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MOLECULAR IDENTIFICATION OF GUT MICROFLORA OF *Bacillus coagulans* SUPPLEMENTED FEED FED *Macrobrachium rosenbergii* POST-LARVAE USING 16S rRNA

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ABSTRACT

Probiotics yield numerous health benefits to the host. In this way the probiotic bacterium, *Bacillus coagulans* has competitively been excluded the pathogenic bacteria, *Streptococcus* spp., and *Klebsiella* spp., from the gut of *Macrobrachium rosenbergii* post-larvae when given through feed (Manjula *et al.*, 2018). The elimination of these two pathogenic bacteria indicated the fact that *B. coagulans* improved the disease resistance capacity of *M. rosenbergii.* The present paper deals with 16S rRNA sequence analysis of gut microbial diversity of *M. rosenbergii* fed with *B. coagulans* (CFU, $2.28x10^{-7}$) supplemented feed, which revealed the presence of *B. coagulans* (1541 bp), *Lactobacillus fermentum* (1516 bp), *Lactobacillus lactis* (1499 bp), *Lactobacillus acidophilus* (1507 bp), *Escherichia coli* (1465 bp) and *Staphylococcus aureus* (1476 bp). The BLAST of these sequences showed almost 100% similarities with the same species retrieved from the NCBI database. The MAS showed 920 identical amino acids residues, 119 similar amino acids residues and 461 variable amino acids sites. The nucleotide sequence divergence and the calculated phylogenetic information clearly discriminate these bacterial species.

Contribution/Originality: This study indicates that the probiotic, *Bacillus coagulans* used as a feed supplement helped in the exclusion of two pathogenic bacteria *Streptococcus* spp., and *Klebsiella* spp., from the gut of *Macrobrachium rosenbergii.* The gut microflora identified have been barcoded and authenticated.

1. INTRODUCTION

The outbreak of bacterial diseases in marine shrimp, led to shift of prawn culture towards freshwater side. In India, the freshwater prawn particularly, *Macrobrachium rosenbergii* (De Man) has great potential as a candidate species for culture due to its fast growth and better environmental tolerance. It has high economic value and consumer acceptability due to its nutritious delicacy, protein rich, low cholesterol and palatable taste (Dayal *et al.*, 2013; Maliwat *et al.*, 2016; FAO, 2018).

In aquaculture, the addition of probiotics would be an integral component of the host and helpful for maintaining the gut bacterial balance, as they act as natural immune enhancers, which provoke the disease resistance by neutralizing the colony establishment of pathogenic bacteria by competitive exclusion (Wu *et al.*, 2010;

Sakkaravarthi *et al*., 2011; Zokaeifar *et al*., 2012; Hassaan *et al*., 2014; Chen *et al*., 2017; Ramirez and Romero, 2017; Le *et al*., 2018; Zoqratt *et al*., 2018). Some studies have been confirmed that the intestinal microbiota (*Photobacterium*, *Aeromonas*, *Vibrio*, *Xanthomonas*, *Enterobacter*, *Bacillus* sp., *B. licheniformis Pediococcus*, *Agrobacterium*, *Corynebacterium*, *Alcaligenes*, *Flavobacterium*, *Pseudomonadaceae* and *Chromobacterium*) act as probiotics and showed antimicrobial activity against some pathogenic bacteria (*Vibrio harveyi*, *Vibrio fischeri*, *E. coli*, *S. aureus*, *Pseudomonas fluorescens*) in aquaculture animals, such as the shrimp, *Litopenaeus vannamei* (Gullian *et al*., 2004; Luis-Villaseñor *et al*., 2015; Sha *et al*., 2016; Vieira *et al*., 2016; Zhang *et al*., 2016; Vargas-Albores *et al*., 2017; Li *et al*., 2018). *Bacillus* S11 has competitively excluded *V. harveyi* in *Penaeus monodon* (Rengpipat *et al*., 2000). *L. acidophilus* worked well against *Vibrio parahaemolyticus*, *Vibrio cholerae*, *V. harveyi* and *Vibrio alginolyticus* in *P. monodo* (Sivakumar et al., 2012). Total heterotrophic bacteria increases, soil quality, water quality and production of *P. monodon* by reducing pathogenic green *Vibrio* and luminous *Vibrio* (Hasan *et al*., 2012). It has been reported that *Lactobasillus* strains, *Enterococcus faecalis* LS1-2 and *Enterococcus faecium* Z1-2, having significant antimicrobial activities against shrimp pathogens (Cui *et al*., 2017). The probiotic organisms *Bacillus subtilis*, *Lactobacillus plantarum*, *P. fluorescens* and *Staphylococcus lactis* isolated from commercial shrimp feed, shrimp gut, water and seaweed showed *in vitro* antimicrobial activity against shrimp pathogens like *V. parahaemolyticus*, *Aeromonas hydrophila*, *V. cholera* and *V. harveyi* (Chelladurai *et al*., 2015). *Paenibacillus* spp., and *Bacillus cereus*, inhibits *Vibrio* spp., in shrimp intestine *P. monodon* (Ravi et al., 2007).

Many studies revealed the beneficial effects of probiotic bacteria on aquaculture animals for improving the health and performance as antimicrobial agents and growth promoters (Deeseenthum *et al*., 2007; Nayak, 2010; Van Hai and Fotedar, 2010; Olmos *et al*., 2011; Mohapatra *et al*., 2012; Lakshmi *et al*., 2013; Korada and Yarla, 2014; Ghosh *et al*., 2016; Prasad and Reddy, 2016; Vieira *et al*., 2016; Das *et al*., 2017). Probiotics enhanced immunity in the form of haemocytes, phagocytic activity and phenoloxidase activity in *L. vannamei* against *V. harveyi* infection (Arisa *et al*., 2015). *Lactobacillus bulgaricus* E20 has enhanced survival and growth of *L. vannamei* and developed immunity in the form of haemocytes, phenoloxidase activity, respiratory burst against *V. parahaemolyticus* (Roomiani *et al*., 2018). *Bacillus subtilis* isolated from mangrove cockle, *Anadara tuberculosa* served as probiotic *in L. vannamei*, improved growth and cellular immune response (Sánchez-Ortiz *et al*., 2015). *Bacillus licheniformis* and *Lactobacillus rhamnosus* improved the growth of *L. vannamei* (Swapna et al., 2015). Even probiotics have been appreciated for its beneficial effects on the host, including anti-allergic and anti-inflammatory effects. Tropomyosin (Tm) is the predominant allergic protein in shellfish. The probiotic, *Bifidobacterium infantis* 14.518 (Binf) effectively suppressed Tm-induced allergic response in a mouse model by both preventive and therapeutic strategies (Fu *et al*., 2017).

Most of the probiotic strains (*Pseudoalteromonas*, *B. cereus*, *Bacillus* sp., *B. subtilis*, *Bacillus thuringiensis*, *Bacillus endophyticus* YC3-b, *B. endophyticus* C2-2 and *Bacillus tequilensis* YC5-2, *Bacillus firmus*, *Lactobacillus*, *Lactobacillus plantarum*, *Rhodobacter capsulatus*, *Photobacterium damselae*, *V. alginolyticus*) isolated from shrimp gut have produced digestive enzymes (proteases, pepsin, amylases, lipases, esterases and chitinases (Yang *et al*., 2015). *Rhodosporidium paludigenum*, red yeast produces pigmentation and reduces oxidative stress in aquatic animals (Scholz *et al*., 1999). Pigmented *Bacillus* produced carotenoids and free-radical scavenging activity (Ngo *et al*., 2016). *Streptomyces* and *Bacillus* have been combined and used as an excellent producer of antibiotics in aquaculture as a probiotic agent (Das *et al*., 2006; Bernal *et al*., 2017).

The gut microbiota of an organism is based on its surrounding environment and health status (Tang *et al*., 2014; Hou *et al*., 2016; Zhang *et al*., 2016; Chen *et al*., 2017; Zeng *et al*., 2017). 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 (Rungrassamee *et al*., 2014; Yan *et al*., 2016; Li *et al*., 2017; Yao *et al*., 2018). Studies on the gut microbiota of *M. rosenbergii* are very limited except few (Zarif and Azin, 2014; Bhavani *et al*., 2015; Karthik and Saravana, 2018; Karthik *et al*., 2018; Manjula *et al*., 2018). Therefore in order to understand its

relationship with host, it is necessary to identify the composition of gut microbiota. The phylogeny of any organism is based on morphological data, and sequences of DNA, RNA or protein. In this study the diversity of gut microbiota of *M. rosenbergii* PL fed with *B. coagulans* (**CFU, 2.28x10^{-7}**) supplemented diet was analysed through sequencing of 16S rRNA gene, which can generate reliable and sufficient information either at genes or species level.

2. MATERIALS AND METHODS

Previously the prawn, *Macrobrachium rosenbergii* post larvae (PL) were supplemented with five different serially diluted concentrations (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}) of the probiotic bacterium, *Bacillus coagulans*. At 10^{-7} (CFU, 2.28x10^{-7}), the presence of *Streptococcus* spp., *Klebsiella* spp., *E. coli* and *Staphylococcus* spp., were recorded in control PL. In the experimental PL, *Bacillus* spp., *Lactobacillus* spp., *E. coli* and *Staphylococcus* spp., were recorded. This revealed that the pathogenic bacteria, *Streptococcus* spp., and *Klebsiella* spp., were found to have competitively been excluded (Manjula *et al*., 2018). Therefore, the gut bacterial diversity of experimental PL fed with *B. coagulans* supplemented feed were subjected to molecular analysis in the present paper.

2.1. Molecular Analysis

2.1.1. 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* and *Staphylococcus* 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). 20 µl of Proteinase K 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 (Sambrook *et al*., 1989).

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 100µl of TE buffer and stored at -20°C for future usage, or at -80°C for long preservation.

2.1.2. 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.

2.1.3. Amplification of 16S r-RNA

The 16S r-RNA gene was amplification in Applied Biosystem Thermo Cycler by using the universal primers with forward and reverse in nature (5'-TGCCAGGCGGCCGCAGAGTRTGATC**M**T**Y**GCT**W**AC-3', and 5'-TGCCAGGCGGCCGCCG**Y**TA**M**CTT**W**TTACG**R**CT-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 rRNA) 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 with 100 µl reaction.

Components	Quantity
DNA	1 µl (100ng)
Forward primer	400ng
Reverse primer	400ng
dNTPs (10mM each)	4µl
10X Chrom Taq RNA Polymerase Assay Buffer	10 µl
Chrom Taq RNA Polymerase Enzyme (3U/µl) 1 µl and Water	93 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	05.00 m	
Final Denaturation	90°C	00.30 m	35
Annealing	50°C	00.30 m	
Extension	72°C	01.30 m	
Final Extension	72°C	7.00 m	

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

2.2. 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). 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

2.2.1. Template Quantity for PCR Product

100-200bp (1-3ng/ µl), 200-500bp (3-10ng/ µl), 500-1000bp (5-20ng/ µl), 1000-2000bp (10-40ng/ µl), Plasmid-Single-stranded (25-50ng/µl), and Double-stranded (150-300 ng/µl). Our desired quantity of PCR product volume of this study: 1000-1500bp (5-20 ng/ µl).

2.2.2. Template Pre-Heat Treatment

The template RNA was heated at 96ºC for 5 minute in a 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 (20 µl)
Ready Reaction Premix	2.5X	4 µl
Bi□ Dye Sequencing buffer	5X	2 µl
Primer	-	3.2 pM (µl)
Template	-	3 µl
Water	-	10 µl
Final Volume	1X	20 µl

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

Table-4. PCR sequencing cycling conditions.

Process	T□mperature	Time	Cycles
Initial Denaturation	96ºC	1 sec	25
Denaturation	96ºC	10 sec	25
Annealing	50ºC	5 sec	25
Elongation	60ºC	4 min	--

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

2.2.3. 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, after completion the data was analyzed.

2.3. Bioinformatics Analysis (Sequence Annotations and Statistics)

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

2.4. Multiple Sequence Alignment

Multiple sequence alignment (MSA) is a sequence alignment of three or more biological sequences, generally protein, DNA, or RNA. MSA is generally the alignment of three or more biological sequences (protein or nucleic acid) of similar length. From the output, homology can be inferred and the evolutionary relationships between the sequences studied.

2.5. T- Coffee Alignment

T-Coffee is a multiple sequence alignment package. Can use T-Coffee to align sequences or to combine the output of your favorite alignment methods (Clustal, Mafft, Probcons, Muscle, etc.) into one unique alignment (M-coffee).T-Coffee can align Protein, DNA and RNA sequences. It is 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.

2.6. 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 is rendered by web browser as a standard web page.

Multiple align show used to highlight the amino acid residues in the sequences. The resulted sequences from T-coffee were uploading in MAS and the following parameters were selected identical amino acid residues in amino acid color, similar residues in black and variables in white color. After selecting the parameters the sequences were submitted and the result was appeared in new window.

2.7. Phylogenetic Analysis

Phylogenetic analysis is the process used to determine the evolutionary relationship between the organisms and species level. The result of the analysis can be 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 variation given by same special like sexual dimorphism, larvae adult coloration and now it is carried out using DNA sequence.

2.8. Synonymous and Non-Synonymous Substitution

Estimation of synonymous (Ks) and non-synonymous (Ka) substitutions was calculated by Li93 method (Muse and Gaut, 1994) 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 (Felsenstein, 1981).

2.9. Saturation

Analysis of sequence saturation was done by using DAMBE V 5.3.10 (Xia, 2013) for calculating the transitional and transvertional substitutions against genetic distance (TN93). The substantial saturation of the sequence was checked by using the method of Xia *et al.* (2003); Xia and Lemey (2009) (DAMBE).

2.10. Molecular Evolutionary Genetic Analysis (MEGA V.6)

MEGA tool is used to find 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 also can be estimated. The estimation was accomplished using

bootstrapping approach. Transition and type of substitution between the sequences can also be used for inferring phylogenetics by the distance based methods, along with bootstrap test. This tool is 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. Sequence 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.

3. RESULTS AND DISCUSSION

The isolated genomic DNA from *B. coagulans*, *L. fermentum*, *L. lactis*, *L. acidophilu,s E. coli* and, *S. aureus* showed >10kb size each Figure 1. Their amplified 16S r-RNA gene with the universal primers have yield ~1500 bp size nucleotide each for *B. coagulans*, *L. fermentum*, *L. lactis*, *L. acidophilu,s E. coli* and, *S. aureus* Figure 2.

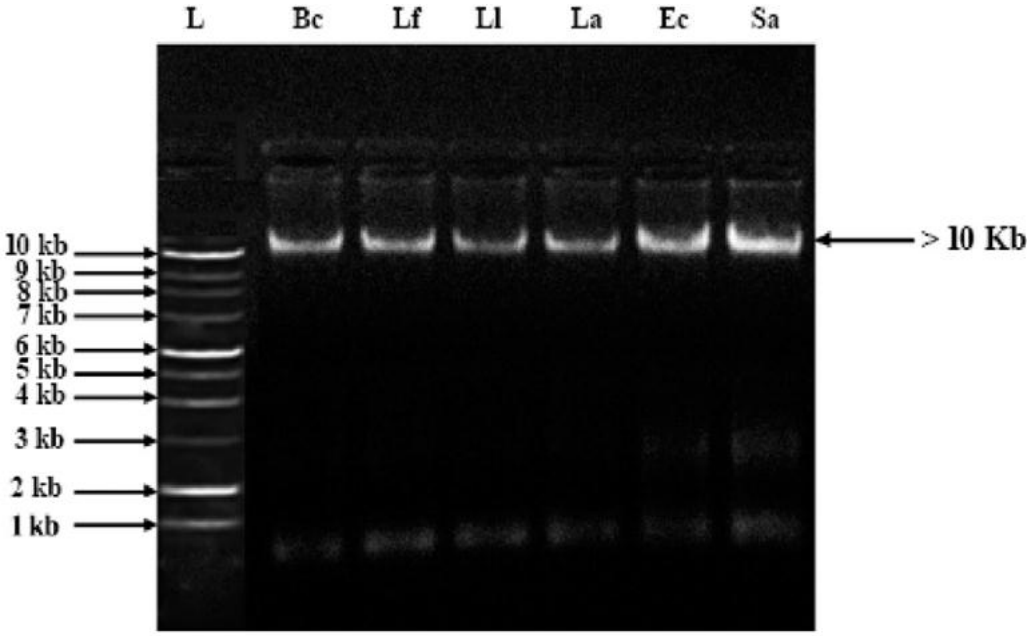

Figure-1. AGE (1%) of bacterial species shows >10 kb genomic DNA. L, Ladder (1 kb); Bc, *B. coagulans*; Lf, *L. fermentum*; Ll, *L. lactis*; La, *L. acidophilus*; Ec, *E. coli*; Sa, *S. aureus.*

Source: Paper authors original work.

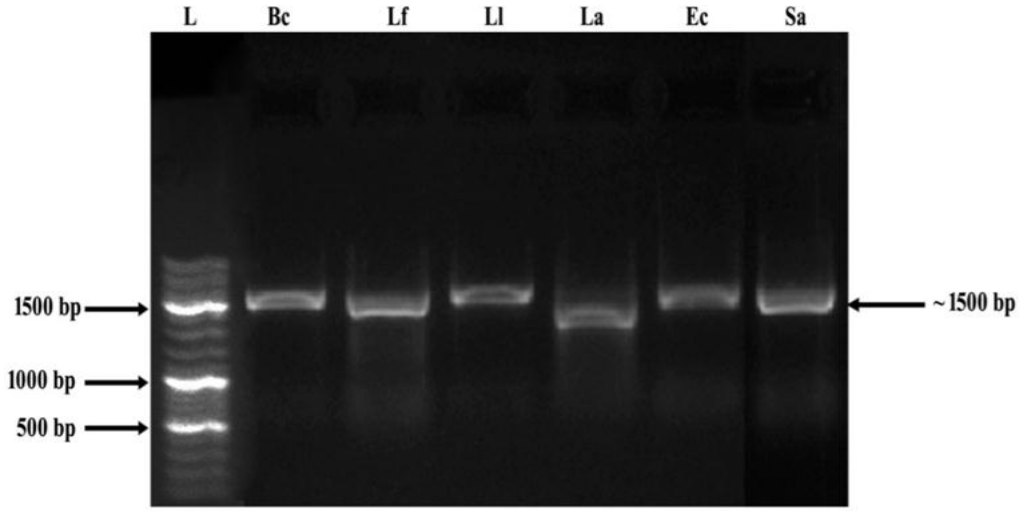

Figure-2. AGE (2%) of Bacterial species shows ~1500 bp of amplified product. L, Ladder (1 kb); Bc, *B. coagulans*; Lf, *L. fermentum*; Ll, *L. lactis*; La, *L.acidophilus*; Ec, *E. coli*; Sa, *S. aureus.*

Source: Paper authors original work.

The amplified product showed 1541 bp, 1516 bp, 1507 bp, 1499bp, 1465 bp, 1499 bp, and 1476 bp nucleotide sequences for *B. coagulans*, *L. fermentum*, *L. lactis*, *L. acidophilus*, *E. coli* and, *S. aureus* respectively Table 5. These sequences were authenticated with the NCBI Gen Bank.

Table-5. 16S rRNA barcodes of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. coagulans* incorporated diet (Base pair values are given in parenthesis with respective Gen Bank accession number).

Bacillus coagulans (1541 bp, MG557779)

TAGAGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGTGCGGACC
TTTTAAAAGCTTGCTTTTAAAAGGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGT
AAGATCGGGATAACGCCGGGAAACCGGGGCTAATACCGGATAGTTTTTTCCTCCGCATGGAGGAAA
AAGGAAAGACGGCTTCGGCTGTCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGGGGTA
ACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGAC
ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAG
CAACGCCGCGTGAGTGAAGAAGGCCTTCGGGTCGTAAAACTCTGTTGCCGGGGAAGAACAAGTGCC
GTTCGAACAGGGCGGCGCCTTGACGGTACCCGGCCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCTT
CTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGTCATTGGAAACTGGGAGGCTTGAGT
GCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAG
TGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTA
GTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACACCTCTGACCTCCCTGGAGACAGGGCCTTCCCCTTCGGGGGACAGAGTGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TGACCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTA
CAAAGGGCTGCGAGACCGCGAGGTTAAGCCAATCCCAGAAAACCATTCCCAGTTCGGATTGCAGGCT
GCAACCCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACC
TTTACGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTA
TCGGAAGGTGCGGTTGGATCACC

Lactobacillus fermentum (1516 bp, MG557782)

CCTGGCTCAGGATGAACGCCGGCGGTGTGCCTAATACATGCAAGTCGAACGCGTTGGCCCAATTGA
TTGATGGTGCTTGCACCTGATTGATTTTGGTYGCCAACGAGTGGCGGACGGGTGAGTAACACGTA
GGTAACCTGCCCAGAAGCGGGGGACAACATTTGGAAACAGATGCTAATACCGCATAACARCGTTGT
TCGCATGAACAACGCTTAAAAGATGGCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAG
CTTGTTGGTGGGGTAAYGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCA
CAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGC
GCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAA
AGAAGAACACGTATGAGAGTAACTGTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAG
AGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAAAC
TGGATAACTTGAGTGCAGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATA
TGGAAGAACACCAGTGGCGAAGGCGGCTACCTGGTCTGCAACTGACGCTGAGACTCGAAAGCATGG
GTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAGGTGTTGGAGG
GTTTCCGCCCTTCAGTGCCGGAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTT
GAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACG
CGAAGAACCTTACCAGGTCTTGACATCTTGCGCCAACCCTAGAGATAGGGCGTTTCCTTCGGGAACG
CAATGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTGTTACTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTAC
AATGGACGGTACAACGAGTCGCGAACTCGCGAGGGCAAGCAAATCTCTTAAAACCGTTCTCAGTTCG
GACTGCAGGCTGCAACTCGCCTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG
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Lactococcus lactis (1499 bp, MG557783)

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTTGAGCGATGAAGATTGGTGCTTGCACCAATT
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ACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATAC
ATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGG
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TTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCATCAAGTG
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GTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTC
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GGGCACTCTAACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGC
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Lactobacillus acidophilus (1507 bp, MG557781)

CCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAACCAACAGA
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GGTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTG
AGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGAT
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ACCTTCGGGAAGGAGCCGTCTAAGGCAGGGCAGATGACTGGGGTGAAGTCGTAACA

Escherichia coli (1465 bp, MG557780)

GGCGTAAAGGTTAGTCATACCAGTGCAAGTCGACGGTAACAGGAAGAAGCTTGCTTCTTTGCTGAC
GAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACG
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AAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGTGCAAGCGTTAATCGGA
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GGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCG
CTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA

CCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTCTCCCAATAGGGG
GTATGGAATTCCG

Staphylococcus aureus (1476 bp, MG557784)
AGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACGGACGAGAAGCTTGCTTCTC
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GAAACCGGAGCTAATACCGGATAATATTTTGAACCGCATGGTTCAAAAGTGAAAGACGGTCTTGCT
GTCACTTATAGATGGATCCGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGA
TGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGA
AGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTT
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GCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTC
CATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCT
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TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACT
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TGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATG
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AAGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAA
TCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTC
ACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCTTTTAGGAGCTAGCCGTCGAAGGT
GGGACAAATGATTGGGGTG

Source: Paper authors original work.

The BLAST of these sequences showed almost 100% similarities with the same species retrieved, 100% for *B. coagulans*, 99% for *L. fermentum*, 100% for *L. lactis*, 99% for *E. coli*, and 100% for *S. aureus* Table 6.

Table-6. BLAST identification of 16S r-RNA gene sequences of subjected and retrieved bacterial species with their Gen Bank accession numbers.

Queried sequences	Author, Country and Accession Number	I (%)	G (%)	M.S	Retrieved/ Matched species	Author, Country and Accession Number
Bacillus coagulans	Paper authors, India MG557779	100	0	Plus	*Bacillus coagulans*	Tanaka *et al.* (2007) Japan AB362709
Lactobacillus fermentum	Paper authors, India MG557782	99	0	Plus	*Lactobacillus fermentum*	Kang (2017) Korea CP016803
Lactococcus lactis	Paper authors, India MG557783	100	0	Plus	*Lactococcus lactis*	Linares (2014) Netherlands CP002094
Lactobacillus acidophilus	Paper authors, India MG557781	100	0	Plus	*Lactobacillus acidophilus*	Nam (2017) Korea CP017062
Escherichia coli	Paper authors, India MG557780	100	0	Plus	*Escherichia coli*	Hagaggi (2017) Egypt KY906967
Staphylococcus aureus	Paper authors, India MG557784	100	0	Plus	*Staphylococcus aureus*	Yamamoto and Wan (2017) Japan AP017891

Source: Paper authors original work, and retrieved from NCBI data base.

The MAS showed 920 identical amino acids residues, 119 similar amino acids residues and 461 variable amino acids sites Figure 3.

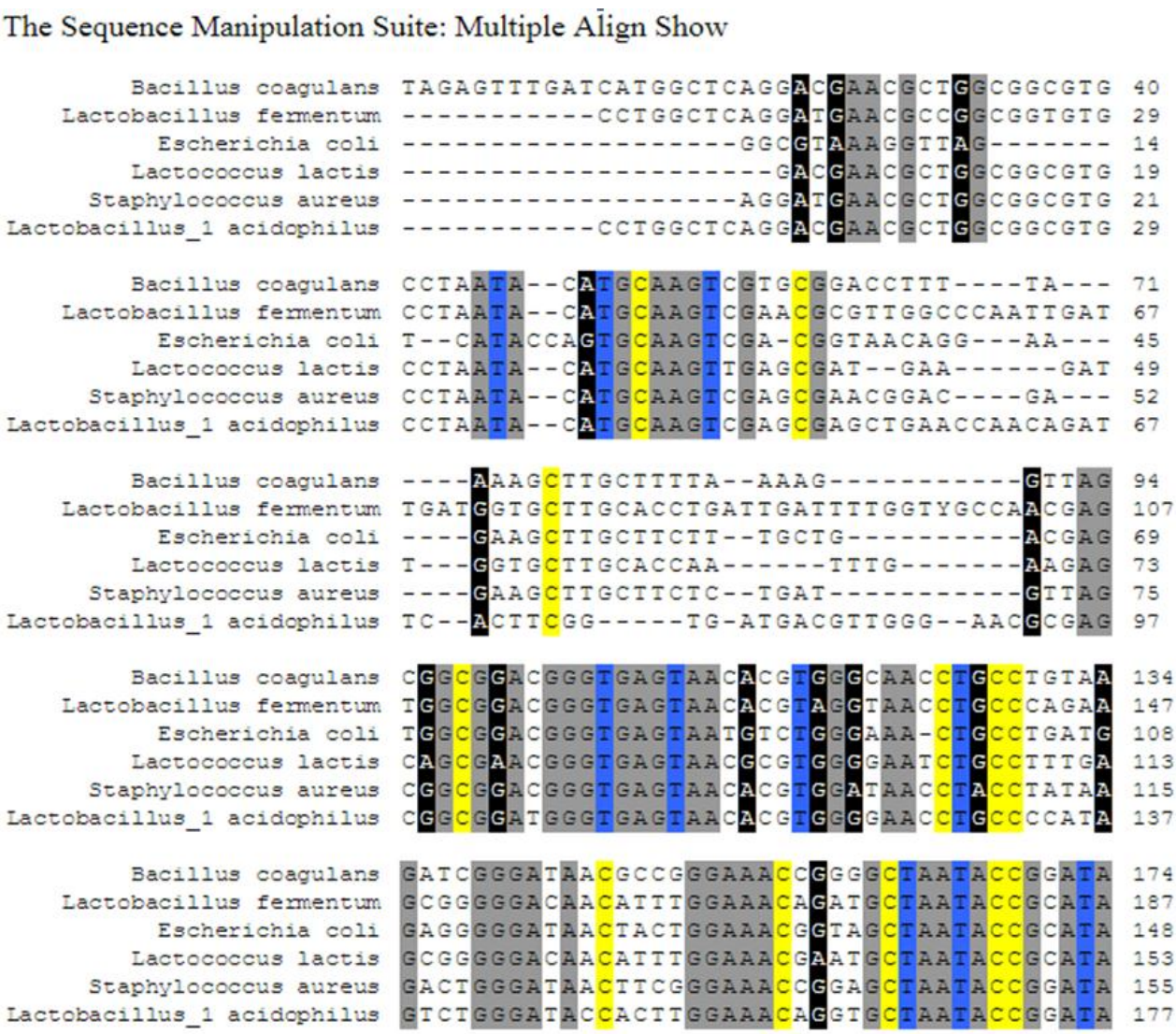


Figure-3. Multiple sequence alignment of 16S r-RNA gene sequences of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. coagulans* incorporated diet. Multiple align show (MAS) with coloured background (identical residues are indicated by amino acid colour and similar residues are black in colour. Gaps and other residues are given in white background).

The nucleotide composition showed more GC biases (51.2-56.2%) and less AT biases (29.2-31.9%), minimum in *S. aureus* and maximum in *B. coagulans* Table 7. The lower AT bias recorded indicates 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 *B. coagulans* incorporated diet.

Name of the species	T	A	C	G	AT	GC
Bacillus coagulans	19.4	24.4	43.8	24.3	31.9	56.2
Lactobacillus fermentum	21.5	25.4	46.9	22.9	30.1	53.1
Lactobacillus acidophilus	20.3	25.9	46.3	23.0	30.8	53.7
Lactococcus lactis	21.6	27.0	48.6	21.3	30.1	51.4
Escherichia coli	19.5	25.9	45.4	23.0	31.6	54.6
Staphylococcus aureus	21.7	27.1	48.8	22.0	29.2	51.2
Avg.	**20.7**	**26.0**	**46.6**	**22.7**	**30.6**	**53.4**

Source: Paper authors original work.

The mean divergent rate of different combinations of these bacterial species was 1.265 with a maximum of 2.365 (between *B. coagulans* vs. *S. aureus*) and minimum of 0.889 (between *S. aureus* vs. *L. acidophilus*) Table 8; Figure 4.

It has been reported >1% intra genus and 0.3% intra species 16S rRNA heterogeneity in filamentous marine cyanobacteria (Engene *et al.*, 2010; Engene and Gerwick, 2011). However, Pei *et al.* (2010) reported that *E. coli* is known to have high intra-specific variation (1.10 %) between multiple 16S rRNA genes in the genome. A high degree of intra-genomic variation has been reported in *Lactobacillus rhamnosus* (0–7.67%), *Caldanaerobacter subterraneus* (0.03–6.23%), *Desulfitobacterium hafniense* (0.06–3.73 %), *Bacteroides ovatus* (0.07–3.30 %), *Yersinia enterocolitica* (0–2.67 %) and *Desulfitobacterium dehalogenans* (0–2.14 %) 16S rRNA gene copies (Engene and Gerwick, 2011).

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

Between Species	Divergence (%)
Bacillus coagulans vs. *Lactobacillus fermentum*	1.432
Bacillus coagulans vs. *Escherichia coli*	1.197
Lactobacillus fermentum vs. *Escherichia coli*	1.054
Bacillus coagulans vs. *Lactococcus lactis*	0.935
Lactobacillus fermentum vs. *Lactococcus lactis*	1.432
Escherichia coli vs. *Lactococcus lactis*	1.008
Bacillus coagulans vs. *Staphylococcus aureus*	2.365
Lactobacillus fermentum vs. *Staphylococcus aureus*	1.487
Escherichia coli vs. *Staphylococcus aureus*	0.962
Lactococcus lactis vs. *Staphylococcus aureus*	0.943
Bacillus coagulans vs. *Lactobacillus acidophilus*	1.265
Lactobacillus fermentum vs. *Lactobacillus acidophilus*	1.248
Escherichia coli vs. *Lactobacillus acidophilus*	1.035
Lactococcus lactis vs. *Lactobacillus acidophilus*	1.730
Staphylococcus aureus vs. *Lactobacillus acidophilus*	0.889
Average	**1.265**

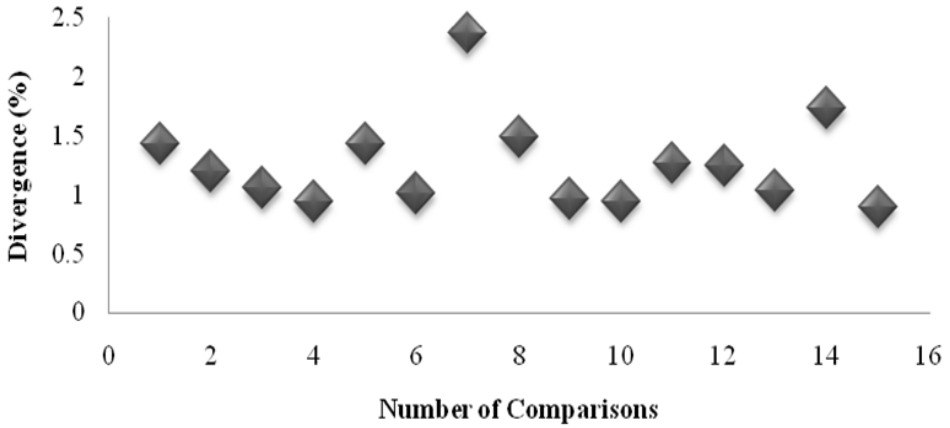


Figure-4. 16S r-RNA nucleotide divergence of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. coagulans* incorporated diet.

The non-synonymous (Ka) substitution was higher (2.243) than that of synonymous (Ks) substitution (0.621), which indicates the possibility of occurrence of more deleterious mutation and less silent mutation. Similarly, the transversional (Tv) substitution was higher (0.14) than that of transitional (Ts) (0.13), which indicates the fact that these sequences have more phylogenetic information. However, saturation was not occurred in these sequences, which was confirmed by the predicted higher critical value of index of substitution saturation (Iss.c), 0.813 than that of index of substitution saturation (Iss), 0.318, and therefore more phylogenetic differences existed between sequences Table 9; Figure 5.

Most animal species are un-described. In phylogeny of life forms, generally there are three clades, each with different clusters. They are (1) archaea (single celled microorganism, the prokaryotes, their cell wall does not contain peptidoglycan, the cell membrane have ether linked lipids), (2) bacteria (the first life form appeared on earth,

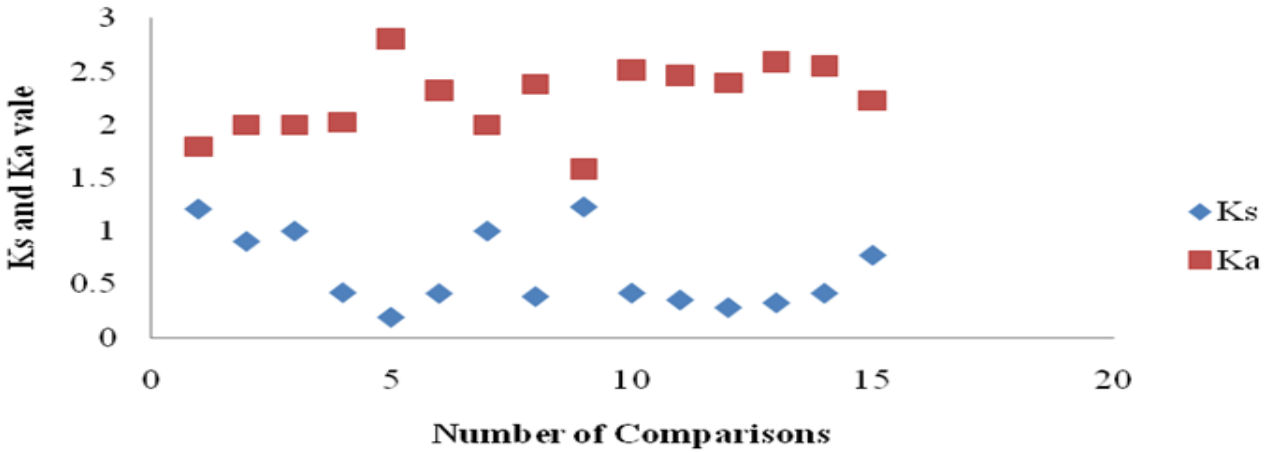
their cell wall contain peptidoglycan and the cell membrane have ester linked lipids), and (3) eukaryota (consisted of all the multicellular organisms having nucleus surrounded by nuclear membrane).

Substitutional saturation decreases phylogenetic information (Lopez *et al*., 1999; Xia *et al*., 2003). When the sequences have experienced full substitution saturation, the similarity between the sequences will depend entirely on the similarity in nucleotide frequencies (Steel *et al*., 1993; Xia *et al*., 2003) which often does not reflect phylogenetic relationships. Similar information has also been reported by us in crabs, prawns and planktons of freshwater and marine species (Rajkumar *et al*., 2015; Udayasuriyan *et al*., 2015; Bhavan *et al*., 2015;2016;2017). Figure 6.

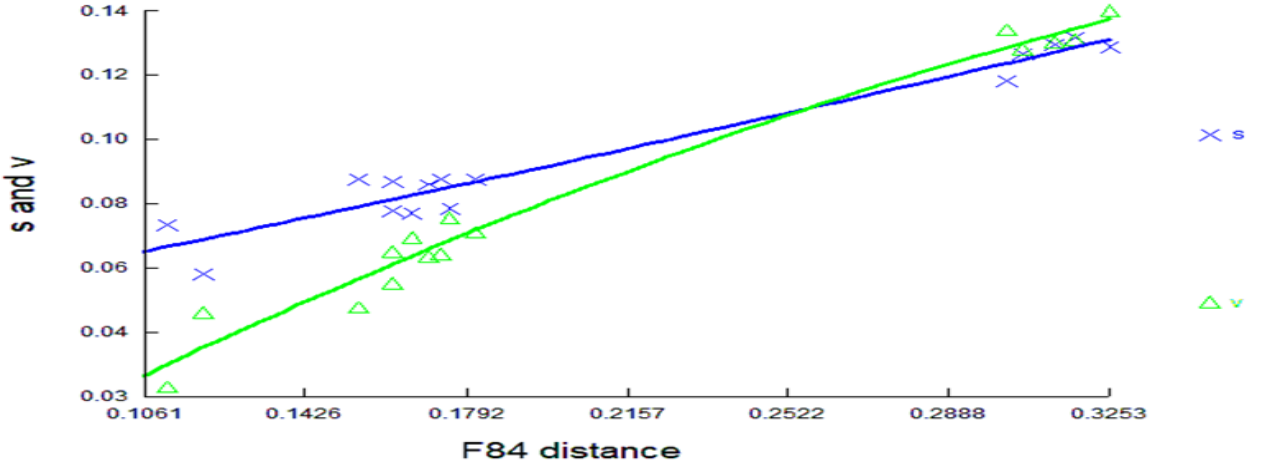
Table-9. Phylogenetic information of bacterial species identified (based on 16S r-RNA) in the gut of *M. rosenbergii* PL fed with *B. coagulans* incorporated diet.

Phylogenetic information	Ks	Ka	Ka-Ks	Ts	Tv	Tv-Ts	Iss	Iss.c	Iss.c-Iss
Subjected species	0.621	2.243	1.622	0.13	0.14	0.01	0.318	0.813	0.495

Ks, Synonymous substitution; **Ka**, Non-synonymous substitution; **Ts**, Transitional substitution; **Tv**, Transversional substitution; **Iss**, Index of substitution saturation; **Iss.c**, Critical value of index of substitution saturation.



A). Number of synonymous (Ks) and non-synonymous (Ka) substitutions occurred at 3rd codon position in nucleotides of 16S r-RNA gene partial sequences of bacterial diversity identified in the gut of *M. rosenbergii* PL fed with *B. coagulans* incorporated diet



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. coagulans* incorporated diet

Figure-5. Synonymous (Ks) and non-synonymous (Ka), transitional (X, blue) and transversional (Δ, green) substitutions occurred in16S r-RNA partial gene sequences of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. coagulans* incorporated diet.

Based on the divergence value, the phylogenetic tree topologies revealed discrimination between identified bacterial species in the gut of *M. rosenbergii* PL.

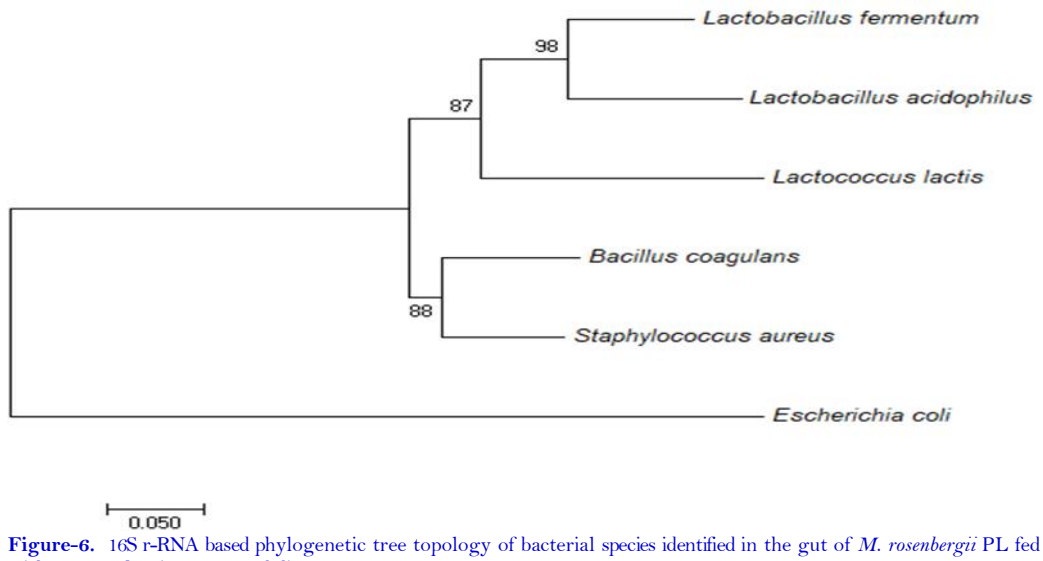


Figure-6. 16S r-RNA based phylogenetic tree topology of bacterial species identified in the gut of *M. rosenbergii* PL fed with *B. coagulans* incorporated diet.

4. CONCLUSION

In *B. coagulans* incorporated feed fed *M. rosenbergii* PL gut, the bacterial diversity identified using 16S rRNA revealed presence of six dominant bacteria, *B. coagulans*, *L. fermentum*, *L. acidophilus L. lactis*, *E. coli* and *S. aureus*. They showed 99-100% similarity with *B. coagulans*, *L. fermentum*, *L. acidophilus L. lactis*, *E. coli* and *S. aureus*, respectively available with NCBI-GenBank data base. Therefore, the identified 16S rRNA sequences of these bacterial species are accurate. The authenticated sequences of these bacteria showed more numbers of identical amino acid residues than variable amino acid sites, and such a variation fixed the phylogenetic tree type.

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Competing Interests: The authors declare that they have no competing interests.
Contributors/Acknowledgement: All authors contributed equally to the conception and design of the study.

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