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# Probiotics in Larvae and Juvenile Whiteleg Shrimp *Litopenaeus vannamei*

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Additional information is available at the end of the chapter

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## 1. Introduction

In penaeid shrimps, *Vibrio* spp. is the main cause of bacterial diseases, such as *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi* (Garriques and Arevalo, 1995) and *V. penaeicida* (Aguirre-Guzmán and Ascencio-Valle, 2001). Possible mode of infection consists of three basic steps: (i) the bacterium penetrates the host cuticle or exoskeleton wound by means of chemotactic motility; (2) within the host tissues the bacterium deploys iron-sequestering systems; e.g., siderophores, to “steal” iron from the host; and (3) the bacterium eventually damages the organisms by means of extracellular products, e.g. hemolysins and proteases (Thompson et al., 2004). Containing high loads of either *Vibrio parahaemolyticus* or *V. harveyi* induced the rounding up and detachment of epithelial cells from the basal lamina of the midgut trunk. Epithelial cell detachment of epithelial was not seen in the presence of non-pathogenic bacteria (probiotics) (Chen et al., 2000; Martin et al., 2004). Pathogens like *Vibrio* spp., which cause detachment of the epithelium in the midgut trunk, can affect high mortality in shrimp by eliminating 2 layers that protect the shrimp from infections: the epithelium and the peritrophic membrane it secretes. In addition, loss of the epithelium may affect the regulation of water and ion outtake into the body (Mykles 1977, Neufeld and Cameron 1994).

Prevention and control of diseases had led to increase the use of antibiotics developing drug resistant bacteria, which are difficult to control and eradicate. An alternative to antibiotic treatment is the use of probiotics or beneficial bacteria that control pathogens. Probiotics are generally defined as viable microorganisms that, when to human or animals, beneficially affect the health of the host by improving the indigenous microbial balance (Fuller, 1989; Havenaar et al., 1992). Generally, probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals (Vine et al., 2004). Probiotics may protect their host from pathogens by producing metabolites that inhibit the colonization or growth of other

microorganisms or by competing with them for resources such as nutrients or space (Vine et al. 2004). Studies of probiotics to improve growth or survival in crustacean larvae are scarce. Recently, methods for improving water quality of hatcheries and application of probiotics has gained momentum (Balcázar et al., 2007a; Gómez et al., 2008; Guo et al., 2009; Van Hai et al., 2009). Daily administration of probiotics based on *Bacillus* spp. during hatchery and farming stages leads to higher feed conversion ratios, improved specific growth rates, and higher final shrimp biomass than controls (Guo et al., 2009; Liu et al., 2009a). Metamorphosis improved with administration of the probiotic *B. fusiformis* (Guo, et al., 2009). Zhou et al. (2009) found that *B. coagulans* SC8168, as a water additive at certain concentrations, significantly increased survival and some digestive enzyme activities of shrimp larvae. *Bacillus* spp. possesses adhesion abilities, produce bacteriocins, and provide immunostimulation (Ravi et al., 2007).

The criteria of probiotic selection to be used in aquaculture systems has been discussed by some authors. Nguyen et al. (2007) suggest that the beneficial effect of the probiotics on the host has been wrongly attributed to what is found during *in vitro* observations, that *in vivo* physiology might be different from *in vitro* metabolic processes. Development of suitable probiotics is not a simple task and requires full-scale trials, as well as development of appropriate monitoring tools and controlled production (Decamp et al., 2008). *In vitro* and *in vivo* studies are needed to demonstrate antagonisms to pathogens and their effect on survival and growth of the host. The main purpose of using probiotics is to maintain or reestablish a favorable relationship between friendly and pathogenic microorganisms that constitute the flora of intestinal or skin mucus of aquatic animals. Since, successful probiotic is expected to have a few specific properties in order to certify a beneficial effect (Ali, 2000).

Bacteria present in the aquatic environment influence the composition of the gut microbiota and vice versa. The genus present in the intestinal tract generally seems to be those from the environment or the diet that can survive and multiply in the intestinal tract (Cahill, 1990). Therefore, probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals. Gram-negative facultative anaerobic bacteria such as *Vibrio* and *Pseudomonas* constitute the predominant indigenous microbiota of a variety of species of marine animals (Onarheim et al., 1994). On the other hand, the indigenous microbiota of freshwater animals tends to be dominated by members of the genera *Aeromonas*, *Plesiomonas*, representatives of the family *Enterobacteriaceae*, and obligate anaerobic bacteria of the genera *Bacteroides*, *Fusobacterium*, and *Eubacterium* (Sakata 1990). Lactic acid producing bacteria, which are prevalent in the mammal or bird gut, are generally sub-dominant in fishes and are represented essentially by the genus *Carnobacterium* (Ringo & Vadstein 1998). Ideally, microbial probiotics should have a beneficial effect and not cause any harm to the host. Therefore, all strains have to be non-pathogenic and non-toxic in order to avoid undesirable side-effects when administrated to aquatic animals (Chukeatirote, 2002).

Some research and products talk about the multifactorial action of the probiotics (Gomez et al., 2007; Tuohy et al., 2003) on aquatic animals. However, the multifactorial effect is not agreed with evidence or is overestimate. Sometimes, this type of publicity about the

potential of those products really affects the perspective of real probiotic designed for aquaculture industry.

Different modes of action or properties are desired on the potential probiotic like antagonism to pathogens (Ringo and Vadstein, 1998), ability of cells to produce metabolites (like vitamins) and enzymes (Ali, 2000), colonization or adhesion properties (Olsson *et al.*, 1992), enhance the immune systems (Perdigon *et al.*, 1995) and others. On the other hand, a criterion to discard potential harmful bacteria is the ability to produce toxins that induce lysis of host cells (Zamora-Rodríguez, 2003).

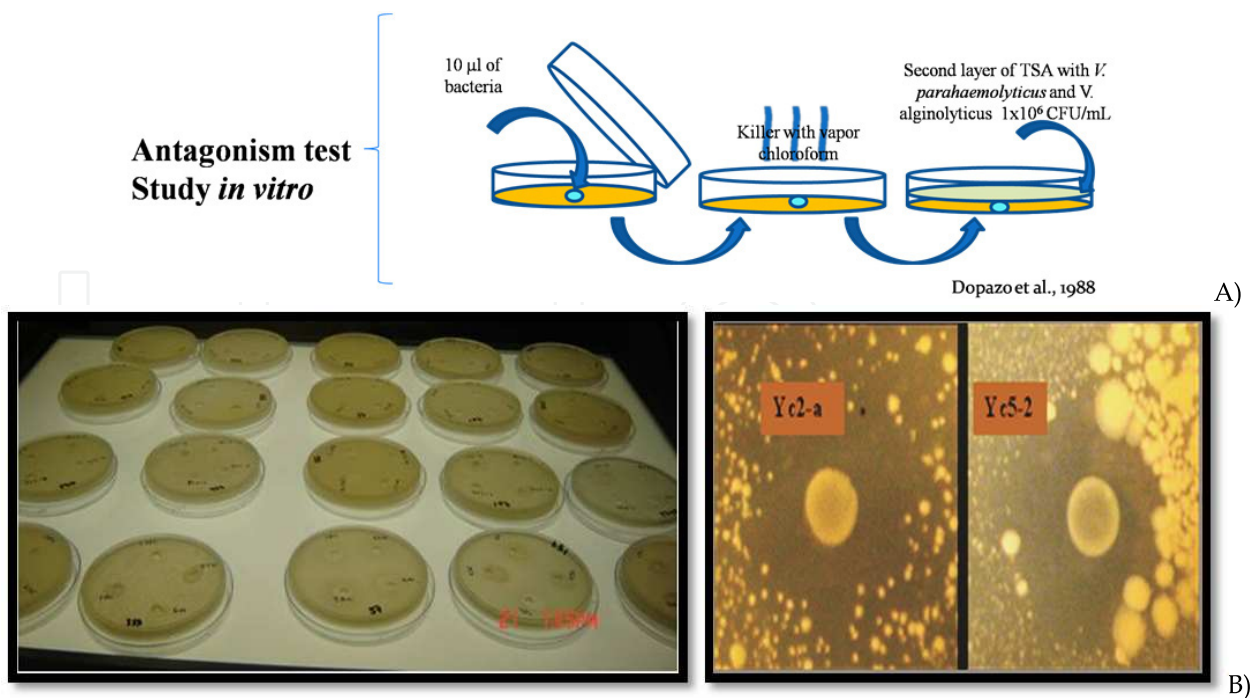
Various mechanisms have been proposed to explain their beneficial effects, including competition for adhesion sites, competition for nutrients, enzymatic contribution to digestion, improved water quality, and stimulation of the host immune response (Kumar Sahu *et al.*, 2008). Selection of probiotics in aquaculture enterprises is usually based on results of tests showing antagonism toward the pathogens, an ability to survive and colonize the intestine, and a capacity to increase an immune response in the host. Adhesion of probiotic microorganisms to the intestinal mucus is considered important for many of the observed probiotic health effects (Ouwehand *et al.*, 2000). Adhesion is regarded a prerequisite for colonization (Alander *et al.*, 1999).

The composition of the bacterial community in an aquaculture environment has a strong influence on the internal bacterial flora of farmed animals, which is vital for their nutrition, immunity and disease resistance (Luo *et al.* 2006). The intestinal microbiota of aquatic organisms in culture is an important factor in maintaining the healthy, either by preventing pathogen colonization, degradation of food, production of antimicrobial compounds, producing nutrients and maintaining normal mucosal immune (Escobar-Briones *et al.*, 2006). The interest in investigating the intestinal microbiota is based on the need for a better understanding of how probiotics can influence the bacterial composition. Another important function was to emerge in recent years suggesting that the effect of the commensal microbiota influence processes such as lipid metabolism and development of the host immune response. The inter-relationship between the microbiota and the host are clearly important in relation to health and the imbalance between these systems results in disease development. Several studies listed the benefits of these probiotics to culture organisms, however, few works that the type of modulation is performed to the intestinal microbiota and its effects on health of the host organism. The interest to investigate the intestinal microbiota is based on the need for a better understanding of how probiotics can influence the bacterial composition. Such studies have been widely performed in vertebrates (Birkbeck, 2005; Austin, 2006; Escobar-Briones *et al.*, 2006; McKellep Bakke, 2007), but in invertebrates is very limited. The intestinal microbiota of aquatic organisms has shown a high dependence of bacterial colonization during early development, environmental conditions and change in diet (Ringo *et al.*, 1995, 2006; Ringo and Birkbeck, 1999; Olafsen, 2001). For that to know the impact that probiotics in the modulation of intestinal microbiota should be studied. We investigated the effect of *Bacillus* probiotics

was showed trait inhibitory to *Vibrio* and ability to adhere and grow, on intestinal mucus on the survival and rate of development of whiteleg shrimp *L. vannamei* larvae to understand mechanisms of how endemic *Bacillus* probiotic strains improve the health of larvae. Moreover, analyzed the composition of bacterial communities in the juvenile shrimp *L. vannamei* know the impact that probiotics in the modulation of intestinal microbiota.

2. Antagonism test

Antagonism in the world of bacteria is a highly prevalent phenomenon: one bacterium species suppresses the development or inhibits the growth of other microorganisms (Egorov, 2004). A common way to select probiotic is to perform *in vitro* antagonism test. *Bacillus* spp. produce polypeptides (bacitracin, gramicidin S, polymyxin, and tyrothricin) that are active against a broad range of Gram positive and Gram negative bacteria, which also explains the inhibitory effect on pathogenic *Vibrio* (Drablos et al., 1999; Morikawa et al., 1992; Perez et al., 1993). The antagonism of *Bacillus* is due mainly to the production of antimicrobial proteins and antibiotics as well as chemical compounds synthesized by secondary metabolism pathways (Hu et al., 2010), competition for essential nutrients and adhesion sites. We scrutinized their ability to inhibit the growth of *Vibrio* species utilized the two-layer method described by Dopazo et al. (1988) (Figure 1), shows that only two isolates *Bacillus tequilensis* and *B. amyloliquefaciens* (YC5-2 and YC2-a) inhibited growth of *V. campbelli* (CAIM 333) and *V. vulnificus* (CAIM 157).



**Figure 1.** A) Schematic from Antagonism test utilized the two-layer method described by Dopazo et al. (1988). B) Zone inhibition obtained by *Bacillus amyloliquefaciens* (strain YC2-a) and *Bacillus tequilensis* (strain YC5-2) against *Vibrios parahaemolyticus*.



The well diffusion test (Balcázar et al., 2007) showed that 24-h cultures of inactivated isolates YC5-2 (*Bacillus tequilensis*), YC2-a (*B. amyloliquefaciens*) YC3-b (*B. endophyticus*) and C2-2 (*B. endophyticus*) were able to inhibit *V. parahaemolyticus* (CAIM 170) and *V. harveyi* (CAIM 1793). *V. alginolyticus* (CAIM 57) showed sensitivity but no inhibition to these probiotic strains (Luis-Villaseñor et al., 2011) (Figure 1, Table 1). *Bacillus* strains isolated from shrimp inhibited vibriosis by a well-diffusion method. The antagonism test showed that probiotic strains were able to inhibit pathogenic strains of *V. harveyi* (CAIM 1793), *V. parahaemolyticus* (CAIM 170), *V. campbelli* (CAIM 333), *V. alginolyticus* (CAIM 57), and *V. vulnificus* (CAIM 157). Similar results were obtained by Balcazar et al. (2007a), where *B. subtilis* UTM 126 was able to inhibit *V. parahaemolyticus* PS-107. Nakayama et al. (2009) found that cell-free supernatant from *B. subtilis*, *B. licheniformis*, and *B. megaterium* inhibited growth of one *V. harveyi* strain for 24 h. Decamp et al. (2008) administered *B. subtilis* and *B. licheniformis* to larval *L. vannamei* and *Penaeus monodon* and this inhibited growth of *Vibrio* strains and increased the survival rate of the shrimp.

Isolate	Gram	Hemolytic activity		Inhibition zone (mm)				
		Erythrocytes	Hemocytes	<i>V. parahaemolyticus</i> CAIM 170	<i>V. harveyi</i> CAIM 1793	<i>V. campbelli</i> CAIM 333	<i>V. vulnificus</i> CAIM 157	<i>V. alginolyticus</i> CAIM 57
YC5-2**	+	γ	NR	17.5±0.7	11±1.8	5±1.4	18±1.4	*
YC2-a**	+	γ	NR	13.5±1.0	12±3.0	9±1.4	6.5±0.2	*
C2-2	+	γ	NR	21.5±1.1	11.5±2.1	NR	NR	*
YC3-B	+	γ	NR	13.5±2.1	11±2.1	NR	NR	*
YC1-A	+	α	4.5±0.7	16.5±2.1	8.85±0.5	9±0.5	21.9±1.6	*
YC3-C	+	α	4.5±1.4	17.5±0.7	10±1.4	9.1±0.1	18.7±1.1	*
YC3-A	+	α	3.5±0.7	13.45±1.1	8±1.4	8.15±1.6	18.1±1.8	*
YC2-B	+	β	8.5±0.7	8.5±2.1	13±1	NR	4.6±0.8	*
YC3-D	+	β	8.7±0.3	9.5±0.7	11±1	NR	10.75±0.4	*

\*\* = Inhibitory effect for the two-layer method (Dopazo et al. 1988). γ = Growth, but not hemolysis. NR = Negative to the test.

**Table 1.** Test of antagonism of probiotics isolates against pathogenic *Vibrio* strains. \* = Bacteriostatic effect.

### 3. Hemolytic activity of *Bacillus* strains

The principal purpose of the use of probiotics in to produce a proper relationship between useful microorganism and the pathogenic microflora and their environment. Probiotics should be of animal-species origin, this criteria is based on ecological reasons, and takes into consideration the original habitat of the selected bacterial (in intestinal flora) (Farzanfar, 2006). One of the most important features of a probiotic is that it does not harm the host (Kesarcodi-Watson et al., 2008). Some *Bacillus* spp. produce hemolysins, which could be a health risk to the host (Liu et al., 2009b). Bernheimer and Grushoff (1967) demonstrated that *B. cereus*, *B. alvei*, *B. laterosporus*, *B. subtilis* contained streptolysin and lysins. To measure hemolytic activity of the various *Bacillus* strains on erythrocytes, nine isolated *Bacillus* probiotic strains were inoculated by streaking on plates containing blood-based agar supplemented with 5% (w/v) human sterile blood and 3% (w/v) NaCl. Plates were incubated at 37 °C for 24 h and results were determined, as described by Koneman et al. (2001), as: α-hemolysis (slight destruction of

hemocytes and erythrocytes with a green zone around the bacterial colonies);  $\beta$ -hemolysis (hemolysin that causes a clean hemolysis zone around the bacterial colonies); and  $\gamma$ -hemolysis (without any change in the agar around the bacterial colonies).

Hemolytic activity in shrimp hemocytes was tested, as described by Chin-I et al. (2000). Briefly, a 1-mL syringe was rinsed with EDTA buffer (450 mmol L<sup>-1</sup> NaCl, 10 mmol L<sup>-1</sup> 5 KCl, 10 mmol L<sup>-1</sup> EDTANa<sub>2</sub>, and 10 mmol L<sup>-1</sup> HEPES at pH 7.3). After disinfecting the surface of the shrimp weighing ~20 g with 70% ethanol; hemolymph was drawn with a sterile needle from between the fifth pair of pereopods; 1 mL hemolymph was immediately transferred to a sterilized tube containing 0.2 mL EDTA buffer and stained with 133  $\mu$ L 3% (w/v) Rose Bengal dye (#R4507, Sigma St. Louis, MO) dissolved in EDTA buffer with gentle shaking to achieve complete mixing. Aseptically, 1 mL of the stained hemolymph preparation was added to 15 mL sterile basal agar medium containing (10 g L<sup>-1</sup> Bacto 12 peptone (#211677, Difco), 5 g L<sup>-1</sup> HCl, and 15 g L<sup>-1</sup> Bacto agar (#214050, Difco) at pH 6.8) cooled to 45–50 °C, followed by gentle mixing and poured into Petri dishes. Shrimp blood agar plates with a rose red color were considered satisfactory because of the homogenously distributed stained hemocytes. When the hemocytes were destroyed by hemolytic bacteria, a clear zone (4 mm) appeared around the colonies. Four *Bacillus* strains isolated from the gut of adult *L. vannamei* (YC2-a, *B. amyloliquefaciens*; YC3-b, *B. endophyticus*; YC5-2, *B. tequilensis* and C2-2; *B. endophyticus*) exhibited type  $\gamma$  hemolytic activity (without any change in the agar around the bacterial colonies), three *Bacillus* strains (*B. licheniformis* strains YC1-a, YC3-a, and YC3-c) exhibited type  $\alpha$  hemolytic activity (slight destruction of hemocytes around the bacterial colonies), and two *Bacillus* strains (YC3-d and YC2-b) having type  $\beta$  hemolytic activity (destruction of hemocytes, showed a clean zone around the bacterial colonies) (Luis-Villaseñor et al., 2011).

#### 4. Mucus adhesion assay and bacterial growth in mucus

The intestinal epithelium is a natural barrier of the gastrointestinal tract providing defense against extrinsic invasions. The resident microflora, especially the beneficial ones, plays a crucial role in maintaining the host healthiness in numerous ways including; preserving the niche balance of intestinal microflora, reducing the colonization and invasion of pathogens, retaining the epithelial integrity and promoting immune function (Ouwehand et al., 1999; Herich and Levkut 2002). The strains with the highest adhesion ability have the greatest effect on host healthiness and performance (Majamaa et al., 1995; Shornikova et al., 1997; Kirjavainen et al., 1998; Ouwehand et al., 1999). Mucus composition varies from site to site. Among its major components is a group of high molecular weight glycoproteins called mucins. Depending upon the location, mucus may also contain various electrolytes, sloughed epithelial cells, plasma proteins, immunoglobulins, lysozyme, bacteria and their products, digested food material, digestive enzymes, epithelial cell membrane glycoproteins, and other components (Gibbons, 1982). The suggested functional properties of mucins are: Lubrication of epithelial surfaces; Diffusion barrier to nutrients, drugs, ions, toxins, and macromolecules; binding of bacteria, virus, parasites; Detoxification by heavy metal binding; Protection of mucosa against proteases; Interaction with immune surveillance system, and Interaction of membrane mucins with microfilaments (actins) (Forstner and Forstner, 1989).

The protective role of mucosal surfaces against potentially harmful substances such as acids, digestive enzyme, food lectins, toxins, bacterial and others infectious agents (Forstner and Forstner, 1989). The cell wall of Gram-positive bacteria is made up of a thick, multilayered peptidoglycan sacculus (also called murein) containing teichoic acids, proteins and polysaccharides (Vinderola et al., 2004). Mucin and cell surface carbohydrate are usually considered to be highly hydrophilic, although like other oligosaccharides, they can probably adopt amphipathic configurations (Sundari et al., 1991) to present a hydrophobic surface for interactions with some bacterial structures (Forstner and Forstner, 1994).

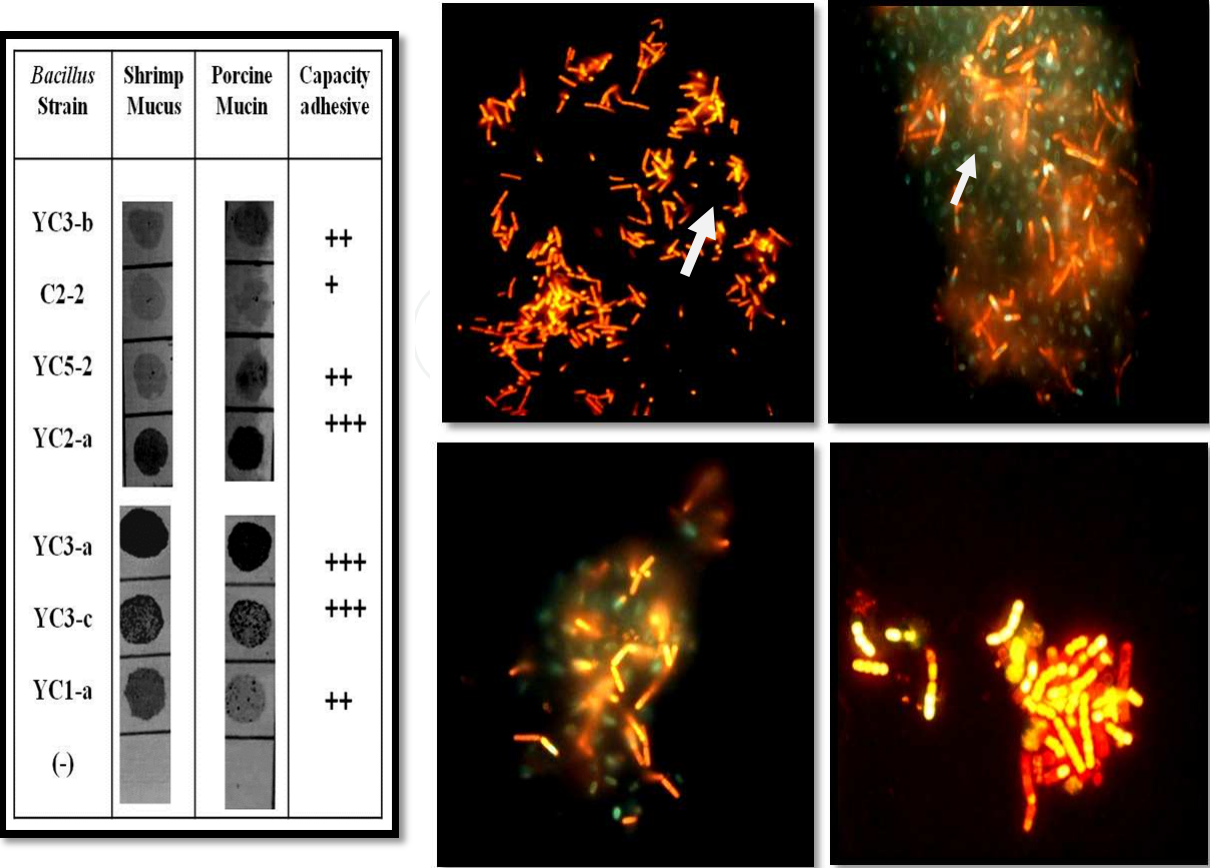
The ability to adhere to the intestinal mucus is considered one of the main criteria in the selection of potential probiotics as adhesion prolongs their permanence in the intestine and thus allows them to exert healthful effect (Apostolou et al., 2001).

During characterization of potential probiotics, we scrutinized their ability to adhere and colonize the intestine of shrimp. The dot-blot assays described in the present report is based on the formation of a complex between adhesion promoting compounds from the cell surface of the bacteria and the enzymatically labeled receptor in gastrointestinal mucus, followed by the visualization of bound components on a solid phase matrix (Rojas et al., 2002). Seven strains (YC2-a, YC3-b, YC5-2, C2-2, YC1-a, YC3-a and YC3-C) adhered to porcine gastric and crudes shrimp mucus (Fig 2). The seven isolates were able to grow in the mucus 24 h after inoculation; after 48 h viable cell counts were tower. These strains were examined for their ability to grown shrimp intestinal mucus. Sterility of mucus was confirmed on specific media. The number of viable cells decreased by ~50% at 48 h; strains22 YC5-2, YC3-a, YC3-c, YC1-a, and YC2-a had viable cell counts between  $18 \times 10^6$  UFC mL<sup>-1</sup> and  $10 \times 10^9$  UFC mL<sup>-1</sup> at 24 h, which decreased to between  $1.3 \times 10^6$  UFC mL<sup>-1</sup> and  $0.126 \times 10^6$  UFC mL<sup>-1</sup> at 48 h; however, abundant free spores were observed in five strains with epifluorescence microscopy (Table 2). Strains YC3-b and C2-2 had viable cell counts between  $1.87 \times 10^6$  UFC mL and  $4.14 \times 10^6$  UFC mL at 24 h, showing a decrease at 48 h with viable bacteria remaining about  $0.18 \times 10^6$  UFC mL<sup>-1</sup> for both strains. Similar studies reported that strains of *Bacillus* spp. able to grow in water and colonize the digestive tract of shrimp. This ability is related to competitive exclusion. However, in vitro activity assays cannot be used to predict a possible in vivo effect (Balcázar et al., 2006).

Bacterial Strains	CFU mL <sup>-1</sup>	
	Time (h)	
	24	48
YC3-B	$1.87 \times 10^6$	$0.18 \times 10^6$
C2-2	$4.14 \times 10^6$	$0.18 \times 10^6$
YC5-2	$>10 \times 10^9$	$0.126 \times 10^6$
YC2-a	$18 \times 10^6$	$1.3 \times 10^6$
YC3-A	$>10 \times 10^9$	$0.27 \times 10^6$
YC3-C	$>10 \times 10^9$	$0.84 \times 10^6$
YC1-A	$>10 \times 10^9$	$0.54 \times 10^6$
Control	0	0

**Table 2.** Growth of bacterial in mucus of shrimp *Litopenaeus vannamei*





**Figure 2.** A) Testing of adhesion of bacterial isolates to shrimp mucus and mucin by the Dot-blot method,(-): negative control (Buffer Hepes-Hanks) Capacity: weak adhesion(+), moderate adhesion (++) , strong adhesion (+++). B) Acridine orange staining of *Bacillus* spp. Adhered to mucus of shrimp observed by fluorescent microscope.

The presence of *Bacillus* species, whether as spores or vegetative cells, within the gut could arise from ingestion of bacteria associated with soil. However, a more unified theory is now emerging in which *Bacillus* species exist in an endosymbiotic relationship with their host, being able temporarily to survive and proliferate within the GIT. In some cases though, the endosymbiont has evolved further into a pathogen, exploiting the gut as its primary portal of entry to the host (*B. anthracis*) or as the site for synthesis of enterotoxins (*B. cereus*, *B. thuringiensis*) (Jensen et al., 2005).

5. Larval culture

Previous studies showed that inoculation with a probiotic strain during cultivation of larval *L. vannamei* (nauplii stage V) prevented colonization by a pathogenic strain, because the probiotic succeeds in colonizing the gut of the larvae (Zherdmant et al., 1997; Gómez-Gil et al., 2000). In this study, the effects of the probiotic strains cultured alone or mixed in the larval culture were evaluated. *Bacillus* strains were tested on larval shrimp using a daily concentration of  $1 \times 10^5$  CFU mL<sup>-1</sup>, starting each bioassay at nauplii V and a density of 225 nauplii L<sup>-1</sup>. Inoculations of four natural, commercial products and antibiotic oxytetracycline were added directly to the

water. Larvae inoculated with potential probiotic isolates at a density of  $1 \times 10^5$  CFU mL<sup>-1</sup> had significantly better survival than the control. The highest larval survival, compared to the control (4.9%) was inoculated with isolate YC5-2 (67.3%) and the commercial probiotic Alibio™ (57.4%). The low survival of the control shrimp (5%) in the second trial reinforced the view that probiotics are highly effective for increasing survival of larvae. Srinivas et al. (2010) showed that traditional practices (large exchange of water, application of disinfectants and antimicrobials, or both) are required to successfully complete the larval cycle; hence, the low survival rate in our control group in our bioassay was expected.

The larvae were sampled to determine the effect of the potential probiotics on larval development and rate of development, using the index of development (ID) described by Villegas and Kanazawa (1979):

$$ID = (\Sigma[i n_i]) / n,$$

Where *i* is the absolute value attributed to each larval stage (3 = ZIII; 4 = MI, 5 = MII; 6 = MIII, and 7 = PL1), *n<sub>i</sub>* is the total number of larvae at stage *i*, and *n* is the number of organisms measured.

A mix of two strains induced the highest rate of development (7.00), followed by Alibio™ (6.35). Highest larval survival occurred with single-strain treatments, but the highest rate of larval development was obtained with the *Bacillus* mix. The onset of exogenous feeding by larvae of penaeid shrimp is a critical phase in survival, growth, and development because the larval gut is exposed to microbes at the transition from nauplii 5 to zoea I (Jones et al., 1997). In our study, *Bacillus tequilensis* (strain YC5-2), *B. endophyticus* (strains C2-2 and YC3-b), and *B. amyloliquefaciens* (strain YC2-a) significantly increased development of larvae (Luis-Villaseñor et al., 2011). Using probiotics, modification of bacterial communities in tank water improves cultivation of larval crustaceans (Balcazar et al., 2007b; Garriques and Arevalo, 1995; Gómez et al., 2008; Guo et al., 2009; Nogami and Maeda, 1992) and bivalves Douillet and Langdon (1993, 1994); Riquelme et al., 1996, 1997, 2001). Our study advances previous work demonstrating that probiotics maintain a balanced and natural bacterial community that improves production of shrimp larvae, which is also reflected in the rate of development, as demonstrated in our two bioassays with *Bacillus* spp.



**Figure 3.** Larvae shrimps of *Litopenaeus vannamei* in stage Zoea III and Mysis I.

Decamp et al. (2008) administered *B. subtilis* and *B. licheniformis* to larval *L. vannamei* and *Penaeus monodon* and this inhibited growth of *Vibrio* strains and increased the survival rate of the shrimp. Inhibitory effects of *Bacillus* are attributed to various causes: alterations of the pH in growth medium, use of essential nutrients, and production of volatile compounds (Chaurasia et al., 2005; Gullian et al., 2004; Yilmaz et al., 2006).

## 6. Modulation of microbiota

Intestinal bacteria thrive in a stable, nutrient rich environment but serve beneficial function to the host including energy salvage of otherwise indigestible complex carbohydrates, vitamin and micronutrient synthesis, activation of immune response, development and competitive exclusion of pathogenic microorganisms (Neish et al., 2010). It is clear that bacterial species of the gut can influence the health and robustness of the host. One of the problems associated with evaluating *Bacillus* products (or indeed any probiotic product) for aquaculture is determining whether the observed effect is due to the action of the bacterium on the host gut or due to an indirect effect on water quality or antagonism of external pathogens. Regardless, sufficient evidence suggests that adding *Bacillus* as spores or vegetative cells to rearing ponds has a beneficial effect. It is important to know the origin of the probiotic strain in order to increase the probability of survives and colonize the gastrointestinal tract of the host (Vine et al., 2004). The interest in investigating the intestinal microbiota is based on the need for a better understanding of how probiotics can influence the bacterial composition. For instance, Oxley et al., 2002, examined the bacterial flora of healthy wild and reared *P. mergulensis* shrimp and found a high abundance of *Vibrio*, the authors also found that the bacterial floras of wild and reared penaeid shrimp are similar and suggested that shrimp may influence and/or select the composition of their gut microbiota. To study the intestinal microbiota composition, culture-dependent methods are considered inadequate because more those 99% of all bacteria cannot yet be cultivated (Amann et al. 1995). Composition of the aquatic bacterial community in ponds has a strong influence on the internal bacterial flora of farmed marine animals, which is vital for their nutrition, immunity, and disease resistance (Luo et al., 2006). At the same time, it also impacts, and is impacted by, the bacterial communities in the nearby marine environments that receive aquacultural effluents (Guo & Xu 1994). Intestinal microbiota of cultivated aquatic organisms is an important factor in maintaining health, either by preventing colonization by pathogens, decomposition of food, production of antimicrobial compounds, releasing nutrients, and maintaining normal mucosal immunity (Escobar-Briones et al., 2006).

Single Strain Conformation Polymorphism (SSCP) is based on sequence-specific separation of polymerase chain reaction (PCR)-derived rRNA gene amplicons in polyacrylamide gels is used to study the diversity of microbes based on the sequence difference of PCR products of 16S rDNA gene amplified from different microbes (Dohrmann and Tebbe, 2004). Our interest in intestinal microbiota is based on the need for understanding how probiotics influence bacterial composition. Similar studies have been performed in vertebrates (Brikbeck et al., 2005; Austin, 2006; Escobar-Briones et al., 2006; Bakke-McKellep, 2007; He et al., 2009; Nayak, 2010; Tapia-Paniagua et al., 2010). In invertebrates, studies are limited; they include Pacific white shrimp *Litopenaeus vannamei* (Johnson et al., 2008), Kuruma shrimp

*Marsupenaeus japonicus* (Liu et al., 2010), European lobster *Homarus gammarus* L. (Daniels et al., 2010), and Chinese shrimp *Fenneropenaeus chinensis* (Liu et al., 2011).

We used probiotic strains of *Bacillus* that are antagonistic to pathogenic strains of *Vibrio*, are not harmful to juvenile shrimp, and adhere to and grow on intestinal mucosa, which is an important factor in colonizing or at least remaining for a moderate amount of time in the shrimp gut (Luis-Villaseñor et al., 2011). In our study, SSCP analysis using universal primers targeting the V4 and V5 regions of the 16S rRNA gene were used to visualize the bacterial diversity and identify the dominant intestinal bacterial in juvenile shrimp *L. vannamei* (Fig. 4). Tanks were stocked with 21 shrimp ( $8 \pm 0.1$  g each), and inoculated daily with one of the following treatments:

1. *Bacillus* mix at a density of  $0.1 \times 10^6$  CFU mL<sup>-1</sup>.
2. Commercial probiotic Alibio® at  $1 \times 10^6$  CFU mL<sup>-1</sup>.
3. Control: Juvenile *L. vannamei* without probiotics.

Each treatment and control was performed in quintuplicate and each replicate was represented by one tank.

A total of 119 bands from four SSCP gels were registered, sequenced, and identified. Analysis of the SSCP fingerprints showed that the composition of the intestinal microbiota of juvenile *L. vannamei* exposed to a *Bacillus* mix was modified. The shrimp treated with *Bacillus* mix showed higher bacterial diversity than the control groups. Liu et al. (2010) reported that the addition of *Bacillus* spp. in feed of the shrimp *Marsupenaeus japonicus* increased individual variation and the total diversity of bacterial species.

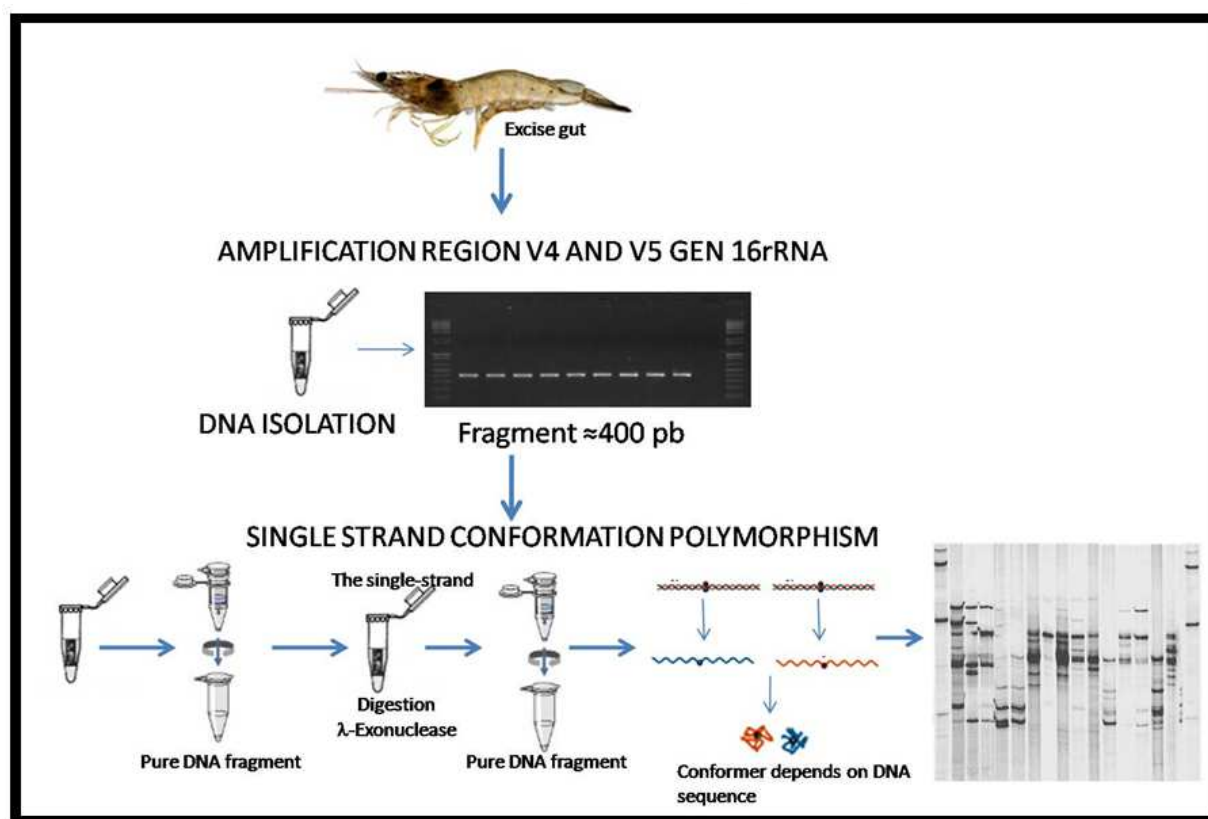
A comparison of the patterns obtained from shrimp gut samples inoculated with probiotics at 5 days showed uniformity in the composition of the microbiota and clustering with high similarity of 71.3% and 71.21% for *Bacillus* mix and Alibio, respectively. However, both exhibited a lower similarity than control group by 23.7% (Fig. 5a).

The dendrogram analysis at day 10 showed that SSCP pattern in samples from shrimp treated with *Bacillus* mix were clustered into one group was 62.3% for M1-M2 and 82.8% for M4-M5, whereas shrimps treated with Alibio were clustered into a different one had similarity of 72.7% (A1-A5). Results were heterogeneous in the Control group, with similarity of 50.6% for C1-C4 and 84.6% for C2-A4 (Fig. 5b). Similarity at day 15 had the highest homogeneity between treatments: 86.9% for the *Bacillus* mix treatments (M1-M3) and 93.2% (M2-M4) and 87.6% for the Alibio treatments (A1-A3) and 93.9% (A1-A5) (Fig. 6a). Similar banding patterns occurred at day 20, reaching 89.9% to 98.5%. Variation in the communities with each treatment group did not vary greatly (Fig. 6b).

In our study, most of the OTUs identified by SSCP gels treated with the probiotics belong to phylogenetic groups class  $\alpha$ - and  $\gamma$ -proteobacteria, flavobacteria, shingobacteria, and fusobacteria, compared with other species of invertebrates, where the microbiota were represented by class  $\alpha$ -,  $\gamma$ -, and  $\epsilon$ -proteobacteria in fleshy prawn *Fenneropenaeus chinensis* (Liu et al., 2011), by fusobacteria and  $\gamma$ -proteobacteria in giant tiger prawn *Penaeus monodon*



(Chaiyapechara et al., 2011), and by derribacteres, mollicutes,  $\gamma$ - and  $\varepsilon$ -proteobacteria, small fractions of firmicutes, cytophaga-flavobacter-bacteroides, verricomicrobiae,  $\beta$ - and  $\delta$ -proteobacteria in vent shrimp (Durand et al., 2010). Furthermore, the gut content of shrimps inoculated with the *Bacillus* mix and Alibio had higher bacterial diversity, compared with the controls, supported by the total number of OTU's.

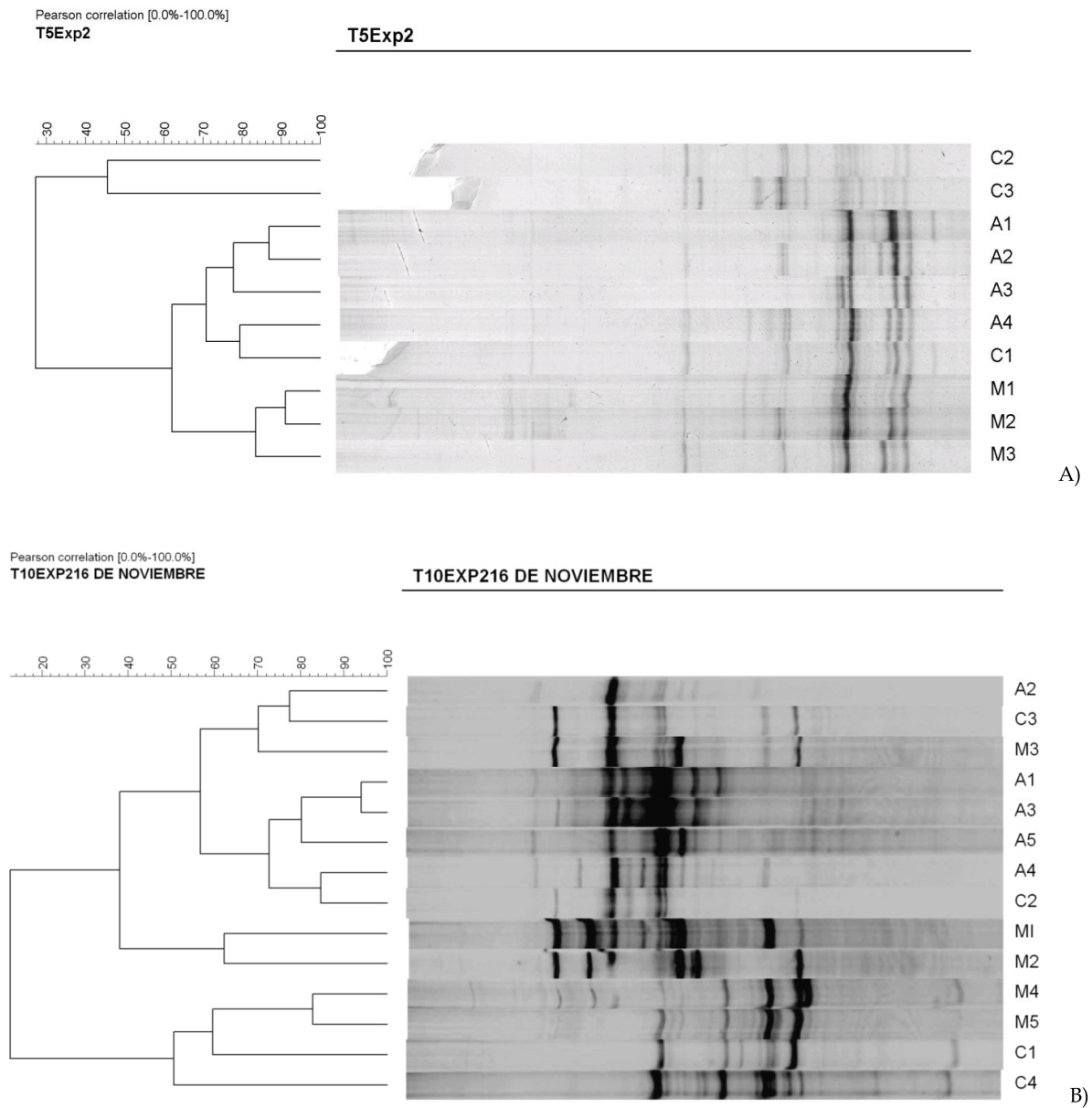


**Figure 4.** Schematic illustrating the process of Single strand conformation polymorphism (SSCP).

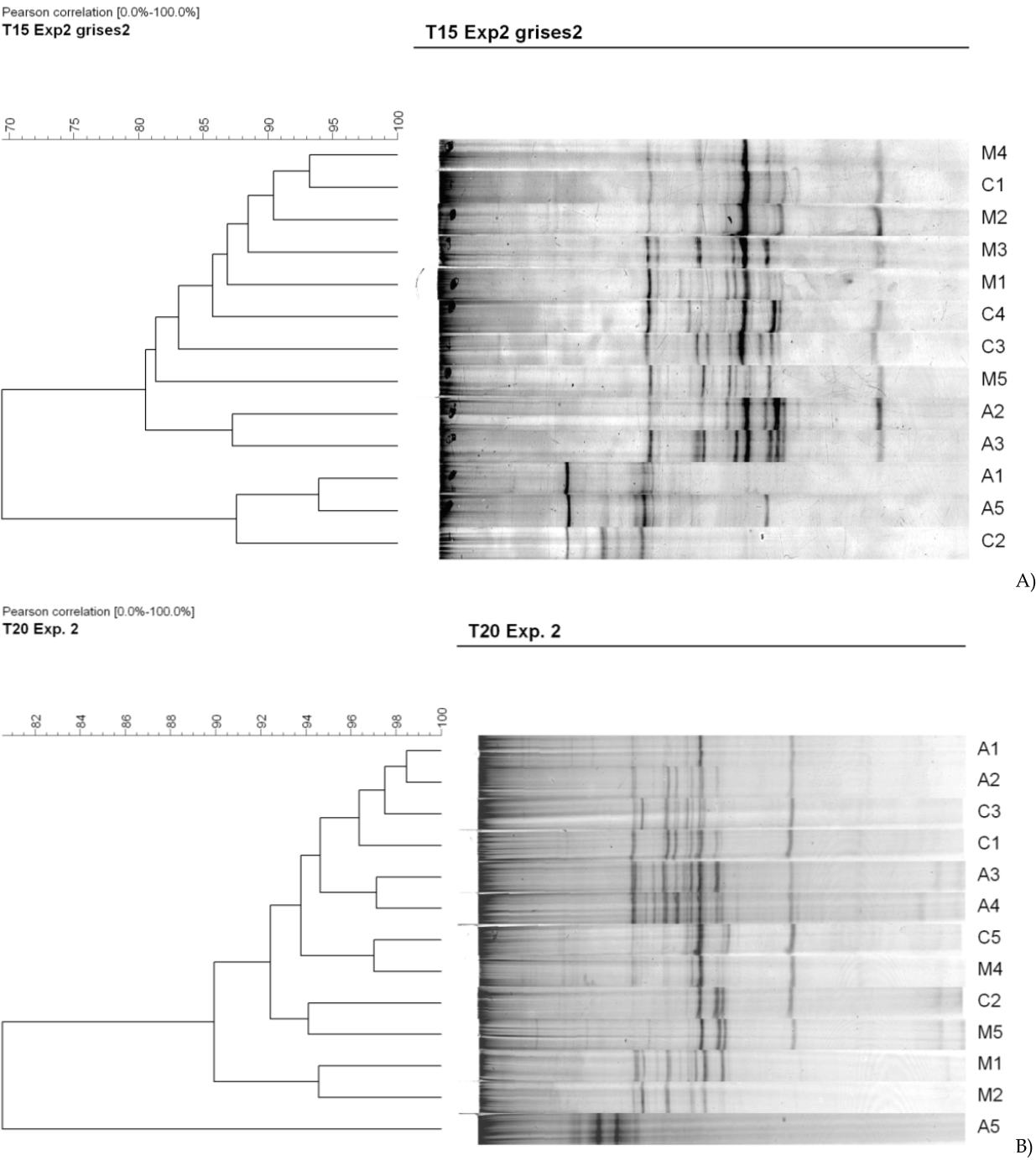
The intestinal bacterial community shows a similar dominance of  $\alpha$ -proteobacteria and flavobacteria at all times in shrimp treated with probiotics. The resident community included *Maribius salinus* and *Donghicola eburneus* ( $\alpha$ -proteobacteria) and *Wandonia haliotis* (flavobacteria) in all treatments. Dominance of  $\gamma$ -proteobacteria occurs in the intestinal community of other crustaceans, including *Fenneropenaeus chinensis* (Liu et al., 2011), ornate rock lobster *Panulirus ornatus* (Payne et al., 2007), *Rimicaris exoculata* (Durand et al., 2009), European lobster *Homarus gammarus* L. (Daniels et al., 2010), and *Penaeus monodon* (Chaiyapechara et al., 2011).

Sequence analysis showed that at day 5, intestines of the shrimp were dominated by phylogenetic groups flavobacteria and  $\alpha$ -proteobacteria. At day 15, the *Bacillus* mix treatment had small populations of  $\alpha$ -proteobacteria and flavobacteria, the Alibio treatment led to the appearance of sphingobacteria and fusobacteria. At day 20,  $\alpha$ - and  $\gamma$ -proteobacteria, sphingobacteria, and flavobacteria were present, with few variations between treatments.

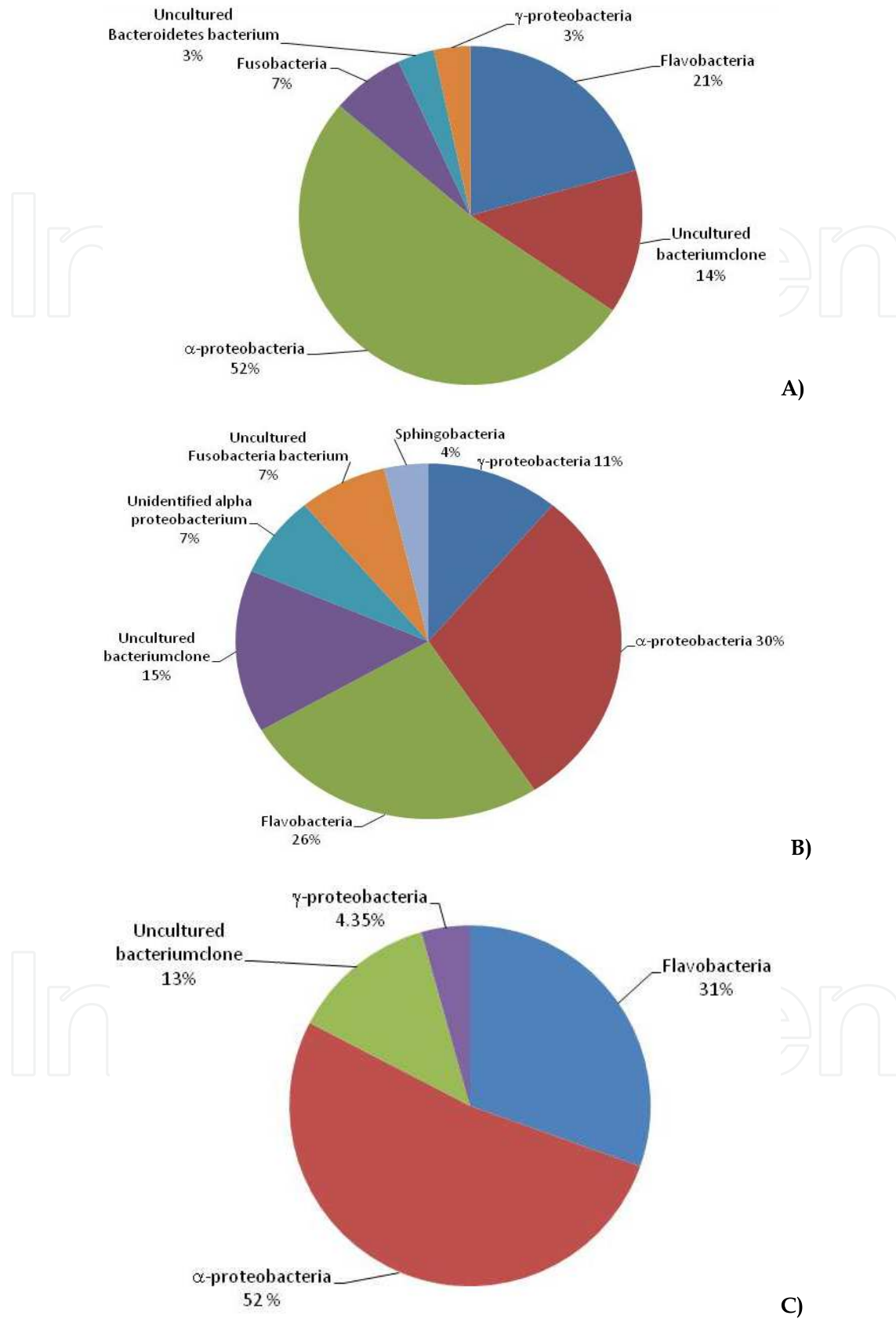




**Figure 5.** Dendrogram illustrating the relationship (percent similarity) between bacterial communities in gut of shrimp at 5 d (a) and 10 d (b) inoculated with probiotics; M1–M5 (*Bacillus*mix), A1–A5 (commercial probiotic), C1–C4 (without probiotics). Scale of dendrogram show similarity percent of clusters. The dendrogram was calculated with UPGMA and Pearson correlation.



**Figure 6.** Dendrogram illustrating the relationship (percent similarity) between bacterial communities in shrimp gut at 15 d (a) and 20 d (b) inoculated with probiotics; M1–M5 (*Bacillus*mix), A1–A5 (commercial probiotic), C1–C4 (without probiotics). Scale of dendrogram showed similarity percent of clusters. The dendrogram was calculated with UPGMA and Pearson correlation.



**Figure 7.** Composition of intestinal bacterial community of individual *L. vannamei* inoculated with probiotics *Bacillus* mix (M5-M20), Alibio (A5-A20), and Control (C5-C20) based on 16S rRNA.

Dempsey et al., (1989) suggest that only one or two phylogenetic groups dominate the shrimp gut and have very low diversity. The most common genera of gut microbiota in aquatic invertebrates are *Vibrio*, *Pseudomonas*, *Flavobacterium*, *Micrococcus*, and *Aeromonas* (Harris, 1993). These reports of gut communities in shrimp were based mainly on culture dependent microbiological techniques. Comparisons with molecular techniques indicate that 10–50% of population is cultivable (Holzapfel et al., 1988; Wilson et al., 1996). Since the SSCP monitors the predominant bacteria in a sample, bands representing *Bacillus* probionts were not detected because the density of probiotic strains was  $<0.1 \times 10^6 \text{CFU mL}^{-1}$ . Smalla et al., (2007) reported that DGGE and SSCP can contribute to the generation of the same bands, hence, leading to an underestimate of diversity. Likewise, Muyzer et al., (2003) shows that DGGE can only detect 1–2% of the microbial population representing the dominant species present in microbial communities.

## 7. Conclusion

*Bacillus* spp. exposed to *L. vannamei* increased survival, and development in larvae, and modulated the intestinal microbiota in juvenile shrimp. This study demonstrated that the management the properly combinations of selected *Bacillus* isolates are a good option to improve health, rate of development, and survival in shrimp. The isolates we tested were antagonistic to pathogenic strains of *Vibrio* and were not harmful to the larvae. Their ability to adhere and grow in intestinal mucosa is an important factor in colonizing or at least remaining for short time periods in the gut of shrimp. More rapid development also occurred when the larvae were treated with mixtures of *Bacillus* strains. Treatment Mix-2 increased survival and larval development, compared to the control group. Similar results were found by Guo et al. (2009), where *B. fusiformis* increased survival and accelerated metamorphosis of *P. monodon* and *L. vannamei* larvae. This study demonstrated that management that combines properly selected *Bacillus* isolates are a good option in larviculture to improve health, rate of development, and rate of survival of whiteleg shrimp.

In summary, analysis of SSCP fingerprints demonstrated that the composition of the intestinal microbiota of shrimp inoculated with the *Bacillus* mix was distinctly different from the control group. The *Bacillus* mix significantly reduced species diversity and richness and increased similarity of the microbial communities within the probiotic replicates, reducing diversity compared to the control, predominantly consisting of  $\alpha$ - and  $\gamma$ -proteobacteria, fusobacteria, sphingobacteria, and flavobacteria.

## Author details

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