Bacterial diversity and potential risk factors associated with *Salmonella* contamination of seafood products sold in retail markets in Bangkok, Thailand

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Consumption of contaminated food causes 600 million cases, including 420,000 of fatal infections every year. Estimated cost from food-borne illnesses is USD 110 billion per year, especially in low- and middle-income countries. Thailand is a leading producer and consumer of seafood, but little is known about bacterial contamination in seafood. In particular, public health agencies need to know the relationship between bacterial diversity in seafood and their risk factors, as assessed with readily available culture-dependent and bacterial phenotyping methods. To address this, levels of indicator bacteria, *Salmonella* and *Vibrio* in various seafood commodities were determined and to identify risk factors associated with *Salmonella* contamination. A total of 335 samples were collected from October 2018 to July 2019 throughout Bangkok, Thailand; overall sample composition was Pacific white shrimp (n = 85), oysters (n = 82), blood cockles (n = 84), and Asian seabass (n = 84). The prevalence was 100% for fecal coliforms and 85% for *E. coli*. In contrast, prevalence was 59% for *V. parahaemolyticus*, 49% for *V. cholerae*, 19% for *V. alginolyticus*, 18% for *V. vulnificus*, and 36% for *Salmonella*. Highest concentrations of fecal coliforms and *E. coli* were in oysters. Highest concentrations of *Salmonella* with Matopeni (31%) being the predominant serotype were in shrimp. *Salmonella* contamination was significantly associated with type of seafood, sampling location, retail conditions, and the presence of *E. coli*, *V. alginolyticus* and *V. vulnificus*. A cutoff value for *E. coli* concentration of 1.3×10^4 MPN/g predicted contamination of *Salmonella*, with a sensitivity of 84% and specificity of 61%. Displaying seafood products on ice, presence of *E. coli* and *Vibrio*, and seafood derived from Eastern Thailand were associated with an increased risk of *Salmonella* contamination.

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19 **Abstract**
20 Consumption of contaminated food causes 600 million cases, including 420,000 of fatal
21 infections every year. Estimated cost from food-borne illnesses is USD 110 billion per year,
22 ~~especially in~~ low- and middle-income countries. Thailand is a leading producer and consumer of
23 seafood, but little is known about bacterial contamination in seafood. In particular, public health
24 agencies need to know the relationship between ~~bacterial diversity~~ in seafood and ~~their~~ risk
25 factors, as assessed with readily available ~~culture-dependent and bacterial phenotyping~~ methods.
26 To address this, levels of indicator bacteria, *Salmonella* and *Vibrio* in various seafood
27 ~~commodities~~ were determined ~~and~~ to identify risk factors associated with *Salmonella*
28 contamination. A total of 335 samples were collected from October 2018 to July 2019
29 throughout Bangkok, Thailand; overall sample composition was Pacific white shrimp (n = 85),
30 oysters (n = 82), blood cockles (n = 84), and Asian seabass (n = 84). ~~The~~ prevalence was 100%
31 for fecal coliforms and 85% for *E. coli*. ~~In contrast,~~ prevalence was 59% for *V.*
32 *parahaemolyticus*, 49% for *V. cholerae*, 19% for *V. alginolyticus*, 18% for *V. vulnificus*, and
33 36% for *Salmonella*. Highest concentrations of fecal coliforms and *E. coli* were in oysters.
34 Highest concentrations of *Salmonella* with Matopeni (31%) being the predominant serotype were
35 in shrimp. *Salmonella* contamination was significantly associated with type of seafood, sampling
36 location, retail conditions, and the presence of *E. coli*, *V. alginolyticus* and *V. vulnificus*. A cutoff
37 value for *E. coli* concentration of 1.3×10^4 MPN/g predicted contamination of *Salmonella*, with a
38 sensitivity of 84% and specificity of 61%. Displaying seafood products on ice, presence of *E.*

39 *coli* and *Vibrio*, and seafood derived from Eastern Thailand were associated with an increased
40 risk of *Salmonella* contamination.
41

Introduction

The global fisheries and aquaculture both inland and marine reached 171 million tonnes in 2016 (*Food and Agriculture Organization of the United Nations, 2018*) and consumption of fish, and fishery products per capita double from 10 kg in 1960 to greater than 20 kg in 2016 (*Food and Agriculture Organization of the United Nations, 2018*). In Southeast Asia, consumption of fish and fishery products varies from 6 to 64 kg per capita per year (*Food and Agriculture Organization of the United Nations, 2015*). In Thailand, consumption of fish and fishery products is about 31 kg per capita per year, which accounts for 12% of total protein consumption per person (*Food and Agriculture Organization of the United Nations, 2015*). Thailand is one of the top ten exporters of fish and fishery products, which accounted for 4% of global exports in 2016 (*Food and Agriculture Organization of the United Nations, 2018*).

~~Due to the rapid growth of global consumption of fish and fishery products, seafood safety is of high public health concern.~~ Foodborne diseases ~~each year~~ afflict a third of the world population (*World Health Organization, 2004*), but data about the number of illnesses from seafood-borne outbreaks is limited for many parts of the world. Most examinations of seafood outbreaks have been done in the United States, where approximately 9.4 million illnesses, almost 56,000 hospitalizations, and 1,351 deaths, were associated with foodborne contamination per year (*Scallan et al., 2011*). Almost half (45%) of foodborne outbreaks reported in the U.S. are from bacteria, and fish are frequently implicated (*Gould et al., 2013*). In Europe, 5,175 foodborne outbreaks were reported in 2019. *Salmonella* spp. cause most of these outbreaks. ~~There were 87,923 and 7,775 confirmed cases of salmonellosis and infections from Shiga-toxin-producing *Escherichia coli*, respectively~~ (*European Food Safety Authority; European Centre for Disease Prevention and Control, 2021*).

Pollution, animal density, and global trading contribute to bacterial contamination of seafood products (*Papadopoulou et al., 2007*). The most common pathogens associated with seafood-borne diseases are *Vibrio*, *Salmonella*, *Shigella*, and *Clostridium botulinum* (*Iwamoto et al., 2010*). Seafood-borne outbreaks caused by *V. parahaemolyticus*, *V. cholera* serogroup O139, *V. vulnificus*, *Salmonella* serotype Weltevreden, and *E. coli* have been reported (Bonnin-Jusserand et al., 2019; Heinitz et al., 2020; Martinez-Urtaza et al., 2016; Raymond & Ramachandran, 2019).

In Thailand, *V. parahaemolyticus* and *Salmonella* spp. are the leading causes of foodborne diarrhea. Even though Thailand is one of the major exporters of seafood products, monitoring of bacterial pathogens of these exports is limited. Therefore, the objectives of this study were: 1) examine the prevalence of bacterial indicators, *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus*, *V. alginolyticus*, and *Salmonella* isolated from Pacific white shrimp, oysters, blood cockle, and Asian seabass in fresh markets in Bangkok, Thailand; 2) identify serotypes of *Salmonella* among various seafood samples; and 3) determine risk factors for *Salmonella* contamination and a ~~potential~~ cutoff value for *E. coli* concentration ~~predicting~~ the presence of *Salmonella* in ~~these samples~~.

82 **Materials & Methods**

83 **Sample collection**

84 Samples of fresh fish and shellfish ($n = 335$) were collected from open-air retail fresh markets

85 between October 2018 and July 2019 from four districts in Bangkok, Thailand, resulting in a

86 sample composition of Pacific white shrimp (*Litopenaeus vannamei*) ($n = 85$), oyster (*Saccostrea*

87 *cuccullata*) ($n = 82$), blood cockle (*Tegillarca granosa*) ($n = 84$), and Asian seabass (*Lates*

88 *calcarifer*) ($n = 84$) (Table 1). Due to varying availability of these four different seafood

89 commodities at each market, there were slightly different total sample sizes for some seafood

90 commodities ranging from $n = 82$ to $n = 85$ (Table 1). Pacific white shrimp, oysters, and blood

91 cockles are raised in saltwater ponds; the majority of Asian seabass are raised in estuaries, but

92 some are raised in saltwater ponds.

93   Individual seafood samples were purchased in the early morning (5 to 7 a.m.). At least

94 200 g of the samples were placed into a double sterile plastic bag. The samples were kept on ice

95 ($< 10\ °C$) during transportation and kept in the cooler. All samples were submitted to the

96 laboratory within 3 h. Microbiological determination was performed within 6 h after receiving

97 samples in the Department of Veterinary Public Health, Faculty of Veterinary Science,

98 Chulalongkorn University.

99   Average and standard deviation (sd) of minimum and maximum ambient air temperature

100 (°C), wind speed (km/h), precipitation (mm), and relative humidity (%) in Bangkok, Thailand,

101 were retrieved from the Thai Meteorological Department (www.tmd.go.th). The average ($\pm$ sd)

102 daily minimum and maximum ambient air temperature was 26.8 ($\pm$ 1.8) °C and 34.1 ($\pm$ 2.6) °C;

103 average ($\pm$ sd) wind speed was 13.0 ($\pm$ 2.0) km/h, average 24-hour precipitation 1.4 ($\pm$ 4.0) mm,

104 and average relative humidity was 75.1 ($\pm$ 7.4) %.

105 **Predictor variables**

106 Risk factors for *Salmonella* contamination included type of seafood (Pacific white shrimp,

107 oyster, blood cockle, or Asian seabass), sampling district (Din Daeng, Huay Kwang,

108 Samphanthawong, or Dusit), regional source of seafood (central, eastern, southern Thailand, or

109 unidentified source), retail storage of fish and shellfish samples (pooling and combining different

110 seafood products for retail display versus keeping each seafood type separate when on display),

111 and retail display condition (on ice or without ice). The concentrations of fecal coliform

112 (MPN/g) and *E. coli* (MPN/g), and the prevalence of *V. parahaemolyticus*, *V. vulnificus*, *V.*

113 *alginolyticus* and *V. cholerae* were evaluated as putative risk factors for *Salmonella*

114 contamination.

115 **Bacterial concentration and phenotyping**

116 Seafood samples were analyzed in triplicate for coliforms, *E. coli*, *Salmonella*, *V.*

117 *parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. cholerae*. Fecal coliform and *E. coli*

118 were enumerated according to the U.S. Food and Drug Administration (U.S. FDA)

119 Bacteriological Analytical Manual (BAM) with slight modification (*Feng et al., 2002*). Briefly, a

120 25 g sample (shrimp and Asian seabass) was aseptically cut into small pieces and placed into 225

121 mL of Buffered Peptone Water (BPW) (Difco, MD, USA). Pieces were then homogenized for 1

122   to 2 min. The resulting suspension was serially diluted using three-tube most probable number

123   (MPN) at different dilutions from 10^{-1} to 10^{-4}. One mL of each solution was diluted in Lactose

124   Broth (LB) (Difco), and then incubated at 35 °C for 24 h. A loopful of the mixture solution was

125   transferred to Brilliant Green Lactose Bile (BGLB) (Difco) and EC broth (Difco), respectively.

126   After overnight incubation, positive tubes were recorded and calculated as concentration of fecal

127   coliforms (MPN/g). One loopful of EC broth was streaked on Eosin Methylene Blue (EMB;

128   Difco) agar plates and reported as *E. coli* concentration (MPN/g).

129   Fecal coliform and *E. coli* were enumerated in oysters and blood cockles following (*Feng*

130   *et al., 2002*). Briefly, 100 g of meat was added into 100 mL of Phosphate Buffered Saline (PBS)

131   (Difco), which was then blended aseptically for 1 to 2 min. The resulting suspension was serially

132   diluted in LB to 10^{-4}. These dilutions were transferred to BGLB and EC broth. Biochemical tests

133   including indole test and Triple Sugar Iron (TSI; Difco) were performed on suspect colonies for

134   all samples. The lower and upper limits of the detection of fecal coliforms and *E. coli* were 1.0

135   and $1.1×10^5$ MPN/g, respectively.

136   *Salmonella* detection followed ISO 6579-1:2017 (*International Organization for*

137   *Standardization, 2017*). Briefly, 25 g of seafood was cut, and added to 225 mL of BPW. The

138   pieces were homogenized for 2 min and incubated at 37 °C for 18 h. After incubation, 0.1 mL of

139   the suspension was inoculated into Modified Semi-solid Rappaport-Vassiliadis (MSRV) (Difco)

140   agar plate and incubated at 42 °C overnight. A loopful from the MSRV plates was restreaked

141   onto Xylose Lysine Deoxycholate (XLD) (Difco) agar. Presumptive colonies of *Salmonella* were

142   pink to red colonies with a black center. Biochemical tests (citrate utilization, TSI reaction,

143   indole test) were used to confirm presumptive *Salmonella* colonies according to a standard

144   protocol from the U.S. FDA BAM (*Andrews et al., 2007*). Three typical colonies of *Salmonella*

145   were selected for serotyping. Slide agglutination test was performed to determine serotype of

146   *Salmonella* followed by Kauffmann-White Scheme, Pasteur Institute (*Grimont and Weill, 2007*).

147   Commercial antiserums (S&A Reagents Lab Ltd., Lat Phrao, Bangkok, Thailand) were used to

148   determine the serotype of *Salmonella*.

149   Isolation of *Vibrio* spp. followed U.S. FDA BAM (*Kaysner et al., 2004*). Briefly, 50 g of

150   each sample was added to 450 mL of PBS, and homogenized for 1 to 2 min. One mL of resulting

151   suspension was added to 10 mL of Alkaline Peptone Water (APW) (Difco) and incubated at 37

152   °C overnight. After incubation, one loopful of solution was streaked on Thiosulfate-Citrate-Bile

153   Salts-sucrose (TCBS) (Difco) agar plate containing 2% of NaCl. Presumptive colonies of *Vibrio*

154   were confirmed using CHROMagar™ *Vibrio* (HiMedia Laboratories, Mumbai, India) agar.

155   TCBS and CHROMagar™ *Vibrio* plates were incubated at 37 °C for 24 h. Colonies with green

156   center on TCBS agar were presumed to be *V. parahaemolyticus*. Colorless colonies were

157   presumed to be *V. vulnificus*. On CHROMagar™ *Vibrio* agar plate, mauve colonies were

158   presumed to be *V. parahaemolyticus*, and green blue to turquoise blue were presumed to be of *V.*

159   *vulnificus*. Colorless colonies were presumed to be *V. alginolyticus*.

160   Isolation of *V. cholerae* followed U.S. FDA BAM (*Kaysner et al., 2004*). Briefly, 25 g of

161   sample was added to 225 mL of APW, homogenized for 1 to 2 min, and incubated at 35±2 °C for

162   8 h. A loopful of solution was streaked to TCBS agar plates. After incubation at 37 °C for 24 h,
163   presumptive colonies of *V. cholerae* were confirmed on CHROMagar™ *Vibrio*. Typical colonies
164   of *V. cholerae* on TCBS agar plate are 2 to 3 mm diameter, yellow, and flat colonies with opaque
165   center, whereas the presumptive colonies of *V. cholerae* in CHROMagar™ *Vibrio* agar were
166   green blue to turquoise blue. Biochemical tests including TSI, oxidase test, and growth in sodium
167   chloride were conducted to confirm *Vibrio* identifications.

168   **Statistical analyses**
169   Chi-square test and odds ratios were used to examine the association between different species of
170   bacterial contamination and different types of seafood. For the odds ratio calculations of the
171   association between bacteria contamination among seafood samples, shrimp was set as the
172   referent category based on its popularity in Thai cuisine and largest sample size ($n = 85$) of the
173   four seafood commodities. In addition, logistic regression was used to determine the association
174   between *Salmonella* contamination and various risk factors. To construct the final logistic
175   regression model, univariate associations were first evaluated for all risk factors for *Salmonella*
176   and an initial multivariable model constructed from only significant univariate risk factors ($P \leq$
177   0.2). A backward stepping algorithm was then used to eliminate non-significant ($P > 0.05$) risk
178   factors based on a likelihood ratio test resulting in a final multivariable logistic regression model
179   with only significant ($P \leq 0.05$) risk factors. Receiver operating characteristic (ROC) analysis
180   was performed to predict contamination of *Salmonella* using estimation of the concentration of
181   *E. coli*. Based on ROC analysis, the optimal cutoff value for the concentration of *E. coli*. was
182   determined. All statistical analyses were performed using Stata version 14.0 (StataCorp, College
183   Station, TX, USA). A *P*-value < 0.05 was considered as statistically difference under the two-
184   sided hypothesis test.

185

186   # Results

187   **Occurrence of indicator bacteria in seafood samples**
188   All seafood products sampled in Bangkok were positive for fecal coliforms with total average
189   concentration ($\pm$ sd) at 9×10^4 ($\pm 4 \times 10^4$) MPN/g (Table 2). The prevalence of *E. coli* was 85%,
190   with total average concentration ($\pm$ sd) of 2×10^4 ($\pm 4 \times 10^4$) MPN/g. Oyster samples had the
191   highest concentrations ($\pm$ sd) of fecal coliforms at 1×10^5 ($\pm 7 \times 10^3$) and *E. coli* at 5×10^4 ($\pm$
192   5×10^3), while blood cockle and seabass had the lowest concentrations of these indicator bacteria
193   (Table 2).

194   **Occurrence of *Vibrio* and *Salmonella* in seafood samples**
195   The overall prevalence for the various bacterial pathogens observed in all 335 seafood samples
196   was 59% for *V. parahaemolyticus*, 18% for *V. vulnificus*, 19% for *V. alginolyticus*, 49% for *V.*
197   *cholerae* and 36% for *Salmonella*. The highest prevalence of *V. parahaemolyticus*, *V. vulnificus*,
198   *V. alginolyticus*, *V. cholerae*, and *Salmonella* were observed in blood cockle (78%), Pacific
199   white shrimp (33%), oyster (29%), Asian seabass (76%), and Pacific white shrimp (40%),
200   respectively (Figure 1). The lowest prevalence ($< 10\%$) of *V. vulnificus* was observed in blood
201   cockle and for *V. alginolyticus* in Asian seabass. Moreover, shrimp were most likely to have any

202   of the four pathogens, followed by oysters (Figure 1). Blood cockles exhibited very high
203   contamination of *V. parahaemolyticus*, while Asian seabass tended to harbor *V. cholerae* (Figure
204   1). Based on Chi-square tests, the was a significant association between different types of
205   samples and the occurrence of *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. cholerae,*
206   and *Salmonella* contamination ($P < 0.0001$).
207   Pacific white shrimp exhibited a high prevalence of *V. parahaemolyticus* (59%), *V.*
208   *cholerae* (53%) and *Salmonella* (47%), whereas oysters were mainly contaminated with *V.*
209   *parahaemolyticus* (45%) and *Salmonella* (38%). In blood cockles, they had low prevalence of *V.*
210   *cholerae, V. vulnificus,* and *V. alginolyticus.* Asian seabass exhibited a high prevalence of *V.*
211   *cholerae* (91%) and *Salmonella* (46%).
212   Matopeni (31%), Corvallis (5%), Give (5%), and Rissen (5%) were the most common
213   serotypes of *Salmonella* isolated from seafood products (Table 3). Matopeni was the
214   predominant serotype (52/56) observed from Asian seabass samples ($n = 56$ isolates), whereas
215   Itami and Leith were common serovars isolated from the shrimp samples ($n = 47$ isolates). For
216   oysters ($n = 43$ isolates) and blood cockles ($n = 24$ isolates), the major serotypes were Give
217   (19%) and Rissen (33%), respectively.
218   **The distribution of *Salmonella, V. parahaemolyticus, V. vulnificus, V. cholerae,* and *V.***
219   ***alginolyticus* among seafood products**
220   Odds of *V. vulnificus* contamination in shrimp was 7.0 (1/0.143) times higher than that for blood
221   cockle ($P = 0.002$) (Table 4). Odds of *V. cholerae* contamination in shrimp were 7.5 (1/0.134)
222   and 23.6 (1/0.043) times higher than for oyster ($P < 0.0001$) and blood cockle ($P = 0.002$),
223   respectively. The presence of *V. parahaemolyticus* in the blood cockle was higher than in shrimp
224   (OR = 9.1, $P < 0.0001$). The odds of *V. alginolyticus* contamination in shrimp was 13.3 (1/0.075)
225   and 20.0 (1/0.050) times higher than in blood cockles and seabass, respectively.
226   **Risk factors associated with *Salmonella* contamination**
227   *Salmonella* contamination of seafood sold throughout Bangkok was significantly associated with
228   type of seafood, sampling district, retail display condition, regional source of seafood, and the
229   presence of *E. coli, V. alginolyticus,* and *V. vulnificus* (Table 5). *Salmonella* contamination in
230   Pacific white shrimp was not different form Asian seabass; in contrast, both oysters and blood
231   cockles had significantly lower odds of *Salmonella* contamination compared to shrimp. Seafood
232   from markets in Huay Kwang (OR = 1.7) and Dusit (OR = 1.4) had a higher odds of *Salmonella*
233   contamination compared to seafood from Din Daeng and Samphanthawong. Seafood displayed
234   on ice (OR = 1.7, $P < 0.0001$) had a higher odds of *Salmonella* contamination compared to retail
235   seafood products not displayed on ice. Seafood products sourced from Eastern Thailand had
236   significantly higher odds of *Salmonella* contamination compared to seafood sourced from other
237   regions (OR = 3.5, $P < 0.0001$). Lastly, the odds of *Salmonella* contamination were positively
238   associated with the presence of *E. coli* and *V. alginolyticus,* but negatively associated with *V.*
239   *vulnificus.*
240   **ROC and area under the ROC curve**

241   The area under the ROC curve (AUC) at 64% with standard error = 0.30 (C.I. = 58% - 70%)
242   (Figure 2). The ROC AUC was statistically significance ($P < 0.0001$) compared to the null value
243   of AUC = 0.5. The presence of *Salmonella* in seafood products was predicted by a concentration
244   of 1.3×10^4 *E. coli* MPN/g, with a sensitivity of 84% and specificity of 61%.
245

Discussion

247   According to the Ministry of Public Health for Thailand, the concentration of *E. coli* should not
248   exceed 10 MPN/g of fresh or frozen seafood and less than 3 MPN/g of seafood consumed raw; in
249   addition, all products must not contain detectable *Salmonella*, *V. cholerae*, *V. parahaemolyticus*
250   in a 25 g sample (*Bureau of Quality and Safety of Food, 2020*). In this study, the concentration of
251   *E. coli* averaged 2×10^4 MPN/g for all the seafood samples. In fact, only 18% ($n = 60/335$) of all
252   seafood samples had concentrations of *E. coli* < 10 MPN/g and only 7% ($n = 6/82$) of oyster
253   samples (often eaten raw) had < 3 MPN/g. Furthermore, the prevalence of *Salmonella* (36%), *V.*
254   *cholerae* (49%), *V. parahaemolyticus* (59%) indicated widespread bacterial contamination of
255   these seafood products, which also violates food safety standards. Therefore, implementation of
256   basic sanitation and evaluation of microbiological contamination of seafood products sold in
257   Bangkok are needed
258   *Salmonella* is an important pathogen that is responsible for seafood-borne illness
259   worldwide (*Barrett et al., 2017; European Food Safety Authority, 2014*). However, *Salmonella*
260   is not a normal flora in finfish and shellfish products. The major sources of *Salmonella*
261   contamination in seafood include aquatic and aquaculture systems, seafood processing facilities,
262   hygiene practices during transport, storage, and handling (*Amagliani et al., 2012; Fernandes et*
263   *al., 2018*). In this study, the prevalence of *Salmonella* ranged from 14% to 47%. This prevalence
264   was similar to the prevalence of 25% *Salmonella* contamination of shrimp cultured in Vietnam
265   (*Phan et al., 2005*), but substantially less than the 90 to 100% prevalence of *Salmonella*
266   contamination in fish (93%) and shrimp (100%) collected from a market in Indonesia (*Pramono*
267   *et al., 2019*).
268   Type of seafood, sampling retail location, use of ice during retail display, regional source
269   of seafood, and presence of *E. coli* and *Vibrio* were all significantly associated with the presence
270   of *Salmonella* (Table 5). These risk factors should be interpreted with caution given the
271   observational nature of this study and the possibility of undetected confounding in the statistical
272   analysis. The presence of *E. coli* in a seafood sample was associated with a 4-fold increase in the
273   odds of *Salmonella* contamination (OR = 4.0, $P < 0.0001$); similarly, the presence of *V.*
274   *alginolyticus* in a seafood sample was associated with a 1.4-fold increase in the odds of
275   *Salmonella* contamination (OR = 1.4, $P < 0.04$).
276   Seafood displayed on ice during retail had almost twice the odds of *Salmonella*
277   contamination (OR = 1.7, $P < 0.0001$) than seafood not displayed on ice. This may seem
278   counterintuitive, but ice used to chill seafood can be contaminated with pathogenic
279   microorganisms (*Falcão et al., 2009*). Ice can be a vehicle for various pathogenic organisms,
280   including diarrheagenic *E. coli*, *Aeromonas*, *S. enteritidis* and fecal coliforms (*Falcão et al.,*

281   *2002*; *Falcão et al., 2004*; *Kirov, 1993*). In this study, most of the ice used to store seafood was
282   at risk of rapidly melting due to high ambient temperatures in open air conditions. Melting ice
283   can spread bacteria from one seafood item to nearby retail items, readily contaminating other
284   seafood left standing in contaminated melt water.
285   In addition, the physical placement of seafood for display in retail markets can spread
286   bacterial contamination between seafood items if seafood handlers do not practice proper
287   sanitation during handling (i.e., bare hands touching multiple seafood items; not replacing latex
288   or plastic gloves at high enough frequency during retail display placement of seafood items).
289   Therefore, maintaining sanitary conditions during the production, storage, and use of ice to
290   prevent microbial contamination should be closely observed. Implementation of programs for
291   food safety and also for prevention and control of diarrheal diseases have reduced mortality and
292   morbidity rates of diarrheal diseases and strengthened food safety in Thailand (*Food Control*
293   *Division, Food and Drug Administration, Thailand. 2004*).
294   Seafood sourced from Eastern Thailand had a 3.5-higher odds of *Salmonella*
295   contamination than seafood from other regions (OR = 3.5, $P < 0.0001$). The coastal area of
296   Eastern Thailand has concentrated areas of industrialization, agricultural development, and
297   tourism-related urbanization, with major concerns of increased water pollution and resource
298   depletion (*Nitivattananon and Srinonil, 2019*). Wastewater quality is a major concern for this
299   area, especially in Chonburi and Rayong Provinces due to several industrial estates. Moreover,
300   Chonburi, Chachoengsao, and Rayong Provinces have been designated for developing the
301   Eastern Economic Corridor (EEC), so reduction of waste and wastewater is of increasing
302   concern.
303   In this study, the diversity of *Salmonella* serovars varied between the different seafood
304   products. Pacific white shrimp had the greatest diversity, 21 different serovars with prevalence
305   per serovar ranging from 1-3%. Eleven serovars from isolated from oysters, with prevalence per
306   serovar ranging from 1-5% similar to Pacific white shrimp. In contrast, only 5 serovars were
307   isolated from blood cockles, with a similar range of prevalence per serovar of 2-5%. Least
308   diverse were isolates from Asian seabass ~~where~~ only two serovars were recovered, with 52 of 56
309   of these *Salmonella* isolates being Matopeni and the remainder being Paratyphi B. Serotype
310   Matopeni has been reported in aquatic pet shops (*Gaulin et al., 2005*) and in food supplements
311   from Germany (*European Commission, 2018*). The infection of *S.* Matopeni has been reported in
312   Malaysian children (*Lee et al., 2003*). *Salmonella* Paratyphi B in raw tuna sushi imported from
313   Indonesia in 2015 caused 65 foodborne cases in the U.S. (*Centers for Disease Control and*
314   *Prevention, 2018*). *S.* Typhimurium, *S.* Enteritidis, *S.* Typhi, and *S.* Paratyphi B were also
315   detected in fresh fish in Iran (*Rahimi et al., 2013*). *S.* Paratyphi B can be classified as d-tartrate
316   fermenting (dT+) and d-tartrate non-fermenting (dT-) strains. The dT+ strain is less virulent and
317   commonly reported with gastroenteritis, while the dT- strain is associated with paratyphoid
318   fever. The dT+ strain is associated with a significant emerging disease worldwide and of public
319   health concern (*Denny et al., 2007*, *Hassan et al., 2018*). Hence, classification of *S.* Paratyphi B
320   biotype should be further investigated.

321   The common serovars in Pacific white shrimp were Itami (11%, $n = 5/47$) and Leith (9%,
322    $n = 4/47$). Itami was first documented as a new serovar from a traveler to Thailand suffering
323   from gastroenteritis (*Sakazaki et al., 1981*). Itami has also been reported from infected humans in
324   Taiwan (*Kuo et al., 2014*). In contrast to serovars isolated during this study, serovars *S.*
325   Weltevreden, *S.* Tennessee, and *S.* Dessau were isolated from shrimp from the Mekong Delta,
326   Vietnam (*Phan et al., 2005*). The most common *S. enterica* serovar isolated from oysters was
327   Give (19%, $n = 8/43$ isolates), which is different from oysters in the U.S. where Newport was the
328   most common serotype (*Brands et al., 2005*). A previous study in Western Thailand found that
329   the most common serovar in cultured oysters (*C. lugubris* and *C. belcheri*) from Phang Nga
330   Province was Paratyphi B (*Jeamsripong et al., 2018*). This suggests that the distribution of
331   *Salmonella* serovars within Thailand depends on geographical location and type of seafood.
332   Serovar Give is an enteric serotype usually isolated from swine and ruminants, but rarely found
333   in humans (*Higgins et al., 1997*). ~~It is possible that the contamination of Give may be the result~~
334   ~~of livestock or agricultural production near the oyster growing site,~~ *S. enterica* Give has been
335   frequently reported in European national laboratories (*Jansen et al., 2005*). The higher virulence
336   of the Give serovar compared to other non-typhoidal *Salmonella* may explain the higher hospital
337   rate associated with human Give infections (*Girardin et al., 2006*).
338   Even though typhoid and paratyphoid salmonellosis are endemic diseases in Thailand,
339   typhoid fever rates declined, and paratyphoid stabilized from 2003 to 2014 in this nation
340   (*Techasaensiri et al., 2018*). In Thailand, *S. enterica* Weltevreden is commonly reported in
341   human, frozen seafood, frozen ducks, and polluted water (*Bangtrakulnonth et al., 2004*). *S.*
342   Weltevreden, *S.* Stanley, *S.* Anatum, and *S.* Rissen are frequently reported in human from
343   northern and central Thailand (*Prasertsee et al., 2019*; *Sirichote et al., 2010*). Therefore,
344   surveillance and monitoring of oysters due to this ~20% prevalence of *Salmonella*
345   contamination, and fully cooking oysters prior to consumption are both needed to reduce the risk
346   of food-borne *Salmonella* infection from Thai-cultured oysters.
347   In this study, the most common *Salmonella* serovar found in blood cockles was Rissen
348   (33%, $n = 8/24$ isolates), similar to a study in India (*Kumar et al., 2009*), but it should be noted
349   that none of the five different serovars isolated from cockles had a prevalence above 5%.
350   Seafood such as cockles can acquire *Salmonella* from contaminated water or other environmental
351   matrices during aquaculture, processing, shipping, and retail display. Good hygiene and basic
352   sanitation together with proper seafood handling and storage should be performed throughout the
353   food chain (farm to fork) to reduce the risk of seafood-related *Salmonella*.
354   According to BQSF for Thailand seafood for human consumption should have no
355   detectable *V. parahaemolyticus* and *V. cholerae* in 25 g of sample; however, 50-60% of samples
356   contained these bacteria. This high prevalence is consistent with previous work demonstrating
357   that between 2003 and 2015 the prevalence of *V. parahaemolyticus* was 64% in oysters, followed
358   by clams (53%), fish (51%), and shrimp (48%) (*Odeyemi, 2016*). *V. parahaemolyticus*, *V.*
359   *cholerae*, and *V. vulnificus* are important seafood-borne pathogens that cause gastroenteritis in
360   humans. *V. alginolyticus* can cause ear infection and intestinal disease in humans. In this study,

361   the main source of *V. parahaemolyticus* was blood cockles (OR = 9.1, *P* < 0.05), while *V.*
362   *cholerae* was commonly found in Asian seabass (OR = 4.0, *P* > 0.05). *V. parahaemolyticus* and
363   *V. vulnificus* have been reported in bivalves in many countries such as Thailand, China, and
364   Korea (*Changchai & Saunjit, 2014*; *Jiang et al., 2019*; *Ryu et al., 2019*). In this study, shrimp
365   and oysters were predominantly contaminated with *V. vulnificus* and *V. alginolyticus*.
366   *Clostridium perfringens*, *Staphylococcus aureus*, *V. parahaemolyticus*, and *Salmonella*
367   spp. are the leading causes of foodborne illnesses in Thailand (*Bureau of Epidemiology, 2019*).
368   In Thailand, human salmonellosis caused 167 illnesses per 100,000 persons in 2019.
369   Contaminated produce and water have been indicated as important sources of *Salmonella*
370   infection (*Bureau of Epidemiology, 2019*). The trend of *V. cholerae* infection decreased from
371   2.51 to 0.02 cases per 100,000 persons during 2010-2019, and contaminated water and seafood,
372   poor sanitation, and dense housing have been blamed as sources of contamination (*Bureau of*
373   *Epidemiology, 2019*).
374   In this study, the determination of bacterial prevalence and abundance was made using
375   culture-dependent methods. For *Salmonella* spp. and *Vibrio* spp. detection this approach has high
376   accuracy and sensitivity compared with certain molecular techniques (*Almeida et al., 2013;*
377   *Eriksson & Aspan 2007; Hara-Kudo et al., 2001; Mainar-Jaime et al., 2013; Yeung &Thorsen,*
378   *2016*). However, these methods can fail to detect viable but nonculturable state (VBNC) strains.
379   VBNC bacteria can preserve metabolic activity and generate virulent proteins (*Alleron et al.,*
380   *2013; Morishige et al., 2015*). Hence, molecular techniques are recommended to determine
381   bacterial contamination, but unfortunately such equipment, needed supplies and training required
382   to implement these molecular techniques are not readily available to many food safety officers,
383   even in the developed world. In the absence of molecular equipment, the use of an appropriate
384   MPN assay is justified and provides an estimate of bacterial load, along with ease of
385   interpretation of the results. In this study, the determination of bacterial species was made using
386   bacterial phenotyping ~~methods, a culture-based method which~~ was justified given our goals for a
387   highly sensitive assay using readily available reagents ~~which could also estimate the number of~~
388   ~~culturable bacteria in Bangkok seafood commodities. Identification of specific serogroups of~~
389   ~~*Vibrio* spp., virulence factors, and bacterial toxins should be examined in future work.~~
390   *E. coli* concentrations appeared ~~well suited to predicting~~ *Salmonella* contamination of
391   seafood. Based on the Youden index ~~that uses the maximal difference between sensitivity and 1-~~
392   ~~specificity~~ (*Ruopp et al., 2008*), a cutoff value for *E. coli* was 1.3×10^4 MPN/g can be
393   implemented for both monitoring seafood for *Salmonella* contamination and to establish
394   threshold control measures at processing or during retail storage. This cutoff is much higher than
395   the microbiological criteria set forth in the Commission Regulation (EC) No 2073/2005
396   (*European Commission, 2005*), and the BQSF, Thailand (*Bureau of Quality and Safety of Food,*
397   *2020*). This ~~may be because~~ high concentrations of *E. coli* in this sample collection ~~generated a~~
398   ~~high cutoff value to discriminate the presence or absence of *Salmonella* in the samples. Lastly,~~
399   given that the detection of *Salmonella* and *Vibrio* spp. is of similar expense and technical
400   difficulty as quantifying *E. coli* concentrations in seafood matrices, it may be more expeditious

401 and more accurate to focus seafood safety monitoring protocols on *Salmonella* and *Vibrio* spp.
402 detection rather than rely on indicator bacteria like *E. coli* that invariably suffer from false-
403 positive and false-negative signals.
404

Conclusions

406 Finfish and shellfish products sold in Bangkok are contaminated with diverse *Salmonella*
407 serovars and species of *Vibrio*. Although the concentration of *E. coli* predicted *Salmonella*
408 contamination for these seafood samples, the high cutoff value (1.3×10^4 MPN/g) for maximal
409 test accuracy will likely prevent this method from being adopted as a food hygiene surveillance
410 tool. ~~Current Thai BQSF regulations require no more than 10 *E. coli* MPN/g for fresh or frozen~~
411 ~~seafood.~~ Bacterial contamination varied by seafood commodity, with substantial differences
412 between Asian seabass, oysters, blood cockle, and Pacific white shrimp. This may reflect
413 different aquaculturing, harvesting, processing, and retail display practices.
414

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418

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Table 1(on next page)

Factors associated with bacterial contamination of seafood products sold in Bangkok, Thailand.

[1] Row totals of percentages may not add to 100% during to rounding of decimals.

1   **TABLE 1** Factors associated with bacterial contamination of seafood products sold in Bangkok,
2   Thailand.

Risk factors	No. of sample (%)[1]				
	Pacific white shrimp	Oyster	Blood cockle	Asian seabass	Total
Type of seafood	85 (25.4%)	82 (24.5%)	84 (25.1%)	84 (25.1%)	335 (100%)
Sampling district					
Din Daeng	11 (30.6%)	7 (19.4%)	4 (11.1%)	14 (38.9%)	36 (100%)
Huay Kwang	17 (22.4%)	21 (27.6%)	24 (31.6%)	14 (18.4%)	76 (100%)
Samphanthawong	28 (25.5%)	26 (23.6%)	28 (25.5%)	28 (25.5%)	110 (100%)
Dusit	29 (25.7%)	28 (24.8%)	28 (24.8%)	28 (24.8%)	113 (100%)
Regional source of seafood					
Central	65 (25.3%)	53 (20.6%)	72 (28.0%)	60 (26.1%)	257 (100%)
Eastern	3 (10.7%)	0 (0%)	16 (57.1%)	9 (32.1%)	28 (100%)
Southern	10 (40.0%)	6 (24.0%)	5 (20.0%)	4 (16.0%)	25 (100%)
Unidentified	7 (28.0%)	7 (28.0%)	7 (28.0%)	4 (16.0%)	25 (100%)
Retail storage					
Pool	4 (4.9%)	0 (0%)	0 (0%)	78 (95.1%)	82 (100%)
Separate	81 (32.0%)	82 (32.4%)	84 (33.2%)	6 (2.4%)	253 (100%)
Retail display condition					
Without ice	39 (31.2%)	28 (22.4%)	47 (37.6%)	11 (8.8%)	125 (100%)
On ice	46 (21.9%)	54 (25.7%)	37 (17.6%)	73 (34.8%)	210 (100%)

3   [1] Row totals of percentages may not add to 100% during to rounding of decimals.

Table 2(on next page)

Concentrations of fecal coliforms and *E. coli* in seafood products sold in Bangkok, Thailand.

Notes sd, standard deviation.

1   **TABLE 2** Concentrations of fecal coliforms and *E. coli* in seafood products sold in Bangkok,
2   Thailand.

Samples	**Fecal coliforms**		***E. coli***	
	Prevalence (%)	**Average ± sd (MPN/g)**	**Prevalence (%)**	**Average ± sd (MPN/g)**
Shrimp ($n = 85$)	85 (100%)	9.43×10^4 (3.4×10^4)	85 (100%)	1.07×10^4 (2.5×10^4)
Oyster ($n = 82$)	82 (100%)	1.10×10^5 (7.1×10^3)	82 (100%)	5.13×10^4 (4.5×10^3)
Blood cockle ($n = 84$)	84 (100%)	5.71×10^4 (4.8×10^4)	68 (81.0%)	5.85×10^3 (2.2×10^4)
Asian seabass ($n = 84$)	84 (100%)	8.70×10^4 (4.1×10^4)	56 (66.7%)	1.30×10^3 (2.6×10^3)
Total ($n = 335$)	335 (100%)	8.70×10^4 (4.1×10^4)	285 (85.1%)	1.85×10^4 (3.7×10^4)

3

4   **Notes**

5   sd, standard deviation.

Table 3(on next page)

Distribution of *Salmonella* serovars in seafood products sold in Bangkok, Thailand ($n =$ 170 isolates).

1 **TABLE 3** Distribution of *Salmonella* serovars in seafood products sold in Bangkok, Thailand (*n*
2 = 170 isolates).

| | Number of positive (%) | | | | |
Serotype	Shrimp	Oyster	Blood cockle	Asian seabass	Total
Agona	-	-	4 (2.35)	-	4 (2.35)
Aminatu	2 (1.18)	-	-	-	2 (1.18)
Australia	1 (0.59)	-	-	-	1 (0.59)
Bardo	-	4 (2.35)	-	-	4 (2.35)
Bonames	3 (1.76)	-	-	-	3 (1.76)
Breukelen II	-	4 (2.35)	-	-	4 (2.35)
Corvallis	-	4 (2.35)	4 (2.35)	-	8 (4.71)
Dabou	-	4 (2.35)	-	-	4 (2.35)
Dresden	1 (0.59)	-	-	-	1 (0.59)
Enteritidis	3 (1.76)	-	-	-	3 (1.76)
Farmingdale	-	4 (2.35)	-	-	4 (2.35)
Give	-	8 (4.71)	-	-	8 (4.71)
Glidji	-	2 (1.18)	-	-	2 (1.18)
Hisingen	2 (1.18)	-	-	-	2 (1.18)
Itami	5 (2.94)	-	-	-	5 (2.94)
Kentucky	3 (1.76)	-	-	-	3 (1.76)
Konongo	1 (0.59)	-	-	-	1 (0.59)
Lansing	1 (0.59)	-	-	-	1 (0.59)
Leith	4 (2.35)	-	-	-	4 (2.35)
Lexington	-	4 (2.35)	-	-	4 (2.35)
Lezennes	-	-	4 (2.35)	-	4 (2.35)
Linguere	2 (1.18)	-	-	-	2 (1.18)
Matopeni	-	-	-	52 (30.59)	52 (30.59)
Oslo	3 (1.76)	-	-	-	3 (1.76)
Paratyphi B2	-	-	-	4 (2.35)	4 (2.35)
Rissen	-	-	8 (4.71)	-	8 (4.71)
Ruiru	-	4 (2.35)	-	-	4 (2.35)
Stanley	-	-	4 (2.35)	-	4 (2.35)
Stuttgart	2 (1.18)	-	-	-	2 (1.18)
Soerenga	-	1 (0.59)	-	-	1 (0.59)
Tounouma	1 (0.59)	-	-	-	1 (0.59)
Typhimurium	1 (0.59)	-	-	-	1 (0.59)
Victoria	3 (1.76)	-	-	-	3 (1.76)
Windermere	3 (1.76)	-	-	-	3 (1.76)
Weltevreden	-	4 (2.35)	-	-	4 (2.35)

Wohlen	2 (1.18)	-	-	-	2 (1.18)
Yeerongpilly	1 (0.59)	-	-	-	1 (0.59)
II/IIIa/IV	3 (1.76)	-	-	-	3 (1.76)
Total	47 (27.65)	43 (25.29)	24 (14.12)	56 (32.94)	170 (100.00)

3

Table 4(on next page)

Comparing the odds of contamination from *Salmonella*, *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, or *V. alginolyticus* among seafood products sold in Bangkok, Thailand.

Notes. [1] referent category, based on shrimp's popularity in Thai cuisine and largest sample size among the four seafood commodities. OR, Odds ratio. S.E., standard error. C.I., confidence interval.

1 **TABLE 4** Comparing the odds of contamination from *Salmonella*, *V. parahaemolyticus*, *V.*
2 *vulnificus*, *V. cholerae*, or *V. alginolyticus* among seafood products sold in Bangkok, Thailand.

Parameters	OR	S.E.	95% C.I.	*P*-value
Salmonella				
Shrimp[1]	1.0	-	-	-
Oyster	0.684	0.155	0.439-1.065	0.093
Blood cockle	0.188	0.388	0.125-0.281	< 0.0001
Asian seabass	0.975	0.767	0.209-4.555	0.974
Constant	0.889	0.394	0.373-2.121	0.791
V. parahaemolyticus				
Shrimp[1]	1.0	-	-	-
Oyster	0.576	0.096	0.413-0.802	0.001
Blood cockle	9.100	1.686	6.329-13.085	< 0.0001
Asian seabass	0.431	0.276	0.122-1.515	0.189
Constant	1.429	0.277	0.977-2.088	0.065
V. vulnificus				
Shrimp[1]	1.0	-	-	-
Oyster	0.353	0.196	0.119-1.045	0.060
Blood cockle	0.143	0.088	0.043-0.477	0.002
Asian seabass	0.100	0.130	0.008-1.285	0.077
Constant	0.635	0.325	0.233-1.731	0.374
V. cholerae				
Shrimp[1]	1.0	-	-	-
Oyster	0.134	0.060	0.056-0.322	< 0.0001
Blood cockle	0.043	0.045	0.006-0.329	0.002
Asian seabass	3.958	5.020	0.329-47.548	0.278
Constant	2.400	1.596	0.652-8.835	0.188
V. alginolyticus				
Shrimp	1.0	-	-	-
Oyster [1]	1.114	0.418	0.534-2.322	0.774
Blood cockle	0.075	0.087	0.008-0.715	0.024
Asian seabass	0.050	0.042	0.009-0.260	< 0.0001
Constant	0.491	0.131	0.292-0.827	0.007

3 **Notes.**

4 [1] referent category, based on shrimp's popularity in Thai cuisine and largest sample size
5 among the four seafood commodities. OR, Odds ratio. S.E., standard error. C.I., confidence
6 interval.

Table 5(on next page)

Final multivariable logistic regression model for risk factors associated with *Salmonella* contamination of seafood products sold in Bangkok, Thailand.

AIC = 368.67

Notes. [1]reference group. OR, Odds ratio. S.E., standard error. C.I., confidence interval. AIC, Akaike information criterion.

1   **TABLE 5** Final multivariable logistic regression model for risk factors associated with

2   *Salmonella* contamination of seafood products sold in Bangkok, Thailand.

Parameters	OR	S.E.	95% C.I.	*P*-value
Type of seafood				
Shrimp[1]	1.0	-	-	-
Oyster	0.48	0.089	0.331-0.689	< 0.0001
Blood cockle	0.19	0.045	0.119-0.303	< 0.0001
Asian seabass	1.06	0.638	0.328-3.450	0.919
Sampling district				
Din Daeng[1]	1.0	-	-	-
Huay Kwang	1.66	0.232	1.259-2.182	< 0.0001
Samphanthawong	0.43	0.046	0.351-0.532	< 0.0001
Dusit	1.42	0.120	1.205-1.676	< 0.0001
Retail display condition				
No ice[1]	1.0	-	-	-
On ice	1.71	0.201	1.360-2.154	< 0.0001
Regional source of seafood				
Central[1]	1.0	-	-	-
Eastern	3.46	0.670	2.325-5.141	< 0.0001
Southern	0.89	0.061	0.780-1.020	0.094
Unidentified	0.81	0.135	0.581-1.120	0.199
Presence of *E. coli*				
No[1]	1.0	-	-	-
Yes	4.02	1.223	2.213-7.295	< 0.0001
Presence of *V. alginolyticus*				
No[1]	1.0	-	-	-
Yes	1.37	0.212	1.008-1.851	0.044
Presence of *V. vulnificus*				
No[1]	1.0	-	-	-
Yes	0.61	0.130	0.405-0.930	0.021
Constant	0.19	0.051	0.109-0.318	< 0.0001

3   AIC = 368.67

4   **Notes.**

5   [1]reference group. OR, Odds ratio. S.E., standard error. C.I., confidence interval. AIC, Akaike

6   information criterion.

Figure 1

The prevalence of *V. parahaemolyticus* (VP), *V. vulnificus* (VV), *V. alginolyticus* (VA), *V. cholerae* (VC), and *Salmonella* spp. in shrimp (n = 85), oyster (n = 82), blood cockle (n = 84), and
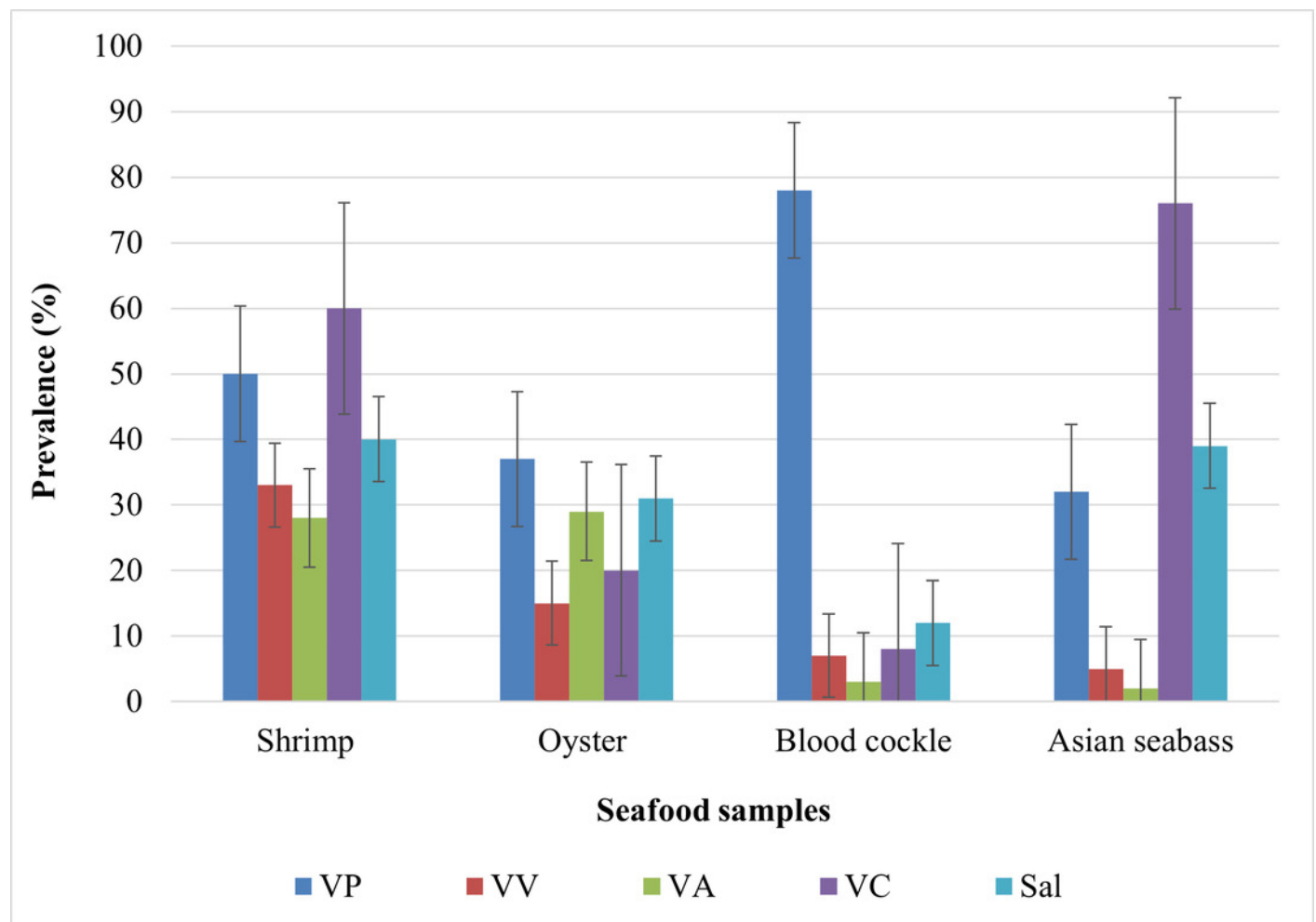
Figure 2

Area under the ROC curve and cutoff value for concentration of *E. coli* were used to maximally predict the contamination of *Salmonella* in retail seafood commodities (*n* = 335).