4. RESULTS

4.1 QUANTITATIVE ANALYSIS OF BACTERIA

To know the importance of quality of fishes caught in the coastal areas, the marine fishes collected from the landing centres of polluted and non polluted areas such as Cuddalore and Parangipettai was taken in the present study with respect to nature, management and standard fish quality for human consumption. In the present study, the quantitative and qualitative analysis of the bacterial isolates, antibiotic resistance and curing of plasmids with acridine orange and plasmid isolation and their molecular weight were analysed during October 2011 to September 2012. The results are as follows,

4.1.1 *Rastrelliger kanagurta*

The samples of *Rastrelliger kanagurta* collected from sampling sites showed higher bacterial counts in Station II i.e. Cuddalore when compared with Parangipettai.

**Station- I (Parangipettai)**

The seasonal bacterial count of skin samples in *Rastrelliger kanagurta* showed maximum \(2.8\times10^{-4}\) in summer and minimum \(5\times10^{-3}\) during premonsoon period. The gill samples ranged from \(1.13\times10^{-6}\) to \(4.0\times10^{-5}\) in summer and postmonsoon seasons, gut samples also showed maximum count
of $2.40 \times 10^{-6}$ in summer and while minimum count of $8.3 \times 10^{-4}$ at pre monsoon which is highly significant ($P<0.001$).

**Station- II (Cuddalore)**

The bacterial count of skin varied from $6.0 \times 10^{-4}$ to $1.97 \times 10^{-6}$ in premonsoon and monsoon seasons. The count in gill samples was maximum of $2.72 \times 10^{-6}$ in postmonsoon and minimum of $1.46 \times 10^{-6}$ in premonsoon while gut bacterial count was found maximum ($2.66 \times 10^{-6}$) in postmonsoon and minimum ($1.58 \times 10^{-6}$) in premonsoon. From the above result, the statistical analysis revealed that it is statistically significant ($P<0.05$).

The bacterial count in skin, gill and gut samples of *Rastrelliger kanagurta* had maximum counts in summer only at station I, however it varied in station II where the count was maximum in monsoon and postmonsoon. The minimum counts showed variations at station I in premonsoon and postmonsoon and in station II it was recorded only in premonsoon for all samples of fish organs. The bacterial counts were found in an increasing order of skin> gill >gut samples of *Rastrelliger kanagurta* (Table: 1 & 2) (Fig. 1).

### 4.1.2 *Lates calcarifer*

The specimens of *Lates calcarifer* collected from both the stations had more bacterial counts in Station II (Cuddalore) when compared with Station I (Parangipettai).
Station- I (Parangipettai)

The seasonal bacterial count of skin samples of *Lates calcarifer* varied from $2.2 \times 10^{-4}$ to $4.0 \times 10^{-4}$ during summer and premonsoon period respectively. The gill samples showed lower value ($1.31 \times 10^{-6}$) in postmonsoon and higher value ($1.43 \times 10^{-5}$) in summer seasons. The gut samples showed minimum count of $2.4 \times 10^{-4}$ in postmonsoon and maximum count of $1.60 \times 10^{-6}$ in premonsoon. Highly significant difference was observed ($P<0.001$).

Station- II (Cuddalore)

The bacterial count of skin ranged $1.17 \times 10^{-6}$ to $5.3 \times 10^{-4}$ in monsoon and summer respectively, while in gill was minimum of $9.1 \times 10^{-6}$ in premonsoon and maximum of $1.84 \times 10^{-6}$ in summer seasons and gut bacterial count was maximum ($2.35 \times 10^{-6}$) in summer and minimum ($4.6 \times 10^{-6}$) in postmonsoon and there was no significant changes in statistical analysis at $P<0.05$.

In station I and II there was a definite trend of seasonal variation of bacteria observed. The bacterial count of samples showed increase in the order of skin, gill and gut regions (Table: 3 & 4) (Fig. 2).

4.1.3 *Lutjanus fulviflamma*

The results of total bacterial count found in skin, gill and gut regions of *Lutjanus fulviflamma* were found to be maximum in Cuddalore and minimum in Parangipettai region.
Station- I (Parangipettai)

The seasonal bacterial counts of skin samples were found between $4.5 \times 10^{-4}$ to $1.5 \times 10^{-4}$, which showed maximum counts in summer and minimum during monsoon period. Whereas in gill samples the bacterial count varied between $1.34 \times 10^{-6}$ to $3.86 \times 10^{-5}$ during summer and premonsoon respectively. The gut samples showed maximum count of $1.24 \times 10^{-6}$ and minimum count of $1.05 \times 10^{-6}$ in summer and post monsoon seasons. The data obtained from the samples showed a significant difference at $P < 0.05$ and was highly significant at $P < 0.001$.

Station- II (Cuddalore)

The bacterial count in skin ranged a maximum of $1.53 \times 10^{-6}$ and minimum of $8.5 \times 10^{-4}$ during summer and post monsoon periods respectively. In gill samples it showed maximum of $2.24 \times 10^{-6}$ and minimum of $1.06 \times 10^{-6}$ during summer and premonsoon, while gut bacterial count varied between $1.91 \times 10^{-6}$ and $1.22 \times 10^{-6}$ in summer and post monsoon respectively. Statistical analysis was not significant ($P < 0.05$).

The data obtained from the present study revealed that both the stations registered maximum bacterial count in skin, gill and gut samples of *Lutjanus fulviflamma* during summer while minimum counts were reported during other
seasons. The bacterial counts were found in an increasing order of skin, gut and gill of *Lutjanus fulviflamma*. (Table: 5 & 6) (Fig: 3).

4.2 QUALITATIVE ANALYSIS OF BACTERIA

4.2.1 Morphological characterization

Gram staining

Gram staining was performed and the organisms isolated were identified as gram negative (*Micrococcus* spp, *Staphylococcus* spp, *Bacillus cereus* and *Paenibacillus alvei*) and gram positive (*Vibrio parahaemolyticus*, *Aeromonas hydrophila* and *Pseudomonas flourescens*).

Motility

The organisms isolated were identified as motile and non-motile which showed diffused growth along the line of inoculation (*Vibrio parahaemolyticus*, *Aeromonas hydrophila* and *Pseudomonas flourescens*, *Bacillus cereus* and *Paenibacillus alvei*) and absence of diffuse growth along the line of inoculation *Micrococcus* spp, *Staphylococcus* spp.
4.2.2 Biochemical Characterization

IMViC TEST

Indole production

The ability of the organisms to break down tryptophan and the release of Indole was tested by loopful of organism in Indole medium containing tryptophan. Indole production is detected as appearance of red colour ring (occurring within a few seconds) or pink colour shows positive result with an addition of Kovacs reagent where as other organisms showed negative results.

Methyl red test (MR)

The organisms inoculated in MR-VP broth were incubated at 37°C for 24-28 hours, to these 5-6 drops of Methyl red reagent was added and gently mixed, which gives the appearance of red colour as positive and organisms with no colour formation or yellow colour indicated as negative results.

Voges- Proskauer test

The organisms were inoculated in VP broth are incubated at 37°C for 24-28 hours. To this reagent A and reagent B were added, red colour indicated as positive results, no colour formation in the organisms indicates as negative results.
**Citrate utilisation test**

The ability of the organisms to utilize citrate as a sole carbon source was tested by streaking a loopful of culture in a simmons citrate slant. The medium turned blue, indicated positive results that the organisms could utilize citrate as a sole carbon source, the medium that has retained the green colour indicates that organism could not utilize citrate as sole carbon source as negative results.

**Catalase test**

The organisms are taken with a loop from 24 hours bacterial culture was transferred to a clean glass slide to this 1-2 drops of the Hydrogen peroxide was added to the bacterial cells, brisk effervescence was observed in organism indicated that they were positive for catalase production and the absence of gas formation indicates as negative results.

**Oxidase test**

The ability of the organism to release oxidative enzymes was tested by Oxidase disc taken in a clean microscopic slide, placed one or two drops of culture on the disc, appearance of deep purple colour in the disc indicates the positive reaction for oxidase production and the organisms which showed no colour change indicated negative reaction.
Nitrate test

Nitrate reduction by the organisms was investigated by transferring 1 ml of the culture grown for 2 – 3 days in nitrate broth into a clean test tube by adding Reagent A and Reagent B. Presence of red colour in an organism indicates as positive for nitrate reduction, whereas no colour change as negative result of nitrate reduction.

Urease test

The ability of the organism to release urease was tested by streaking a loopful of culture in agar medium, turned as yellow to red which indicated the release of urease in the organism; no colour change indicates the negative results.

TSI test

A loopful of culture was streaked and stabbed on the TSI slants and was incubated at 37°C for 24 hours, black colouration of some organisms indicates the presence of H₂S production, and some showed negative reaction. Some organisms showed acid production due to glucose or lactose fermentation.

4.2.2.1 Bacterial flora of fishes

Biochemical characteristics of the gram positive and gram negative bacterial spp. were isolated from the examined fishes. Results indicated that from the three edible fishes of two stations nearly 16 bacterial species were
identified as gram positive and gram negative bacteria such as *Micrococcus lylae, Micrococcus luteus, Micrococcus sedentarius, Micrococcus nishimoniaiseis, Staphylococcus aureus, Staphylococcus saprophyticus, Pseudomonas fluorescens, Aeromonas hydrophila, Vibrio parahaemolyticus, Bacillus cereus* and *Paenibacillus alvei*.

The bacterial flora isolated from *Lutjanus fulviflamma, Rastrelliger kanagurta, Lates calcarifer* of skin, gill and gut samples collected from Parangipettai and Cuddalore were same but varied in their bacterial counts.

### 4.2.3 Molecular characterization

In the present study 16s rRNA sequencing was used to differentiate between the isolates, as it is difficult to identify the representatives of bacterial flora of fishes by using morphological and biochemical criteria. Thus, 16s rRNA gene is a highly preserved region with small changes that can be the characteristic of different species. Ribosomal genes are compared with bacteria in most of the taxonomical studies. Molecular identification of the isolates was done by the sequencing of 16S rRNA gene. The presence of 1,500 bp product was observed by agarose gel electrophoresis confirmed the amplification of 16S rRNA gene.

**BLAST- sequence analysis**

The PCR product was gel eluted and sequenced. The partial 16S rRNA sequencing analysis of the bacterial isolate N3 and N12 was compared for
homology from the NCBI GenBank databases using Basic Local Alignment

**Nucleotide accession number**

The nucleotide sequence of 16S rRNA gene of bacteria isolated from
fishes was deposited in Gene bank.

### 4.3 DETERMINATION OF ANTIBIOTIC SUSCEPTIBILITY

Nowadays antibiotic resistance is a significant worldwide health
problem. From the present study wide incidence of antibiotic resistance in the
bacterial strains randomly collected and isolated from marine fishes of skin, gill
and gut regions of N3 and N12 were selected from the 13 bacterial isolates.

Bacterial isolate N3 and N12 were tested with antibiotics disc containing
ampicillin (10µg), oxacillin (5µg), methicillin (5µg), amikacin (30µg) and
rifampicin (5µg) in which the isolate N3 showed sensitivity to all the
antibiotics used where as N12 showed resistant pattern to antibiotics. N3-
*Micrococcus sedentarius* were highly sensitive to ampicillin and rifampicin
followed by oxacillin, amikacin and methicillin.

### 4.4 CURING OF PLASMID USING ACRIDINE ORANGE

When acridine orange mediated plasmid curing was done, resistant
against antibiotics disc containing ampicillin, oxacillin, methicillin, amikacin
and rifampicin to N12 - *Paenibacillus alvei* bacterial isolate were lost which indicated the presence of plasmids. The principle behind this test to use the acridine orange is to knock out plasmid factor responsible for the resistance of the isolate.

4.5 PLASMID DNA ISOLATION BY ALKALINE LYSIS METHOD

Plasmid was extracted from the multiple drug resistance *Paenibacillus alvei* and run on agarose gel electrophoresis along with the 1 kb ladder and the bands were observed in the gel documentation system. The molecular weight of the isolated plasmid DNA was found to be 2750 bp and 6000 bp.

4.6 BIOINFORMATICS ANALYSIS.

Multiple sequence alignment, distances and clustering with Neighbor-joining method were performed using MEGA5 for the obtained inter and intra species relationships. Advancement in molecular biology and DNA sequencing techniques has enabled us to characterize the genomes of various organisms rapidly. Thus, analyses of the DNA sequences of various species are providing valuable information about their taxonomy, gene makeup and utilizations (Fig. 11 & 12). The phylogenetic analysis of 16S rDNA sequence of the isolates along with the sequences retrieved from the NCBI was carried out with MEGA 5 using the neighbor-joining method. The result of phylogenetic analysis showed distinct clustering of the isolates and confirms the results of the sequence similarity analysis.
Micrococcus sedentarius showing the phylogenetic neighborhood in a 16S rRNA based tree showing (fig.11). Analysis of the 16S rRNA gene copies in the genome differed by one nucleotide from another, and by up to two nucleotides from the previously published 16S rRNA sequence. The tree was rooted with members of the neighboring family. The branches are scaled in terms of the expected number of substitutions per site.
A phylogenetic tree was constructed using the Neighbor-Joining method for partial sequences of genes encoding 16S rRNA derived from sequenced genomes of *Paenibacillus alvei*, along with the sequences of members from the *Bacillus* spp. Phylogenetic analysis of *Paenibacillus alvei* was performed using MEGA5 with the Neighbor-Joining method. The bar represents the unit length of the number of nucleotide substitutions per site.