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Original Research Article

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, $935x10^{-6}$). 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 indentified 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 finfish, 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 *Penaeus 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. amyloliquefaciens* 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^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10}). 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, *Pesudomonas* 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-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

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'-TGCCAGGCGGCCGCAGAGTRTGATCMTYGCTW AC-3, and 5'-TGCCAGGCGGCCGCCGYTAMCTTWTTACGRCT-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	1µl (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/

µl (500-1000bp), 10-40ng/ µl (1000-2000bp), 25-50ng/µl for single stranded plasmid, and 150-300 ng/µl 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/ µl.

Template Pre-Heat Treatment

The template RNA was heated at 96ºC for 5 minute in ABI Thermal Cycler and cooled in ice bath immediately and stored at 4ºC 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 -20ºC. 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 ºC	1.0 seconds	25
Denaturation	96 ºC	10.0 seconds	25
Annealing	50 ºC	5.0 seconds	25
Elongation	60 ºC	4.0 min.	--

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

Reactions Clean Up by Ethanolic Precipitation

After completion of the PCR program, the sample was processed for ethanolic precipitation. From PCR tube, the samples were transferred to 96 well microlitre plates and 5 µl of 125 mM EDTA was added to each well. 60 µl of ice cold 100% ethanol (from -20ºC) 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 × g for 30 minutes at 4ºC. The supernatant was carefully removed by inverting the plate and spun up to 180 × g, then removed from the centrifuge. The pellet was rinsed once with 60 µl of ice cold 70% ethanol (-20ºC) by centrifugation at 1650 × g for 15 minutes at 4ºC. 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 95ºC 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, larvae/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 transvertional 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.

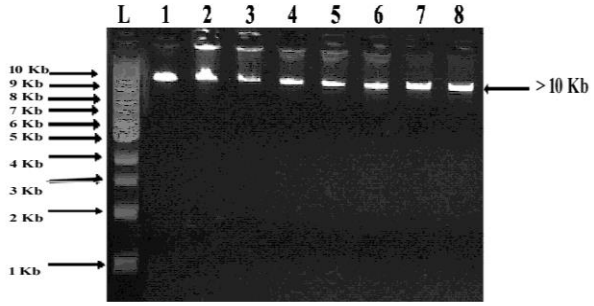


Fig-1: AGE (1%) shows >10 kb genomic DNA for bacterial colonies established in the gut of control prawns. L. Ladder (1kb); 1. *Pseudomonas* sp.; 2. *Klebsiella oxytoca*; 3. *Escherichia coli*; 4. *Bacillus coagulans*; 5. *Streptococcus thermophilus*; 6. *Staphylococcus aureus*; 7. *Citrobacter koseri*; 8. *Acinetobacter* sp.,
Source: Paper authors original work.

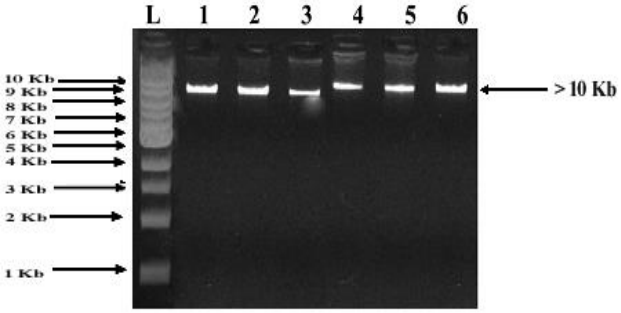


Fig-2: AGE (1%) shows >10 kb genomic DNA for bacterial colonies established in the gut of experimental prawns. L. Ladder (1kb); 1. *Bacillus* sp.; 2. *Bacillus licheniformis*; 3. *Lactobacillus plantarum*; 4. *Escherichia coli*; 5. *Streptococcus iniae*; 6. *Citrobacter* sp.,
Source: Paper authors original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

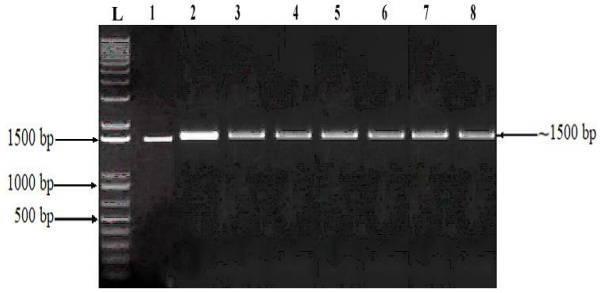


Fig-3: AGE (2%) shows ~1500 bp of amplified product of 16S rRNA of bacterial colonies established in the gut of control prawns. L. Ladder (100 bp); 1. *Pseudomonas* sp.,; 2. *Klebsiella oxytoca*; 3. *Escherichia coli*; 4. *Bacillus coagulans*; 5. *Streptococcus thermophilus*; 6. *Staphylococcus aureus*;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

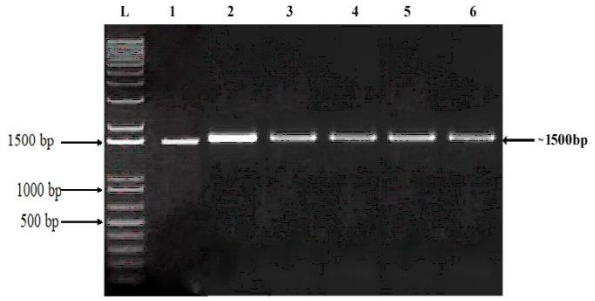


Fig-4: AGE (2%) shows ~1500 bp of amplified product of 16S rRNA of bacterial colonies established in the gut of experimental prawns. L. Ladder (100 bp); 1. *Bacillus* sp.; 2. *Bacillus licheniformis*; 3. *Lactobacillus plantarum*; 4. *Escherichia coli*; 5. *Streptococcus iniae*; 6. *Citrobacter* sp.,
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 Chille KU204888.1
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*	Mo, *et al*., 2018 Switerland Mg755342.1
Staphylococcus aureus (1313 bp)	Paper authors, India MK955475	100	0	Plus	*Staphylococcus aureus*	Buszewski, *et al*., 2019 Poland MK681353.1
Citrobacter koseri (1466 bp)	Paper authors, India MK955476	100	0	Plus	*Citrobacter koseri*	Cairns 2017 Finland LT899939.1
Acinetobacter sp., (1289 bp)	Paper authors, India MK955477	100	0	Plus	*Acinetobacter* sp.,	Boivin-Jahns, *et al*., 1995 France X86572.1

I: Identity; G: Gap; M.S: Matched strand

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 Egypth 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 KU204888.1
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

I: Identity; G: Gap; M.S: Matched strand

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

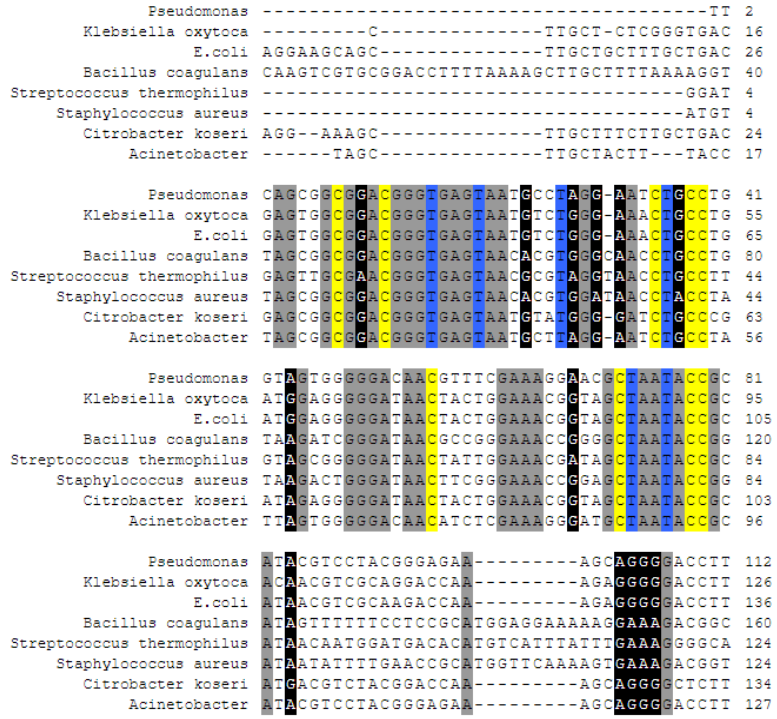


Fig-7: Multiple sequence alignment of 16S r-RNA gene sequences of bacterial species identified in the gut *M. rosenbergii* PL fed with control diet. Multiple align show (MAS) with coloured background (identical residues are indicated by amino acid colour and similar residues are black in colour. Gaps and other residues are given in white background)

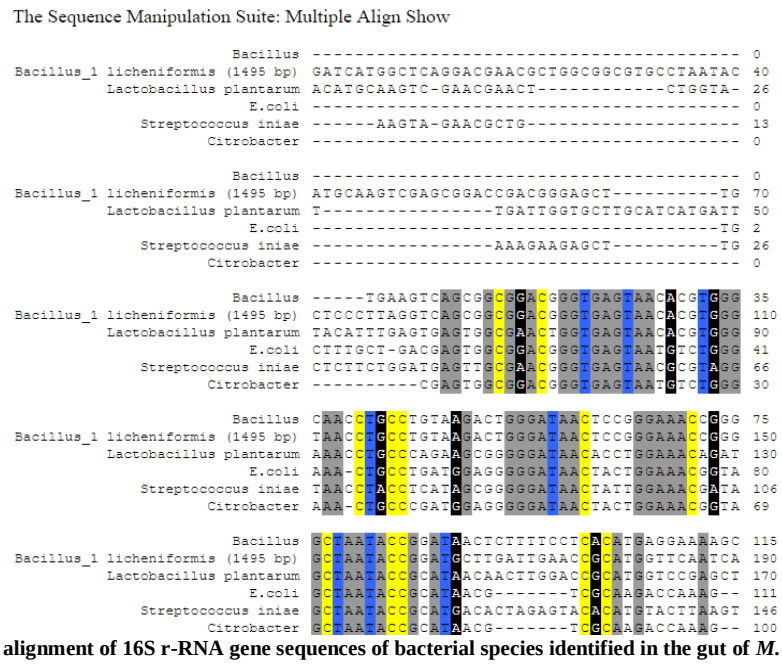


Fig-8: Multiple sequence alignment of 16S r-RNA gene sequences of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. licheniformis* incorporated diet. Multiple align show (MAS) with coloured background (identical residues are indicated by amino acid colour and similar residues are black in colour. Gaps and other residues are given in white background)

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 mt-DNA (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

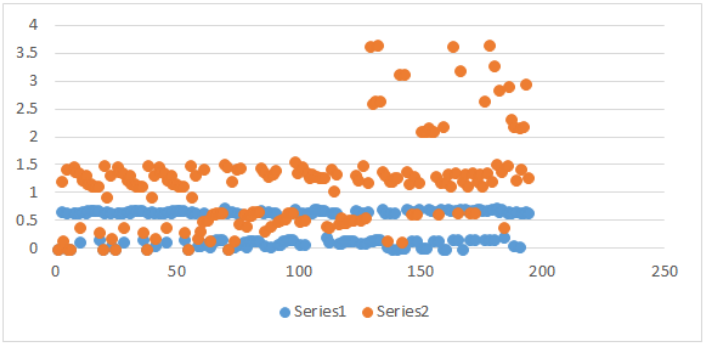
Between Species	Divergence (%)
Bacillus licheniformis vs. Bacillus sp.,	0.044
Bacillus licheniformis vs.Citrobacter sp.,	0.334
Bacillus sp., vs. *Citrobacter* sp	0.319
Bacillus licheniformis vs. Escherichia coli	0.327
Bacillus sp., vs. *Escherichia coli*	0.315
Citrobacter sp., vs. *Escherichia coli*	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*

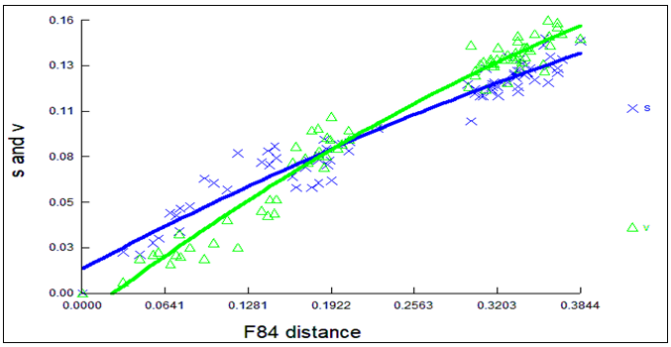
In the control prawns, the non-synonymous (Ka) substitution was higher (2.413) than that of synonymous (Ks) substitution (0.751), which indicates the possibility of occurrence of more deleterious mutation and less silent mutation. Similarly, the transversional (Tv) substitution was higher (0.16) than 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

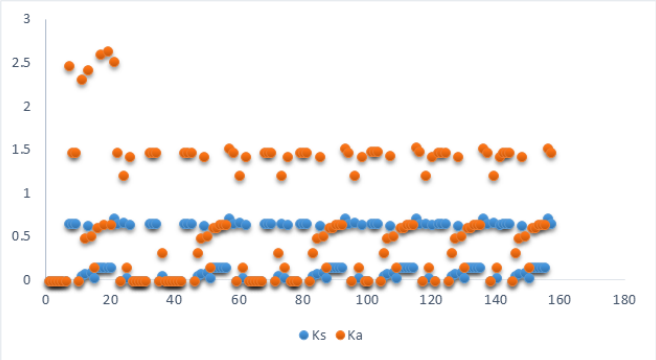


A. Number of synonymous (Ks) and non-synonymous (Ka) substitutions occurred at 3rd codon position in the nucleotides of 16S r-RNA gene partial sequences of control prawns

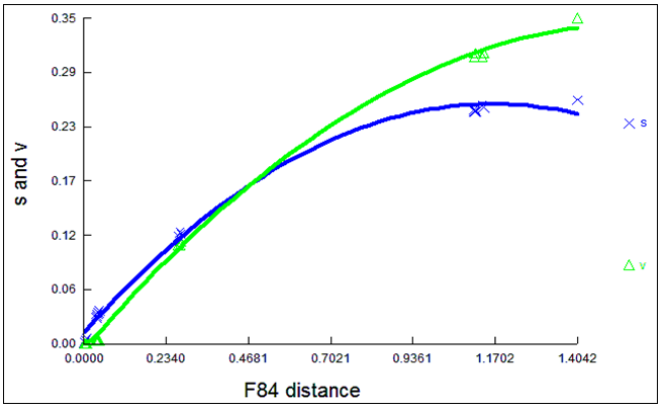


B. Scattergram of transitional (X, blue) and transversional (Δ, green) type substitutions occurred in 16S r-RNA gene partial sequences of bacterial diversity identified in the gut of control prawns

Fig-9: Synonymous (Ks) and non-synonymous (Ka), transitional (X, blue) and transversional (Δ, green) substitutions occurred in16S 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 in16S 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).

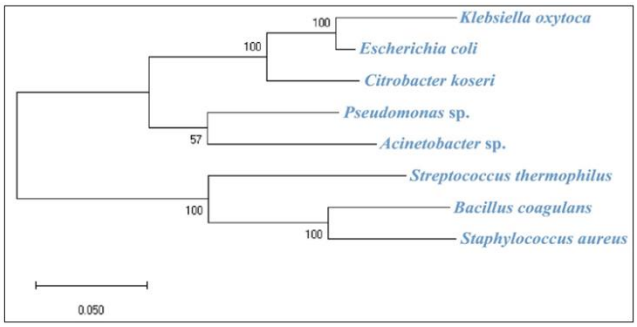


Fig-11: 16S r-RNA based phylogenetic tree topology of bacterial species identified in the gut of control prawns

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).

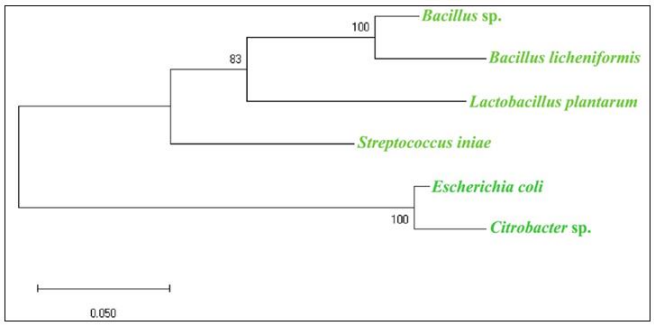


Fig-12: 16S r-RNA based phylogenetic tree topology of bacterial species identified in the gut of *M. rosenbergii* PL fed with B. *licheniformis* incorporated diet

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. inae* 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).

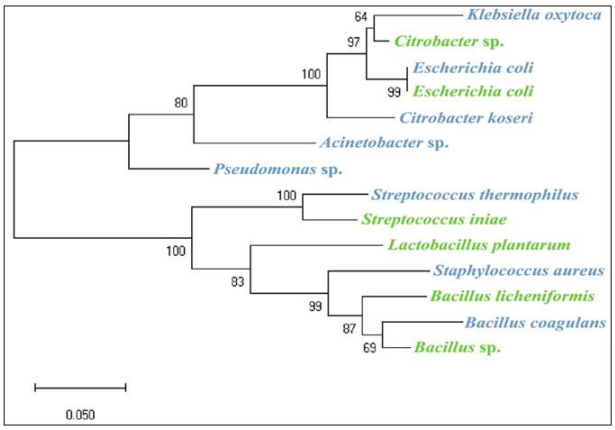


Fig-13: 16S r-RNA based phylogenetic tree topology of bacterial species identified in the gut of *M. rosenbergii* PL fed with control diet and *B. licheniformis* incorporated diet

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 LactoBacil® plus and ViBact* 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* sp., *Klebsiella oxytoca*, *E. coli*, *Bacillus coagulans*, *Streptococcus thermophilus*, *Staphylococcus aureus*, *Citrobacter koseri* and *Acinetobacter* sp. In the case of experimental prawn gut, the presence of six dominant bacteria, such as *Bacillus* sp., *Bacillus licheniformis*, *Lactobacillus plantarum*, *E. coli*, *Streptococcus iniae* and *Citrobacter* sp., were identified. This indicated the fact that the pathogenic bacteria *Pseudomonas* sp., *Klebsiella* sp., *Staphylococcus* sp., and *Acinetobacter* sp., were competitively been 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 using16S r-RNA gene sequencing.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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