Molecular Identification of Gut Microflora of the Prawn *Macrobrachium rosenbergii* Fed with Probiotic Bacterium *Bacillus licheniformis* Supplemented Diet

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**Abstract**

Probiotics offer a wide range of health benefits to the host. The present paper deals with 16S r-RNA sequence analysis of gut microbial diversity of *M. rosenbergii* fed with *B. licheniformis* (MTCC 429; NCBI-GenBank accession number, MK158065 supplemented diet (CFU, 935x10^9)). The >10 kb genomic DNA yield ~1500 pb PCR amplified products against specific 16S r-RNA primers. The aligned sequences of the gut of control prawns showed 1337 bp, 768 bp, 1334 bp, 1419 bp, 1315 bp, 1313 bp, 1466 bp and 1289 bp 16S r-RNA for *Pseudomonas sp.*, *Klebsiella oxytoca*, *Escherichia coli*, *Bacillus coagulans*, *Streptococcus thermophilus*, *Staphylococcus aureus*, *Citrobacter koseri* and *Acinetobacter sp.*, respectively. The gut of experimental prawns showed 1350 bp, 1495 bp, 1464 bp, 1307 bp, 1446 bp, and 1347 bp 16S r-RNA for *Bacillus sp.*, *Bacillus licheniformis*, *Lactobacillus plantarum*, *Escherichia coli*, *Streptococcus iniae* and *Citrobacter sp.*, respectively. The biochemical tests confirmed that the pathogenic bacteria, like *Pseudomonas sp.*, *Klebsiella sp.*, *Staphylococcus sp.*, and *Acinetobacter sp.*, have competitively been excluded from the gut of experimental prawns due to colony establishment of *B. licheniformis* and produced good growth [1]. The BLAST of these sequences showed almost 100% similarities with the same species retrieved from the NCBI database. The MAS showed 460 identical amino acids residues, 79 similar amino acids residues and 308 variable amino acids sites for control prawns, and 879 identical amino acids residues, 85 similar amino acids residues and 396 variable amino acids sites for experiment prawns. These sequences have less number of AT biases and more number of GC biases. Overall, the nucleotide divergence and the phylogenetic information calculated were clearly discriminated these bacterial species. Therefore, 16S r-RNA sequencing provides accurate identification of bacterial species. Thus, the phylogenetic tree topology showed very close/parallel alignment genera indenfified from both control and experimental prawns.

**Keywords:** Prawn, Gut microflora, Probiotics, *Bacillus licheniformis*, 16S r-RNA.

**INTRODUCTION**

Aquaculture has evolved as one of the fastest growing food production systems in the world, contributing significantly to global food security, and after fish, a major share of the global aquaculture production is contributed by crustaceans [2]. The giant freshwater prawn, *Macrobrachium rosenbergii* (de Man 1879), is considered as a candidate species among crustaceans for aquaculture in different parts of tropical and subtropical belts and has economical importance due to its fast growth, high market demand and tolerance to environmental condition [3, 4]. Moreover, *M. rosenbergii* shows resistance to most of the viral diseases that have devastated marine shrimp farming [5].

The gut microbiota of an organism reflects its health status, and it is based on its surrounding environment [6-10]. However, the shift in microbial composition and structure is less affected by the surrounding environment, and the host itself is mainly shaped the stable gut microbial environment [11-14]. The intestinal microflora can help in converting the complex molecules into the simple one by their metabolic activities and serve as an important factor in nutrition, physiology and welfare of the host [15-17]. The available literature in related to gut microbiota of *M. rosenbergii* is moderate [18-22]. Therefore studies are required to understand the probiotic relationship of any bacteria with host.

Probiotics are live microorganisms which when administered in adequate amounts confer a health...
benefit on the host [23]. Recent studies have shown that certain strains of *Bacillus licheniformis* confer significant probiotic actions and enhance the potential functional capacity of gut microbiota, promoting the growth and general immune response in human and animals [24-27, 1]. *B. licheniformis* is a gram-positive, oxidase-positive and catalase-positive endospore forming non-pathogenic bacterium belonging to the genus *Bacillus* [28, 29]. It produces a wide range of extracellular enzymes [30, 31, 26]. It has been reported that *B. licheniformis* competitively excluded pathogenic bacteria from the gut with increased immune response in the white shrimp, *Litopenaeus vannamei* [32], in *Peneaus japonicus* [33] and in *M. rosenbergii* [1].

Mostly, *Bacillus* spp., are used as probiotic bacteria in aquaculture field, but accurate identification for these closely related species is difficult and laborious because conventional phenotypic tests fail to distinguish different strains. In addition, *Bacillus* spp., strains have identical 16S r-RNA gene sequences (~99.2 - ~99.6% sequence similarity) [34, 35]. DNA-based identification methods like 16S r-RNA gene sequencing have been commonly/ widely used as a framework for identification, classification and quantification of microorganisms isolated from natural environments and gut samples [36, 37]. The 16S r-RNA gene is a highly conserved component of the transcriptional machinery of all DNA-based life forms and thus is highly suited as a target gene for sequencing of different bacterial species for constructing their phylogenetic relationships [38, 39]. However, it often shows limited variation for members of closely related taxa [40, 41]. Recent taxonomic studies have indicated that *B. licheniformis* is closely related to *B. subtilis* and *B. amyloydofaciens* according to comparisons of 16S r-DNA and 16S-23S internal transcribed spacer (ITS) nucleotide sequences [42]. In general, 16S r-DNA sequences are used for *Bacillus* classification as a framework of species delineation [43]. Partial 16S r-DNA sequences near the 5' end region (approx 275 bp) have been useful parameters for the identification or grouping of *Bacillus* species [44]. Xu & Cote [42] reported phylogenetic relationships between *Bacillus* and related genera based on sequence of a 220 bp region covering the highly conserved 150 bp sequence at the 3' end of the 16S r-RNA coding region and the conserved 70 bp sequence at 5' end of 16-23S ITS region. A fast sensitive real time PCR assay for simultaneous detection of *B. licheniformis*, members of the *B. cereus* group and *B. fumarioli* in gelatin have been reported [45]. Moreover, it was accepted that species showing 70% or greater DNA homology usually have more than 97% 16S r-RNA gene sequence similarities [46].

Previously the prawn, *M. rosenbergii* post larvae were supplemented with five different serially diluted concentrations of *B. licheniformis* (10^7, 10^6, 10^5, 10^4 and 10^3). At 10^6 (CFU, 935x10^6), the presence of *Pseudomonas* sp., *Klebsiella* sp., *E. coli*, *Bacillus* sp., *Streptococcus* sp., *Staphylococcus* sp., *Citrobacter* sp., and *Acinetobacter* sp., were recorded in control prawns. In the experimental prawns, *Bacillus* sp., *Lactobacillus* sp., *E. coli*, *Streptococcus* sp., and *Citrobacter* sp., were observed. This revealed that the pathogenic bacteria, *Pseudomonas* sp., *Klebsiella* sp., *Staphylococcus* sp., and *Acinetobacter* sp., were found to have competitively been excluded from the gut of experimental prawns due to establishment of *B. licheniformis* colony, and produced good growth [1]. In the present paper the molecular identification of these bacterial species were done by analysing the DNA barcoding of 16S rRNA.

**MATERIALS AND METHODS**

The probiotic bacterium, *B. licheniformis* was procured from Microbial Type Culture Collection (MTCC 429), Chandigarh, India, in lyophilized powder form. It was subjected to broth culture [1], sequenced and authenticated in NCBI-GenBank with the accession number, MK158065.

**DNA Barcoding of Gut Microbial Consortium of *M. rosenbergii* Fed with *B. licheniformis* Supplemented Diet**

Isolation and Purification of Genomic DNA

Bacterial genomic DNA was isolated from individual culture of *Bacillus* spp., (one colony), *Lactobacillus* spp., (three colonies), *E. coli*, *Streptococcus* spp., and *Citrobacter* spp., (one colony) by using phenol, chloroform, iso-amyl alcohol method (PCI) and they were homogenized in pre cooled mortar and pistol with 2 volume of cold TE buffer (500 µl). Proteinase K (20 µl) was added and incubated at 56°C for 1-8 hours until the tissue was totally dissolved. Equal volume of PCI was added with concentration of (25:24:1) and mixed thoroughly for few minutes. The sample was centrifuged for 10 minutes at 12,000 rpm. The upper phase was transferred to new 1.5 ml tube, equal volume of Chloroform: Iso water or 1X TAE buffer (500 µl). Proteinase K (20 µl) was added and incubated at 56°C for 1-8 hours until the tissue was totally dissolved. Equal volume of PCI was added with concentration of (25:24:1) and mixed thoroughly for few minutes. The sample was centrifuged for 10 minutes at 12,000 rpm. The upper phase was transferred to new 1.5 ml tube, equal volume of Chloroform: Iso-amyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 10 minutes. The upper layer was transferred to a freshly sterilized micro centrifuge tube and double volume of child absolute ethanol was added. This preparation was kept at -20°C over night for precipitation then centrifuged for 10 minutes at 10,000 rpm. The supernatant was discarded and 500 µl of 70% ethanol was added. The sample was again centrifuged at 7,000 rpm for 10 minutes and the supernatant was removed. The pellet was kept for air dry under the laminar flow. The pellet was re-suspended in 100 µl of nuclease free water or 1X TAE buffer [47].

To the sample 500µl of PCI was added and mixed slowly then it was incubated at 25°C for 5 minutes and centrifuged at 12,000 rpm for 5 minutes at 4°C. The aqueous phase was carefully removed into new centrifuge tube and treated two more times with PCI. The residual protein was eliminated from the

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aqueous phase by adding 400μl of chloroform, mixed slowly and centrifuged at 12,000 rpm for 10 minutes at 4°C. The upper aqueous phase was recovered and the DNA was precipitated by adding 10μl of 4M ammonium acetate and 500 μl of cold absolute ethanol, then incubated at -20°C for 20 minutes and centrifuged at 15,000 rpm for 15 minutes at 4°C. The precipitated DNA was cleaned with ethanol and the pellet was air dried. The pellet (containing Genomic DNA) was dissolved in 100 μl of TE buffer and stored at -20°C for future usage, or at -80°C for long preservation.

**Agarose Gel Electrophoresis (AGE)**

Tank buffer, 1X TAE was prepared, i.e, 365=350 (tank capacity) +15 ml (boat capacity). The presence of genomic DNA was confirmed by 1% agarose gel. Agarose (150mg) was dissolved in 15ml of TAE buffer (the agarose was melted in TAE buffer under micro oven for 1 minute). A drop of ethidium bromide was added, casted at room temperature and poured into the boat. Then the comb was placed. After polymerization, the comb was carefully removed without damaging the wells. The boat was fixed into the tank filled with 350 ml of 1X TAE buffer. The sample DNA was mixed with loading dye (containing Bromophenol blue and Glycerol in 2:6 ratio), and carefully loaded into the wells of the casted gel. The gel was given 100 volts DC for 30 minutes, safely removed and placed under UV transilluminator / GEL Documentation for viewing the DNA bands.

**Amplification of 16S r-RNA**

The 16S r-RNA gene was amplified in a Thermo Cycler (Applied Biosystem) by using these universal primers with forward and reverse in nature 5’-TGCCAGCGGCGCCAGAGTRTGATCMTYGCTWAC-3’, and 5’-TGCCAGGCGCCGCCGYTAMCTTWTTACGRCT-3’.

PCR was carried out with a final reaction volume of 50 μl in 200 μl capacity thin walled PCR tube. Composition of reaction mixture for PCR is given in Table-1. The PCR tubes containing the mixture were tapped gently and spined briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The condition for PCR is given in Table-2.

To confirm the targeted PCR amplification, 4μl of PCR product from each tube was mixed with 2μl of 6X gel loading dye. The 2% gel was constantly supplied with 50V/cm for 20 min in 1X TAE buffer. The amplified product (16S r-RNA) was visualized as a single compact band of expected size under UV light and documented by gel documentation system (Medicare, UK).

**Table-1: Composition of reaction mixture for PCR with100 μl reaction**

| Components | Quantity |
|------------|----------|
| DNA        | 1 μl (100 ng) |
| Forward primer | 0.5 μl (400 ng) |
| Reverse primer | 0.5 μl (400 ng) |
| dNTPs (10 mM each) | 4 μl |
| 10X Chrom Taq RNA Polymerase Assay Buffer | 10 μl |
| Chrom Taq RNA Polymerase Enzyme (3U/μl) 1μl and Water | 84 μl |
| Total reaction volume: | 100 μl |

**Source:** Chromous Biotech Pvt. Ltd. Bengaluru, India (Manufacturer’s protocol).

**Table-2: Steps and conditions of thermal cycling for PCR**

| Steps       | Temperature(T) | Time     | Cycles |
|-------------|----------------|----------|--------|
| Initial Denaturation | 90°C          | 5.00 min. |        |
| Final Denaturation | 90°C          | 30 seconds | 35     |
| Annealing   | 50°C           | 30 seconds |        |
| Extension   | 72°C           | 1.30 min. |        |
| Final Extension | 72°C         | 7.00 min. |        |

**Source:** Chromous Biotech Pvt. Ltd. Bengaluru, India (Manufacturer’s protocol).

**Sequencing Reaction Preparations**

Sanger sequencing was adapted in which target RNA is denatured and annealed to an oligonucleotide primer, which is then extended by RNA polymerase using a mixture of deoxynucleotide triphosphates (normal dNTPs) and chain-terminating di-deoxynucleotide triphosphates (ddNTPs). The ddNTPs lack the 3’ OH group to which the next dNTP of the growing RNA chain is added. Without the 3’ OH, no more nucleotides can be added, and RNA polymerase falls off. The resulting newly synthesized RNA chains will be a mixture of lengths, depending on how long the chain was when a ddNTP was randomly incorporated.

**Template Quantity for PCR Product**

The following are the template quantity required to yield desired number of base pair sequences, 1-3ng/ μl (100-200bp), 3-10ng/ μl (200-500bp), 5-20ng/
ml (500-1000bp), 10-40ng/ ml (1000-2000bp), 25-50ng/ml for single stranded plasmid, and 150-300 ng/ml for double stranded plasmid. In this study the desired number of base pairs was 1000-1500bp, and therefore we used template volume of 5-20 ng/ml.

**Template Pre-Heat Treatment**

The template RNA was heated at 96°C for 5 minute in ABI Thermal Cycler and cooled in ice bath immediately and stored at 4°C until use. First PCR machine was switched and the program was set. Thawed the BDT v 3.1 kit on ice and aliquot 10 μl of RR mix into sterile 0.2 ml microfuge tubes on ice and stored at -20°C. Sequencing reactions was prepared in 0.2 ml PCR thin wall tube or micro plate well by placing the tube on ice. Addition was made in the order listed in the table below and the reagents was thawed and mixed thoroughly before use. The reaction content was mixed briefly in tube/plate, covered the plate with plate seal film and centrifuged for a quick spin of 20 seconds. The plates/tubes were transferred to the PCR machine and the PCR program was started as follows (Table 3 and 4).

| Table-3: Template pre-heat treatment |
|--------------------------------------|
| **Reagent**                        | **Concentration** | **Volume** |
| Ready Reaction Premix               | 2.5 X             | 4.0 μl     |
| Big Dye Sequencing buffer           | 5.0 X             | 2.0 μl     |
| Primer                              | -                 | 3.2 pM/ μl |
| Template                            | -                 | 3.0 μl     |
| Water                               | -                 | 10.0 μl    |
| **Final Volume**                    | 1X                | 20.0 μl    |

Source: Chromous Biotech Pvt. Ltd. Bengaluru, India (Manufacturer’s protocol).

| Table-4: PCR sequencing cycling conditions |
|--------------------------------------------|
| **Process** | **Temperature** | **Time** | **Cycles** |
| Initial Denaturation | 96 °C          | 1.0 seconds | 25          |
| Denaturation               | 96 °C          | 10.0 seconds | 25          |
| Annealing                  | 50 °C          | 5.0 seconds | 25          |
| Elongation                 | 60 °C          | 4.0 min.   | --          |

Source: Chromous Biotech Pvt. Ltd. Bengaluru, India (Manufacturer’s protocol)

**Reactions Clean Up by Ethanol precipitation**

After completion of the PCR program, the sample was processed for ethanol precipitation. From PCR tube, the samples were transferred to 96 well microfibre plates and 5 μl of 125 mM EDTA was added to each well. 60 μl of ice cold 100% ethanol (from -20°C) was added to each reaction, the plate was sealed and mixed by vortexing for 20-30 seconds and incubated at room temperature for 15 minutes. The sample plate was spun at 3,000 x g for 30 minutes at 4°C. The supernatant was carefully removed by inverting the plate and spun up to 180 x g, then removed from the centrifuge. The pellet was rinsed once with 60 μl of ice cold 70% ethanol (-20°C) by centrifugation at 1650 × g for 15 minutes at 4°C. The plate was inverted and spun up to 180 × g for 1 minute, and then removed from the centrifuge. The sample was re-suspended in 10 μl of Hi-Di formamide and incubated for 15 minutes at room temperature. The re-suspended samples were transferred to the appropriate wells of the sample plate. Ensured each sample was positioned at the bottom of its tube or well. The samples were denatured at 95°C for 5 minutes with snap chill and the plate was loaded into sequencer, and after completion, the data were analyzed.

**Bioinformatics Analysis (Sequence Annotations and Statistics)**

The sequence statistical analysis was conducted by various software’s and online tools. The sequences were aligned with FASTA format, submitted to NCBI – GenBank database and authenticated. Before, the sequences were involved to found the nucleotide information, both forward and reverse sequences were merged (Contigs) with PRABI-Doua: CAP3 online tool. The sequences were subjected to basic local alignment search tool (BLAST) to find out the internal stop codon and reading frame shift. Finally, the starting codon was found for detecting the translated protein by using ORF finder. Ban kit sequence submission tool was used to submit the sequence to GenBank.

**Multiple Sequence Alignment**

Multiple sequence alignment (MSA) tool was used for aligning three or more biological sequences of similar length, generally protein, DNA, or RNA. From the output, sequence homology and evolutionary relationship between sequences was inferred.

**T-Coffee Alignment**

T-Coffee is a multiple sequence alignment package used to align sequences of protein, DNA and RNA or to combine the output of our favourite alignment methods (Clustal, Mafft, Probcons, Muscle,
etc.) into one unique alignment (M-coffee). It can also
able to combine sequence information with protein
structural information (Expresso), profile information
(PSI-Coffee) or RNA secondary structures (R-Coffee).
This multiple sequence alignment web server has been
introduced in 2011 NAR web server issue.

Multiple Align Show (MAS)

The Sequence Manipulation Suite is a
collection of web-based programs for analyzing and
formatting DNA and protein sequences. The output of
each program is a set of HTML commands, which
rendered by web browser as a standard web page.

The multiple align show (MAS) was used to
highlight the amino acid residues in the sequences. The
resulted sequences from T-coffee were uploaded in
MAS and the following parameters were selected: (i)
identical amino acid residues in amino acid colour, (ii)
similar amino acid residues in black colour, and, (iii)
variable amino acids in white colour. After selecting
these parameters, the sequences were submitted to
NCBI-GenBank, and the subsequent data was appeared
in new window.

Phylogenetic Analysis

Phylogenetic analysis is the process used to
determine the evolutionary relationship between the
organisms at the species level. The result of the analysis
was drawn in a hierarchical diagram called ‘Cladogram’
or ‘phylogram’ (phylogenetic tree). The branch of the
tree denotes the hypothesized evolutionary relationship
(phylogeny). Each member in a branch, also known as a
monophyletic group assumed to be descendants from a
common ancestor. Originally, phylogenetic tree was
created by morphological variations like sexual
dimorphism, larva/adult coloration of a given species,
and now, it is carried out using DNA sequence.

Synonymous and Non-Synonymous Substitution

Estimation of synonymous (Ks) and non-
synonymous (Ka) substitutions were calculated by Li93
method [48] of DAMBE for 3rd codon position. The maximum
likelihood (ML) analysis for the synonymous
and non-synonymous substitutions was produced by
joint reconstructions of ancestral states by Muse-Gaut
model of codon substitution and Felsenstein model of
nucleotide substitution [49].

Saturation

Analysis of sequence saturation was done by
using DAMBE V 5.3.10 [50] for calculating the
transitional and transversional substitutions against
 genetic distance (TN93). The substantial saturation of
the sequence was checked by using the method of [50,
51].

Molecular Evolutionary Genetics Analysis (MEGA
V.6)

MEGA tool was used to find out the evolutionary
relationship between the species using homologous
sequences. It is based on the statistical analysis of
genes, the percentages of conservedness, variance and
parsimony of the sequences. The distance between
groups was also estimated. The estimation was
accomplished by bootstrapping approach. The
information regarding transition and types of
substitution between the sequences were used for
inferring phylogenetics by distance based methods,
along with bootstrap test. This tool was used for
estimating evolutionary distance, constructing
phylogenetic trees, testing tree reliability, making genes
and domains, testing for selection, grouping, sequence
computing and constructing tree from distance data.
Sequences were aligned (Multiple align) using Bio Edit
and the resulted sequences were converted in to MEGA
format, which was used for reconstruction of
phylogenetic tree topology.

RESULTS AND DISCUSSION

Molecular Characterization of Probiotic, B. licheniformis (MTCC 429) and Other Gut Bacteria
of M. rosenbergii

The isolated genomic DNA from control
prawn showed greater than 10 kb size (Figure-1) and
the PCR amplified DNA for 16S rRNA gene showed
1500 bp (Figure-3). Actually the aligned sequence
showed 1516 bp for B. licheniformis sub-culture
(MTCC 429; NCBI-GenBank accession number,
MK158065 which showed 100% similarity with the
colony of B. licheniformis (1495 bp; MK955479) that
was established in the gut of M. rosenbergii, and further
showed 99% similarity with the same species which
existed in NCBI database. The aligned sequences from
the gut of control showed 1337 bp for Pseudomonas
sp., 768 bp for Klebsiella oxytoca, 1334 bp for Escherichia
coli, 1419 bp for Bacillus coagulans, 1315 bp for
Streptococcus thermophilus, 1313 bp for Staphylococcus
aureus, 1466 bp for Citrobacter koseri and 1289 bp for
Acinetobacter sp. The details of BLAST for these
sequences, the similarity data (99.9-100%) retrieved
from the NCBI database and the GenBank accession
numbers are presented in Table-5.

The isolated genomic DNA from experimental
prawn also showed greater than 10 kb size (Figure-2)
and the PCR amplified DNA for 16S rRNA gene also
showed 1500 bp (Figure-4). Here, the aligned sequence
showed 1350 bp for Bacillus sp., 1495 bp for Bacillus
licheniformis, 1464 bp for Lactobacillus plantarum,
1307 bp for Escherichia coli, 1446 bp for Streptococcus
iniae and 1347 bp for Citrobacter sp. The details of
BLAST for these sequences, the similarity data (100%)
retrieved from the NCBI database and the GenBank
accession numbers are presented in Table-6.
Fig 1: AGE (1%) shows >10 kb genomic DNA for bacterial colonies established in the gut of control prawns. L. Ladder (1kb); 1. Pseudomonas sp.; 2. Klebsiella oxytoca; 3. Escherichia coli; 4. Bacillus coagulans; 5. Streptococcus thermophilus; 6. Staphylococcus aureus; 7. Citrobacter koseri; 8. Acinetobacter sp., Source: Paper authors original work.

Fig 2: AGE (1%) shows >10 kb genomic DNA for bacterial colonies established in the gut of experimental prawns. L. Ladder (1kb); 1. Bacillus sp.; 2. Bacillus licheniformis; 3. Lactobacillus plantarum; 4. Escherichia coli; 5. Streptococcus iniae; 6. Citrobacter sp., Source: Paper authors original work.

Fig 3: AGE (2%) shows ~1500 bp of amplified product of 16S rRNA of bacterial colonies established in the gut of control prawns. L. Ladder (100 bp); 1. Pseudomonas sp.; 2. Klebsiella oxytoca; 3. Escherichia coli; 4. Bacillus coagulans; 5. Streptococcus thermophilus; 6. Staphylococcus aureus; 7. Citrobacter koseri; 8. Acinetobacter sp., Source: Paper authors original work.

Fig 4: AGE (2%) shows ~1500 bp of amplified product of 16S rRNA of bacterial colonies established in the gut of experimental prawns. L. Ladder (100 bp); 1. Bacillus sp.; 2. Bacillus licheniformis; 3. Lactobacillus plantarum; 4. Escherichia coli; 5. Streptococcus iniae; 6. Citrobacter sp., Source: Paper authors original work.
Table 5: BLAST identification of 16S rRNA gene sequences of control, and retrieved bacterial species

| Queried Sequences | Author, Country and Accession Number | I (%) | G (%) | M.S | Retrieved/Matched species | Author, Country and Accession Number |
|-------------------|--------------------------------------|-------|-------|-----|---------------------------|--------------------------------------|
| *Pseudomonas* sp., (1337 bp) | Paper authors, India MK955470 | 100 | 0 | Plus | *Pseudomonas* sp., | He, 2017 China KY927414.1 |
| *Klebsiella oxytoca* (768 bp) | Paper authors, India MK955471 | 100 | 0 | Plus | *Klebsiella oxytoca* | Jaddo, et al., 2018 (Iraq) MH295829.1 |
| *Escherichia coli* (1334 bp) | Paper authors, India MK955472 | 100 | 0 | Plus | *Escherichia coli* | Pulgar, et al., 2015 Chile K1204888.1 |
| *Bacillus coagulans* (1419 bp) | Paper authors, India MK955473 | 100 | 0 | Plus | *Bacillus coagulans* | Tanaka, et al., 2007 Japan AB362709.1 |
| *Streptococcus thermophilus* (1316 bp) | Paper authors, India MK955474 | 99.92 | 0 | Plus | *Streptococcus thermophilus* | Buszewski, et al., 2019 Poland MK681353.1 |
| *Staphylococcus aureus* (1313 bp) | Paper authors, India MK955475 | 100 | 0 | Plus | *Staphylococcus aureus* | Cairns 2017 Finland LT899939.1 |
| *Citrobacter koseri* (1466 bp) | Paper authors, India MK955476 | 100 | 0 | Plus | *Citrobacter koseri* | Boivin-Jahns, et al., 1995 France X86572.1 |
| *Acinetobacter sp.* (1289 bp) | Paper authors, India MK955477 | 100 | 0 | Plus | *Acinetobacter sp.* | |

Table 6: BLAST identification of 16r RNA gene sequences for experimental group and retrieved bacteria species and their GenBank accession numbers

| Queried sequences | Author, Country and Accession Number | I (%) | G (%) | M.S | Retrieved/Matched species | Author, Country and Accession Number |
|-------------------|--------------------------------------|-------|-------|-----|---------------------------|--------------------------------------|
| *Bacillus* sp., (1350 bp) | Paper authors, India MK955478 | 100 | 0 | Plus | *Bacillus* sp | Lu, 2017 China MG309335.1 |
| *Bacillus licheniformis* (1495 bp) | Paper authors, India MK955479 | 100 | 0 | Plus | *Bacillus licheniformis* | Aboelnaga, et al., 2018 Egypt MK028349.1 |
| *Lactobacillus plantarum* (1464 bp) | Paper authors, India MK955480 | 100 | 0 | Plus | *Lactobacillus plantarum* | Zhang, 2018 China MH016559.1 |
| *Escherichia coli* (1307 bp) | Paper authors, India MN121246 | 100 | 0 | Plus | *Escherichia coli* | Mandakovic, et al., 2015 Chile K1204888.1 |
| *Streptococcus iniae* (1446 bp) | Paper authors, India MN121247 | 100 | 0 | Plus | *Streptococcus iniae* | Hoshino, et al., 2008 Japan AB470235.1 |
| *Citrobacter sp.* (1347 bp) | Paper authors, India MN121248 | 100 | 0 | Plus | *Citrobacter sp.* | Haque, et al., 2019 Bangladesh MK695712.1 |

The MAS showed 460 identical amino acid residues, 79 similar amino acid residues and 308 variable amino acid sites for control prawns (Figure-7), and 879 identical amino acid residues, 85 similar amino acid residues and 396 variable amino acid sites for experiment prawns, which are presented in Figure-8.

The Sequence Manipulation Suite: Multiple Align Show
The nucleotide composition showed more GC biases, 50.6-56.4% (Staphylococcus aureus and Bacillus coagulans) and less AT biases, 43.6-49.4% (Bacillus coagulans and Staphylococcus aureus) in the control prawns (Table-7). In the experimental prawns as well there were more GC biases, 51.2-55.7% (Lactobacillus plantarum and Bacillus licheniformis) and less AT biases, 44.3-48.8% (Bacillus licheniformis and Lactobacillus plantarum) (Table-8). The lower AT biases recorded in control and experimental prawns indicate the less abundance of nuclear copies of mtDNA (NUMTs) known as pseudogenes, homologs or paralogs.
Table 7: 16S r-RNA nucleotide composition of bacterial species identified in the gut of *M. rosenbergii* PL fed with control diet

| Prawn category | Bacterial Species           | A   | T   | AT  | C   | G   | GC  |
|----------------|-----------------------------|-----|-----|-----|-----|-----|-----|
| *M. rosenbergii* PL fed with control diet | *Pseudomonas sp.* | 25.5 | 20.8 | 46.3 | 22.5 | 31.2 | 53.7 |
|         | *Klebsiella oxytoca*       | 24.1 | 20.4 | 44.5 | 22.9 | 32.6 | 55.5 |
|         | *Escherichia coli*         | 24.7 | 20.0 | 44.8 | 23.3 | 31.9 | 55.2 |
|         | *Bacillus coagulans*       | 24.4 | 19.2 | 43.6 | 24.8 | 31.6 | 56.4 |
|         | *Streptococcus thermophilus* | 25.5 | 22.1 | 47.5 | 22.0 | 30.5 | 52.5 |
|         | *Staphylococcus aureus*    | 27.3 | 22.1 | 49.4 | 21.7 | 28.9 | 50.6 |
|         | *Citrobacter koseri*       | 25.9 | 20.9 | 46.8 | 21.8 | 31.4 | 53.2 |
|         | *Acinetobacter sp.*        | 25.8 | 21.7 | 47.6 | 21.6 | 30.9 | 52.4 |
| **Average** |                              | **25.4** | **20.9** | **46.3** | **22.5** | **31.1** | **53.6** |

Source: Paper authors original work.

Table 8: 16S r-RNA nucleotide composition of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. licheniformis* incorporated diet

| Prawn category | Bacterial Species           | A   | T   | AT  | C   | G   | GC  |
|----------------|-----------------------------|-----|-----|-----|-----|-----|-----|
| *M. rosenbergii* PL fed with *B. licheniformis* incorporated diet | *Bacillus sp.* | 25.3 | 19.6 | 44.9 | 24.2 | 30.9 | 55.1 |
|         | *Bacillus licheniformis*    | 24.5 | 19.8 | 44.3 | 24.1 | 31.5 | 55.7 |
|         | *Lactobacillus plantarum*   | 26.5 | 22.3 | 48.8 | 21.9 | 29.4 | 51.2 |
|         | *Escherichia coli*          | 24.7 | 20.2 | 44.9 | 23.0 | 32.1 | 55.1 |
|         | *Streptococcus iniae*       | 26.5 | 21.8 | 48.3 | 21.8 | 29.9 | 51.7 |
|         | *Citrobacter sp.*           | 25.4 | 19.8 | 45.2 | 22.9 | 31.8 | 54.8 |
| **Average** |                              | **25.5** | **20.6** | **46.1** | **23.0** | **30.9** | **53.9** |

Source: Paper authors original work.

The inter species divergence of control prawns was lower (0.059) between *Klebsiella oxytoca* vs. *Escherichia coli* and higher (0.366) between *Streptococcus thermophilus* vs. *Acinetobacter sp.* (Table 9). The inter species divergence of experiment prawns was lower (0.032) *Citrobacter sp.*, vs. *Escherichia coli* and high (0.345) between *Citrobacter sp.*, vs. *Streptococcus iniae* (Table 10). These results indicated very close relationship between the species.

Table 9: 16S r-RNA nucleotide divergence of bacterial species identified in the gut of control prawns

| Between Species | Divergence (%) |
|-----------------|----------------|
| *Pseudomonas sp.*, vs. *Klebsiella oxytoca* | 0.206 |
| *Pseudomonas sp.*, vs. *Escherichia coli* | 0.174 |
| *Klebsiella oxytoca* vs. *Escherichia coli* | 0.059 |
| *Pseudomonas sp.*, vs. *Bacillus coagulans* | 0.310 |
| *Klebsiella oxytoca* vs. *Bacillus coagulans* | 0.335 |
| *Escherichia coli* vs. *Bacillus coagulans* | 0.306 |
| *Pseudomonas sp.*, vs. *Streptococcus thermophilus* | 0.299 |
| *Klebsiella oxytoca* vs. *Streptococcus thermophilus* | 0.358 |
| *Escherichia coli* vs. *Streptococcus thermophilus* | 0.320 |
| *Bacillus coagulans* vs. *Streptococcus thermophilus* | 0.205 |
| *Pseudomonas sp.*, vs. *Staphylococcus aureus* | 0.303 |
| *Klebsiella oxytoca* vs. *Staphylococcus aureus* | 0.360 |
| *Escherichia coli* vs. *Staphylococcus aureus* | 0.323 |
| *Bacillus coagulans* vs. *Staphylococcus aureus* | 0.120 |
| *Streptococcus thermophilus* vs. *Staphylococcus aureus* | 0.191 |
| *Pseudomonas sp.*, vs. *Citrobacter koseri* | 0.187 |
| *Klebsiella oxytoca* vs. *Citrobacter koseri* | 0.112 |
| *Escherichia coli* vs. *Citrobacter koseri* | 0.083 |
| *Bacillus coagulans* vs. *Citrobacter koseri* | 0.315 |
| *Streptococcus thermophilus* vs. *Citrobacter koseri* | 0.342 |
| *Staphylococcus aureus* vs. *Citrobacter koseri* | 0.337 |
| *Pseudomonas sp.*, vs. *Acinetobacter sp.* | 0.144 |
| *Klebsiella oxytoca* vs. *Acinetobacter sp.* | 0.227 |
| *Escherichia coli* vs. *Acinetobacter sp.* | 0.197 |
| *Bacillus coagulans* vs. *Acinetobacter sp.* | 0.317 |
| *Streptococcus thermophilus* vs. *Acinetobacter sp.* | 0.366 |
| *Staphylococcus aureus* vs. *Acinetobacter sp.* | 0.342 |
| *Citrobacter koseri* vs. *Acinetobacter sp.* | 0.162 |
Table-10: 16S r-RNA nucleotide divergences of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. licheniformis* incorporated diet

| Between Species | Divergence (%) |
|-----------------|----------------|
| *Bacillus licheniformis* vs. *Bacillus* sp. | 0.044 |
| *Bacillus licheniformis* vs. *Citrobacter* sp. | 0.334 |
| *Bacillus* sp., vs. *Citrobacter* sp. | 0.319 |
| *Bacillus licheniformis* vs. *Escherichia coli* | 0.327 |
| *Bacillus* sp., vs. *Escherichia coli* | 0.315 |
| *Citrobacter* sp., vs. *Escherichia coli* | 0.032 |
| *Bacillus licheniformis* vs. *Lactobacillus plantarum* | 0.150 |
| *Bacillus* sp., vs. *Lactobacillus plantarum* | 0.138 |
| *Citrobacter* sp., vs. *Lactobacillus plantarum* | 0.334 |
| *Escherichia coli* vs. *Lactobacillus plantarum* | 0.339 |
| *Bacillus licheniformis* vs. *Streptococcus iniae* | 0.177 |
| *Bacillus* sp., vs. *Streptococcus iniae* | 0.165 |
| *Citrobacter* sp., vs. *Streptococcus iniae* | 0.345 |
| *Escherichia coli* vs. *Streptococcus iniae* | 0.336 |
| *Lactobacillus plantarum* vs. *Streptococcus iniae* | 0.183 |

**Phylogenetic Relationship of Gut Bacterial Consortium of *M. rosenbergii***

In the control prawns, the non-synonymous (Ka) substitution was higher (2.413) than that of synonymous (Ks) substitution (0.751), which indicates the possibility of occurrence of more deleterious mutation and less silent mutation. Similarly, the transversional (Tv) substitution was higher (0.16) than that of transitional (Ts) (0.13), which indicates the fact that these sequences have more phylogenetic information. However, saturation had not occurred in these sequences, which was confirmed by the predicted higher critical value of index of substitutional saturation (Iss.c), 0.716 than that of index of substitution saturation (Iss), 0.338, and therefore more phylogenetic differences existed between sequences (Table 11; Figure 9). The same trend was observed in the experimental prawns as well (Ka = 0.879, Ks = 2.457, Tv = 0.35, Ts = 0.25 Iss.c = 0.889, Iss = 0.448) (Table-11 & Figure-10).

Table-11: Phylogenetic information of bacterial species identified (based on 16S r-RNA) in the gut of *M. rosenbergii* PL fed with *B. licheniformis* incorporated diet

| Phylogenetic information | Ks  | Ka  | Ka-Ks | Ts  | Tv  | Tv-Ts | Iss  | Iss.c | Iss.c - Iss |
|--------------------------|-----|-----|-------|-----|-----|-------|------|-------|------------|
| Control                  | 0.751 | 2.413 | 1.662 | 0.13 | 0.16 | 0.03  | 0.338 | 0.716 | 0.378      |
| Experimental             | 0.879 | 2.457 | 1.578 | 0.25 | 0.35 | 0.1   | 0.448 | 0.889 | 0.441      |

A. Number of synonymous (Ks) and non-synonymous (Ka) substitutions occurred at 3rd codon position in the nucleotides of 16S r-RNA gene partial sequences of control prawns
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B. Scattergram of transitional (X, blue) and transversional (Δ, green) type substitutions occurred in 16S r-RNA gene partial sequences of bacterial diversity identified in the gut of control prawns

Fig-9: Synonymous (Ks) and non-synonymous (Ka), transitional (X, blue) and transversional (Δ, green) substitutions occurred in 16S r-RNA partial gene sequences for control prawns

A. Number of synonymous (Ks) and non-synonymous (Ka) substitutions occurred at 3rd codon position in the nucleotides of 16S r-RNA gene partial sequences of experimental prawns

B. Scattergram of transitional (X, blue) and transversional (Δ, green) type substitutions occurred in 16S r-RNA gene partial sequences of bacterial diversity identified in the gut of M. rosenbergii PL fed with B. lichiformis incorporated diet

Fig-10: Synonymous (Ks) and non-synonymous (Ka), transitional (X, blue) and transversional (Δ, green) substitutions occurred in 16S r-RNA partial gene sequences for experimental prawns

The phylogenetic tree topology of bacterial species identified in the gut of control prawns shows three clusters. The first cluster was formed by S. aureus and B. coagulans as sister taxa with bootstrap value of 100, and S. thermophilus was sat alone. The second cluster was formed by Acinetobacter sp., and Pseudomonas sp., as sister taxa with bootstrap value of 57. The third cluster was formed by C. koseri as a lone clade, and E. coli and K. oxytoca were aligned as sister taxa at the top of the tree with bootstrap value of 100 (Figure-11).
The phylogenetic tree for the experimental prawns shows two major clusters. The first cluster at bottom of the tree was with *Citrobacter* sp., and *E. coli* aligned as a sister taxa with bootstrap value of 100. The second major cluster includes 2 clades and a cluster. *S. iniae* and *L. plantarum* were separately aligned as distinct clades, and *B. licheniformis* and *Bacillus* sp., were aligned as a sister taxa with bootstrap value of 100 (Figure-12).

When the bacterial species identified in the gut of both control and experimental prawns were pooled together, the phylogenetic tree topology appears with two major clusters, each of which was with five clades. In the first cluster, the identified *Bacillus* sp. aligned at the bottom of the tree as a sister clade with *B. coagulan*. The remaining species, such as *B. licheniformis*, *S. aureus* and *L. plantarum* were aligned in three separate clades. *S. iniae* of experimental prawns and *S. thermophiles* of control prawns aligned as a sister taxa at the top of the first cluster (Figure-13).

The second cluster, there were three separate clades with *Pseudomonas* sp., *Acinetobacter* sp., and *C. koseri*, respectively. There were two sister taxa, first with *E. coli* of control and experimental prawns, and second sister taxa was formed by *Citrobacter* sp., of experimental prawns and *K. oxytoca* of control prawns with bootstrap value of 64. In other way *Citrobacter*, *E. coli* and *Klebsiella* of both control and experimental prawns formed a separate cluster at top of the tree. Therefore, species with same genus aligned phylogenetically very close (Figure-13).
Microbial identification in animal husbandry and agriculture is meaningful only when microbiota can be classified to the level of genus or species. The classification of 35–40% of the reads to a genus or species is considered as a successful result, considering that only a small fraction of all 16S gene sequences belonging to uncultivable bacteria are registered in databases [52, 53]. Similarly, the metagenomic analyses using 16S r-DNA genes against *Lactobacillus*<sup>® plus</sup> and *Vibact*<sup>®</sup> supplemented diets fed *M. rosenbergii*, the colonies established were unknown Proteobacteria of 1355 bp and 1760 bp respectively and have been reported to match and confirmed with genus *Ralstonia* and *Comamonas* respectively [54, 55, 20, 21]. In the present study, the identified bacterial species using 16S r-RNA sequences showed 99-100% similarities with the database available with GenBank. Therefore, 16S r-RNA sequence analyses were accurate here.

**CONCLUSION**

The bacterial diversity identified using 16S r-RNA revealed presence of eight dominant bacteria in the control prawn gut. They were *Pseudomonas*<sup>sp.</sup>, *Klebsiella oxytoca*, *E. coli*, *Bacillus coagulans*, *Streptococcus thermophilus*, *Staphylococcus aureus*, *Citrobacter koseri* and *Acinetobacter*<sup>sp.</sup>. In the case of experimental prawn gut, the presence of six dominant bacteria, such as *Bacillus*<sup>sp.</sup>, *Bacillus licheniformis*, *Lactobacillus plantarum*, *E. coli*, *Streptococcus iniae* and *Citrobacter*<sup>sp.</sup> were identified. This indicated the fact that the pathogenic bacteria *Pseudomonas*<sup>sp.</sup>, *Klebsiella*<sup>sp.</sup>, *Staphylococcus*<sup>sp.</sup> and *Acinetobacter*<sup>sp.</sup> were competitively excluded by the establishment of *B. licheniformis* colony. Therefore, adhesion and colonization of the probiotic bacterium, *B. licheniformis* in the gut of *M. rosenbergii* has accurately been identified using 16S r-RNA gene sequencing.

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**CONFLICT OF INTEREST**

The authors have no conflict of interest.

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