



International Journal of Fisheries and Aquatic Studies

E-ISSN: 2347-5129

P-ISSN: 2394-0506

(ICV-Poland) Impact Value: 5.62

(GIF) Impact Factor: 0.549

IJFAS 2019; 7(6): 316-321d

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www.fisheriesjournal.com

Received: 01-09-2019

Accepted: 05-10-2019

Ramya Deepika K

Department of Marine Living
Resources, Andhra University,
Visakhapatnam, Andhra
Pradesh, India

Janakiram P

Department of Marine Living
Resources, Andhra University,
Visakhapatnam, Andhra
Pradesh, India

Sunil kumar D

Department of Marine Living
Resources, Andhra University,
Visakhapatnam, Andhra
Pradesh, India

Surendran V

Department of Marine Living
Resources, Andhra University,
Visakhapatnam, Andhra
Pradesh, India

Madhuri M

Department of Marine Living
Resources, Andhra University,
Visakhapatnam, Andhra
Pradesh, India

Geetha S

Department of Marine Living
Resources, Andhra University,
Visakhapatnam, Andhra
Pradesh, India

Corresponding Author:

Janakiram P

Department of Marine Living
Resources, Andhra University,
Visakhapatnam, Andhra
Pradesh, India

Gut bacterial diversity of wild and cultured *penaeus vannamei*

Ramya Deepika K, Janakiram P, Sunil kumar D, Surendran V, Madhuri M and Geetha S

Abstract

The pacific white shrimp, *Penaeus vannamei* is a marine crustacean which has economic importance in the world market. To ensure sustainability of the shrimp industry, production capacity and disease outbreak prevention must be improved. Understanding healthy microbial balance inside the shrimp gut and Hepatopancreas can provide a benchmark study and facilitates to adapt better farming practices. Gut micro biome of cultured and wild *P vannamei* were analyzed and obtained eight bacterial isolates from the former and four from the latter. Out of the eight isolates obtained from the gut of cultured shrimp, four isolates were identified as *Vibrio tubiashii*, *Vibrio gazogenes*, *Vibrio proteolyticus*, *Vibrio natriegens* and the remaining from Hepatopancreas (HP) were identified as *Vibrio ordalii*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Corynebacterium kutscheri*. Four types of bacterial isolates were obtained from the wild shrimp, out of them two gut isolates were identified as *Vibrio vulnificus*, *Vibrio furnissii* and the remaining isolates from hepatopancreas were identified as *Lactobacillus fermentum* and *Staphylococcus aureus*. All the 12 isolates were subjected to antibiotic sensitivity tests and found that Ciprofloxacin (Cf) showed highest inhibitory zone (34mm) against *Vibrio harveyi* and Norfloxacin (NX) showed lowest (10) mm against *Vibrio vulnificus*. All the 12 bacterial isolates were resistant to penicillin-G. Ampicillin showed inhibitory activity against *Vibrio harveyi* and rest of the isolates were resistant. *Vibrio tubiashii* was resistant to Ampicillin, Furadazole, Penciling-G, and chloramphenicol.

Keywords: Microbiome, Shrimp gut, *Peneaus vannamei*, Bacteria, Hepato pancreas

Introduction

Penaeus vannamei, commonly known as Pacific white leg shrimp, is one of the most principal crustacean cultured shrimp species (>70%) worldwide, and contributing importantly to the economic development of coastal wetland areas [6]. The pacific white shrimp, *Penaeus vannamei* is becoming more and more important for aquaculture as one of the most profitable species in shrimp farming, with the production being more than 3 million tons per year [40]. The main bottleneck to the sustainable production is disease outbreak. Economic losses due to the disease in shrimp more than \$1 billion per year [23, 21, 14]. As in many areas of animal nutrition, a great emphasis is now placed on understanding the roles of micro biota in the health, growth, and survival of cultured organisms [2]. In marine organisms, gut microbes are associated with digestive enzyme production [36, 28, 17, 19] competitive exclusion of pathogenic bacteria and generation of essential elements for host metabolism. In krill *Meganyctiphanes norveica* [9], both the stomach and hepatopancreas were found to contain saprophytic bacteria related to enzymatic activity. Facultative anaerobic strains initially dominate in the intestine after which variation in micro biota population depends on diet, age, geographic location, medical treatment and overall organism condition [5, 16]. Their trophic functions promote cell growth and differentiation, as well as stimulation of the immune system. Their protective functions are present from birth since they act as the first line of defense against pathogenic, exogenous or opportunist microorganisms, creating a barrier effect [16]. Some bacteria in the intestine can be pathogenic to their host, whereas others are beneficial to the development, morphology, and physiology of the host, contributing to nutrient absorption, immune responses, and gut epithelial development [11, 29]. The micro biota in the aquatic environment is usually in equilibrium and is composed by bacteria that are either beneficial or neutral to cultured animals, or by harmful obligate and opportunistic pathogenic bacteria [34]. The hepatopancreas is an essential organ for digestion and absorption of nutrients, and plays a role

in innate immunity in invertebrates [30]. Bacteria in the γ -Proteobacteria class were the only common bacteria group found in the intestinal tracts of shrimp from all farms. The dominant bacterial genera in the intestinal population of each shrimp varied among different farms, and these genera were *Vibrio*, *Photobacterium*, *Aeromonas* or *Propionigenium* (phylum *Fusobacteria*). Other commonly found genera included *Antinomyces*, *Anaerobaculum*, *Halospirulina*, *Pseudomonas*, *Mycoplasma*, and *Shewanella*. Twelve groups of bacteria including *Proteobacteria*, *Firmicutes*, *Fusobacteria*, *Actinobacteria*, *Cyanobacteria*, *Tenericutes*, *Deinococcus-Thermus*, *Planctomycetes*, *Spirochaetes*, *Synergistetes*, *Thermotogae* and *Verrucomicrobia* were represented in the sequences. Additionally, strictly anaerobic bacteria such as *Propionigenium* and *Fusibacter* were found [33]. The gut flora of *Neocaridina denticulate* shrimp the predominant bacteria was found to be Proteobacteria with more than 80% reads from the gut flora at the early gonad development belonged to a coxiella type bacterium [8]. Changes in the micro biota of shrimp species, such as *Penaeus monodon* [31], *Fenneropenaeus chinensis* (Chinese shrimp) [24], *Penaeus paniculatus* [38], *Penaeus merguensis* (banana shrimp) [26], and *Penaeus vannamei* [14], have been described under a variety of growth and water quality conditions. However, the micro biota from natural environments has only been studied in the intestine of *P. monodon* [31]. Micro biota of the intestine and hepatopancreas and their potential functions in wild type (wt) *P. vannamei* captured in the Pacific Ocean are unknown. Thus, the characterization of the natural *P. vannamei* micro biota, to be used as a reference for comparison with healthy and diseased cultured shrimp in hatcheries, is necessary. Hence, modification of the macrobiotic could be utilized as a new approach against shrimp diseases [39]. The intestinal macrobiotic of pacific blue shrimp and black tiger shrimp have been well investigated [31, 7], while most reports about pacific white shrimp focus on the microbial community of the surrounding water [37, 12] and the effect of diet on intestinal macrobiotic [41]. Previous studies revealed that many bacterial diseases are associated with the shifts and imbalance of intestine macrobiotic in other aquaculture animals [27, 22] and the probiotic addition is helpful for maintaining the intestinal bacterial balance [15, 3]. Very few studies have quantified how the micro biota was affected by the emergence of disease under aquaculture conditions [18]. There is scarce information about the micro biota in shrimp organs other than the intestine, leaving aside the important connection that exists between the function of this organ and the hepatopancreas because the crustacean digestive tract is continuous [8]. Knowledge of the intestinal micro biota of pacific white shrimp at different culture stages is still limited, hence the present investigation

Materials and Methods

Ethics Statement

An ethics statement is not required for this work. No specific permits were required for the described field studies. The field studies did not involve endangered or protected species.

Sample collection

Wild (wt. 50±1g) and cultured (wt. 32±1g) *Penaeus vannamei* were obtained from Vaisakhi bio marine hatchery of Srikakulam 18°11'6.12"N, 83°52'16.95"E and culture ponds at Bheemili 17°54'6.09"N 83°26'59.17"E respectively. They

were transported to the laboratory under continuous oxygenated condition. Each shrimp sample (2 individuals/sample) was anaesthetized on ice for 5-10 min, and their gut and hepatopancreas (HP) were aseptically dissected. Pre-weighed gut (0.25g) and HP (2.11g) were homogenized with 2% sterile NaCl solution

Total Bacterial count (TBC)

The homogenized samples were serially diluted with sterile normal saline water for 6 dilutions (10^{-1} to 10^{-6}). One ml of sample was taken from 10^{-3} and 10^{-5} dilutions separately and added to the Petridishes and poured the Zobell's Marine Agar (ZMA) medium and incubated for 24 hrs at $32\pm 1^\circ\text{C}$. Number of colonies were counted and expressed in CFU/g.

Total Vibrio count (TVC)

Total *Vibrio* count was estimated by pour plate method (APHA, 1992). The homogenized samples were serially diluted with sterile normal saline water for 6 dilutions (10^{-1} to 10^{-6}). One ml of sample was taken from 10^{-3} and 10^{-5} separately and added to the Petridishes. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar medium was poured into the Petridishes containing the sample and incubated for 24 hrs at $32\pm 1^\circ\text{C}$. Number of colonies were counted and expressed in CFU/g. Upon incubation those colonies which appeared dominant and distinct were selected for further study.

Identification of isolated bacteria

Various morphological and biochemical characterization tests were carried out *viz.*, Grams staining, Motility of bacteria by hanging drop method, Spore staining, Acid fast staining, Aerobic and Anerobic, Oxidative and Fermentative, Acid production from glucose, Oxidase, Catalase, following the protocols given in the Bergey's manual of Systemic Bacteriology [20] to identify the Bacterial isolates upto species level.

Species level characterization tests

Growth on TCBS, Decarboxylation of Amino-acids (Arginine, Ornithine, Lysine), MRVP test, Starch hydrolysis, Urea hydrolysis, Aesculin hydrolysis, Utilization of carbohydrates (L-Arabinose, Dextrose, Fructose, Lactose, Mannose, Galactose, Sucrose, Trehalose, Cellobiose, Melibiose, Salicin, Xylose), Citrate utilization, Nitrate reduction, ONPG hydrolysis.

Antibiotic sensitivity tests

All the isolates obtained were subjected to antibiotic sensitivity tests *viz.* Ox tetracycline (O), Norfloxacin (NX), Streptomycin(S), Ciprofloxacin (Cf), Ampicillin (A), Furozolidone (Fr), Penicillin-G (P), Chloramphenicol (c), Tetracycline (T) and Erythromycin (E). The sensitivity of the isolates to various antibiotics was tested following the method [4]. Young cultures (18 hrs.) of the isolates were spread evenly over Mueller's Hinton Agar (MHA) dishes. Antibiotic discs (Himedia, Mumbai), were placed over the spread culture carefully and incubated overnight at 32°C . Upon incubation, the inhibition zones obtained around the discs were measured by using Kirby bauer scale.

Results

Bacterial count

The total bacterial counts (TBC) in the Hepatopancreas (HP) and gut of the wild brooder were found to be 0.62×10^6 CFU/g

and 0.76×10^8 CFU/g respectively. The total vibrio counts (TVC) in Hepatopancreas and gut of wild shrimp brooder were 0.4×10^6 CFU/g and 0.6×10^8 CFU/g respectively (Table 1). The total bacterial counts (TBC) in HP and Gut in cultured shrimp were 0.7×10^5 CFU/g and 0.5×10^7 CFU/g respectively. The total vibrio counts (TVC) in HP and Gut of cultured shrimp 0.3×10^1 CFU/g and 0.25×10^5 CFU/g respectively (Table. 2).

Table 1: TBC and TVC of wild shrimp

Sample no.	TBC(CFU/g)		TVC(CFU/g)	
	HP	Gut	HP	Gut
1	0.67×10^6	0.81×10^8	0.35×10^4	0.67×10^8
2	0.65×10^6	0.72×10^8	0.46×10^4	0.56×10^4
3	0.54×10^6	0.75×10^8	0.41×10^4	0.59×10^4
Average	0.62×10^6	0.76×10^8	0.40×10^4	0.6×10^8

Table 2: TBC and TVC of cultured shrimp

Sample no.	TBC(CFU/g)		TVC(CFU/g)	
	HP	Gut	HP	Gut
1	0.75×10^5	0.50×10^7	0.35×10	0.19×10^5
2	0.65×10^5	0.55×10^7	0.27×10	0.3×10^5
3	0.70×10^5	0.45×10^7	0.28×10	0.26×10^5
Average	0.70×10^5	0.50×10^7	0.30×10	0.25×10^5

Colony characterization and differentiation of the isolates

Based on the colony morphology (Colour, pigmentation, size, Appearance, optical property, Form, Elevation, Margin and texture) 12 different types of bacterial colonies were observed. All the 12 different bacterial colonies were isolated

and designated as (G1,G3-V,G-a,G-b,G-V(a),G-V(b),Hp-V(a),HP-V(b),HP-a,HP-b,HP-1,HP-2). Colony characteristics of the bacterial isolates were summarized in Table 3. Most of these isolates were white in color, circular form and shiny in appearance with opaque optical property. The texture of all these isolates were smooth, entire margin, and raised elevation except G-b, HP-a and G (V)a.

Taxonomical characterization of the bacterial isolates

Following gram staining procedure all of the 12 isolates were observed under microscope. Nine isolates such as G1,G3-V,G-a,G-b,G-V(a),G-V(b),Hp-V(a),HP-V(b),HP-a were gram negative and three isolates,HP-b,HP-1,HP-2 were gram positive. Based on the Gram staining, further required biochemical tests were performed and tabulated the results (Table nos. 3 and 4). Based on biochemical tests gram negative bacteria were identified as G1- *Vibrio vulnificus*, G3-V- *Vibrio parahaemolyticus*, G-a - *Vibrio tubiashii*, G-b- *Vibrio gazogenes*, G-V(a)- *Vibrio proteolyticus*, G-V(b) - *Vibrio natriegen*, and Hp-V(a)-*Vibrio ordalii*, HP-V(b) - *Vibrio harveyi*, HP-a- *Vibrio tubiashii*. Gram positive bacteria identified as HP -1- *Lactobacillus fermentum*, HP-2 - *Staphylococcus aureus*, HP-b -*Corynebacterium kutscheri*.

Antibiotic sensitivity

Inhibition zones obtained in the sensitivity assay were presented in the Fig. 1 and their values in Table Nos. 4, 5 categorized into highly sensitive, moderately sensitive, sensitive and resistant based on the zone of inhibition. All isolates were found resistant to Penicillin G.

Table 3: Antibiotic sensitivity of Gram negative bacterial isolates

S.no	Name of Antibiotics	Diameter of Zone of inhibition(mm)								
		G1	G3-V	G-a	G-b	Hp-a	G(V)a	G(V)b	Hp(V)a	Hp(V)b
1	Oxytetracycline (O)	25	23	22	28	24	26	19	0	19
2	Norfloxacin (NX)	21	22	16	18	17	21	18	12	10
3	Streptomycin (S)	19	17	14	15	14	19	19	13	11
4	Ciprofloxacin (Cf)	35	29	34	28	28	29	26	27	20
5	Ampicillin (A)	21	0	0	0	0	0	0	0	0
6	Furozolidone (Fr)	24	22	0	22	17	24	19	19	18
7	Penicillin-G (P)	0	0	0	0	0	0	0	0	0
8	Chloramphenicol (c)	34	27	0	29	31	27	27	31	25
9	Tetracycline (T)	29	25	29	26	28	28	24	29	24
10	Erythromycin (E)	12	15	17	19	16	18	17	11	12

Highly Sensitive: 20 mm, moderately sensitive: 15-20 mm, Sensitive: 1-15 mm, Resistant- 0 mm

All the Gram negative isolates were highly sensitive to Ox tetracycline, Ciprofloxacin, Chloramphenicol, Furozolidone and Tetracycline and moderately sensitive to Norfloxacin, Streptomycin and Erythromycin. All the isolates were resistant to Penicillin -G and Ampicillin, except G1- *Vibrio vulnificus* which proved to be highly sensitive to Ampicillin. The isolates Hp (v) a- *vibrio ordalii* was resistant to ox tetracycline, G-a- *vibrio tubiashii* to chloramphenicol and Furozolidone. The isolates G1-*vibrio vulnificus*, G3-V- *vibrio parahaemolyticus*, Hp (v) a-*vibrio ordalii* and Hp (v)b- *vibrio harveyi* were sensitive to Erythromycin. Hp(v)a- *vibrio ordalii* and Hp(v)b- *vibrio harveyi* to Norfloxacin and G-a- *vibrio tubiashii*, G-b- *vibrio gazogenes*, Hp-a- *vibrio tubiashii*, Hp(v)a- *vibrio ordalii* and Hp(v)b- *vibrio harveyi* were sensitive to Streptomycin. The isolates G(v)b- *vibrio natriegen* and Hp(v)b- *vibrio harveyi* were moderately sensitive to ox tetracycline and G-a- *vibrio tubiashii*, G-b- *vibrio gazogenes*, Hp-a- *vibrio tubiashii*, G(v)b- *vibrio*

natriegen to Norfloxacin. The isolates G1-*vibrio vulnificus*, G3-v- *vibrio parahaemolyticus*, G (v)a- *Vibrio proteolyticus* are highly sensitive to Norfloxacin.

Table 4: Antibiotic sensitivity of Gram positive bacterial isolates

S. No	Name of Antibiotics	Diameter of Zone of inhibition(mm)		
		Hp-1	Hp-2	Hp-b
1	Oxytetracycline O)	21	22	25
2	Norfloxacin (NX)	18	16	15
3	Streptomycin (S)	16	20	16
4	Ciprofloxacin (Cf)	26	25	30
5	Ampicillin (A)	0	0	0
6	Furozolidone (Fr)	16	20	16
7	Penicillin-G (P)	R	R	R
8	Chloramphenicol (c)	20	23	24
9	Tetracycline (T)	26	27	27
10	Erythromycin (E)	14	14	17

Highly Sensitive - 20 mm, Moderately Sensitive - 15-20mm, Sensitive -10-15mm, Resistant - 0mm

All the Gram positive isolates tested were highly sensitive to Ox tetracycline, Ciprofloxacin and Tetracycline and moderately sensitive to Norfloxacin, Streptomycin and Erythromycin, whereas highly sensitive to Furozolidone. Hp-

1-lactobacillus fermentum and Hp-2-staphylococcus aureus were sensitive to erythromycin except Hp-b-corynebacterium kutscheri which was moderately sensitive to it.

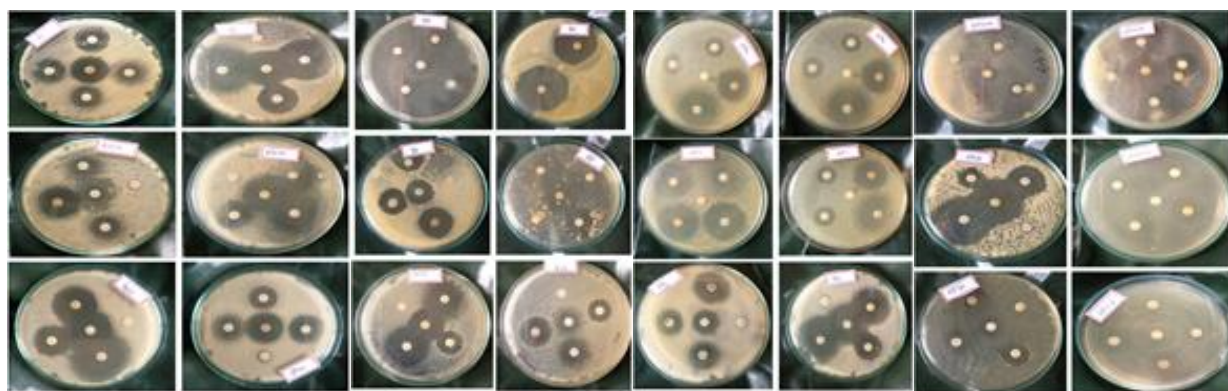


Fig 1: Inhibitory activity of different commercial antibiotics against the gut microbial isolates(G1,G(v)A,G3(V) Ga, Gb, G-V(b) HPa,HP1,HP2 HP(V)a, HP (b)HP(V)b)

Discussion

Gut microbiome of *P. monodon* was more diverse and dissimilar to that of *P. merguensis* [25, 32]. Sequences from (Denaturing Gradient Gel Electrophoresis) bands showed that *Vibrio*, *Photobacterium*, and *Fusobacteria* constituted core bacterial members in both the shrimp guts *Clostridia* [35], *Mollicutes* and *Ferrimonas* spp. were only found in the gut of *P. monodon* [10]. Characterized the micro biota from the Pacific Whiteleg shrimp hepatopancreas and intestine of wild type and cultured shrimp by using sequencing of seven hypervariable regions of the 16S rRNA gene. The identified bacteria from wild shrimp belonged to *Proteobacteria* *Cyanobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Bacteroidetes* and *Firmicutes*, *Vibrio* and *Pseudomonas*. *Photobacterium* and *Acinetobacter* were the most abundant genera in the hepatopancreas and intestine. From cultured shrimp *Faecalibacterium prausnitzii* and *pantoea agglomerans*, which belonging to *Firmicutes* and *proteobacteria* were found [26]. characterized the Bacterial flora from the gut of the wild and cultured *Penaeus merguensis* (Banana prawn), the identified bacterial floral compositions, which included members from the genera *Aeromonas*, *Plesiomonas*, *Photobacterium*, *Pseudoalteromonas*, *Pseudomonas* and *Vibrio* [31]. Characterized intestinal Bacteria in Wild and cultured *Penaeus monodon* by employing barcode Pyrosequencing analysis of V3-4 regions of 16S rRNA genes to examine intestinal bacteria communities in wild and cultured shrimp and identified the bacterial communities belonging to the *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes* groups.

Isolates in the present investigation were *Proteobacteria* and *Actinobacteria*. As mentioned earlier the six isolates were identified from the group *Proteobacteria* i.e., *Vibrio tubiashii*, *Vibrio gazogenes*, *Vibrio proteolyticus*, *Vibrio natriegens*, *Vibrio ordalii*, *Vibrio vulnificus*, and one from the *Actinobacteria* i.e *Corynebacterium kutscheri*. In the present study two bacterial isolates *Proteobacteria* and *Firmicutes* from the gut and hepatopancreas of cultured *Penaeus vannamei* were identified. Two bacteria were found from the *Proteobacteria* belonging to *Vibrio harveyi*, *Vibrio furnissii*. Similarly two isolates were identified from the group *Firmicutes* viz. *Lactobacillus fermentum* and *Staphylococcus aureus*.

The sensitivity of *V. vulnificus* (18 strains), *V. parahaemolyticus* (12 strains), *V. fluvialis* (19 strains) and *V. metschnikovii* (3 strains) to various antibiotics and confirmed that all the 52 isolates were found resistant to Ampicillin and sulfamethoxazole, and sensitive to imipenem, meropenem and Norfloxacin¹. Antibiotic sensitivity of the isolate in the present study also revealed that all the species were resistant to Penicillin and also Ampicillin showed 21 mm zone against *Vibrio vulnificus* rest of all were resistant it.

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