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Effects of different shrimp extracts on *Vibrio parahaemolyticus* growth and virulence

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Abstract:

***Vibrio parahaemolyticus* is the main causative agent of acute hepatopancreatic necrosis disease (AHPND) in shrimp. This study aimed to investigate how shrimp extracts affect the growth and virulence of an AHPND-causative strain known as *V. parahaemolyticus* XN9. To this end, the bacteria was cultured in media containing 3% extract of each of five shrimp types and their growth kinetics were compared against that from bacteria grown in brain-heart infusion (BHI) media. Eight-hour growth curves were constructed using the plate-counting method. The activity of five extracellular enzymes that contribute to bacterial virulence was examined using the agar-based method. The results showed that *V. parahaemolyticus* XN9's growth was strongly enhanced in all five shrimp extract media with the highest increase (25% greater than the BHI medium) found in the giant tiger prawn extract. Additionally, all the shrimp extracts boosted the extracellular enzymatic activity of *V. parahaemolyticus* XN9, although to different extents. In summary, the shrimp extracts, particularly that from the prawns, not only promoted the viability and growth of *V. parahaemolyticus* XN9 but also its extracellular enzymatic activities.**

Keywords: **extracellular enzymes, growth curve, shrimp extracts, viability, *Vibrio parahaemolyticus*, virulence factors.**

Classification number: **3.5**

Introduction

Vibrio parahaemolyticus is a Gram-negative, halophilic, non-spore-forming, curved, rod-shaped marine bacterium [1]. This bacterium is versatile in its ability to live as a free organism or as a parasite of marine or fresh-water zooplankton, fish, shellfish, and shrimp [2]. It has been reported as the leading food-born gastroenteritis causative agent in humans [3]. In addition, *V. parahaemolyticus* was also identified as the pathogen of AHPND, previously named the early mortality syndrome (EMS), which has caused serious damage to the global shrimp production in the last decade [4]. The disease was first identified in China in 2009 where it quickly spread to Vietnam in 2010, followed by Malaysia in 2011, Thailand in 2012, and finally expanded to the Western hemisphere after that [5]. A Vietnamese study in 2018 showed that this bacterium is highly ubiquitous in water samples (78.1%) and seafood (86.2%) from the Mekong delta [6]. In the region of Hanoi, *V. parahaemolyticus* was also found to be dominantly present in shrimp samples (>90%) [7]. Fortunately, not all of the *V. parahaemolyticus* strains are pathogenic. It has been proven that only a specific set of *V. parahaemolyticus,* those that produce the $PirA^{vp}$/$PirB^{vp}$ toxin, can cause AHPND [4, 8]. The ability to generate this binary toxin is determined by the genes located in a large plasmid residing within the bacterial cells. Genotyping analysis of the plasmid revealed the close relationship of Vietnamese AHPND isolates with the ones from China and Thailand [9]. However, the ability of *V. parahaemolyticus* to cause disease may also have contributions from other virulence factors [5]. Previous data indicated that extracellular enzymes are an important part of the bacterium's virulence [10-13]. Unfortunately, the study of the expression and activity of these enzymes is limited as the experimental outcome is greatly affected by the culture conditions. Many studies have shown that taking *V. parahaemolyticus* out of its inherent natural environment, specifically a shrimp or oyster body, would significantly affect its physiology, growth and virulence when stored or grown in nutrient media [14-18]. Therefore, in this study, we used the extracts of different shrimps that are potential hosts of *V. parahaemolyticus* to investigate the impact of these extracts on the growth and extracellular enzymatic activity of *V. parahaemolyticus*. Five shrimp types were used in the study, including white-leg shrimp (*Litopenaeus vannamei* or formerly called *Penaeus vannamei*), red-leg shrimp (a variety of *Penaeus vannamei*), giant tiger prawn (*Penaeus monodon*), giant river prawn (*Macrobrachium rosenbergii*), and greasyback shrimp (*Metapenaeus ensis*), which are AHPND-affected and major cultured shrimp. The results of this study would assist virulence research of *V. paraheamolyticus*.

Materials and methods

Materials

V. parahaemolyticus XN9 was generously provided by Nha Trang University, Khanh Hoa, Vietnam. The bacterial identity and association to AHPND was confirmed in the previous study [19]. Five kinds of shrimp including white-leg shrimp (*Litopenaeus*

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vannamei), red-leg shrimp (a variety of *Litopenaeus vannamei*), greasyback shrimp (*Metapenaeus ensis*), giant river prawn (*Macrobrachium rosenbergii*), and giant tiger prawn (*Penaeus monodon*) were purchased fresh from the Binh Dien market, Ho Chi Minh city, Vietnam in 2018.

Preparation of shrimp-extract containing media

The preparation of the shrimp extract media was adapted from the chopped meat culture protocol with modifications [20]. In brief, the shrimp were washed in distilled water, then grounded into a paste using blender and cooked at 60°C for 1 h. The obtained shrimp pastes were dried at 80°C and ground again to achieve shrimp extract powders. The drying time was adjusted to be around 3 h so that the net weight of the achieved shrimp extract powder was about 30% of the initial fresh shrimps' weight. The powders were then stored at 4°C for later use. The media was prepared with 3% shrimp extracts and 2.5% NaCl. The control medium to be used was BHI (Himedia, India) with 2.5% NaCl supplemented. All the media were adjusted to pH 7.0.

Growth analysis of V. parahaemolyticus under different shrimp extracts

The plate counting method was used to build the growth curve of *V. parahaemolyticus*. The formula $\mu=(\log_{10} X-\log_{10} X_0)/t\times2.303$ was applied where μ is the specific growth rate (SGR), X is the cell number or cell mass at a certain time, X_0 is the initial cell number or cell mass, and t is the time [21]. In short, an overnight culture at 30°C of *V. parahaemolyticus* XN9 in BHI broth (Himedia, India) with 2.5% NaCl supplemented was used to inoculate 50 ml in either BHI broth (Himedia, India) with 2.5% NaCl supplemented or in the medium containing 3% extract of either white-leg shrimp, red-leg shrimp, greasyback shrimp, giant river prawn, or giant tiger prawn. The initial OD was adjusted to an $OD_{620\ nm}$ of 0.05. The culture was incubated at 30°C under static conditions for 8 h. After each hour, 100 µl of culture was taken out to be plated on BHI agar.

Extracellular enzymatic activity analysis of V. parahaemolyticus under different shrimp extracts

Caseinase, hemolysin, lipase, gelatinase, and chitinase were examined using the agar-based method [22, 23]. To achieve this, *V. parahaemolyticus* XN9 was inoculated and cultured in either BHI broth (Himedia, India) with 2.5% NaCl supplemented or in a medium containing 3% extract of either white-leg shrimp, red-leg shrimp, greasyback shrimp, giant river prawn, or giant tiger prawn. The culture was incubated at 30°C, under static conditions and overnight. After that, the overnight culture was diluted to OD_{620nm}=0.05 and 10 µl of this suspension was dropped onto the surface of the BHI agar containing either 1.5% (w/v) skim milk, 2.0% gelatin (w/v), sheep blood agar, tributyrin agar, or 5% colloidal chitin for caseinase, gelatinase, hemolysin, lipase, and chitinase testing, respectively. The plates were incubated at 30°C overnight for the caseinase and gelatinase testing and for 48 h for the hemolysin, lipase, and chitinase testing. While *Vibrio cholerae* VCTC2012 (obtained from Vietnam type culture collection, kindly provided by Namkhoa Ltd, Vietnam) was used as a positive control for caseinase, gelatinase, hemolysin, and chitinase testing, *Staphylococcus aureus* ATCC 29213 (purchased from Lan Oanh Ltd, Vietnam) was used for lipase testing. After incubation, clear halos around the colonies were observed, which indicated that the activity of the testing enzyme was measurable. In the case of gelatinase, to observe the activity of the enzyme, the agar surface was immersed in saturated ammonium sulfate [$(NH_4)_2SO_4$] (JHD, China) that forms a cloudy precipitate with the remaining gelatin and results in a clear halo zone indicating where the gelatin was digested. The experiments were performed in triplicate.

Data analysis

The enzymatic activity (EA) was calculated as $EA=\frac{D-d}{2}$, in which *D* is the diameter of both the bacterial drop and the zone around the bacterial drop (mm) and *d* is the diameter of the bacterial drop itself (mm). The EA is graded as follows: (–) when no visible halo is present, (+) when the EA value is limited to 1-2 mm, and (++) when the EA value is greater than or equal to 2 mm [22]. Each test was performed in triplicate and the obtained data were analysed using one-way ANOVA (Excel software, Microsoft). The means were also analysed using t-tests ($p<0.05$) to compare the variation between the extracellular enzymatic activity of each test and the corresponding standard condition (2.5% NaCl, pH 8.5, 120 rpm and 30°C).

Results

Growth kinetics of V. parahaemolyticus XN9 in BHI and in media containing different shrimp extracts

The growth curves of *V. parahaemolyticus* XN9 in the different tested media are constructed and presented in Fig. 1. In all the tested media, *V. parahaemolyticus* reached a stationary phase after 5 h. After 8 h, *V. parahaemolyticus* XN9 reached a much higher CFU in all the shrimp extract media compared to the BHI medium (Fig. 1). The growth in BHI was monitored further for more than 24 h in the optimizing step, but no particular

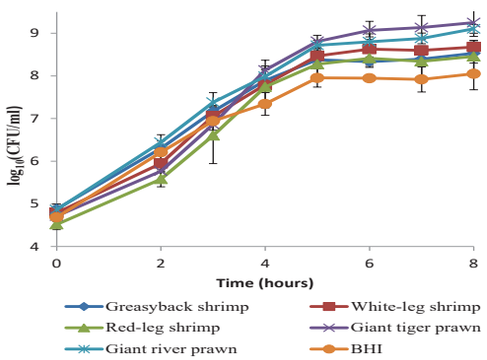

Fig. 1. Growth of *V. parahaemolyticus* XN9 in different media. *V. parahaemolyticus* XN9 was cultured in either BHI or a medium containing either greasyback shrimp, white-leg shrimp, red-leg shrimp, giant river prawn, or giant tiger prawn extract. The culture was carried out at 30°C, under static conditions, and observed for 8 h.

activity was observed after the bacteria reached its stationary phase (data not shown).

The calculated SGR also showed that *V. parahaemolyticus* XN9 grew very well in all shrimp extract media compared to the BHI medium (Table 1). While SGR in BHI was only 1.51±0.05 (h^{-1}), SGR in greasyback shrimp, white-leg shrimp, red-leg shrimp, giant river prawn, and giant tiger prawn media were all higher with SGR values of 1.61±0.01, 1.69±0.04, 1.73±0.10, 1.81±0.03, and 1.89±0.04 (h^{-1}), respectively. Therefore, except for greasyback shrimp, the SGR of *V. parahaemolyticus* XN9 in the other four tested shrimp extracts were significantly higher when compared to the BHI medium ($p<0.05$). The giant tiger prawn was the best medium, which brought about an increase of SGR by 25% followed immediately by giant river prawn at 20%.

Table 1. Effect of different shrimp extracts on specific growth rate (SGR) of *V. parahaemolyticus* XN9. The specific growth rate was calculated after 5 h, just before reaching the stationary phase. The values are expressed in mean ± standard deviation (SD).

Types of medium	SGR (h^{-1})
BHI	1.51±0.05
White-leg shrimp	1.69±0.04
Red-leg shrimp	1.73±0.10
Giant tiger prawn	1.89±0.04
Greasyback shrimp	1.61±0.01
Giant river prawn	1.81±0.03

Extracellular enzymatic activity of V. parahaemolyticus XN9 in BHI and in media containing different shrimp extracts

The extracellular enzymatic activity of *V. parahaemolyticus* (caseinase, gelatinase, lipase, hemolysis, and chitinase) in the presence of 5 different shrimp extracts media were examined. As a result, activity was observed in caseinase, gelatinase, and lipase for all the tested media but no activity was observed in hemolysin and chitinase (Fig. 2). While caseinase and gelatinase showed no significant difference in activity between the BHI and other shrimp extract media, the lipase activity, in contrast, was significantly higher in all shrimp extracts compared to BHI ($p<0.05$). Besides, the giant tiger prawn and giant river prawn, again, were the best media to promote the activity of the extracellular enzymes in *V. parahaemolyticus* (Fig. 2).

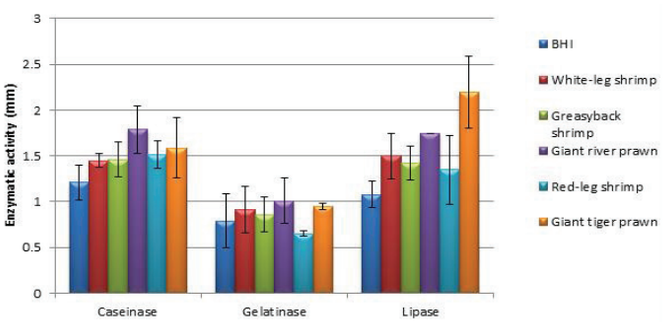

Fig. 2. Extracellular enzymatic activity of *V. parahaemolyticus* XN9 in different tested media. Hemolysin and chitinase did not express activity in any media thus were not shown.

Discussion

V. parahaemolyticus is ubiquitous in marine and estuarine environments. It can colonize multiple aquatic animals and cause disease in these animals and sometimes in humans, too. While the human isolates can survive well with *in vitro* culturing conditions, the strains that cause the disease in shrimp normally do not and require different optimal culture conditions [14, 24]. Their growth and viability are highly affected by culture conditions like salinity, pH, temperature, and medium type [25, 26]. In this study, we saw a significant improvement in the viability and growth of *V. parahaemolyticus* XN9, a shrimp disease-causing strain, when cultured in shrimp extract media compared to the optimal artificial culture medium [14]. Especially, prawn extracts including giant tiger and river prawn were the two extracts that strongly promoted the growth rate of the bacterium. The data suggests that the shrimp-adapted isolates should be cultured and studied using host-extract containing media.

Regarding extracellular enzymatic activity, our results also present strong activity of lipase, caseinase, and gelatinase when *V. parahaemolyticus* was precultured in shrimp extract media. As the number of bacteria was adjusted before the enzymatic testing, the activity of the enzymes seemed to be due to the bacterial cells themselves and not their quantity. The increase of lipase, caseinase, and gelatinase can provide an explanation for the improved growth and viability of *V. parahaemolyticus* when cultured in shrimp extracts. Additionally, the observed increase also implicated that the virulence of *V. parahaemolyticus* can be increased when existing with its hosts. Lipase, for example, was a common virulence factor of *V. parahaemolyticus* with about 80% of *V. parahaemolyticus* having lipase [13], while proteases (caseinase, gelatinase) were shown to be important to the pathogenesis of *Vibrio* [11, 27]. The increase of the enzyme is associated with the increased pathogenicity of the *Vibrio* species [11, 27].

On the other hand, no activity from hemolysin and chitinase was observed in *V. parahaemolyticus* XN9 in our study. Regarding hemolysin of *V. parahaemolyticus*, both thermostable direct hemolysin (TDH) or TDH-related hemolysin (TRH) are considered as major virulence factors of *V. parahaemolyticus* because of their ability to effectively induce cell lysis [2]. However, not all pathogenic strains contained hemolysins and, while 90% of human clinical isolates are hemolysin producers, shrimp disease-causing isolates are not [6, 19, 28]. Chitinase, on the other hand, was not investigated much as a virulence factor in *V. parahaemolyticus*. It seems that the chitinase-encoding gene lies on a prophage-like element and multiple genes are involved in the chitin-degrading ability of *V. parahaemolyticus* [29, 30]. The chitinase activity requires proper conditions to be induced [30] and thus it is understandable if it was not observed under our study settings.

Conclusions

In summary, our study showed that the media containing shrimp extract not only enhanced the viability of *V. parahaemolyticus*

XN9, but also improved its production of virulence factors, especially the giant tiger prawn and giant river prawn extracts. Therefore, it is suggested that shrimp extracts have the potential to replace conventional nutrient ingredients in preparation media, which assists the study of *V. parahaemolyticus* in laboratory conditions.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

[1] C.A. Broberg, T.J. Calder, K. Orth (2011), "*Vibrio parahaemolyticus* cell biology and pathogenicity determinants", *Microbes. Infect.*, **13(12-13)**, pp.992-1001.

[2] D. Ceccarelli, L. Calegari, F. Asconeguy, I.A. Conti-Diaz (2013), "Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors", *Front. Cell. Infect. Microbiol.*, **3**, DOI: 10.3389/fcimb.2013.00097.

[3] E. Scallan, P.M. Griffin, F.J. Angulo, R.V. Tauxe, R.M. Hoekstra (2011), "Foodborne illness acquired in the United States-major pathogens", *Emerg. Infect. Dis.*, **17(1)**, pp.7-15.

[4] C.T. Lee, I.T. Chen, Y.T. Yang, T.P. Ko, Y.T. Huang, J.Y. Huang, M.F. Huang, S.J. Lin, C.Y. Chen, S.S. Lin, D.V. Lightner, H.C. Wang, A.H. Wang, H.C. Wang, L.I. Hor, C.F. Lo (2015), "The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin", *Proc. Natl. Acad. Sci. U.S.A*, **112(34)**, pp.10798-10803.

[5] P. Li, L.N. Kinch, A. Ray, A.B. Dalia, Q. Cong, L.M. Nunan, A. Camilli, N.V. Grishin, D. Salomon, K. Orth (2017), "Acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* strains maintain an antibacterial type VI secretion system with versatile effector repertoires", *Appl. Environ. Microbiol.*, **83(13)**, DOI: 10.1128/AEM.00737-17.

[6] T.T.H. To, Y. Haruka, N.K. Thuan, H.K. Yukiko, T. Takahide, H. Hideki (2018), "Prevalence of *Vibrio parahaemolyticus* in seafood and water environment in the Mekong delta, Vietnam", *J. Vet. Med. Sci.*, **80(11)**, pp.1737-1742.

[7] V.T. Tra, L. Meng, D. Pichpol, P.H. Ngan, M. Baumann, T. Alter, S. Huehn (2016), "Prevalence and antimicrobial resistance of *Vibrio* spp. in retail shrimps in Vietnam", *Berl. Munch. Tierarztl. Wochenschr.*, **129(1-2)**, pp.48-51.

[8] R. Sirikharin, S. Taengchaiyaphum, P. Sanguanrut, T.D. Chi, R. Mavichak, P. Proespraiwong, B. Nuangsaeng, S. Thitamadee, T.W. Flegel, K. Sritunyalucksana (2015), "Characterization and PCR detection of binary, pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp", *PLOS ONE*, **10(5)**, DOI: 10.1371/journal.pone.0126987.

[9] J.E. Han, K.F. Tang, D.V. Lightner (2015), "Genotyping of virulence plasmid from *Vibrio parahaemolyticus* isolates causing acute hepatopancreatic necrosis disease in shrimp", *Dis. Aquat. Organ.*, **115(3)**, pp.245-251.

[10] K. Kadokura, A. Rokutani, M. Yamamoto, T. Ikegami, H. Sugita, S. Itoi, W. Hakamata, T. Oku, T. Nishio (2007), "Purification and characterization of *Vibrio parahaemolyticus* extracellular chitinase and chitin oligosaccharide deacetylase involved in the production of heterodisaccharide from chitin", *Appl. Microbiol. Biotechnol.*, **75(2)**, pp.357-365.

[11] S. Miyoshi (2013), "Extracellular proteolytic enzymes produced by human pathogenic *vibrio* species", *Front. Microbiol.*, **4**, DOI: 10.3389/fmicb.2013.00339.

[12] R. Wang, L. Sun, Y. Wang, Y. Deng, Z. Fang, Y. Liu, Q. Deng, D. Sun, R. Gooneratne (2018), "Influence of food matrix type on extracellular products of *Vibrio parahaemolyticus*", *BMC Microbiol.*, **18(1)**, DOI: 10.1186/s12866-018-1207-7.

[13] R.A. Costa, L.M. Conde Amorim, R.L. Araujo, R.H. dos Fernandes Vieira (2013), "Multiple enzymatic profiles of *Vibrio parahaemolyticus* strains isolated from oysters", *Rev. Argent. Microbiol.*, **45(4)**, pp.267-270.

[14] P.T.L. Anh, L.Q. Khang, N.T. Thuc, D.N.P. Chau, N.T.T. Hoai (2018), "Optimizing conditions for *Vibrio parahaemolyticus* culture and preservation", *7th International Conference on the Development of Biomedical Engineering in Vietnam*, pp.681-684.

[15] H. Fujikawa, B. Kimura, T. Fujii (2009), "Development of a predictive program for *Vibrio parahaemolyticus* growth under various environmental conditions", *Biocontrol. Sci.*, **14(3)**, pp.127-131.

[16] B. Liu, H. Liu, Y. Pan, J. Xie, Y. Zhao (2016), "Comparison of the effects of environmental parameters on the growth variability of *Vibrio parahaemolyticus* coupled with strain sources and genotypes analyses", *Front. Microbiol.*, **7**, DOI: 10.3389/fmicb.2016.00994.

[17] W.B. Whitaker, M.A. Parent, L.M. Naughton, G.P. Richards, S.L. Blumerman, E.F. Boyd (2010), "Modulation of responses of *Vibrio parahaemolyticus* O3:K6 to pH and temperature stresses by growth at different salt concentrations", *Appl. Environ. Microbiol.*, **76(14)**, pp.4720-4729.

[18] S.A. Soto-Rodriguez, R.L. Olvera, D.A. Palacios-Gonzalez, C. Bolan-Mejia, K.G. Aguilar-Rendon (2019), "Characterization and growth conditions of *Vibrio parahaemolyticus* strains with different virulence degrees that cause acute hepatopancreatic necrosis disease in *Litopenaeus vannamei*", *J. World Aquacult. Soc.*, **50(4)**, pp.1-14.

[19] N.N. Vu, P.T.T. Huyen, L.N.M. Tien, D.N.P. Chau, H. Tung, N.T.T. Hoai (2017), "Investigating the production of extracellular enzymes of various *Vibrio parahaaemolyticus* isolates in Vietnam", *J. Biotechnol.*, **15(4)**, pp.703-710.

[20] M.K. Bacic, C.J. Smith (2008), "Laboratory maintenance and cultivation of bacteroides species", *Curr. Protoc. Microbiol.*, **13**, DOI: 10.1002/9780471729259.mc13c01s9-13C.1.

[21] I. Pepper, C. Gerba, J. Brendecke (2011), *Environmental Microbiology: A Laboratory Manual, 2nd Edition*, Elsevier, 232pp.

[22] A.B. Vermelho, M.N. Meirelles, A. Lopes, S.D. Petinate, A.A. Chaia, M.H. Branquinha (1996), "Detection of extracellular proteases from microorganisms on agar plates", *Mem. Inst. Oswaldo. Cruz.*, **91(6)**, pp.755-760.

[23] K. Ohishi, K. Murase, T. Ohta, H. Etoh (2000), "Cloning and sequencing of a chitinase gene from *Vibrio alginolyticus* H-8", *J. Biosci. Bioeng.*, **89(5)**, pp.501-505.

[24] R. Paranjpye, O.S. Hamel, A. Stojanovski, M. Liermann (2012), "Genetic diversity of clinical and environmental *Vibrio parahaemolyticus* strains from the Pacific Northwest", *Appl. Environ. Microbiol.*, **78(24)**, pp.8631-8638.

[25] J. Lee (2009), *Laboratory Studies of Growth Conditions of Vibrio parahaemolyticus in Pacific Oyster (Crassostrea gigas) with International Considerations to Shellfish-associated Illnesses in South Korea*, An Undergraduate Thesis, Oregon State University, 153pp.

[26] J.A. Gooch, A. DePaola, J. Bowers, D.L. Marshall (2002), "Growth and survival of *Vibrio parahaemolyticus* in postharvest American oysters", *J. Food Prot.*, **65(6)**, pp.970-974.

[27] G. Osei-Adjei, X. Huang, Y. Zhang (2018), "The extracellular proteases produced by *Vibrio parahaemolyticus*", *World J. Microbiol. Biotechnol.*, **34(5)**, DOI:10.1007/s11274-018-2453-4.

[28] P. Raghunath (2014), "Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*", *Front. Microbiol.*, **5**, DOI:10.3389/fmicb.2014.00805.

[29] D. Castillo, K. Kauffman, F. Hussain, P. Kalatzis, N. Rorbo, M.F. Polz, M. Middelboe (2018), "Widespread distribution of prophage-encoded virulence factors in marine *Vibrio* communities", *Sci. Rep.*, **8(1)**, DOI: 10.1038/s41598-018-28326-9.

[30] T. Hirano, M. Okubo, H. Tsuda, M. Yokoyama, W. Hakamata, T. Nishio (2019), "Chitin heterodisaccharide, released from chitin by chitinase and chitin oligosaccharide deacetylase, enhances the chitin-metabolizing ability of *Vibrio parahaemolyticus*", *J. Bacteriol.*, **201(20)**, DOI: 10.1128/JB.00270-19.