5. Mechanisms of probiotic actions in shrimp: Implications to tropical aquaculture

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Abstract. The shrimp aquaculture industry had been very dependent to synthetic antimicrobials as disease control agents. This approach was considered unsustainable and not eco-friendly because drug-resistance and horizontal transmission following excessive and indiscriminate use posed serious threats. The industry was then confronted to search suitable and effective alternatives, and the use of beneficial microorganisms or probiotics was identified to be very promising. Probiotics are live or dead, or even a component of microorganisms capable of conferring beneficial effects to the host and/or its environment. Interestingly, probiotic actions are multifaceted in nature as benefits are delivered in several mechanisms. They are not only capable of protecting the farmed animals from diseases but they can also influence growth, nutritional status and the quality of the rearing environment. The application of probiotics in shrimp has been demonstrated to 1) influence the soil and water quality, 2) enhance growth, 3) inhibit pathogen proliferation, 4) modulate the

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host microbiota, 5) contribute essential nutrients and beneficial enzymes, and 6) modulate the host immunity. The chapter synthesizes the identified mechanisms of probiotic actions in shrimp and provides future perspectives in support of the continual search and development of probiotics for shrimp aquaculture. In addition, a microcosm discussion is provided citing the current status and issues on probiotics research and applications in Philippine shrimp aquaculture. This paper affirms the importance of probiotics in the effective health management of shrimp aquaculture.

Introduction

Shrimp aquaculture is a profitable venture but its economic potential is challenged by several issues and concerns that considerably hinder sustainable growth and development. Viral and bacterial diseases are among the commonly associated limiting factors of a successful shrimp aquaculture. The use of antibiotics was a conventional strategy to prevent and control disease outbreaks. There had been an excessive and indiscriminate use of antibiotics (Mohapatra et al., 2013), and this practice still persists until now despite several precautionary standards set in a number of countries and politico-geographical regions. One of the biggest concerns was the development of drug-resistance and multiple antibiotic resistance (MAR) in bacteria, which resulted in resistance transfer and reduced efficacy of antibiotic treatment (Tendencia and de la Peña, 2001). In addition, the prevalence of antibiotic residues in the ponds was considered a serious environmental risk. Following incidences primarily on these concerns, the excessive use of antibiotics as disease control agents in shrimp aquaculture had been reduced and efforts in searching alternative strategies were encouraged.

The exploration of alternative strategies paved way to the development and application of probiotics as a thematic aspect of health and welfare management in aquaculture. In light of new trends towards eco-friendly aquaculture production, the promise of probiotics was welcomed with positivity as evidenced by numerous papers discussing and advancing their beneficial properties, relevance and application (Guo et al., 2009; Lazado & Caipang, 2014a; Ninawe & Selvin, 2009; van Hai & Fotedar, 2010).

Probiotics was primarily considered a disease control agent in shrimp aquaculture. In this disease control context, probiotics have been demonstrated capable of directly inhibiting the growth and proliferation of pathogens and also stimulating the dominance of the beneficial microbiota of the host and its rearing environment (Chythanya & Karunasagar, 2002; Lazado & Caipang, 2014c; Lazado et al., 2011; Lazado et al., 2010b; Li et al., 2007; Preetha et al., 2007; Vaseeharan and Ramasamy, 2003). It is important
to mention that the host animal and its immediate environment impose decisive factors for these benefits to be delivered optimally. Thus, the selection of appropriate probiotics particularly by taking into consideration the physiological and biochemical features of the host and the physico-chemical conditions of the immediate environment is crucial (Lazado et al. 2015). Interestingly, several other beneficial properties have been documented in probiotics hence expanding the understanding of their use in farmed aquatic animals. The application of probiotics has a significant advantage not only by being a disease control agent but notably as alternatives that promote health and welfare in farmed animals in a larger scope (Lazado & Caipang, 2014a).

The use of microorganisms and their metabolites is not a new approach in health management. Nevertheless, one of the major rationales affirming the importance of probiotics in aquaculture is its credibility in fostering sustainability by having insignificant risk of harm to the farmed animals and their environment. Most importantly, they offer effective and viable alternative solution to a problem impeding the industry. The chapter provides a synthesis of the current knowledge on the applications of probiotics in shrimp focusing on how this group of microorganisms confers their benefits to the host and its environment. Several probiotic mechanisms have been identified through the years, but this chapter discusses how they 1) improve the soil and water quality, 2) enhance growth, 3) inhibit pathogens, 4) influence host microbiota, 5) contribute nutrients and enzymes, and 6) modulate host immunity. At the end of the chapter, a separate discussion is provided on the current status of probiotics research and application in the Philippines.

**Probiotics as beneficial microorganisms.** Probiotics is coined from the Latin word *PRO* meaning for and from the Greek word *BIOS* meaning life (Zivkovic, 1999). The word *probiotics* was introduced by Lilly and Stillwell in 1965 as substances produced by a microorganism that prolong the logarithmic growth phase in other species (Lilly & Stillwell, 1965). The idea was re-introduced in the context of microbial feed/food supplements by Parker in 1974 (Parker, 1974). One of the most significant definitions of probiotics was coined by Fuller by defining probiotics as “live microbial food supplement that benefits the host (human or animal) by improving the microbial balance of the body” (Fuller, 1989). This definition of probiotics had been the most popular and widely referred to both in human and veterinary studies for several years (Lazado & Caipang, 2014a).

The empirical concept of probiotic application in aquaculture was made by Kozasa in mid 1980s when he used the spores of *Bacillus toyoi* as feed additive to increase the growth rate of yellow tail, *Seriola quinqueradiata*
(Kozasa, 1986). However, it did not trigger much attention as evidenced by the insignificant amount of research studies published within that aspect thereafter. Probiotics research started to become very prominent in aquaculture during the late 1990s. This approach was evoked when the active call for alternatives to antibiotics had become very resolute. In the early 2000s, Verschuere and his colleagues proposed an aquaculture-based definition stating that “A probiotic is defined as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment” (Verschuere et al., 2000). This definition allowed a broader application of the term “probiotic” and addressed concerns on Fuller’s definition particularly on the complex interaction between the culture environment and the host that characterized the aquatic environment. A joint expert panel from Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), defined probiotics as “live microorganisms, which when consumed in adequate amounts, confer a health benefit for the host” (FAO/WHO, 2001). The complexity of the aquatic environment makes these pioneering definitions disputable and their application could be on a case-by-case basis (Lazado & Caipang, 2014a). This is particularly relevant if we introduce the concepts of 1) application to the rearing water (Makridis et al., 2005; Spanggaard et al., 2001) and 2) bacterial viability (Díaz-Rosales et al., 2006; Lazado & Caipang, 2014b; Lazado et al., 2010a; Salinas et al., 2006). The unprecedented amount of researches generated on probiotics in aquaculture put forth the need to have a working definition in an aquaculture point of view. Necessary considerations must be made in order that a definition encompasses the physico-chemical and biological prerequisites of a dynamic aquatic system including the organisms thriving in it. In response to the point raised by Merrifield et al. (2010) that abovementioned concerns need to be considered in the understanding of probiotics for farmed aquatic animals, a simplified yet broad definition of probiotics was proposed by Lazado & Caipang (2014a) stating that “live or dead, or even a component of the microorganisms that act under different modes of action in conferring beneficial effects to the host or its environment”. This definition could be applied both in fish and in penaeids. For this chapter, the term “probiotics” is referenced to the aforementioned definition.

Mechanisms of probiotic actions. Interestingly, probiotics application is not an approach based on a single mechanism. In fact, these beneficial microorganisms are capable of delivering their benefits in scores of means (Farzanfar, 2006; Gomez-Gil et al., 2000; Lazado & Caipang, 2014a,d;
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Mohapatra et al., 2013; Ninawe & Selvin, 2009). The probiotic action can either be direct (i.e. host) or indirect (i.e. environment) or a combination of both. This broad perspective of probiotic action is a product of a diversified understanding of probiotics in an aquatic setting. It is now withdrawing from the traditional understanding of being just disease control agents. The principal consideration that probiotics as disease control agents will still persist however their other mechanisms of action provide cognizance of their numerous applications. The beneficial effects of probiotics are products of several interrelated or dependent mechanisms.

Most studies on probiotic applications in aquaculture were conducted in fish (Lazado et al. 2015). Nevertheless, there are still considerable amount of researches in shrimp demonstrating the mechanisms observed in the piscine model. In the succeeding sections, documented mechanisms of probiotic actions in penaeid model are enumerated and described. A pictorial representation of the different mechanisms highlighting the interrelationship of the probiotic actions is given in Figure 1.

**Improvement of soil and water quality.** The physico-chemical properties of the rearing soil and water are crucial for the success of shrimp culture. For instance, persistent infections could be actually due to poor water quality and low water exchange rates (Zokaeifar et al., 2014). The significance of environmental conditions can be introduced in the discussion of probiotics in two ways: 1) optimum conditions support the biological requirements of the host making them less susceptible to diseases, and 2) the quality of the rearing system should promote the dominance of beneficial microorganisms rather than the pathogenic population. One of the indirect mechanisms of probiotic actions in shrimp is the improvement of the culture pond conditions especially modifying the physico-chemical properties of water and soil.

The genus *Bacillus* is one of the widely known shrimp probiotics capable of modifying the quality of the rearing environment. *Bacillus* spp. are generally more efficient in converting organic matter back to CO₂ than other gram-negative bacteria, which would convert a greater percentage of organic carbon to bacterial biomass or slime (Stanier et al., 1963). *Bacillus* spp. were oftentimes used when the main aim of probiotic application was to manipulate the rearing conditions. *Bacillus* were administered either freeze-dried or microencapsulated to the rearing water of shrimp larval and post-larval stages and were shown to decrease the level of ammonia, nitrite and pH of the water (Nimrat et al., 2012). There was also a reduction of water ammonia, nitrite, nitrate and phosphate in the water of *Penaeus monodon* culture pond treated with a commercial probiotics (Lakshmanan & Soundarapandian, 2008). The reduction of ammonia and nitrite is attributed
to the capacity of the members of the *Bacillus* genera in mineralizing nitrogenous wastes via nitrification and/or denitrification (Joong et al., 2005; Zhao et al., 2010). Further, the nitrification process discharges hydrogen ion leading to a reduction in pH (Camargo & Alonso, 2006). Probiotic addition can also modify the chemistry of benthic community as shown by the lowering of total nitrogen and total carbon concentrations in *P. monodon* culture ponds (Shishehchian et al., 2001). Application of commercial probiotics composed of *Bacillus* sp., *Nitrosomonas* sp., *Nitrobacter* sp. and *Lactobacillus* to *Penaeus vannamei* ponds resulted to the mitigation of nitrogen and phosphate pollution in pond sediments as shown by the reduction of total phosphorus, total inorganic phosphorus, total nitrogen and total organic carbon (Wang & He, 2009).

**Figure 1.** Mechanisms of probiotic actions in shrimp. Probiotics are known to confer benefits through different mechanisms. These beneficial microorganisms are capable of producing inhibitory substances that could interact directly to bacterial and viral pathogens (Iyapparaj et al., 2013; Nhan et al., 2010; Vaseeharan and Ramasamy, 2003). Probiotics are also capable of manipulating the pond ecosystem such as the microflora (Boonthai et al., 2011) and the physico-chemical conditions (Zokaeifar et al., 2014). Dietary supplementation of probiotics enhances growth (Johansson et al., 2000) through enzyme contribution that increases digestibility (Leonel Ochoa-Solano and Olmos-Soto, 2006). Probiotics are also capable of manipulating the host microbiota (Makridis et al., 2005; Preetha et al., 2007). The protection from pathogens after probiotic treatment could be attributed to the direct inhibition of pathogens or to the capability of probiotics in modulating the immune system of shrimp (Chiu et al., 2007; Fu et al., 2011).
**Pathogen inhibition.** The direct inhibition of pathogens via probiotics could be through the production of antagonistic metabolites (Chythanya and Karunasagar, 2002; Iyapparaj et al., 2013; Lazado et al., 2010b) and/or interference of adhesion (Lazado et al., 2011). These characteristics are commonly used as primary tests in selecting and identifying candidate probiotics. Most of the probiotics in shrimp are characterized by their capability to inhibit the growth of a wide range of pathogens. An isolate identified as *Pseudomonas* sp. from the digestive tract of healthy juvenile *Litopenaeus vannamei* exhibited strong antibacterial activity against *Vibrio parahaemolyticus, V. alginolyticus* and *V. cholera* (Far et al., 2013). A novel strain of *Bacillus pumilus* was isolated from the midgut of healthy black tiger shrimp and showed strong inhibitory activity against *V. alginolyticus,* *V. mimicus,* and *V. harveyi.* In addition, the suitability of the isolate as probiotics was supported by the absence of any known *B. cereus* enterotoxin genes (Hill et al., 2009). Several candidate Bacillus probiotics demonstrated strong inhibition on the growth of pathogenic *V. harveyi* strains isolated from different origins (Thailand, Philippines and LMG4044). It was further shown that toxin production of *V. harveyi* is decreased by the addition of *B. licheniformis* or *B. megaterium* supernatant (Nakayama et al., 2009).

One of the areas in the study of probiotics in shrimp that needs to be explored further is the identification of the antagonistic metabolites. Works on the identification of antagonistic activity of candidate probiotics in most cases halted after observing the inhibitory activity, thus often failed to explore the identity of the metabolites that render such effect. *Pseudomonas* I-2 strain exhibited strong inhibitory activity against several strains of pathogenic *Vibrios.* The inhibitory compound produced was characterized as low molecular weight, heat stable, soluble in chloroform and resistant to proteolytic enzymes (Chythanya & Karunasagar, 2002). *Lactobacillus* sp. MSU3IR, a goat milk isolate and a candidate shrimp probiotics, produced bacteriocin as an inhibitory substance. The production of bacteriocin of this isolate could be optimized by manipulating the culture conditions of the bacteria (Iyapparaj et al., 2013).

Quorum sensing was demonstrated as one of the virulence mechanisms of many pathogenic bacteria including *V. harveyi* (Lilley & Bassler, 2000). This bacterial phenomenon allows single bacterial cells to measure the concentration of bacterial signal molecules and works in either of the two known systems: the Autoinducer 1 system (AI-1) for the intraspecies communication using different Acyl-homoserine lactones (AHL) or AI-2 for the interspecies communication (Jacobi et al., 2012). Application of N-acyl homoserine lactone-degrading bacterial enrichment cultures (EC) directly to the water or via *Artemia* nauplii of *Macrobrachium rosenbergii* elicited
protective effect following *V. harveyi* infection. The quorum quenching feature of the EC is attributed for this observed protection (Nhan et al., 2010). Microorganisms capable of producing compounds that inhibit the quorum-sensing signals could be established as new generation of antimicrobial agents that would be useful for veterinary science and agriculture (Chu et al., 2011).

In an *in vivo* setting, the antagonistic mechanism of probiotics was demonstrated by the decrease in number of the presumptive pathogenic group in the rearing environment. The luminous bacterial count in the rearing system of *P. monodon* decreased significantly after probiotic treatments (Janeo and Corre Jr, 2011). The presumptive count of *Vibrio* in the rearing water decreased significantly in the shrimp treated with probiotics with known antagonistic activity (Boonthai et al., 2011). In another study, the colonization of *V. harveyi* in *P. monodon* was inhibited significantly by exposure to probiotics and the mechanism of inhibition was by competitive exclusion (Gullian et al., 2004).

Most of the inhibition studies used bacteria as pathogen targets. However, some reports showed that the inhibitory spectrum of probiotics is not only limited to bacterial pathogens as they have demonstrated antiviral properties as well. *Pediococcus pentosaceus* isolated from the gut of wild brown shrimp (*Farfantepeneus californiensis*) were administered through feeds to *L. vannamei* to determine its antiviral activity. Probiotic administration showed a decrease in the prevalence of white spot syndrome virus (WSSV) but not the hematopoietic necrosis virus (IHHNV) in naturally infected shrimp (Leyva-Madrigal et al., 2011). Protective effect against WSSV was also demonstrated in another study in *L. vannamei* using *Bacillus OJ* as probiotics. Increasing dosage of probiotics resulted in an increasing trend in shrimp survival. Interestingly, a positive synergistic effect was observed when probiotics were administered with 0.2% isomatooligosaccharides (Li et al., 2009). Using a recombinant technology approach, *Bacillus subtilis* spores harboring a viral protein VP28 was given to *L. vannamei* and extremely high survival (83.3%) was observed following challenge with WSSV (Fu et al., 2011).

**Growth enhancement.** The most popular, common and practical method of probiotic application in aquaculture is through oral administration as feed supplements (Gomes et al., 2009; Huang et al., 2006). Not surprisingly, their growth-enhancing property was also explored. There is no universal consensus on how probiotics influence growth of cultivated species including fish and shrimp. However, this beneficial influence is being proposed to be an outcome of the increase in the appetite, or in the improvement of digestibility (Martinez Cruz et al., 2012), wherein the latter is the most explored in shrimp probiotic studies.
The widely used probiotics in shrimp with growth-enhancing capability belongs to the *Bacillus* genera. It was shown that administration of *Bacillus* sp. in *L. vannamei* influenced the apparent digestibility coefficients (ADC) of dry matter, crude protein, lipid, phosphorus, essential amino acids, non-essential amino acids and fatty acids (Lin et al., 2004). Further, the significant increase in the ADC was dependent on the concentration of the probiotics in the administered diets. The influence of probiotics in the growth performance of shrimp was also observed in *Fenneropenaeus indicus* fed with commercial *Bacillus* probiotic cocktail (Ziae-Nejad et al., 2006). The feed conversion ratio and specific growth rate were significantly higher in probiotic-fed group than the control group. In another study using *Bacillus* isolates from the intestine of the host, morphometric changes were observed in *M. rosenbergii* fed with probiotics. Besides the increase in weight, the body length of probiotic-fed prawn was significantly longer than those in the control group (Deeseenthum et al., 2007). These significant changes were also exhibited in a study with *P. monodon* (Soundarapandian & Sankar, 2008). Weight gain as an indicator of growth enhancement by probiotic feeding was also demonstrated in *P. monodon* (Rengpipat et al., 1998; Rengpipat et al., 2003), *P. vannamei/L. vannamei* (Gómez & Shen, 2008; Gullian et al., 2004; Wang and Gu, 2010; Wang, 2007; Zokaeifar et al., 2012), *M. japonicus* (Dong et al., 2013), *M. rosenbergii* (Mujeeb Rahiman et al., 2010) and *F. brasiliensis* (De Souza et al., 2012). Probiotics could facilitate the efficient utilization of nutrients and minerals from the feed which eventually being used by the host for their biological requirements. For example in *Penaeus indicus* fed with lactic acid bacteria, there was almost 20% increase on the digestibility when the shrimp were fed with probiotics (Fernandez et al., 2011). Indeed, the observed changes in growth performance could be attributed to the enhancement of different parameters like feed efficiency and feed conversion efficiency (Boonthai et al., 2011; Zokaeifar et al., 2014; Zokaeifar et al., 2012).

**Enzymatic contributions.** In relation to the capability of probiotics in enhancing growth performance, their influence on the enzymatic physiology of the gut is believed to be one of the significant contributors of this beneficial outcome. The nutritional condition of farmed animals is a consequence of both the given feeds and their digestive physiology (Becerra-Dórame et al., 2012). Digestive enzymes are necessary to break complex compounds into simpler and absorbable molecules that could be utilized by the host (Lazado et al., 2012). In fact, the utilization of the feed-related substances is greatly dependent on whether they can be easily absorbed or not by the host and used ultimately for their physiological processes. In some cases, the digestive physiology of the host could be influenced by several
factors such as the environment, types of feed and health status. In the context of probiotic applications, enzymatic influence could either be through stimulation (i.e. promotion of endogenous enzyme production) or direct contribution (i.e. production of exogenous enzymes by the administered microorganism).

Protein is considered a major limiting nutrient for shrimp growth (Kureshy and Allen Davis, 2002), therefore probiotics producing proteases are regarded as potential candidates. The increase in feed digestibility associated to probiotic administration has been linked to the capability of these microorganisms in facilitating the digestion of protein constituents through their enzymes that contribute to the host endogenous proteolytic activity (Leonel Ochoa-Solano and Olmos-Soto, 2006). Concomitant to the improvement in growth, significant increase in digestive protease due to probiotic application was observed in *P. vannamei* (Gómez & Shen, 2008; Liu et al., 2009; Wang, 2007) and *Fenneropenaeus indicus* (Ziaei-Nejad et al., 2006). Besides protease, probiotics can also influence other digestive enzymes of shrimp such as amylase (Castex et al., 2008; Gómez & Shen, 2008; Wang, 2007; Ziaei-Nejad et al., 2006), cellulase (Wang, 2007), lipase (Wang, 2007; Ziaei-Nejad et al., 2006) and trypsin (Nimrat et al., 2013). The influence of probiotics on the digestive physiology of shrimp is continuously considered a desirable feature because their contribution has associated positive consequences.

**Modulation of host microbiota.** The role of the host gastrointestinal tract microbiota can be divided into 2 major distinctive functions. First is on the context of immunity as the microbiota is crucial for the maintenance of the mucosal immunity and serves as an important defensive barrier against invading pathogens (Lazado & Caipang, 2014a,d). The second function is on nutrition as these commensal microorganisms provide nutrients and beneficial enzymes to the host (Lazado et al. 2015; Nayak, 2010b).

Probiotics are known to modulate the microbial community structure of the host. As discussed in a recent review paper in Atlantic cod, the discussion of probiotics and gut microbiota should not be mutually exclusive. This is because gut microbiota is a determinant of the success of probiotic applications while probiotics are modulators of the gut microbial community (Lazado & Caipang, 2014a). This inference is particularly interesting in aquaculture species where application of probiotics is mainly through diets and the gastrointestinal tract is the organ where host-probiotics interaction is highly prominent. There are two central philosophies on how probiotics influence the microbial community structure of the host: 1) determinism, which states that a well-defined dose-response relationship is required; and 2) stochasticism, which describes chance favors organisms which happen to be in the right place at the right time (Verschuere et al., 2000).
It was suggested by Dempsey that one or two phylogenetic groups dominate the shrimp gut and have very low diversity (Dempsey et al., 1989). The profile shifts in the microbial structure following probiotic applications can easily be observed because primarily, the normal gut microbiota of shrimp is not as diverse as compared to fish. Shrimp fed with a commercial probiotic cocktail (contained different strains of *Bacillus*) revealed the shift from the dominance of *Vibrio* to *Bacillus* in the gut after 2 h and after 24 h in the hepatopancreas (Janeo & Corre Jr, 2011). The change on microbial community structure was also observed in *L. vannamei* exposed to probiotics of different origins. The gut microbial community of the probiotic fed group was mainly consisted of α- and γ-proteobacteria, fusobacteria, sphrobacteria and flavobacteria while the control group was principally dominated by α-proteobacteria and flavobacteria (Luis-Villaseñor et al., 2013). Though there was no significant change in the total bacterial count in *L. vannamei*-fed with *B. licheniformis*, the application of probiotics lowered the *Vibrio* count in the gut tract of shrimp (Li et al., 2007). This significant reduction of *Vibrio* in the gut was also documented in *P. monodon* fed with *Synechocystis MCCB* (Preetha et al., 2007) and in *Marsupenaeus japonicus* fed with *Bacillus* (Dong et al., 2013). The administration of probiotics also increased the total microbial count and decreased the population of *Vibrio* in the gut of *P. japonicus* (Zhang et al., 2011). These observations exemplified the competitive exclusion mechanism of probiotics in modifying the microbial population of the gut specifically by lowering the count of the pathogenic group.

**Immunomodulation.** The interest on the importance of probiotics to host immunity became very prominent in the early 1990s when significant evidence demonstrated several positive health-related responses to the manipulation of the gastrointestinal microflora (Huis in 't Veld, 1991; Smirnov et al., 1993). The studies in human models became the baseline information in exploring the same effects in fish and other invertebrates including shrimp. Particularly in fish, probiotic application can modulate both the adaptive and innate immunity (Lazado & Caipang, 2014d; Nayak, 2010a). Shrimp are believed to be lacking an adaptive immunity but they still possess an interesting innate immune system that effectively protects them from harmful microorganisms (Young Lee & Söderhäll, 2002). Reports showed that shrimp innate immunity responded to probiotic treatments through the modulation of the cellular and humoral immune responses (Lazado et al. 2015; Lakshmi et al., 2013; Ninawe & Selvin, 2009; van Hai & Fotedar, 2010). Though the current knowledge is fragmentary on how probiotics stimulate the innate immunity of shrimp, a growing number of researches provide, to some extent, the immunomodulatory mechanisms of probiotics through treatment-response approaches. There are two apparent
reasons why the immunomodulatory features of probiotics have been of great interest in shrimp aquaculture in the last years. During the early days of applications in shrimp, protection observed following pathogen challenge was commonly used gauge of a positive consequence of probiotics application. This result was traditionally explained through the direct antagonism of the probiotics towards the pathogens (Figure 1). By looking into a different perspective, this could be described by the capability of probiotics in boosting the immunity to elicit potent responses against the pathogens (Figure 1). Secondly, sustainable aquaculture is gearing towards a more pro-active management. The immunomodulatory capabilities of probiotics have been considered an effective prophylactic strategy. Foremostly, probiotics are greatly considered alternative to antibiotics and this attribute made their application more enviable (Figure 2).

**Figure 2.** Model of immunomodulation in shrimp by probiotics. Pattern recognition receptors are necessary in identifying microbe associated molecular patterns (PAMPs) of both the pathogens and the probiotics. Though several PAMPs have already been identified in shrimp, none of them have been demonstrated so far to be involved in the probiotics-host recognition in this species (Lazado et al., 2015). The application of probiotics influences the humoral and cellular defenses of the shrimp. Following probiotic treatment, increase in the number of hemocytes can be observed (Rengpipat et al., 2000; Zhang et al., 2011). Probiotics stimulate the production of reactive oxygen species (Mujeeb Rahiman et al., 2010). In addition, probiotic treatment increases phenoloxidase activity (Chiu et al., 2007; Gullian et al., 2004) and hemocytic phagocytosis (Rengpipat et al., 2000; Tseng et al., 2009). The transcription of several immune-related genes can also be modulated by probiotic treatment (Antony et al., 2011; Dong et al., 2013). These stimulated immune defenses play a crucial role in the responses and eventual protection during pathogen exposure.
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Hemocytes play important functions in shrimp immune responses including recognition, phagocytosis, melanization, cytotoxicity and cell–cell communication (Johansson et al., 2000). They are characterized as hyaline cell, small-granule cell and large-granule cell (Heng & Wang, 1998; Tsing et al., 1989). Studies on the effects of probiotics in shrimp immunity are mainly focused on the responses of hemocytes. Total hemocyte count (THC) was increased following probiotic application in *M. rosenbergii* (Mujeeb Rahiman et al., 2010), *P. monodon* (Rengpipat et al., 2000), *P. japonicus* (Zhang et al., 2011) and *L. vannamei* (Li et al., 2007). However, it was observed in *L. vannamei* that THC decreased significantly following feeding with *Lactobacillus plantarum* (Chiu et al., 2007); no significant effect was observed after feeding with *B. subtilis* (Tseng et al., 2009). This effect was also observed in *Pseudomonas*-fed *P. monodon* (Alavandi et al., 2004).

Phagocytosis is a mechanism of most cells including hemocytes in combating and eliminating pathogens. In an interesting study, hemocytic phagocytosis was enhanced by *B. subtilis* harboring a viral protein (VP28) and this was speculated as a significant factor in the protection observed against WSSV infection (Fu et al., 2011). Hemocytic phagocytosis was also enhanced in *P. monodon* fed with *Bacillus* S1 (Rengpipat et al., 2000) and *L. vannamei* fed with *B. subtilis* E20 (Tseng et al., 2009). In addition, respiratory burst of hemocytes could also be enhanced by probiotic addition (Mujeeb Rahiman et al., 2010).

Despite the absence of immunoglobulins, shrimp have developed complex immune defenses that could counteract intruding pathogens and one of them is through the prophenoloxidase (proPO) cascade system (Fagutao et al., 2009). Phenoloxidase plays a pivotal function in controlling bacterial load in the haemolymph (Fagutao et al., 2009), in self non-recognition (Hernández-López et al., 1996) and protection against pathogenic bacteria (Amparyup et al., 2009). The application of probiotics resulted to the stimulation of phenoloxidase activity in *L. vannamei/P. vannamei* (Chiu et al., 2007; Li et al., 2007; Nimrat et al., 2013; Tseng et al., 2009; Wang & Gu, 2010), *P. monodon* (Rengpipat et al., 2000), *P. japonicus* (Zhang et al., 2011) and *M. rosenbergii* (Mujeeb Rahiman et al., 2010). In most cases, the increase in the activity of phenoloxidase was concurrent to the protection after pathogen challenge. Probiotics were also shown to modulate the activity of other innate immune defenses such as superoxide dismutase (Castex et al., 2009; Gullian et al., 2004; Li et al., 2007; Wang and Gu, 2010; Zhang et al., 2011), catalase (Castex et al., 2009), lysozyme and nitric oxide synthase (Zhang et al., 2011). In addition, probiotic treatment did not only enhance the antioxidant defenses of shrimp but it could also alleviate
oxidative stress (lower malondialdehyde and carbonyl protein) induced by pathogen exposure (Castex et al., 2009, 2010).

Immunomodulatory functions of probiotics were also demonstrated in their capacity to influence the transcription of several immune-related genes in shrimp. Upregulated expression was observed in prophenoloxidase paralogs, antimicrobial peptides, peroxinectin, serine protease, lipopolysaccharide-and β-1,3-glucan binding protein and lysozyme (Antony et al., 2011; Dong et al., 2013; Liu et al., 2010; Zokaeifar et al., 2014; Zokaeifar et al., 2012). The stimulated expression of these immune genes was implicated to the observed robust responses and protection during pathogen challenge and stress exposure.

**Development of probiotics.** The selection, evaluation and development of probiotics is a multi-step procedure predominantly focused on their safety, functionality and technological characteristics (Reid, 2006; Sanders & Huis In’t Veld, 1999; Verschuere et al., 2000). Probiotics applications in aquaculture had been for some time dependent on the use of terrestrial probiotics especially the lactic acid bacteria (LAB). Though there is no universal acceptance that probiotics should be from the host (Verschuere et al., 2000), the utilization of host-derived microorganism has become a popular and enviable approach at present (Lazado et al., 2015; Lazado & Caipang, 2014a). There is no standard protocol that is universally accepted and approved on the development of probiotics in shrimp though Lakshmi and company proposed a procedure (Lakshmi et al., 2013) principally based on previously published suggested strategies (Balcázar et al., 2006; Verschuere et al., 2000). The approach presented is good however it did not show the importance of ensuring the identity of the microorganisms which was suggested in the FAO-WHO guidelines of 2002 (FAO/WHO, 2002). Secondly, it did not highlight the importance of both in vitro and in vivo approaches in profiling the probiotic features of the candidate microorganism. The multi-step approach especially in delineating the in vitro and in vivo features is among the core areas of probiotic development. Figure 3 shows a simplified proposed strategy for probiotic development in shrimp, taking into consideration what have been done in shrimp and some issues that should be addressed. The 2 main elements, both mutually inclusive, on having a systematic approach in developing probiotics for aquaculture are: 1) biological aspect: it is essential that the candidate microorganism is identified properly and the mechanisms of probiotic actions are known; 2) commercial aspect: solid evidences on the biological activities of the candidate microorganisms will substantiate the claim and will serve as deciding factors to be granted authorization by the regulatory agencies. In general, contemporary researches on the
development of probiotics for aquaculture only reached the biological aspect and they did not advance into a commercial commodity as envisioned.

In a global perspective, there are no international standard and regulatory guidelines for the usage of probiotics in aquaculture (Ninawe & Selvin, 2009). Usually, the regulatory agency of each country is the one setting the guidelines on the use of probiotics in aquaculture. For example in the European Union, the European Food Safety Authority set the guidelines and Bactocell®, a lyophilized live _Pediococcus acidalactici_ was the first probiotics that was given approval for use in aquaculture (E.F.S.A., 2012).

**Probiotics in a Philippine perspective.** In 2004, the Bureau of Food and Drug Administration (now Food and Drug Administration) released Bureau Circular No. 16 stating the guidelines of probiotic drug use in the Philippines (BFAD, 2004). It was enumerated in the order that the approved bacterial strains as probiotics in the Philippines are the following: _Lactobacilli, Bifidobacteria_, nonpathogenic strains of _Streptococcus, Saccharomyces boulardi_ and _Bacillus causii_. The order was particularly directed for human application and did not mention its applicability for veterinary use in the country. The main law governing Philippine fisheries is Republic Act 8550 also known as The Philippine Fisheries Code of 1998. The implementing rules and guidelines of this act did not mention any provisions on the application of probiotics in aquaculture. It is understandable that this law did not able to cover the applications of beneficial microorganisms because at the time of drafting until promulgation, probiotics was just an emerging thematic area in aquaculture. To our knowledge, there is no existing law or implementing guidelines governing research and application of probiotics for aquaculture species in the Philippines. This is one of the main challenges concerning the status of probiotics in Philippine aquaculture. In reference to Figure 3, one of the main components in the development of probiotics is its/their compliance to laws or existing guidelines. If we follow this systematic approach, it could not be achieved in the Philippines at present because there are no rules that will guarantee this provision. Even though the national veterinary drug residues control programme is under R.A. 3720 or the “Food, Drugs and Devices and Cosmetics Act” and R.A. 7394 or the “Consumer Act of the Philippines”, they still lack implementing guidelines on the process of developing a probiotic product for use in aquaculture. Despite of this statutory deficit, probiotics are being used in a number of aquaculture farms in the Philippines (Cruz et al., 2008; Moriarty, 1999). In Negros Island, the use of probiotics is being combined with green water technology in a tilapia-shrimp polyculture (Cruz et al., 2008). This system
is a current practice to reduce the expenses in using probiotics alone and aeration which they stated as among the costliest inputs in shrimp culture in that region. The present authors tried to gather statistical information from concerned agencies on the number of fish/shrimp farm in the Philippines that are using probiotics as part of their production management. Unfortunately, there are no existing reports that could be provided at the moment. Even though probiotics are believed to be “harmless”, having a specific set of guidelines on their application is a must to avoid inappropriate use in the future and to ensure that they are being applied as per their intent.

Figure 3. Modified diagram of the selection of probiotics for shrimp.
There are several readily available probiotics for aquaculture use in the Philippine market. Biomin®, an international company locally operated by Ariela Marketing Co. Inc., has an array of probiotics and bioactive compounds that are being marketed for the aquaculture industry (www.biomin.net). For example, Aquastar® is a multispecies microorganism cocktail with several published modes of action such as enhancement of immune response, production of inhibitory substances and improvement of water quality. BZT® Bio is a commercial cocktail of microencapsulated bacteria and enzymes and is being marketed as having the capability of reducing organic bottom solids and improving water quality (www.atcvietnam.com.vn).

It is important to mention that there is no extensive probiotics research in Philippine aquaculture. There is no identified research group that is working on probiotics for aquaculture use unlike in other Asian countries such as Japan, Korea, Thailand and India. This observation is supported by a limited number of published peer-reviewed papers on probiotics from Philippine universities and research institutions.

The Scopus database (www.scopus.com) covers nearly 21,000 titles from 5,000 publishers around the world. Searching the database using search terms 1 (probiotics AND shrimp AND Philippines), search terms 2 (probiotics AND fish AND Philippines) and search terms 3 (probiotics AND aquaculture AND Philippines) generated only 4, 3 and 1 document results respectively (as of February 5, 2015). We acknowledge that there might be a number of papers not covered in this quick literature survey or there are some that are unpublished data. Despite the fact that probiotics are being used in Philippine aquaculture, this statistics reflects that its research component is lagging behind hence should be incisively encouraged. A concerted effort from the academe, the state and public sectors must be put forward for the advancement and development of probiotics research in the Philippines.

Nevertheless, it is also important to highlight some probiotic studies conducted by Philippine-based researchers for they serve as foundation of future studies. Torres et al. (1997) showed that commercial probiotics demonstrated inhibitory activity against Philippine strains of pathogenic Vibrio. In another study, the biocontrol property of commercial probiotics against luminous Vibrio was demonstrated as well (Lio-Po et al., 2007). Trials were also conducted in combining commercial probiotics and “green water” technology in shrimp and the results showed promise yet did not develop into a full scale technology (Corre Jr et al., 2000). Janeo and Corre (2011) demonstrated the effects of commercial probiotics in shrimp reared in a bioaugmented system. Recently, a group from the University of the
Philippines Visayas demonstrated that applications of commercial probiotics to saline tilapia could enhance growth, phytoplankton production and water quality (Mohamed et al., 2013). These data clearly show that Philippine-based researchers are inclined to the use of commercial probiotics. To our knowledge, there are no published reports characterizing and discussing the probiotic potential of a microbial candidate isolated from the Philippines for aquaculture. In the future, this has to be considered by Philippine-based researchers with interest on probiotics for aquaculture because publications from other countries have clearly demonstrated the potential of developing probiotics from the indigenous microbiota of aquaculture species (Balcázar et al., 2006; Lazado et al., 2015; Lazado & Caipang, 2014a; Martinez Cruz et al., 2012; Merrifield et al., 2010; Mohapatra et al., 2013).

**Summary.** Shrimp aquaculture is an important industry thus management should not be only profitably sound but it should be ecologically sustainable as well. The industry is taking the right direction in reducing the use of antibiotics and promoting the adoption of eco-friendly alternatives like probiotics as disease control agents. Probiotics is a group of microorganisms possessing a number of beneficial features in promoting better health status of shrimp. These beneficial features are conferred through different mechanisms. Probiotics promote better health status by modifying the rearing environment and by influencing host’s physiological responses leading to improved growth and disease resistance. Our understanding of probiotics in shrimp as a prophylactic measure is limited and mostly speculative. The published reports discussing the mechanisms of probiotic actions in shrimp corroborate this conclusion. In particular, the status of probiotics research and application in the Philippines partly represents a microcosm of the global position of probiotics in shrimp culture. There are numerous issues that need to be addressed particularly in research approaches and legislations. Thus, several initiatives must be upheld in response to the present knowledge gap. The promise of probiotics for an effective and sustainable shrimp culture is enormous. Therefore, documented mechanistic understanding on how these microorganisms deliver their benefits is a must as this will be pivotal in their advancement as potential, valuable and compelling alternative to antibiotics.

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