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Identification and Phylogenetic Analysis of Bacterial Isolates from *Litopenaeus vannamei* Shrimp Culture System and Gut Environment Based on 16SrRNA Gene Sequence Data

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Selected bacterial isolates from a *Litopenaeus vannamei* shrimp culture system and gut environment were assessed using 16S rRNA gene sequencing method to identify their identity and to construct their phylogenetic relationship. In a preliminary study, a total of 19 isolates were selected as probiotics. These isolates were prepared using freeze and heat-shock method to obtain the DNA template. PCR amplification of 16S ribosomal RNA gene of isolates was carried out using bacterial universal primers 9F and 1510R and was sequenced using an automated DNA sequencer. These gene sequences were compared with other gene sequences in the GenBank database (NCBI) using a BLAST search to find closely related sequences. Alignment of these sequences with sequences available from GenBank database was carried out to construct a phylogenetic tree for these bacteria. Most of the isolates obtained, *i.e.* 17 out of the 19 isolates, belonged to different species of *Bacillus*, sharing 95 to 99% 16S ribosomal RNA identity with the respective type-strain, whereas the remaining 2 isolates belonged to *Micrococcus* sp. and *Micrococcus luteus*, with 97 to 99% 16S rRNA homology, consecutively.

Key words: *Bacillus*, *Micrococcus,* PCR amplification of 16S rRNA gene, phylogeny

The growing economic importance of the aquaculture industry worldwide and the need to find a new approach for a probiotic method has led to increasing interest in rapid and reliable methods for detection and identification of bacteria (Nilsson and Strom 2002). Recently, nucleic acid-based assays have been studied intensively. One of methods being developed is that of 16S ribosomal RNA genes sequencing. These assays are generally considered to be more accurate, more sensitive, inexpensive and more rapid to perform than those of more conventional variety, such as morphological, biochemical/physiological and serological assays, which led to various results of species resemblance in physiology and susceptibility to environmental exchanges (Macrae 2000).

Broad-range PCR amplification of 16S rDNA with universal bacterial primers and subsequent sequence analysis of cloned products is a widely used method in molecular biology. The method can be used for identification of microorganism identity to the genus or species level (Tiirola *et al*. 2002) and construct phylogenetic analysis which plays on important role in taxonomic positioning. It can be undertaken by comparing the gene sequences with other bacteria sequences in the GenBank database using basic local alignment search tool (BLAST) search and continue with method of neighbor-joining to construct a phylogenetic tree (Saitou and Nei 1987; Holmes 2003).

In this paper, we identified the selected bacterial isolates from *L. vannamei* culture system and gut environment using the 16S ribosomal RNA gene sequence and constructed and analyzed the phylogenetic trees using the program Clustal X. It is important to understand that genetic diversity of selected isolates of probiotic bacteria and their taxonomic position provides basic information for developing disease control strategies in the shrimp culture industry.

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MATERIALS AND METHODS

Sampling of Bacteria and Isolation. Bacterial isolates were obtained from the water, sediments and shrimp's gut using healthy wild shrimps (15 ± 1 g) collected from four districts, *i.e.,* Pandeglang, Serang, Tangerang and Karawang. Sampling stations were determined randomly with 5 to 7 spots per pond. Substrates were taken from 3 substrates, *i.e.* pond water, mud and shrimp' gut following the procedures of Hjelm *et al*. (2004). Water aliquots of 500 mL and mud aliquot of 50 to100 g were taken using sterile plastic bags and stored in refrigerator at 4 °C. Samples from shrimp' guts were conducted by taking living shrimps to the laboratorium using closed transportation with air and water at a ratio 3:1.

Sample of 5% of obtained material were taken and then isolated and enriched on TSB medium with 2% NaCl and incubated for 24 h at 30 °C before streaking bacteria on marine agar. Samples from mud of 50% (w/v) were diluted using sterile distilled water, whereas samples from shrimp's guts were taken by gut-scraping.

All subsequent isolates were streaked on marine agar and incubated for 24 h at 30 °C. Representative colonies were selected based on phenotype, Gram staining and acidity of the medium. The selected isolates were cultured on slash-marine-agar and stored at 4-10 °C after separation into three groups, i.e. original culture, stock culture and working culture.

Development of Pure Cultures of Isolates. Nineteen selected isolates from the slash-agar stock were streaked in

duplicate on the respective media (marine agar, DIFCO, 55.1 $g \cdot l^{-1}$) plates and incubated at 30 ºC for 48 h. The isolates were then stored in a refrigerator for short periods until further use.

Preliminary Characterization of Isolated Bacteria. Characterization of isolates was done following the method of Akhtar *et al.* (2008). Colonies on respective media plates were examined using the light microscope and their characteristics *i.e.* color, form, elevation and margin of colonies were recorded. Isolates were also characterized on the basis of Gram staining.

DNA Template Preparation. DNA templates were prepared by using the freeze and heat-shock method (Sjamsuridzal and Oetari 2003). Incubated isolates (48 h) were taken using sterile microtips and put into 1.5 mL microtube containing 500 µL of nuclease-free water. The cell suspension was vortexed for 1 min and then frozen for 24 h. The suspension was then boiled using a water bath (>95 ºC) for 20 min. The cells suspension was centrifuged at 13 000 rpm for 15 min. The supernatant containing the DNA was stored in a refrigerator until further use.

Phylogenetic Analysis of Isolates. Polymerase chain reaction amplification of 16S ribosomal RNA of isolates were carried out using following primers and set of conditions: forward primer (9F; 5'-GAGTTTGATCCTGGCTCAG-3' and reverse primer (1510R; 5'GGCTACCTTGTTACGA-3') (Nilsson and Strom 2002). Each vial contained 1.25 µL of each primer, 15 µL RTG suspended with nuclease free water and 7.5 µL DNA template in a total 25 µL reaction volume. The reaction mixture was heated for 3 min at 95 °C and then amplification was carried out in 35 cycles. Each cycle was comprised of 30 sec at 95 °C, 15 sec at 55 °C and 1 min at 72 °C. The final extension was for 5 min at 72 °C followed by storage at 25 °C. The PCR product was purified using sodium acetate/ethanol precipitation to remove excess primers and free nucleotides.

The next step was cycle-sequencing reaction, in which a similar process to amplification of 16S ribosomal RNA was performed. Each vial contained 1 µL Big Dye Terminator Ready Reaction Mix V.3.1, 0.5 µL primer 9F, 7.0 µL sequence buffer, 0.5 µL nuclease free water and 1 µL DNA template in a total of 10 µL reaction volume. The reaction mixture was heated for 1 min at 96 °C and then amplification was carried out over 30 cycles. Each cycle was comprised of 10 sec at 96 °C, 5 sec at 55 °C and 90 sec at 60 °C. The final step was storage at 25 °C. The PCR product was re-purified using sodium acetate/ethanol precipitation to remove excess dye-terminators, followed by denaturation using 15 µL Hi-DI Formamide and heat denaturation at 95 °C for 5 min and then immediately transferred on ice.

Amplified 16S ribosomal RNA fragments of about 1500 bp size were obtained from each isolate and were partially sequenced (around 500 bp) using an automated DNA sequencer (ABI 310 PRISM), following the procedure given by the manufacturer. The gene sequences were compared with others in the GenBank databases using the NCBI BLAST (www.ncbi.nlm.nih.gov). Gene sequences of 16S ribosomal RNA of selected organisms were obtained from GenBank and aligned with gene sequence of our isolates using a multiple sequence alignment program CLUSTAL X software (Thompson 1997). The aligned sequences were used to construct a distance matrix, after the generation of 100 bootstrap sets, which was subsequently used to construct a phylogenetic tree using the neighbor-joining method (Holmes 2003).

RESULTS

Colony and Cell Morphologies of Isolates. Nineteen isolates of selected putative probiotic bacteria were examined for studies on the basis of the colony color and bacterial morphology (Table 1). Colony pigmentation of the isolates included white, light pink, pink-orange, light yellow, yellow and pinkish red. These colonies were dominated by circular forms and only 4 isolates had circular-tending to a irregular form. Colony elevation was convex in all cases. All isolates had entire margins and gave Gram positive staining.

PCR Amplification of 16S rRNA Genes. Electrophoretic visualization of amplified 16S rRNA genes from nineteen selected probiotics using primer 9F and 1510R is shown in

Table 1 Some characteristics of different isolates of selected putative probiotic bacteria

Isolates code	Substrate	Macroscopic observation of colony					Gram staining
		Color	Surface	Forms	Margins	Elevation	
S_{18}	water	White	Glossy	Circular	Entire	Convex	Positive
T_{1}	water	White	Glossy	Circular	Entire	Convex	Positive
T_{17}	gut	White	Glossy	Circular	Entire	Convex	Positive
T_{28}	gut	White	Glossy	Circular	Entire	Convex	Positive
P_{18}	gut	White	Glossy	Circular	Entire	Convex	Positive
T_{9}	gut	Light Pink	Glossy	Circular	Entire	Convex	Positive
P_{43}	water	Light Pink	Glossy	Circular	Entire	Convex	Positive
T_{21}	gut	Pink Orange	Glossy	Circular	Entire	Convex	Positive
T_{26}	gut	Pink Orange	Glossy	Circular	Entire	Convex	Positive
K_{48}	sediment	Pink Orange	Glossy	Circular tend to Irregular	Entire	Convex	Positive
P_{10}	sediment	Pink Orange	Glossy	Circular tend to Irregular	Entire	Convex	Positive
P_{16}	water	Pink Orange	Glossy	Circular tend to Irregular	Entire	Convex	Positive
P_{11}	water	Pink Orange	Glossy	Circular tend to Irregular	Entire	Convex	Positive
S_{9}	gut	Light yellow	Glossy	Circular	Entire	Convex	Positive
T_{23}	gut	Yellow	Glossy	Circular	Entire	Convex	Positive
S_{23}	sediment	Pinkish red	Glossy	Circular	Entire	Convex	Positive
T_{18}	water	Pinkish red	Glossy	Circular	Entire	Convex	Positive
K_{52}	water	Pinkish Red	Glossy	Circular	Entire	Convex	Positive
P_{7}	sediment	Pinkish Red	Glossy	Circular	Entire	Convex	Positive

S18 until P1 shown the isolates code of selected indigenous bacteria

Fig 1. The characteristic bacterial 16S gene amplicon of about 1500 bp was clearly visible in all cases.

Identity of Isolates Based on 16S-rRNA-Sequence Method. Nucleotides BLAST search of partial sequences (500 bp) of the 16S rRNA for various isolates showed that most of the isolates had variable percentage identity with *Bacillus* (95-99%), whereas the remainder, *i.e.* isolate S_{23} and $S_{9,}$ had 97% and 99% homology to *Micrococcus* sp. and *Micrococcus luteus*, respectively (Table 2).

Construction of Phylogenetic Tree. A phylogenetic tree was developed by aligning 16S rRNA sequences of different bacteria taken from GenBank, NCBI and sequences of the isolates from this study (Fig 2).

Isolate S_9 was related to *Micrococcus luteus* clone B14. It shared 99% similarity in its 16S ribosomal RNA sequence, whereas isolate T_{23} was related to *Micrococcus* sp. Mali2 and *Kocuria* sp. with 97% similarity. Based on the morphology of colony (Table 1), both isolates had similarity in patters of surface, form, margin, elevation and Gram staining *i.e.,* glossy, circular, entire, convex and Gram positive, consecutively. The defining feature of both isolates was that isolate S_9 had a light yellow color, whereas T_{23} was yellow.

Meanwhile, the cluster of the *Bacillus* group was divided into six clusters. The first cluster consisted of isolates P_{18}, S_{18}, T_{28}, T_1 and T_{17}, which were closely related to *Bacillus pumilus* (DURCK14, S6-05, AU39 and NUC-F) and *Bacillus antrachis* (me-12), with similarity of 98, 98, 99, 99, 95%, consecutively, and bootstrap value 99.9%. The second cluster consisted of two isolates, *i.e.* T_{21} and T_{26}. Both isolates related to *Bacillus* sp. WRB-4, with similarity 95 and 97% consecutively with bootstrap value of 100%. The third cluster was divided into two sub-clusters. The first was isolate P_7, which was related to *Bacillus* sp. Mali10 with similarity of both 97% while the second sub-cluster was isolates S_{23} and K_{52}, which were close to *Bacillus* sp. By231Ydz-fq with a similarity of 96-97%. The fourth cluster was isolate T_{18}. This was close to *Bacillus* sp. K38T with a 99% similarity. The fifth cluster was represented by T_9 and P_{43} which were related to *Bacillus subtilis* S8-04 and *Bacillus megaterium* TK1 with a similarity of 96 and 98% consecutively. The sixth cluster was again divided into sub-clusters. The first sub-cluster consisted of isolate P_{10} with a 99% similarity and related to *Bacillus flexus* LLH. The second sub-cluster was represented by isolates K_{48}, P_{16} and P_{11}, and related to *Bacillus flexus* GS11 and *Bacillus* sp. Bsi20565 with similarities of 95, 97, 98 and bootstrap values higher than 60%.

DISCUSSION

This is a preliminary study on the characteristics of nineteen isolated colonies. Colonies were grown on agar media and were visible after 12 h incubation at 30 °C. This is due to the presence of sufficient nutrient, appropriateness of the agar used and appropriateness of the culture period. Marine agar (DIFCO; 55.1 g l^{-1}) is a specific agar for heterotrophic marine bacteria. It contains 2% (w/v) NaCl. Such conditions were appropriated for isolates which were obtained from shrimp ponds with a salinity of about 25 ppt. Thus the physiological activity from the bacteria was not disrupted. The 48 h-incubation-period was adequate enough for bacterial growth. Our study showed the colonies grew

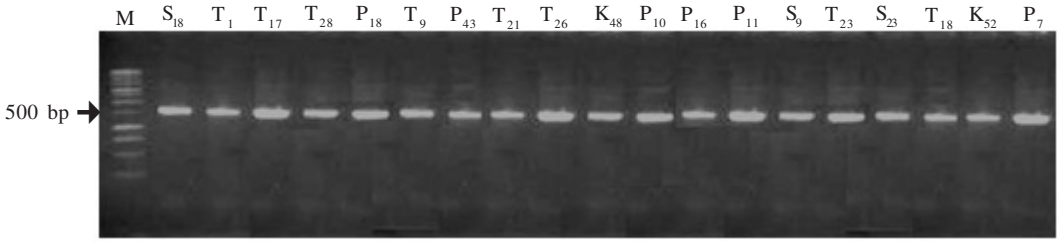

Fig 1 Visualization of amplification of 16S rRNA genes from 19 selected using primer 9F and 1510R. M, Marker; bp, base pair; S18 until P1 shown the code of selected indigenous bacteria isolates.

Table 2. Nearest relative of various selected isolates of probiotic bacteria

Isolate	Substrate	Nearest relative	Accession Number	Homology (%)
S_{18}	water	*Bacillus pumilus* isolate NUC-F	DQ833752	98
T_1	water	*Bacillus pumilus* strain S6-05	EU624429	99
T_{17}	gut	*Bacillus pumilus* strain AU39	EF032679	95
T_{28}	gut	*Bacillus pumilus* strain DURCK14	AM778191	99
P_{18}	gut	*Bacillus pumilus* isolate EGU275	EF633222	98
T_9	gut	*Bacillus subtilis* strain S8-04	EU620412	96
P_{43}	water	*Bacillus megaterium* strain TK1	EU586034	98
T_{21}	gut	*Bacillus* sp. WRB-4	EF636891	95
T_{26}	gut	*Bacillus* sp. WRB-4	EF636891	97
K_{48}	sediment	*Bacillus flexus* strain GS11	DQ365587	95
P_{10}	sediment	*Bacillus flexus* ISOLAT LLH	DQ333292	99
P_{16}	water	*Bacillus flexus* strain GS11	DQ365587	97
P_{11}	water	*Bacillus* sp. BSi20565	EU330341	98
S_9	gut	*Micrococcus luteus* clone B14	EU196531	99
T_{23}	gut	*Micrococcus* sp. "Mali2"	AY211096	97
S_{23}	sediment	*Bacillus* sp. By231Ydz-fq	EU070372	96
T_{18}	water	*Bacillus* sp. K38T	AM983525	99
K_{52}	water	*Bacillus* sp. 'Mali10'	AY211104	97
P_7	sediment	*Bacillus* sp. 'Mali10'	AY211104	97

S18 until P1 shown the isolates code of selected indigenous bacteria

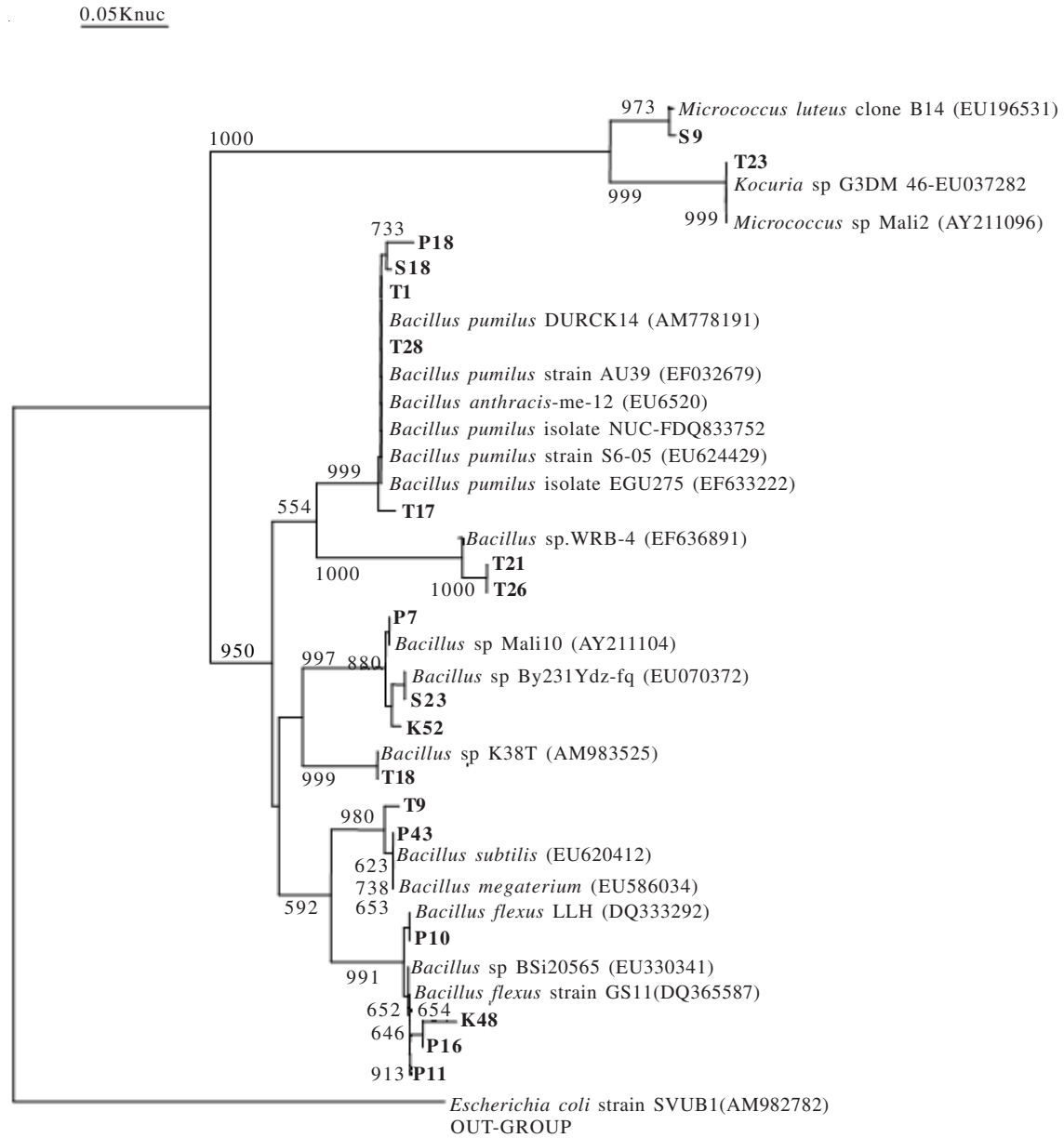

Fig 2 The inferred relationship based on partial 16S ribosomal RNA sequence, of selected putative probiotic bacteria (boldface) to other bacteria. The tree was rooted with *Escherichia coli* as an out-group. Scale bar represents the number of inferred nucleotide substitution per site. Bootstrap values (1000 replicates) are shown at the nodes.

well, with the typical color of bacteria. The results were in line with Akhtar *et al.* (2008), in that the growth of bacteria will also be determined by the period of incubation by giving enough time to reach the optimum stage.

On a molecular basis, all selected isolates belonged to the Gram positive group. Most of the isolates (17 isolates) were bacilli (89.7%), with the remaining two isolates (10.53%) being Micrococci (Table 1). It indicated that bacteria belonging to Bacilli group were more prevalent in healthy *L. vannamei* shrimp ponds. This finding was in line with those of Verschuere *et al*. (2000), Balcázar *et al*. (2006) and Geovanny *et al.* (2007) in which several bacteria were identified and can be considered as probiotic bacteria in aquaculture such as: *Bacillus*, *Vibrio*, *Pseudomonas*, *Micrococcus*, *Bacteroides* and *Clostridium*.

Gram negative bacteria were not found in the 19 culture isolates, after identification based on the sequence of 16S ribosomal RNA. It was probably due to the fact Gram negative bacteria which were successful obtained during sampling of bacteria and isolation were not showing the characteristics of probiotic bacteria *i.e.* do not improve shrimps' survival rate during challenge test (Rengpipat *et al*. 2000).

Isolates related to Bacilli were represented by *Bacillus* sp. (isolates code: T_{21}, T_{26}, T_{18}, K_{52}, P_7, P_{11} and S_{23}), *B. pumilus* (isolates code: S_{18}, T_1, T_{17}, T_{28} and P_{18}), *B. subtilis* (isolate code: T_9), *B. megaterium* (isolate code: P_{43}), and *B. flexus* (isolates codes: K_{48}, P_{10} and P_{16}). Meanwhile, *Micrococcus*, was represented by *Micrococcus* sp. (isolate code: T_{23}) and *M. luteus* (isolate code: S_9). As the major species found, the dominance of Bacilli can be comprehended since they have a wide range of diversity in physiological ability with respect to heat, acidity and salinity tolerance. bacilli can be found in water, soil and the digestive tracts of several organisms, including shrimps (*L. vannamei*) (Rengpipat *et al.* 2000).

Although the genus *Micrococcus* has not a wide range of tolerance compared to *Bacillus*, it is however considered

as a halotolerant group. *Micrococcus* can grow at 5% (w/v) NaCl, and manage to survive in the digestive tract as well. Geovany *et al*. (2007) reported that *M. luteus* was the major component of the population of the gut of Rainbow Trout (*Oncorhynchus mykiss*).

Phylogenetic analysis indicated that nineteen strains isolated from selected local probiotic bacteria can be identified as Gram positive bacteria of the *Bacillus* and *Micrococcus* species (Fig 2). When the type of *Escherichia coli* used for an out-group, it was clearly seen that the distribution of the nineteen of the isolates fell into two big clusters, i.e. *Bacillus* cluster and *Micrococcus* cluster. One was composed of seventeen *Bacillus* genera consisted of isolate P_{18}, S_{18}, T_{28}, T_1, T_{17}, T_{21}, T_{26}, P_7, S_{23}, K_{52}, T_{18}, T_9, P_{43}, P_{10}, K_{48}, P_{16} and P_{11}, indicated 95% in bootstrap value. The other was composed of two micrococcal strains, S_9 and T_{23}, indicated 100% in bootstrap value. The higher bootstrap value (≥95%) indicated that both groups were clearly distinct (Meerak *et al*. 2007).

Based on the findings above, it may be concluded that the 19 isolates of bacteria can be used as probiotic bacteria and are useful for developing shrimp culture systems. *Bacillus* and *Micrococcus* are frequently used in shrimp aquaculture to improve the health status toward disease outbreaks through diet or giving out into water culture (Gatesoupe 1999; Verschuere *et al*. 2000; Balcázar *et al*. 2006).

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