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

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Abstract :

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 LMG2850T) at 105 CFU.mL−1 and then exposed by balneation to 106 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 may be 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

Introduction

Vibrio parahaemolyticus is naturally found in marine estuaries and coasts, and in molluscan shellfishes (Odeyemi, 2016). It is one important causative bacterial agent of acute gastroenteritis in humans worldwide associated to consumption of raw or undercooked seafood, especially in Asia and in the United States (Newton et al., 2012; Miller et al., 2021; Chen et al., 2022). So far rare cases have been reported in Europe (Martinez-Urtaza et al., 2018; Amato et al., 2022), however climate change projections and rising temperature sea water may increase the risk of *Vibrio* sp. infections in this area (Huehn S. et al., 2014; Fleischmann et al., 2022).

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 (Satish Kumar et al., 2011; Le and Yang, 2018), and *in vivo* models such as mice (Wang et al., 2022) or marine organisms (Liu et al., 2015; Girija et al., 2018;). 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 (Douillet and Langdon, 1994; Gibson et al., 1998; Campa-Córdova et al., 2011; 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 the 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, few studies have investigated the inhibition activities of bioprotective agents against *V. parahaemolyticus* as PHP during oyster depuration (Xi et al., 2014; Khouadja et al., 2017; Kang et al., 2018). Khouadja *et al.* (2017) showed approximately 2 log $CFU.mL^{-1}$ reduction of *V. parahaemolyticus* concentration in oysters challenged by injection with a *V. parahaemolyticus* strain (10^6 CFU/ml) and exposed to *Lactobacillus* sp. strains (10^6 $CFU.mL^{-1}$) after two days (Khouadja et al., 2017). However, in such experiments, using an injection method to contaminate oysters with *V. parahaemolyticus* is questionable and 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 *Lactiplantibacillus 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, the virulence of *E. faecium* makes its use as a biocontrol agent against *V. parahaemolyticus* questionable (Vancanneyt et al., 2002). 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 or resistance 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 with a depuration process (Lee et al., 2008) that could potentially be implemented in oyster farms and plants.

Materials and methods

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.

***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).

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).

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's recommendation with 1µM of each primer. Thermal cycling reactions were: 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 (Hall, 1999). Strain identification was performed using nucleotide BLAST on the NCBI website and n/r databank (Altschul et al., 1990).

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}. One milliliter of diluted-cultures were seeded onto square Petri dishes (120 x 120 mm) containing 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 (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. Partial inhibitions consisted in a clear halo where some individual colonies were present or a halo with lower density of cells.

Lactic acid bacteria persistence in filtered and sterile seawater

To verify LAB persistence in filtered and sterile seawater (FSSW, natural seawater filtered through 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.

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**).

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.

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 with *Vp-gfp*$^{+}$ and LAB strains as factors. To evaluate the difference of *Vp-gfp*$^{+}$ reduction in oyster haemolymph between the control condition (without LAB) and conditions with each LAB strains, a post hoc Tukey test was performed for each *Vp-gfp*$^{+}$ strain.

Results

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.

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 at 19°C, all four 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.

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 as 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 significantly impact growth of these strains.

*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**).

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 (**Table S2**). 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**).

Discussion

Probiotics are widely used in aquaculture as a replacement for 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 the depuration process when other methods fail to completely eliminate *V. parahaemolyticus,* are detrimental for oysters, or are 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 LABs may be caused by various compounds such as organic acids, bacteriocins or small molecules such as hydrogen peroxide, diacetyl, reuterin … (Ibrahim et al., 2021). 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 is 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 (Kirs et al., 2011; Jones et al., 2014). 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 (Xi et al., 2014; Kang et al., 2018). 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., 2018) 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* (Kumar et al., 2016; Ferreira et al., 2021) or *Vibrio cholerae* (VidyaLaxme et al., 2014). In the same idea, bioprotective agents could be used in a synergistic manner with other 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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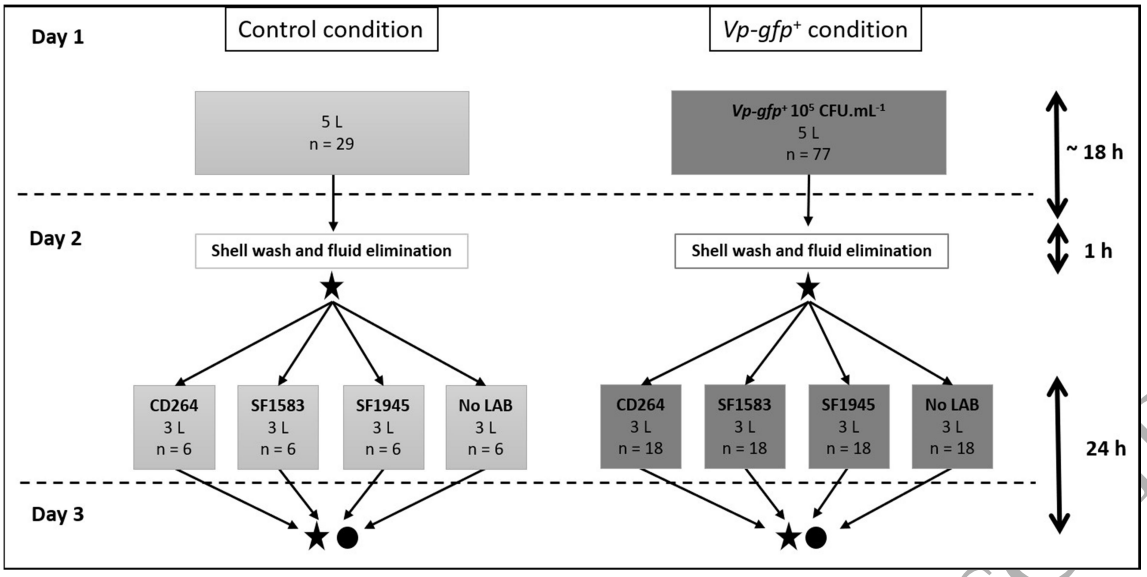

Figure 1. Experimental protocol of challenge tests of oysters with Vp-*gfp*$^+$ and LAB strains. Stars: *Vp-gfp*$^+$ quantification by flow cytometry in haemolymph. Circles: LAB enumeration in haemolymph on agar plates. n: number of oysters.

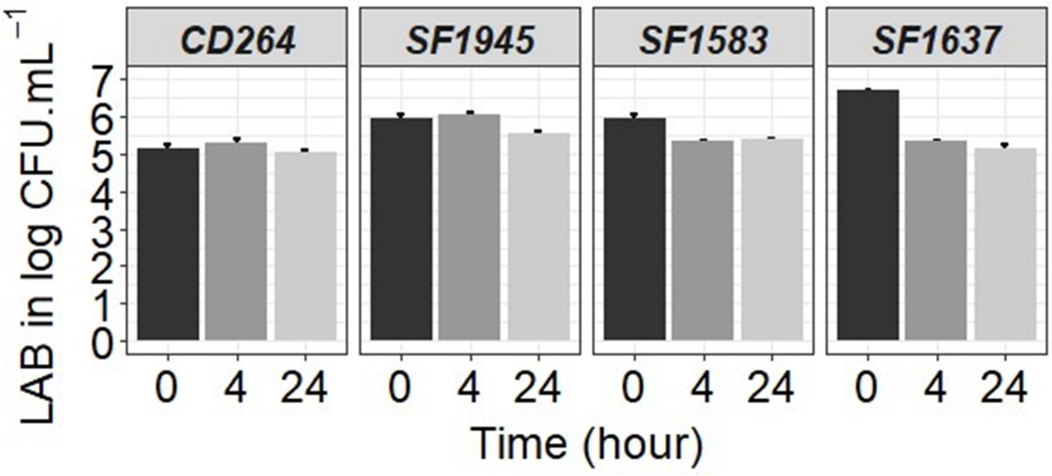

Figure 2. LAB strains persistence in seawater, at 19°C, during 24 h. *Vagococcus fluvialis* CD264, *Latilactobacillus sakei* SF1583, *Weissella hellenica* SF1637 and *Lactococcus lactis* SF1945. Data represents mean ± standard error**.**

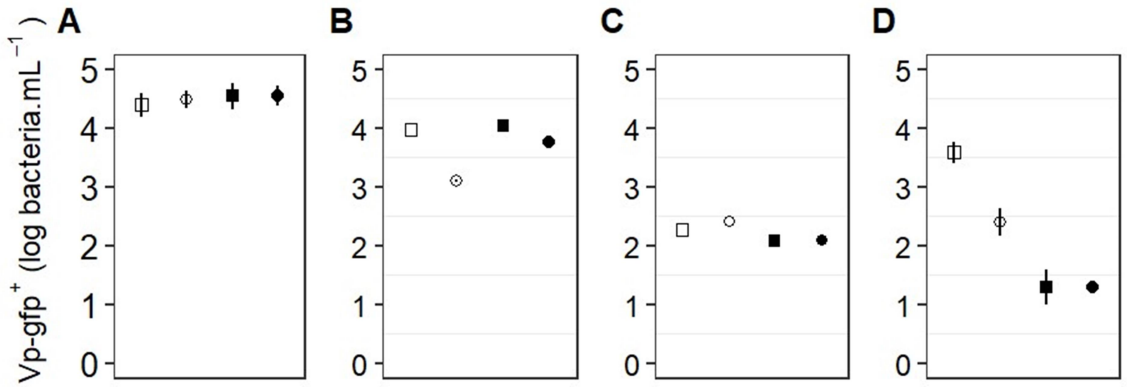

Figure 3. Concentrations of *Vp-gfp*$^+$ in seawater and haemolymph during challenge test. (A) Initial concentration of *Vp-gfp*$^+$ in seawater. (B) Concentrations of *Vp-gfp*$^+$ in seawater after the overnight balneation with oysters. (C) Concentrations of *Vp-gfp*$^+$ in oyster haemolymph after the 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.

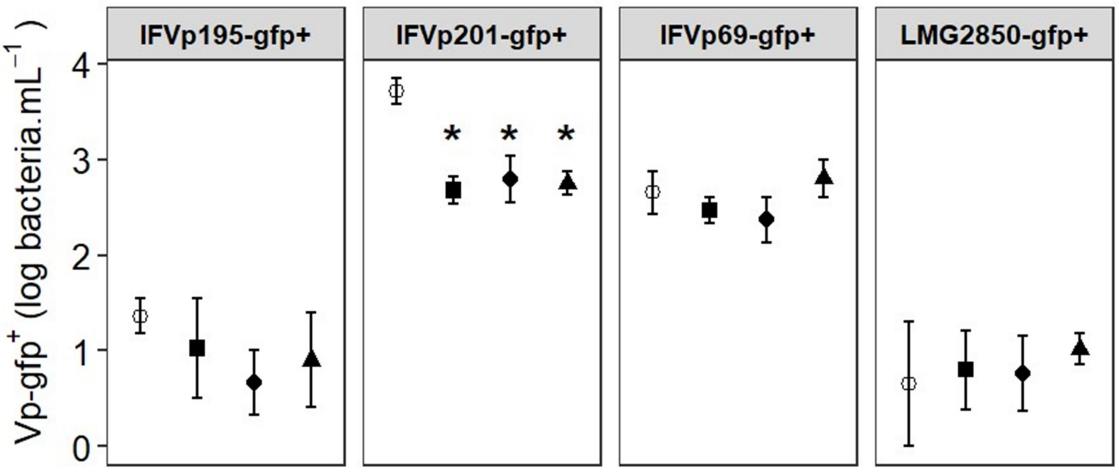

Figure 4. *Vp-gfp*$^+$ quantification in oyster haemolymph after an overnight exposure of oysters followed by 24h-incubation with different LAB. (○): Vp-*gfp*$^+$ alone, (■): Vp-*gfp*$^+$ and *V. fluvialis* CD264, (●): Vp-*gfp*$^+$ and *L. sakei* SF1583, and (▲): Vp-*gfp*$^+$ and *L. lactis* SF1945. 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. *: $p < 0.05$

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

Species	Strain	Origin	Medium
Carnobacterium divergens	V41	Salmon viscera	BHIS
Carnobacterium divergens	SF 1372	Smoked salmon	Elliker
Carnobacterium divergens	SF 1486	Smoked salmon	Elliker
Carnobacterium divergens	SF 1514	Smoked salmon	Elliker
Carnobacterium divergens	SF 1516	Smoked salmon	Elliker
Carnobacterium divergens	SF 1518	Smoked salmon	Elliker
Carnobacterium divergens	SF 1533	Smoked salmon	Elliker
Carnobacterium inhibens	CD 344	Peeled shrimp	BHIS
Carnobacterium inhibens	MIP 2551	Whole shrimp	BHIS
Carnobacterium maltaromaticum	SF 1371	Smoked salmon	Elliker
Carnobacterium pleistocenium	EU 2258	Rough-head grenadiers	BHIS
Carnobacterium viridans	EU 2254	Sea bream	BHIS
Enterococcus faecalis	CD 267	Peeled shrimp	BHIS
Lactococcus lactis	SF1945	Smoked salmon	BHIS
Latilactobacillus curvatus	SF 1574	Smoked salmon	MRS
Latilactobacillus fuchuensis	SF 1527	Smoked salmon	MRS
Latilactobacillus sp.	SF 1535	Smoked salmon	MRS
Latilactobacillus sakei	SF1583	Smoked salmon	MRS

Latilactobacillus sakei	EU 2203	Smoked salmon	MRS
Vagococcus fluvialis	CD264	Peeled shrimp	BHIS
Vagococcus fluvialis	CD 273	Peeled shrimp	BHIS
Vagococcus fluvialis	CD 294	Peeled shrimp	BHIS
Vagococcus fluvialis	CD 295	Peeled shrimp	BHIS
Weissella hellenica	SF1637	Smoked salmon	MRS
Weissella viridescens	SF 1628	Smoked salmon	MRS
Weissella viridescens	SF 1632	Smoked salmon	MRS
Weissella viridescens	SF 1639	Smoked salmon	MRS
Weissella viridescens	SF 1641	Smoked salmon	MRS
Weissella viridescens	SF 1642	Smoked salmon	MRS
Weissella viridescens	SF 1643	Smoked salmon	MRS
Weissella sp.	SF 1635	Smoked salmon	MRS
Vibrio parahaemolyticus	IFVp5	Clinical	BHIS
Vibrio parahaemolyticus	IFVp18	Mussel (*Mytilus edulis*)	BHIS
Vibrio parahaemolyticus	IFVp22	Mussel (*M. edulis*)	BHIS
Vibrio parahaemolyticus	IFVp69	Mussel (*M. edulis*)	BHIS
Vibrio parahaemolyticus	IFVp136	Mussel (*Mytilus galloprovincialis*)	BHIS
Vibrio parahaemolyticus	IFVp177	Oyster (*C. gigas*)	BHIS
Vibrio parahaemolyticus	IFVp182	Seawater	BHIS

Vibrio parahaemolyticus	IFVp195	Seawater	BHIS
Vibrio parahaemolyticus	IFVp201	Mussel (*M. edulis*)	BHIS
Vibrio parahaemolyticus	IFVp203	Mussel (*M. edulis*)	BHIS
Vibrio parahaemolyticus	IFVp408	Oyster (*C. gigas*)	BHIS
Vibrio parahaemolyticus	LMG2850T	Clinical	BHIS
Vibrio parahaemolyticus	IFVp201-gfp+	GFP-tagged	LBS50
Vibrio parahaemolyticus	IFVp69-gfp+	GFP-tagged	LBS50
Vibrio parahaemolyticus	LMG2850-gfp+	GFP-tagged	LBS50
Vibrio 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

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

3: high inhibition, 2: average inhibition, 1: low inhibition, 0: no inhibition