependant, l'application de probiotiques est complexifiée pour la production aquacole en milieu naturel telle que la conchyliculture. Seulement quelques études ont montré que l'utilisation de souches de LAB permettaient une meilleure dépuration de *V. parahaemolyticus* chez l'huître (Kang et al., 2018; Xi et al., 2014). Il reste cependant encore beaucoup de travail à réaliser afin de déterminer les paramètres optimums pour l'utilisation de LAB pour l'élimination de *V. parahaemolyticus* lors de la dépuration d'huîtres.

Publication IV

Titre de l'article	**"Screening of marine lactic acid bacteria for *Vibrio parahaemolyticus* inhibition and application to depuration in Pacific oysters (*Crassostrea gigas*)"**
Statut de l'article	Accepté chez *Journal of Applied Microbiology* DOI : 10.1093/jambio/lxac081
Objectif de l'étude	Etudier l'utilisation de bactéries lactiques (LAB) comme potentiel traitement post-récolte pour la dépuration de *V. parahaemolyticus* de l'huître *C. gigas.*
Résultats principaux	Un screening de 30 LAB isolées de produits de la mer et supposées adaptées à des conditions de croissance en eau de mer (Projet Vi-LAB) a permis de sélectionner 3 souches d'espèces différentes capables d'inhiber *in vitro* 10 souches de *V. parahaemolyticus* avec des efficacités variables. Les résultats suggèrent que cette inhibition est principalement due à de l'acidification. L'intérêt de ces 3 LAB quant à l'élimination de *V. parahaemolyticus* lors de la dépuration d'huîtres a été étudiée chez des d'huîtres contaminées avec 4 souches de *V. parahaemolyticus*. En plus de montrer des profils de dépuration variables selon les souches de *V. parahaemolyticus*, cette étude a permis de montrer jusqu' a 1 log CFU.mL^{-1} de diminution de *V. parahaemolyticus* par rapport à la condition contrôle.
Conclusions	L'ajout de LAB lors de la dépuration d'huîtres semblent être une voie intéressante à explorer pour l'élimination de *V. parahaemolyticus*.

TITLE: Screening of marine lactic acid bacteria for *Vibrio parahaemolyticus* inhibition and application to depuration in Pacific oysters (*Crassostrea gigas*).

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ABBREVIATED RUNNING HEADING:

LAB to eliminate *V. parahaemolyticus* from oysters

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SUMMARY:

Aims: This study aims to assess the use of marine lactic acid bacteria (LAB) to reduce *V. parahaemolyticus* levels during oyster depuration process.

Methods and Results: The inhibitory effect of thirty marine LAB strains against *V. parahaemolyticus* strains was evaluated by *in vitro* assays. Three positive strains (*Latilactobacillus sakei* SF1583, *Lactococcus lactis* SF1945 and *Vagococcus fluvialis* CD264) were selected for *V. parahaemolyticus* levels reduction during oyster depuration. Pacific oysters *Crassostrea gigas* were artificially and independently contaminated by four GFP-labelled *V. parahaemolyticus* strains (IFVp201, IFVp69, IFVp195 and LMG2850^T) at 10^5 CFU.mL^{-1} and then exposed by balneation to 10^6 CFU.mL^{-1} of each LAB strains during 24 h, at 19°C. Quantification of *V. parahaemolyticus* in haemolymph by flow cytometry revealed variations in natural depuration of the different *V. parahaemolyticus* strains alone. Furthermore, the addition of LABs improved up to 1-log bacteria.mL^{-1} the reduction of IFVp201 concentration in comparison to the control condition.

Conclusions: Although further optimizations of procedure are needed, addition of marine LABs during oyster depuration is an interesting strategy to reduce *V. parahaemolyticus* levels in *C. gigas*.

Significance and impact of the study: Our study provides promising ways to develop a depuration process which could potentially be implemented in oyster farms.

Keywords: *V. parahaemolyticus*, Lactic acid bacteria, flow cytometry, oyster depuration, challenge test, seafood safety

1. Introduction

Vibrio parahaemolyticus is the first causative bacterial agent of acute gastroenteritis in humans worldwide associated to consumption of raw or undercooked seafood, especially oysters. *V. parahaemolyticus* can naturally be found in marine estuaries and coasts, and in molluscan shellfishes (Odeyemi, 2016). Probiotics, firstly investigated as replacement for antibiotics to avoid increase of antibiotic-resistant bacteria (Hossain et al., 2022), were shown to inhibit *V. parahaemolyticus* growth using *in vitro* co-culture methods (Charernjiratragul et al., 2010; Girija et al., 2018) or inhibition tests in cell lines (Le and Yang, 2018; Satish Kumar et al., 2011), and *in vivo* models such as mice (Wang et al., 2022) or marine organisms (Girija et al., 2018; Liu et al., 2015). Indeed, probiotics are now widely used in aquaculture, resulting in growth increase of shellfish, nutritional advantage and immunostimulant effects as prophylactic (Ringø, 2020). Use of probiotics with *Crassostrea* spp. oyster, improved larval growth and increased resistance against oyster pathogens (Campa-Córdova et al., 2011; Douillet and Langdon, 1994; Gibson et al., 1998; Karim et al., 2013).

To ensure the safety of oysters, depuration is performed before commercialization by transferring oysters for 24 to 48 h in UV-treated seawater (Lee et al., 2008). Oyster safety for consumers is assessed by determination of *Escherichia coli* and coliform levels but not *V. parahaemolyticus* levels (Lee et al., 2008) except in some countries such as Japan (Hara-Kudo and Kumagai, 2014). However, it is known that traditional depuration process is not efficient enough for the removal of *V. parahaemolyticus* from oysters (Shen et al., 2019). Various post-harvest processes (PHP) such as irradiation (Mahmoud and Burrage, 2009), high pressure (Berlin et al., 1999), low temperature pasteurization (Andrews et al., 2000) and fast freezing (Liu et al., 2009) were also developed to reduce *V. parahaemolyticus* levels in oysters before commercialization. Due to economic investment in such materials and to regular oyster death occurring during processes, there is a need to develop new PHP to reduce *V. parahaemolyticus* levels in oysters. New approaches were investigated using biological treatments. Until now, only few studies investigated the inhibition activities of bioprotective agents against *V. parahaemolyticus* as PHP during oyster depuration (Kang et al., 2018; Khouadja et al., 2017; Xi et al., 2014). Khouadja *et al.* (2017) showed a reduction of *V. parahaemolyticus* concentration in oysters challenged by injection with a *V. parahaemolyticus* strain (10^6 CFU/ml) and exposed to *Latilactobacillus* sp. strains (10^6 CFU.mL^{-1}) after nine days (Khouadja et al., 2017). However, in such experiments, using an injection method to contaminate oysters with *V. parahaemolyticus* is questionable that is why balneation is generally favoured. Xi *et al.*

(2014) showed a *V. parahaemolyticus* reduction of 0.65 log MPN.g^{-1} in oyster tissues after a balneation with 10^4 CFU.mL^{-1} of *V. parahaemolyticus* for 20 h followed by a balneation with 10^7 CFU.mL^{-1} of *Lactilactobacillus plantarum* for four days when compared to controls. Moreover, Kang *et al*. (2018) showed that a simultaneous exposure of oysters by balneation to 10^5 CFU.mL^{-1} of *V. parahaemolyticus* and *Enterococcus faecium* at 25°C for 24 h resulted in a *V. parahaemolyticus* reduction of 0.88 log CFU.g^{-1}. However, if of interest as a biocontrol agent against *V. parahaemolyticus*, virulence of *E. faecium* is questionable (Vancanneyt et al., 2002), thus may be not suitable for food application. Further studies are needed to determine optimal conditions for use of bioprotective agents to reduce *V. parahaemolyticus* concentration in oysters during depuration, in particular for the selection of bioprotective agents and *V. parahaemolyticus* strains. Indeed, Lactic Acid Bacteria (LAB) which are Generally Recognized As Safe (GRAS organisms) and are largely used for fermented food production (Wang et al., 2021) and food biopreservation (Borges et al., 2022) could be further investigated for depuration process. Furthermore, as different *V. parahaemolyticus* strains can exhibit different depuration profiles in oysters (Aagesen et al., 2018), several strains of *V. parahaemolyticus* should be selected for PHP evaluation.

In this study, we screened a collection of salt and cold tolerant marine LAB strains isolated from refrigerated seafood products for their growth inhibition capacities of *V. parahaemolyticus* strains *in vitro*. Three of the LAB strains tested *in vitro* and four different *V. parahaemolyticus* strains selected according to their origin, genetic content and their sensitivity to LAB strains were used to evaluate depuration in spat *C. gigas* oysters. We established a simple and accurate depuration procedure consisting of an initial exposure of oysters to *V. parahaemolyticus* strains followed by 24 h of depuration with or without LAB strains in balneation, in accordance to the depuration process (Lee et al., 2008) which could potentially be implemented in oyster farms and plants.

2. Materials and methods

a. Bacterial strains and growth conditions

Marine lactic acid bacteria (LAB) strains used in this study are listed in **Table 1**. LAB strains were isolated from seafood products by the laboratory of Microbial Ecosystems and Marine Molecules for Biotechnology (EM3B, MASAE, Ifremer). LAB strains were cultivated starting from a -80°C glycerol stock in the appropriate medium as indicated in **Table 1**, *i.e.* Man Rogosa Sharpe (MRS, Biokar Diagnostics) or Brain Heart Infusion

supplemented with 1.5 % NaCl (BHIS, Biokar Diagnostics), for two successive cultures at 30°C for 24 h.

Table 1. Bacterial strains, sample origins and culture media.

Species	Strain	Origin	Medium
Vagococcus fluvialis	CD264	Peeled shrimp	BHIS
Latilactobacillus sakei	SF1583	Smoked salmon	MRS
Lactococcus lactis	SF1945	Smoked salmon	BHIS
Weissella hellenica	SF1637	Smoked salmon	MRS
Vibrio parahaemolyticus	IFVp5	Clinical	BHIS
V. parahaemolyticus	IFVp18	Mussel (*Mytilus edulis*)	BHIS
V. parahaemolyticus	IFVp22	Mussel (*M. edulis*)	BHIS
V. parahaemolyticus	IFVp69	Mussel (*M. edulis*)	BHIS
V. parahaemolyticus	IFVp136	Mussel (*Mytilus galloprovincialis*)	BHIS
V. parahaemolyticus	IFVp177	Oyster (*C. gigas*)	BHIS
V. parahaemolyticus	IFVp182	Seawater	BHIS
V. parahaemolyticus	IFVp195	Seawater	BHIS
V. parahaemolyticus	IFVp201	Mussel (*M. edulis*)	BHIS
V. parahaemolyticus	IFVp203	Mussel (*M. edulis*)	BHIS
V. parahaemolyticus	IFVp408	Oyster (*C. gigas*)	BHIS
V. parahaemolyticus	LMG2850^T	Clinical	BHIS
V. parahaemolyticus	IFVp201-*gfp*$^+$	GFP-tagged	LBS50
V. parahaemolyticus	IFVp69-*gfp*$^+$	GFP-tagged	LBS50
V. parahaemolyticus	LMG2850-*gfp*$^+$	GFP-tagged	LBS50
V. parahaemolyticus	IFVp195-*gfp*$^+$	GFP-tagged	LBS50

MRS: Man Rogosa Sharpe, BHIS: Brain Heart Infusion Salt, LBS50: Luria Bertani 3% NaCl supplemented with 50 µg.mL^{-1} of trimethoprim

***V. parahaemolyticus* strains** used in this study were selected among the *Vibrio* collection of the laboratory of health environment and microbiology (LSEM – Ifremer) in Brest (France) (Table 1). The *V. parahaemolyticus* LMG2850^T (ATCC17802^T) strain was used in this study as a reference strain. *V. parahaemolyticus* strains were cultivated in BHIS for 24 h at 30°C starting from -80°C glycerol stock.

GFP-tagged *V. parahaemolyticus* strains. IFVp195, IFVp69 and LMG2850 strains were electroporated with the pFD086 plasmid harbouring *gfp* gene and trimethoprim-resistance expression cassettes as previously described for IFVp201-*gfp*$^+$ (Sorée et al., 2022). The *Vp-gfp*$^+$ strains were cultivated in Luria Bertani 3% NaCl supplemented with

50 µg.mL^{-1} of trimethoprim (LBS50) starting from -80°C glycerol stock for approx. 18 h at 37°C. Mutants were validated by plasmid stability determination and growth comparison between wild type and *gfp*$^{+}$ clone as previously described (Sorée et al., 2022).

b. Quantification of bacterial strains

LAB concentration was measured by calculation of colony forming units per mL of culture log transformed (log CFU.mL^{-1}) in their appropriate medium agar plates (**Table 1**).

Vp-gfp^{+} cultures were quantified using a Cyflow Space (Sysmex-Partec, Munster, Germany) by detection of their green fluorescence (FL1 detector of the flow cytometer) and their relative size and granularity (Forward SCatter [FSC]; Side SCatter [SSC]). The bacterial counting was performed according to the number of cells detected by the flow cytometer in a volume of 200 µL of sample and was expressed in log of bacteria.mL^{-1} (Sorée et al., 2022).

c. Identification of marine LAB strains by 16S rRNA gene sequencing

LAB strains presenting inhibition capacities against *V. parahaemolyticus* LMG2850^{T} were identified using partial 16S rRNA gene sequencing. Briefly, 500 µL of a 24 h culture was centrifuged for 3 min at 13,000 g and the pellet was washed with 300 µL of phosphate buffered- saline (PBS, Sigma-Aldrich, France). To avoid degradation of genomic DNA, 200 µL of Tris-EDTA (10 mM Tris-HCl, 1mM EDTA, pH 7.5) was added into the pellet. Nucleic acids were extracted by thermal lysis (15 min at 95°C) using a thermoblock (ThermomiMixer® C, Eppendorf, Germany). After another centrifugation for 3 min at 13,000 g, 200 µl of the supernatant was conserved at -20°C. The 16S rRNA gene was amplified from 1 µL of supernatant using with PCR universal primers, 8F (5'-AGAGTTTGATCATGGCTCAG-3') and 1489R (5'-GTTACCTTGTTACGACTTCAC-3') (Lane, 1991; Weisburg et al., 1991). PCR amplification was performed using the DreamTaq Green polymerase Master mix (Thermo Fisher Scientific, France) following the manufacturer recommendation and the following thermal conditions: initial denaturation at 95°C for 5 min, 30 cycles of amplification (95°C for 30 s, 52°C for 30 s, 72°C for 1 min), and final extension for 10 min at 72°C using a T100TM Thermal Cycler (Bio-Rad, Hercules, U.S.A). The Sanger sequencing of amplified products was carried out by Genoscreen (Lille, France). A consensus sequence was built using forward and reverse sequences with BioEdit software. Strain identification was performed using nucleotide BLAST on the NCBI website and n/r databank.

d. Vibrio parahaemolyticus growth inhibition

The spot-on lawn method was used to assay inhibition of *V. parahaemolyticus* by LAB strains. Briefly, *V. parahaemolyticus* strains were grown in BHIS for 24 h at 37°C and stationary cultures were 100-fold diluted with BHIS to reach an initial concentration of ~10^4 CFU.mL^{-1}. Diluted-cultures were seeded (1 mL) onto Zobell (4 g.L^{-1} of tryptone, 1 g.L^{-1} of yeast extract, 33.3 g.L^{-1} of NaCl) supplemented with 2% glucose and 1% agar plates (Z2G 1%), and these plates were air dried for 45-50 min. LAB strains were cultivated twice successively in appropriate medium (**Table 1**) in 96-deep well plate; 4 replicates for each strain (2 biological and 2 technical replicates to avoid repeatability issues) were carried out at 25-30°C for 24 h. LAB cultures, supernatant and buffered supernatants were spotted (5 µL) on the surface of Z2G 1% agar plates seeded with one of the *V. parahaemolyticus* strain. The supernatant was obtained by filtering LAB cultures using nitrocellulose filter of 0.22 µm (VWR, USA) and buffered using NaOH 2N to pH 6- pH 7 determined with pH-indicator strips pH 0-14 (VWR, Belgium).

Inhibition halos (clear zones around the spot) were observed after a 48-hour incubation at 30°C, indicating an inhibition. Halo intensity was described as: 0 for no growth inhibition, 1 for an inhibition at the colony spot, 2 for a weak or partial inhibition and 3 for a high inhibition.

e. Lactic acid bacteria persistence in filtered and sterile seawater

To verify LAB persistence in filtered and sterile seawater (FSSW, natural seawater filtered with 1 µm polypropylene mesh and treated with UV), bacterial strains were grown as previously described. The optical density at 600 nm of the washed-culture was measured to estimate the concentration and was inoculated in 1 L of FSSW to a final concentration of 10^6 CFU.mL^{-1} and maintained at 19°C. LAB concentrations were measured at 0 h, 4 h, and 24 h, in seawater samples after inoculation onto appropriate agar medium plates (**Table 1**) at 30°C for 24 h.

f. Challenge tests with oysters

Spat *Crassostrea gigas* diploid oysters were produced according to a methodology that allowed pathogen-free oysters (« Naissain standardisé Ifremer », (Petton et al., 2013)) and maintained in controlled inland experimental platforms in Argenton (Finistère, France) and Bouin (Vendée, France) using sand-filtered and UV-treated seawater. These oysters were produced in 2020; they were 12.7 ± 4.0 g of total weight (tissues and shell) and 4.5 ± 0.5 cm long), and have never been exposed to environmental marine natural microflora. Effects of three LAB strains (CD264, SF1583 and SF1945) on *Vp-gfp*$^+$

elimination during oyster depuration were investigated during *in vivo* experimental bacterial challenge tests with oysters (**Figure 1**).

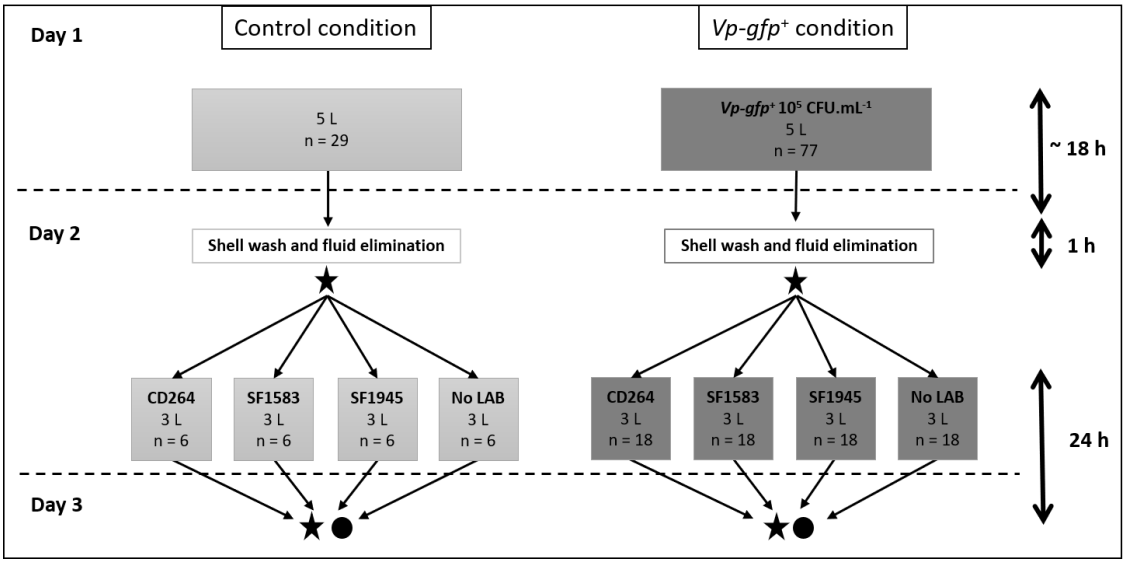

Figure 1. Experimental protocol of challenge tests of oysters with *Vp-gfp*⁺ and LAB strains. Tanks of 5 L and 3 L for of filtered and sterile seawater. Stars: *Vp-gfp*⁺ quantification by flow cytometry in haemolymph. Circles: LAB enumeration in haemolymph on agar plates. n: number of oysters.

Overnight accumulation of *Vp-gfp*⁺ in oysters. Before experimentation with oysters, stationary cultures of *Vp-gfp*⁺ were centrifuged and the pellets were washed twice in buffered physiological-water (BPW, 0.4 g.L^{-1} of $K_2H_3PO_4$; 4.5 g.L^{-1} of Na_2HPO_4, $12H_2O$; 7.2 g.L^{-1} of NaCl). Each washed-culture of *Vp-gfp*⁺ was inoculated in separate 5 L tank of FSSW with an initial concentration of 10^5 CFU.mL^{-1} of seawater that was confirmed by flow cytometry. Oysters (n = 77) were transferred in contaminated tanks and exposed to *Vp-gfp*⁺ for approximately 18 h at 20°C. A control tank with oysters (n = 29) not exposed to *Vp-gfp*⁺ was maintained in the same conditions. Aeration was provided to all tanks. After the overnight accumulation, concentration of *Vp-gfp*⁺ in seawater was determined by flow cytometry and oysters were transferred one hour into "fresh" FSSW to remove potential *Vp-gfp*⁺ present on shells and in shell fluid. After this hour, concentrations of *Vp-gfp*⁺ in haemolymph (one pool of five oysters for each condition) were determined by flow cytometry. Haemolymph samples were collected using sterile tools and workstation. Each oyster was shucked, and abductor muscles were cautiously cut with a scalpel to allow opening of the shell and avoid damaging of the pericardial cavity. Haemolymphs were collected into the pericardial cavity using a 25G needle and 1 mL insulin syringe. Collected haemolymphs were filtered on a 30 µm nylon mesh to remove aggregates and/or debris (Sorée et al., 2022).

Oyster exposure to LAB strains. Before experimentation with oysters, stationary LAB cultures were centrifuged and the pellet was washed twice in BPW, and plated onto respective medium (**Table 1**) to determine culture concentrations (48 h at 30°C). Washed-LAB cultures were inoculated in separate 3 L tanks of FSSW at a final concentration of 10^6 CFU.mL^{-1} as described in **Figure 1**. Seawater samples from each tank were plated onto MRS or BHIS agar (48 h at 30°C) to enumerate the initial concentrations of LAB in seawater. Oysters previously exposed (n = 18) and not exposed to *Vp-gfp*$^+$ (n = 18) were transferred in separate 3 L LAB inoculated tanks for 24 h at 20°C. Oysters exposed to *Vp-gfp*$^+$ (n = 6) and control oysters (n = 6) were maintained as negative controls in the same conditions but not exposed to LAB strains (**Figure 1**). After the 24 h exposure to LAB, haemolymphs were collected from three pools of five oysters for LAB-exposed oysters and from one pool of five oysters for non-exposed oysters. LAB enumerations were performed by plating of haemolymph onto MRS or BHI agar (48 h at 30°C) and *Vp-gfp*$^+$ quantifications were performed by flow cytometry. Seawater pH was determined using pH-indicator strips pH 0-14 (VWR) in each tank.

g. Statistical analyses

All statistical analyses were performed using RStudio 2021.09.0+351 "Ghost Orchid" Release (2021-09-20) for Windows. Data are presented as means ± standard error and a $p < 0.05$ indicated a statistically significant difference. Normality and homogeneity of variables were verified using Shapiro test and Levene test, respectively.

Concentrations of *Vp-gfp*$^+$ in oyster haemolymph after 24 h of depuration were analysed using an ANOVA and post hoc Tukey test grouped by *Vp-gfp*$^+$ strain and Bonferroni adjusted with the condition (with or without LAB during the depuration) as factor.

3. Results

a. Inhibition of *Vibrio parahaemolyticus* strains by LAB

The inhibition capacities of 30 seafood LAB against the *V. parahaemolyticus* LMG2850^T strain were assessed using miniaturized spot-on lawn method (**Table S1**). Strain identification by 16S rRNA gene sequencing revealed that no inhibition was observed for *Enterococcus* sp. and *Vagococcus* sp. in the screening conditions. Fifteen positive strains were identified: 4 among 12 (4/12) *Carnobacterium* sp. strains, 1/1 *Lactococcus lactis* strain, 2/5 *Latilactobacillus* sp. strains and 8/8 *Weissella* sp. strains. Three strains from different genera were selected for further inhibition tests: *Latilactobacillus sakei* SF1583, *Lactococcus lactis* SF1945 and *Weissella hellenica* SF1637. The capacity of these three LABs to inhibit eleven *V. parahaemolyticus* growth was then investigated, using the

miniaturized spot-on lawn method. Results suggested variable inhibition profiles of *V. parahaemolyticus* growth by LAB strains (**Table 2**).

In these experimental conditions, the *L. sakei* SF1583 and *W. hellenica* SF1637 cultures and supernatants displayed inhibitory activities against most *V. parahaemolyticus*. On the contrary, *L. lactis* SF1945 culture was inactive against four *V. parahaemolyticus* strains while supernatant was inactive against all strains but IFVp195. Five *V. parahaemolyticus* (IFVp22, IFVp182, IFVp201, IFVp203, and IFVp408) showed the same behaviour as LMG2850^T towards LAB cultures, independently of their clinical or environmental origin (**Table 1**). Results showed that IFVp195 appeared particularly sensitive to the three LAB cultures whereas IFVp177 seemed resistant. This could be probably related to acid resistance; indeed, the loss of supernatant activity when adjusted at pH 7 suggested an inhibitory effect by acid production (**Table 2**). These results were in accordance with the pH of cultures. SF1583 and SF1637 exhibiting average to high inhibitory activities, reached a pH of 4 in MRS medium at the stationary phase. The SF1945 strain, exhibiting null to low inhibitory activities, had a pH of 6 when cultured in BHI medium.

Table 2. Inhibitory activity profiles of LAB strains according to *V. parahaemolyticus* strains, by spot-on lawn method. Inhibitory activity was related to halo size. 0: no inhibition, 1 to 3: weak to strong inhibition.

		LAB whole cultures			**LAB supernatant (SN)**			**LAB buffered-SN**		
		SF1583	SF1945	SF1637	SF1583	SF1945	SF1637	SF1583	SF1945	SF1637
***V. parahaemolyticus* strains**	LMG2850^T	2	1	1	3	0	3	0	0	0
	IFVp5	1	0	1	3	0	3	0	0	0
	IFVp18	1	0	1	3	0	3	0	0	0
	IFVp22	2	1	1	3	0	3	0	0	0
	IFVp136	1	0	1	3	0	3	0	0	0
	IFVp177	1	0	0	3	0	3	0	0	0
	IFVp182	2	1	1	3	0	3	0	0	0
	IFVp195	3	2	3	3	1	3	0	0	0
	IFVp201	2	1	1	3	0	3	0	0	0
	IFVp203	2	1	1	3	0	3	0	0	0
	IFVp408	2	1	1	3	0	3	0	0	0

b. Persistence of LAB strains in seawater

Persistence of selected LAB strains in seawater needed to be considered before investigating them for oyster depuration. The strain *Vagococcus fluvialis* CD264 was added to the analysis although none of the *Vagococcus* strains tested in the *in vitro* screening assay inhibited *V. parahaemolyticus* (**Table S1**) because this strain previously showed inhibition capacity against *Vibrionacaeae* such as *Photobacterium phosphoreum*

(Wiernasz et al., 2017). Inoculated between 5.1 and 6.7 log CFU.mL^{-1} in seawater à 19°C, all three LAB strains persisted at approximately 5 log CFU.mL^{-1} after 24 h of experimentation (**Figure 2**). CD264 remained at 5.1 log CFU.mL^{-1} from 0 h to 24 h of persistence. SF1583 and SF1637 significantly decreased at 5.3 log CFU.mL^{-1} after 4 h, and at 5.4 and 5.1 CFU.mL^{-1} after 24 h, respectively. SF1945 concentration remained stable during 4 hours and a significant decrease was observed to 5.5 CFU.mL^{-1} after 24 h.

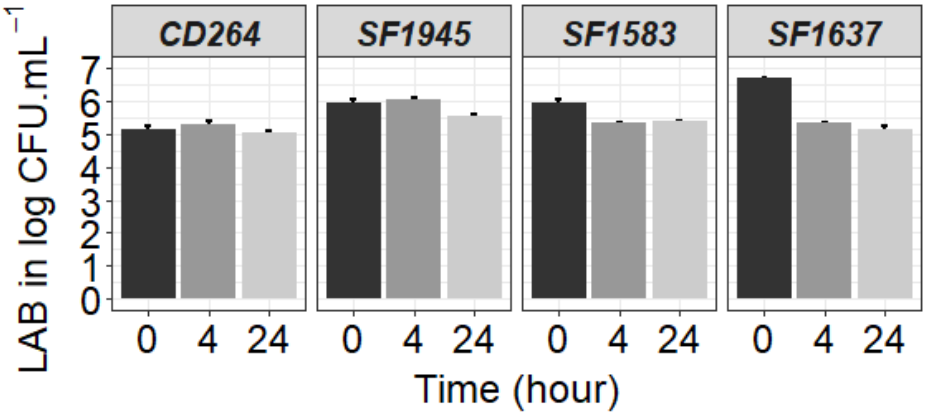

Figure 2. LAB strains persistence in seawater, at 19°C, during 24 h. ***Vagococcus fluvialis*** **CD264,** ***Latilactobacillus sakei*** **SF1583,** ***Weissella hellenica*** **SF1637 and** ***Lactococcus lactis*** **SF1945**. Data represents mean ± standard error

c. Validation of *Vp-gfp*$^{+}$ mutants

Four strains of *V. parahaemolyticus* were selected for this experiment: the reference strain LMG2850^{T}, and IFVp18, IFVp195 and IFVp201 displaying different virulence gene content (Sorée *et al*., in preparation), origins (**Table 1**) and LAB sensitivities (**Table 2**). GFP-tagged *V. parahaemolyticus* (*Vp-gfp*$^{+}$) strains were then constructed to quantify them by flow cytometry according to a method previously described (Sorée at al., 2022). For unknown reasons, electroporation of IFVp18 failed, and it was thus replaced by the IFVp69 strain which harboured the same genetic profile concerning *tdh, trh* and T3SS genes than IFVp18 (Sorée *et al*., in preparation). Assays of plasmid stability showed that pFD086 plasmid persisted after 24 h at 100% in IFVp195-*gfp*$^{+}$ and IFVp69-*gfp*$^{+}$, and at 96% in LMG2850-*gfp*$^{+}$ strains after an initial inoculation of 10^{5} CFU.mL^{-1} in FSSW. The differences of doubling time between wild type and GFP-tagged strains were of 0 min (doubling time = 43 min) for LMG2850^{T}, 1 min (35 and 34 min, respectively) for IFVp69 and 5 min (60 and 65 min, respectively) for IFVp195, showing that the pFD086 plasmid did not impact growth of these strains.

d. Challenge tests of oysters with IFVp201-*gfp*⁺ and LAB strains

Effects of three LAB strains (CD264, SF1583 and SF1945) on *Vp-gfp*⁺ removal during oyster depuration were investigated during *in vivo* experimental bacterial challenge tests with oysters. Because of experimental costs and time, only one of the two *Lactobacillaceae* SF1583 and SF1637 was retained. Spat *C. gigas* oysters were exposed to an initial concentration of *Vp-gfp*⁺ in seawater that ranged from 4.4 to 4.6 log bacteria.mL^{-1} (**Figure 3A**). Concentrations of *Vp-gfp*⁺ after the balneation with oysters ranged from 3.1 to 4.0 log bacteria.mL^{-1} in seawater (**Figure 3B**) and from 2.1 to 2.4 log bacteria.mL^{-1} in oyster haemolymph (**Figure 3C**), corresponding to a 100-fold decrease when compared to initial concentration. Oysters were then washed and put in clean seawater for natural depuration, containing LAB strains or not. After 24 h, *Vp-gfp*⁺ concentrations in oyster haemolymph ranged from 1.3 to 3.6 log bacteria.mL^{-1} in the absence of LAB strains (**Figure 3D**).

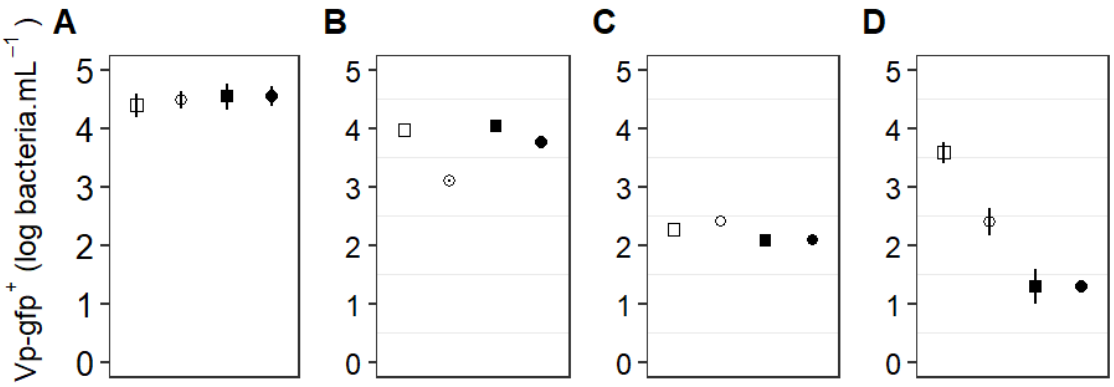

Figure 3. Concentrations of *Vp-gfp*⁺ in seawater and haemolymph during challenge test. (A) Initial concentration of *Vp-gfp*⁺ in seawater. (B) Concentrations of *Vp-gfp*⁺ in seawater after the overnight balneation with oysters. (C) Concentrations of *Vp-gfp*⁺ in oyster haemolymph after the overnight exposure with oysters. (D) Concentration of *Vp-gfp*⁺ in oyster haemolymph after 24 h of natural depuration. (□): IFVp201-*gfp*⁺, (○): IFVp69-*gfp*⁺, (■): IFVp195-*gfp*⁺, (●): LMG2850-*gfp*⁺. Data represents the mean ± standard deviation.

LAB strains were inoculated in seawater at concentrations ranging from 5.2 to 5.7 log CFU.mL^{-1} for CD264, from 5.4 to 5.9 log CFU.mL^{-1} for SF1583 and from 5.8 to 6.1 log CFU.mL^{-1} for SF1945. After LAB exposure (24 h), no oyster mortality was recorded, neither in the *Vp-gfp*⁺ exposed tanks, nor in control tanks, and concentrations of CD264, SF1583 and SF1945 in oyster haemolymph were of 3.6, 3.9 and 4.4 log CFU.mL^{-1}, respectively. Only IFVp201-*gfp*⁺ depuration was significantly higher with CD264 (p = 0.01), SF1583 (p = 0.03) and SF1945 (p = 0.02) compared to control condition, without LAB (**Figure 4**). IFVp195-*gfp*⁺ and LMG2850-*gfp*⁺ were rapidly eliminated by the oyster

after 24 h of depuration but *V. parahaemolyticus* concentrations were slightly lower in presence of LAB strains in comparison with the control condition ($p > 0.05$). IFVp69-*gfp*$^+$ depuration was not promoted by LAB presence with concentrations ranging from 2.4 to 2.7 log bacteria.mL^{-1} in haemolymph after 24 h of depuration, and concentration was even slightly higher in presence of SF1945 compared to the control condition (**Figure 4**).

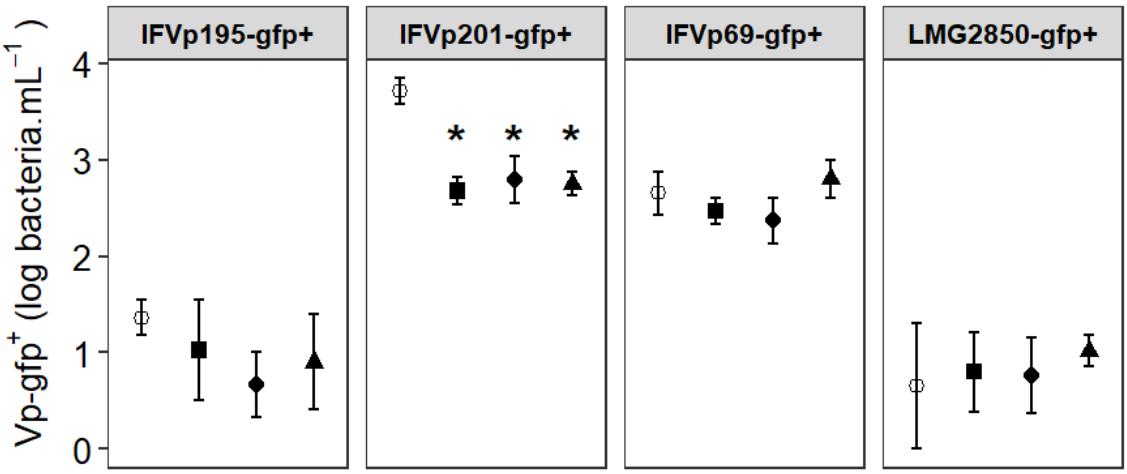

Figure 4. *Vp-gfp*$^+$ quantification in oyster haemolymph after an overnight exposure of oysters followed by 24h-incubation with different LAB. (○): *Vp-gfp*$^+$ alone, (■): *Vp-gfp*$^+$ and *V. fluvialis* CD264, (●): *Vp-gfp*$^+$ and *L. sakei* SF1583, and (▲): *Vp-gfp*$^+$ and *L. lactis* SF1945. Control: data are represented as the mean of technical replicates of one pool of five oysters ± standard error. CD264, SF1583 and SF1945: data are represented as the mean of three pools of five oysters ± standard error. *: $p < 0.05$

4. Discussion

Probiotics are widely used in aquaculture as replacement of antibiotics. Growth of *V. parahaemolyticus*, marine bacteria known as foodborne pathogen, was shown to be inhibited *in vitro* by a range of probiotics (Charernjiratragul et al., 2010; Girija et al., 2018). Bioprotective agents could be helpful in depuration process when other methods either failed to completely eliminate *V. parahaemolyticus,* or are detrimental for oysters or too expensive for oyster farmers. In this study, marine and thus, salt and cold resistant LAB strains were investigated for their anti-*V. parahaemolyticus* activities *in vitro* and for *V. parahaemolyticus* removal during *Crassostrea gigas* depuration.

In our study, among the six LAB genera investigated, some strains of *Latilactobacillus* sp., *Carnobacterium* sp., and *Lactococcus lactis,* and all tested strains of *Weissella* sp. showed inhibitory activities against *V. parahaemolyticus.* Antimicrobial activities of LAB may be caused by acid, hydrogen peroxide or bacteriocin production (Lindgren and Dobrogosz, 1990). In our study, LAB cultures adjusted to pH 7 no longer exhibited inhibition activity

against *V. parahaemolyticus* growth, suggesting an inhibition by acid production. Indeed, *V. parahaemolyticus* strains were shown to be acid sensitive (Beuchat, 1976; Fan et al., 2022). Moreover, variations in acid sensitivity of *V. parahaemolyticus* could explain the variations in inhibition between *V. parahaemolyticus* strains observed in our study (Chiang et al., 2012). To our knowledge, it was the first study showing antimicrobial activity of *Weissella* sp. strains against *V. parahaemolyticus*, but as some species of *Weissella* can cause infections in human and rainbow trout (Fusco et al., 2015), the SF1637 was not selected for the following oyster experimentations. According to these results, the two strains, *Latilactobacillus sakei* SF1583 and *L. lactis* SF1945 as well as the *Vagococcus fluvialis* CD264 strain were selected for practical application for oyster depuration.

The LAB strains used in this study were isolated from refrigerated marine seafood. Their origins probably explain their persistence in seawater, seafood matrix is an ecological niche characterized by low temperature and depleted in carbohydrates easily assimilable. Oysters were firstly contaminated with *V. parahaemolyticus* and exposed to LAB strains to investigate a depuration procedure which would answer to PHP and regulation issues, and would be easily accessible to oyster farmers and processing oyster plants. Oysters were contaminated in average by 2.25 log bacteria.mL^{-1} of *V. parahaemolyticus* corresponding to concentrations found in environmental oysters (Jones et al., 2014; Kirs et al., 2011). As the depuration profiles in oysters was shown to vary according to *V. parahaemolyticus* strains (Aagesen et al., 2018), we thus tested the LAB efficiency against four different *V. parahaemolyticus* strains. Indeed, results showed higher depuration of IFVp195 and LMG2850^{T} from oysters than of IFVp201 and IFVp69 to a lesser extent. LAB strains were able to concentrate in oyster tissues after 24 h with approximately 4 log CFU.ml^{-1}, which is slightly less than the concentration used for other oyster bioprotective agents (Xi et al., 2014). LAB strains did not promote *V. parahaemolyticus* proliferation in oysters, even if a slightly higher concentration of IFVp69 with SF1945 was observed when compared to the control condition. Furthermore, in our conditions, LAB effects varied according to *V. parahaemolyticus* strains. The natural depuration of IFVp195 and LMG2850^{T} allowed to observe a slight LAB effect with lower *V. parahaemolyticus* concentrations in presence of LAB in comparison to control conditions. IFVp69 depuration was not enhanced by the presence of LAB strains. These results were a little more promising to those of previous experimentations (Kang et al., 2018; Xi et al., 2014). Indeed, we obtained similar results to Kang *et al.* (2018) but with a seawater temperature of 20°C, more suitable for depuration process in oyster plants and farms than 25°C. With the use of a *L. plantarum*

strain, Xi *et al.* showed a difference of 0.12 and 0.65 log CFU/ml at 15°C and 10°C, respectively, suggesting that these low temperatures could not be optimal for LAB activity. LAB strains used in our study were isolated from refrigerated seafood products, which could be an advantage for their survival in seawater and for the reduction of *V. parahaemolyticus* in oysters as it was shown that *V. parahaemolyticus* depuration was higher at lower temperatures (15°C) (Chae et al., 2009). Differences in LAB concentrations inoculated in seawater, 10^5 (Kang *et al.*) and 10^6 CFU.mL^{-1} (our study), did not seem to impact depuration efficacy. Similarly, the exposure of oysters with *V. parahaemolyticus* and LAB strains at the same time (Kang *et al., 2018*) or successively (our study) did not seem to impact depuration efficacy.

Prophylactic treatment of oyster larvae with probiotics could improve effects on *V. parahaemolyticus* by lowering contamination of spat and adult oysters in natural environment and facilitate reduction of *V. parahaemolyticus* levels during depuration. Indeed, previous studies showed that prophylactic use of probiotics in oyster larvae production increased growth, survival, resistance against pathogens and immune system (Campa-Córdova et al., 2011; Fdhila et al., 2017; Sánchez-Ortiz et al., 2020). Moreover, bioprotective agent cocktails previously showed a stronger inhibition response against human pathogens such as *Escherichia coli* (Ferreira et al., 2021; Kumar et al., 2016) or *Vibrio cholerae* (VidyaLaxme et al., 2014). In the same idea, bioprotective agents could be used in a synergistic manner with another PHP to increase oyster depuration. With this in mind, further studies could focus on combination of prophylactic (larval hatchery) and therapeutic (depuration process) treatments with bioprotective agents, and on bioprotective agents cocktails against *V. parahaemolyticus* ; this would allow to comply with the National Shellfish Sanitation Program (NSSP) from the US Food and Drug Administration (FDA) which recommends a reduction of *V. parahaemolyticus* to non-detectable levels (< 30 MPN.g^{-1}) and to achieve a minimum 3.52 log reduction (FDA, 2019) to validate a PHP.

In our study, we evaluated inhibition activities of marine LAB strains against *V. parahaemolyticus* growth. Use of LAB strains during oyster depuration is an interesting way to overcome the issues with existing post-harvest processes concerning *V. parahaemolyticus* elimination from oysters before commercialization. However, further studies are needed to determine optimal conditions and improve and validate appropriate depuration methods for an efficient reduction of *V. parahaemolyticus* levels in oysters like the use of bioprotective agent cocktails or combined-prophylactic treatments. Moreover, before implementation in oyster plants and farms, sensory

analyses will be needed to evaluate the impact of LAB strains on the smell and taste of oysters (Wiernasz et al., 2017).

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AUTHOR CONTRIBUTIONS

Conceptualization: DHH, CDL, VVB, DP. Methodology: MS, EH, SL, LK. Investigation and result analysis: MS, EH, SL, DHH, LK, DP. Writing – Original draft preparation: MS, DP, LK. Writing – review and editing: MS, CDL, VVB, DHH, DP. Supervision: DHH, DP.

CONFLICTS OF INTEREST

No conflict of interest declared.

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SUPPLEMENTARY TABLES

Table S1. LAB Inhibition capacities against *V. parahaemolyticus* LMG2850 strain by spot on lawn method, on Z2G medium agar (Zobell broth supplemented with 2% glucose and 1% agar). 0 :no inhibition, 1 to 3: weak to strong inhibition. R1, R2: biological replicates, a and b : technical replicates.

Species	strain	growth medium	R1a	R1b	R2a	R2b
Carnobacterium divergens	V41	BHI NaCl 2%	0	0	0	0
Carnobacterium divergens	SF 1372	Elliker	0	0	0	0
Carnobacterium divergens	SF 1486	Elliker	0	0	0	0
Carnobacterium divergens	SF 1514	Elliker	0	0	0	0
Carnobacterium divergens	SF 1516	Elliker	0	0	0	0
Carnobacterium divergens	SF 1518	Elliker	0	0	0	0
Carnobacterium divergens	SF 1533	Elliker	0	0	0	0
Carnobacterium inhibens	CD 344	BHI NaCl 2%	2	1	2	0
Carnobacterium inhibens	MIP 2551	BHI NaCl 2%	3	2	2	3
Carnobacterium maltaromaticum	SF 1371	Elliker	0	0	0	0
Carnobacterium pleistocenium	EU 2258	BHI NaCl 2%	1	0	0	0
Carnobacterium viridans	EU 2254	BHI NaCl 2%	1	1	1	0
Enterococcus faecalis	CD 267	BHI NaCl 2%	0	0	0	0
Lactococcus lactis	**SF 1945**	**BHI NaCl 2%**	0	0	2	2
Latilactobacillus curvatus	SF 1574	MRS	0	0	0	0
Latilactobacillus fuchuensis	SF 1527	MRS	0	0	0	0
Latilactobacillus fuchuensis/curvatus	SF 1535	MRS	0	0	2	0
Latilactobacillus sakei	EU 2203	MRS	0	0	0	0
Latilactobacillus sakei	**SF 1583**	**MRS**	2	2	0	0
Vagococcus fluvialis	CD 273	BHI NaCl 2%	0	0	0	0
Vagococcus fluvialis	CD 294	BHI NaCl 2%	0	0	0	0
Vagococcus fluvialis	CD 295	BHI NaCl 2%	0	0	0	0
Weissella hellenica	**SF 1637**	**MRS**	3	3	2	3
Weissella viridescens	SF 1628	MRS	3	3	2	3
Weissella viridescens	SF 1632	MRS	3	3	3	3
Weissella viridescens	SF 1639	MRS	3	3	3	3
Weissella viridescens	SF 1641	MRS	3	3	3	3
Weissella viridescens	SF 1642	MRS	3	3	3	3
Weissella viridescens	SF 1643	MRS	3	3	3	3
Weissella viridescens/minor	SF 1635	MRS	3	3	3	3

Table S2. Initial concentrations of LAB strains in seawater for each condition

	LAB strains		
***V. parahaemolyticus* strains**	**CD264**	**SF1583**	**SF1945**
IFVp69	5.23 ± 0.04	5.94 ± 0.02	5.79 ± 0.06
IFVp201	5.24 ± 0.14	5.70 ± 0.20	5.93 ± 0.07
IFVp195	5.66 ± 0.21	5.45 ± 0.07	6.07 ± 0.07
LMG2850	5.57 ± 0.18	5.42 ± 0.04	6.01 ± 0.01

Conclusion

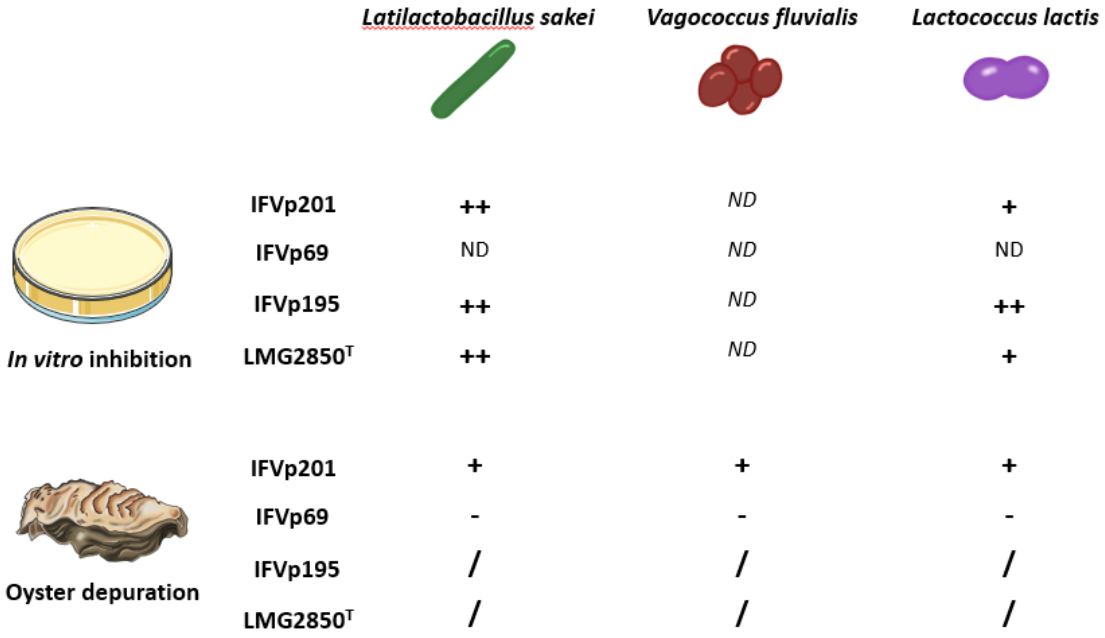

Figure 11. Résumé graphique de l'activité d'inhibition des bactéries lactiques sur la croissance *in vitro* de souches de *V. parahaemolyticus* et lors de la dépuration d'huîtres *C. gigas*. ND : non déterminé. / : dépuration naturelle donc effet des LAB faiblement visibles, **++** : forte inhibition, + : inhibition (*in vitro* inhibition) ou élimination (oyster depuration) moyenne, - : pas d'inhibition (*in vitro* inhibition) ou d'élimination (oyster depuration)

General discussion, conclusions and perspectives

I. General discussion

Vibrio parahaemolyticus is a marine bacterium naturally present in coastal and estuarine environment and represents a public health issue in many countries (Iwamoto et al., 2010). In USA, *V. parahaemolyticus* infection cases need to be mandatory declared to health authorities (CDC, 2017). In Europe, no regulation about mandatory declaration is established for now although vigilance concerning *Vibrio* risk is settled with the *Vibrio* map viewer https://www.ecdc.europa.eu/en/publications-data/vibrio-map-viewer). In France, incidence of *V. parahaemolyticus* infection is really low, while prevalence of *V. parahaemolyticus* in oysters and surrounding environment is high and can rise 100% of positive samples during the summer (Deter et al., 2010; Esteves et al., 2015; Robert-Pillot et al., 2014). Many studies suggested that surface seawater temperature (SST) is the main factor involved in variation of *V. parahaemolyticus* concentration in oysters and their surrounding environment (Johnson et al., 2012). In the global change and the increased SST context, many authors warned about the increase of *Vibrio* spp. proliferation and *Vibrio* infection cases (Baker-Austin et al., 2013; Froelich et al., 2019). Indeed, *V. parahaemolyticus* was isolated in oysters at higher latitudes (Baltic sea) and during a longer period than what was observed 20 years ago (Baker-Austin et al., 2013; Vezzulli et al., 2016), these observations were reinforced by forecasting models for the following 100 years (Hartwick et al., 2019). In this context, efforts should be made in order to further characterize environmental strains and their virulence (Publication I – *In silico* genetic analysis and *in vivo* virulence of *Vibrio parahaemolyticus* strains), as well as *V. parahaemolyticus* risk factors associated to shellfish (Publication II and III – Life history and ploidy of *Crassostrea gigas* and *Vibrio parahaemolyticus* contamination) and decrease *V. parahaemolyticus* risk during oyster consumption (Publication IV – Lactic acid bacteria to eliminate *V. parahaemolyticus* from oysters before commercialization).

1. *In sillico* genetic analyses and *in vivo* virulence of *Vibrio parahaemolyticus* strains

The Laboratory of Health Environment and Microbiology (LSEM - Ifremer, Brest, France) isolated more than 300 environmental *V. parahaemolyticus* strains between 1999 and 2012 from various samples (shellfishes, seawater, sediment…) collected from French coastal areas (Hervio Heath et al., 2002; Tall, 2013). Ten environmental and one clinical *V. parahaemolyticus* strains were selected, based on virulence gene content by qPCR methods, and their whole genome was sequenced. The genotypic diversity of these 11 *V. parahaemolyticus* strains was characterized *in silico* according to composition of core

and accessory genomes, presence of various presumably and known virulence genes, and prediction of prophages and pathogenicity islands (Publication I).

A parallel is made between the origin of *V. parahaemolyticus* isolates (clinical or environmental) and pathogenicity according to presence of *tdh* and/or *trh* genes but clinical isolates were once environmental isolates, and nothing say that an environmental isolate could not be involved in clinical cases in the future. Indeed, studies showed that haemolysin genes can be absent in clinical isolates (Jones et al., 2012), and conversely haemolysin genes can be present in environmental isolates (DePaola et al., 2003a; Jones et al., 2012). Percentage of strains harbouring the haemolysin genes could be biased by the technology employed such as PCR primers (Gutierrez West et al., 2013). In this thesis work, we showed during enumerations of indigenous *V. parahaemolyticus* in 2N and 3N oysters that 100% of oyster samples tested were *trh2*$^+$ in July and 96% were *tdh*$^+$ in November (Publication III). Presence of these genes in environmental isolates suggested the possibility that haemolysins and/or other virulence factors could be part of adaptative strategies for their survival in response to environmental stress or bacterial competition and that *V. parahaemolyticus* could simply be an opportunistic pathogen in humans and other animals. The 11 environmental *V. parahaemolyticus* strains sequenced in our study harbour various haemolysin gene content concerning *tdh/trh* combinations and variants (Publication I – Table 2 and Figure S2). The strains exhibit also some characteristics common to all *V. parahaemolyticus* strains, such as presence in all strains of T3SS1, T6SS2, flagella and type IV pili clusters. Moreover, all the *trh*$^+$ strains harboured the urease operon and the T3SS2β while the other strains harboured the T3SS2α (Park et al., 2000), except for IFVp18 and IFVp69 which did not harbour a T3SS2 cluster.

However, some particularities were identified in these 11 strains. Firstly, IFVp201 and IFVp203 exhibited a VPIII organization of the T3SS1 cluster resulting in the replacement of the VP1676-VP1679 region by three other genes (Wu et al., 2020). These three new genes were also identified by BLASTp on chromosome I of five other *V. parahaemolyticus* genomes (IFVp18, IFVp22, IVp177, IFVp195 and IFVp408). This region was not be involved in cytotoxicity in HeLa cells using deleted mutant of the region VP1676-VP1679 (Ono et al., 2006) and in fish cells by comparison of strains harbouring different T3SS1 organizations (Wu et al., 2020). Analyses of their putative function suggested that they were part of a tripartite efflux pump operon with a major facilitator superfamily (MFS) transporter, a periplasmic adaptor protein and a TetR regulator, while the outer membrane factor was encoded outside of this operon, as frequently seen in other gram-

negative bacteria (Alav et al., 2021). Tripartite efflux pumps were shown to be mainly involved in antibiotic resistance but also in virulence, quorum sensing or biofilm formation (Alav et al., 2021). Furthermore, a biosynthesis cellulose operon (*bcs*) was identified in strains lacking the T6SS1 (IFVp201, IFVp203 and IFVp177). Meparambu Prabhakaran *et al.* suggested that this operon could be involved in environmental fitness in place of the T6SS1 (Meparambu Prabhakaran et al., 2021) and presented a similar organization to the one of *E. coli* and *Salmonella enterica* serovar *Typhimurium* (Krasteva et al., 2017). Cellulose was identified as a major component of biofilm involved in environmental survival (Augimeri et al., 2015) and in virulence as shown for *V. cholerae* which exhibited a hyper-infectious phenotype when bacteria were grown in biofilm (Tamayo et al., 2010). As suggested above, presence of these genes does not mean a virulent phenotype, thus *in vivo* experiments are needed to determine pathogenicity of *V. parahaemolyticus* strains.

Using the infant rabbit model, IFVp201 (*tdh3*$^+$ *trh1*$^+$) showed a highly virulent phenotype while IFVp195 (*tdh1*$^+$) an avirulent phenotype. These results obtained with IFVp195 were interesting especially since this strain harbours the T3SS2α, shown to play an important role in virulence of the RIMD2210633 strain in rabbit model (Ritchie et al., 2012). This result could be explained by different parameters. First, an effector, which can be translocated by the T3SS2 only, can be present in RIMD2210633 but absent in IFVp195, thus explaining differences in virulence phenotype between these two strains. Second, low IFVp195 concentrations in intestinal homogenates 120 HPI could reflect the absence of colonization of the small intestine by the bacteria and/or a low survival rate in intestinal tracts of infant rabbits. Bacterial survival in intestinal tract can be associated to different processes, and in particular to acid sensitivity. Indeed, as shown in Chapter III, IFVp195 showed a particular high sensitivity to acid production by LAB strains during *in vitro* inhibition tests in comparison to the other *V. parahaemolyticus* strains (Publication IV). The pH of rabbit stomach is of 1-2 (Merchant et al., 2011) which could have a negative impact on IFVp195 survival, and thus on intestine colonization. In 2019, Wang *et al.*, using *in vitro* simulated digestive fluid, demonstrated that among the four genotypes (*tdh*$^+$ *trh*$^+$, *tdh*$^+$ *trh*$^-$, *tdh*$^-$ *trh*$^+$ and *tdh*$^-$ *trh*$^-$), *tdh*$^+$ *trh*$^+$ *V. parahaemolyticus* strains had a greater survival rate than *tdh*$^+$ *trh*$^-$ strains (Wang et al., 2019). These results could explain why IFVp201 (*tdh*$^+$ *trh*$^+$) appeared more virulent than RIMD2210633 (*tdh*$^+$ *trh*$^-$) (Ritchie et al., 2012). It is important to note that the absence of virulence of IFVp195 (*tdh1*$^+$) strain in infant rabbit question about reliability of haemolysins as criteria of potential pathogenicity of *V. parahaemolyticus*, especially since controls of seafood based

their methods on *tdh/trh* detection in samples resulting in the removal of the seafood product and/or farm closure (DGAl, 2019; NSSP, 2017)

The wax moth *Galleria mellonella* appeared as a promising model to study virulence potential of various bacterial species (Bokhari et al., 2017; Miyata et al., 2003; Tran et al., 2010). We showed that systemic infection of larvae by *V. parahaemolyticus* was partially induced by the T3SS1 using historical mutants of RIMD2210633 (Ritchie et al., 2012). However, the systemic infection was not entirely explained by the T3SS1, because infection with the mutant resulted in only 38% of larvae survival. Other mechanisms should be involved in *V. parahaemolyticus* virulence in larvae after an infection by injection, among which the presence and expression of VP1678 gene or T6SS cluster (Brodmann et al., 2021; Liaw et al., 2019).

2. Life history and ploidy level of *Crassostrea gigas* oysters, and *Vibrio parahaemolyticus* contamination

Presence and concentration of *V. parahaemolyticus* in seafood, *e.g.* oysters, is a critical turning point in public health issue. Abiotic factors influencing abundance of *V. parahaemolyticus* in oysters were widely studied in comparison to biotic factors associated to host as oysters.

Here, we firstly investigated the life history of oysters on accumulation and depuration of *V. parahaemolyticus* in *C. gigas* (Publication II) using three batches of diploid *C. gigas* oysters maintained year-round and all their life in Ifremer secured infrastructures (naive oysters) and three batches of oysters grown in the intertidal environment (environmental oysters). Experimental IFVp201 accumulation and depuration between naive and environmental oysters were compared. Then, the impact of ploidy levels of oysters on accumulation and depuration of *V. parahaemolyticus* in *C. gigas* (Publication III) was investigated with three batches of each ploidy (2N and 3N) maintained in intertidal environment and collected once a month from May to November 2021. Natural *V. parahaemolyticus* contamination (indigenous *V. parahaemolyticus*), and experimental IFVp201 accumulation and depuration between 2N and 3N oysters were compared. Life history appeared to impact IFVp201 experimental accumulation but not depuration, while ploidy level did not seem to have an impact on natural contamination with *V. parahaemolyticus* (except with a tendency in autumn/winter), or on experimental accumulation and depuration.

Given that *V. parahaemolyticus* is not an oyster pathogen, it is conceivable to think that oysters did not respond to the presence of *V. parahaemolyticus* in their tissues. Indeed, it

was shown in another oyster species, *C. virginica*, that haemocytes were not chemoattracted by live or heat-killed *V. parahaemolyticus* ATCC17802^T (Cheng and Howland, 1979). However, other studies showed that *C. virginica* haemocytes were able to kill *V. parahaemolyticus in vitro* (Genthner et al., 1999; Volety et al., 2001). Taken together, these results suggested that oyster haemocytes may be able to recognize and eliminate *V. parahaemolyticus*. Moreover, haemocyte activity was suggested to be modulated by environmental parameters such as temperature and salinity (Gagnaire et al., 2006), and thus could explain the re-accumulation observed for 2N and 3N oysters in November between 24 h and 48 h of depuration. Indeed, it was shown that haemocytes killed less *V. parahaemolyticus* in winter than in summer (Genthner et al., 1999). Furthermore, difference between environmental temperature at the time of collection (10-11°C) and experimental temperature (19°C) could have also favoured *V. parahaemolyticus* proliferation. Furthermore, oysters harbour an immune memory allowed by a phenomenon called immune priming which results in a higher immune response when exposed a second time to a pathogen. To our knowledge, this phenomenon has not been investigated yet for human pathogens such as *V. parahaemolyticus* but it was shown for oyster pathogens like *Vibrio splendidus* in *C. gigas* (Zhang et al., 2014). According to the concentrations of indigenous *V. parahaemolyticus* observed in 2N and 3N oysters (Publication III), we could hypothesize that environmental oysters were exposed to natural *V. parahaemolyticus* during the summer prior to experimentations (life history of *C. gigas*, October 2021). This first exposure to indigenous *V. parahaemolyticus* could have led to higher immune response unlike naive oysters and thus resulted in elimination of *V. parahaemolyticus*. Moreover, presence of indigenous *V. parahaemolyticus* in oysters during this experimentation could result in niche competition with IFVp201. However, this could not be confirmed as no difference was observed in IFVp201 accumulation in 2N and 3N oysters whatever the concentration of indigenous *V. parahaemolyticus*. Nevertheless, depuration rates of 2N and 3N oysters were inversely proportional to concentrations of indigenous *V. parahaemolyticus*. Indigenous *V. parahaemolyticus* could prevent colonization by IFVp201 and thus result in rapid depuration, as shown with *V. vulnificus* (Froelich and Oliver, 2013; Groubert and Oliver, 1994).

Differences in oyster microbiota composition and dynamics could partially explain these results. Indeed, Offret *et al.* (2020) showed that after 3.5 month of implantation of naive oysters from Ifremer hatchery in the intertidal environment, their microbiota were drastically changed (Offret et al., 2020). Although oyster microbiota was close to clam

microbiota it was surprisingly distinct from the one of surrounding seawater and sediments. Microbiota can be also influenced by oyster genetic, environmental conditions, temporal variations and spatial location (Lokmer et al., 2016b; Nguyen et al., 2020). Indeed, a recent study showed that microbiota can be mainly shaped by host genetics even when oysters were maintained in the same environment (Unzueta-Martínez et al., 2022). Whereas determination of "core" microbiota is a difficult thing, identification of opportunistic bacteria which colonized oysters during summer mortalities resulted in more consensus results. Indeed, oysters susceptible to summer mortalities always harboured *Vibrio* genus (Clerissi et al., 2020; Corr et al., 2007; de Lorgeril et al., 2018; King et al., 2019a; King et al., 2019b; Lokmer and Wegner, 2015; Richard et al., 2021), were often associated with *Photobacterium* (Clerissi et al., 2020; King et al., 2019a; Lokmer and Wegner, 2015) and *Arcobacter* genera (de Lorgeril et al., 2018; King et al., 2019b; Lokmer and Wegner, 2015; Richard et al., 2021), and sometimes with *Streptococcus* (King et al., 2019a; Li et al., 2022) and *Roseovarius* genera (King et al., 2019a; Ramachandran et al., 2018). These increased levels in opportunistic bacteria during summer mortalities could result in higher susceptibility to bacterial colonization, including *V. parahaemolyticus*, and thus, could partly explain the seasonal dynamic of *V. parahaemolyticus* concentrations observed in 2N and 3N oysters. However, dynamics of *tdh*$^+$ and *trh*$^+$ strains were not similar to dynamic of total *V. parahaemolyticus*.

Indeed, prevalence of *tdh*$^+$ *V. parahaemolyticus* in oysters was higher in autumn (November) than in summer (July), suggesting a higher environmental fitness of these strains or less bacterial competition at this season. Similar results were previously observed in *C. virginica* (DePaola et al., 2003b; López-Hernández et al., 2015; Williams et al., 2017) and in *C. gigas* (Flynn et al., 2019) with higher potentially pathogenic *V. parahaemolyticus* in autumn/winter than in spring/summer. Added to these observations, authors of previous studies concluded that prevalence of *tdh*$^+$ *V. parahaemolyticus* was higher when concentration of *tlh*$^+$ (total *V. parahaemolyticus*) was lower (DePaola et al., 2003b; Flynn et al., 2019). In Europe, consumption of oysters is high during autumn/winter for Christmas and New Year celebrations. This higher prevalence of potentially pathogenic *V. parahaemolyticus* strains at this period highlights the need of *V. parahaemolyticus* detection in seafood control year-round. Indeed, the IFVp201 strain (*tdh*$^+$ *trh*$^+$), isolated from mussels (*Mytilus edulis*) on the French Atlantic coast in December 2009, was shown to be highly virulent in rabbit model (Publication I).

In this chapter (Publication II and III), some factors such as life history and ploidy level of oysters could slightly impact *V. parahaemolyticus* accumulation and/or depuration in

oysters. However, the natural occurrence of *V. parahaemolyticus* in oysters requires treatments or methods to eliminate them from oysters before commercialization.

3. Lactic acid bacteria to eliminate *Vibrio parahaemolyticus* before commercialization

As elimination of *V. parahaemolyticus* by classical methods of depuration was not successful or sufficient, we investigated the potential of probiotics like lactic acid bacteria (LAB) to inhibit growth of *V. parahaemolyticus in vitro* and to promote elimination of *V. parahaemolyticus* from oysters during depuration (Publication IV).

In our study, LAB strains showed varying inhibitory capacities against *V. parahaemolyticus* growth by acid production. A previous study observed variations in acid tolerance (pH 4 to pH 7) between *V. parahaemolyticus* strains (Chiang et al., 2012). In our study, IFVp195 was the most sensitive to inhibition by LAB strains, suggesting a higher acid sensitivity than the other strains. This result can be linked to lower survival in infant rabbit intestines of IFVp195 than IFVp201 (Chapter 1). According to these results and the genetic profiles (Publication I), four *V. parahaemolyticus* strains (IFVp201, IFVp195, IFVp69 and LMG2850^T) were selected to investigate LAB use during oyster depuration.

Experimental accumulation and depuration of *V. parahaemolyticus* in oysters showed varying depuration rate between strains, as previously described (Aagesen et al., 2018). Persistence of *V. parahaemolyticus* in oysters appeared to be associated to type IV pili and flagella (Aagesen et al., 2013) even though no correlation was made between persistence in oysters, motility and biofilm formation (Aagesen et al., 2018). In our study, no correlation was observed between the persistence of the four *V. parahaemolyticus* strain in oysters, biofilm formation and motility assays (unpublished data).

Only few studies investigated use of LAB strains for *V. parahaemolyticus* elimination during oyster depuration (Kang et al., 2018; Xi et al., 2014). Comparison of our results as well as the experimental parameters with results of these other studies will be useful to identify optimal parameters to ensure the maximal *V. parahaemolyticus* elimination. Three major parameters were usually investigated concerning *V. parahaemolyticus* depuration: time of processing, and seawater salinity and temperature (Campbell et al., 2022), added to LAB species selection and type of process (balneation or injection). In comparison to our study, Kang *et al.* simultaneously exposed oyster to *V. parahaemolyticus* and *Enterococcus faecium*, which could result in an inhibition of *V. parahaemolyticus* directly in seawater before entering in oysters, which is not what we

wanted to investigate. Xi *et al.* showed little to no difference in log reduction between control condition and LAB treatment. In Xi *et al*., the time of depuration was of four days, non-adapted to classical depuration process (24 – 48 h) in oyster farms, in comparison to 24 h in our study. However, it was shown that a longer time of depuration increase the log reduction of *V. parahaemolyticus* in oysters (Phuvasate et al., 2012), suggesting that the longer LAB exposure time should not be involved in the lower log reduction of *V. parahaemolyticus*. Since seawater salinities and bacterial concentrations were similar between the two studies, the last parameter which could explain the lower LAB efficacy during depuration in Xi *et al*. was the seawater temperature. Indeed, Xi *et al.* exposed oysters to LAB at 10 and 15°C, in comparison to 20°C in our study. Experiment temperatures were previously shown to impact LAB efficiency (Douillet and Langdon, 1994). Although some parameters need to be optimally determined, use of LAB strains for elimination of *V. parahaemolyticus* seems a promising method.

II. Conclusions

This thesis work provided many interesting insights concerning virulence of *V. parahaemolyticus* strains, factors involved in accumulation and depuration of *V. parahaemolyticus* in oysters and method for elimination of *V. parahaemolyticus* during post-harvest depuration. We can firstly conclude that presence of virulence factors such as TDH and T3SS2 did not necessarily result in a virulent phenotype in rabbit model (Publication I). Then, life history but not ploidy impact *V. parahaemolyticus* experimental accumulation but not depuration (Publication II and III). However, interesting results were observed in November 2021 with less natural contamination in 3N than in 2N oysters and presence of potentially more virulent *V. parahaemolyticus* strains (Publication III). Finally, use of LAB as post-harvest process to eliminate *V. parahaemolyticus* appeared to be a promising method (Publication IV).

III. Perspectives

Some perspectives could be considered following this thesis work, to further characterize potential virulence genes in *V. parahaemolyticus* and determine their phenotype *in vivo*. Then, to identify factors associated to oysters involved in *V. parahaemolyticus* accumulation, persistence and depuration. Finally, to develop an efficient and reliable method to eliminate *V. parahaemolyticus* from oysters in order to

respect the 3.52 log reduction rule requested by the US FDA through the National Shellfish Sanitation Program (NSSP, 2017).

1. *In sillico* and *in vivo* virulence of *Vibrio parahaemolyticus* strains

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is increasingly used for identification of bacteria. It is rapid, low in cost and suitable for high-throughput characterization (Mellmann et al., 2009; Mougin et al., 2020; Seng et al., 2009). Although Li *et al.* (2018) asked for caution for the use of MALDI-TOF to distinguish serovariants of *V. parahaemolyticus* due to its taxonomic resolution often over-estimated (Li et al., 2018), identification of potentially pathogenic strains appeared to be promising (Paauw et al., 2014; Rahmani et al., 2021). However, as for genomic analyses, MALDI-TOF analyses need to be associated to *in vivo* virulence experiments to identified proteins only present in pathogenic strains (Rahmani et al., 2021).

Considering our results with infant rabbit model, virulence characterization of *V. parahaemolyticus* appeared complex and experimentations with more strains with diversified *in sillico* potential virulence gene profiles are needed to decipher pathogenicity of *V. parahaemolyticus*. It is known that sub-variants of *trh2*$^{+}$ exist (Bechlars et al., 2015) and, to our knowledge, their virulence phenotype was never determined using *in vivo* models, thus it could be interesting to characterize virulence phenotype of *V. parahaemolyticus* strains with various *trh2*$^{+}$ sub-variant. Moreover, strains lacking haemolysins but with T3SS2 (typically IFVp182 in our study) could be also interesting for *in vivo* experiments to investigate the role of T3SS2 in virulence by avoiding use of deletion mutants. Moreover, GFP-tagged *V. parahaemolyticus* strains could be used for *in vivo* experiments to investigate the localization, colonization and structural changes in rabbit epithelial cells, as previously investigated with RIMD2210633 (Ritchie et al., 2012). All these experiments could help to identify more factors involved in virulence, and thus, refine identification of potential virulence factors besides haemolysins and T3SS.

Though we suggested a role of T3SS1 in *V. parahaemolyticus* virulence in *G. mellonella* larvae, other factors can be involved especially since the deletion of T3SS1 resulted in only 38% of larvae survival. For this purpose, we could investigate involvement of T6SS using deletion mutants like those used in Salomon *et al.* (2013). Moreover, the important factor which can bias results in *in vivo* models is the infection route. Indeed, as observed for murine model, factors involved in virulence in intra-peritoneally and orogastrically infected mice were different (Hiyoshi et al., 2010; Yang et al., 2019). The same assumptions can be made concerning *G. mellonella* larvae. This way, we could investigate

V. parahaemolyticus virulence in larvae using an oral infection (Ramarao et al., 2012) and study factors involved in virulence using deletion mutants as used in our study.

2. Life history and ploidy level of *Crassostrea gigas* and *Vibrio parahaemolyticus* accumulation

One of the limits of experimental accumulation and depuration is the fact that after 18 h or 24 h of *V. parahaemolyticus* accumulation, the interaction was transient, thus do not reflect the real association between oyster and autochthonous bacteria (Froelich and Oliver, 2013). As a continuous contamination by *V. parahaemolyticus* concentrations reflecting those in the environment could be technically difficult, we could, for example, investigate accumulation of *V. parahaemolyticus* directly in environment. In this context, oysters grown in secured infrastructures, *i.e.* naive oysters, could be implemented in environment before summer and analysed after summer for *V. parahaemolyticus* levels, in comparison to oysters grown in environment. As investigated in a previous study (Offret et al., 2020), 3.5 months implementation resulted in microbiota composition changes. Thus, if naive oysters accumulated *V. parahaemolyticus* as much as environmental oysters after this implementation, microbiota could be the major factor influencing *V. parahaemolyticus* accumulation. Whereas, if naive oysters maintained their "permissiveness" toward *V. parahaemolyticus* in comparison to environmental oysters, it could mean that oyster genetic or immune response could be major factors involved in *V. parahaemolyticus* accumulation. In all cases, microbiota analyses of naive and environmental oysters and surrounding waters before and after the summer could be performed in order to further characterize stable and transient microbiota of oysters. In the second case, immune priming assays could be considered to investigate immune response of oysters following *V. parahaemolyticus* accumulation. If these assays revealed an immune priming, identification of recognition and elimination mechanisms of *V. parahaemolyticus* by the oyster immune system could be useful to consider "vaccination" of oysters during their larval stage. Indeed, it was shown that exposure of larval *C. gigas* oysters to microorganism-enriched environment increased survival rate during summer mortalities during adult stage and that this resistance was transmitted in the following generation (Fallet et al., 2022). Vaccination in aquaculture consisted in exposure to either entire or part of the fish pathogen (Mondal and Thomas, 2022). With this in mind, we could imagine an immersion vaccination using formalin inactivated bacteria according to vaccines already commercially available for fin-fish (Brudeseth et al., 2013), and because oral or injected vaccines would be out of the question for oysters.

Selection of oyster families harbouring resistance against OsHV-1 and *Vibrio aestuarianus* (Azéma et al., 2017) was performed to counteract summer mortalities of juvenile oysters on French Atlantic coast. A transcriptomic study showed that oyster families resistant to OsHV-1 exhibited an early induction of genes involved in antiviral defences (de Lorgeril et al., 2018). Recent studies identified a significant region associated to host resistance to (survival and viral load) located in chromosome 6 of *C. gigas* oysters (Gutierrez et al., 2018) and a transcript encoding a Toll-like Receptor (TLR), involved in immune response. This transcript was overrepresented in three OsHV-1-resistant families over OsHV-1-sensitive families (De Lorgeril et al., 2020). These results represent interesting ways for future studies to understand the mechanisms underlying in oyster resistance to summer mortalities. These resistant oyster families could represent an economic advantage for oyster farmers. In that respect, investigation of the resistance or susceptibility to OsHV-1 of these oyster families to human pathogens such as *V. parahaemolyticus* is an upmost sanitary importance.

The difference in indigenous *V. parahaemolyticus* levels between 3N than 2N oysters in November was an intriguing result (even if non-significant). In this context, it would be interesting to collect more 2N and 3N oysters during autumn/winter to validate or not these results. If these results were confirmed, 3N oysters, besides representing an economic interest due to their faster growth, could represent a sanitary interest over 2N oysters concerning *V. parahaemolyticus* risk. To understand this potential difference, characterization of their physiological parameters, *e.g.* filtration rate or clearance rate, could be useful. Indeed, a lower filtration rate or higher clearance rate of 3N oysters could explain the lower contamination by *V. parahaemolyticus* than in 2N oysters. Thus, characterization of oyster microbiota in 2N and 3N oysters could be useful, firstly, in identification of core microbiota of the two oyster types and, secondly, in understanding factors involved in *V. parahaemolyticus* accumulation in oysters.

While biotic and abiotic parameters shaping total *V. parahaemolyticus* levels in environment were well studied (Blackwell and Oliver, 2008; Johnson et al., 2012; Parveen et al., 2008), factors governing proliferation of potentially pathogenic *V. parahaemolyticus* in environment, which are the real threat to human health, were slightly analysed. As discussed previously, more potentially pathogenic (like IFVp201) *V. parahaemolyticus* were detected in November than during the other months and independently of ploidy level. It would be useful to identify biotic and abiotic factors involved in variations of potentially pathogenic *V. parahaemolyticus* proliferation to determine periods with higher infection risks for human during complementary experiments.

3. Elimination of *Vibrio parahaemolyticus* before commercialization

In oyster farming, probiotics were often used at the larval stage in hatchery to increase growth and survival to environmental pathogens such as *Vibrio tubiashii* or *Vibrio coralliilyticus* (Gibson et al., 1998; Kesarcodi-Watson et al., 2012). To our knowledge, such experiments were also performed using human pathogenic *Vibrio* but to a lesser extent, thus, prophylactic treatment of oyster larvae with probiotics to decrease potential future *V. parahaemolyticus* contamination could be considered in future experiments. Especially since use of probiotics with shrimp larvae against acute hepatopancreatic necrosis disease (AHPND)[10]-induced *V. parahaemolyticus* showed higher survival to AHPND (Lim et al., 2020; Ramirez et al., 2022). Indeed, early life treatments with enriched-microorganisms seawater (Fallet et al., 2022) or probiotics (Yin et al., 2021) appeared to increase immune response in *C. gigas* and in large yellow croaker (*Larimichthys crocea*), respectively. This prophylactic treatment performed in hatchery at the larval stage could be associated to post-harvest treatment with probiotics to maximize elimination of *V. parahaemolyticus* in oysters. In this context, optimal conditions (temperature, salinity, concentration, time of processing, administration mean, seawater system, food or starvation…) need to be further determined by doing new experiments with varying conditions. It was shown that marine aggregates[11] showed significantly higher bacterial up take *in vitro* than bacteria alone (Froelich et al., 2013; Kach and Ward, 2008). Thus, we could consider to integrate probiotic bacteria into marine aggregates to ensure a maximal accumulation in oysters and maximal *V. parahaemolyticus* elimination. Furthermore, synergistic antimicrobial activities between different probiotics could be explored in the future (Ferreira et al., 2021; Lim et al., 2020; VidyaLaxme et al., 2014) to increase inhibitory activities up to 3.52 log reduction of *V. parahaemolyticus* levels (NSSP, 2017). Finally, use of LAB in food can result in modification of sensorial aspect, due to their acidification activity. With this in mind, a sensory evaluation should be performed to evaluate the impact of LAB on smell and taste of oysters (Wiernasz et al., 2017; Wiernasz et al., 2020).

[10] Acute hepatopancreatic necrosis disease (AHPND) affects shrimp production worldwide resulting in shrimp mortalities (Kumar et al., 2021).

[11] These particles naturally consist of fecal pellets, larvacean houses, phytoplankton, microbes, and inorganics. In the mentioned study, these aggregates were laboratory-created using deionized water and hyaluronic acid

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Appendixes

Appendix 1.

Function and localization of each gene from T3SS cluster.

T3SS part	Information	Protein
Translocon	pore	VopB
	pore	VopD
	chaperone	VcrH
Structural	Tip	VcrV
	chaperone	VcrG
	body	VscF
	chaperone	VscG
	chaperone	VscE
	chaperone	VscR
	polymerization control	VscH
	lenght control	VscP
	OM ring	VscC
	chaperone	VscX
	inner rod	VscI
	IM ring exterior	VscD
	IM ring interior	VscJ
	C-ring, socket/Cup	VscR
	C-ring, socket/Cup	VscS
	C-ring, socket/Cup	VscT
	C-ring, socket/Cup	VscU
	C-ring, socket/Cup	VcrD/VscV
	C-ring, secretion specificity	VscX
	C-ring, secretion specificity	VscY
	ATPase	VscN
Regulators	cytoplasmic	VscK
	cytoplasmic	VscL
	cytoplasmic	VscQ
	Secretion	VopN
	Secretion	Vcr1
	Secretion	VscB
	Secretion	Vcr2
	Substrate recycling?	VscO
	T3SS transcription	ExsA
	T3SS transcription	ExsC
	T3SS transcription	ExsD
	T3SS transcription	ExsE

Appendix 2.

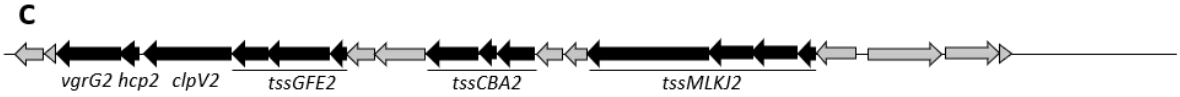
T6SS genes according to official nomenclature (A), and T6SS1 (B) and T6SS2 (C) operons in RIMD 2210633.

A

T6SS subunit	Localization
TssA	putative cytosolic protein
TssB	soluble protein, OM associated
TssC	soluble protein, OM associated
TssD/Hcp	soluble, forms hexameric rings, putative pilus
TssE	putative soluble protein
TssF	putative soluble protein
TssG	putative soluble protein
TssH/ClpV	cytosolic protein, ATPase
TssI/VgrG	cell puncturing device, forms trimers
TssJ/SciN	outer membrane lipoprotein
TssK	putative cytosolic protein
TssL	inner membrane
TssM/IcmF	inner membrane

B

C



Appendix 3.

ChiRP (A, *pil* genes) and MSH (B, *msh* genes) operons in RIMD2210633.

A

B

Table of content

Titre : Vers une meilleure approche de la virulence et de la gestion du risque de *Vibrio parahaemolyticus*, bactérie marine potentiellement pathogène pour l'Homme

Mots clés : *Vibrio parahaemolyticus*, virulence, génomique, *Crassostrea gigas*, accumulation, dépuration

Résumé : *Vibrio parahaemolyticus* (*Vp*) est une bactérie marine considérée comme la principale cause bactérienne de gastroentérites liées à la consommation de produits de la mer crus ou insuffisamment cuits chez l'Homme dans le monde entier. L'absence de facteurs de virulence connus (TDH, TRH, système de sécrétion de type III (T3SS)) chez des souches *Vp* isolées de cas cliniques souligne la nécessité de mieux caractériser ces facteurs et leur expression dans des modèles *in vivo*. De plus, un réel manque existe concernant l'implication de facteurs biotiques associés à l'hôte, ici l'huître *Crassostrea gigas*, sur l'accumulation et la dépuration de *Vp* dans leurs tissus. Les objectifs de cette thèse sont de mieux caractériser le potentiel pathogène des souches environnementales de *Vp* et identifier les facteurs associés aux huîtres qui pourraient avoir un impact sur les niveaux de contamination de ces bactéries dans leurs tissus.

Dans un premier temps, une analyse génomique a été combinée à une caractérisation phénotypique de la virulence grâce à deux modèles *in vivo* : le lapin et larves de *Galleria mellonella*. Les résultats ont montré que, d'une part, la TDH n'est pas un indicateur pertinent de la pathogénicité de *Vp* chez le modèle lapin, et d'autre part, que le T3SS-1 de *Vp* joue un rôle dans la mortalité des larves. Il s'agit de nouvelles observations très intéressantes qui devront être examinées davantage. Les expérimentations sur *C. gigas* ont montré que les huîtres cultivées dans des structures sécurisées accumulaient expérimentalement plus de *Vp* que les huîtres cultivées en milieu intertidal et que, le niveau de ploïdie des huîtres (diploïde vs triploïde) n'a pas eu d'impact sur la contamination naturelle, l'accumulation expérimentale et la dépuration de *Vp*. Enfin, l'utilisation de bactéries lactiques présentant des activités d'inhibition de la croissance de *Vp in vitro*, a permis d'obtenir des résultats prometteurs concernant l'élimination de *Vp* pendant la dépuration d'huîtres.

Title : Towards a better overview of virulence and risk management of *Vibrio parahaemolyticus*, a marine bacterium potentially pathogenic for humans

Keywords : *Vibrio parahaemolyticus*, virulence, genomic, *Crassostrea gigas*, accumulation, depuration

Abstract : *Vibrio parahaemolyticus* (*Vp*) is a marine bacterium considered as a leading cause of gastroenteritis associated with raw or undercooked seafood consumption in humans worldwide. Isolation of *Vp* strains lacking known virulence factors (TDH, TRH, type III secretion system (T3SS)) in clinical cases highlighted the need to better characterize these factors and their expression in *in vivo* models. Moreover, factors associated to the host, the oyster, *Crassostrea gigas*, should be considered to better accumulation and depuration of *Vp* from their tissues. The objectives of this thesis were to better characterize the pathogenic potential of environmental strains of *Vp* and to identify factors associated to oyster which could impact the contamination levels of these bacteria in their tissues.

The genomic analysis combined to phenotypic characterization of virulence in the infant rabbit and *Galleria mellonella* larvae models revealed that, the TDH does not seem to be the most relevant indicator of *Vp* pathogenicity, and that larvae mortality was probably explained by *Vp*'s T3SS-1. These are very interesting new observations which need to be further addressed. Experiments on *C. gigas* showed that oysters grown in secured structures accumulated experimentally more *Vp* than oysters grown in intertidal environment and that the ploidy level of oysters (diploid vs. triploid) did not impact the natural contamination and the experimental accumulation and depuration of *Vp.* Finally, the use of Lactic acid bacteria which inhibited the growth of *Vp in vitro*, provided promising results concerning *Vp* elimination during oyster depuration.