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16S RRNA PROFILING OF THE GUT MICROFLORA IN EUTHYNNUS AFFINIS AND THUNNUS ALBACARES COLLECTED FROM THE SOUTHERN PART OF INDIA

A. Nasreem[1*], D. Deleep Packia Raj[2]

[1*]Department of Zoology and Research centre,Scott christian College (Autonomous)Nagercoil 629001 (Affiliated to Manonmaniam Sundaranar University), Tirunelveli, TamilNadu, India. Email: anasreem@gmail.com
[2]Department of Zoology and Research centre,Scott christian College (Autonomous)Nagercoil 629001 (Affiliated to Manonmaniam Sundaranar University), Tirunelveli, TamilNadu, India.

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ABSTRACT
The intestinal microflora of fishes is essential for digestion, absorption of nutrients, immunity, and general well-being of the hosts. Gut microbiota composition depends on both host and environmental conditions. It greatly contributes to fish physiology and adaptation. This study focused on determining the bacterial diversity of two economically relevant tuna fish species, i.e., Euthynnus affinis (Kawakawa) and Thunnus albacares (Yellowfin tuna) that were sourced from different geographic regions of the south-west coast of India, which includes Thoothoor, Kanyakumari, and Trivandrum. Intestinal tissue was dissected aseptically, homogenized, serially diluted, and inoculated in Nutrient Agar plates for isolation of bacteria. Isolates were then subjected to DNA extraction and 16S rRNA PCR amplification followed by Sanger sequencing. The six isolated bacteria included Microbacterium sp. and Bacillus cereus (Trivandrum), Bacillus cereus and Lacrimispora amygdalina (Thoothoor), and Escherichia coli and Oceanisphaera ostreae (Kanyakumari). The results offer baseline information regarding composition of the gut microbiota associated with E. affinis and T. albacares from the Indian south coast and also provide knowledge on host-microbe relationships in natural environments.

KEYWORDS: gut bacteria, microorganisms, microflora, 16S rRNA, PCR amplification, tuna fish

1. INTRODUCTION

Microbiomes in fish guts play a vital role in the physiology of the host, helping in digestion, nutrient uptake, metabolism, immune system, and resisting diseases (Egerton et al., 2018). The microbial composition and diversity of fish gut microbiomes depend on various host factors as well as environmental factors. The host factors include fish species, genetic makeup, and age, while environmental factors comprise geographical location, water chemistry, temperature, and habitat type (Lokesh & Kiron, 2016). As fishes are continuously exposed to their environment, any change in environmental conditions causes significant alterations in the gut microbial community composition that ultimately influences fish health and physiology (Sylvain et al., 2020).

Tuna species constitute one of the most ecologically and economically valuable groups of marine fish, playing a critical role in marine ecosystems and global fisheries. These highly migratory pelagic fishes are widely distributed in tropical and temperate oceans, where they act as apex predators and play an important role in marine food webs. Euthynnus affinis and Thunnus albacares are two commercially viable fishes that commonly found in the Indian Ocean. The body of E. affinis is streamlined and fusiform in shape with distinctive dark oblique stripes on its dorsal side, while T. albacares is easily distinguished by its metallic blue colored dorsum, silver belly, and extended yellow dorsal and anal fins. Both species serves as commercially valuable fish resources and provide a good source of protein for human consumption. Due to their wide geographic distribution, migratory behaviour, and ecological significance, these species provide suitable model for studying gut microbiota and host-microbiome relationships.

The development of molecular biology technology has greatly contributed to research on microbial diversity and taxonomy. In the existing range of molecular technologies, DNA sequencing of the 16S ribosomal RNA gene is one of the most popular and successful methods for bacterial identification and phylogenetic investigation. The gene is characterized by highly conserved and highly variable areas, allowing precise differentiation between various bacteria taxa at the genus and species levels. Hence, the DNA sequencing of the 16S rRNA gene became a standard method for bacterial isolate description and diversity assessment. Bacterial identification and taxonomic classification can be significantly refined through sequence comparisons utilizing database searches, including NCBI BLAST databases (Janda & Abbott, 2007; Clarridge, 2004).

Despite significant advances in studies on the fish gut microbiota, data concerning culturable bacterial diversity of wild tuna from the coastal region of India is relatively sparse. Previous studies on the bacterial microbiota have concentrated

on farmed species or employed a non-culture method in regions like the Pacific Ocean, Atlantic Ocean, and South China Sea. Hence, there is a relative dearth of information regarding the composition and geographical variations in culturable gut bacteria associated with E affinis and T albacares from the south-west coast of India is limited. Isolation and molecular characterization of culturable bacteria are crucial since some of these bacteria may have probiotic characteristics, facilitate nutrient digestion, improve fish wellbeing, and produce industrially relevant molecules (Gupta et al., 2020).

Thus, the present study was designed to isolate and characterize the culturable gut bacteria associated with E affinis and T albacares collected from three geographically distant coasts of South-Western coastal region of India, i.e., Thoothoor, Kanyakumari, and Trivandrum. The bacterial strains isolated were characterized based on the identification of 16S rRNA sequences through BLAST analysis. This investigation will contribute to the existing literature with baseline information on the culturable gut microbiota of tuna fish.

2. MATERIALS AND METHODS

2.1 Sample Collection and Bacterial Isolation

Samples of tuna fish were collected from three geographically different regions located in the south-west coastal region of India, such as Trivandrum, Thoothoor, and Kanyakumari. The samples collected were taken to the lab and processed on the very same day. The gut tissues were aseptically removed and sterilized with sterile distilled water and 70% ethanol, followed by homogenization in 1 mL of sterile distilled water. Serial dilution was performed up to 10-5 using sterile distilled water.

The aliquots of 10-3 and 10-4 dilutions were spread on Nutrient Agar plates and incubated at 37°C for 24 hours. Colonies with morphological difference were isolated and sub-cultured and further grown in Nutrient Broth at 37°C overnight.

2.3 Isolation of Genomic DNA

Extraction of genomic DNA was done from overnight cultures of bacteria through the sodium dodecyl sulfate (SDS) extraction method but with minor adjustments. Pelleting of the bacterial cells was achieved by centrifugation at 21,500 × g for 5 minutes at 4°C then suspension in TE buffer followed by lysozyme and RNase A treatment to allow cell lysis and RNA degradation, respectively.

The DNA extraction procedure involved phenol:chloroform alcohol (25:24:1, v/v/v) and chloroform alcohol (24:1, v/v) extractions to purify the cell lysate. Precipitation was carried out through the addition of sodium acetate and ice-cold ethanol, washed with 70% ethanol, dried using an air stream, and finally resuspended in deionized water for further processing.

2.4 Agarose Gel Electrophoresis of Genomic DNA

To determine the purity and integrity of the isolated genomic DNA, agarose gel electrophoresis analysis was done according to existing protocols. For this process, an 0.8% agarose gel was made by mixing 0.4 g agarose with 50 mL of 1× Tris-Acetate-EDTA (TAE) buffer solution. The mixture was warmed to dissolve the agarose completely followed by cooling and addition of ethidium bromide.

The cooled agarose was poured into a gel mold containing a comb. After the agarose had been solidified, the gel was transferred to an electrophoresis unit containing 1× TAE buffer solution. DNA samples were loaded onto individual wells after mixing with loading dye. Electrophoresis was performed at 100 V for migration of the tracking dye to about two-thirds of the total distance covered by the gel.

2.5 Polymerase Chain Reaction (PCR) Amplification of the 16S rRNA Gene

Identification of the bacteria was achieved through PCR amplification of the 16S ribosomal RNA (rRNA) gene, which is conserved within all bacterial genera and serves as a basis for bacterial taxonomy and classification.

PCR amplification was done using universal primers for 16S rRNA of bacteria:

Forward primer: AGAGTTTGATCMTGGCTC

Reverse primer: AAGGAGGTGWTCCARCC

PCR amplification mix contained 25 µL Red Taq Master Mix, 2 µL forward primer, 2 µL reverse primer, 10 µL template DNA (50 ng µL-1), and 11 µL nuclease-free water in a total volume of 50 µL.

The PCR thermocycling parameters were as follows: initial denaturation step at 94˚ C for 5 min; then 35 cycles with 94 ˚C for 30 s, 55 ˚C for 30 s, and 72°C for 45 s; followed by 72˚ C for 7 min.

2.6 Identification of PCR Products

PCR products obtained in the study were analyzed through gel electrophoresis using agarose gels. A 1% agarose gel was prepared by mixing 0.5 g of agarose in 50 mL of 1x TAE buffer. After solidifying the agarose gel, the PCR products were run together with a 1 kb DNA ladder at 80 volts for 45 minutes. Amplified DNA fragments of about 1500 bp in size were detected under UV light and photographed through a gel documentation system.

2.7 DNA Sequencing and Sequence Analysis

PCR products displaying positive amplification reactions were isolated and sequenced by Sanger sequencing methods. Approximately 1500 bp internal regions of the bacterial 16S rDNA gene were sequenced using the same primer pair utilized for amplifying the genes.

The sequenced nucleotide sequences were then subjected to BLAST analyses using the Basic Local Alignment Search Tool found in the NCBI database. Bacteria were classified based on the highest homology between the obtained sequence and the sequences available in the GenBank database. Identified bacteria were used to evaluate the culturable diversity of the gut bacteria associated with tuna fish collected from various geographic origins.

3. RESULTS AND DISCUSSION

3.1 Isolation of Gut Bacteria from Tuna Fish

Tuna specimens belonging to E affinis and T albacares were collected from three different geographical sites, viz. Thoothoor, Kanyakumari, and Trivandrum, which were used right away after being collected in order to maintain the indigenous microbiome of the fish samples. Samples of the tuna fish used in the present study are depicted in Figure 1. After performing aseptic dissection and sterilizing the surface, gut tissues were homogenized and subjected to serial dilution in order to isolate bacteria.

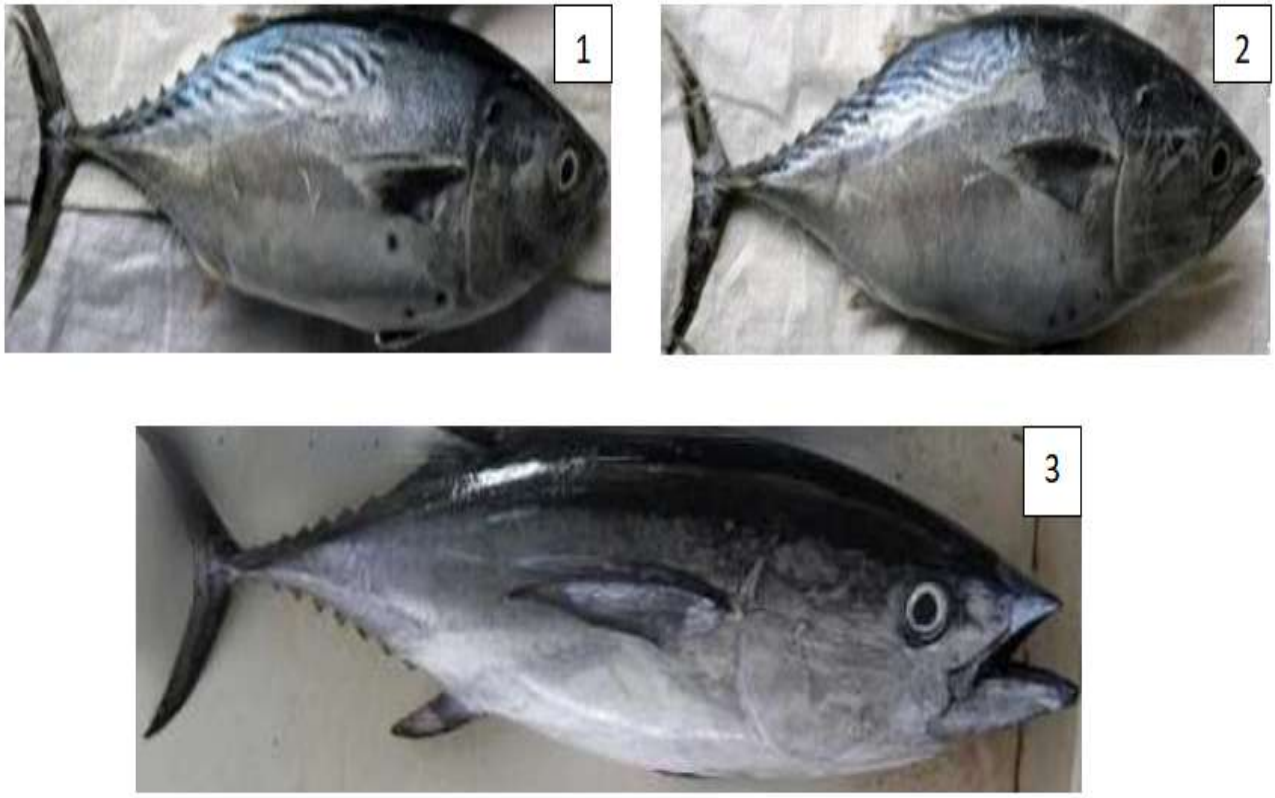

Figure 1. Samples of the tuna fish collected for the present study: (1) Thoothoor, (2) Kanyakumari, and (3) Trivandrum.

Bacterial colonies formed upon incubation in Nutrient Agar media and were characterized in terms of size, shape, elevation, and texture, thereby indicating that the samples contained diverse bacteria populations. Typical examples of isolated bacterial colonies are illustrated in Figure 2.

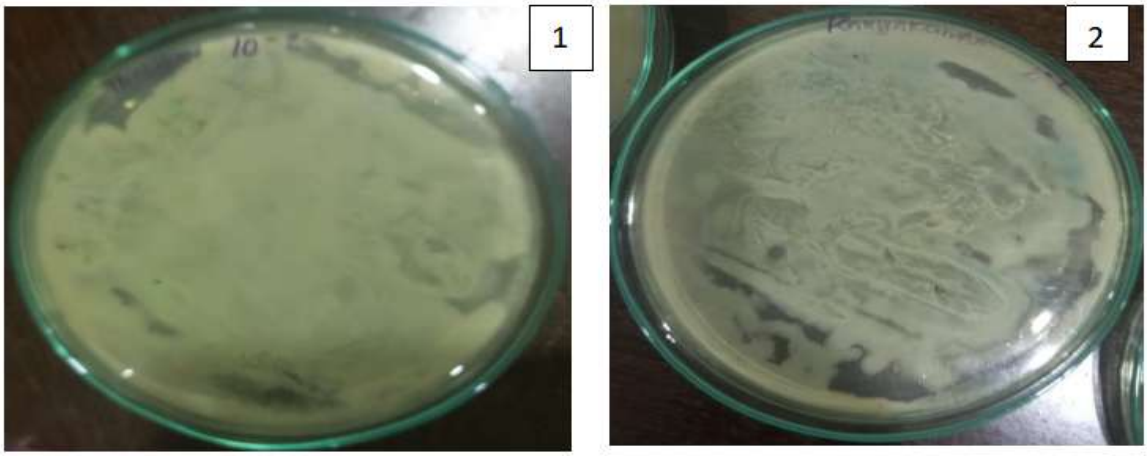

Figure 2. Isolated colonies of bacteria isolated from tuna gut after serial dilution. (1) Thoothoor, (2) Kanyakumari, and (3) Trivandrum.

The occurrence of colonies exhibiting different morphologies indicates a heterogeneous microbial population within the tuna gut. This finding is consistent with previous studies showing that the intestinal microbiota of marine fishes is shaped by dietary, environmental, and habitat-related factors (Egerton et al., 2018; Tarnecki et al., 2017). The highly migratory nature and diverse feeding habits of tuna may further influence the composition and diversity of their gut microbiota.

3.2 Genomic DNA Extraction

The genomic DNA was successfully extracted from chosen bacterial isolates using the SDS extraction technique. The extracted genomic DNA was then subjected to analysis by agarose gel electrophoresis. The electrophoresis apparatus employed in analysing the DNA is illustrated in Figure 3.

Figure 3. The set-up for agarose gel electrophoresis for DNA analysis.

Clear and distinct DNA bands were observed following agarose gel after electrophoresis, indicating the successful extraction of the bacterial genomic DNA. The agarose gel picture captured during bacterial genomic DNA isolation is provided in Figure 4.

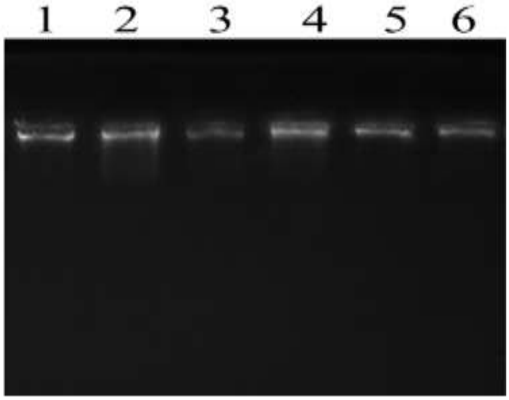

Figure 4. Agarose gel picture depicting bacterial genomic DNA extracted from tuna gut bacteria. Lanes 1-2: Thoothoor isolates; Lanes 3-4: Kanyakumari isolates; Lanes 5-6: Trivandrum isolates.

Identification of clear DNA bands without much degradation suggested that the genomic DNA extracted was good enough for PCR amplification and sequencing analysis. Good quality genomic DNA is required for bacterial identification through molecular techniques (Teletchea, 2009).

3.3 Amplification of the 16S rRNA Gene

Amplification of the 16S rRNA gene from the extracted genomic DNA was carried out using universal bacterial primers. Sequences of the primers used in this study are shown in Table 1.

Table 1. Universal bacterial 16S rRNA primers for amplification

Primer	Sequence (5'-3')
Forward Primer	AGAGTTTGATCMTGGCTC
Reverse Primer	AAGGAGGTGWTCCARCC

The PCR products were amplicons of ~1500 bp in all bacterial isolates. The amplicons were resolved by running the samples on 1% agarose gels and stained with ethidium bromide followed by UV observation.

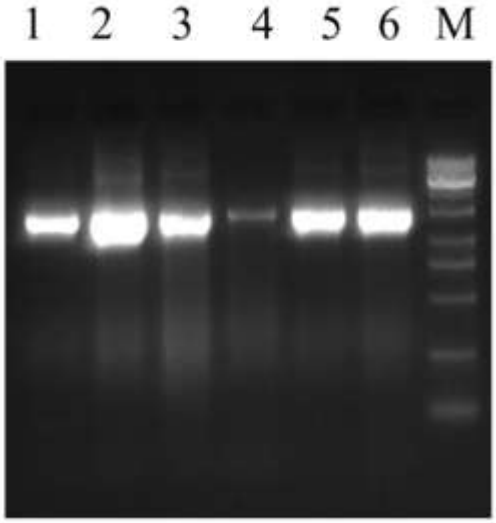

Figure 5. PCR products (~1500 bp) amplified by universal bacterial 16S rRNA primers. Lane 1-2: Thoothoor isolates; Lane 3-4: Kanyakumari isolates; Lane 5-6: Trivandrum isolates.

This result indicated that the purified genomic DNA was ready for further sequencing and taxonomic characterization. The use of 16S rRNA is one of the best methods for identification of bacteria due to the conserved character of this gene (Alikunhi et al., 2017).

3.4 Sequence Analysis and Identification of Bacterial Isolates

The amplified PCR products were subjected to Sanger sequencing and the sequences generated were analyzed via NCBI BLAST database. Sequence analysis revealed that Trivandrum isolate 1 had a close resemblance with Microbacterium species. The graphical representation of BLAST hits, significant sequence alignments, and sequence comparison have been illustrated in Figures 6, 7, and 8.

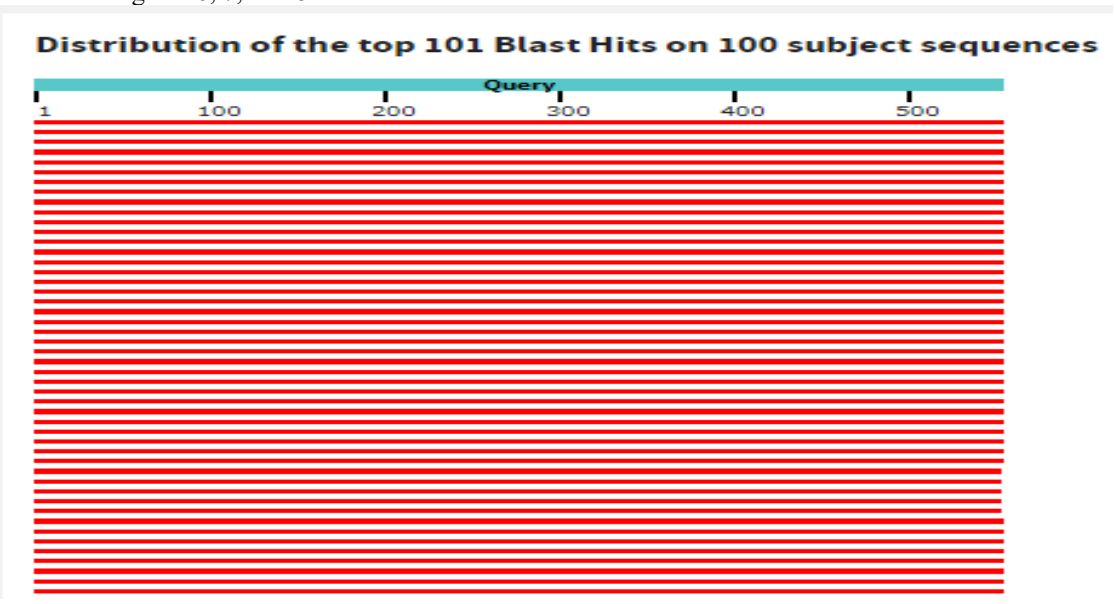

Figure 6. Graphic Representation of Top BLAST Hits Obtained for Trivandrum Isolate 1.

Figure 7. Significant sequence alignments obtained for Trivandrum isolate 1.

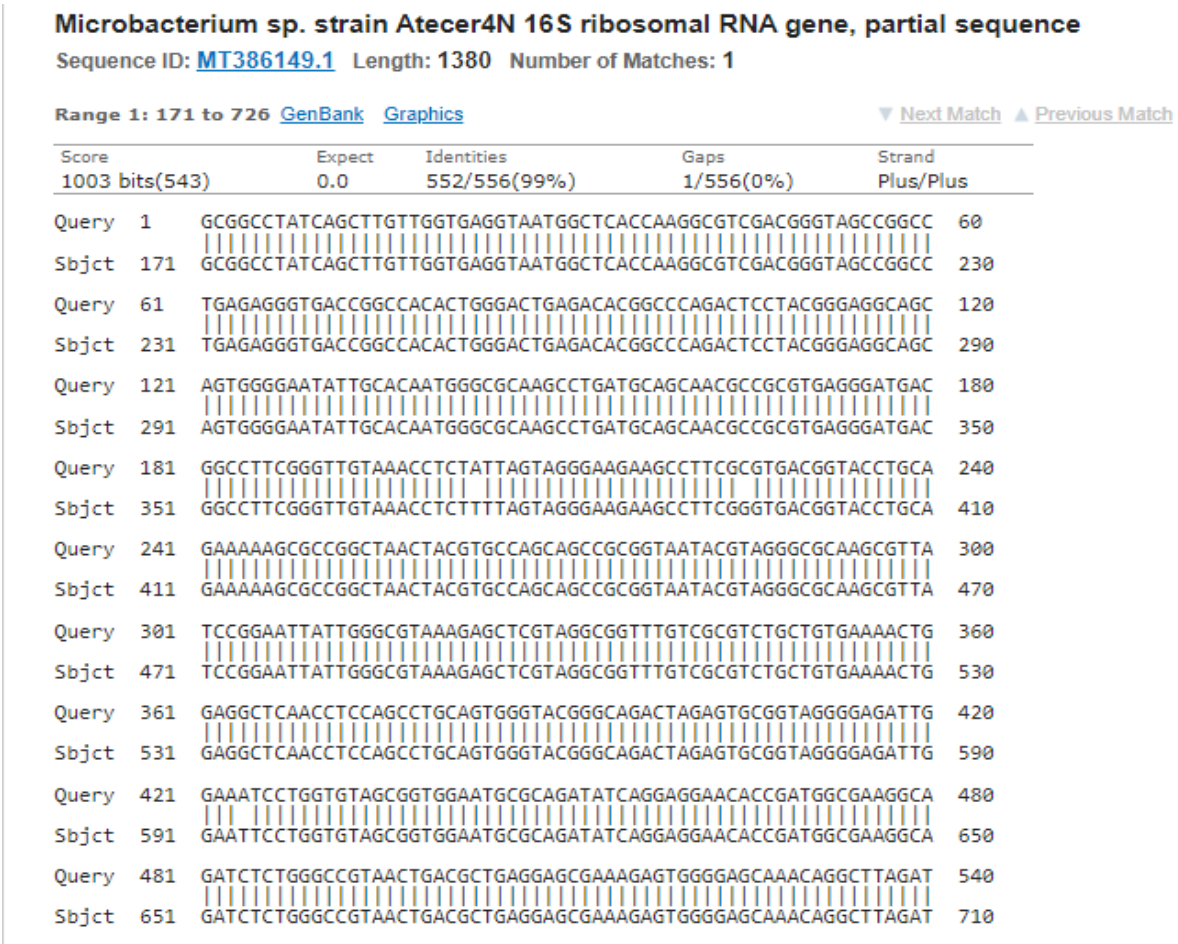

Figure 8. Sequence alignment showing similarity between Trivandrum isolate 1 and the nearest bacterial match.

Identification of Microbacterium species indicates the possible existence of environmental bacteria having survival and adaptation ability to marine host-associated environment. Environmental bacteria belonging to genus Microbacterium are common inhabitants of aquatic environments, involved in the cycling of nutrients (Zhou et al., 2022). In the same way, Trivandrum isolate 2 had maximum similarity with Bacillus cereus.

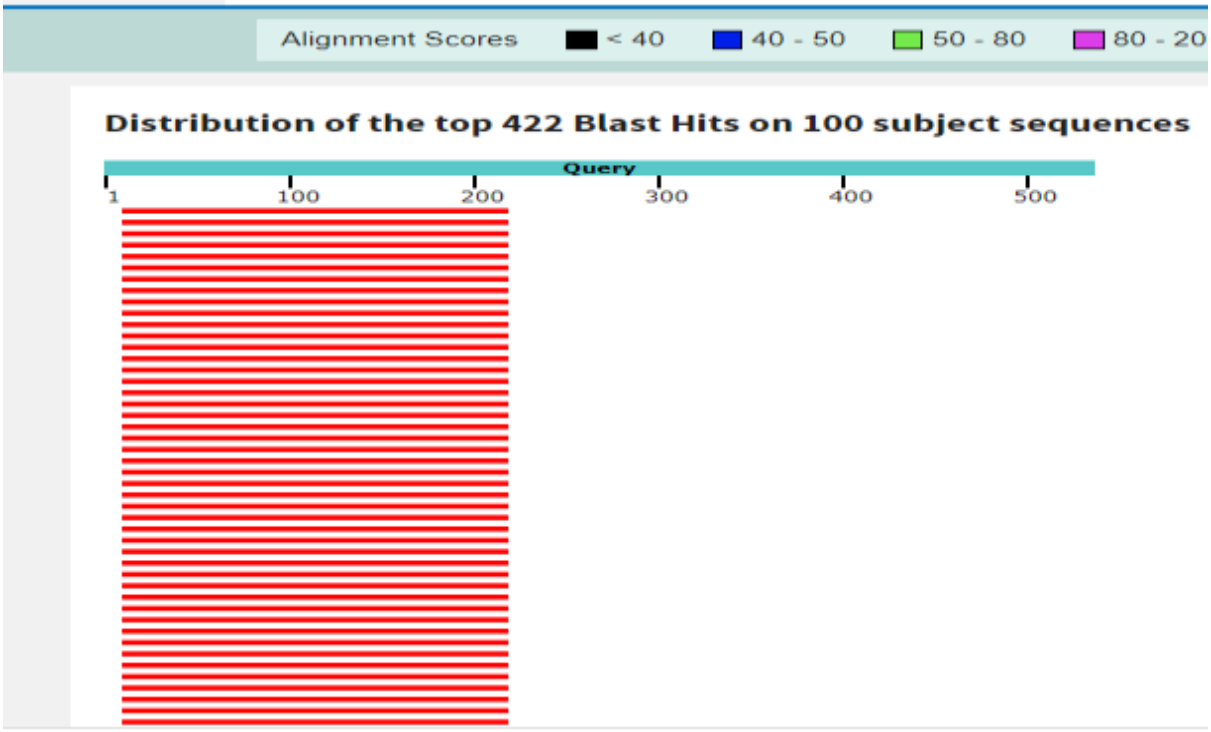

Figure 9. Graphic Representation of Top BLAST Hits Obtained for Trivandrum Isolate 2.

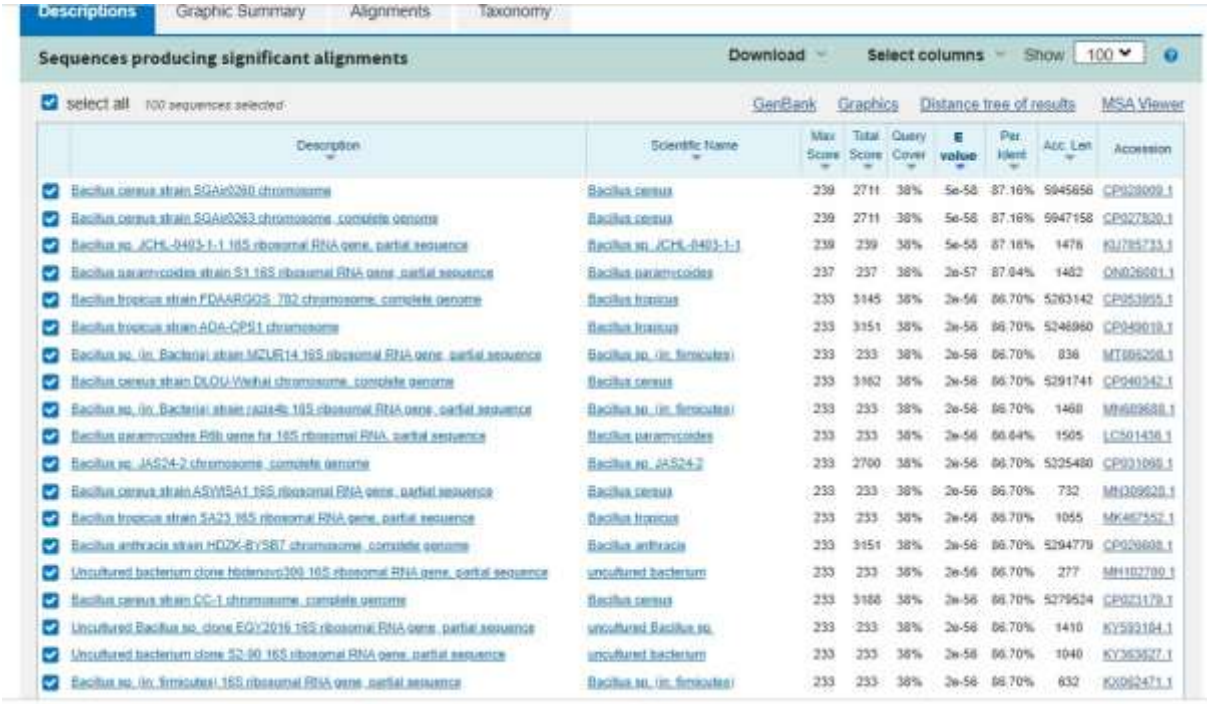

Figure 10. Significant sequence alignments obtained for Trivandrum isolate 2.

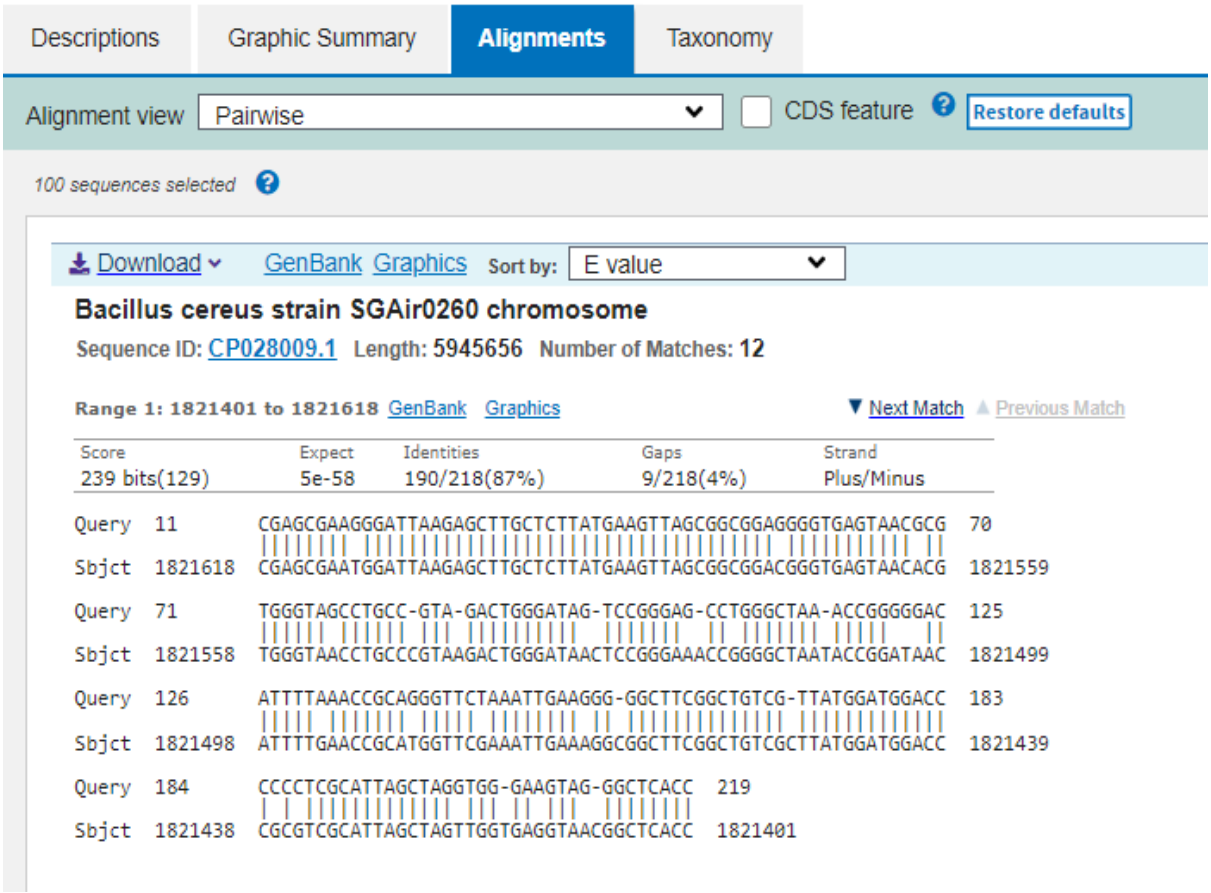

Figure 11. Sequence alignment showing similarity between Trivandrum isolate 2 and Bacillus cereus.

The bacterial species within the genus Bacillus have been isolated from fish gut tracts and are characterized by their ability to secrete extracellular enzymes responsible for the breakdown and digestion of nutrients (Ray et al., 2012). Furthermore, Thoothoor isolate 1 was found to be Bacillus cereus through BLAST search results.

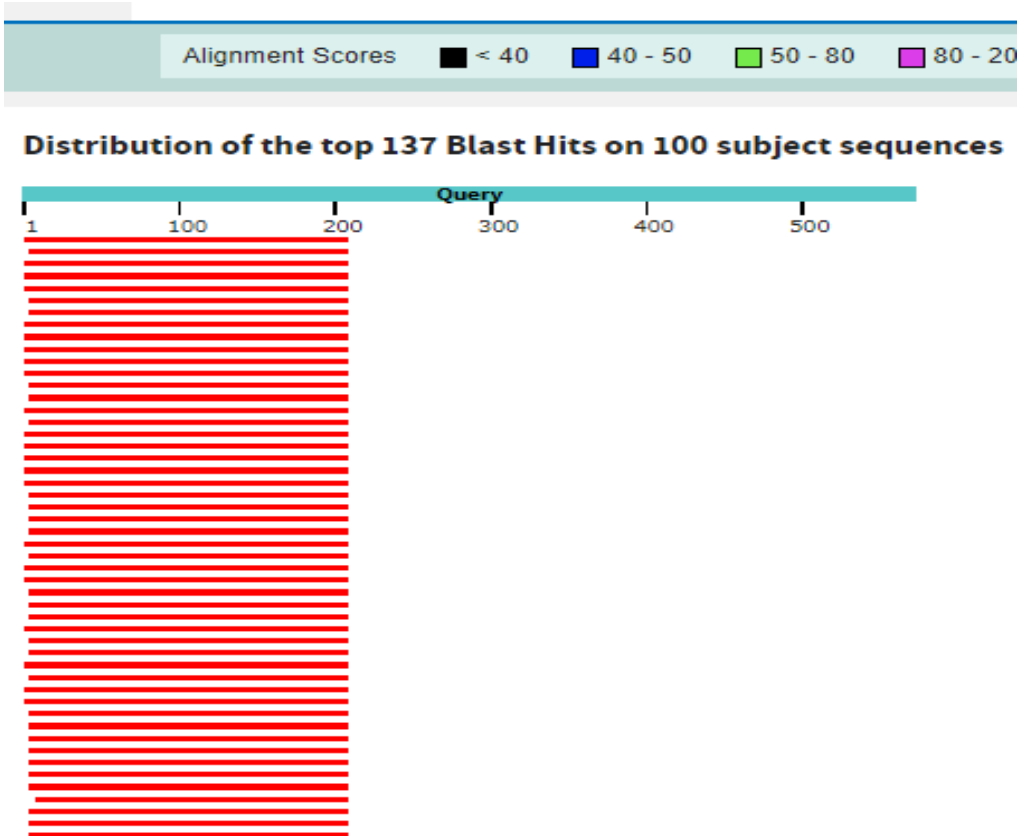

Figure 12. Top BLAST matches for Thoothoor isolate 1.

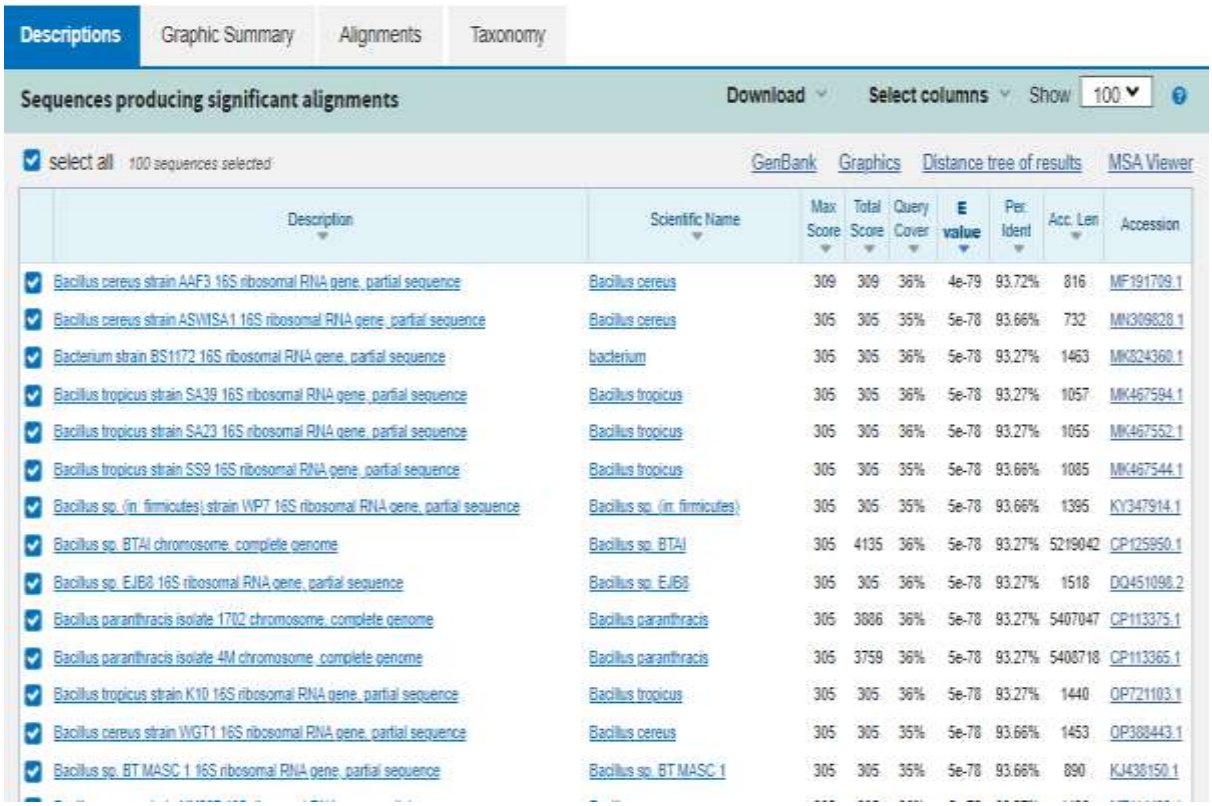

Figure 13. BLAST matches with significant alignments for Thoothoor isolate 1.

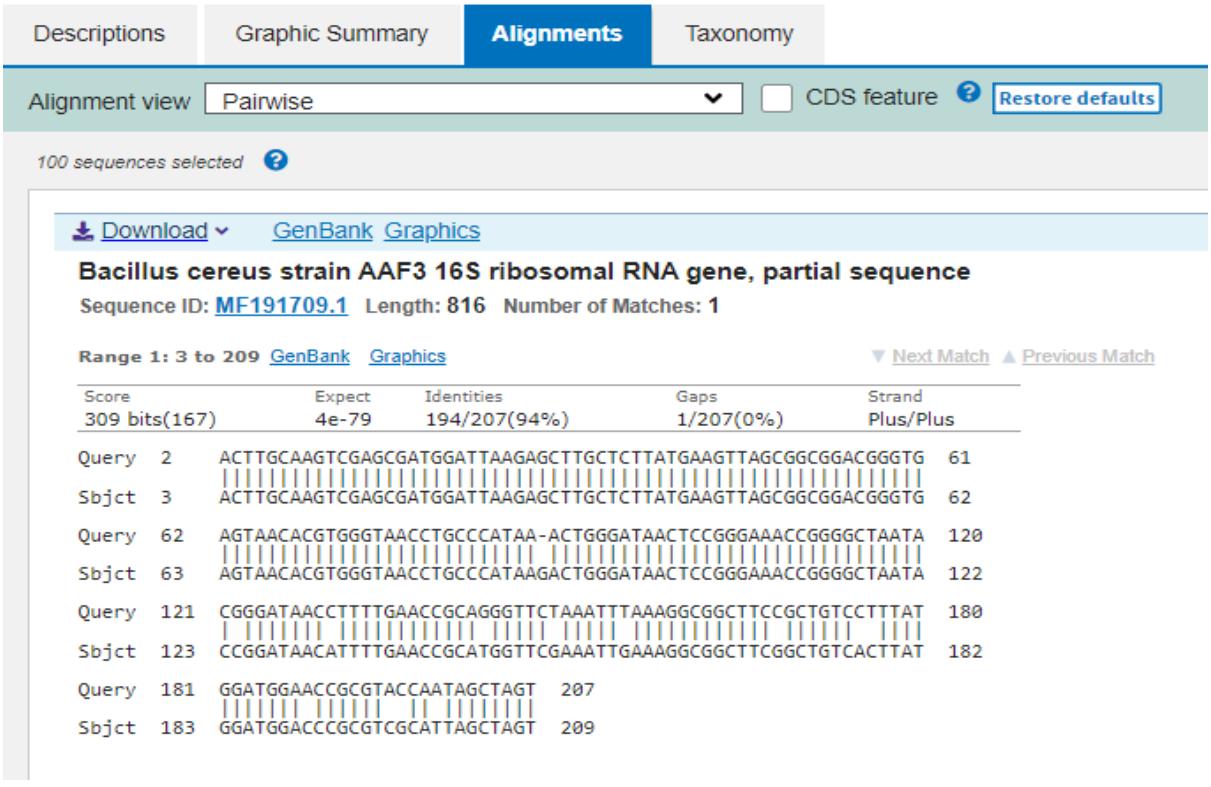

Figure 14. Sequence alignments for Thoothoor isolate 1 and Bacillus cereus.

In view of the findings obtained, it is likely that Bacillus cereus is a common culturable member of the tuna gut microbiome population. Second Thoothoor isolate was found to show highest identity at the sequence level with Lacrimispora amygdalina.

Figure 15. Graphical representation of top BLAST hits for Thoothoor isolate 2.

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Lacrimispora amygdalina strain JB20 16S ribosomal RNA gene, partial sequence	Lacrimispora amygdalina	985	985	100%	0.0	99.27%	1437	MT264911.1
Lacrimispora amygdalina strain JB12 16S ribosomal RNA gene, partial sequence	Lacrimispora amygdalina	985	985	100%	0.0	99.27%	1394	MT264792.1
Lacrimispora amygdalina strain JB4 16S ribosomal RNA gene, partial sequence	Lacrimispora amygdalina	985	985	100%	0.0	99.27%	1414	MT264780.1
Clostridium indicum strain PI-S10-A1B 16S ribosomal RNA gene, partial sequence	Clostridium indicum	985	985	100%	0.0	99.27%	1509	MH193604.3
Bacterium enrichment culture clone OTU1 16S ribosomal RNA gene, partial sequence	bacterium enrichment culture	985	985	100%	0.0	99.27%	1169	KP715407.1
Clostridium indicum strain PI-S10-A1B 16S ribosomal RNA, partial sequence	Clostridium indicum	985	985	100%	0.0	99.27%	1509	NR_180048.1
Lacrimispora amygdalina strain BR-10 16S ribosomal RNA gene, partial sequence	Lacrimispora amygdalina	985	985	100%	0.0	99.27%	1485	ON754640.1
Clostridium sp. AIP 321.05 16S ribosomal RNA gene, partial sequence	Clostridium sp. AIP 321.05	985	985	100%	0.0	99.27%	1362	DQ507245.1
Lacrimispora amygdalina strain BR-10 16S ribosomal RNA, partial sequence	Lacrimispora amygdalina	985	985	100%	0.0	99.27%	1485	NR_115211.1
Uncultured bacterium clone RMAL69 16S ribosomal RNA gene, partial sequence	uncultured bacterium	983	983	99%	0.0	99.26%	1274	JF681680.1
Uncultured bacterium clone RMAL68 16S ribosomal RNA gene, partial sequence	uncultured bacterium	983	983	99%	0.0	99.26%	1270	JF681679.1
Uncultured bacterium clone RMAL61 16S ribosomal RNA gene, partial sequence	uncultured bacterium	983	983	99%	0.0	99.26%	1274	JF681672.1
Uncultured bacterium clone RMAL45 16S ribosomal RNA gene, partial sequence	uncultured bacterium	983	983	99%	0.0	99.26%	1284	JF681656.1
Uncultured bacterium clone RMAL43 16S ribosomal RNA gene, partial sequence	uncultured bacterium	983	983	99%	0.0	99.26%	1274	JF681654.1

Figure 16. Important sequence alignments for Thoothoor isolate 2.

Lacrimispora amygdalina strain JB20 16S ribosomal RNA gene, partial sequence

Sequence ID: MT264911.1 Length: 1437 Number of Matches: 1

Range 1: 272 to 816 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
985 bits(533)	0.0	541/545(99%)	0/545(0%)	Plus/Plus

```
Query  1    CCGACCTGAGAGGGTGACCGGCCACATTGCGACTGAGACACGGCCCAAACTCCTACGGGA  60
            ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  272  CCGACCTGAGAGGGTGACCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGA  331

Query  61   GGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCGACGCCGCGTGACT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  332  GGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCGACGCCGCGTGACT  391

Query  121  GAAGAAGTATTTCGGTATGTATAGGTCTATCAGCAGGGAAGAATATGACGGTACCTGACT  180
            |||||||||||||||||||| |||||||||||||||||||| ||||||||||||||||||
Sbjct  392  GAAGAAGTATTTCGGTATGTAAAGGTCTATCAGCAGGGAAGAAAATGACGGTACCTGACT  451

Query  181  AAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  452  AAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTAT  511

Query  241  CCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGCGATGCAAGTCTGGAGTGAAAGCCCG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  512  CCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGCGATGCAAGTCTGGAGTGAAAGCCCG  571

Query  301  GGGCTCAACCCCGGGACTGCTTTGGAAACTGTGTTGCTAGAGTGCAGGAGAGGTAAGTGG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  572  GGGCTCAACCCCGGGACTGCTTTGGAAACTGTGTTGCTAGAGTGCAGGAGAGGTAAGTGG  631

Query  361  AATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTCGCGAAGGCGG  420
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  632  AATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGG  691

Query  421  CTTACTGGACTGTAACTGACGTTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  692  CTTACTGGACTGTAACTGACGTTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATA  751

Query  481  CCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTTGGGGAGCAAAGCTCTTCGGT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  752  CCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTTGGGGAGCAAAGCTCTTCGGT  811

Query  541  GCCGC  545
```

Figure 17. Sequence alignment depicting identity between Thoothoor isolate 2 and Lacrimispora amygdalina.

Lacrimispora genera consist of anaerobes which undergo fermentation metabolism and can break down organic compounds. Presence of such isolates suggests that anaerobic niches are present in fish intestines (Sivasubramanian et al., 2012). Sequence analysis for Kanyakumari isolate 1 showed identification of Escherichia coli.

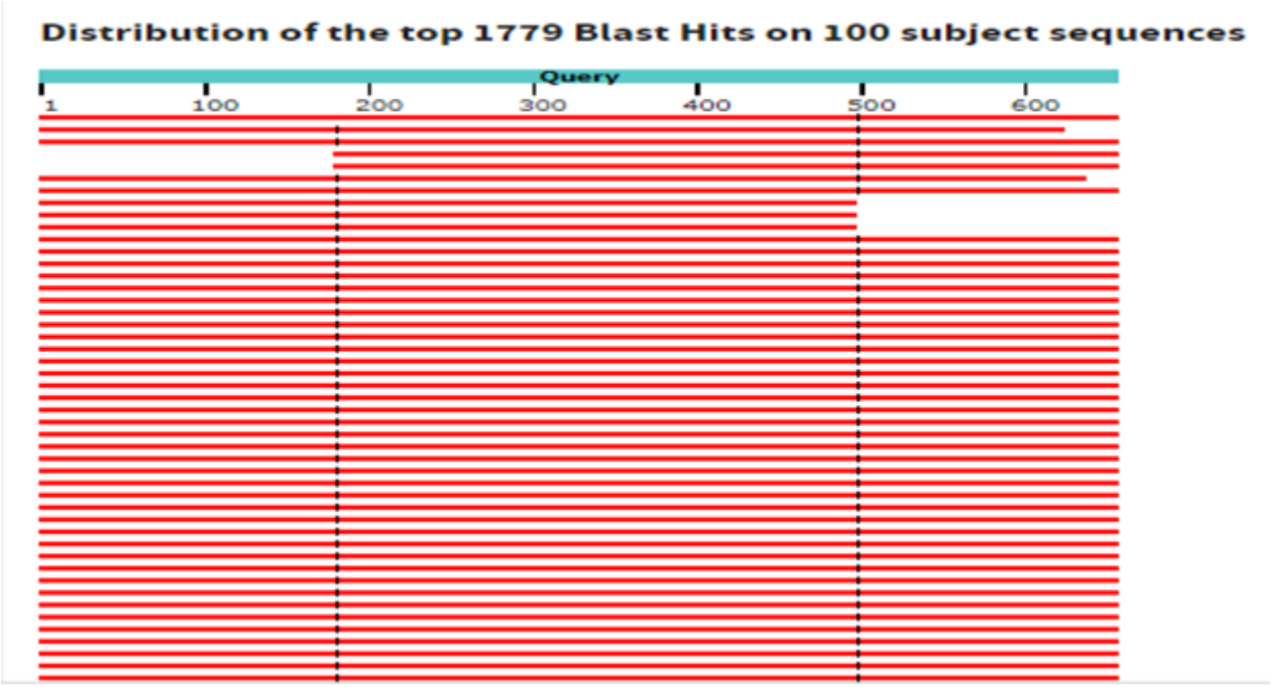

Figure 18. Graphical representation of top BLAST hits for Kanyakumari isolate 1.

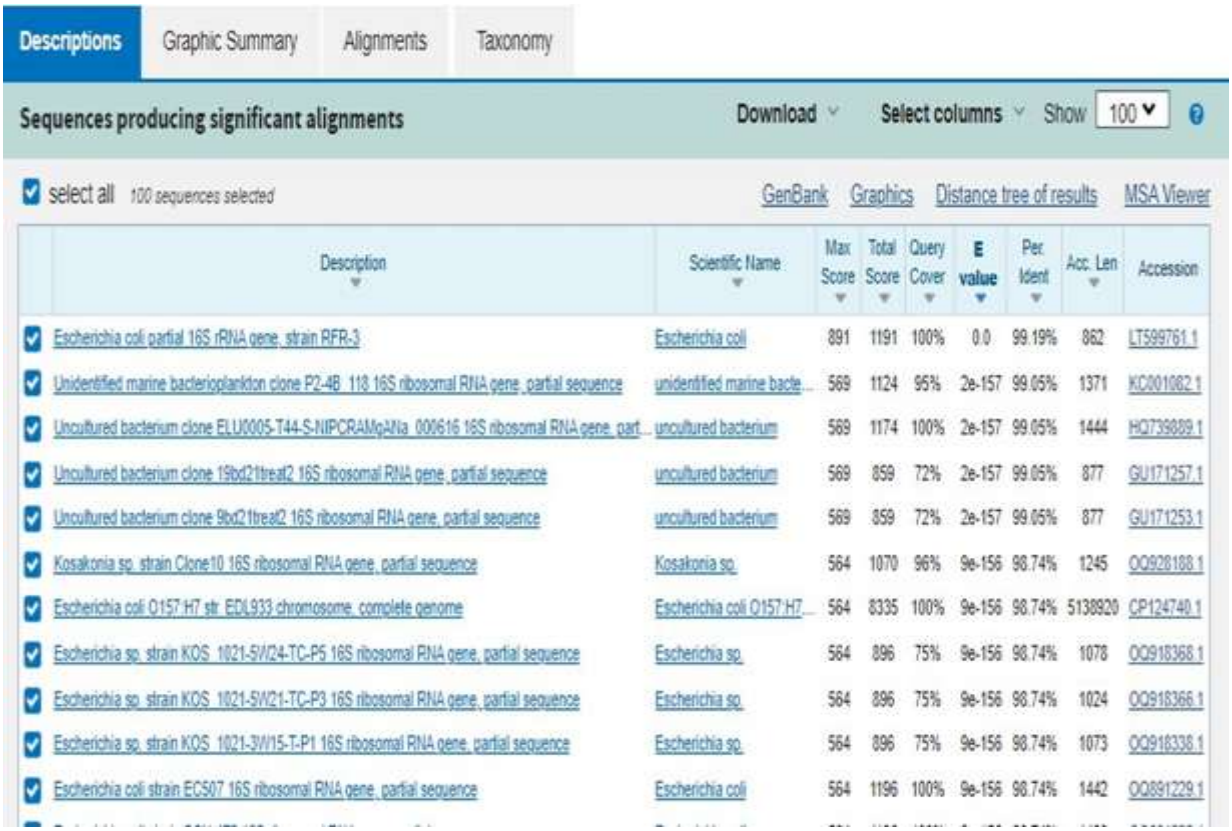

Figure 19. Important sequence alignments for Kanyakumari isolate 1.

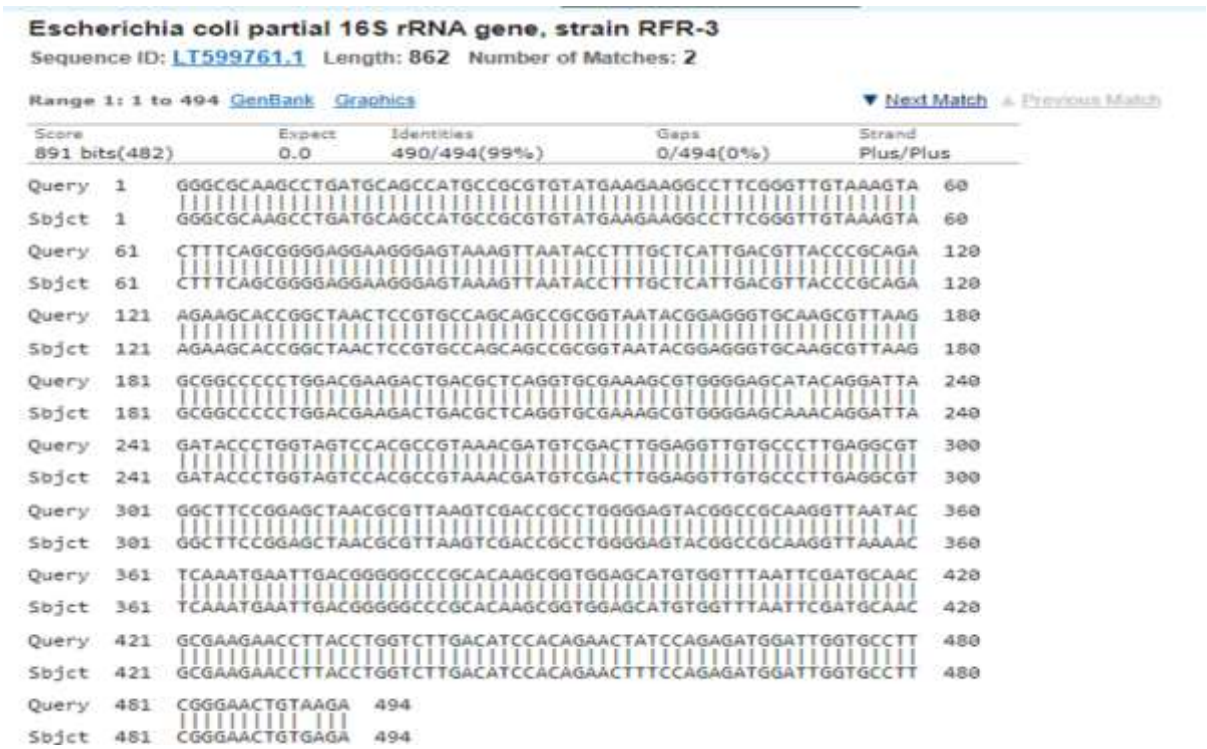

Figure 20. Sequence alignment indicating identity between Kanyakumari isolate 1 and Escherichia coli.

Presence of E. coli could either be due to environmental contact or transient colonization via food consumption, which has been shown earlier in marine fishes (Luna et al., 2012). Maximum similarity of second isolate from Kanyakumari is observed with Oceanisphaera ostreae.

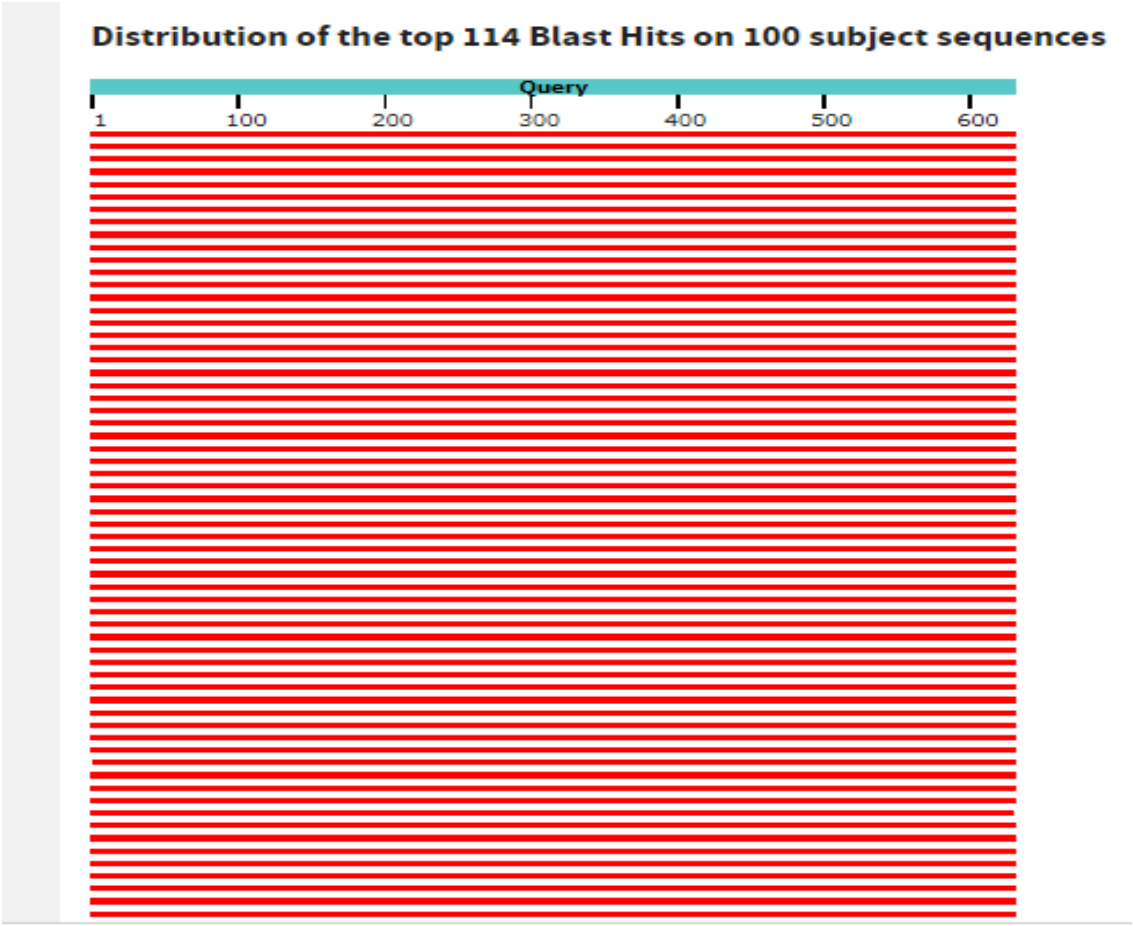

Figure 21. Graphical representation of top BLAST hits for Kanyakumari isolate 2.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Oceanisphaera ostreae strain 20_KNS1_Sed_R1 16S ribosomal RNA gene, partial sequence	Oceanisphaera ostreae	1120	1120	100%	0.0	98.88%	1408	MN080177.1
Oceanisphaera ostreae strain 4_KNS1_Sed_R4 16S ribosomal RNA gene, partial sequence	Oceanisphaera ostreae	1112	1112	100%	0.0	98.72%	1402	MN080153.1
Oceanisphaera ostreae strain T-w6 16S ribosomal RNA, partial sequence	Oceanisphaera ostreae	1112	1112	100%	0.0	98.72%	1465	NR_109099.1
Oceanisphaera sp. 48(2010) 16S ribosomal RNA gene, partial sequence	Oceanisphaera sp. 48(2010)	1112	1112	100%	0.0	98.72%	1498	GQ891117.1
Oceanisphaera sp. SS1.15 16S ribosomal RNA gene, partial sequence	Oceanisphaera sp. SS1.15	1107	1107	100%	0.0	98.56%	1504	KC160849.1
Oceanisphaera sp. SS1.16 16S ribosomal RNA gene, partial sequence	Oceanisphaera sp. SS1.16	1103	1103	100%	0.0	98.41%	1505	KC160850.1
Oceanimonas sp. SS1.31 16S ribosomal RNA gene, partial sequence	Oceanimonas sp. SS1.31	1079	1079	100%	0.0	97.77%	1503	KC160864.1
Oceanimonas sp. SS1.27 16S ribosomal RNA gene, partial sequence	Oceanimonas sp. SS1.27	1079	1079	100%	0.0	97.77%	1503	KC160861.1
Oceanimonas sp. SS1.18 16S ribosomal RNA gene, partial sequence	Oceanimonas sp. SS1.18	1079	1079	100%	0.0	97.77%	1503	KC160852.1
Oceanisphaera sp. SS1.13 16S ribosomal RNA gene, partial sequence	Oceanisphaera sp. SS1.13	1079	1079	100%	0.0	97.77%	1503	KC160847.1
Uncultured bacterium clone XJDN-868 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1074	1074	100%	0.0	97.61%	1485	KJ454331.1
Uncultured bacterium clone XJDN-350 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1074	1074	100%	0.0	97.61%	1516	KJ454073.1
Oceanimonas sp. SS1.23 16S ribosomal RNA gene, partial sequence	Oceanimonas sp. SS1.23	1074	1074	100%	0.0	97.61%	1503	KC160857.1
Oceanisphaera profunda strain SM1222 chromosome, complete genome	Oceanisphaera profunda	1068	8348	100%	0.0	97.45%	3508860	CP021377.1
Uncultured bacterium clone XJDN-562 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1068	1068	100%	0.0	97.45%	1485	KJ454178.1
Uncultured bacterium clone XJDN-146 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1068	1068	100%	0.0	97.45%	1483	KJ453971.1
Oceanimonas sp. SS1.26 16S ribosomal RNA gene, partial sequence	Oceanimonas sp. SS1.26	1068	1068	100%	0.0	97.45%	1504	KC160860.1

Figure 22. Significant sequence alignments identified for Kanyakumari isolate 2.

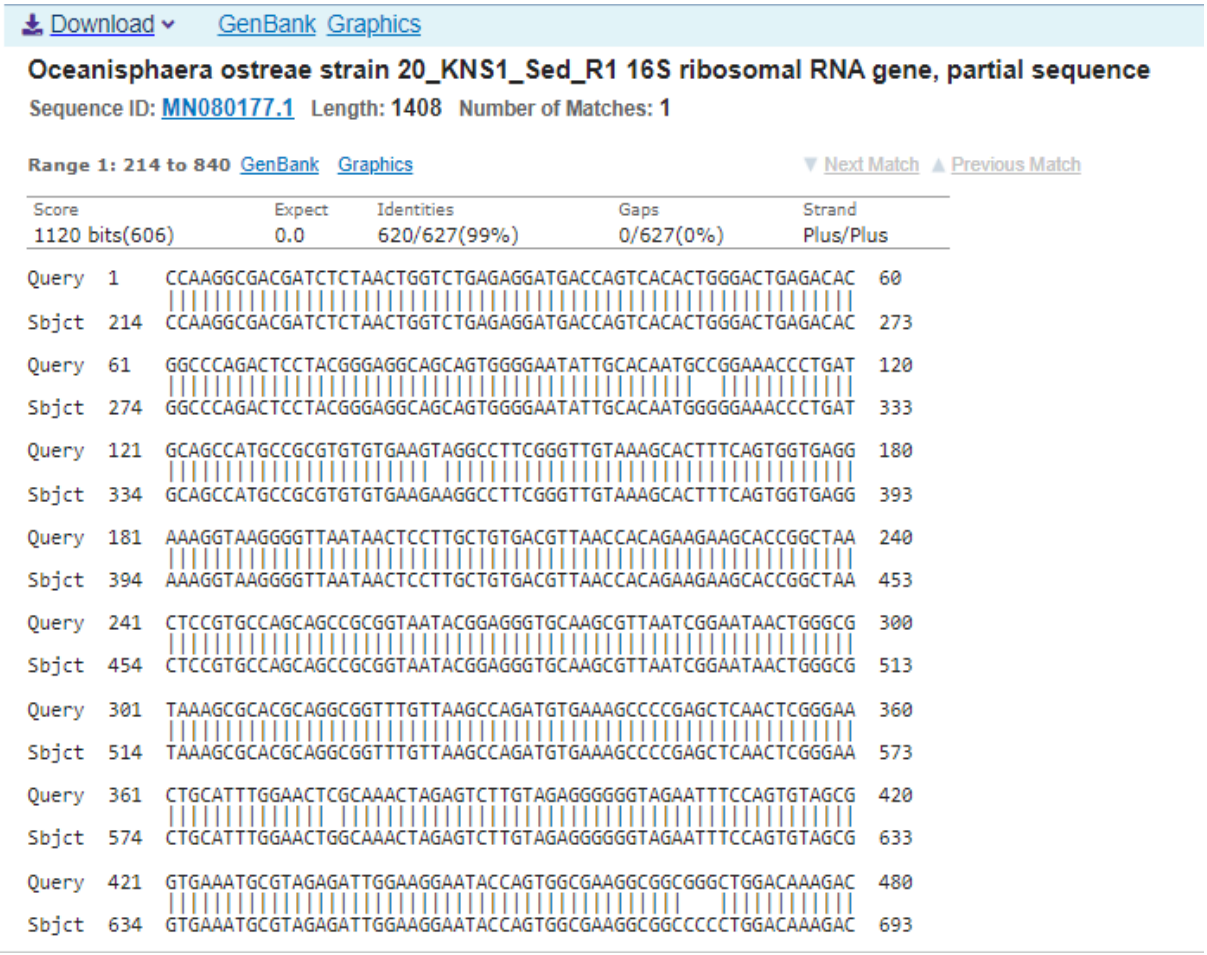

Figure 23. Alignment of sequence demonstrating similarity to Kanyakumari isolate 2 and Oceanisphaera ostreae.

Oceanisphaera ostreae is a bacterium associated with the marine environment that can be found in seawater and marine life forms. It emphasizes yet again the impact of the surrounding marine environment on the bacteria community within the gut of tunas.

Table 2. Bacterial isolates identified through 16S rRNA gene sequencing

Location	Isolate	Identified Species
Trivandrum	Colony 1	Microbacterium sp.
Trivandrum	Colony 2	Bacillus cereus
Thoothoor	Colony 1	Bacillus cereus
Thoothoor	Colony 2	Lacrimispora amygdalina
Kanyakumari	Colony 1	Escherichia coli
Kanyakumari	Colony 2	Oceanisphaera ostreae

The bacterial taxa identified in the present study are consistent with existing literature describing diverse gut microbial communities in marine fishes. Members of the genus Bacillus have frequently been observed within the intestinal tracts of fish and are recognized for their involvement in nutrient breakdown and the production of enzymes (Ray et al., 2012). Similarly, Microbacterium species are commonly found in aquatic environments while Oceanisphaera bacteria are linked to the existence in the marine environment. The detection of different groups of bacteria in samples taken from Thoothoor, Kanyakumari, and Trivandrum suggests the potential impact of environmental factors on gut microbial diversity. Such findings are also supported by previous studies where habitat, food preference, and environment parameters played significant roles in determining the gut microbiome of fish species (Lokesh & Kiron, 2016; Egerton et al., 2018; Cao, 2025).

4. CONCLUSION

Gut microorganisms associated with E affinis and T albacares were isolated and identified from specimens obtained from three geographic regions, which included Thoothoor, Kanyakumari, and Trivandrum. The gut tissues were homogenized, serially diluted, and then inoculated in Nutrient Agar medium to isolate pure colonies of bacteria. The selected colonies were purified through subcultures, preserved as stocks, and grown in liquid media for further molecular studies. Genomic DNA was extracted from bacterial cultures and subjected to molecular characterization by 16S rRNA gene amplification and sequencing. Sequences were compared with the NCBI BLAST database to identify bacteria. Sequence analyses showed the presence of Bacillus cereus and Microbacterium species in samples from Trivandrum, while those from

Thoothoor had Bacillus cereus and Lacrimispora amygdalina. On the other hand, Kanyakumari samples had isolates such as Escherichia coli and Oceanisphaera ostreae. These results show that there are diverse culturable bacterial communities found inside the intestines of tuna fishes, with differences in bacterial community composition from different geographic locations. The study provides baseline information of culturable bacteria in the gut microbiome of E. affinis and T. albacares, and helps us gain more knowledge regarding microbe diversity in tuna fish populations.

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