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RESEARCH ARTICLE

Development of enhanced selective media for detection of *Vibrio parahaemolyticus* in oysters

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Abstract This study was undertaken to develop enhanced selective media for detection of *Vibrio parahaemolyticus* in oysters. Primarily, tryptic soy agar (TSA) was supplemented with 4.5–5% NaCl, 0.1–0.5% oxgall, and/or 1–2% sodium citrate, and adjusted to pH 8–9. A total of 21 *Vibrio* spp., 24 indicators, and 26 food-borne isolates were streaked on the modified media, followed by 24 h of incubation at 37 °C. While all the indicators and isolates failed to grow on TSA containing 5% NaCl, 0.5% oxgall, and 2% sodium citrate (TSA_{OSS1}; pH 9), *V. parahaemolyticus* was culturable on this selective medium. Particularly, the ability of TSA_{OSS1} to quantify *V. parahaemolyticus* in oysters was superior to thiosulphate citrate bile salts sucrose (TCBS) agar. *V. parahaemolyticus* distinctly produced its white-yellowish, round, and edge-pointed colony on TSA_{OSS1}. $TSAO_{SS1}$ with high selectivity potentials over TCBS may be a promising alternative for detection of *V. parahaemolyticus* in seafoods or natural reservoirs.

Keywords Detection · Food safety · Seafood · Selective medium · *Vibrio parahaemolyticus*

Introduction

Vibrio parahaemolyticus has been recognized as one of the leading pathogens associated with food-borne diseases and illnesses globally. *V. parahaemolyticus* is commonly found in marine estuarine ecosystems and readily isolated from a wide variety of marine products, including clam, eel, mackerel, octopus, oyster, salmon, shrimp, and squid (Chen et al., 2017; Xu et al., 2014; Yu et al., 2013). Consumption of raw or undercooked marine products contaminated with pathogenic *V. parahaemolyticus* may result in a significant manifestation of clinical symptoms ranging from abdominal pain, diarrhea, nausea, and vomiting to septicemia (Alipour et al., 2014; Nair et al., 2007; Su and Liu, 2007). Particularly, *V. parahaemolyticus* is

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characterized to express several virulence determinants, such as thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), and some effector proteins produced via type III secretion system (T3TSS), involved in severe infectious potentials, thereby threatening public health-hygiene concerns (Baffone et al., 2005; Vieira et al., 2011).

Standard bacteriological methods are widely used to investigate the abundance and incidence of *V. parahaemolyticus* in raw or processed seafood commodities (Korean Ministry of Food and Drug Safety, 2017; The International Organization for Standardization, 2017; U.S. Food and Drug Administration, 2004). The laboratory methodologies possess some stepwise procedures, including enrichment, isolation, enumeration, screening, and confirmation. Once serial diluents of food homogenates re-suspended in either phosphate buffered saline (PBS) or alkaline peptone water (APW) are enriched overnight at 35 °C, the bacterial aliquots are spread on selected culture media such as thiosulphate citrate bile salt sucrose (TCBS) and modified colistin polymyxin B cellobiose (mCPC) agars for enumerating viable numbers of *V. parahaemolyticus*. Suspect or presumptive colonies of *V. parahaemolyticus* can be further confirmed by appropriate biochemical and/or molecular tests. Alternatively, some studies employed not only most probable number (MPN), but also hydrophobic grid membrane filtration (HGMF) methods for estimating the actual number of pathogenic *V. parahaemolyticus* strains or recovering these bacteria from estuarine seawaters (Alam et al., 2001; Gooch et al., 2001; Hagen et al., 1994). Nevertheless, there have been no gold standard models for accurate detection of *V. parahaemolyticus* in natural environments or food products and estimation of its health impact most likely due to the limited information about highly selective bacteriological media for pathogenic *V. parahaemolyticus* strains (Dickinson et al., 2013; Pinto et al., 2011). Essentially, such a detection platform for *V. parahaemolyticus* is preferentially based on the utilization of differential-selective culture media due to their easy-to-use and economic feasibility (Duan et al., 2006; Nigro and Steward, 2015). However, previous studies emphasized some limitations of the commercially purchased medium used for detection of *V. parahaemolyticus* in environments and foodstuffs (Banerjee and Farber, 2017; Kriem et al., 2015; Pinto et al., 2011; Rosec et al., 2012; Yoon et al., 2017). In a study of Pinto et al., (2008), TCBS showed 36 false positive isolates of *V. parahaemolyticus* out of fifty shell fishes. While *V. parahaemolyticus* was detected from 97 out of 300 oysters by real time quantitative polymerase chain reaction (qPCR), TCBS yielded 78 positive results of *V. parahaemolyticus*; however, more than 19 isolates were identified as false negative (Cai et al., 2006). A total of 320 colonies were isolated from estuarine environments along Neuse and Pamlico Rivers in North Carolina using TCBS, and 125 (39%) colonies were identified as non-*Vibrio* spp. (Prefer and Oliver, 2003).

As the growth of *V. parahaemolyticus* is very sensitive to several extrinsic factors, such as the acidity and osmolarity of menstruum, nutrient-availability, and temperature, the ecological modification of this bacterium in response to various environmental conditions can significantly affect its detectability (Blanco-Abad et al., 2009; Yoon et al., 2016). Indeed, the detectability of *V. parahaemolyticus* tends to become concentrated during warming season, when the incidence of disease and illness is the highest. An increase in the plankton abundance and estuarine surface water temperature significantly correlated the prevalence of specific gene clusters such as *trh*, *tdh*, and *ORF8* in *V. parahaemolyticus* (Turner et al., 2014). In contrast, *V. parahaemolyticus* was seldom detected during cold weather (Alam et al., 2001). *V. parahaemolyticus* and *Vibrio vulnificus* account for a significant proportion of food-borne outbreaks associated with seafoods, mainly responsible for sporadic gastroenteritis symptoms (Kriem et al., 2015). Hence, there is a strong need for the establishment of specific screening strategies to detect pathogenic *V. parahaemolyticus* in marine products and further prevent human infections involved in *Vibrio* spp. As the true incidence of *V. parahaemolyticus* might be underestimated significantly by its complex interaction with environmental surroundings, enhanced microbiological diagnosis tools for monitoring *V. parahaemolyticus* should be necessarily established to reduce a food safety hazard posed by pathogenic *V. parahaemolyticus*. The development of an accurate and efficient selective medium for evaluating the distribution of *V. parahaemolyticus* in food products may be of great value to ensure public health hygiene practices in food service industries. Therefore, this study was undertaken to develop enhanced selective media for detecting and identifying *V. parahaemolyticus* in raw oysters.

Materials and methods

Preparation of bacterial inoculums

A total of 21 *Vibrio* spp., such as *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 33844, *V. parahaemolyticus* ATCC 27969, *V. vulnificus* ATCC 27562, *V. vulnificus* ATCC 33815, *V. alginolyticus* ATCC 17749, *V. alginolyticus* ATCC 40829, *V. carchariae* ATCC 35084, *V. fluvialis* ATCC 33809, *V. furnissii* ATCC 35016, *V. gazogenes* ATCC 29988, *V. harveyi* ATCC 14126, *V. hollisae* ATCC 33564, *V. liquefaciens* ATCC 17058, *V. marinagilis* ATCC 14398, *V. marinovulgaris* ATCC

Table 1 Evaluation of the ability of *V. parahaemolyticus* and indicators to grow on TSA containing 0.5–5% NaCl (pH 7–9) at 37 °C for 24 h

Bacterium	ATCC number	NaCl concentration (pH 7)			NaCl concentration (pH 8)			NaCl concentration (pH 9)		
		0.5%	3%	5%	0.5%	3%	5%	0.5%	3%	5%
Vibrio parahaemolyticus	27969	+	+	+	+	+	+	+	+	+
Vibrio parahaemolyticus	33844	+	+	+	+	+	+	+	+	+
Number of positive strains		2	2	2	2	2	2	2	2	2
Acinetobacter calcoaceticus	ND[a]	+	+	+	+	+	+	+	+	+
Chryseobacterium balustinum	ND	+	−	−	+	−	−	−	−	−
Citrobacter freundii	8090	+	+	+	+	+	+	+	+	−
Cronobacter sakazakii	12868	+	+	+	+	+	+	+	+	+
Dickeya zeae	ND	+	+	+	+	−	−	+	−	−
Escherichia coli	25922	+	+	+	+	+	+	+	+	−
Escherichia coli O157:H7	35150	+	+	+	+	+	+	+	+	−
Hafnia alvei	29927	+	+	+	+	+	−	+	+	−
Klebsiella pneumoniae	13883	+	+	+	+	+	+	+	+	−
Pantoea agglomerans	ND	+	+	+	+	+	+	+	−	−
Pseudomonas aeruginosa	15692	+	+	+	+	+	+	+	+	−
Salmonella Typhimurium	19586	+	+	+	+	+	−	+	+	−
Salmonella Enteritidis	13076	+	+	+	+	+	+	+	+	+
Stenotrophomonas maltophilia	ND	+	+	+	+	+	+	+	+	+
Yersinia enterocolitica	55075	+	+	−	+	−	−	+	−	−
Bacillus cereus	10876	+	+	+	+	+	+	+	+	+
Clavibacter michiganensis	ND	+	+	+	+	+	+	+	+	+
Enterococcus faecalis	19433	+	+	+	+	+	+	+	+	+
Lactococcus lactics	11454	+	+	+	+	+	+	+	−	−
Leuconostoc mesenteroides	27258	+	+	+	+	+	+	+	+	+
Listeria monocytogenes	19115	+	+	+	+	+	+	+	+	−
Pediococcus acidilactici	33314	+	+	+	+	+	−	+	+	−
Staphylococcus aureus	12600	+	+	+	+	+	+	+	+	+
Weissella confusa	10881	+	+	+	+	+	+	+	+	+
Number of positive strains		24	23	22	24	21	18	23	19	10

[a]Not determined

14394, *V. metschnikovii* ATCC 700040, *V. mimicus* ATCC 33653, *V. natriegens* ATCC 14048, *V. nereis* ATCC 25917, and *V. proteolyticus* ATCC 15338, were purchased from the Korean Culture Center of Microorganisms (Seoul-si, Republic of Korea). Bacterial stocks were maintained at − 75 °C and activated in tryptic soy broth (TSB; Difco® Laboratories Inc., Detroit, MI, USA) supplemented with 3% NaCl, following 24 h of incubation at 37 °C.

As shown in Table 1, both 26 indicators and 24 food-borne isolates used in this study were obtained from the bacterial collections of Chung-Ang University (Anseong-si, Republic of Korea). Preliminarily, these bacteria were analyzed with 16S rRNA sequencing by SolGent (SolGent Co., Ltd., Yuseong-gu, Daejeon-si, Republic of Korea) and confirmed. Individually, all bacterial stocks were maintained at − 75 °C and cultured in TSB at 37 °C overnight.

Effect of individual respective selective agents on the growth of *V. parahaemolyticus*

Commercially purchased culture media, such as TCBS (Difco®), mCPC (HiMedia® Laboratories, Bombay, India), cellobiose-colistin (CC; HiMedia®), and sodium dodecyl sulphate polymyxin sucrose agar (SDS; HiMedia®), developed for detection of *V. parahaemolyticus* were prepared according to the instructions provided by manufacturers. As stated above, the overnight cultures were streaked onto these media using by a sterile 1-mm-volume loop and were incubated at 37 °C for 24–48 h.

Tryptic soy agar (TSA; Difco®) was supplemented with 5 g/L oxgall (Difco®), 2 g/L X-gall (Difco®), 3 g/L sodium cholate (Sigma-Aldrich Co., St Louis, MO, USA), 5 g/L sodium taurocholate (Sigma-Aldrich), and 10 g/L cellobiose (Sigma-Aldrich), respectively. Each of the bacterial cultures grown overnight was streaked onto these media using a sterile 1-mm-volume loop, followed by 24 h of incubation at 37 °C.

Formulation of enhanced selective media

Microbiological culture media, such as brain heart infusion (BHI; Difco®) agar, Luria–Bertani (LB; Oxoid Ltd., Basingstoke, UK) agar, and nutrient (NA; Oxoid) agar, were prepared with some modifications by adding 1–10% NaCl and/or 0.1–0.5% oxgall, and adjusted to pH 8–9 using membrane-filtered 1 mol NaOH solution (Kanto chemical, Tokyo, Japan). Furthermore, either TSA or BHI was supplemented with 1–5% NaCl, 0.1–0.5% oxgall, and 1–5% sodium citrate (Sigma-Aldrich), and adjusted to pH 8–9. The overnight culture of *V. parahaemolyticus* and non-Vibrio spp. (indicators and food-borne isolates) was streaked on these media using a sterile 1-mm-volume loop, and all agar plates were incubated at 37 °C for 24–48 h.

Practical food application of the formulated selective media

Raw oyster, mussel, and mackerel were purchased from a market in Anseong-si, the Republic of Korea. Marine products were cut lengthwise with a sterile knife, and the slices weighing to 25 g were inoculated by the overnight culture of *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 27969, and *V. parahaemolyticus* ATCC 33844 individually. After 1–2 h of drying in a laminar flow biosafety hood at 25 °C, each of the slices was transferred to a stomacher bag containing 225 mL of APW and homogenized in a BagMixer® 400 stomacher (INTERSCIENCE, Saint-Nom-la-Breteche, France) for 2 min. The bacterial fluids were serially ten-fold diluted in APW, followed by plating on the formulated selective media. After 1–2 days of incubation at 37 °C, the presumptive or suspect colonies of *V. parahaemolyticus* from the formulated selective media were enumerated and further confirmed via polymerase chain reaction (PCR) analyses.

PCR assay

TSB containing 3% NaCl was inoculated by a single colony obtained from the formulated selective medium, followed by 24 h of incubation at 37 °C. After centrifugation at 12,000 × *g* for 3 min, the overnight culture (1 mL) was washed twice with 1 mL of PBS and re-suspended in 1 mL of PBS. Genomic DNA was extracted from the pure culture using QIAamp® DNA Mini Kit (QIAGEN Ltd., GmbH, Hilden, Germany). The PCR reaction mixture had a total volume of 20 μL containing 1.2 μL of a template DNA, 2.0 μL of each 10 pmol primer, 1.0 μL of 0.5 U Taq DNA polymerase, 0.8 μL of 2.5 mM dNTPs, 2.0 μL of 10 × PCR buffer, 1.6 μL of 25 mM $MgCl_2$, and 11.9 μL of sterile deionized water. Three different primer sets, such as *tdh* (F, GGCTCAAAATGGTTAAGCG; R, CATTTCC GCTCTCATATGC), *trh* (F, TGGTTGACATCCTACATG ACTGTG: R, GGGGATCCCTCAGTACAAAGCCTT), and *toxR* (F, GTCTTCTGACGCAATCGTTG; R, ATACG AGTGGTTGCTGTCATG) were used in this study. The reaction conditions for *tdh* (251 bp) and *trh* (250 bp) were as follows: initial denaturation at 96 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55°C for 1 min, and extension 72 °C for 1 min. The *toxR* (368 bp) was amplified with an initial denaturation at 96 °C for 5 min, followed by 20 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 1.5 min, and extension 72 °C for 1.5 min. The finished products were determined by electrophoresis on 2% agarose gels.

Results and discussion

Optimization of NaCl and pH

During the past several decades, a wide range of enrichment broths and selective agars for the detection of *V. parahaemolyticus* have been developed to reduce or prevent a food safety hazard posed by this pathogen (Bisha et al., 2012; Donovan and van Netten, 1995). In this study, various differential-selective media, such as TCBS, mCPC, CC, and SDS, were employed to compare their effects on the growth of *V. parahaemolyticus*. Among the commercial media tested, TSBS was demonstrated for its higher capacity to recover *V. parahaemolyticus* from seafoods. However, previous literatures (Blanco-Abad et al., 2009; Eddabra et al., 2011) highlighted that some selective media may not distinguish *V. parahaemolyticus* with other *Vibrio* spp. Particularly, *V. parahaemolyticus* produces its greenish, round, and opaque colony as the same as those of *V. vulnificus*, and *V. mimicus* on TCBS. The accuracy of TCBS for targeting *V. parahaemolyticus* in shellfishes was estimated by approximately 51% (Pinto et al., 2011). Furthermore, the ability of ChromagarVibrio to differentiate *V. parahaemolyticus* from commensal microorganisms found in mussel and lake water was highly unreliable though this bacterium becomes viable capable of producing its mauve colonies on ChromagarVibrio following less than 24 h of incubation at 37 °C (Blanco-Abad et al., 2009;

Table 2 Evaluation of the ability of *V. parahaemolyticus* and indicators to grow on TSA supplemented with 5% NaCl and individual selective agents at 37 °C for 24 h

Bacterium	ATCC number	Selective agent[a]					
		OG	XG	SC	ST	PT	CB
Vibrio parahaemolyticus	27969	+	+	+	+	+	+
Vibrio parahaemolyticus	33844	+	+	+	+	+	+
Number of positive strains		2	2	2	2	2	2
Acinetobacter calcoaceticus	ND[b]	–	+	+	+	+	+
Chryseobacterium balustinum	ND	–	–	–	–	–	–
Citrobacter freundii	8090	–	+	+	+	–	+
Cronobacter sakazakii	12868	–	+	+	+	–	+
Dickeya zeae	ND	–	–	–	–	–	–
Escherichia coli	25922	–	–	–	–	–	+
Escherichia coli O157:H7	35150	–	–	+	–	–	+
Hafnia alvei	29927	–	+	+	+	+	+
Klebsiella pneumoniae	13883	–	+	+	+	–	+
Pantoea agglomerans	ND	–	+	+	–	–	+
Pseudomonas aeruginosa	15692	+	+	+	+	+	+
Salmonella Typhimurium	19586	–	+	–	+	–	+
Samonella Enteritidis	13076	+	+	+	+	+	+
Stenotrophomonas maltophilia	ND	+	+	+	+	+	+
Yersinia enterocolitica	55075	–	–	–	–	–	–
Bacillus cereus	10876	–	+	+	+	+	+
Clavibacter michiganensis	ND	+	+	+	+	+	+
Enterococcus faecalis	19433	–	+	+	+	+	+
Lactococcus lactics	11454	–	+	+	+	+	+
Leuconostoc mesenteroides	27258	–	+	+	+	+	+
Listeria monocytogenes	19115	–	+	+	+	+	+
Pediococcus acidilactici	33314	–	–	–	–	–	+
Staphylococcus aureus	12600	+	+	+	+	+	+
Weissella confusa	10881	+	+	+	+	+	+
Number of positive strains		6	18	18	17	13	21

[a]CB, 10 g/L cellobiose; OG, 5 g/L oxgall; PT, 10 g/L potassium tellurite; SC, 3 g/L sodium cholate; ST, 5 g/L sodium taurocholate; XG, 2 g/L X-gal

[b]Not determined

Nigro and Steward, 2015). The growth of *V. parahaemolyticus* and *V. vulnificus* can be significantly different, depending on the application of various suspension solutions (Yoon et al., 2016). When *V. parahaemolyticus* ATCC 17802 was re-suspended and serially diluted in the modified PBS containing 3% NaCl (pH 6), followed by plating on TCBS, significantly higher populations of *V. parahaemolyticus* ATCC 17802 were counted in comparison with those in PBS containing less than 1% NaCl. Accordingly, there is an urgent need to reinforce either selectivity or sensitivity of currently used culture media to monitor the ecology and epidemiology of pathogenic *V. parahaemolyticus* more efficiently.

The ability of *V. parahaemolyticus* and indicators to grow on TSA supplemented with 0.5–5% NaCl (pH 7–9) is characterized in Table 1. Two strains of *V. parahaemolyticus* were progressively culturable on all modified media, irrespective of NaCl and pH levels. Among the modified media tested, TSA supplemented with 5% NaCl (pH 9) facilitated the growth of *V. parahaemolyticus* ATCC 27969 and *V. parahaemolyticus* ATCC 33844, while inhibiting 14 indicators mostly.

Preliminarily, a wide range of base ingredients constituting the commercialized differential-selective media for *V. parahaemolyticus* are characterized in this study (supplementary material 1). Most of the media contain 0.5–5% NaCl and remain slightly alkaline (pH 8–9) independently. Some selective ingredients, such as oxgall, x-gall, polymyxin B, polymyxin E, sodium cholate, sodium taurocholate, potassium tellurite, and cellobiose, are shown to play an important role in controlling non-*Vibrio* spp. Although various differential-selective media such as

Table 3 Evaluation of the ability of *V. parahaemolyticus* and indicators to grow on TSA supplemented with 5% NaCl and varying concentrations (0.1–0.5%) of oxgall (pH 9) at 37 °C for 24 h

Bacterium	ATCC number	Oxgall concentration					
		0.0%	0.1%	0.2%	0.3%	0.4%	0.5%
Vibrio parahaemolyticus	27969	+	+	+	+	+	+
Vibrio parahaemolyticus	33844	+	+	+	+	+	+
Number of positive strains		2	2	2	2	2	2
Acinetobacter calcoaceticus	ND[a]	+	−	−	−	−	−
Chryseobacterium balustinum	ND	−	−	−	−	−	−
Citrobacter freundii	8090	−	−	−	−	−	−
Cronobacter sakazakii	12868	+	−	−	−	−	−
Dickeya zeae	ND	−	−	−	−	−	−
Escherichia coli	25922	−	−	−	−	−	−
Escherichia coli O157:H7	35150	−	+	+	+	+	−
Hafnia alvei	29927	−	−	−	−	−	−
Klebsiella pneumoniae	13883	−	−	−	−	−	−
Pantoea agglomerans	ND	−	−	−	−	−	−
Pseudomonas aeruginosa	15692	−	+	+	+	+	+
Salmonella Typhimurium	19586	−	−	−	−	−	−
Samonella Enteritidis	13076	+	+	+	−	−	−
Stenotrophomonas maltophilia	ND	+	+	+	+	+	−
Yersinia enterocolitica	55075	−	−	−	−	−	−
Bacillus cereus	10876	+	−	−	−	−	−
Clavibacter michiganensis	ND	+	+	+	+	+	+
Enterococcus faecalis	19433	+	+	+	+	+	−
Lactococcus lactics	11454	−	−	−	−	−	−
Leuconostoc mesenteroides	27258	+	−	−	−	−	−
Listeria monocytogenes	19115	−	+	+	+	+	−
Pediococcus acidilactici	33314	−	+	+	+	+	+
Staphylococcus aureus	12600	+	+	+	+	+	+
Weissella confusa	10881	+	+	+	+	+	+
Number of positive strains		10	10	10	9	9	5

[a]Not determined

TCBS, mCPC, CC, and SDS were widely recommended to detect *Vibrio* spp., *V. parahaemolyticus* ATCC 27969 and *V. parahaemolyticus* ATCC 33844 grew on TCBS and SDS, whereas only *V. parahaemolyticus* ATCC 27969 produced its colony on mCPC and CC (supplementary material 2). It was also found that a total of 8, 4, 5, and 11 indicators were culturable on TCBS, mCPC, CC, and SDS, respectively. So far, TCBS containing 0.5% oxgall was demonstrated to be the most effective selective medium for detection of *V. parahaemolyticus*.

Screening of selective agents

In order to confirm the effect of oxgall on the growth of *V. parahaemolyticus* and non-*Vibrio* spp. in comparison with that of X-gal, sodium cholate, sodium taurocholate, potassium tellurite, and cellobiose, each of the selective agents was added to TSA containing 5% NaCl (pH 9), and then a total of 26 bacteria were plated on these media, followed by 24 h of incubation at 37 °C (Table 2). Although *V. parahaemolyticus* ATCC 27969 and *V. parahaemolyticus* ATCC 33844 remained culturable on all of the media used, TSA supplemented with 5% NaCl and 0.5% oxgall (pH 9) successfully supported the growth of *V. parahaemolyticus*, while repressing most leading food-borne pathogens selectively, including *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica*, *Bacillus cereus*, and others.

On TSA supplemented with 5% NaCl (pH 9), the effect of 0.1–0.5% oxgall on the culturability of *V. parahaemolyticus* and non-*Vibrio* spp. is presented in Table 3. As expected, increasing proportions of oxgall resulted in higher growth-inhibitory effects to non-*Vibrio* spp. Obviously, the addition of 0.5% oxgall onto TSA containing 5% NaCl (pH 9) contributed to the highest selective growth of *V. parahaemolyticus* strains.

Table 4 Evaluation of the ability of *V. parahaemolyticus* and indicators to grow on TSA, BHI, LBA or NA supplemented with 5% NaCl and 0.5% oxgall (pH 9)

Bacterium	ATCC number	TSA[a]	BHI	LBA	NA
Vibrio parahaemolyticus	27969	+	+	+	+
Vibrio parahaemolyticus	33844	+	+	+	+
Number of positive strains		2	2	2	2
Acinetobacter calcoaceticus	ND[b]	–	–	–	–
Chryseobacterium balustinum	ND	–	–	–	–
Citrobacter freundii	8090	–	–	–	–
Cronobacter sakazakii	12868	–	–	–	–
Dickeya zeae	ND	–	–	–	–
Escherichia coli	25922	–	–	–	–
Escherichia coli O157:H7	35150	–	–	–	–
Hafnia alvei	29927	–	–	–	–
Klebsiella pneumoniae	13883	–	–	–	–
Pantoea agglomerans	ND	–	–	–	–
Pseudomonas aeruginosa	15692	+	–	–	+
Salmonella Typhimurium	19586	–	–	–	–
Salmonella Enteritidis	13076	–	–	–	–
Stenotrophomonas maltophilia	ND	–	–	–	–
Yersinia enterocolitica	55075	–	–	–	–
Bacillus cereus	10876	–	–	–	–
Clavibacter michiganensis	ND	+	+	+	+
Enterococcus faecalis	19433	–	–	–	–
Lactococcus lactics	11454	–	–	–	–
Leuconostoc mesenteroides	27258	–	–	–	–
Listeria monocytogenes	19115	–	–	–	–
Pediococcus acidilactici	33314	+	–	–	–
Staphylococcus aureus	12600	+	–	–	–
Weissella confusa	10881	+	–	–	–
Number of positive strains		5	1	1	2

[a]BHI, brain heart infusion agar; LBA, Luria–Bertani agar; NA, nutrient agar; TSA, tryptic soy agar

Formulation of enhanced selective media

To determine the most suitable basal medium involved in the selective growth of *V. parahaemolyticus*, the ability of *V. parahaemolyticus* and indicators to grow on TSA, BHI, LBA, and NA supplemented with 5% NaCl and 0.5% oxgall (pH 9) is evaluated in Table 4. Commonly, two strains of *V. parahaemolyticus* grew on all of the modified media. In contrast, *Clavibacter michiganensis* was found to produce its colony on BHI and LBA supplemented with 5% NaCl and 0.5% oxgall (pH 9), respectively. The use of TSA containing 5% NaCl and 0.5% oxgall (pH 9) also failed to inhibit the growth of 5 indicators, including *Cl. michiganensis*, *Pseudomonas aeruginosa* ATCC 15692, *Pediococcus acidilactici* ATCC 33314, *Staphylococcus aureus* ATCC 12600, and *Weissella confusa* ATCC 10881. The modified BHI yielded strong selectivity for *V. parahaemolyticus* equal to or higher than that of the modified TSA.

Table 5 shows the effect TSA supplemented with 5% NaCl, 0.5% oxgall, and 2% sodium citrate (pH 9) on the colony-forming ability of *Vibrio parahaemolyticus* strains, indicators, and food-borne isolates in compassion with TCBS. Three strains of *V. parahaemolyticus* and 24 food-borne isolates were capable of forming their own colonies on TCBS. On TSA supplemented with 5% NaCl, 0.5% oxgall, and 2% sodium citrate (pH 9), *V. parahaemolyticus* was successfully culturable, whereas neither 24 food-borne isolates nor 26 indicative microorganisms were able to colonize. No growth of 23 food-borne isolates was observed on BHI supplemented with 5% NaCl, 0.5% oxgall, and 2% sodium citrate (pH 9) albeit this modified medium did not facilitate the growth of *V. parahaemolyticus* ATCC 17802.

Table 5 Effect of TSA, BHI, LBA or LA supplemented with 5% NaCl, 0.5% oxgall, and 2% sodium citrate (pH 9) on the colony-forming ability of *V. parahaemolyticus*, indicators, and bacterial isolates in compassion with TCBS after 24 h of incubation at 37 °C

Bacterium	ATCC/origin	TCBS[a]	TSA	BHI	LBA	NA
Vibrio parahaemolyticus	17802	+	+	−	−	−
Vibrio parahaemolyticus	27969	+	+	+	−	−
Vibrio parahaemolyticus	33844	+	+	+	−	−
Number of positive strains		3	3	2	0	0
Acinetobacter calcoaceticus	ND[b]	− [b]	−	−	−	−
Bacillus cereus	10876	−	−	−	−	−
Chryseobacterium balustinum	ND	−	−	−	−	−
Clavibacter michiganensis	ND	−	−	−	−	−
Cronobacter sakazakii	12868	+	−	−	−	−
Escherichia coli	25922	−	−	−	−	−
Escherichia coli O157:H7	35150	−	−	−	−	−
Enterococcus faecalis	19433	+	−	−	−	−
Dickeya zeae	ND	−	−	−	−	−
Hafnia alvei	29927	−	−	−	−	−
Klebsiella pneumoniae	13883	+	−	−	−	−
Lactococcus lactis	11454	−	−	−	−	−
Leuconostoc mesenteroides	27258	−	−	−	−	−
Listeria monocytogenes	19115	−	−	−	−	−
Pantoea agglomerans 1	ND	−	−	−	−	−
Pantoea agglomerans 2	ND	−	−	−	−	−
Pediococcus acidilactici	33314	−	−	−	−	−
Pseudomonas	15692	+	−	−	−	−
Salmonella Enteritidis	13076	+	−	−	−	−
Salmonella Typhimurium	19585	+	−	−	−	−
Stenotrophomonas maltophilia 1	ND	−	−	−	−	−
Stenotrophomonas maltophilia 2	ND	−	−	−	−	−
Staphylococcus aureus	23235	+	−	−	−	−
Staphylococcus saprophyticus	15305	+	−	−	−	−
Yersinia enterocolitica	55075	−	−	−	−	−
Weissella confusa	10881	−	−	−	−	−
Number of positive strains		8	0	0	0	0
Aeromonas hydrophila/caviae/sobria 1	Plaice	+	−	−	−	−
Aeromonas hydrophila/caviae/sobria 2	Rockfish	+	−	−	−	−
Burkholderia cepacia	Plaice	+	−	−	−	−
Citrobacter freundii	Plaice	+	−	−	−	−
Cedecea davisae	Rockfish	+	−	−	−	−
Cedecea lapagei	Shrimp	+	−	−	−	−
Cronobacter spp.	Plaice	+	−	−	−	−
Enterobacter aerogenes	Rockfish	+	−	−	−	−
Enterobacter cloacae	Rockfish	+	−	+	+	−
Ewingella americana	Clam	+	−	−	−	−
Pantoea spp. 1	Shrimp	+	−	−	−	−
Pantosa spp. 3	Rockfish	+	−	−	−	−
Pseudomonas luteola	Plaice	+	−	−	−	−
Pseudomonas oryzihabitans	Rockfish	+	−	−	−	−
Salmonella spp.	Rockfish	+	−	−	−	−
Serratia ficaria	Plaice	+	−	−	−	−

Table 5 continued

Bacterium	ATCC/origin	TCBS[a]	TSA	BHI	LBA	NA
Serratia marcescens	Rockfish	+	–	–	–	–
Serratia odorifera 1	Rockfish	+	–	–	–	–
Serratia plymuthica	Rockfish	+	–	–	–	–
Raoultella ornithinolytica	Rockfish	+	–	–	–	–
Staphylococcus lentus	Rockfish	+	–	–	–	–
Staphylococcus saprophyticus	Rockfish	+	–	–	–	–
Staphylococcus xylosus	Rockfish	+	–	–	–	–
Vibrio ordalii	Rockfish	+	–	–	–	–
Number of positive strains		24	0	1	1	0

[a]BHI, brain heart infusion agar; LBA, Luria–Bertani agar; NA, nutrient agar; TSA, tryptic soy agar

[b]Not determined

Table 6 Quantification (log CFU/g) of *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 27969 or *V. parahaemolyticus* ATCC 33844 in raw oysters using formulated selective media, followed by 24 h of incubation at 37 °C

Medium	Ingredient[a]					Strain		
	Base	OG (%)	SC (%)	pH	NaCl (%)	ATCC 17802	ATCC 27969	ATCC 33844
TCBS	–	0.5	1	8	1	2.90	2.60	2.48
TSA_{OSS1}	TSA	0.5	2	9	5	3.30	3.42	3.53
TSA_{OSS2}	TSA	0.2	1	8	5	3.38	3.49	3.54
TSA_{OSS3}	TSA	0.2	2	8	5	3.00	3.00	3.35
TSA_{OSS4}	TSA	0.5	1	8	5	2.60	–[b]	3.58
TSA_{OSS5}	TSA	0.5	2	8	5	3.18	–	3.48
TSA_{OSS6}	TSA	0.2	1	9	5	–	2.30	3.02
TSA_{OSS7}	TSA	0.2	2	9	5	2.00	2.65	2.70
TSA_{OSS8}	TSA	0.5	1	9	5	2.30	2.00	3.28
BHI_{OSS1}	BHI	0.5	2	9	5	3.40	3.43	3.53
BHI_{OSS2}	BHI	0.2	1	8	5	3.50	3.27	3.37
BHI_{OSS3}	BHI	0.2	2	8	5	3.28	2.30	3.24
BHI_{OSS4}	BHI	0.5	1	8	5	–	2.81	–
BHI_{OSS5}	BHI	0.5	2	8	5	2.40	2.40	2.70
BHI_{OSS6}	BHI	0.2	1	9	5	–	3.16	3.24
BHI_{OSS7}	BHI	0.2	2	9	5	2.40	2.00	2.95
BHI_{OSS8}	BHI	0.5	1	9	5	3.51	–	2.70

[a]BHI, brain heart infusion agar; OG, oxgall; SC, sodium citrate; TSA, tryptic soy agar

[b]No growth

Food application

Of which the aforementioned selective agents, oxgall is a mixture of taurocholic acid and glycocholic acid, mainly suppressing the growth of Gram-positive bacteria. In this study, neither 26 indicator bacteria nor 23 food-borne isolates were capable of growing on TSA_{OSS1-2}, but these media stimulated the growth of three *V. parahaemolyticus* strains selectively. The ability of TSA_{OSS1-8} and BHI_{OSS1-8}, consisting of 1–5% NaCl, 0.2–0.5% oxgall, 1–2% and sodium citrate (pH 8–9), to quantify the viable number of *V. parahaemolyticus* in raw oysters is presented in Table 6. Primarily, we observed the inability of TCBS to distinguish between *V. parahaemolyticus* strains and background microbiota in oysters and mackerels, thereby resulting in a greater underestimation for quantifying the number of *V. parahaemolyticus*. Suspect or presumptive colonies towards *V. parahaemolyticus* on TCBS were further identified to be non-*Vibrio* spp. as determined by PCR analyses with three different primer sets (*tdh*, *trh* or *toxR*). Nevertheless, *V. parahaemolyticus* in oysters was estimated to grow on TCBS at levels of approximately 2.48–2.90 log CFU/g. In contrast, TSA_{OSS1-3} and BHI_{OSS1-3} enabled three *V. parahaemolyticus* strains to colonize at levels of

3.00–3.54 and 2.30–3.53 log CFU/g, respectively (Table 6). Once *V. parahaemolyticus* was inoculated in raw mackerels, neither TSA_{OSS1-8} nor BHI_{OSS1-8} supported the growth of *V. parahaemolyticus* ATCC 17802, whereas *V. parahaemolyticus* ATCC 27969 and *V. parahaemolyticus* ATCC 33844 grew on these media at levels of 3.48–4.13 and 2.48–5.14 log CFU/g, respectively (*data not shown*). Moreover, *V. vulnificus* ATCC 27562 and *V. vulnificus* ATCC 33815 cannot grow on TSA_{OSS1}. Particularly, *V. parahaemolyticus* was recovered from raw oysters on TSA_{OSS1} at levels of 3.30–3.54 log CFU/g, while inhibiting the indigenous microbial community selectively. Importantly, *V. parahaemolyticus* distinctly produced its white-yellowish, round, and edge-pointed colony on TSA_{OSS1-3} and BHI_{OSS1-3} (supplementary material 3). Taken together, the results indicate that the formulated selective media, TSA_{OSS1} and $BHIO_{SS1}$ strongly supported the growth of *V. parahaemolyticus* strains, while suppressing most of the background microorganisms, concomitantly with an increase in the selectivity for *V. parahaemolyticus* higher than did TCBS.

As mentioned earlier, the recognition of *V. parahaemolyticus* as a leading pathogen closely linked to human infections has forced the establishment of efficient monitoring methods for rapid detection of this bacterium in seafoods and natural reservoirs. For instance, a simple multi-plating method of TCBS coupled with Chromagar resulted in a remarkable reduction in the incidence of false positive isolates of *V. parahaemolyticus*, contributing to an improvement in the estimation of its abundance (Nigro and Steward, 2015). Rosec et al. (2012) showed that the international standard ISO/TS 21872–1 method for detection of *V. parahaemolyticus* in oyster exerted 35 (approximately 35%) false negative results out of 103 raw samples. The authors further used ChromagarVibrio and TCBS concurrently, and presumptive colonies were analyzed for the presence of a *toxR* fragment (368 bp) specifically found in *V. parahaemolyticus*. Such a modification in the protocol led to an improvement in the sensitivity of ISO/TS 21872–1 program for better quantifying *V. parahaemolyticus* in raw seafoods. In a study of Duan et al. (2006), wherein evaluated the efficacy of double layer agar plate (DLAP) methods for quantifying *V. parahaemolyticus*, the heat- or cold-injured cells were incapable of recovering on TCBS and BioChrome Vibrio medium (BCVM), while remaining culturable on a DLAP with TSA and BCVM. The results were in accordance with Wang et al. (2015).

Considering that currently used detection platforms for *V. parahaemolyticus* include one or more culture-based methods prior to subsequent molecular analyses, including DNA hybridization, PCR, and loop-mediated isothermal amplification (Bisha et al., 2012), we formulated an enhanced selective medium, TSA_{OSS1} with high selectivity potentials over TCBS for culturing and quantifying *V. parahaemolyticus* in raw oysters. TSA_{OSS1} could be a promising alternative to detect *V. parahaemolyticus* in natural reservoirs, providing valuable insights in understanding the ecology of *Vibrio* spp.

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Compliance with ethical standards

Conflict of interest No conflict of interests was declared.

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