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Vibrios associated with *Penaeus chinensis* (Crustacea: Decapoda) larvae in Chinese shrimp hatcheries

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Abstract

Bacteriological surveys were performed in 1995 and 1996 in three shrimp hatcheries located in the north of the People's Republic of China. Samples were taken from routine productions of healthy *Penaeus chinensis* larvae, their environment and from diseased larvae. A total of 186 isolates from the dominant bacterial flora was characterized by Biolog metabolic fingerprinting and identified by comparison to a database of 850 *Vibrio* type- and reference strains. Representative *Vibrio harveyi* strains were further genotypically analyzed by AFLP fingerprinting of whole-genomes. An overwhelming predominance of *V. alginolyticus* and *V. harveyi* was observed in the larval developmental stages from zoea stage on. The flora associated with larvae is not very stable and is influenced by the bacterial flora of the administered food and by the environment. In the 1995 survey, the bacterial flora of successful *P. chinensis* larvae productions was mainly dominated by *V. alginolyticus* and unidentified Gram negative strains, while *V. harveyi* was absent. The bacterial numbers gradually increased from nauplii stage to post-larval stage, but few vibrios were isolated from nauplii stage. High *V. harveyi* numbers (up to 10^5 CFU/larva) in the

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larvae are correlated with weak larvae and mass mortalities. Between *V. harveyi* strains, isolated from healthy and diseased larvae, no phenotypic or genotypic differences were found. The presence of *V. alginolyticus* might influence the pathogenicity of *V. harveyi* or might have an impact on the resistance of larvae to bacterial pathogens.

Keywords: Shrimp hatcheries; Post-larval stage; Mass mortalities

1. Introduction

Due to bacterial and viral disease incidence in farmed penaeid shrimp in China, industrial productions have dropped to 55,000 tons in 1994 which is only 25% of the 1992 production level (Wang et al., 1997). The collapse has been caused by the 'White spot syndrome virus' (also referred to as HHNBV, SEMBV, WSBV or RVPJ) which became epizootic due to a deteriorated ecological environment (Wang et al., 1997). For nearly as long as penaeid shrimp have been cultured, reports of bacterial infections and diseases caused by *Vibrio* spp. have been by far the most numerous (Lightner, 1993). Outbreaks of vibriosis have been reported and have become a severe barrier for further development of shrimp aquaculture in PRC. Numerous *Vibrio* species have been reported as causal agents of diseases in various penaeid shrimp species: *Vibrio harveyi* infections in *Penaeus indicus* larvae (Prayitno and Latchford, 1995) and in *P. monodon* larvae (Lavilla-Pitogo et al., 1990), *V. parahaemolyticus* infections in *P. monodon* juveniles and adults (Ruangpan and Kitao, 1991; Nash et al., 1992; Chanratchakool et al., 1995) and in *P. orientalis* juveniles and adults (Xu et al., 1994), *V. vulnificus* infections in *P. monodon* juveniles and adults (Song et al., 1990; Ruangpan and Kitao, 1991; Nash et al., 1992; Chanratchakool et al., 1995), *V. damsela* infections in *P. monodon* juveniles and adults (Nash et al., 1992), *V. campbellii* infections in *P. orientalis* larvae (Xu et al., 1994) and *V. splendidus* infections in *P. monodon* larvae (Lavilla-Pitogo et al., 1990; Prayitno and Latchford, 1995). Reports on the involvement of *Vibrio* species in diseased *P. chinensis* larvae exist, but are published in PRC, in Chinese. Moreover, only Xu et al. (1994) and Prayitno and Latchford (1995) mentioned the verification of Koch's postulates and succeeded the reisolation of the presumed pathogens from diseased larvae.

Because vibrios are also isolated from healthy penaeid shrimp, the hypothesis of the opportunistic nature of vibrios associated with penaeid shrimp has become widely accepted (Ruangpan and Kitao, 1991; Hameed, 1993; Lightner, 1993;). Opportunistic vibrios may cause serious problems in larvae when they are suffering from stress caused by (i) suboptimal or unstable environment, (ii) high stocking densities and (iii) inadequate management (Nash et al., 1992). Prayitno and Latchford (1995) have shown that the pathogenicity of *V. harveyi* and *V. splendidus* strains is related to the age of the larvae of *P. indicus* and *P. monodon* suggesting that different pathogenic strains might be involved at different larval developmental stages. Vibriosis in penaeid shrimp hatcheries might be due either to an opportunistic *Vibrio* flora or to pathogenic *Vibrio* strains, specifically infective at one or more larval developmental stages.

In this paper, we report on the dominant microflora associated with the larval developmental stages during *P. chinensis* productions in People's Republic of China.

2. Material and methods

2.1. Sample collection

In spring 1995, *P. chinensis* larvae of different stages and their environment, were collected at Dahua and Sanshandao hatcheries both located in Laizhou (Shandong Province, PRC) (Table 1). The larval food (egg yolk emulsion), the larval environment, i.e., tankwater, inletwater, wastewater, and nearshore seawater were sampled.

The spring 1996 sampling was focused on healthy and diseased larvae of different developmental stages. Samples were collected at Dahua and Fengcheng hatcheries, located in Laizhou and Jimo, respectively (Shandong Province, PRC) (Table 1). From water samples, serial dilutions were made in 0.85% sterile saline solution and duplicate 0.1 ml aliquots were plated on Marine Agar (MA) containing Marine broth 2216 (Difco, Detroit, USA) supplemented with 1.5% (w/v) bacteriological agar no. 1 (Oxoid, Hampshire, England) or on Thiosulphate Citrate Bile Sucrose Agar (TCBS) (Oxoid).

Larvae were washed three times with 100 ml of sterile seawater on sterile filters and transferred to sterile 0.85% saline solution. For nauplii, zoea and mysis stages and post-larval stages, respectively 50, 20 and 10 to 15 larvae were collected and homogenized in a sterile Potter blender and diluted serially using 0.85% saline solution. Duplicate aliquots (0.1 ml) of the dilutions were plated on MA and on TCBS. All plates were incubated at 28°C.

2.2. Isolation and storage of the isolates

Colony counts were made after 2 to 7 days of growth. One to three dominant colonies were selected based on their morphological appearance and further purified on MA. Pure cultures were stored in a deepfreezer at −80°C or in a nitrogen container at −140°C after addition of Marine broth to which 20% (w/v) glycerol was added.

2.3. Characterization of the isolates

Gram staining was performed using the method described by Smibert and Krieg (1981). The further phenotypic characterization was performed by the Biolog GN technique microplates (Austin et al., 1995a). Strains were grown on Brain Heart Infusion (BHI) (Difco, Detroit, USA) supplemented with 1.5% (w/v) bacteriological agar no.1 and with 1.5% (w/v) NaCl or on MA for 24 h at 25°C. Inocula were prepared in 1.5% (w/v) NaCl solution and cell densities were photometricaly standardized between 0.261 and 0.300 OD using a photometer at 590 nm. The wells of the Biolog GN microplates (Biolog, Hayward, CA, USA) were inoculated with the cell suspension and the microplates were incubated for 24th at 25°C. Changes in colour were measured using a Multiscan Multisoft filter photometer (Labsystems, Helsinki, Finland) at 550 nm. For

Table 1
List of the isolates from routine productions of *P. chinensis* in Chinese hatcheries

Hatchery	Date	Source	Number of isolates	Isolation medium	Identification (number of isolates)
Dahua	Spring 1995	Healthy larvae	27	MA or TCBS	*V. alginolyticus* (7), *V. ichtyoenterii* (1), *Vibrio* STD3-139 (1), Unidentified STD3-366 (5), Unidentified STD3-437 (2), Other Unidentified (11)
Dahua	Spring 1995	Environment healthy larvae	59	MA	*V. alginolyticus* (2), Unidentified STD3-366 (10), Unidentified STD3-437 (5), Unidentified STD3-399∗ (1), Other Unidentified (41)
Dahua	Spring 1995	Diseased larvae	4	MA or TCBS	Unidentified STD3-366 (1), Other Unidentified (3)
Sanshandao	Spring 1995	Healthy larvae	7	MA or TCBS	V. alginolyticus (1), Unidentified STD3-366 (3), Other Unidentified (3)
Sanshandao	Spring 1995	Environment healthy larvae	14	MA	*V. alginolyticus* (1), *Vibrio* STD3-348 (1), Unidentified STD3-366 (2), Unidentified STD3-437 (1), Other Unidentified (9)
Sanshandao	Spring 1995	Diseased larvae	6	MA or TCBS	*V. alginolyticus* (3), Unidentified (3)
Dahua	Spring 1996	Healthy larvae	52	MA or TCBS	*V. alginolyticus* (18), *V. harveyi* (13), *Vibrio* STD3-1085 (2), *Vibrio* STD3-348 (3), *Vibrio* STD3-1057 (2), *Vibrio* STD3-1058 (1), Unidentified STD3-399∗ (4), Other Unidentified (9)
Fengcheng	Spring 1996	Diseased larvae	17	TCBS	V. harveyi (9), Vibrio STD3-348 (1), *Vibrio* STD3-1008 (1), *Vibrio* STD3-1018 (2), *Vibrio* STD3-1085 (2), *Vibrio* STD3-1057 (1), Unidentified (1)

The strains were identified by Biolog metabolic fingerprinting and by comparison to databases of reference strains.

identification, the metabolic fingerprints of the strains grown on BHI agar were compared to a database containing Biolog fingerprints of 850 *Vibrio* reference strains, including all the *Vibrio* type strains. Comparison of the strains was performed by numerical analysis using the Pearson product moment correlation coefficient and the UPGMA (Sneath and Sokal, 1973) clustering method. Clusters were delineated at 80%r and isolates clustering with typestrains were considered to belong to the same species.

Fatty acid methyl ester (FAME) gas chromatographic analysis was used to identify non-vibrios according to the method described by Vandamme et al. (1992). Strains were grown on Trypticase Soy Broth (Becton Dickinson, Cockeysville, USA) supplemented with 1.5% (w/v) bacteriological agar no. 1 (Oxoid, Hampshire, England) at 28°C for 24 h. Fame fingerprints were identified by using the Microbial Identification System software package (MIS version no. 3.9) obtained from Microbial ID, Netwark, DE (USA), and a calibration mixture of known standards (Hewlett-Packard).

Genotypic characterization of the isolates, STD3-999, STD3-1002, STD3-1020, STD3-1035, STD3-1041 and STD3-1060, was performed by AFLP fingerprinting of whole-genomes employing two oligonucleotide primers, H02 and T02 according to the protocols reported by Janssen et al. (1996).

3. Results and discussion

3.1. Identification of the isolates

All 186 isolates were Gram negative. Seventy-two isolates were identified as *Vibrio* (Tables 1 and 2). Three *Vibrio* species were identified, i.e., *V. alginolyticus* (32 strains), *V. harveyi* (21 strains) and *V. ichtyoenterii* (1 strain). Seven other *Vibrio* groups were delineated and labeled according to the reference strain of the cluster (*Vibrio* STD3-348, *Vibrio* STD3-139, *Vibrio* STD3-1008, *Vibrio* STD3-1018, *Vibrio* STD3-1057, *Vibrio* STD3-1085 and *Vibrio* STD3-1058). The other 114 strains were characterized using Biolog, but remained unidentified (Tables 1 and 2). Also with Fatty Acid Methyl Ester (FAME) analysis, no unambiguous identification of these strains could be obtained.

The identity of six representative *Vibrio* isolates, previously identified as *V. harveyi* using the Biolog GN technique, is questioned by the AFLP genomic fingerprinting result. The AFLP patterns of these strains (Fig. 1) indicate that they are genotypically very similar, but they show only 30%r similarity with the cluster containing the typestrain and reference strains of *V. harveyi*. The exact taxonomic position of these *V. harveyi* strains needs further clarification.

3.2. Distribution of the isolates according to the larval developmental stages

In this survey, an overwhelming predominance of *V. alginolyticus* and *V. harveyi* was observed in the larval developmental stages of *P. chinensis* larvae from zoea stage on (Table 2). Bacterial counts for both species vary between 10^2 to 10^5 CFU/larva. *V. alginolyticus* was mostly isolated from healthy and only in a few cases from diseased larvae. *V. harveyi* were as frequently isolated from healthy or diseased individuals, although this species was absent in the samplings of 1995 (Table 3). Increasing bacterial

Table 2
Distribution of the isolates of healthy and diseased *P. chinensis* larvae of different developmental stages and their environment

	Nauplii		Zoea		Mysis		Post larvae		Environment		Total	
	H	D	H	D	H	D	H	D	H	D	H	D
Vibrio strains												
Vibrio alginolyticus	1	0	6	3	8	0	11	0	3	0	29	3
Vibrio harveyi	0	1	3	4	1	2	9	2			13	9
Vibrio ichtyoenterii							1	0			1	0
Vibrio STD3-348			1	0	1	0	1	1	1	0	4	1
Vibrio STD3-139							1	0			1	0
Vibrio STD3-1008							0	1			0	1
Vibrio STD3-1018			0	1	0	1					0	2
Vibrio STD3-1057	1	0	1	1							2	1
Vibrio STD3-1085			2	1	0	1					2	2
Vibrio STD3-1058			1	0							1	0
Subtotal *Vibrio* strains	2	1	14	10	10	4	23	4	4	0	53	19
Unidentified G-strains												
Unidentified STD3-366			5	1			3	0	12	0	20	1
Unidentified STD3-437							2	0	6	0	8	0
Unidentified STD3-399*	1	0	1	0	1	0	1	0	1	0	5	0
Other unidentified	4	0	10	6	2	1	7	0	50	0	73	7
Subtotal unidentified G-strains	5	0	16	7	3	1	13	0	69	0	106	8
Total of strains analysed	8		47		18		40		73		159	27

H: Isolates from healthy larvae.
D: Isolates from diseased larvae.
Environment H&D: isolates from the inletwater, wastewater, tankwater, nearshore seawater and food of healthy and diseased larvae, respectively.

numbers were found from nauplii to post-larval stages. The dominant bacteria associated with nauplii remained unidentified and only few vibrios were found associated with this larval developmental stage.

3.3. Comparison of the microbial flora of the different hatcheries

In 1995, samplings were done at Dahua and Sanshandao hatcheries. The *Vibrio* diversity was low and bacterial counts were low and did not exceed 10^2 CFU/larva. The bacterial flora was dominated by *V. alginolyticus* and unidentified Gram negative strains (groups STD3-366 and STD3-437). These bacteria were characteristic for successful productions of *P. chinensis* larvae.

In 1996, samplings were done from healthy and diseased larvae at Dahua and Fengcheng hatcheries. In both hatcheries, the *Vibrio* diversity was higher than in 1995. Eight different *Vibrio* species were isolated, i.e., *V. alginolyticus*, *V. harveyi*, *Vibrio* STD3-348, *Vibrio* STD3-1008, *Vibrio* STD3-1018, *Vibrio* STD3-1057, *Vibrio* STD3-1085 and *Vibrio* STD3-1058 (Table 3).

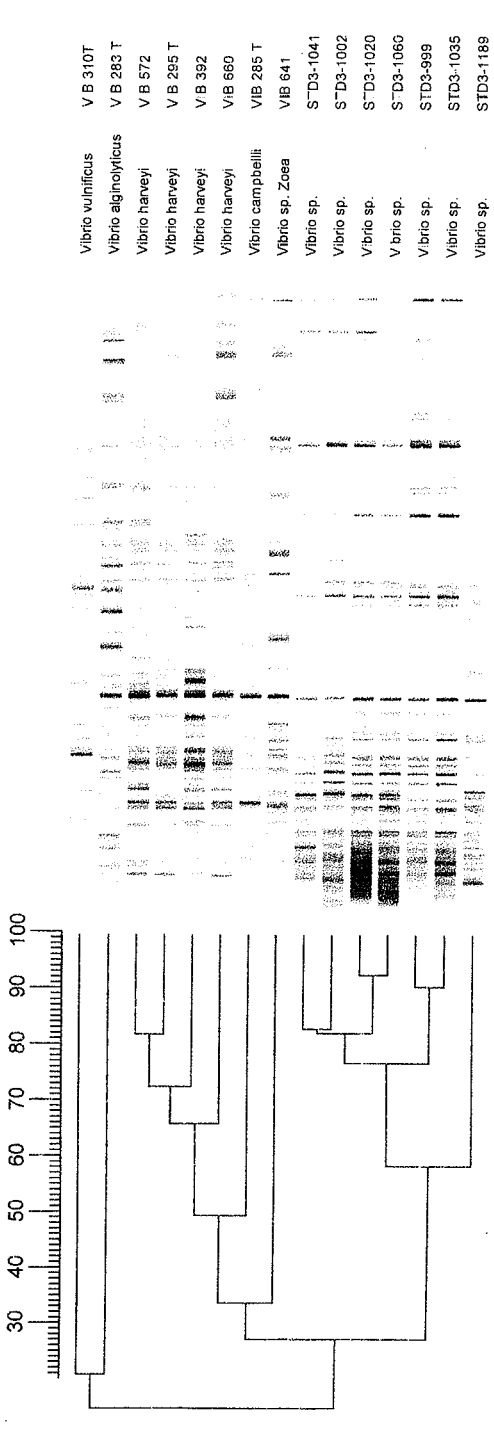

Fig. 1. AFLP fingerprints of whole-genomes of *V. harveyi* strains isolated from diseased and healthy *P. chinensis* larvae and from *Vibrio* reference strains. For comparison of the AFLP fingerprints, the Pearson product moment correlation coefficient was used. The isolates were grouped by UPGMA.

Table 3
Distribution of the isolates of healthy and diseased *P. chinensis* larvae from Dahua, Sanshandao and Fengcheng hatcheries during two production cycles

	1995				1996					
	Dahua		Sanshandao		Dahua		Fengcheng		Total	
	H	D	H	D	H	D	H	D	H	D
Vibrio strains										
Vibrio alginolyticus	9	0	2	3	18	0			29	3
Vibrio harveyi					13	0	0	9	13	9
Vibrio ichtyoenterii	1	0							1	0
Vibrio STD3-348			1	0	3	0	0	1	4	1
Vibrio STD3-139	1	0							1	0
Vibrio STD3-1008							0	1	0	1
Vibrio STD3-1018							0	2	0	2
Vibrio STD3-1057					2	0	0	1	2	1
Vibrio STD3-1085					2	0	0	2	2	2
Vibrio STD3-1058					1	0			1	0
Subtotal *Vibrio* strains	11	0	3	3	39	0	0	16	53	19
Unidentified G-strains										
Unidentified STD3-366	15	1	5	0					20	1
Unidentified STD3-437	7	0	1	0					8	0
Unidentified STD3-399 *	1	0			4	0			5	0
Other unidentified	52	3	12	3	9	0	0	1	73	7
Subtotal unidentified G-strains	75	4	18	3	13	0	0	1	106	8
Total of strains analysed	90		27		52		17		159	27

H: Isolates from healthy larvae and their environment.
D: Isolates from diseased larvae and their environment.

In Dahua hatchery, no disease outbreaks occurred in 1996. Variations in the presence of vibrios were found between the different larval developmental stages. Only very few vibrios were isolated from nauplii, but they become the dominant taxon from stage zoea 3 to post-larvae 5 stages. *V. alginolyticus* was the dominant species isolated from healthy zoea 3 to mysis 3 stages and bacterial counts varied between 10^2 to 10^3 CFU/larva. *V. harveyi* became, instead of *V. alginolyticus*, predominant during healthy mysis 3 to post-larvae 5 stages, with bacterial counts varying between 10^2 to 10^4 CFU/larva. Nevertheless, *V. alginolyticus* was always present as the second dominant taxon.

Disease outbreaks hit Fengcheng hatchery quite often in 1996. Symptoms of the rapidly spreading disease were: inactivity, anorexia and opaqueness of the larvae, loss of setae and finally the larvae settled on the bottom of the tanks and died. Neighboring hatcheries had already experienced similar disease outbreaks, preventing the cultivation of larvae to post-larvae. Larvae of all developmental stages of nine diseased tanks were examined: in all cases *V. harveyi* was isolated from larvae in high numbers (up to 10^5 CFU/larva). Histological studies showed a massive bacterial colonization of the feeding

appendages and the foregut, suggesting an oral infection route, followed by infection of the midgut, the hepatopancreas and a terminal septicemia. Lightner (1993) has described similar infections caused by other vibrios in Ecuador. Very surprising is the absolute absence of *V. alginolyticus* in the samples from Fengcheng, although this species was always isolated from healthy larvae in Dahua hatchery during the same sampling period.

3.4. Relationship between V. harveyi and V. alginolyticus

Many publications report on the isolation of *V. harveyi* from diseased penaeid shrimp (Lavilla-Pitogo et al., 1992; Owens et al., 1992; Lightner, 1993; Prayitno and Latchford, 1995) and other marine animals (Kraxberger-Beatty and McGarey, 1990). The presence of *V. harveyi* seems to have an impact on the health status of the larvae. In 1996, *V. harveyi* appeared in Dahua hatchery and in Fengcheng hatchery. Only Fengcheng was hit by massive mortalities in all larval stages while Dahua was not. The pathogenicity of the *V. harveyi* strain STD3-1002 (isolated from diseased larvae in Fengcheng hatchery) was confirmed by oral challenge infection trials on *P. chinensis* larvae, causing more than 80% mortality in zoea stages after 24 h, using a bacterial density of 10^4 CFU/ml (Xu et al., unpublished data). Symptoms of the disease were the same as those observed during the disease outbreak in Fengcheng hatchery. Although individual genetic differences exist between the *V. harveyi* isolates STD3-1035, STD3-1041 and STD3-1060, isolated from healthy larvae at Dahua hatchery in 1996, and *V. harveyi* strains STD3-999, STD3-1002 and STD3-1020, isolated from diseased larvae in Fengcheng hatchery in 1996, no correlation between AFLP banding patterns (Fig. 1) and origin is found. Also Pedersen et al. (1997), who did an extensive taxonomic study on *V. harveyi*, could not find any correlation between genotypic and phenotypic characteristics and pathogenicity. This result supports the statement that some vibrios, which are normal saprophytic bacteria of estuarine environments, are opportunistic bacteria capable of infecting larvae (Egidius, 1987).

Since no phenotypical or genotypical differences were observed amongst the *V. harveyi* strains isolated from diseased larvae in Fengcheng hatchery and from healthy larvae in Dahua hatchery, another parameter might be involved in preventing disease outbreaks, e.g., the presence of an antagonistic strain. The water quality, e.g., COD, pH, NO_2–N, temperature, dissolved oxygen and salinity was measured at Fengcheng and Dahua hatchery, but no significant differences could be observed (Xu et al., unpublished data). These observations suggest that the water quality did not enhance disease outbreaks. All of the *V. harveyi* strains occurred at Dahua hatchery together with *V. alginolyticus*. In Fengcheng hatchery, *V. alginolyticus* was absent while *V. harveyi* were dominating the bacterial flora in the larvae in high numbers. The mechanism by which *V. alginolyticus* influences the pathogenicity of *V. harveyi* or the ability of the larvae to resist against bacterial pathogens is unknown. Austin et al. (1995b) has described the antagonistic properties of a *V. alginolyticus* strain towards the pathogens *Aeromonas salmonicida*, *V. anguillarum* and *V. ordalii*. In Ecuador, a *V. alginolyticus* strain with antagonistic properties towards the pathogen *V. harveyi* is found and will be applied as a probiont in *P. vannamei* hatcheries (San Miguel et al., 1997). Because of the dominance of *V. harveyi* bacteria during disease outbreaks in Fengcheng hatchery, we assume that

the *Vibrio* spp., which were always present in low numbers, had a minor effect on the health status of the larvae.

3.5. Relationship between the environmental and the larval microflora

The 114 strains that remained unidentified were mainly isolated from the environment, i.e., from the inletwater, tankwater, nearshore seawater, wastewater and the food, but the groups STD3-366, STD3-437 and STD3-399* were also recovered from the shrimp larvae (10^2 to 10^5 CFU/ml).

Egg yolk emulsion is frequently used as a larval food for the production of *P. chinensis* larvae. Strain STD3-399*, which was dominantly present in the food, was also isolated from healthy larvae of all developmental stages fed with egg yolk emulsion (10^2 to 10^5 CFU/larva). Whether this strain has a positive effect on the healthy status of the larvae can only be tested by further experiments. That this strain was isolated less from diseased larvae could be due to the fact that diseased larvae stop feeding. Strains STD3-366 and STD3-437, isolated as the dominant bacteria in the inletwater and waste water, proliferated in high numbers in healthy larvae (10^3 to 10^4 CFU/larva) in 1995 and showed a strong correlation with healthy zoea and post-larval stages. These strains were never recovered in 1996. Future artificial infection experiments will verify their influence on the larval health status. That both the type of food and the environment influence the microflora associated with marine fish larvae has been demonstrated by Verdonck et al. (1994) and by Verdonck and Swings (1995) in marine fish.

4. Conclusions

An overwhelming predominance of *V. alginolyticus* and *V. harveyi* was observed in the *P. chinensis* larval developmental stages from zoea stage on. The flora associated with the larvae is not very stable and is influenced by the bacterial flora of the administered food and the environment. The dominant larval flora of nauplii remained largely unidentified and very few vibrios were isolated from this developmental stage. The larval bacterial load gradually increased from nauplii stage to post-larval stage.

V. harveyi was the sole dominant bacterium isolated from diseased zoea, mysis and post-larval stages. The bacterial flora of healthy larvae was dominated both by *V. harveyi* together with *V. alginolyticus*. No phenotypical or genotypical differences between *V. harveyi* strains, isolated from healthy and diseased larvae, were found. The results suggest antagonistic (probiotic) properties of *V. alginolyticus* or an ability to enhance larval resistance towards pathogenic strains. Other unidentified strains are also related with successful larval productions and might have antagonistic (probiotic) properties. Whether the presence of *V. alginolyticus* and/or other strains can prevent larval diseases caused by *V. harveyi* bacteria has to be further tested.

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