LWT - Food Science and Technology 158 (2022) 113182

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Global expansion of *Vibrio parahaemolyticus* threatens the seafood industry: Perspective on controlling its biofilm formation

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ARTICLE INFO

Keywords:
Climate change
Biofilm
Life cycle
Molecular mechanisms
Control strategies

ABSTRACT

As global warming increases the geographical range and frequency of *Vibrio parahaemolyticus* infections, its formation of biofilms providing bacteria greater resistance to stress and contributing to the persistence of pathogens, is threatening the seafood industry. *V. parahaemolyticus* has a number of advantages leading to biofilm formation. This study reviews recent advances in understanding *V. parahaemolyticus* biofilm formation on biotic and abiotic surfaces, discusses research gaps in the mechanism of biofilm formation and examines promising biofilm control strategies to overcome current limitations of chemical disinfectant. This information will deepen our understanding of *V. parahaemolyticus* biofilm formation, as well as help design and optimize *V. parahaemolyticus* biofilm control strategies for the seafood industry.

1. Introduction

V. parahaemolyticus is a curved rod, Gram-negative bacterium that naturally exists in the marine environment. It can be prevalent in oysters, clams, fish, shrimps, mussels, scallop and periwinkle (Odeyemi, 2016), and infections involve the consumption of raw or undercooked seafood. *V. parahaemolyticus* survives at 5-45 °C and achieves substantial growth when seawater temperatures are over 14-19 °C. This explains why this pathogen is prevalent in summer and autumn seasons. Global warming has caused an increasing geographical range and frequency of *V. parahaemolyticus* infections. Repeated cases of infection and outbreaks have been reported in unexpected areas where there were previously no or only sporadic cases (Table 1).

V. parahaemolyticus has advantages enabling biofilm formation in seafood environments. *V. parahaemolyticus* forms biofilm assisted by a dual flagellar system - polar and lateral flagella (Kim & McCarter, 2000), which is not the case with *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes* pathogens. This dual flagellar system allows *V. parahaemolyticus* to move under various conditions, thereby adjusting to different environments and attaching onto surfaces. *V. parahaemolyticus* can produce an active chitinase, enabling it to adsorb onto chitin- and copepod-surfaces (Makino et al., 2003). This helps *V. parahaemolyticus* initiate colonisation of seafood due to the capability to degrade and utilize chitinous materials of seafood surfaces. Biofilm communities are covered by extracellular polymeric substances (EPS) and show facilitated persistence within the seafood plant surfaces. Biofilms are posing challenges for hygienic treatments and risks of pathogen outbreaks.

Understanding *V. parahaemolyticus* biofilm formation will help develop biofilm decontamination techniques in seafood scenarios and reduce risks of *V. parahaemolyticus* infections. This study reviews new findings and conclusions about *V. parahaemolyticus* biofilm formation in seafood and processing plant environments, as well as describes recent advances in understanding the mechanisms of *V. parahaemolyticus* biofilm formation. It will contribute to overcoming the limitations of current chemical disinfectant treatments and help develop novel cost-effective control strategies to meet the requirements of achieving and maintaining product safety and quality in the seafood industry.

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https://doi.org/10.1016/j.lwt.2022.113182
Received 6 October 2021; Received in revised form 3 January 2022; Accepted 29 January 2022
Available online 3 February 2022
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Table 1
Emerging *Vibrio* food poisoning in unexpected regions.

Region	Year	No. of cases	Infection route	Reference
France	2010–2019	91	Seafood	FAO (2021)
Canada	2020	21	Oysters	
Canada	2015	82	Oysters	
England	2010–2020	22	Seafood	
New Zealand	2019–2020	40	Mussels	

2. Overview of *V. parahaemolyticus* biofilm formation in seafood related environments

2.1. Fundamentals of *V. parahaemolyticus* biofilm formation in seafood related environments

V. parahaemolyticus forms biofilms on marine biotic surfaces and abiotic surfaces under appropriate incubation conditions, functioning as a source of pathogenic bacteria with 10-1000 times the resistance to hygiene treatments than planktonic counterparts. Table 2 summarises published methods for detecting *V. parahaemolyticus* biofilm. While these methods have contributed to a deeper understanding of *V. parahaemolyticus* biofilm, each method has limitations. Certain techniques may concentrate exclusively on a single or a few aspects of biomass, cell viability, VBNC population, matrix structure or biofilm composition. Therefore, researchers commonly employ a combination of methods to detect and describe biofilm characteristics.

Odeyemi (2016) examined the prevalence of *V. parahaemolyticus* based on 48 published studies and discovered its presence in oysters, clams, fish, shrimp, and mussels was 63.4 %, 52.9 %, 51.0 %, 48.3 %, and 28.0 %, respectively. *V. parahaemolyticus* can form biofilms on these surfaces (Table 3), especially uneven seafood surface areas where pits and edges provide protections for biofilm communities from biocidal treatments (Han, Mizan, Jahid, & Ha, 2016). *V. parahaemolyticus* biofilm was identified on crab and shrimp surfaces by Han et al. (2016), and on shrimp and mussel surfaces by Ashrafudoulla et al. (2019). However, Rosa, Conceição, Conceição, and Timm (2018) discovered that *V. parahaemolyticus* did not form biofilm on shrimp shells, the different results occurred which could be a result of different isolates examined.

Several studies have documented that *V. parahaemolyticus* forms biofilms on abiotic surfaces (Table 3). Stainless steel, polystyrene, fibreboard, polypropylene boxes, and glass are among materials that have the potential to support the biofilm production. *V. parahaemolyticus* can form biofilms on the internal pipe surfaces of water distribution networks in seafood processing plants, which may contaminate the entire processing plant when the water is used. Seawater is frequently utilised as the initial stage in washing seafood instead of freshwater, avoiding the depletion of restricted freshwater reserves. Many pathogens do not grow well in seawater, but *V. parahaemolyticus* is an exception. Notably, washing water is routinely recirculated, allowing *V. parahaemolyticus* to accumulate and potentially develop biofilms on interior pipe surfaces (Di Pippo, Di Gregorio, Congestri, Tandoi, & Rossetti, 2018). Although chlorine and ultraviolet (UV) treatments are commonly used to disinfect industrial water, they are ineffective at removing biofilms from the water distribution network and seafood processing facilities. Moreover, cells may detach from bacterial biofilms and enter the water distribution system as a result of these treatments (Shikongo-Nambabi, Kachigunda, & Venter, 2010).

2.2. Biofilm life cycle

2.2.1. Surface attachment

V. parahaemolyticus forms biofilms using polar and lateral flagella. Polar flagella (driven by sodium ions) are utilised for swimming, while lateral flagella (driven by protons) are used for swarming (Kim and McCarter, 2000). Polar flagella function via the *fla* gene system, which is

Table 2
Different methods to determine *V. parahaemolyticus* biofilm.

Method	Application	Limitations	References
Colony formation Units (CFU)	Enumeration of culturable cells in the biofilm matrix	Only detects culturable biofilm populations, but not dormant, viable but non culturable communities; Underestimates biofilm populations due to cell aggregation; Time consuming.	(Chen et al., 2020; Guo et al., 2020; Han et al., 2016, 2017; Li et al., 2020; Mougin et al., 2019; Ning et al., 2021; Roy et al., 2021; Sun et al., 2019; Tan et al., 2021)
Microtiter plate assay - crystal violet dye & biofilm formation index (BFI)	Quantification of biomass through crystal violet stain	Lack of sensitivity; Lack of consistency; Dead cells will be stained and included as the biomass volume.	
Microtiter plate assay - Calgary device	Quantification of biomass	Calgary device lid and bottom plate are needed; CFU assay to enumerate cell numbers inside biofilm matrix still needs to be performed.	
PMA-qPCR method	Quantification of viable cell numbers in the biofilm matrix	Expensive; Need to use CFU assay to enumerate cultivable cell population to obtain VBNC cell population.	
Fluorescence microscopy	Enumeration of living and dead cell numbers through living and dead cells stains	Expensive use of fluorescence dye; Cell number counts are limited by microscopic view scopes.	
Confocal laser scanning microscopy (CLSM) imaging	Biofilm matrix observation; Structural detection	High price of fluorescence dye; Interference of self-fluorescence from the matrix.	
Scanning electoral microscopy (SEM) imaging	Observe the biofilm morphology	General observation of biofilm morphology, not useful if used to compare biofilms with limited differences.	
XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (Sigma Aldrich, UK) method	Determination of biofilm metabolic activity	Detection limit of 10^3–10^8 CFU/biofilm; Comparing the metabolic activity within the biofilm formed by same strain, strain variations may induce differences of metabolic activity.	
Phenol-sulphoacid method	Chemical method to quantify extracellular polymeric substance (EPS) production	Can only detect extracellular polysaccharides; Low accuracy; Quantification of EPS requires standard curves, or EPS production can	

(*continued on next page*)

Table 2 (*continued*)

Method	Application	Limitations	References
		only be compared by OD value.	
RT-qPCR	Quantifies expression levels of biofilm relative genes	Expensive; Multiple steps to extract clean RNA.	

highly regulated and comprises three distinct types of gene clusters. The polar flagella function as a mechano-sensor, resulting in a reduction in flagellar rotation and activation of the *laf* gene-encoded lateral flagella expression (Kim and McCarter, 2000). Swarming via lateral flagella requires bacterial cells to reach certain numbers, and the morphology of *V. parahaemolyticus* becomes elongated as it transitions from swimming to swarming cells (Freitas, Glatter, & Ringgaard, 2020). *V. parahaemolyticus* swarms, twitches, and glides across surfaces through lateral flagella, pili and the secretion of slime surface adhesins, respectively. The motility facilitates cell interaction with the surface, biofilm formation as well as virulent infection of host cells (Rossi, Paroni, & Landini, 2018). However, it is unknown whether the flagellum is lost and/or degraded following surface attachment, or whether flagella serve as structural components of the biofilm, and little is understood about how the various forms of motility interact and initiate biofilm development in *V. parahaemolyticus*.

2.2.2. Microcolony formation and the matrix

Swarm motility promotes surface colonisation through a decrease in flagellar locomotion, adhesin protein secretion and exopolysaccharide synthesis. Microcolonies are aggregates of 50 or fewer cells that form as a forerunner to biofilm formation; small colony variants promote biofilm aggregate production and antimicrobial tolerance, acting as a survival strategy with a low reproduction rate (Steenackers, Parijs, Dubey, Foster, & Vanderleyden, 2016). Following microcolony development, the cells become stronger and more stable due to the secretion of structural components, exopolysaccharides, matrix proteins, and eDNA that work as a "molecular glue" to aid attachment. The mechanism by which individual cells transform into cell aggregates, on the other hand, is not completely understood.

Biofilm maturation results in cells buried deep within biopolymer layers with a variety of mature matrix structures, including: 1) monolayer biofilms - *V. parahaemolyticus* was reported to form this structure at 4 and 10 °C (Han et al., 2016); 2) multilayer biofilms with large aggregates of bacterial cells; 3) a matrix structure previously described for other species but not yet been observed for *V. parahaemolyticus*, consist of multi-layered biofilms with small aggregates at base and motile cells covering the surface, and associate with a late mature biofilm and dispersion (Houry et al., 2012). Chemical components contained within the *V. parahaemolyticus* biofilm matrix, such as polysaccharides, proteins and eDNA, shape the biofilm architecture by changing the biovolume, porosity and mean thickness of the three-dimensional matrix (Tan et al., 2018), but the mechanisms are poorly understood.

2.2.3. Dispersion

Dispersion is the final stage of the biofilm life cycle; cells inside the biofilm actively escape from the extracellular matrix, resulting in eroded biofilm matrices and bacterial cells that can migrate to new nutrition and resource-rich environments (Steenackers et al., 2016). While it is well established that dispersion is related with cell death and lysis, little is known about the information in *V. parahaemolyticus*.

2.3. Environmental factors influencing biofilm formation

Attachment and biofilm formation of *V. parahaemolyticus* to biotic and abiotic surfaces are complicated processes that are influenced by a variety of factors including temperature, composition of the attachment medium, contact surface, cell surface, strain variants and so on.

2.3.1. Temperature

Temperature has been claimed to play a key role in influencing *V. parahaemolyticus* biofilm formation. Song et al. (2017) reported that *V. parahaemolyticus* produced better biofilm at 25 °C than at 15 °C and

Table 3
Biofilm formulation of *V. parahaemolyticus* on biotic and abiotic surfaces.

Surface	Strain	Inoculum size	Incubation parameters	Biofilm formulation results	References
Biotic - crab - shrimp Abiotic - Stainless steel	Cocktail of *V. parahaemolyticus* KCTC 2471, KCTC 2729, ATCC 33844	Crab & shrimp: 1: 2500 dilution of OD 1.0 cell suspension SS: 1:50 dilution of OD 1.0 cell suspension	Time: 24 h Temperature: 4, 10, 15, 20, 25, 30, 35, and 37 °C	The higher CFU levels were observed on the crab surfaces (almost 8 log CFU/cm^2) than on the shrimp surfaces (7 log CFU/cm^2) at 25-37 °C. 30 °C was the optimum condition for biofilm formation (>8 log CFU/cm^2).	Han et al. (2016)
Biotic - shrimp - mussel	*V. parahaemolyticus* clinical isolates (ATCC17802, ATCC27969) and 8 other environmental isolates	10^5 CFU/mL, 10 mL	Time: 24 h Temperature: 30 °C	For shrimp surfaces, environmental isolates formed 6.21-6.89 $\log_{10}$ CFU/cm^2, clinical isolates formed 5.59-6.19 $\log_{10}$ CFU/cm^2. For mussel surfaces, environmental isolates formed 5.91-6.40 $\log_{10}$ CFU/cm^2, clinical isolates formed 5.29-5.72 $\log_{10}$ CFU/cm^2.	(Ashrafudoulla et al., 2019),
Biotic - shrimp - Fish, white mouth croaker	*8 V. parahaemolyticus* environmental isolates	Original concentration of overnight culture	Time: 240 h (replace inoculum broth each 48 h) Temperature: 37 °C	No biofilms on shrimp shells, but on operculum of fish (5-6 $\log_{10}$ CFU/cm^2).	Rosa et al. (2018)
Abiotic - Stainless steel	*V. parahaemolyticus* ANSES collection 14-B3PA-0046	10^8 CFU/mL, 11 mL	Time:3 h, 24 h, 48 h Temperature: 8 °C, 37 °C	No big difference of biofilm formation at 8 and 37 °C, ranging from 6 to 9 $\log_{10}$ CFU/cm^2.	Mougin et al. (2019)
Abiotic - Stainless steel - glass	*V. parahaemolyticus* ST55, 16 clinical and 12 environmental isolates	1:2 dilution of OD 1.5 cell suspension	Time: 1–8 h Temperature: 25 °C	Clinical strains attached better on stainless steel surface than did environmental strains. The cell density reached a peak at 6 or 8 h (6-8*10^5 CFU/mL) on stainless steel and glass surfaces and declined thereafter.	Wong (2002)
Abiotic - glass	*V. parahaemolyticus* VP-C7	1:100 dilution of OD 0.4 cell suspension	Time: 2 h, 8 h, 12 h, 24 h and 48 h Temperature: 15, 25 and 37 °C	When cultured at 15 °C, a mature biofilm only forms after 48 h (biofilm thickness of 19.73 μm), while a mature biofilm forms between 12 and 24 h at 25 C° (biofilm thickness of 18.94-19.80 μm).	Song et al. (2017)

37 °C. According to Han et al. (2016), *V. parahaemolyticus* develops multi-layered biofilms at 15 and 37 °C, but monolayers at 4 and 10 °C. It has been concluded that biofilm formation could be the main cause of food safety problems at higher temperature (25-37 °C) (Han et al., 2016).

2.3.2. Sodium chloride and glucose

Sodium chloride (NaCl) and glucose influence adhesion and maturation of *V. parahaemolyticus* biofilms. For example, the adsorption of *V. parahaemolyticus* onto plankton or chitin-containing materials was observed at salinities as low as 1.7 % in estuary waters while salinity in open ocean seawater is approximately 3.5 %. *V. parahaemolyticus* formed the best biofilm in tryptic soy broth (TSB) containing 2 % NaCl and the least biofilm in TSB containing 5 % NaCl. Glucose concentrations of 0.005-0.015 % in TSB promoted biofilm formation, whereas high glucose concentrations of 0.05 % inhibited the formation (Mizan, Ashrafudoulla, Sadekuzzaman, Kang, & Ha, 2018).

2.3.3. Contact surface (charge, hydrophobicity)

The charge on the cell surface varies under different physiological conditions, influencing bacterial attachment to surfaces. Cells of *V. parahaemolyticus* are frequently negatively charged, and thus prefer to attach to positively charged surfaces (Mizan et al., 2016). Besides, *V. parahaemolyticus* biofilm formation is positively correlated with cell surface hydrophobicity and is associated with the flagellar and proteins on the bacterial cell outer membrane (Mizan et al., 2016). The hydrophobicity of contact surface (stainless steel, polystyrene and glass) exhibited a negative correlation with the amount of eDNA, extracellular protein and biofilm biomass in *V. parahaemolyticus* biofilms (Guo et al., 2020). Instead of hydrophobic stainless steel, *V. parahaemolyticus* grows a better biofilm on surfaces such as polystyrene and glass; this phenotype was related with different survival adaptation mechanisms on these surfaces (Guo et al., 2020; Wong, 2002).

3. Molecular mechanisms of *V. parahaemolyticus* biofilm formation

3.1. Positive regulators

The mechanisms by which *V. parahaemolyticus* biofilms form are regulated by a systemic and integrated regulatory network. Though there are numerous genes responsible for EPS biosynthesis and production, the *cps* locus has been extensively studied and will be discussed in Positive regulators and Negative regulators (Fig. 1).

CpsQ can activate the expression of the capsular polysaccharide genes *cpsA-cpsJ* (*vpa1403-1412*) in *V. parahaemolyticus*, thereby regulating biofilm production. CpsQ is a c-di-GMP binding protein that is regulated by intracellular c-di-GMP concentrations, and has been demonstrated to be a positive, regulator of capsular polysaccharide CPSA expression. It was reported that quorum sensing regulators OpaR and AphA bind to the promoter region of the *mfpABC* operon to enhance and repress *mfpABC* transcription, as well as repress and enhance *cpsQ-mfpABC* operon expression, and influence biofilm development (Zhou et al., 2013). Due to their accumulation at high cell density, it was hypothesised that CpsQ and MfpABC might play a role in the middle/late stages of growth and pathogenesis. CpsQ contributes to capsule expression when c-di-GMP levels are elevated, but it is not solely responsible for biofilm formation; therefore, deletion of CpsQ does not eliminate biofilm formation (Kimbrough, Cribbs, & McCarter, 2020).

CpsR is another transcription regulator that regulates the formation of the biofilm matrix. CpsR is also not solely required for biofilm formation in *V. parahaemolyticus,* but it is critical for the increased CPSA

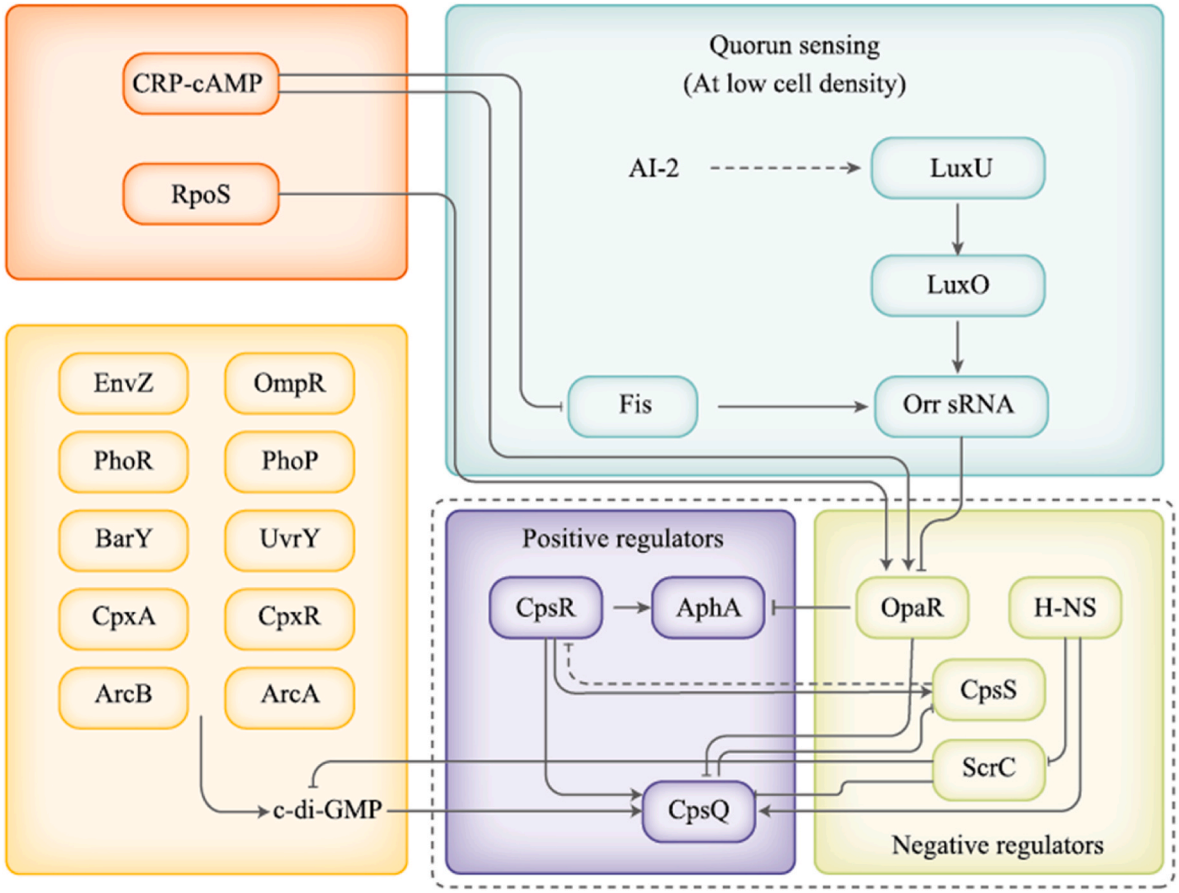

Fig. 1. The transcriptional network of *V. parahaemolyticus* biofilm formation.

expression elicited by c-di-GMP (Yildiz & Visick, 2009). It is required in the ScrABC-dependent pathway to regulate swarming and EPS production. Introduction of the *cpsR1*::Tn5 allele into the rugose *scrA* mutant resulted in the transformation of a rugose colony to smooth colony, the elimination of exopolysaccharide production, and a decreased capacity for biofilm formation (Guvener & McCarter, 2003). CpsR acts prior to CpsQ and can activate CpsQ and CpsS; the CpsS-CpsR-CpsQ regulatory cascade is responsible for the EPS production (Guvener & McCarter, 2003).

Quorum sensing governs sets of cellular pathways, including motility, virulence and biofilm formation through its master regulators-AphA and OpaR. AphA is a small PadR family expression regulator with an N-terminal winged-helix DNA-binding domain; AphA is activated at low cell density, to promote the transcription of virulence, flagella-mediated motility and biofilm formation (Zhou et al., 2013). In the presence of low cell density, redundant $Qrr_{1\text{-}4}$ sRNAs regulated by LuxQ-LuxU-LuxO pathway inhibit *opaR* and *cpsQ-mfpABC* transcription, thereby influencing the formation of *V. parahaemolyticus* biofilms (Liu, Zhu, Zhang, & Zhao, 2021; Zhou et al., 2013).

3.2. Negative regulators

Signalling via chemotaxis ScrABC is involved in competitive colonisation and the development of the biofilm matrix. ScrA is found in the *scrABC* operon and promotes the expression of *laf* genes while repressing the transcription of *cps* genes in *V. parahaemolyticus* (Boles & McCarter, 2002). ScrC functions as a diguanylate cyclase (DGC), retains GGDEF and EAL domains and is regulated by ScrA and ScrB translation, it can promote CpsQ repression and c-di-GMP degradation (Kimbrough et al., 2020). A *scrABC* operon mutation has a profound effect on gene expression, resulting in decreased swarming activity, increased cellular c-di-GMP levels, overproduced CPSA, crinkly colonies and enhanced biofilm formation (Kimbrough et al., 2020).

CpsS is the dominant negative regulator in *V. parahaemolyticus* (Yildiz & Visick, 2009). It is another member of the CsgD family and contains a DNA-binding domain similar to that of CsgD. CpsS can inhibit *cpsR* expression, while *cpsR* activates *cpsQ*, and CpsQ inhibits *cpsS*. CpsS represses CpsQ, but it is unknown whether it directly or indirectly regulates CpsQ. Deletion of *cpsS* results in capsule overexpression and wrinkly colonies (Enos-Berlage, Guvener, Keenan, & McCarter, 2005).

OpaR governs biofilm formation through regulating CpsQ (Zhou et al., 2013). The histone-like nucleoid structuring protein H-NS is a "transcriptional silencer", it is involved in the transcription of genes and the folding of DNA and an expression activator of the *cpsA-cpsJ* operon (Enos-Berlage et al., 2005). Mutants of *hns* fail to trigger CPSA production and polar flagellar activity, thereby resulting in decreased biofilm formation in *V. parahaemolyticus* (Enos-Berlage et al., 2005).

3.3. EPS synthesis genes

Numerous gene loci/genes have been identified as being involved in the formation of the extracellular matrix of *V. parahaemolyticus* biofilms. *cpsA-J* (*vpa1403-1412*) is required for the synthesis of capsular polysaccharide A (CPSA), a major component of the *V. parahaemolyticus* biofilm (Yildiz & Visick, 2009). *cpsA-J* is also required for the formation of opaque colonies, rugose phase transmission of the colony and biofilm development (Enos-Berlage & McCarter, 2000). *vp1476-1458* is an ortholog of the *syp* locus that is conserved in *Vibrio fischeri*, this locus was reported to be responsible for wrinkled colonies, pellicle formation and matrix production in *V. fischeri* (Yildiz & Visick, 2009). *V. parahaemolyticus* shares 85.9 and 75.8 % similarities with the glycosyltransferase gene *sypQ* from *Vibrio alginolytious* 12G01 and *Vibrio harveyi* ATCC BAA-1116 (Ye, Zheng, & Zheng, 2014), indicating the relatedness with poly-N-acetylglucosamine (PNAG) biosynthesis and function as an intercellular adhesin. Cellulose is typically found in flagella or pili of Gram-negative bacteria; however, the cellulose synthase gene cluster is limited known in *V. parahaemolyticus*. Another locus involved in *V. parahaemolyticus* biofilm formation is *vp0214-0237*, which results in translucent colonies, decreased adherence to surfaces, inhibition of swarming motility and interruption of biofilm maturation (Enos-Berlage et al., 2005). Proteins and eDNA in the extracellular matrix contribute to the structure and stability, but they are poorly studied in *V. parahaemolyticus*.

3.4. Will pathogenicity influence biofilm formation?

Virulence factors are typically associated with bacterial pathogenicity. *V. parahaemolyticus* produces a variety of virulence factors, including thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) and two distinct type III secretion systems (T3SS1 and T3SS2) (Makino et al., 2003). The pathogenicity of a strain and its ability to form biofilms are related properties. Song et al. (2017) discovered that pathogenic *V. parahaemolyticus* accumulates more biofilm matrix than non-pathogenic strains. Similarly, Wong (2002) found that clinical strains adhered more readily to stainless steel than environmental strains, and that decreased c-di-GMP levels within cells can promote biofilm formation and pathogenicity in *V. parahaemolyticus*. Zhang et al. (2019) revealed that the transcription factor QsvR works in conjunction with QS system to regulate the expression of virulence genes, T3SS1 and pathogenicity island (PAI, T3SS2 and TDH), in *V. parahaemolyticus*. AphA has a role in the initial colonisation stage, it activates T3SS1 genes expression but inhibits the expression of PAI genes, thus boosting *V. parahaemolyticus* cytotoxicity, whereas OpaR and QsvR act at a higher cell density by activating PAI transcription, thereby enhancing enterotoxicity and causing severe gastroenteritis. QsvR can also maintain basal levels of T3SS1 expression despite OpaR negatively regulates it.

4. Limitation of chemical disinfectants and promising strategies to overcome

4.1. Limitation of chemical disinfectants

Chemical disinfectant treatment is a simple, cost-effective, and widely used strategy for pathogen contamination control in the food industry. Numerous studies to determine the efficacy of various chemical disinfectants against *V. parahaemolyticus* biofilms have been conducted (Table 4). While cleaning chemicals and disinfectants remove soils and inactivate biofilm cells from seafood and plant surfaces, using disinfectants alone at recommended concentrations makes it difficult to control biofilm effectively; what is worse, recurrence and recolonization of pathogen communities with increased acquired resistance may occur (Rosa et al., 2018). Increased disinfectant concentrations are discouraged, as they may corrode plant surfaces, influence seafood sensory attributes and produce chlorine and/or other by-products that are hazardous to human health. Additionally, routine exposure of biofilm populations to chemical disinfectants may also result in an increase in VBNC state cells, which aids in the persistence of biofilm communities on seafood processing plants. For instance, the routine use of chemical disinfectants in cleaning and sanitation in smoked salmon processing plants has resulted in the development of *L. monocytogenes* VBNC populations that are resistant to environmental stress and difficult to eradicate (Brauge et al., 2020).

4.2. Promising strategies

As chemical disinfectant treatments fail to remove *V. parahaemolyticus* biofilms effectively, alternative strategies for mitigating these risks are required (Fig. 2).

4.2.1. Plant surface modification

Processing plant surface modifications have been studied to inhibit biofilm colonisation and development. For example, it has been

Table 4
The effect of chemical disinfectant treatment on *V. parahaemolyticus* biofilm.

Disinfectants	Biofilm	Reduction	Reference
Sodium hypochlorite - 20 ppm chlorine (~20 mg/L chlorine), 10min	240 h old biofilms formed on biotic and abiotic surfaces	*V. parahaemolyticus* biofilm communities of 2-5 $\log_{10}$ CFU/cm^2 were remained after treatment (~6 $\log_{10}$ CFU/cm^2 biofilm as control).	Rosa et al. (2018)
Sodium hypochlorite - 4 mg/L chlorine, 1h	72 h old biofilm on glass slides	The bacterial density dropped from 7.90 to 3.97 $\log_{10}$ CFU/cm^2;	Shikongo-Nambabi et al. (2010)
Hydrogen peroxide (H_2O_2) - 0.08 %, 1h		Inactivated cells to non-detectable levels from over 7 $\log_{10}$ CFU/cm^2;	
Ozone - 1.6 mg/L, 1h		Reduced ~ 2 $\log_{10}$ CFU/cm^2 biofilm cell densities.	
Sodium hypochlorite - 50-300 ppm, 6 % w/v chlorine, 5min Strong acidic electrolyzed water (SAEW) - 30 ppm chlorine (~30 mg/L chlorine), 1–15 min	24 h old biofilm on shrimp and crab surfaces	Sodium hypochlorite rendered maximum reductions of 3.78 and 3.32 $\log_{10}$ CFU/cm^2 on shrimp and crab surfaces (6.87 and 7.37 $\log_{10}$ CFU/cm^2 as control); SAEW achieved reductions of 1.42-3.05 and 1.14 to 2.56 $\log_{10}$ CFU/cm^2 on shrimp and crab surfaces.	Roy et al. (2021)
Strong acidic electrolyzed water (SAEW) - pH 2.3, 136.33 mg/L chlorine, 30s	48 h old biofilm	Decreased viable *V. parahaemolyticus* from 6.90 to 3.33 $\log_{10}$ CFU/cm^2.	Han et al. (2017)
Acidic electrolyzed water - pH 2.28, 52.26 mg/mL chlorine, 10 min	48 h old biofilms on polystyrene surfaces	The biovolume, eDNA, protein and polysaccharide content of *V. parahaemolyticus* matrix was significantly reduced.	Li et al. (2020)

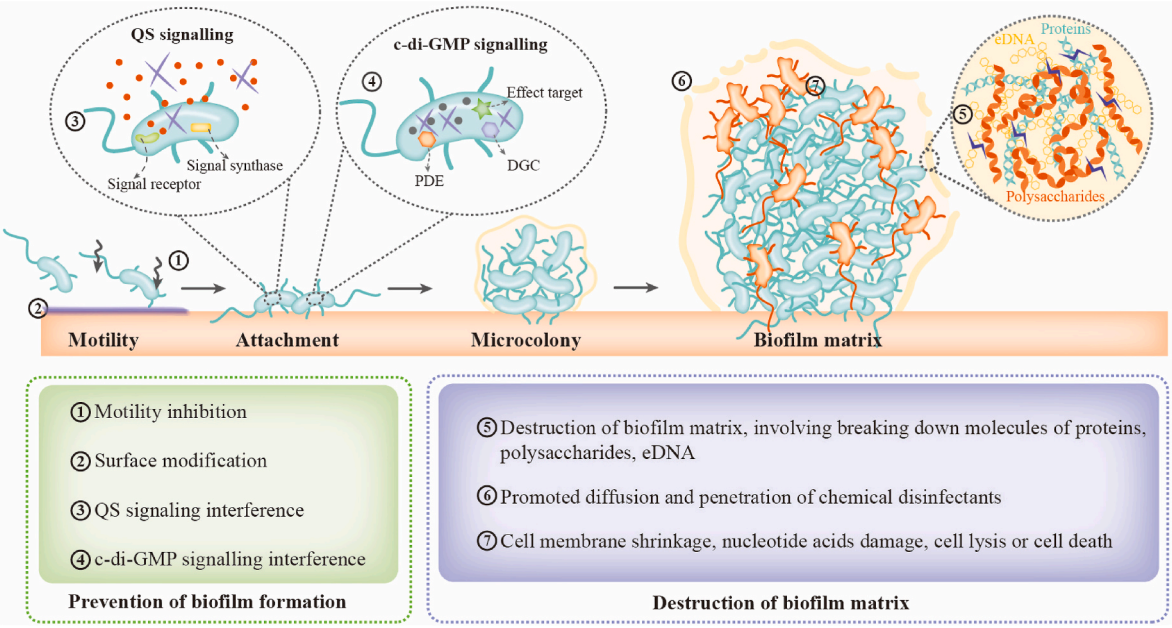

Fig. 2. Promising inactivation forms of *V. parahaemolyticus* biofilm communities.

demonstrated that sol-gel modification, in which the material is transformed from a liquid to a gel state, reached to reduce the average biomass weight attached. This was employed on milk processing plant surfaces to reduce biomass, and this investigation indicated that the biomass weight on stainless steel 316L and sol-gel-modified coupons in a benchtop plate heat exchanger was 19.21 mg/cm^2 and 0.37 mg/cm^2, respectively (Liu, Jindal, Amamcharla, Anand, & Metzger, 2017). Biosurfactants are surface-interactive molecules that exhibit both hydrophobic and hydrophilic properties, for instance, BS-SLSZ2, the biosurfactant derived from the marine bacterium *Staphylococcus lentus*, which has been shown to hinder bacterial attachment, impair bacteria-bacteria interactions, and prevent the formation of biofilms. At a dose of 20 μg, *V. harveyi* and *Pseudomonas aeruginosa* biofilm formation were inhibited by 80 % and 82 %, respectively (Hamza, Satpute, Banpurkar, Kumar, & Zinjarde, 2017).

4.2.2. Physical strategies

4.2.2.1. Bubbles. The bubble technique is being developed for anti-biofilm applications. Laser-induced vapour bubbles are used to enhance the distance between sessile cells. Millimetre-sized air bubbles in water significantly reduced the number of biofilm cells attached to stainless steel and polypropylene surfaces by 1.6 and 0.9 $\log_{10}$, respectively, and also removed the carbohydrate, protein and fat residues from stainless steel surfaces (Burfoot, Limburn, & Busby, 2017). Shiroodi, Schwarz, Nitin, and Ovissipour (2021) employed bubbles to treat *V. parahaemolyticus* biofilms on plastic and stainless-steel coupons, achieving reductions of 2.5 and 1.4 $\log_{10}$ CFU/cm^2 biofilm cells in 2 min, respectively, and cell elimination of >7 $\log_{10}$ CFU/cm^2 in 5 min.

4.2.2.2. Cold plasma. Cold plasma has received much attention for

biofilm controlling due to its non-thermal, rapid, green and waterless properties. The reactive species generated have been identified as the primary components for antibiofilm abilities, as they can destroy biofilm matrix, penetrate the structures and eventually lead the bacterial cells to death. High-voltage cold plasma (80 kV) treatment of 60 s reduced *L. monocytogenes*, *Salmonella enterica* and *Pseudomonas fluorescens* biofilm cells by 3.76, 4.14 and 2.6 $\log_{10}$ CFU/mL from initial cell concentrations of 5.4 ± 0.4 $\log_{10}$ CFU/mL, respectively (Patange et al., 2019). Dielectric barrier discharge plasma treatment (1.1 kV, 30 min) reduced *E. coli* in fresh oysters by 1.01 $\log_{10}$, while having no effect on the glycogen content or texture of oyster meat (Choi et al., 2022), demonstrating the potential for cold plasma application in seafood environments. However, the removal efficacy of *V. parahaemolyticus* biofilms is limited known and requires further investigations.

4.2.2.3. Low-frequency and high-intensity ultrasound treatment. The ultrasound with a low frequency and a high intensity has been proposed to be more effective at decontaminating biofilms, and the biofilm removal is based on mechanical oscillation, free radicals (H · and .OH) and hydrogen peroxide (H_2O_2) generated, as well as localized heating. 10 s treatment of 40 kHz flat ultrasonic transducer achieved to remove *E. coli* and *Staphylococcus aureus* dairy biofilms from stainless steel surfaces. The high-intensity ultrasound has been examined for its application on fish, involving salmon (*S. salar*), mackerel (*S. scombrus*), cod (*G. morhua*) and hake (*M. merluccius*) fillets. The microorganism on fish surfaces were significantly reduced, but no significant changes of lipid content, the moisture level remained stable except hake (Pedrós-Garrido et al., 2017). Additional research of low-frequency and high-intensity ultrasound treatment on *V. parahaemolyticus* biofilm cells should be conducted.

4.2.2.4. Ultraviolet-C (UV–C) treatment. UV-C spectrum (250-270 nm) is lethal to a wide range of microorganisms, including bacteria, yeasts and viruses. UV-C (5, 10 min) combined with 50-500 μg/mL peroxyacetic acid reduced *Salmonella* Enteritidis biofilm cells from stainless-steel and silicone rubber surfaces by 3.10-6.41 $\log_{10}$ CFU/mL, UV-C (5, 10 min) in combination with 0.5-2.0 % lactic acid reduced by 3.35-6.41 $\log_{10}$ CFU/mL (Byun et al., 2022). UV-C treatment of 5, 10 and 30 mW*s/cm^2 decreased *V. parahaemolyticus* biofilm communities by 1.37-2.53 and 0.75-1.94 $\log_{10}$ CFU/cm^2 on shrimp and crab surfaces, respectively; UV-C (60 mW*s/cm^2) along with sodium hypochlorite (300 ppm) reached decrease of biofilm cells by 3.78 and 3.32 $\log_{10}$ CFU/cm^2; UV-C (60 mW*s/cm^2) combined with slightly acidic electrolyzed water reduced *V. parahaemolyticus* biofilm cells by 4.41 and 4.06 $\log_{10}$ CFU/cm^2, respectively (Roy et al., 2021).

4.2.2.5. High hydrostatic pressure (HHP) treatment. HPP is another nonthermal approach that has been demonstrated to be effective at inactivating *V. parahaemolyticus* in the seafood industry. 400 MPa HHP (20 °C, 3 min) decreased *V. parahaemolyticus* in oysters to undetectable level from ~5 $\log_{10}$ CFU/g, and the characteristic taste of oyster meat can retain for up to 15 days if refrigerated at –20 °C (Liu et al., 2022). However, it is limited known about HPP efficacy on *V. parahaemolyticus* biofilm cells in seafood, despite reports of its use against other food pathogens. For example, *Enterobacter sakazakii* biofilm formation was significantly reduced by 45 % when incubated for 24 h with 400 MPa HHP (Liao, Tao, Li, Xu, & Wang, 2021).

4.2.2.6. Photodynamic inactivation (PDI). PDI involves the use of a photosensitizer (PDI) activated by visible light, leading to reactive oxygen species (ROS) production and consequently inactivation of microbial cells. Inactivation of microbes is caused by ROS which has potential to deconstruct EPS, proteins, lipids and nucleic acids in biofilm matrix (Tan et al., 2021). Chen et al. (2020) found that PDI treatment (5 min, 1.14 J/cm^2) with 1.0 μM curcumin as the photosensitizer (PS) decreased *V. parahaemolyticus* (~8 $\log_{10}$ CFU/mL) to non-detectable levels, it could downregulate virulence genes (*tdh* and *toxR*) and biofilm formation related genes (*oxyR*, *aphA*, *luxR* and *opaR*) thereby controlling *V. parahaemolyticus* biofilm contaminations, they also found that 20.0 μM curcumin PDI treatment (60 min, 13.68 J/cm^2) achieved to eradicate 48 h old *V. parahaemolyticus* biofilm on polystyrene surfaces.

4.2.3. Chemical strategies

4.2.3.1. Essential oils. Essential oils are plant-based extracts that contain a variety of antibacterial and antibiofilm components. Citrus peel essential oils inhibit *V. parahaemolyticus* biofilm formation by repressing flagella gene transcription, T3SS effector function and quorum sensing activities (Sun et al., 2019); at a concentration of 3.125 and 6.25 μg/mL, citral reduced 42 and 58 % autoinducer-2 (AI-2) quorum sensing activity, respectively; 6.25 and 12.5 μg/mL of citral decreased *V. parahaemolyticus* swimming and swarming by 20-47 % and 35-50 %, respectively; citral at a concentration of 12.5 g/mL was observed to disrupt the three-dimensional *V. parahaemolyticus* biofilm matrix with dispersed cells.

4.2.4. Biocontrol strategies

4.2.4.1. Small-molecule signal blocker. Interfering biofilm formation with small molecules is a novel strategy; these molecules are referred to as flagella inhibitors, quorum sensing inhibitors and c-di-GMP signalling blockers. For example, diguanylate cyclase (DGC) has been identified as a critical target for modulating the c-di-GMP process and biofilm formation phenotypes, Christen et al. (2019) demonstrated that 4-(2, 5-dimethylphenoxy)-N-(4-morpholin-4-ylphenyl) butanamide and six other small molecules in the low μM inhibited c-di-GMP signalling and biofilm formation via regulation of the DGC in a non-competitive manner. Similarly, 2,6-Di-*tert*-butyl-4-methylphenol (DTBMP) from *Chroococcus turgidus* was found to inhibit initial *V. parahaemolyticus* biofilm formation by interfering with hydrophobic activity, swarming motility and quorum sensing, 250 μg/mL of DTBMP reduced 74 % EPS production; DTBMP was also examined *in vivo* on pacific white shrimp, and demonstrated anti-adherence efficacy without causing fatal effects on the shrimp (Santhakumari et al., 2018).

4.2.4.2. Enzymes. Another alternative to biofilm contamination control in the food industry are non-toxic and disruptive enzymes. For example, endolysins are peptidoglycan hydrolases that can deconstruct biofilm matrix and hydrolyse bacterial cell wall peptidoglycan, the combination of endolysin Lysqdvp001 (60 U/g) and ε-PL (ε-poly-lysine, 0.20 mg/g) can significantly reduce *V. parahaemolyticus* in *Gadus macrocephalus*, *Penaeus orientalis* and oyster by 3.75, 4.16 and 2.50 $\log_{10}$ CFU/g, respectively. The sole usage of ε-PL reached elimination of *V. parahaemolyticus* biofilms by 28.67 %, 14.27 % and 12.67 %, respectively, from polystyrene, glass and stainless-steel surfaces, though Lysqdvp001 had no substantial effect; the cocktail of these two enzymes removed 55.13 %, 44.43 % and 68.00 % of the biofilms, indicating the synergistic effect of enzyme treatment may be due to the attack of different *V. parahaemolyticus* target sites (Ning, Lin, & Wang, 2021).

4.2.4.3. Bacteriophages. Bacteriophages can destroy biofilms by breaking down the biofilm matrix. For example, phage cocktails were prepared by mixing phage isolated from cattle faeces; the phage cocktails inhibited *E. coli* O177 biofilm formation at 25 °C within 24 h, and reduced pre-formed biofilms to undetectable levels within 5 h (Montso, Mlambo, & Ateba, 2021). Bacteriophage OMN was reported to kill 90 % and 99 % *V. parahaemolyticus* communities on oyster meat surfaces after 48 and 72 h of treatment, respectably, showing the viability of bacteriophage application in seafood contexts (Zhang et al., 2018). However, as much of the bacteriophage studies are limited to laboratory

conditions, additional work is required to apply in the industry.

5. Conclusions and perspectives

The persistence of the *V. parahaemolyticus* biofilm in the seafood plant may result in pathogen recurrence and complicate hygienic treatment. It is necessary to understand the mechanism by which *V. parahaemolyticus* biofilms form. Specific questions include the following: 1) Is the flagellum lost and/or degraded, or is flagella function structural in the biofilm following surface attachment? 2) Do various forms of motility collaborate, interact and trigger biofilm formation in *V. parahaemolyticus*? 3) What are the mechanisms underlying the formation of *V. parahaemolyticus* biofilm structures? 4) What are the polysaccharides and protein components of the matrix at various stages of biofilm development? 5) What is the mechanism behind biofilm dispersal? 6) What are the interactions of transcriptional regulators and biological pathways in biofilm formation and pathogenicity?

The most effective way to control *V. parahaemolyticus* biofilm contamination is to inhibit *V. parahaemolyticus* colonisation and biofilm formulation. In the seafood industry, biological-control, and safe and cost-effective strategies are preferred but require more investigation. Notably, while most novel biofilm decontamination strategies have been evaluated in the laboratory, it is necessary to consider their regulatory status and how they will be applied on an industrial scale.

Author contributions

Dan Wang: writing - original draft, review & editing; **Steve Flint, Jon Palmer**, **Dragana Gagic**, **Graham Fletcher** and **Stephen On**: supervision, writing - review & editing.

Funding

This study is funded by Massey University, the grant number is P963141222WangD.

Declaration of competing interest

The authors have no competing interests to declare.

Acknowledgement

Authors convey their appreciation to the support from School of Food and Advanced Technology (Massey University), School of Fundamental Sciences (Massey University), Plant & Food Research (New Zealand) and Faculty of Agriculture and Life Sciences (Lincoln University).

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