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Distribution and expression of virulence genes in potentially pathogenic bacteria isolated from seafood in Thailand

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ABSTRACT

Since seafood can be a reservoir for diverse pathogenic bacteria, the risk to human health associated with seafood merits evaluation. The potentially pathogenic bacteria were isolated from seafood in Thailand. The 40 obtained strains were distinguished by ERIC-PCR fingerprints and identified to the species level by the VITEK 2 system. Their 16S rRNA gene had 97.74–99.79% nucleotide sequence identities to those of bacteria in 11 genera including *Aeromonas*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Morganella*, *Salmonella*, *Proteus*, *Providencia*, and *Vibrio*. Among the eight detected virulence genes, the *tlh* gene was expressed under all conditions, which, contrary to the *cnf2*, *LTI*, *ssaO*, and *vt2e* genes. Nutrient enrichment had a positive effect on the expression of *kfu* and *uge* genes, as well as a negative effect on the expression of the *csgD* gene. NaCl conferred a positive effect on the expression of the *uge* gene but negatively affected the expression of the *csgD* gene.

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Distribución y expresión de genes de virulencia en bacterias potencialmente patógenas aisladas de mariscos y pescados en Tailandia

Debido a que los mariscos y los pescados pueden operar como reservorio de diversas bacterias patógenas, es necesario evaluar el riesgo asociado que los alimentos de origen marino conllevan para la salud humana. Con este objetivo, en el presente estudio se aislaron bacterias potencialmente patógenas de los alimentos de origen marino disponibles en Tailandia. Las 40 cepas obtenidas se distinguieron por huellas reconocidas mediante ERIC-PCR y se identificaron a nivel de especie por medio del sistema VITEK 2. Su gen 16S de ARNr tiene una identidad de secuencia de nucleótidos de 97.74 a 99.79% en comparación con las de las bacterias de 11 géneros, entre ellos *Aeromonas*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Morganella*, *Salmonella*, *Proteus*, *Providencia* y *Vibrio*. A diferencia de los genes *cnf2*, *LTI*, *ssaO* y *vt2e*, entre los ocho genes de virulencia detectados el gen *tlh* se expresó en todas las condiciones. El enriquecimiento de nutrientes provocó un efecto positivo en la expresión de los genes *kfu* y *uge*, y un efecto negativo en la expresión del gen *csgD*. El NaCl confirió un efecto positivo a la expresión del gen *uge*, pero afectó negativamente la expresión del gen *csgD*.

1. Introduction

Seafood has numerous positive effects on human health and regular consumption of seafood is recommended by dietary guidelines (Farmery et al., 2018). However, the main problem associated with seafood consumption is the contamination by chemicals, heavy metals, toxins, and pathogenic microorganisms. Seafood has been recognized as an important source of food poisoning epidemics worldwide. Microbial contamination in seafood can occur in every step from exposures to marine water and weeds, harvesting and processing, packaging, storing, transporting, and cooking. Examples of important seafood-associated pathogenic bacteria are *Clostridium*, *Enterococcus*, *Escherichia*, *Salmonella*, *Shigella*, and *Vibrio* (Iwamoto et al., 2010). These bacteria have been reported as causative agents of foodborne outbreaks and therefore are indicators of microbial contamination in food and water. Within an individual genus, only some species and strains are pathogenic (Duvall et al., 2017; Iwamoto et al., 2010). Each species has a broad

variety of different degrees of virulence, ranging from avirulence to lethality. The ability of bacteria to cause diseases is controlled by several virulence genes which may be located on transmissible genetic elements including transposons, plasmids, and specific regions of the bacterial chromosomes, termed pathogenicity islands (PAI) (Hacker et al., 1997). Virulence genes play roles in controlling related mechanisms such as synthesis and release of virulence factors, adherence factors, survival factors, putative regulators, proteins, enzymes, toxins, iron-binding compounds, and biofilm (Bahador et al., 2019; Liu et al., 2019; Mannion et al., 2018; Zughaier & Cornelis, 2018); formation of lesions in intestinal epithelial cells (Capeda-Molero et al., 2017); suppression of host immune systems; infection in urinary tracts and bloodstream (Daga et al., 2019); adaptation to acidic and anaerobic conditions in intestines; adaptation for survival in the presence of intestinal microbiota (Jubelin et al., 2018); host-bacterial interaction (Niu et al., 2013); and determination of host specificity

(Pan et al., 2014). These mechanisms are essential for inhabitation, survival, adaptation, and multiplication in hosts. Virulence genes are consequently important for bacterial infection and pathogenesis. The expression of virulence genes has been found to be affected by several factors such as nutrient starvation (Chandra et al., 2017; Paytubi et al., 2017); concentrations of NaCl (Larsen & Jespersen, 2015), oxygen (Melson & Kendall, 2019), and glucose (Valdes et al., 2018); temperature (Guijarro et al., 2015); growth phase (Koohsari et al., 2017); kind of carbon source (Ferrando et al., 2014; Kentache et al., 2016); pH (Do et al., 2019); sodium glycocholate (Joffe et al., 2019); and L-glutamine (Haber et al., 2017).

The aims of this work was to investigate the presence of virulence genes in seafood-associated bacteria and to determine the environmental conditions that stimulate the expression of virulence genes.

2. Materials and methods

2.1. Isolation of target bacteria from seafood

Thirty-five samples of fresh seafood, which belonged to 15 species listed in Table 2, were collected from three central seafood markets in Thailand, including the Talaythai market in Samutsakhon province, Bankhaotakiab market in Prachuapkhirikhun province, and Banphe market in Rayong province, in August 2014. All samples were placed in sterile zip-lock plastic bags, kept in ice buckets prior to isolation of target bacteria belonging to genera *Escherichia*, *Salmonella*, *Shigella*, and *Vibrio*. The isolation was performed within 6 h after sample collection (Da Silva et al., 2012). Twenty-five grams of flesh samples were aseptically excised, transferred to stomacher bags containing 225 ml of peptone water, and then homogenized at 200 rpm for 2 min by using a Seward stomacher 400 circulator (Seward Limited, West Sussex, UK). Homogenates were serially diluted in peptone water to make dilutions of 10^{-1} , 10^{-2} , and 10^{-3} . These dilutions were pour plated on three selective media including eosin methylene blue (EMB) agar (HiMedia Laboratories, Mumbai, India), *Salmonella-Shigella* (SS) agar (Pronadisa, Madrid, Spain), and thiosulfate citrate bile salts sucrose (TCBS) agar (HiMedia Laboratories, Mumbai, India). Plates were incubated at 35°C for 2 days. Presumptive *Escherichia coli* formed metallic sheen colonies with diameters of 2–3 mm on EMB agar. Presumptive *Salmonella* formed colorless, transparent colonies with or without black centers on SS agar. Presumptive *Shigella* formed colorless, transparent colonies on SS agar. Presumptive *Vibrio* isolates were considered those displaying yellow, green, or green-blue colonies on TCBS agar. The numbers of presumptive target bacteria in each seafood sample were quantified as a log colony-forming unit (CFU)/g. Pure cultures of selected isolates were maintained on Luria-Bertani (LB) slants at 4°C and in 20% glycerol at –80°C.

2.2. Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) fingerprinting

ERIC-PCR was performed to analyze genotypic diversity and relatedness among bacterial isolates as well as to distinguish individual strains. Genomic DNA of each isolate was extracted

from an exponentially grown culture using a GF-1 bacterial DNA extraction kit (Vivantis Technologies Sdn. Bhd., Selangor Darul Ehsan, Malaysia) and used as a template DNA for PCR using a pair of primers ERIC2 (5' AAG TAA GTG ACT GGG GTG AGC G 3') and ERIC1R (5' ATG TAA GCT CCT GGG GAT TAC C 3') as described previously (Ogutcu et al., 2009). DNA was amplified in a 25- μ l reaction volume containing 2 μ l of template DNA, 1.25 mM dNTP, 2.5 μ l of 10X buffer, 1.5 mM MgCl₂, 2 mM of each primer, and 2.5 units of *Taq* DNA polymerase. Negative controls (no DNA added) were included in all sets of reactions. PCR cycles consisted of the first denaturation at 95°C for 7 min, and then 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 65°C for 8 min, followed by a final extension at 65°C for 16 min. The presence and sizes of the amplified fragments were determined by agarose [1% in Tris-borate-EDTA (TBE) buffer] gel electrophoresis. Gels containing SafeView FireRed gel casting dye (Applied Biological Materials Inc., Richmond, Canada) were visualized with a Molecular Imager® Gel Doc™ XR+ system (Bio-Rad Laboratories, Hercules, CA, USA). The unweighted pair groups using mathematical averages (UPGMA) dendrogram was constructed using the Phoretix ID Pro. software (Total Lab Ltd., Newcastle upon Tyne, UK). The strains generating individual ERIC-PCR fingerprints were selected for further steps of the study.

2.3. Sequence analysis of partial 16S rRNA gene

Genomic DNA of each strain was extracted from an exponentially grown culture using a GF-1 bacterial DNA extraction kit (Vivantis Technologies Sdn. Bhd., Selangor Darul Ehsan, Malaysia) and used as a template DNA for PCR using a pair of universal primers UN16S 926 f (5' AAA CTY AAA KGA ATT GAC GG 3') and UN16S 1392 r (5' ACG GGC GGT GTG TRC 3') (Lane, 1991) as described previously (Pongsilp et al., 2002). Partial 16S rRNA gene was amplified in a 25- μ l reaction volume containing 2 μ l of template DNA, 0.2 mM dNTP, 2.5 μ l of 10X buffer, 1.5 mM MgCl₂, 2 mM of each primer, and 1.25 units of *Taq* DNA polymerase. Negative controls (no DNA added) were included in all sets of reactions. PCR cycles consisted of the first denaturation at 95°C for 5 min, and then 34 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The presence and sizes of the amplified fragments were determined as described above. The PCR products were purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and subsequently sequenced by Bio Basic, Inc. (Markham, Ontario, Canada). The nucleotide sequences were aligned with the reference 16S rRNA gene sequences using the nucleotide BLAST (BLASTn) program of the National Center for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the closest genera.

2.4. Species identification by the VITEK 2 system

Strains were identified to the species level by the GN card of the VITEK 2 system version 07.01 (bioMérieux, Inc., Durham, NC, USA).

2.5. Detection and sequence analysis of virulence genes

Genomic DNA of each strain was extracted as described above and used as a template DNA for PCR to detect the presence of 22 virulence genes listed in Table 1. The rationale for selecting these virulence genes was based on their prevalence in the identified bacterial genera and their potential impact on human and animal health. The presence and sizes of the amplified fragments were determined as described above. The PCR products were purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and subsequently sequenced by Bio Basic, Inc. (Markham, Ontario, Canada). The nucleotide sequences were aligned with reference sequences using the BLASTn program of NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.6. Factors affecting the expression of virulence genes

Representative strains harboring virulence genes were selected to monitor the expression of virulence genes by a reverse-transcription PCR (RT-PCR) assay. The factors that we examined were as follows: 1) concentrations of NaCl (0 M, 0.17 M, and 0.3 M); 2) nutrient enrichment [LB medium and minimal M63 medium (Skyberg et al., 2007)]; 3) aeration conditions (microaerophilic and aerobic conditions); 4) temperatures (25°C and 37°C); and 5) growth phases [early exponential phase (6 h of growth), mid-exponential phase (12 h of growth)], and stationary phase (16 h of growth). Overnight cultures were subcultured by inoculating 1/100 volumes into LB broth without NaCl. Subcultures were grown at 37°C without aeration for 12–16 h and then inoculated into either LB broth or M63 broth. Cultures were grown under the tested conditions. The 10 conditions investigated were as follows: 1) LB broth without NaCl under the microaerophilic condition at 37°C for 12 h; 2) LB broth with 0.17 M NaCl under the microaerophilic condition at 37°C for 12 h; 3) LB broth with 0.3 M NaCl under the microaerophilic condition at 37°C for 12 h; 4) M63 broth without NaCl under the microaerophilic condition at 37°C for 12 h; 5) M63 broth with 0.17 M NaCl under the microaerophilic condition at 37°C for 12 h; 6) M63 broth with 0.3 M NaCl under the microaerophilic condition at 37°C for 12 h; 7) LB broth with 0.17 M NaCl under the aerobic condition at 37°C for 12 h; 8) LB broth with 0.17 M NaCl under the microaerophilic condition at 25°C for 12 h; 9) LB broth with 0.17 M NaCl under the microaerophilic condition at 37°C for 6 h; and 10) LB broth with 0.17 M NaCl under the microaerophilic condition at 37°C for 16 h. After cultivation, total RNA was extracted from cell pellets using a ZR Fungal/Bacterial RNA Miniprep. kit (Zymo Research Corporation, Irvine, CA, USA) and immediately used as a template DNA for RT-PCR. RT-PCR was performed using a SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and pairs of primers specific to each virulence gene. The PCR mixtures were prepared according to the manufacturer's instructions. Negative controls (DNase, RNase free ultrapure distilled water) and positive controls (PCR products of each gene) were included in all sets of reactions. The presence and sizes of the synthesized cDNA were determined by agarose (1% in TBE buffer) gel electrophoresis as described above.

2.7. Statistical analysis

Experiments were conducted in triplicate. Means and standard deviations were calculated by the Excel program version 16.0.

3. Results and discussion

3.1. Prevalence of presumptive target bacteria

Among 35 seafood samples, the numbers of presumptive *E. coli*, *Salmonella-Shigella*, and *Vibrio* were above the detection limit (1.2 log CFU/g) in seven (20.0%), 13 (37.1%), and 10 (28.6%) samples, respectively. The numbers of presumptive *E. coli*, *Salmonella-Shigella*, and *Vibrio* per sample that were above the detection limit ranged from 1.5 ± 1.1 to 1.9 ± 0.9 , from 2.3 ± 1.3 to 2.4 ± 1.3 , and from 1.2 ± 0.6 to 2.2 ± 1.5 log CFU/g, respectively. The average numbers of presumptive target bacteria per sample of the same seafood species sold in three central seafood markets in Thailand are shown in Table 2. As reported in our previous publication, 89% (31/35) of these seafood samples contained presumptive enterobacteria, as estimated by total plate counts using violet red bile glucose (VRBG) agar. The average numbers of presumptive enterobacteria per sample ranged between 1.3 ± 0.2 and 5.4 ± 0.1 log CFU/g (Pongsilp & Nimnoi, 2018). Forty-two presumptive isolates from three media were selected from different seafood species and collection sites. The isolates were purified and stored. The isolates from EMB agar, SS agar, and TCBS agar were abbreviated as SFEMB, SFSS, and SFTCBS, respectively.

The numbers of these potentially pathogenic bacteria are considered as an important issue for food safety. Among pathogenic bacterial species, the minimum infective doses (MID), which cause diseases, vary considerably. The MIDs are relatively high for different serovars of *Salmonella* ($>10^5$ cells), *E. coli* ($>10^5$ cells), and *Vibrio cholerae* (10^3 – 10^8 cells). By contrast, the MIDs are apparent low for enterohemorrhagic *E. coli* (EHEC) (about 10 cells) and some *Shigella* (less than 10 cells) (Kothary & Babu, 2001; Schmid-Hempel & Frank, 2007).

3.2. ERIC-PCR fingerprinting of presumptive isolates of target bacteria

The 42 presumptive isolates of target bacteria generated 40 distinct ERIC-PCR patterns ranging in number from 1–7 and in size from approximately less than 500–4,000 base pairs (bp). This result suggests that 40 individual strains were obtained. The UPGMA dendrogram constructed from ERIC-PCR patterns of 40 individual strains is presented in Figure 1.

3.3. Identification of the closest genera based on partial 16S rRNA gene sequences

Partial 16S rRNA gene sequences (approximately 500 bp) of 40 presumptive strains were employed for the identification of their closest genera. Alignments of the obtained sequences (GenBank accession numbers MT422214–MT422253) revealed 97.74–99.79% identities to those of potentially pathogenic bacteria in 11 genera including *Aeromonas* (nine strains), *Enterobacter* (seven strains), *Citrobacter* (five strains), *Morganella* (four strains),



Table 1. Targeted virulence genes and related information.

Table 1. Genes de virulencia seleccionados e información relacionada.

Virulence gene	Mechanism	Primer sequence	Size of the amplified fragment (bp)	Bacteria harboring virulence gene	Reference
<i>aerA</i>	synthesis of aerolysin	aerAF (5' AAC CGA ACT CTC CAT) aerAR (5' CGC CTT GTC CTT GTA 3')	301	<i>A. hydrophila</i>	Li et al. (2011)
<i>ahp</i>	synthesis of cytotoxic enterotoxin	ahpF (5' ATT GGA TCC CTG CCT A 3') ahpR (5' GCT AAG CTT GCA TCC G 3')	911		
<i>alt</i>	synthesis of serine protease	altF (5' TGA CCC AGT CCT GG 3') altR (5' GGT GAT CGA TCA CC 3')	442		
<i>csgD</i>	synthesis of curli	csgDF (5' TGA AAR YTG GCC GCA TAT CAA TG 3') csgDR (5' ACG CCT GAG GTT ATC GTT TGC C 3')	355	<i>E. cloacae</i>	Akbari et al. (2015)
<i>cesT/eee</i>	interaction with host cell	cesT/eeeF (5' GTT TGC AGA GAA TGG TGG CCC 3') cesT/eeeR (5' TAG CTT ATG CTT GTG CCG GGT 3')	333	<i>E. coli</i>	Sandner et al. (2001)
<i>espB</i>		espBF (5' GCC GCT CTG ATT GGT GGT GCT 3')	387		
<i>cnf1</i>	synthesis of cytotoxic necrotizing factor	espBR (5' TGG CGT TGA ACC GGA AAT CCT 3') cnf1F (5' AAG ATG GAG TTT CCT ATG CAA GGA G 3') cnf1R (5' CAT TCA GAT CCT GCC CTC ATT ATT 3')	498		Yamamoto et al. (1995)
<i>cnf2</i>		cnf2F (5' GTG AGG CTC AAC GAG ATT ATG CAC TG 3') cnf2R (5' CCA CGC TTC TTC AGT TGT TCC TC 3')	839		Pass et al. (2000)
<i>eeeA</i>	interaction with host cell	eeeAF (5' TGA GCG GCT GGC ATG AGT CAT AC 3')	241		
<i>LTI</i>	synthesis of thermolabile toxin	eeeAR (5' TCG ATC CCC ATC GTC ACC AGA GG 3') LTI F (5' TGG ATT CAT CAT GCA CCA CAA GG 3') LTI R (5' CCA TTT CTC TTT TGC CTG CCA TC 3')	360		
<i>STII</i>		STII F (5' CCC CCT CTC TTT TGC ACT TCT TTC C 3')	423		
<i>vt1</i>	synthesis of verotoxin	STIIR (5' TGC TCC AGC AGT ACC ATC TCT AAC CC 3') vt1F (5' ACG TTA CAG CGT GTT GCR GGG ATC 3')	121		
<i>vt2e</i>		vt1R (5' TTG CCA CAG ACT GCG TCA GTR AGG 3')	322		
<i>kfu</i>	iron uptake system	vt2eF (5' CCA GAA TGT CAG ATA ACT GGC GAC 3') vt2eR (5' GCT GAG CAC TTT GTA ACA ATG GCT G 3')	797	<i>Klebsiella</i>	Aher et al. (2012)
<i>magA</i>	synthesis of capsular polysaccharide	kfuF (5' GAA GTG ACG CTG TTT CTG GC 3')	1,280		
<i>uge</i>	synthesis of lipopolysaccharide	magAR (5' GCA ATG GCC ATT TGC GTT AG 3')	534		
<i>ssaO</i>	type III secretion system apparatus	ugeF (5' TCT TCA CGC CTT CCT TCA CT 3')	378	<i>Salmonella</i>	Bhowmick et al. (2011b)
<i>sscB</i>	synthesis of chaperone	ugeR (5' GAT CAT CCG GTC TCC CTG TA 3')	435		
<i>sseG</i>	secretion system effector	ssaOF (5' ATG GAA ACT TTG CTG GAG A 3') ssaOR (5' TCA ACT TTG GTA ATA CGC AT 3')	690		
<i>tdh</i>	synthesis of thermolabile haemolysin	sscBF (5' ATG ATG ATG AAA GAA GAT CA 3') sscBR (5' TTA AGC AAT AAG AGT ATC AA 3')	269	<i>Vibrio</i>	Panicker et al. (2004)
<i>tth</i>		sseGF (5' ATG AAA CCT GTT AGC CCA AA 3') sseGR (5' TTA CTC CGG CGC ACG TTG TT 3')	450		
<i>trh</i>		tdhF (5' GTA AAG GTC TCT GAC TTT TGG AC 3') tdhR (5' TGG AAT AGA ACC TTC ATC TTC ACC 3')	500		
		tthF (5' AAA GCG GAT TAT GCA GAA GCA CTG 3') tthR (5' GCT ACT TTC TAG CAT TTT CTC TGC 3')			
		trhF (3' TTG GCT TCG ATA TTT TCA GTA TCT 3') trhR (5' CAT AAC AAA CAT ATG CCC ATT TCC G 3')			

Table 2. Average numbers of presumptive target bacteria per sample of the same seafood species sold in Thailand.**Tabla 2.** Número promedio de bacterias presuntamente seleccionadas por muestra de la misma especie de marisco o pescado vendida en Tailandia.

Seafood species		Average number of presumptive target bacteria per sample of the same seafood species (log CFU/g)* (number of positive sample)		
Common name (number of sample)	Scientific name	Presumptive <i>E. coli</i> on EMB agar	Presumptive <i>Salmonella</i> and <i>Shigella</i> on SS agar	Presumptive <i>Vibrio</i> on TCBS agar
Blue swimming crab (6)	<i>Portunus pelagicus</i>	0.7 ± 1.1 (1)	under the detection limit (< 1.2)	0.9 ± 1.1 (2)
Banana shrimp (5)	<i>Fenneropenaeus merguensis</i>	0.9 ± 1.2 (1)	2.0 ± 2.1 (2)	0.8 ± 1.1 (1)
Splendid squid (4)	<i>Loligo formosana</i>	1.1 ± 1.4 (1)	1.7 ± 2.0 (1)	1.3 ± 1.6 (1)
Spotted babylon (3)	<i>Babylonia areolata</i>	under the detection limit	1.9 ± 2.2 (1)	0.8 ± 1.0 (1)
Scallop (3)	<i>Argopecten purpuratus</i>	under the detection limit	1.9 ± 2.1 (1)	under the detection limit
Barramundi (2)	<i>Lates calcarifer</i>	1.5 ± 1.6 (1)	2.3 ± 0.8 (2)	1.5 ± 1.6 (1)
Bigfin reef squid (2)	<i>Sepioteuthis lessoniana</i>	1.8 ± 0.7 (2)	2.3 ± 1.0 (2)	2.1 ± 1.5 (2)
Short-bodied mackerel (2)	<i>Rastrelliger brachysoma</i>	under the detection limit	2.1 ± 2.3 (1)	under the detection limit
Silver pomfret (2)	<i>Pampus argenteus</i>	under the detection limit	under the detection limit	1.5 ± 1.6 (1)
Kuruma prawn (1)	<i>Marsupenaeus japonicus</i>	under the detection limit	under the detection limit	under the detection limit
Blood cockle (1)	<i>Tegillarca granosa</i>	under the detection limit	under the detection limit	under the detection limit
Mangrove crab (1)	<i>Scylla serrata</i>	under the detection limit	under the detection limit	under the detection limit
Cuttle fish (1)	<i>Sepia officinalis</i>	under the detection limit	2.3 ± 1.4 (1)	under the detection limit
John's snapper (1)	<i>Lutjanus johnii</i>	1.8 ± 0.9 (1)	2.4 ± 1.3 (1)	1.7 ± 1.4 (1)
Green tiger prawn (1)	<i>Penaeus semisulcatus</i>	under the detection limit	2.4 ± 1.5 (1)	under the detection limit

*In cases that there were more than one sample of the same seafood species, the values shown are the means of all samples assayed ± standard deviations. In cases that there was only one sample of the same seafood species, the values shown are the means of three replicates ± standard deviations.

*En los casos en que hubo más de una muestra de la misma especie de mariscos o pescados, los valores mostrados son las medias de todas las muestras ensayadas ± desviaciones estándar. En los casos en que sólo hubo una muestra de la misma especie de mariscos o pescado, los valores mostrados son las medias de tres réplicas ± desviaciones estándar.

Klebsiella (three strains), *Vibrio* (three strains), *Escherichia* (two strains), *Hafnia* (two strains), *Proteus* (two strains), *Salmonella* (two strains), and *Providencia* (one strain). The sequences of the gene encoding small-subunit ribosomal RNA (16S rRNA) provide valuable data and the sequence comparisons have been generally employed as a powerful tool for the identification of bacteria to the genus level at a sequence identity threshold of 95% (Pongsilp, 2012; Schloss & Handelsman, 2005). The obtainment of potentially pathogenic bacteria in 11 genera suggests that EMB agar, SS agar, and TCBS agar were not sufficiently specific to isolate only *E. coli*, *Salmonella-Shigella*, and *Vibrio*, respectively. Both *Citrobacter* and *Escherichia* exhibited similar colonial characteristics on EMB agar. Seven genera, including *Aeromonas*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Morganella*, *Proteus*, and *Salmonella*, that formed colonies with similar morphologies, were isolated from SS agar. *Providencia* and *Vibrio* grew with similar characteristics on TCBS agar.

3.4. Species identification based on the VITEK 2 system

The VITEK 2 system was employed for species identification of 40 strains. The VITEK results are consistent with the 16S rRNA gene sequencing results. The VITEK results revealed that the strains had 91–99% probabilities of being members of 17 species including *Aeromonas caviae* (five strains), *Aeromonas enteropelogenes* (two strains), *Aeromonas salmonicida* (one strain), *Aeromonas sobria* (one strain), *Citrobacter braakii* (two strains), *Citrobacter freundii* (two strains), *Citrobacter koseri* (one strain), *Enterobacter cloacae* (five strains), *Enterobacter ludwigii* (two strains), *E. coli* (two strains), *Hafnia alvei* (two strains), *Klebsiella pneumoniae* (three strains), *Morganella morganii* (four strains), *Proteus hauseri* (two strains), *Providencia rustigianii* (one strain), *Salmonella enterica* (two strains), and *Vibrio parahaemolyticus* (three strains).

The 11 bacterial genera identified in this study have been reported as severe pathogens. Their occurrence in seafood indicates poor sanitary quality and therefore represents a risk to public health. Acceptable limits of some of these bacteria

have been regulated by international and national health organizations. For example, the Ministry of Public Health, Thailand, has announced microbiological standards for seafood. *Salmonella* spp., *V. cholerae*, and *V. parahaemolyticus* shall not be detected in 25 g while *E. coli* must not exceed 3 CFU/g. Previous studies have reported the contamination of seafood by some of these bacteria. The highest prevalence of *Aeromonas hydrophila* was observed in fish (19.5%), followed by shrimp (9.2%), lobster (9.3%), and crab (6.7%) caught off the south coast of Iran, in which 57 strains out of 62 (91.9%) harbored the cytolytic enterotoxin gene (Rahimi et al., 2014). The prevalence of *Citrobacter* (45.56%), *Proteus* (31.79%), *E. coli* (7.64%), *Shigella* (7.64%), *Salmonella* (3.35%), *Klebsiella* (2.44%), and *Enterobacter* (1.52%) in horse mackerel (*Trachurus trachurus*) sold in Istanbul, Turkey was annually recorded (Tosun et al., 2016). All 20 seafood samples from landing centers and retail markets in Mumbai, India were contaminated with fecal coliforms ranging in number from 150 to 1.1×10^3 CFU/100 g. Among 329 isolates of *E. coli*, 175 isolates (53.19%) harbored virulence genes such as Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), intimin (*eaeA*), and hemolysin (*hlyA*) (Prabhakar et al., 2017). The prevalence of Shiga toxin-producing *E. coli* (STEC) in seafood from the Americas region, the European region, and the Western Pacific region was 0.42%, 1.70%, and 0.00%, respectively [WHO & FAO, 2018]. The prevalence of *Vibrio* in 160 retail seafood samples in Berlin, Germany was 55% (Vu et al., 2018). *Vibrio* was characterized as one of the top 10 most abundant genera among 4,953 operational taxonomic units (OTUs) derived from seawater in nine sampling sites in the upper gulf of Thailand (Nimnoi & Pongsilp, 2020).

3.5. Presence of virulence genes among strains

Detection of the 22 virulence genes by PCR revealed the presence of eight genes including *cnf2*, *csgD*, *kfu*, *LTI*, *ssaO*, *tlh*, *uge*, and *vt2e*. The numbers of positive strain in each genus or species that possessed at least one virulence gene are

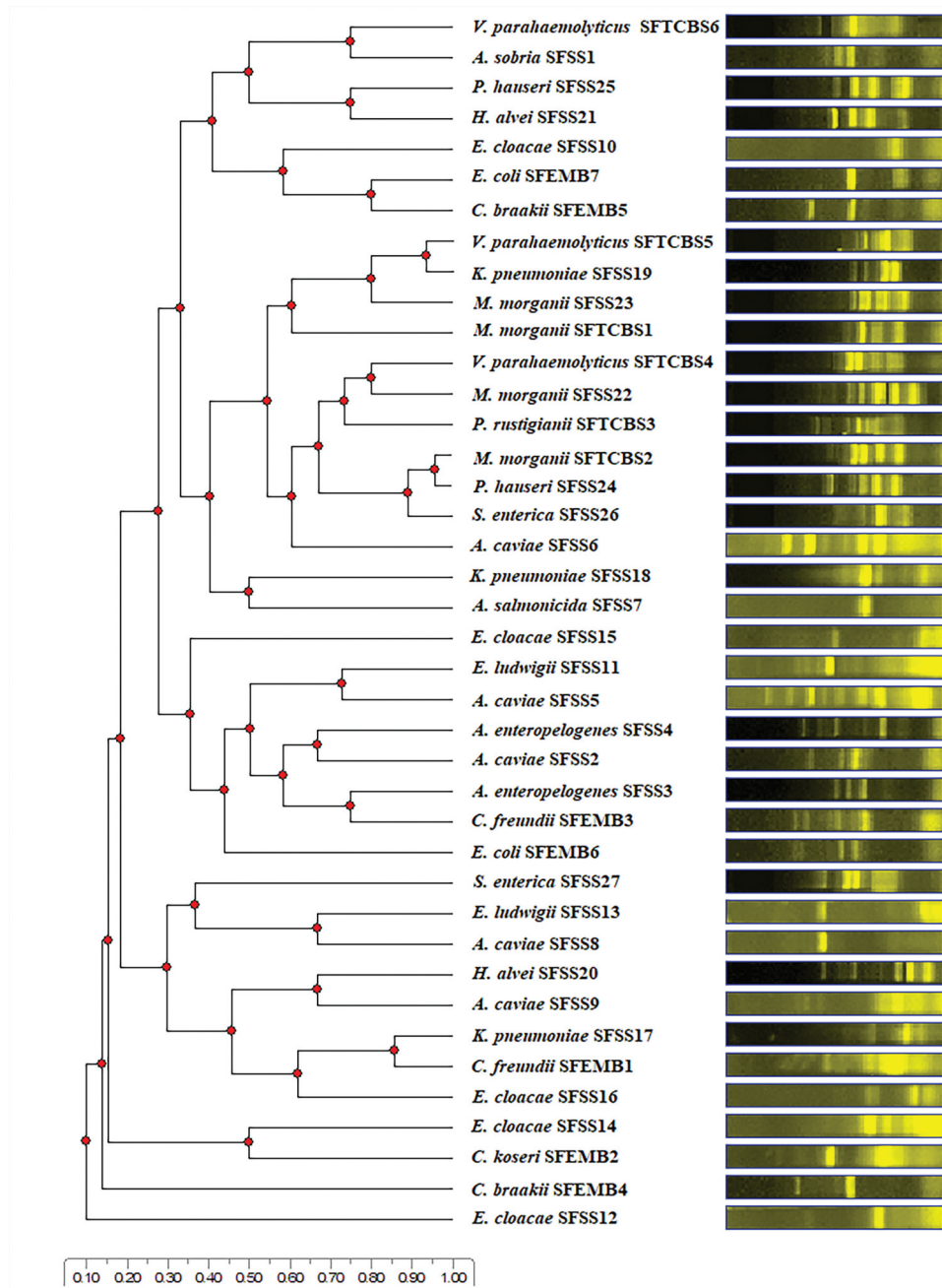


Figure 1. The UPGMA dendrogram constructed from ERIC-PCR patterns of 40 individual strains.

Figura 1. Dendrograma UPGMA construido a partir de patrones ERIC-PCR de 40 cepas individuales.

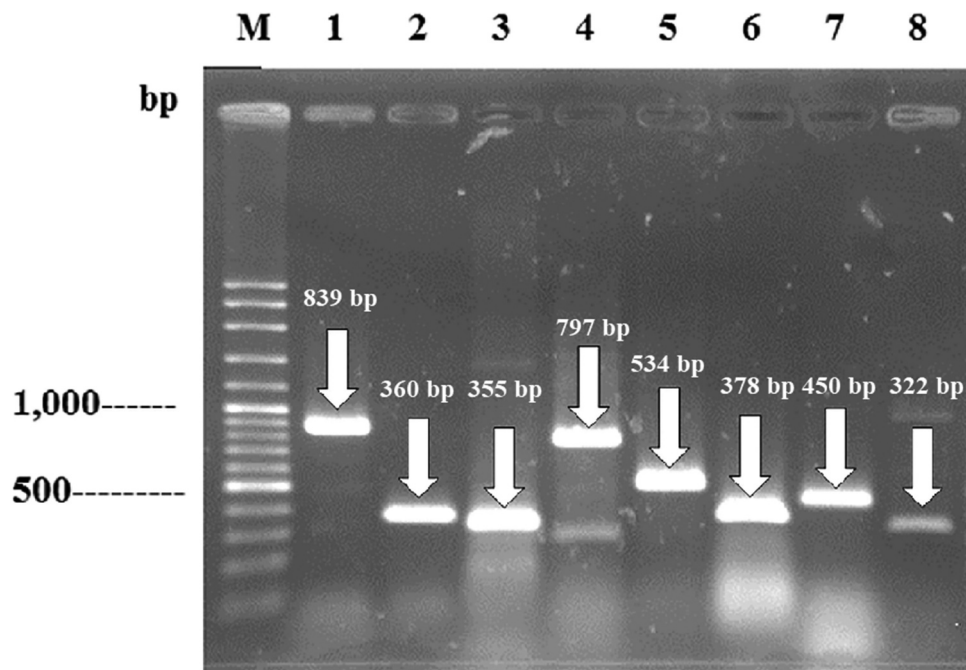
presented in Table 3. The PCR products of the detected virulence genes are shown in Figure 2. Sequencing of PCR-amplified fragments from a representative strain of each species (a total of 11 strains) reinforced the presence of these genes based on 82.82–100% identities to the reference sequences. The 17 sequences of virulence genes were deposited in NCBI under GenBank accession numbers MT483947–MT483963. Table 4 presents the detected virulence genes and their % identities to the reference sequences. Bacteria that have been previously reported to harbor these virulence genes are listed in Table 1. In this study, 24 strains out of 40 (60.0%) possessed at least one virulence gene. The *cnf2* and *vt2e* genes were present in both strains of *E. coli*. The *csgD* gene was distributed among six species of three genera including *Citrobacter* (all five strains of *C. braakii*, *C. freundii*, and *C. koseri*), *Enterobacter* (all seven strains of *E. cloacae* and *E. ludwigii*), and *K. pneumoniae* (two strains out of three). Three species

including *C. braakii* (one strain out of two), *E. coli* (both strains), and *H. alvei* (both strains) harbored the *LTI* gene. *E. cloacae* (three strains out of five), *E. ludwigii* (one strain out of two), and *K. pneumoniae* (one strain out of three) harbored the *uge* gene. While the *kfu*, *ssaO*, and *tth* genes were confined to *K. pneumoniae* (one strain out of three), *S. enterica* (both strains), and *V. parahaemolyticus* (all three strains), respectively. The cooccurrence of three virulence genes, including the *cnf2*, *LTI*, and *vt2e*, was found in both strains of *E. coli*. The existence of virulence genes provides a proper mean of risk assessment of potentially pathogenic bacteria.

Previous studies have reported the impact of these detected virulence genes on disease progression and their presence in potentially pathogenic bacteria associated with seafood. The *cnf2* gene was associated with severe dysenteric syndromes caused by necrotic *E. coli* (NTEC). The *csgD* gene has been reported to have a great impact on

Table 3. The number of strain in each genus or species that possessed virulence gene.**Tabla 3.** Número de cepas de cada género o especie que poseen el gen de virulencia.

Genus/Species	Number of strain	Number of strain possessing virulence gene							
		<i>cnf2</i>	<i>csgD</i>	<i>kfu</i>	<i>LTI</i>	<i>ssaO</i>	<i>tlh</i>	<i>uge</i>	<i>vt2e</i>
<i>Aeromonas</i> spp.	9	0	0	0	0	0	0	0	0
<i>Citrobacter</i> spp.	5	0	5	0	1	0	0	0	0
<i>Enterobacter</i> spp.	7	0	7	0	0	0	0	0	0
<i>E. coli</i>	2	2	0	0	2	0	0	0	2
<i>H. alvei</i>	2	0	0	0	2	0	0	0	0
<i>K. pneumoniae</i>	3	0	2	1	0	0	0	1	0
<i>M. morgani</i>	4	0	0	0	0	0	0	0	0
<i>P. hauseri</i>	2	0	0	0	0	0	0	0	0
<i>P. rustgiani</i>	1	0	0	0	0	0	0	0	0
<i>S. enterica</i>	2	0	0	0	0	2	0	0	0
<i>V. parahaemolyticus</i>	3	0	0	0	0	0	3	0	0
Total	40	2 (5.0%)	14(35.0%)	1 (2.5%)	5 (12.5%)	2(5.0%)	3(7.5%)	5 (12.5%)	2(5.0%)

**Figure 2.** The PCR products of the detected virulence genes.**Figura 2.** Productos de la PCR de los genes de virulencia detectados.

Lane M: 100 bp DNA ladder.; 1: 839-bp PCR product of the *cnf2* gene; 2: 360-bp PCR product of the *kfu* gene; 3: 355-bp PCR product of the *csgD* gene; 4: 797-bp PCR product of the *LTI* gene; 5: 534-bp PCR product of the *uge* gene; 6: 378-bp PCR product of the *ssaO* gene; 7: 450-bp PCR product of the *tlh* gene; 8: 322-bp PCR product of the *vt2e* gene. Arrows indicate PCR products of virulence genes.

Carril M: escalera de ADN de 100 pb [par de bases]. 1: 839 pb producto de PCR del gen *cnf2*; 2: 360 pb producto de PCR del gen *LTI*; 3: 355 pb producto de PCR del gen *csgD*; 4: 797 pb producto de PCR del gen *kfu*; 5: 534 pb producto de PCR del gen *uge*; 6: 378 pb producto de PCR del gen *ssaO*; 7: 450 pb producto de PCR del gen *tlh*; 8: 322 pb producto de PCR del gen *vt2e*. Las flechas indican los productos de la PCR de los genes de virulencia.

the natural lifestyle of *Salmonella* such as the *rdar* morphotype that is correlated with invasion of the intestinal epithelial cells (Mackenzie et al., 2019). The *csgD* gene was detected in all 14 isolates of seafood-associated *S. enterica* serovar Weltevreden (Bhowmick et al., 2011a). The *kfu* and *uge* genes have been reported as ones of the important virulence genes in invasive *K. pneumoniae* strains which cause mastitis (Osman et al., 2014). The *LTI* gene has been reported as a signature gene responsible for the virulence of enterotoxigenic *E. coli* (ETEC) which causes diarrhea (Tomar et al., 2015). The *vt2e* gene was associated with edema disease caused by *E. coli* (Mallorquí et al., 2018). The *ssaO* gene was involved in systematic virulence of *Salmonella* in a mouse typhoid fever model. The binding of EIIA^{Glc} protein to type three secretion system 2 (TTSS-2), which also included SSaO protein, switched *Salmonella* from growth arrest to acute virulence through activation of virulence

factor secretion (Maze et al., 2014). The *ssaO* gene was detected in all 57 seafood-associated serovars of *Salmonella* (Bhowmick et al., 2011b). The *tlh* gene encodes thermolabile hemolysin (TLH) which causes the lysis of red blood cells and therefore contributes to pathogenicity (Praja & Safnurbati, 2018). Although the *tlh* gene is considered species-specific for *V. parahaemolyticus*, it is also widespread in other vibrios including *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio diabolicus*, *Vibrio fischeri*, *Vibrio harveyi*, *Vibrio mimicus*, *Vibrio natriegens*, *Vibrio proteolyticus*, and *Vibrio vulnificus* (Wang et al., 2007; Xie et al., 2005; Yanez et al., 2015). The *tlh* gene was employed for molecular confirmation of 22 *V. parahaemolyticus* isolates from seafood in the south-west coast of India (Meparambu Prabhakaran et al., 2020) and 104 *V. parahaemolyticus* isolates from seafood in Polish market (Lopatek et al., 2018). The association of virulence genes with outbreaks caused by *V. parahaemolyticus*

Table 4. Detected virulence genes and % identities to the reference sequences.**Tabla 4.** Genes de virulencia detectados y porcentaje de identidades respecto a las secuencias de referencia.

Detected virulence gene	A representative strain of each species that harbored virulence gene	% identity to the reference sequence	GenBank accession number of the reference sequence
<i>cnf2</i>	<i>E. coli</i> SFEMB6	99.15%	U01097
	<i>C. braakii</i> SFEMB5	99.68%	CP010376
	<i>C. freundii</i> SFEMB1	97.41%	CP010376
	<i>C. koseri</i> SFEMB2	96.66%	CP024680
	<i>E. cloacae</i> SFSS14	100%	CP010376
	<i>E. ludwigii</i> SFSS13	100%	CP001918
<i>csgD</i>	<i>K. pneumoniae</i> SFSS17	99.02%	CP024675
	<i>K. pneumoniae</i> SFSS17	99.20%	AP018753
<i>kfu</i>	<i>C. braakii</i> SFEMB5	99.71%	EU113255
	<i>E. coli</i> SFEMB6		
<i>LTI</i>	<i>H. alvei</i> SFSS20		
	<i>S. enterica</i> SFSS26	99.17%	CP043662
<i>ssaO</i>	<i>V. parahaemolyticus</i> SFTCBS4	100%	CP006007
<i>tlh</i>	<i>E. cloacae</i> SFSS14	82.82 to 98.38%	CP001891
	<i>E. ludwigii</i> SFSS13		
	<i>K. pneumoniae</i> SFSS19		
	<i>E. coli</i> SFEMB6		
<i>ugt</i>			
<i>vt2e</i>		98.03%	FM998849

Strain names refer to kinds of media used for isolation of the strains. SFEMB: EMB agar; SFSS: SS agar; SFTCBS: TCBS agar.

Los nombres de las cepas hacen referencia a los tipos de medios utilizados para su aislamiento. SFEMB: agar EMB; SFSS: agar SS; SFTCBS: agar TCBS.

Table 5. Expression of virulence genes under the desired conditions.**Tabla 5.** Expresión de los genes de virulencia en las condiciones deseadas.

Condition of cultivation	Gene expression							
	<i>cnf2</i>	<i>csgD</i>	<i>kfu</i>	<i>LTI</i>	<i>ssaO</i>	<i>tlh</i>	<i>ugt</i>	<i>vt2e</i>
1. LB without NaCl, 37°C, microaerophilic, 12 h	-	-	+	-	-	+	-	-
2. LB with 0.17 M NaCl, 37°C, microaerophilic, 12 h	-	-	+	-	-	+	+	-
3. LB with 0.3 M NaCl, 37°C, microaerophilic, 12 h	-	-	+	-	-	+	+	-
4. M63 without NaCl, 37°C, microaerophilic, 12 h	-	+	-	-	-	+	-	-
5. M63 with 0.17 M NaCl, 37°C, microaerophilic, 12 h	-	-	-	-	-	+	-	-
6. M63 with 0.3 M NaCl, 37°C, microaerophilic, 12 h	-	-	-	-	-	+	-	-
7. LB with 0.17 M NaCl, 37°C, aerobic, 12 h	-	-	+	-	-	+	-	-
8. LB with 0.17 M NaCl, 25°C, microaerophilic, 12 h	-	-	+	-	-	+	-	-
9. LB with 0.17 M NaCl, 37°C, microaerophilic, 6 h	-	-	-	-	-	+	-	-
10. LB with 0.17 M NaCl, 37°C, microaerophilic, 16 h	-	-	+	-	-	+	-	-

+: gene was expressed; -: gene was not expressed.

+: el gen se expresó; -: el gen no se expresó.

has been proposed. The *tdh* and *trh* genes are considered to be connected to the ability to cause illness. The considerable difference in genetic marker distribution between clinical and environmental strains of *V. parahaemolyticus* in Thailand during 1998–1999 has been reported. Either or both of the *tdh* and *trh* genes were found in 92.78% of the clinical strains, whereas they were found in only 0.68% of the environmental strains (FAO & WHO, 2020).

3.6. Factors influencing the expression of virulence genes

To determine the influence of factors on the expression of virulence genes, six representative strains of different genera that harbored most of the virulence genes were selected. The representative strains were as follows: 1) *C. braakii* SFEMB5 that harbored the *csgD* and *LTI* genes; 2) *E. ludwigii* SFSS13 that harbored the *csgD* and *ugt* genes; 3) *E. coli* SFEMB6 that harbored the *cnf2*, *LTI*, and *vt2e* genes; 4) *K. pneumoniae* SFSS17 that harbored the *csgD* and *kfu* genes; 5) *S. enterica* SFSS26 that

harbored the *ssaO* gene; and 6) *V. parahaemolyticus* SFTCBS4 that harbored the *tlh* gene. The cDNA PCR products synthesized from total RNA from cells grown under varied conditions would represent gene expression. Table 5 presents the expression of virulence genes under the desired conditions. Among the 10 conditions tested, the *tlh* gene was expressed under all conditions, which, contrary to the *cnf2*, *LTI*, *ssaO*, and *vt2e* genes. The remaining three genes (*csgD*, *kfu*, and *ugt*) were expressed under some conditions. The *csgD*-inducing condition was growth in M63 medium without NaCl, suggesting that nutrient and NaCl deprivation had a positive effect on the expression of the *csgD* gene. The *kfu* gene was expressed under six conditions in which the strains were grown in LB medium but was not expressed under all three conditions in which the strains were grown in M63 medium. Additionally, the expression of the *kfu* gene occurred when cells were in mid-exponential and stationary growth phases. Therefore, the *kfu*-inducing condition was growth during mid-exponential and stationary phases in the enriched medium. While concentrations of NaCl (0 M, 0.17 M, and 0.3 M), aeration conditions (microaerophilic and aerobic conditions), temperatures (25°C and 37°C) had no effect on the expression of the *kfu* gene. The *ugt* gene was expressed under two conditions. The factors influencing its expression included NaCl, nutrient enrichment, aeration condition, temperature, and growth phase. The *ugt*-inducing condition was growth during the mid-exponential phase in enriched medium containing 0.17 M or 0.3 M NaCl under the microaerophilic condition at 37°C.

Previous studies have reported the factors stimulating the expression of virulence genes. Low temperature (9°C) had both negative and positive effects on the expression of the *tlh* gene in different strains of *V. parahaemolyticus*, indicating the expression was irregular depending on variations in environmental conditions (Zhao et al., 2015). The *csgD* gene was expressed under different environmental conditions such as growth at low temperature (<32°C), low osmolarity, and starvation (Gerstel & Romling, 2001). The expression of the *csgD* gene was low in the exponential growth phase but increased in the stationary growth phase (Ogasawara et al., 2010). The osmotic agents (NaCl and sucrose) had a negative effect on the expression of the *csgD* gene (Jubelin et al., 2005; Prigent-Combaret et al., 2001; Zhou et al., 2013). Our finding supports the previous reports that nutrient and NaCl deprivation

stimulate the expression of the *csgD* gene. In this study, the expression of the *csgD* gene could occur during growth at 37°C when cells were grown under nutrient and NaCl deprivation conditions. The *kfu*-inducing and *uge*-inducing condition of pathogenic bacteria has not been previously described. In addition, it has been reported that environmental conditions trigger virulence gene expression and therefore contribute to the development of the disease. For example, an increase to 37°C and a depletion of extracellular iron are universal invasion signals to the bacteria and enable fine-tuning of virulence factor expression, resulting in the promotion of survival and proliferation of bacteria within their hosts (Lam et al., 2014).

4. Conclusion

This study underlines a potential risk of seafood-associated bacteria. Seafood was found to harbor diverse groups of potentially pathogenic bacteria, of which a large proportion belonged to enterobacteria. Among eight detected virulence genes, the *csgD* gene was mostly prevalent. The expression of four virulence genes occurred under the possible environmental conditions, especially the *tlh* gene of *V. parahaemolyticus*, which was expressed under broad conditions. This may raise more concerns about public health. Nevertheless, *V. parahaemolyticus* strains found in this study still remained a characteristic of environmental strains as they lacked the *tdh* and *trh* genes which are virulence marker genes for clinical strains.

Disclosure statement

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