JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ISOLATION OF POTENTIAL PROBIONTS FROM BRACKISHWATER ENRICHED WITH HIGH LEVELS OF CARBON SOURCE

Christopher Marlowe A. Caipang[1,5], Kathleen Mae P. Trebol[2], Indra Suharman[3], Rolando V. Pakingking Jr.[4], Joel E. Deocampo Jr.[5]*

Address(es):
[1] Division of Biological Sciences, College of Arts and Sciences, University of the Philippines Visayas, Miag-ao 5023, Iloilo, Philippines.
[2] Marmi Agricultural Corporation, Silay City, Negros Occidental, Philippines.
[3] Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Universitas Riau, Pekanbaru-Riau, Indonesia.
[4] Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFDEC/AQD), Tigbauan, Iloilo 5021, Philippines.
[5] Department of Biology, College of Liberal Arts, Sciences, and Education, University of San Agustin, Iloilo City 5000, Philippines.

*Corresponding author: cmacaipang@gmail.com

https://doi.org/10.55251/jmbfs.9819

ARTICLE INFO

Received 18. 1. 2023
Revised 8. 7. 2023
Accepted 1. 8. 2023
Published 1. 12. 2023

Regular article

OPEN ACCESS

ABSTRACT

The majority of shrimp producers utilize probiotics derived from terrestrial sources as part of their aquaculture management. The beneficial effects of terrestrial probiotics on shrimp may be affected due to environmental differences between the cultivated species and the source of the probiotics. To ensure maximum effects on the host, it is essential to use probionts derived from the host or the environment of the cultured organism. Consequently, the objective of this study was to isolate and characterize potential probionts from brackishwater by enriching the water with organic sources containing a high ratio of carbon to nitrogen (C:N). Six 10-li containers were filled with brackishwater from an estuary for a mesocosm experiment. To stimulate bacterial growth, water was enriched with either molasses or brown sugar at a C:N ratio of 15. After twenty days, all heterotrophic bacteria in the enriched water were enumerated. The *in vitro* antagonistic activities of distinct bacterial colonies against *Vibrio harveyi*, a crustacean pathogen, were evaluated on fresh Nutrient Agar plates containing 1% sodium chloride. There were 10 bacterial isolates with *in vitro* antibacterial activity. These bacterial isolates are categorized as belonging to the putative genera *Acinetobacter*, *Pseudomonas*, *Sphingobium*, and *Rheinheimera*. The implications of this study suggest that enriching brackishwater with organic carbon sources at high C:N ratios may increase the likelihood of isolating and developing potential probionts for shrimp aquaculture.

Keywords: aquaculture, biofloc, disease control, organic matter, shrimp

INTRODUCTION

Biofloc technology (BFT) is a cutting-edge technology anchored on zero water exchange and recycling of wastes generated within a cultured system. BFT is a promising technology for ensuring the sustainability of marine shrimp aquaculture in shrimp culture **(Krummenauer *et al.*,** 2011; **Ferreira *et al.*,** 2015). The bacterial community that maintains stable levels of nutrients in the water is crucial to BFT-based shrimp aquaculture systems (**Wasielesky *et al.*,** 2006). This bacterial community associated with bioflocs in the culture system can inhibit the growth and proliferation of pathogens through competitive exclusion (**Crab *et al.*,** 2010), as well as supplement the nutrition of the cultured stock (**McIntosh *et al.*,** 2000). However, this microbial community may also harbor pathogenic and opportunistic bacteria that are harmful to farm animals (**Schulze *et al.*,** 2006).

Vibrios stand out among the opportunistic bacteria in the marine environment (**Song & Lee**, 1983). *Vibrio harveyi* and *V. parahaemolyticus* are members of the Vibrionaceae family, which is linked to a number of bacterial diseases in crustaceans, including luminous vibriosis, early mortality syndrome (EMS), and acute hepatopancreatopancreatic necrosis syndrome (AHPNS) (**Leaño & Mohan**, 2012). Vibrios are opportunistic bacteria that influence the growth and survival of farmed shrimp at various life stages (**Costa *et al.*,** 2008). Consequently, the threat posed by Vibrios during shrimp culture is evident. It is essential to manipulate the rearing water to promote the dominance of beneficial bacteria, which in turn reduces the population of these opportunistic bacterial pathogens that threaten the growth and health of cultured shrimp.

Though recently used in aquaculture, most of the probiotics that fish and shrimp farmers utilize are of terrestrial origins (**Lazado & Caipang, 2014**). There are differences in the environment of the shrimp and the source of the probionts; thus, the beneficial effects of terrestrial probiotics may be affected when applied during the culture of shrimp. In view of these limitations, there is a need to explore the possibility of identifying and characterizing host-derived probionts or from the environment of the cultured organism to ensure higher efficiency of the probionts (**Lazado & Caipang, 2014; Zorriehzahra *et al.*, 2016**). Hence, this study aimed to isolate and characterize potential probionts from brackishwater that is used in shrimp culture by enriching the water with organic sources with high carbon to nitrogen (C:N) ratio. These organic carbon sources include the use of brown sugar and molasses in stimulating biofloc production in brackishwater for the isolation of bacterial isolates that can be further developed as probiotics in shrimp aquaculture.

MATERIAL AND METHODS

Collection of water samples and screening of bacterial isolates

This research was conducted in the Biology laboratory of the University of San Agustin College of Liberal Arts, Sciences, and Education in
Iloilo City, Philippines. Brackishwater samples from Iloilo River were transported to the laboratory and poured in six 10-li plastic containers with a capacity of 80%. At the time of sampling, the salinity was 28 ppt and the pH of the water was 7.8. The production of biofloc was accomplished by adding either brown sugar or molasses in triplicate containers at a carbon-to-nitrogen (C: N) ratio of 15 in accordance with the procedures of **Caipang *et al.*** (2022), with minor modifications for small tank systems. Biofloc was maintained in all containers for a duration of 25 days with minimal water exchange.

The biofloc culture water from treatments containing either brown sugar or molasses was serially diluted and plated onto nutrient agar (NA) containing 1% sodium chloride (NaCl). The agar plates were incubated between 27 and 30 degrees Celsius for twenty-four hours. Individual bacterial colonies with diverse morphological characteristics were restreaked onto fresh NA-1% NaCl plates, incubated at 27-30ºC for 24 hours, and stored at 8ºC until further characterization. *Vibrio harveyi*, a known crustacean pathogen, was isolated in a previous study (**Pakingking *et al.*,** 2018). A single colony of this bacterium was inoculated into 10 ml of nutrient broth (NB) supplemented with 1% NaCl and cultivated overnight at 27-30 degrees Celsius with gentle shaking. The overnight culture of the bacterium was diluted with normal saline solution (NSS) to a concentration of 10^3 colony forming units (CFU) ml^{-1}, and 100 µl of the bacterial suspension was plated onto a NA-1% NaCl plate and allowed to adhere for 1 hour. Following this was the spot on-lawn assay (**Pilet *et al.*,** 1995) with individual bacterial colonies obtained from the biofloc water. The agar plates were incubated at 27-30 ° C for 24 hours and then the zones of inhibition were observed.

Characterization of bacterial isolates

The bacterial isolates that exhibited inhibition zones on NA plates seeded with *V. harveyi* were subjected to standard morphological, physiological, and biochemical assays. The phenotypic characters and biochemical properties of the different bacterial isolates were determined based on the descriptions provided in the Bergey's Manual of Systematic Bacteriology **(Holt *et al*., 2000**).

Molecular identification of the isolates was accomplished by extracting bacterial genomic DNA from an overnight culture of the isolates in 5 ml Trypticase Soy Broth (TSB) using a commercial kit (Purelink Genomic DNA Mini, Thermo Fisher Scientific, California, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the 16S rRNA was done using the eubacterial universal primers (Forward: GAGAGTTTGATCCTGGCTCAG; Reverse: CTACGGCTACCTTGTTACGA) of **Bianciotto *et al*.** (2003) in a 25 µL PCR reaction. This consisted of: 2 µL (10-15 ng) of DNA as the template, 2 µL of each primer (5 pmol), 2.5 µL of 10 PCR buffer, 1.5 µL of 2 mM dNTP, 1µL of 50 mM $MgCl_2$ and scaled up to the desired volume using distilled water. The PCR conditions described by **Caipang *et al*.** (2010) were utilized for the amplification. The PCR products were cleaned and sent for sequencing (Macrogen, Korea). Using publicly available data from NCBI GenBank (blast.ncbi.nlm.nih.gov), sequenced data were aligned and analyzed to determine the closest homolog of bacterial isolates.

Co-incubation assay

The anti-*Vibrio harveyi* activity of the bacterial isolates was determined utilizing a modified co-incubation assay (**Caipang *et al*.**, 2008). Using normal saline solution (NSS) as a diluent, an overnight culture of *V. harveyi* and the putative probiotics were diluted to a concentration of 10^3 CFU ml^{-1}. In a 1.5 ml microfuge tube, 100 µl of each probiotic isolate and *V. harveyi* were transferred and thoroughly mixed. The control consisted of *V. harveyi* added to an equal volume of nutrient broth containing 1% NaCl. All mixtures were placed on a rotary agitator and incubated between 27 and 30 degrees Celsius for 24 hours.

Following a 24-hour incubation, a 10-fold serial dilution of each mixture was prepared, and 100 µl aliquots of each dilution were plated onto Thiosulfate–citrate–bile salts–sucrose (TCBS) agar plates for the counting of *V. harveyi*. The probiotic candidates did not grow on TCBS plates, as determined by a preliminary assay; therefore, only *V. harveyi* grew on TCBS plates. The agar plates were placed in an incubator at 27 to 30 degrees Celsius for 24 hours. Colonies of bacteria were enumerated, and the results were expressed as $\log_{10}$ CFU ml^{-1}. Following the procedures outlined by **Caipang *et al.*** (2008), the reduction in *V. harveyi* counts in the co-incubation groups was expressed as a percentage reduction relative to the *V. harveyi* count in the control group. Each experiment was performed three times. Bactericidal activity was defined as a decrease in bacterial counts of at least 1.5 $\log_{10}$ units.

Data analyses

Co-incubated samples and the control were compared using one-way ANOVA (Systat version 8; Systat Software Inc., San Francisco, CA, U.S.A.). Bacterial counts were expressed as $\log_{10}$ CFU ml^{-1}. If the differences were significant, the Tukey test was further used for the analysis. All statistical calculations were performed at a significance level of 0.05.

RESULTS AND DISCUSSION

Bacterial isolates from bracksihwater enriched with high carbon source

A total of 200 bacterial isolates were obtained from brackishwater with biofloc using either molasses or brown sugar as sources of carbon. From these isolates, there were 10 bacterial strains that possessed *in vitro* antagonistic activity against *V. harveyi* as shown by the zones of inhibition on the NA plates. Molecular identification showed that five isolates, namely, *Acinetobacter* (2 isolates), *Rheinheimera*, and *Pseudomonas* (2 isolates) were obtained from biofloc water with molasses as carbon source, while *Sphingobium*, *Pseudomonas* and three isolates of *Acinetobacter* were identified from biofloc water with brown sugar as carbon source (Table 1).

Bioflocs are composed of flocculated organic matter colonized by heterotrophic bacteria, filamentous cyanobacteria, dinoflagellates, ciliates, flagellates and rotifers (**Ballester *et al*., 2007**; **Avnimelech, 2009**). This diverse microbial community includes pathogenic and opportunistic bacteria, as well as neutral and beneficial bacteria (**Schulze *et al*., 2006**; **Ferreira *et al*., 2015**). A previous study by **Anand *et al.*** (2014) used wheat flour as carbon source in the production of biofloc at a C:N ratio of 10:1. Their results showed that microbial succession in the shrimp ponds occurred and the main microorganisms were *Vibrio*, *Lactobacillus*, *Bacillus* and some fungal species. This indicates that dominant bacterial isolates can be identified in biofloc water during enrichment with carbon sources at higher C:N ratio.

Table 1 Bacterial isolates obtained from biofloc water enriched with either molasses or brown sugar as carbon source

Isolate Number	Source	Closest match
SB-C1-2021	Biofloc with molasses	*Acinetobacter indicus*
SB-C2-2021	Biofloc with molasses	*Acinetobacter indicus*
SB-C3-2021	Biofloc with molasses	*Rheinheimera* sp.
SB-C4-2021	Biofloc with molasses	*Pseudomonas aeruginosa*
SB-C5-2021	Biofloc with molasses	*Pseudomonas plecoglossicida*
SB-C6-2021	Biofloc with brown sugar	*Sphingobium* sp.
SB-C7-2021	Biofloc with brown sugar	*Acinetobacter* sp.
SB-C8-2021	Biofloc with brown sugar	*Pseudomonas stutzeri*
SB-C9-2021	Biofloc with brown sugar	*Acinetobacter* sp.
SB-C10-2021	Biofloc with brown sugar	*Acinetobacter* sp.

Table 2 Morphological characteristics of the bacterial isolates obtained from biofloc water enriched with either molasses or brown sugar as carbon source

Characteristics	Isolate Number									
	1	2	3	4	5	6	7	8	9	10
Gram stain	-	-	-	-	-	-	-	-	-	-
Cell Shape[1/]	CB	CB	CB	B	B	CB	CB	B	CB	CB
Colony description										
1. Margin										
Entire (smooth)	+	+	+	+	+	+	+	+	+	+
Undulate (wavy)										
2. Colour										
Orange			+						+	+
Opaque or white	+	+					+			
Milky (yellowish)				+	+	+		+		
3. Elevation										
Flat										
Convex	+	+	+	+	+	+	+	+	+	+
4. Texture										
Slimy,Moist										
Matte	+	+	+				+		+	+
Mucoid				+	+	+		+		
5. Shape										
Round	+	+	+		+		+			
Punctiform				+		+		+	+	+
6. Motility	-	-	-	+	+	-	-	+	-	-
7. Endospore	-	-	-	-	-	-	-	-	-	-
8. Acid fast	-	-	-	-	-	-	-	-	-	-

Legend: [1/]CB- coccobacillus; B – bacillus

Characterization of bacterial isolates

All of the identified bacterial isolates were either rod-shaped or coccobacilli and Gram-negative (Table 2). The biochemical characterization of these isolates revealed that they contained catalase (Table 3). In addition, all isolates, with the exception of isolates 9 and 10 (*Acinetobacter*), can ferment carbohydrates. The addition of higher quantities of carbon sources to the rearing water could be responsible for the isolation of sugar-fermenting bacteria, as these bacteria catabolize the complex carbon molecules present in the water as their population increases. Acetoin was only found in isolates 9 and 10. *Bacillus* species, lactic acid-producing bacteria, and members of the Enterobacteriaceae family are well-known acetoin-producing microorganisms (**Xiao & Lu**, 2014). *Bacillus* is the most well-known acetoin producer among these families due to its high production efficiency and safety. Isolates 9 and 10 are putative *Acinetobacter* in this study. Though this bacterial group is not known for its ability to produce acetoin, **Lee *et al.*** (2009) isolated *Acinetobacter* capable of acetoin production from tobacco plant roots during the screening of antiviral substances that possessed inhibitory effects against Tobacco mosaic virus (TMV).

Table 3 Biochemical characteristics of the bacterial isolates obtained from biofloc water enriched with either molasses or brown sugar as carbon source

Biochemical Characteristics	Isolate Number									
	1	2	3	4	5	6	7	8	9	10
β-galactosidase						+			+	+
Arginine dihydrolase					+					
Citrate utilization				+	+			+		
Detection of acetoin									+	+
Gelatinase			+	+				+		
Fermentation of glucose				+	+	+		+		
Fermentation of melibiose				+	+			+		
Fermentation of arabinose	+	+	+	+			+	+		
Oxidase				+	+			+		
Catalase	+	+	+	+	+	+	+	+	+	+

Reduction of *Vibrio harveyi* in a co-incubation assay

Table 4 shows the counts of *V. harveyi* on NA plates when co-incubated with various bacterial isolates. Using a co-incubation assay, all 10 isolates significantly reduced the population of *V. harveyi in vitro*. Except for isolates 8 and 10, co-incubation with the bacterial isolates resulted in at least a 1.5 $\log_{10}$ reduction of *V. harveyi*. Similarly, the population of *V. harveyi* was reduced by 17.7% to 23.9% relative to the control in the co-incubation assay. With the exception of isolates 8, 9, and 10, all other isolates reduced *V. harveyi* by more than 20%.

Sphingobium is a recently described genus (**Takeuchi *et al.***, 2001) that is thought to be a component of the crustacean intestinal microbiota (Hu et al., 2019). This bacterium has been previously isolated from both untreated and treated water (**Sheu *et al.***, 2013; **Corre *et al.***, 2019) as well as the gut of Pacific white shrimp fed a yeast cell wall-based feed additive (**Servin Arce *et al.***, 2021). This group of bacteria can degrade polycyclic aromatic hydrocarbons; therefore, it is utilized in soil bioremediation (**Chen *et al.***, 2016). It was also isolated from an aquaponics system and linked to antibiotic resistance in an aquaculture facility (**Colombo *et al.***, 2016). In contrast, **Brisou & Prévot** (1954) were the first to describe *Acinetobacter*, a genus of non-motile, aerobic, Gram-negative bacteria. Some species of *Acinetobacter*, specifically *A. baumannii*, are regarded as human and marine pathogens (**Bergogne-Berezin & Towner**, 1996; **Xia *et al.***, 2008). Recent studies, however, have shown its denitrifying activity in the elimination of nitrite in wastewaters (Cao et al., 2012) and its probiotic activities for juvenile catfish by enhancing lysozyme and respiratory activities (**Bunnoy *et al.***, 2019). While the present investigation demonstrated *in vitro* anti-*V. harveyi* activity of *Acinetobacter* sp., **Verschuere *et al.*** (2000) recommend evaluating this bacterial isolate for pathogenicity if it is to be used as probiotics in shrimp aquaculture in the future.

The presence of *Pseudomonas*, *Acinetobacter*, *Sphingobium*, and *Rheinheimera* in brackishwater that had been supplemented with high carbon sources may indicate the significance of these substances in promoting the growth and dominance of beneficial bacterial species that possess anti-*V. harveyi* activities. Particularly, *Pseudomonas* and *Acinetobacter* were isolated from brackish water enriched with brown sugar and molasses. These bacterial isolates are capable of producing substances with antagonistic activity against *V. harveyi*, or they can directly compete with the bacterial pathogen via mechanisms such as competition, exclusion, and displacement (**Lazado *et al.***, 2011; **El-Saadony *et al.***, 2020). Since the bacterial pathogen and probiotic candidates were introduced at comparable concentrations at the same time, the reduction in *V. harveyi* counts is likely due to competition, according to **Lazado *et al.*** (2011). However, a previous study in fish demonstrated that displacement is also one of the mechanisms that inhibited the proliferation of bacterial pathogens in the gut (**Lazado *et al.***, 2011). Future research will investigate what specific mechanism is responsible for inhibiting shrimp bacterial pathogens in water.

Table 4 *Vibrio harveyi* counts* and their reduction[a], expressed as Mean ± SD, during a co-incubation assay with the different bacterial isolates

	Isolate										
	1	2	3	4	5	6	7	8	9	10	Control
Vibrio harveyi counts (Log_{10} CFU/ml)	6.26 ± 0.05	6.23 ± 0.21	6.24 ± 0.06	6.30 ± 0.02	6.37 ± 0.32	6.46 ± 0.41	6.43 ± 0.31	6.74 ± 0.23	6.63 ± 0.25	6.71 ± 0.02	8.19 ± 0.31
Reduction in *V. harveyi* counts (%) relative to Control	23.6 ± 0.05	23.9 ± 0.21	23.8 ± 0.06	23.1 ± 0.02	22.2 ± 0.32	21.2 ± 0.41	21.5 ± 0.31	17.7 ± 0.23	19.0 ± 0.25	18.1 ± 0.02	

Legend: *Co-incubation of the bacterial isolates with *V. harveyi* (pathogen) had significantly lower counts of the pathogen than the control at $p < 0.05$. N=3.
[a]Reduction of *V. harveyi* co-incubated with the different bacterial isolates was computed relative to the population of *V. harveyi* grown in Nutrient Broth supplemented with 1% NaCl.

CONCLUSION

In conclusion, the addition of high carbon sources to brackish water facilitated the isolation of bacterial isolates with potential probiotic applications in shrimp aquaculture. The isolation of *Acinetobacter* and *Pseudomonas* from brackishwaters enriched with brown sugar and molasses suggests that these bacteria could be the dominant isolates in a biofloc system. The ability of the various bacterial isolates to inhibit *in vitro* the proliferation of *V. harveyi*, a known crustacean bacterial pathogen, demonstrates their probiotic potential. If these isolates are to be used as probionts in shrimp culture, it is necessary to conduct additional research into their probiotic mechanisms.

Acknowledgments: This work was supported by the research project, "Biofloc-based Nursery Tank Production of Shrimp for Quality and Sustainable Supply of Aquaculture Products in the New Normal" funded by the Department of Science and Technology (DOST) through the Science for Change Program (S4CP) – Collaborative Research and Development to Leverage Philippine Economy (CRADLE) and monitored by DOST – Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (PCAARRD) with Project Number 8444 awarded to CMA Caipang. The authors of this paper also appreciate the support provided by their respective institutions; namely, the University of the Philippines Visayas, Marmi Agricultural Corporation, SEAFDEC Aquaculture Department, Universitas Riau and the University of San Agustin during the preparation of the manuscript.

Data Availability: The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest: No conflict of interest declared.

REFERENCES

Anand, P. S., Kohli, M. P. S., Kumar, S., Sundaray, J. K., Roy, S. D., Venkateshwarlu, G., Sinha, A., & Pailan, G. H. (2014). Effect of dietary supplementation of biofloc on growth performance and digestive enzyme activities in *Penaeus monodon*. *Aquaculture, 418*, 108-115. https://doi.org/10.1016/j.aquaculture.2013.09.051

Avnimelech, Y. (2009). Biofloc Technology-a Practical Guide Book. The World. Aquaculture Society, Baton Rouge.

Ballester, E. L. C., Wasielesky, W., Cavalli, R. O., & Abreu, P. C. (2007). Nursery of the pink shrimp *Farfantepenaeus paulensis* in cages with artificial substrates: biofilm composition and shrimp performance. *Aquaculture, 265*, 355–362. https://doi.org/10.1016/j.aquaculture.2007.04.003

Bergogne-Berezin, E., & Towner, K. J. (1996). *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical and epidemiological features. *Clinical Microbiology Reviews , 9*, 148-165

Brisou, J., & Prévot, A. R. (1954). Études de systématique bactérienne. X. Révision des espèces reunites dans le denre *Achromobacter*. *Annales de l'Institut Pasteur (Paris), 86*, 722-728.

Bunnoy, A., Na-Nakorn, U., Kayansamruaj, P., & Srisapoome, P. (2019). *Acinetobacter* Strain KUO11TH, a unique organism related to *Acinetobacter pittii* and isolated from the skin mucus of healthy bighead catfish and its efficacy against several fish pathogens.. *Microorganisms, 7 (11)*, 549. https://doi.org/10.3390/microorganisms7110549

Caipang, C. M. A., Brinchmann, M. F., & Kiron, V. (2010). Antagonistic activity of bacterial isolates from intestinal microbiota of Atlantic cod, *Gadus morhua*, and an investigation of their immunomodulatory capabilities. *Aquaculture Research, 41*, 249-256. https://doi.org/10.1111/j.1365-2109.2009.02327.x

Caipang, C. M. A., Hynes, N., Brinchmann, M. F., Korsnes, K., & Kiron, V. (2008). Antimicrobial activity in serum of Atlantic cod *Gadus morhua* L.: comparison between cultured and wild fish. *Journal of Fish Biology, 73*, 115-122. https://doi.org/10.1111/j.1095-8649.2008.01914.x

Caipang, C.M., Trebol, K.M.P., Abeto, M.J.S., Coloso, R.M., Pakingking, R., Calpe, A.T. & Deocampo Jr, J.E. (2022). An innovative biofloc technology for the nursery production of Pacific whiteleg shrimp, Penaeus vannamei in tanks. *International Journal of Biosciences, 21*(4), 71-79. DOI: http://dx.doi.org/10.12692/ijb/21.4.71-79

Cao, H., Wang, H., He, S., Ou, R., Hou, S., & Yang, X. (2012). Isolation and characterization of a denitrifying *Acinetobacter baumannii* H1 using NO2--N as nitrogen source from shrimp farming ponds. *African Journal of Microbiology Research, 6*, 2258-2264.

Chen, C., Lei, W., Lu, M., Zhang, J., Zhang, Z., Luo, C, Chen, Y., Hong, Q., & Shen, Z. (2016). Characterization of Cu (II) and Cd (II) resistance mechanisms in *Sphingobium* sp. PHE-SPH and *Ochrobactrum* sp. PHE-OCH and their potential application in the bioremediation of heavy metal-phenanthrene co-contaminated sites. *Environmental Science and Pollution Research, 23*, 6861-6872. https://doi.org/10.1007/s11356-015-5926-0

Colombo, S., Arioli, S., Guglielmetti, S., Lunelli, F., & Mora, D. (2016). Virome-associated antibiotic-resistance genes in an experimental aquaculture facility. *FEMS Microbiology Ecology, 92*, fiw003. https://doi.org/10.1093/femsec/fiw003

Corre, M. H., Delafont, V., Legrand, A., Berjeaud, J. M., & Verdon, J. (2019). Exploiting richness of environmental waterborne bacterial species to find natural *Legionella pneumophila* competitors. *Frontiers in Microbiology, 9*, 3360. https://doi.org/10.3389/fmicb.2018.03360

Costa, R. A., Vieira, G. H. F., Silva, G. C., Peixoto, J. R. O, Silva dos Fernandes Vieira, R. H., & Saker-Sampaio, S. (2008). *Vibrio* microfauna associated with different development stages of the marine shrimp *Litopenaeus vannamei*. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases, 29*, 49-51.

Crab, R., Lambert, A., Defoirdt, T., Bossier, P., & Verstraete, W. (2010). The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*. *Journal of Applied Microbiology, 109*, 1643-1649. https://doi.org/10.1111/j.1365-2672.2010.04791.x

El-Saadony, M. T., Alagawany, M., Patra, A. K., Kar, I., Tiwari, R., Dawood, M. A., Dhama, K., & Abdel-Latif, H. M. (2021). The functionality of probiotics in aquaculture: an overview. *Fish & Shellfish Immunology, 117*, 36-52. https://doi.org/10.1016/j.fsi.2021.07.007

Ferreira, G. S., Bolivar, N. C., Pereira, S. A., Guertler, C., do Nascimento Vieira, F., Mouriño, J. L. P. & Seiffert, W. Q. (2015). Microbial biofloc as source of probiotic bacteria for the culture of *Litopenaeus vannamei*. *Aquaculture, 448*, 273-279. https://doi.org/10.1016/j.aquaculture.2015.06.006

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., & Williams, S. T. (2000). Bergey's Manual of Determinative Bacteriology. Lippincott Williams and Wilkins, New York, USA.

Hu, X., Yang, H. L., Yan, Y. Y., Zhang, C. X., Ye, J. D., Lu, K. L., Hu, L. H., Zhang, J. J., Ruan, L., & Sun, Y. Z. (2019). Effects of fructooligosaccharide on growth, immunity and intestinal microbiota of shrimp (*Litopenaeus vannamei*) fed diets with fish meal partially replaced by soybean meal. *Aquaculture Nutrition, 25*, 194-204. https://doi.org/10.1111/anu.12843

Krummenauer, D., Peixoto, S., Cavalli, R. O., Poersch, L. H., & Wasielesky Jr, W. (2011). Superintensive culture of white shrimp, *Litopenaeus vannamei*, in a biofloc technology system in southern Brazil at different stocking densities. *Journal of the World Aquaculture Society, 42*, 726-733.

Lazado, C. C., & Caipang, C. M. A. (2014). Atlantic cod in the dynamic probiotics research in aquaculture. *Aquaculture, 424*, 53-62. https://doi.org/10.1016/j.aquaculture.2013.12.040

Lazado, C. C., Caipang, C. M. A., Brinchmann, M. F., & Kiron, V. (2011). In vitro adherence of two candidate probiotics from Atlantic cod and their interference with the adhesion of two pathogenic bacteria. *Veterinary Microbiology, 148*, 252-259. https://doi.org/10.1016/j.vetmic.2010.08.024

Leaño, E. M., & Mohan, C. V. (2012). Early mortality syndrome threatens Asia´s shrimp. *Global Aquaculture Advocate, 4*, 38-39.

Lee, J. S., Lee, K. C., Kim, K. K., Hwang, I. C., Jang, C., Kim, N. G., Yeo, W. H., Kim, B. S., Yu, Y. M., & Ahn, J. S. (2009). *Acinetobacter antiviralis* sp. nov., from tobacco plant roots. *Journal of Microbiology and Biotechnology, 19*, 250-256.

McIntosh, D., Samocha, T. M., Jones, E. R., Lawrence, A. L., McKee, D. A., Horowitz, S., & Horowitz, A. (2000). The effect of a commercial bacterial supplement on the high-density culturing of *Litopenaeus vannamei* with a low-protein diet in an outdoor tank system and no water exchange. *Aquacultural Engineering, 21*, 215-227. https://doi.org/10.1016/S0144-8609(99)00030-8

Pakingking Jr, R., Bautista, N. B., Catedral, D., & de Jesus-Ayson, E. G. (2018). Characterisation of *Vibrio* isolates recovered from the eyes of cage-cultured pompano (*Trachinotus blochii*) infested with caligid parasites (*Lepeophtheirus spinifer*). *Bulletin of the European Association of Fish Pathologists, 38*, 35-41.

Pilet, M. F., Dousset, X., Barre, R., Novel, G., Desmazeaud, M., & Piard, J. C. (1995). Evidence of two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. *Journal of Food Protection, 58*, 256-262. https://doi.org/10.4315/0362-028X-58.3.256

Schmautz, Z., Graber, A., Jaenicke, S., Goesmann, A., Junge, R., & Smits, T. H. (2017). Microbial diversity in different compartments of an aquaponics system. *Archives of Microbiology, 199*, 613-620. https://doi.org/10.1007/s00203-016-1334-1

Schulze, A. D., Alabi, A. O., Tattersall-Sheldrake, A. R., & Miller, K. M. (2006). Bacterial diversity in a marine hatchery: balance between pathogenic and potentially probiotic bacterial strains. *Aquaculture, 256*, 50-73. https://doi.org/10.1016/j.aquaculture.2006.02.008

Servin Arce, K., de Souza Valente, C., do Vale Pereira, G., Shapira, B., Davies, S. J. (2021). Modulation of the gut microbiota of Pacific white shrimp (*Penaeus vannamei* Boone, 1931) by dietary inclusion of a functional yeast cell wall-based additive. *Aquaculture Nutrition, 27*, 1114-1127. https://doi.org/10.1111/anu.13252

Sheu, S. Y., Shiau, Y. W., Wei, Y. T., & Chen, W. M. (2013). *Sphingobium fontiphilum* sp. nov., isolated from a freshwater spring. *International Journal of Systematic and Evolutionary Microbiology, 63*, 1906-1911. https://doi.org/10.1099/ijs.0.046417-0

Song, Y. L., & Lee, S. P. (1983). Characterization and ecological implication of luminous *Vibrio harveyi* isolated from tiger shrimp *(Penaeus monodon*). *Bulletin of the Institute of Zoology Academia Sinica, 3*, 217-220.

Takeuchi, M., Hamana, K., & Hiraishi, A. (2001). Proposal of the genus *Sphingomonas sensu stricto* and three new genera, *Sphingobium, Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. *International Journal of Systematic and Evolutionary Microbiology, 51*, 1405-1417. https://doi.org/10.1099/00207713-51-4-1405

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews, 64*, 655-671. https://doi.org/10.1128/MMBR.64.4.655-671.2000

Wasielesky Jr, W., Atwood, H., Stokes, A., & Browdy, C. L. (2006). Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei*. *Aquaculture, 258*, 396-403. https://doi.org/10.1016/j.aquaculture.2006.04.030

Xia, L., Xiong, D., Gu, Z., Xu, Z., Chen, C., Xie, J., & Xu, P. (2008). Recovery of *Acinetobacter baumannii* from diseased channel catfish (*Ictalurus punctatus*) in China. *Aquaculture, 284*, 285-288. https://doi.org/10.1016/j.aquaculture.2008.07.038

Xiao, Z., & Lu, J. R. (2014). Strategies for enhancing fermentative production of acetoin: a review. *Biotechnology Advances, 32*, 492-503. https://doi.org/10.1016/j.biotechadv.2014.01.002

Zorriehzahra, M. J., Delshad, S. T., Adel, M., Tiwari, R., Karthik, K., Dhama, K., & Lazado, C. C. (2016). Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: a review. *Veterinary Quarterly, 36*, 228-241. https://doi.org/10.1080/01652176.2016.1172132