

A study on bacteria associated with the intestinal tract of farmed yellow seahorse, *Hippocampus kuda* (Bleeker, 1852): characterization and extracellular enzymes

Tanu¹, Deepti D Deobagkar², Rakhee Khandeparker¹, Rayadurga A Sreepada^{1*}, Sushant V Sanaye¹ & Hrishikesh B Pawar¹

¹Aquaculture Laboratory, National Institute of Oceanography, Council for Scientific & Industrial Research (CSIR) Dona Paula–403 004, Goa (India).

²Molecular Biology Research Laboratory, Centre of Advance Studies, Department of Zoology, University of Pune, Pune–411 007 (India).

Running title: Intestinal bacteria in *Hippocampus kuda*

Abstract

The microenvironment of bacteria associated with the gastrointestinal tract of an animal influences the host in many ways, including the metabolism of several nutrients. Isolation, molecular characterisation and enzymatic activities of culturable bacteria associated with the gastrointestinal tract of hatchery-reared oceanic yellow seahorse, *Hippocampus kuda*, were investigated in the present study. 16S rRNA sequencing of bacterial isolates yielded 10 different bacterial genera belonging to two major groups; Proteobacteria (62.9%) and Firmicutes (37.03%). The predominant microbial flora belonged to the genera *Vibrio* spp., *Enterovibrio* sp. and *Bacillus* spp. *Burkholderia cenocepacia*, hitherto unreported from the gastrointestinal tract of marine fish, was retrieved from the seahorse gut, albeit in small numbers. Enzymatic studies indicated that the bacterial isolates possess the ability to degrade lipids, cellulose, xylan, starch and proteins. Lipase activity was exhibited by a majority of the isolates (70.4%) reflecting that the bacterial flora associated with the intestinal tract of seahorses is capable of digesting foods rich in lipids. The possible implications for the role of these bacteria in the digestion and physiological processes are discussed. To our knowledge, this is the first time that the characterisation and enzymatic activities of bacteria associated with the intestinal tract of farmed seahorses have been reported.

Key words: Intestinal bacteria, enzymatic activity, 16S rRNA, *Hippocampus kuda*

*Correspondence: Aquaculture Laboratory, National Institute of Oceanography (CSIR), Dona Paula–403004, Goa (India). Tel/fax: +91-832-2450426/606; E-mail: sreepada@nio.org

Introduction

As a consequence of overexploitation, degradation of habitats and inadequate management, natural populations of many marine species have experienced drastic reductions in numbers during the last few decades. Seahorses constitute a group of marine fish that is often considered as a popular flagship species for engendering support for marine conservation and sustainable management (Sreepada, Desai & Naik 2002). At least 25 million seahorses (>20 metric tonnes, dry weight) are traded annually for traditional Chinese medicine (TCM), as aquarium fish and curios, and nearly 77 nations and territories are involved in the seahorse trade (Project Seahorse 2006; Salin & Mohankumaran 2006). The oceanic yellow seahorse, *Hippocampus kuda* (Bleeker 1852), is widely distributed throughout the tropical Indo-Pacific region (Lourie, Vincent & Hall 1999), inhabiting shallow inshore habitats such as mangroves, seagrass beds and estuaries. It is one of the most heavily traded seahorse species in many Southeast Asian countries for TCM (Job, Do, Meeuwig & Hall 2002). Its conservation status is currently listed by the International Union for the Conservation of Nature (IUCN) as ‘vulnerable’ (IUCN, 2010).

Several attempts to culture seahorses on a mass-scale in the recent past have met with limited success as a result of severe problems of mass mortality due to inadequate nutrition and disease (Truong 1998; Murugan, Dhanya, Sreepada, Rajagopal & Balasubramanian 2009). Provision of feeds to different stages of seahorses has been the major problem due to their unusual sit-and-wait ambush feeding strategy. This species use the snout to suck their prey and swallow whole animals which later on pass through their primitive digestive system consisting of a simple alimentary canal. Seahorses need to feed continuously as they lack a masticatory organ and a true stomach, and because the digestion period is relatively short (Bergert & Wainwright 1997).

In aquatic animals, the intestinal microflora has been reported to aid in the digestion of algal cells, the production of amino acids and the secretion of inhibitory substances that prevent colonization by bacterial pathogens (Fong & Mann 1980; Rimmer & Wiebe 1987; Onarheim & Raa 1990; Berg 1996). Extensive studies have been carried out to understand the role of gut microflora in invertebrates and fishes (Spanggaard, Huber, Nielsen, Nielsen, Appel & Gram 2000; Austin 2002; Ringo, Sperstad, Myklebust, Refstie & Krogdahl 2006). Marine fishes are known to possess a characteristic gut microflora (Westerdahl, Olsson, Kjelleberg & Conway 1991) which is often influenced by the genetic constitution of the host organism. But there are also some transitory bacteria that travel through the gut

without establishing themselves onto the gut wall. These transitory bacteria are affected by the physiochemical conditions within a particular host and by external factors such as diet, environmental influences and stress (Purchase 1977). The gut microflora in fishes and crustaceans can metabolize several nutrients that the host cannot, and can convert them to end products (such as short-chain fatty acids) - a process that has a direct impact on digestive physiology (Suzer, Kamaci, Coban, Saka, Firat & Ozkara 2007; Hoyoux, Zbinden, Samadi, Gaill & Compère 2009). The influence of the resident microflora on mucosal immune function and gut health has become an area of scientific and clinical importance (Galdeano, Leblanc, Vinderola, Bonet & Perdigon 2007).

It is becoming increasingly apparent that the intestinal microflora and its metabolic activities can be an important contributing factor in nutrition, physiology and animal welfare. Practically no information on the composition of bacterial communities associated with the gastrointestinal tract of seahorses is available. However, such information is of paramount importance in live-feed management in seahorse aquaculture, since the lack of a true stomach in this species leads to a relatively fast digestion in this animal. A better knowledge of intestinal microflora of seahorses could be helpful for the formulation of optimal live feed for the growth and development of *H. kuda*, which is necessary for the mass-scale rearing of this 'vulnerable' seahorse species.

Materials and methods

Test animals

The study was carried out at the pilot-scale seahorse hatchery adjoining the aquaculture laboratory complex of the National Institute of Oceanography, Goa (India) where techniques for the standardization of captive breeding, rearing and culture of Indian seahorse species are being established. The juveniles of *H. kuda* used in this study were hatchery-bred animals. Juveniles were reared in two large fibreglass reinforced plastic (FRP) tanks (1500 L) secured with rope (4 mm diameter) holdfasts. Juveniles were fed *ad libitum* three times per day (06:00h, 14:00h and 22:00h) with different live prey organisms such as mix zooplankton dominated with copepods (>80%), *Artemia* nauplii (*Artemia salina*), mysids (*Mesopodopsis orientalis*). Optimum water quality conditions were maintained in the juvenile rearing tanks: temperature ($27 \pm 2^\circ\text{C}$), salinity ($26 \pm 1 \text{ g L}^{-1}$), dissolved oxygen ($6.1 \pm 0.6 \text{ mg L}^{-1}$), pH (7.6 ± 0.3), $\text{NO}_2\text{-N}$ ($<0.02 \text{ mg L}^{-1}$) and NH_3/NH_4 (0 mg L^{-1}) (Murugan *et al.* 2009). The photoperiod of 16 L

(0700 h–2300 h):8 D (23:00–07:00 h) was maintained using a fluorescent bulb of light intensity ~1500 lx at water surface.

Sample collection and preparation

Healthy seahorse juveniles (180 days after birth; mean height, 80 ± 5 mm) of the F1 generation were randomly netted from FRP rearing tanks. Seahorses from two different FRP tanks were used to avoid a possible tank effect. The juveniles were starved for 24 h and thereafter held in sterilized seawater (UV-treated and autoclaved) in order to defecate their digestive tract and to prevent any contamination from the undigested food and faecal matter. A starvation period of 24 h was found to be sufficient for complete gut clearance as the digestion period in this species was found to be < 3 h (personal observation). Before dissection, the body surface of euthanized fish was thoroughly scrubbed with 70% ethanol. The trunk region was incised from the lateral side, aseptically with sterilized instruments under laminar airflow and the gut was excised. Since, the gut in Syngnathids is a small tube like structure with no differentiation between the stomach and intestines (Tipton & Bell 1988), the entire gut of *H. kuda* was examined. In order to retrieve the bacteria associated with the inner walls of the intestine, 5 ml of normal saline (0.9%) was gently flushed through the intestine from anterior to posterior end with a syringe. The eluted saline was collected in a sterilized tube and plated onto different media. From repeated trials, 5 ml of saline was found sufficient to dislodge all gut-adhered bacteria.

Bacteriological analysis

In order to isolate the bacteria, the eluted saline sample was plated directly on four different types of media, nutrient agar (NA), Zobell marine agar (ZMA), nitrate agar and xylose lysine deoxycholate agar (XLDA) (Hi Media Mumbai, India). Plates were inoculated and incubated at 32 ± 2 °C and growth was observed after 24 h and 48 h of incubation. Colonies with different morphological characters (colony size, elevation and pigmentation) were isolated from different media plates and sub-cultured to obtain pure bacterial cultures. For further maintenance of cultures, only NA and ZMA were used.

16S rRNA sequencing

The DNA was extracted from isolates by suspending the bacterial cells into 200 μ l of lysis buffer (10 mM Tris, 5 mM EDTA, pH 5.0) along with 2 mg ml⁻¹ lysozyme, and incubating at room temperature (26

± 2°C) for 5 min. Then, 10% SDS (w/v) was added along with proteinase K, which was further extracted twice with phenol chloroform treatment. 2 µl of supernatant was amplified using PCR (Applied Biosystems, Verity 96 well thermal cycler). The amplification was carried out for 35 cycles of 94 °C, for 45 s, 60 °C for 45 s and 72 °C for 45 s, 72 °C for 7 min with initial 05-min denaturation at 94 °C and the amplified DNA was sequenced at Genei Laboratories, Bangalore (India). The sequence of the primer used was 27 F (5'AGATTTGATCCTGGCTCAG3') and 1492 R (5'GGTTACCTTGTTACGACTT3') (Weisburg, Barns, Pelletier & Lane 1991). The sequences were submitted to NCBI Gene Bank databases and accession numbers obtained (Table 1).

Detection of extracellular enzymes

Bacteria were screened for production of extracellular enzymes using various substrates such as casein, carboxymethylcellulose, starch, xylan and Tween 80. For detection of protease, cellulase, xylanase and amylase, media plates were prepared by adding 0.5% substrate and 1.5% agar in basal salt solution (Khandeparker & Bhosle 2006). Isolates were streaked onto media plates and incubated at 32 ± 2°C. After 24 h, clear zone around the colony indicated the presence of respective enzymes. For protease detection no dye was used as clear zone is easily visible while to check the cellulase and xylanase , Congo red (1%) was used to illustrate the clear the zone around the colony and iodine solution was used for amylase (Sa-Pereira, Costa-Ferreira & Atres-Barros 2002; Kasana, Salwan, Dhar, Dutt & Gulati 2008;). For the detection of lipase, media plates were prepared by adding peptone (1%), NaCl (0.5%), CaCl₂ (0.02%), Tween 80 (1%) and agar (1.5%). Isolates were streaked and incubated as mentioned earlier. Appearance of precipitation zone around the colony showed the presence of lipase (Sierra 1957; Dempsey & Kitting 1987).

Results

16S rRNA sequencing and Phylogenetic analysis

Altogether, 27 bacterial isolates (S1-S27) were retrieved from the intestinal tract of *H. kuda*. Bacterial isolates with different morphological characteristics belonging to both gram positive and gram negative groups were observed. The isolated colonies showed different types of pigmentation; mustard-yellow (S4), pinkish-orange (S5), orange (S19), bright white (S6), rest of the colonies were cream or pale white in colour. The majority of the colonies were mucoid. All colonies produced pungent smell. The

sequences obtained by 16S rRNA sequencing were aligned using basic local alignment search tool (BLAST) and the bacterial genera identified are listed in Table 1. The culturable gut microflora from the studied seahorse species was diverse and consisted of *Bacillus* spp., *Vibrio* spp., *Staphylococcus*.sp., *Burkholderia* sp., *Providencia* spp., *Enterococcus* sp., *Pseudoalteromonas* sp., *Phaeobacter* spp., *Enterovibrio* sp., and *Shewanella* spp. The composition was dominated by *Bacillus* spp. followed by *Vibrio* spp., *Shewanella* spp. and *Providencia* spp. *Phaeobacter* spp. *Staphylococcus* sp. *Burkholderia* sp. *Enterococcus* sp. *Pseudoalteromonas* sp. and *Enterovibrio* sp. constituted <10% population (Table 2).

A phylogenetic tree constructed by the neighbour-joining method (ClustalX and MEGA4) showed that all 27 isolates could be classifiable to four major groups, α -Proteobacteria (S21, S23), β -Proteobacteria (S8), γ -Proteobacteria (S2, S3, S4, S5, S12, S14, S16, S17, S18, S19, S20, S22, S24 and S27) and Firmicutes (S1, S6, S7, S9, S10, S11, S13, S15, S25 and S26) (Fig. 1).

Extracellular enzymes

The majority of the isolates (71.4%) exhibited lipid degradation ability, whilst only a small proportion (17.9%) was amylase positive. More than 50% of the isolates produced xylanase, whereas protease and cellulase were produced by 35% and 25% of isolates, respectively. Many of isolates (33.3%) showed single enzyme activity and rest possessed multienzyme activity. Isolate S21, S24 and S10 showed activity for four enzymes (Table 1).

Discussion

The microenvironment of bacteria associated with the gastrointestinal tract of an animal influences the host in many ways, including the metabolism of nutrients. Given the importance in digestion and health, the composition, diversity and morphological characteristics of the gut microflora in many species of marine and freshwater fishes and invertebrates have been extensively researched (Sera, Ishida & Kadota 1974; Cahill 1990; Westerdahl *et al.* 1991; Spanggaard *et al.* 2000; Austin 2002; Holben, Williams, Saarinen, Sarkilahti & Apajalahti 2002; Ringo *et al.* 2006; Izvekova, Izvekov & Plotnikov 2007; Mondal, Roy, Sen & Ray 2008; Hoyoux *et al.* 2009). The relationship between intestinal microflora and diet as well as their possible role in the digestion in both wild and cultured fish species have been studied (Sakata, Nakaji & Kakimoto 1978; Ringo, Strom & Tabachek 1995; Grisez, Reyniers &

Verdonck 1997; Kuz'mina & Skvortsova 2002). In contrast to several studies describing bacteria associated with marine fishes (Cahill 1990; Hansen & Olafsen 1999; Sun, Yang, Ling, Chang & Ye 2009), there is lack of information on the bacterial communities associated with seahorse. In view of commercial importance of seahorse the present investigation was undertaken. The bacteria isolated from the intestinal tract of seahorse belonged to *Bacillus* spp., *Vibrio* spp., *Staphylococcus* sp., *Burkholderia* sp., *Providencia* spp., *Enterococcus* sp., *Pseudoalteromonas* sp., *Phaeobacter* spp., *Enterovibrio* sp. and *Shewanella* spp. which is in agreement with the microflora that have been reported previously from the guts of different species of fish and crustaceans : *Acinetobacter* spp., *Enterobacter* spp. and *Pseudomonas* spp. in trout (*Oncorhynchus* sp.); *Aeromonas* spp., *Flavobacterium* spp. and *Lactobacillus* spp. in Arctic charr (*Salvelinus alpinus*) (Ringo *et al.* 1995), *Enterovibrio* spp. from the intestinal tract of turbot (*Scophthalmus maximus*) (Thompson, Hoste, Thompson, Goris, Gomez-Gil, Huys, De Vos & Swings 2002) and *Vibrio* spp., *Bacillus* sp., *Pseudomonas* sp., *Photobacterium* sp. and *Plesiomonas* sp. in white shrimp, *Fenneropenaeus (Penaeus) indicus* (Shakila, Vyla, Saravana Kumar, Jeyasekaran & Indra Jasmine 2006). From these studies, it appears that different species of fish and crustaceans have a specific resident gut microbiota (Olafsen 2001; Vine, Leukes & Kaiser 2006), the composition of which may change with fish age, nutritional status and environmental conditions (Olafsen 2001).

There are few studies dealing with bacterial communities associated with captive-reared seahorses. Phylogenetic characterisation and *in situ* detection of bacterial communities associated with the long-nouted seahorse, *Hippocampus guttulatus*, in captivity, have been described by Balcazar, Lee, Pintado & Planas (2010a). These authors reported that Vibrionaceae was the dominant bacterial flora in live prey (*Artemia*) and intestinal content, while Rhodobacteraceae was the dominant bacterial flora from the aquaculture system and in the cutaneous mucus of the seahorse (Balcazar *et al.* 2010a). Molecular characterization and identification of *Vibrio* spp. isolated from captive-bred seahorses with disease symptoms using 16S rRNA sequencing has revealed the predominance of *V. alginolyticus* and *V. splendidus* (Balcazar, Gallo-Bueno, Planas & Pintado 2010b). Further, Balcazar, Pintado & Planas (2010c) have isolated a new species, *Vibrio hippocampi* sp. nov., from faeces of wild-captured *H. guttulatus*. Very recently, bacteria possessing antibacterial activities have been identified and characterized from *H. guttulatus* by Balcazar, Loureiro, Da Silva, Pintado & Planas (2010d). However, substrate degradation abilities of the gut microflora in seahorses have not previously been investigated.

Results of such studies are of practical relevance because seahorses lack a true stomach and therefore most of the digestion takes place in a simple digestive tract (Murugan *et al.* 2009).

Interestingly, a few bacterial genera (*Providencia* sp. and *Shewanella* sp), previously reported in freshwater fishes (Izvekova *et al.* 2007) and unreported from marine fish, were isolated from the intestinal tract of *H. kuda*. This indicates that the intestinal tract of seahorses is capable of supporting diverse groups of bacteria. *Burkholderia cenocepacia* (S8), hitherto unreported from the intestinal tract of all fishes, was, however, recorded in the present study albeit in low numbers (3.70%). Furthermore, majority of the bacterial isolates retrieved from the intestinal tract of *H. kuda* are important symbionts with few exceptions. Bacteria belonging to the genera *Providencia*, *Burkholderia* and *Vibrio* are known to be pathogenic in fish and crustaceans (Alcaide, Gil-Sanz, Sanjuan, Esteve, Amaro & Silveria 2001; Ramsey, Skonieczny, Coolidge, Kurzynski, Proctor & Davis 2001; Camus & Hawke 2002). *V. harveyi* has already been reported to cause mass mortality in seahorse culture (Alcaide *et al.* 2001; Tendencia 2004). Species belonging to *Burkholderia* are nosocomial pathogen and have been reported to cause melioidosis disease in humans (Huang, Jang, Liu, Fung, Yu & Wong 2001; Anon 2004) but its role in the guts of aquatic organisms is little understood. In a comparative study of the gut microbiota of fast- and slow-growing batches of juvenile grouper, *Epinephelus coioides*, Sun *et al.* (2009) reported that the abundance of *B. cepacia* was one order of magnitude higher in the slow-growing batch. Future investigations looking into the role of *Burkholderia* spp. in influencing the growth of seahorses under captivity would therefore be helpful.

In comparison to the vast published literature on the bacterial communities associated with other fish species (Cahill 1990; Izvekova *et al.* 2007), reports on bacteria associated specifically with the gastrointestinal tract of seahorses are not available. From the phylogenetic tree it is seen that alpha, beta and gamma proteobacteria are segregated from each other and form separate clades. All *Bacillus* sp. cluster together and show close relation with *Enterococcus* sp. (S13). While *Vibrio* sp. form three separate clusters and two distinct groups are seen in *Shewanella*. Major bacterial groups such as γ -Proteobacteria, Firmicutes (low G+C content), α -Proteobacteria and β -Proteobacteria contributed, respectively 51.85%, 37.03%, 7.4% and 3.7%, of the total number of isolates retrieved. Taxonomically similar composition has been reported for the intestinal tracts of other aquatic organisms (Trust & Sparrow 1974; Hatha, Kuruvilla & Cheriyan 2000; Spanggaard *et al.* 2000; Romero & Navarrete 2006; Sun *et al.* 2009). The dominant bacterial genera were *Vibrio* and *Shewanella* from class γ -Proteobacteria

and *Bacillus* from the class Bacilli of phylum Firmicutes. Bacteria belonging to the family Vibrionaceae have been reported to be the most abundant in the gastrointestinal tract of fish and crustacean species (Dempsey, Kitting & Rosson 1989; Spanggaard *et al.* 2000; Oxley, Shipton, Owens & McKay 2002; Ringo *et al.* 2006; Sun *et al.* 2009) and were also the dominant bacteria isolated from intestinal contents of *H. guttulatus* in captivity (Balcazar *et al.* 2010a). From this very limited information, it can be deduced that the bacterial flora associated with the intestinal tract of seahorses appear to be taxonomically similar to that associated with the guts of other fish and crustacean species.

Resident intestinal bacteria in fish are known to accelerate the digestion process by producing extracellular enzymes (Stickney & Shumway 1974; Lesel, Fromageot & Lesel 1986; Kono, Matsui & Shimizu 1987; Rimmer & Wiebe 1987; Cahill 1990; Sugita, Kawasaki & Deguchi 1997). Assessment of the substrate degrading ability of gut microflora is important in understanding the nutrition and physiology of the host organism and may help in formulating appropriate feeds. However, the precise nutritional role played by these bacteria is not clearly understood. The culturable bacteria isolated from the intestine of *H. kuda* showed degradative abilities and produced many enzymes, particularly lipases, proteases and carbohydrases. More than 70% of the bacterial isolates showed the presence of lipase activity. Abundance of lipase-producing bacteria in the guts of *H. kuda* was not unexpected since seahorses prey on a variety of live organisms (copepods, mysids, amphipods) rich in highly unsaturated fatty acids (HUFA) (Murugan *et al.* 2009). It has been documented that n-3 HUFA are essential for optimal growth and survival of *Hippocampus* spp. (Chang & Southgate 2001). Relatively higher lipid degradability exhibited by bacterial isolates from *H. kuda* supports this view point.

Most of the isolates retrieved from the intestinal tract of *H. kuda* were observed to degrade a variety of substrate such as cellulose, xylan, proteins and lipids efficiently while only few isolates exhibited amylase activity. In this sense, these bacteria certainly would have an important role in enhancing the digestibility of food and making nutrients available to the fish. Selective enhancement of resident gut bacteria could assist in feed formulation for mass-scale rearing of seahorses. In addition, isolates showing multienzyme activity could be a good source for development of probiotics.

The composition of enzyme producing bacterial flora in the fish digestive tracts are correlated to their feeding habits. To test this hypothesis, Kar & Ghosh (2008) studied the bacterial extracellular enzymes in the intestinal tracts of two freshwater teleosts. They found that higher densities of proteolytic

bacterial strains in carnivore bottom feeder *Channa punctatus* and cellulolytic strains in herbivore column feeder, *Labeo rohita*. From this limited data, it appears that protease and cellulase activities dominate in carnivorous and herbivorous fish, respectively. On contrary, few authors report that herbivorous fish do not possess endogenous cellulase but may pick up cellulase- and amylase-producing bacteria from the feed (Fish 1951; Barrington 1957; Yokoi & Yasumasu 1964). Seahorses are carnivorous fish. Detection of cellulase in the bacterial isolates from the intestinal tract of 24 h starved *H. kuda* highlight that these cellulase producing bacteria may be resident bacteria of seahorse gut.

In conclusion, the results of the present study have practical significance in seahorse husbandry with regards to feed management and disease prevention. The results here may not be representative of all cultured and wild seahorse species, particularly those grown under different feeding regimes. Nevertheless, the present study provides the first insight on the molecular analysis of bacterial communities and degradative abilities of major groups of bacteria associated with the intestinal tract in farmed seahorse. Further studies on the composition of gut microflora from different life stages of seahorses reared under different feeding regimes and their enzymatic activities would assist in understanding role of symbiotic microflora in the digestive process of seahorses.

Acknowledgements

The authors are grateful to Dr. Satish R. Shetye, Director, National Institute of Oceanography (CSIR), Goa (India) for encouragement and facilities. Dr. D. Deobagkar is thankful to CSIR offering a Visiting Scientist Fellowship at NIO, Goa. We are thankful to Dr. Jane Claricoates of University of Swansea (UK) for critically going through the manuscript and two anonymous reviewers for constructive comments. Financial support came from the Department of Biotechnology (Govt. of India). Permission granted by the Ministry of Environment & Forests (Govt. of India) for collection of seahorses is gratefully acknowledged. This represents Contribution No. XXXX of the National Institute of Oceanography (NIO), Goa (India).

References

- Alcaide E., Gil-Sanz C., Sanjuan E., Esteve D., Amaro C. & Silveria L. (2001) *Vibrio harveyi* causes disease in seahorse, *Hippocampus* sp. *Journal of Fish Diseases* **24**, 311–313.
- Anon. (2004) *Morbidity and mortality weekly report (MMWR) Weekly Report*, September 3, vol. **53**. Centre for Disease Control and Prevention, Atlanta, USA
- Austin B. (2002) The bacterial microflora of fish. *Scientific World Journal* **2**, 558–572.
- Balcazar J.L., Lee N.M., Pintado J. & Planas M. (2010a) Phylogenetic characterisation and *in situ* detection of bacterial communities associated with seahorse (*Hippocampus guttulatus*) in captivity. *Systematic and Applied Microbiology* **33**, 71–77.
- Balcazar J.L., Gallo-Bueno A., Planas M. & Pintado J. (2010b) Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from captive-bred seahorses with disease symptoms. *Antonie Van Leeuwenhoek* **97**, 207–210.
- Balcazar J.L., Pintado J. & Planas M. (2010c) *Vibrio hippocampisp.* Nov., a new species isolated from wild seahorse (*Hippocampus guttulatus*). *FEMS Microbiology Letters* **307**, 30–34
- Balcazar J.L., Loureiro S., Da Silva Y.J., Pintado J. & Planas M. (2010d) Identification and characterization of bacteria with antibacterial activities isolated from seahorses (*Hippocampus guttulatus*). *The Journal of Antibiotics* **63**, 271–274
- Barrington E.J.W. (1957) The alimentary canal and digestion. In: *The physiology of fishes* Vol. 1, (ed. By Brown M.E.), pp. 109–161. Academic press, New York, NY, USA.
- Berg R. (1996) The indigenous gastrointestinal microflora. *Trends in Microbiology* **4**, 430–435.
- Bergert B.A. & Wainwright P.C. (1997) Morphology and kinematics of prey capture in the syngnathid fishes *Hippocampus erectus* and *Syngnathus floridae*. *Marine Biology* **127**, 563–570.
- Bleeker P. 1852. Bijdragc tot de kennis der ichtyologische fauna van Singapore. *Natuurkundig-lid.schnj Lioor Nederlandsch hdi?* 3: 82-83.
- Cahill M.M. (1990) Bacterial Flora of Fishes: A Review, *Microbial Ecology* **19**, 21–41.
- Camus A.C. & Hawke J.P. (2002) *Providencia rettgeri* associated septicaemia and meningoencephalitis in juvenile farmed American Alligators *Alligator mississippiensis*. *Journal of Aquatic Animal Health* **14**, 149–153.
- Chang M. & Southgate P.C. (2001) Effects of varying dietary fatty acid composition on growth and survival of seahorse, *Hippocampus* sp. juveniles. *Aquarium Science and Conservation* **3**, 205–214.

- Dempsey A.C. & Kitting C.L. (1987) Characteristic of bacteria isolated from penaeid shrimp. *Crustaceana* **52**, 90–94.
- Dempsey A.C., Kitting C.L. & Rosson R.A. (1989) Bacterial variability among individual penaeid shrimp digestive tracts. *Crustaceana* **56**, 267–278.
- Fish G.K. (1951) Digestion in *Tilapia esculenta*. *Nature* **167**, 900–901.
- Fong W. & Mann K.H. (1980) Role of gut flora in the transfer of amino acids through a marine food chain. *Canadian Journal of Fisheries and Aquatic Sciences* **37**, 88–96.
- Galdeano C., Leblanc A., Vinderola G., Bonet M.G. & Perdigon G. (2007) Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. *Clinical and Vaccine Immunology* **14**, 485–492.
- Grisez L., Reyniers J. & Verdonck L. (1997) Dominant intestinal microflora of seabream and seabass larvae, from two hatcheries during larval development. *Aquaculture* **155**, 387–399.
- Hansen G.H. & Olafsen J.A. (1999) Bacterial interactions in early stages of marine cold-water fish. *Microbial Ecology* **38**, 1–26.
- Hatha A.A.M., Kuruvilla S. & Cheriyan S. (2000) Bacterial flora of intestines of farm raised freshwater fishes *Catla catla*, *Labeo rohita* and *Ctenopharyngodon idella*. *Fishery Technology* **37**, 59–62.
- Holben W.E., Williams P, Saarinen M., Sarkilahti L.K. & Apajalahti J.H. (2002) Phylogenetic analysis of intestinal microflora indicates a novel mycoplasma phylotype in farmed and wild salmon. *Microbial Ecology* **44**, 175–185.
- Hoyoux C., Zbinden M., Samadi S., Gaill F. & Compère P. (2009) Wood-based diet and gut microflora of a galatheid crab associated with Pacific deep-sea wood falls. *Marine Biology* **156**, 2421–2439.
- Huang C., Jang T., Liu C., Fung C., Yu K. & Wong W. (2001) Characteristics of patients with *Burkholderia cepacia* bacteremia. *Journal of Microbiology, Immunology and Infection* **34**, 215–219.
- IUCN, 2010. IUCN Red List of Threatened Species. Version 2010.3. www.iucnredlist.org. Downloaded on 20 October 2010.
- Izvekova G.I., Izvekov E.I. & Plotnikov A.O. (2007) Symbiotic microflora in fishes of different ecological groups. *The Biological Bulletin* **34**, 610–618.
- Job S.D, Do H.H., Meeuwig J.J. & Hall H.J. (2002) Culturing the oceanic seahorse, *Hippocampus kuda*. *Aquaculture* **214**, 333–341.
- Kar N. & Ghosh K. (2008) Enzyme producing bacteria in the gastrointestinal tracts of *Labeo rohita* (Hamilton) and *Channa punctatus* (Bloch). *Turkish Journal of Fisheries and Aquatic Sciences* **8**, 115–120.

- Kasana R.C., Salwan R., Dhar H., Dutt S. & Gulati A. (2008). A Rapid and easy method for the detection of microbial cellulases on agar plates using Gram's Iodine. *Current Microbiology* **57**, 503–50.
- Khandeparker R. & Bhosle N.B. (2006) Isolation, purification and characterization of the xylanase produced by *Arthrobacter* sp. MTCC 5214 when grown in solid state fermentation. *Enzyme and Microbial Technology* **39**, 732–742.
- Kono M., Matsui T. & Shimizu C. (1987) Chitin-decomposing bacteria in digestive tracts of cultures Red Sea bream and Japanese eel. *Nippon Suisan Gakkaishi* **53**, 305–310.
- Kuz'mina V.V. & Skvortsova E.G. (2002) Gastrointestinal bacteria and their role in digestion process in fish. *Uspekhi Sovremennoi Biologii* **122**, 569–579.
- Lesel R., Fromageot C. & Lesel M. (1986) Cellulose digestibility in grass carp and in goldfish. *Aquaculture* **3**, 229–236.
- Lourie S.A., Vincent A.C.J. & Hall H.J. (1999) Seahorses: an identification guide to the world's species and their conservation. Project seahorse, London, UK. 214 pp.
- Mondal S., Roy T., Sen S.K. & Ray A.K. (2008) Distribution of enzyme-producing bacteria in the digestive tracts of some freshwater fish. *Acta Ichthyologica Et Piscatoria* **38**, 1–8.
- Murugan A., Dhanya S., Sreepada R.A., Rajagopal S. & Balasubramanian T. (2009) Breeding and mass-scale rearing of three spotted seahorse, *Hippocampus trimaculatus*, Leach under captive conditions. *Aquaculture* **290**, 87–96.
- Olafsen J. (2001) Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture* **200**, 223–247.
- Onarheim A.M. & Raa J. (1990) Characteristics and possible biological significance of an autochthonous flora in the intestinal mucosa of sea water fish, In: *Microbiology in poecilotherms* (ed. R. Lesel), pp.197–201. Elsevier, Amsterdam, The Netherlands..
- Oxley A.P.A., Shipton W., Owens L. & McKay D. (2002) Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguensis*. *Journal of Applied Microbiology* **93**, 214–223.
- Project Seahorse 2006. Project Seahorse, Annual Report, University of British Columbia, Canada, p. 20.
- Purchon R.D. (1977) The biology of the mollusca, Second ed. Pergamon, Oxford. p. 560.
- Ramsey A.H., Skonieczny P., Coolidge D.H., Kurzynski T.A., Proctor M.E. & Davis J.P. (2001) *Burkholderia cepacia* lower respiratory tract infection associated with exposure to a respiratory therapist. *Infection Control and Hospital Epidemiology* **22**, 423–426.

- Rimmer D.W. & Wiebe W.J. (1987) Fermentative microbial digestion in herbivorous fishes. *Journal of Fish Biology* **31**, 229–236.
- Ringo E., Strom E. & Tabachek J.A. (1995) Intestinal microflora of Salmonids: A review. *Aquaculture Research* **26**, 773–789.
- Ringo E., Sperstad S., Myklebust R., Refstie S. & Krogdahl A. (2006) Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.) the effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture* **261**, 839–841.
- Romero J. & Navarrete P. (2006) 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of Coho salmon (*Oncorhynchus kisutch*). *Microbial Ecology* **51**, 422–430.
- Sakata T., Nakaji M. & Kakimoto D. (1978) Microflora in the digestive tract of marine fish-I. General characterization of the isolates from yellowtail. *Memories of the Faculty of Fisheries of Kagoshima University* **27**, 67–71.
- Salin K.R. & Mohankumaran N.C. (2006) Resources and biodiversity of seahorses and the need for their conservation in India. *Aquaculture Asia* **10**, 3–8.
- Sa-Pereira P., Costa-Ferreira M. & Atres-Barros M.R. (2002) Enzymatic properties of a neutral endo-1, 3(4)-b-xylanase Xyl II from *Bacillus subtilis*. *Journal of Biotechnology* **94**, 256–275.
- Sera H., Ishida Y. & Kadota H. (1974) Bacterial flora in the digestive tracts of marine fish. In: *Effect of the ocean environment on microbial activities* (ed. Colwell R.R., Morita, R.Y.), pp. 467–490, University of Park Press, Baltimore.
- Shakila R.J., Vyla S.A.P., Saravana Kumar R., Jeyasekaran G. & Jasmine I. (2006) Stability of chloramphenicol residues in shrimp subjected to heat processing treatments. *Food Microbiology* **23**, 47–51.
- Sierra G. (1957) A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates Antonie van Leeuwenhoek **23**, 1, 15–22.
- Spanggaard B., Huber I., Nielsen J., Nielsen T., Appel K.F. & Gram L. (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification, *Aquaculture* **182**, 1–15.
- Sreepada R.A., Desai U.M. & Naik S. (2002) The plight of Indian seahorses: need for conservation and management, *Current Science* **82**, 377–378.
- Stickney R.R. & Shumway S.E. (1974) Occurrence of cellulase activity in the stomachs of fishes. *Journal of Fish Biology* **6**, 779–790.

- Sugita H., Kawasaki J. & Deguchi Y. (1997) Production of amylase by the intestinal microflora in cultured freshwater fish. *Letters in Applied Microbiology* **24**, 105–108.
- Sun Y., Yang H., Ling Z., Chang J. & Ye J. (2009) Gut microbiota of fast and slow growing grouper *Epinephelus coioides*. *African Journal of Microbiology Research* **3**, 713–720.
- Suzer C., Kamaci H.O., Coban D., Saka S., Firat K. & Ozkara A. (2007) Digestive enzyme activity of the red porgy (*Pagrus pagrus*, L.) during larval development under culture conditions. *Aquaculture Research* **38**, 1778–1785.
- Tendencia E.A. (2004) The first report of *Vibrio harveyi* infection in the seahorse *Hippocampus kuda* Bleeker, 1852 in the Philippines. *Aquaculture Research* **35**, 1292–1294.
- Thompson F.L., Hoste B., Thompson C.C., Goris J., Gomez-Gil B., Huys L., De-Vos P., & Swings J. (2002) *Enterovibrio norvegicus* gen. nov., sp. nov., isolated from the gut of turbot (*Scophthalmus maximus*) larvae: a new member of the family Vibrionaceae. *International Journal of Systematic and Evolutionary Microbiology* **52**, 2015–2022.
- Tipton K., & Bell S (1988) Foraging patterns of two Syngnathid fishes: importance of harpacticoid copepods. *Marine Ecology Progressive Series* **47**, 31–43.
- Truong S.K. (1998) Prospects of community-based aquaculture in Vietnam. In: *The marine biology of the south China sea III* (ed. Morton B.), pp. 465–474, Hong Kong University Press, Hong Kong.
- Trust T.J. & Sparrow R.A.H. (1974) The bacterial flora in the alimentary tract of freshwater salmonid fishes. *Canadian Journal of Microbiology* **20**, 1219–1228.
- Vine N.G., Leukes W.D. & Kaiser H. (2006) Probiotics in marine larviculture. *FEMS Microbiology Reviews* **30**, 404–427.
- Weisburg W.G., Barns S. M., Pelletier D.A., Lane D. J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**, 697–703
- Westerdahl A., Olsson J.C., Kjelleberg S. & Conway P.L. (1991) Isolation and characterization of turbot (*Scophthalmus maximus*) associated bacteria with inhibitory effects against *Vibrio anguillarum*. *Applied and Environmental Microbiology* **57**, 2223–2228.
- Yokoi Y. & Yasumasu I. (1964) The distribution of cellulase in invertebrates. *Comparative Biochemistry and Physiology* **13**, 223–228.

Table 1 Composition and substrate degrading ability of bacteria isolated from the intestinal tract of farmed yellow seahorse, *Hippocampus kuda*.
 ‘+’ indicates positive activity; ‘-’ indicates negative activity.

Bacterial sp.	NCBI No.	Enzymes				
		Xylanase	Amylase	Cellulase	Lipase	Protease
<i>Bacillus pumilus</i> (S6)	GU969591	+	-	-	-	+
<i>Bacillus pumilus</i> (S7)	GU969592	+	-	-	-	+
<i>Bacillus pumilus</i> (S9)	GU969594	+	-	+	+	-
<i>Bacillus pumilus</i> (S10)	GU969595	+	-	+	+	+
<i>Bacillus pumilus</i> (S11)	GU969596	+	-	-	+	-
<i>Bacillus pumilus</i> (S15)	GU969599	+	-	+	+	-
<i>Bacillus firmus</i> (S25)	GU969609	-	-	-	+	+
<i>Bacillus firmus</i> (S26)	GU969610	+	-	-	+	+
<i>Vibrio sp.</i> (S3)	GU969588	-	+	-	-	-
<i>Vibrio sinaloensis</i> (S4)	GU969589	-	+	-	+	-
<i>Vibrio harveyi</i> (S16)	GU969600	-	+	+	+	-
<i>Vibrio parahaemolyticus</i> (S19)	GU969603	-	+	-	+	-
<i>Vibrio sp.</i> (S20)	GU969604	+	-	-	-	-
<i>Shewanella sp.</i> (S5)	GU969590	-	+	-	-	-
<i>Shewanella sp.</i> (S22)	GU969606	+	-	-	-	-
<i>Shewanella sp.</i> (S24)	GU969608	+	-	+	+	+
<i>Shewanella sp.</i> (S27)	GU969611	+	-	-	+	+
<i>Enterococcus faecalis</i> (S13)	GU969597	-	-	-	+	-
<i>Phaeobacter sp.</i> (S21)	GU969605	+	-	+	+	+
<i>Phaeobacter sp.</i> (S23)	GU969607	+	-	+	-	+
<i>Providencia rettgeri</i> (S17)	GU969601	-	-	-	+	+
<i>Providencia sp.</i> (S12)	GU980965	-	-	-	+	-
<i>Providencia sp.</i> (S14)	GU969598	-	-	-	+	-
<i>Burkholderia cenocepacia</i> (S8)	GU969593	+	-	+	+	-
<i>Enterovibrio nigricans</i> (S2)	GU969587	-	-	-	+	-
<i>Pseudoalteromonas piscicida</i> (S18)	GU969602	+	-	-	+	-
<i>Staphylococcus pasteurii</i> (S1)	GU969586	+	-	-	-	-

Table 2. Relative abundance of major bacterial groups isolated from the intestinal tract of farmed yellow seahorse, *Hippocampus kuda*. Values in parentheses indicate percentage of total isolates.

Bacterial group	Number of isolates
<i>Bacillus</i>	8 (29.62%)
<i>Vibrio</i>	5 (18.51%)
<i>Shewanella</i>	4 (14.81%)
<i>Providencia</i>	3 (11.11%)
<i>Phaeobacter</i>	2 (7.40%)
<i>Enterococcus</i>	1 (3.70%)
<i>Burkholderia</i>	1 (3.70%)
<i>Enterovibrio</i>	1 (3.70%)
<i>Pseudoalteromonas</i>	1 (3.70%)
<i>Staphylococcus</i>	1 (3.70%)
Total	27

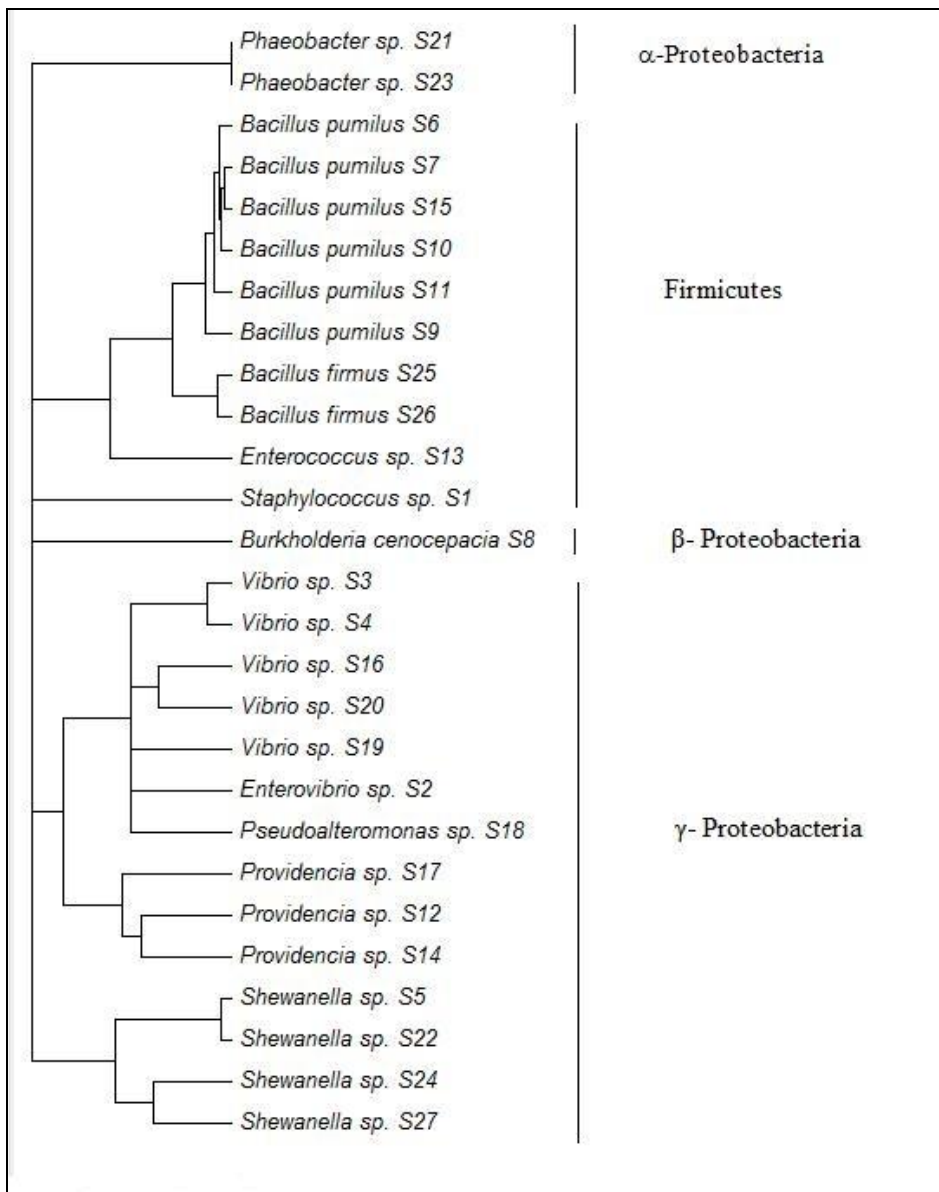


Fig. 1 The phylogenetic tree constructed by the neighbour-joining method based on the partial sequence of the 16S rRNA gene for the bacteria isolated from the intestinal tract of farmed yellow seahorse, *Hippocampus kuda*.