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METABOLOMIC VARIATION STUDY ON THE GENETICALLY VARIANT MICROBIAL STRAINS OF *BORASSUS FLABELLIFER* SAP COLLECTED FROM KANYAKUMARI AND TIRUNELVELI DISTRICTS

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ABSTRACT

Borassus flabellifer (Palmyra tree) has a unique specificity with gallons of medicinal properties in it. Contemporary science has proved that consumption of fruits, leaves, bark, and roots has a role to play in boosting as well as disease-preventing benefits because of a few substances, namely phytochemicals, viz., polyphenols, vitamins, minerals, proteins, etc. (Prasad *et al.* 2016). Palm sap is a non-alcoholic, refreshing natural beverage that has a plethora of health benefits of various natures. As the sap is rich in microflora, it is vulnerable to fermentation instantaneously after the harvest. Toddy palm nectar (TPN) is a naturally fermented sap from young and matured inflorescences of *Borassus flabellifer* Linn. The palm sap fermentation process is controlled by a complex microbial community that evolved during natural fermentation. In the present research, the bacterial strains present in the Palmyra sap responsible for the fermentation of Palmyra sap to toddy were collected from two different geographical communities of Kanyakumari and Tirunelveli Districts, Tamilnadu and were investigated using metagenomics DNA molecular studies and their phylogenetic variation were analyzed using Bioinformatics Tools. The results reported that the Bacterial strains responsible for fermentation was present predominantly in the Palmyra sample collected from Kanyakumari District compared to the samples collected from Tirunelveli District. Comparative metagenomics analysis revealed the presence of beneficial probiotic bacteria in the sap, with a predominance of Proteobacteria and Firmicutes, highlighting the saps potential probiotic benefits.

Introduction

B. flabellifer, a dioecious monocotyledonous woody perennial tree in the family Arecaceae, is a massive palm with a single stem reaching 30 m in height and large fan-shaped leaves spanning 4–6 m in diameters. In India the wealth of Palmyra palm is very rich with a population nearly 122 million palms (Vengaiah *et al.,* 2012). India's rich biodiversity and ethno culinary traditions offer a vast landscape for microbial bioprospecting (Tamang *et al.,* 2021). Among traditional beverages, palmyra palm nectar, locally known as

Pathaneer in Tamil Nadu, is a naturally fermenting sap extracted from the inflorescence of *B. flabellifer Linn,* a culturally significant palm species prevalent in South and Southeast Asia. Fresh Pathaneer is a mildly sweet, translucent liquid with a near-neutral pH (6.0–7.0), rich in carbohydrates, amino acids, vitamins, organic acids, and polyphenols (Lasekan and Abbas, 2010; DebMandal and Mandal, 2011). The quality and quantity of palm sap collected in each cylinder vary based on factors such as hygiene maintenance, preservatives used during harvesting, environmental conditions, tapping duration, palm variety, and soil fertility (Hebbar *et al.,* 2018; Sarkar *et al.,* 2023). Fermented palm nectar is a relatively untapped source for exploring exopolysaccharide (EPS) production and β-galactosidase activity. The commercial interest in β-galactosidase-producing probiotics has grown due to their potential application in managing lactose intolerance (Srinivash *et al.,* 2023). Probiotic strains are often characterized by their ability to withstand harsh gastrointestinal conditions, including low pH, bile salts, and digestive enzymes. In addition, their functional efficacy is evaluated through properties such as co-aggregation, auto-aggregation, cell surface hydrophobicity, antioxidant function, adhesion to intestinal epithelial cells, possession of inhibitory effects towards enteric pathogens, and immunomodulatory potential (Saadat *et al.,* 2019). During natural fermentation, the composition of microbiota in palm saps may change their dynamics (Kouamé *et al.,* 2020) due to the fluctuation of micro-environments (pH, alcohol content, sugar profile and organic acids) (Karamoko *et al.,* 2012), Predictive functional profiles of microbial communities in fermented foods is an appropriate approach to annotate the predictive metabolic pathways in gene sequences of bacteria and fungi (Ortiz-Estrada *et al.,* 2019). Hence, in our present study, we aimed to study the bacterial community present in the natural fermentation of *Palmyra sap* collected from Kanyakumari and Tirunelveli Districts of Tamilnadu in India by high-through put sequencing tool and predict the functional profiles of bacterial genes by using the bioinformatics pipelines.

Materials and Methods

Collection of Palmyra palm sap samples

Fresh palmyra palm sap samples were collected from two geographically distributed areas, namely Kanyakumari and Tirunelveli districts of Tamil Nadu. *Borassus flabellilfer* is extensively found in the Kanyakumari region, particularly in Karungal, where the

extraction of sap reaches its peak from August to November. In the Tirunelveli region, it is widely distributed in the area of Aral, and the extraction of sap is high from April to June. Palm sap is tapped from the mature, unopened inflorescence of the palm. The palm sap was collected by cutting the head of the inflorescence. In rural areas, palm sap was traditionally collected from palmyra trees by organized practice for its local consumers.

The fresh samples were collected in the early morning by professional tappers. As soon as the collected fresh palmyra sap samples were brought down from the palmyra tree, they were transferred to sterilized bottle, packed in a sterile ice box to avoid fermentation during transportation, and transported to the laboratory for analysis. On reaching the laboratory, the palmyra sap samples were stored in the refrigerator at about 4ºC for further analysis.

Figure. 1. Collection of sap from *Borassus flabellifer*

Metagenomic Analysis

Metagenomic analysis was performed to detect the presence and diversity of microbes in the Palmyra sap sample collected from two different geographical locations viz., Kanyakumari and Tirunelveli Districts. Initially, the total DNA was isolated from the Palmyra sap sample using the soil DNA isolation kit from MagGenome. Following this, the DNA was isolated using the manufacturer's

protocol (http://www.maggenome.com/wp-content/uploads/2022/04/XpressDNA-Soil-kit.pdf).The quality of the obtained DNA was analysed using the absorbance readings in a nanodrop. A_{260}/A_{280} ratio of 1.8 was considered of high quality and a total yield of 1 µg was considered fit for sequencing.

Sequencing of the isolated DNA

The analysis of the sequenced fragments was done by Avant 3100 Gene Analyzer. DNA sequences were determined by the chain termination method using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems).Gene sequence of closely related species retrieved from GenBank was used to compare the 16S rRNA gene sequence of strain.

Analysis of the metagenome sequences

The tool used for comparison of 16S rRNA analysis of organisms isolated from palmyra sap sample and classification using the Quantitative Insights into Microbial Ecology (QIIME) software package (Cole *et al.* 2014). The gene sequences of 16S rRNA reads from palmyra sap sample metagenomes were compared with the computational resources and speed of QIIME 2's q2-feature-classifier in generating the three main 16S rRNA databases: Green genes, SILVA, and RDP.

Result and Discussion

A metagenomic approach was used to find out the phylogenetic relationships of the sequenced gene with taxonomic groups of microorganisms known in the database. In this case, phylogenetic clusters like the 16S rRNA gene sequence are targeted where operational taxonomic units (OTUs) (Franzén *et al.* 2015) are compared against their amplitude to estimate the microbial species abundance in that particular environment (Edgar, 2018). Probiotics, which are live beneficial bacteria or yeasts, have gained significant attention in recent years due to their numerous health-promoting properties, such as competitive inhibition of pathogenic bacteria, immune response regulation, assistance in nutrient metabolism, and even improvement of human cognitive function (Rianda *et al.*2019). In the past decades, probiotic research has rapidly increased due to the improvement of multi-omics approaches such as culturomics, genomics, and

transcriptomics (Rebollar *et al.* 2016). Palmyra palm sugar is a local beverage produced by palmyra palm (*Borassus flabellifer* Linn.). The juice is popular in tropical Asian countries, including the south of Thailand (Naknean *et al.* 2010). The juice and products from palmyra palm sugar are an important source of natural bacteria, including LAB. Lactic acid bacteria isolated from fermented palmyra palm sap are considered to be probiotics. (Sornsenee *et al.* 2021). Several studies have investigated the diversity of *Lactobacillus* species present in palmyra sap. For instance, a study conducted by Mitsuwan *et al.* (2022) characterized different lactic acid bacteria species from palmyra sap collected from 30 Palmyra sugar samples. The study identified three different lactobacillus species, namely *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus fermentum,* from the palmyra sap samples. Das and Tamang (2021) carried out computational analysis of metagenomes based on KEGG and MetaCyc databases in the fresh palm saps and fermenting saps during toddy fermentation and found the presence of Firmicutes (78.25%) was the most abundant phylum, followed by Proteobacteria (21.57%). Leuconostoc was the most abundant genus in the early stages of fermentation. Torulaspora, Lachancea, and Starmerella showed their heterogeneous distribution throughout the fermentation. Another study conducted by Pammi *et al.* (2021) also investigated the *Lactobacillus* diversity in palmyra sap from various regions in Tamil Nadu. The study identified four indigenous lactic acid bacteria strains, mainly belonging to the Lactobacillus *plantarum* group. Prathiviraj *et al.* (2022) revealed the bacterial diversity and metabolic profile of Indian palm wine using next-generation sequencing of the V3-V4 regions of the 16S rRNA gene. The metagenomic sequencing reveals the dominance of the phyla Proteobacteria, Firmicutes, and Tenericutes in the Indian palm wine.

The progress of research on metagenomics over the years aims in understanding the genome of uncultured microbes isolated from the environment. In the present study, the metagenome workflow was based on culture-based profiling of the bacteria present in the palmyra sap samples collected from two different localities, viz., Kanyakumari and Tirunelveli District. This study represents the experimental data generation and analysis of the 16S rDNA-based metagenome of the DNA extracted from the Palmyra sap samples for metagenomics analysis. For culture-independent metagenomics profiling of Palmyra sap samples, the region of 16SrRNA was amplified for the selected Palmyra sap samples collected from Kanyakumari and Tirunelveli Districts. After pre-processing the raw data, the 16S amplicon analysis revealed 129,128 raw reads, 77,595,585 raw total bases, 77.60 raw data's in MB, 88,613 high-quality sequence reading region (HQ reads), 39,015,757 HQ total bases and 39.02 HQ Data in MB in palmyra sap samples isolated from Kanyakumari District and the 16s Amplicon analysis of the palmyra sap samples isolated from Tirunelveli District represent 127,120 raw reads, 76,583,582 raw total bases, 76.60 raw data's in MB, 87,632 high-quality sequence reading region (HQ reads), 38,023,768 HQ total bases and 38.02 HQ data. The result of the data statistics was represented in Table 1.

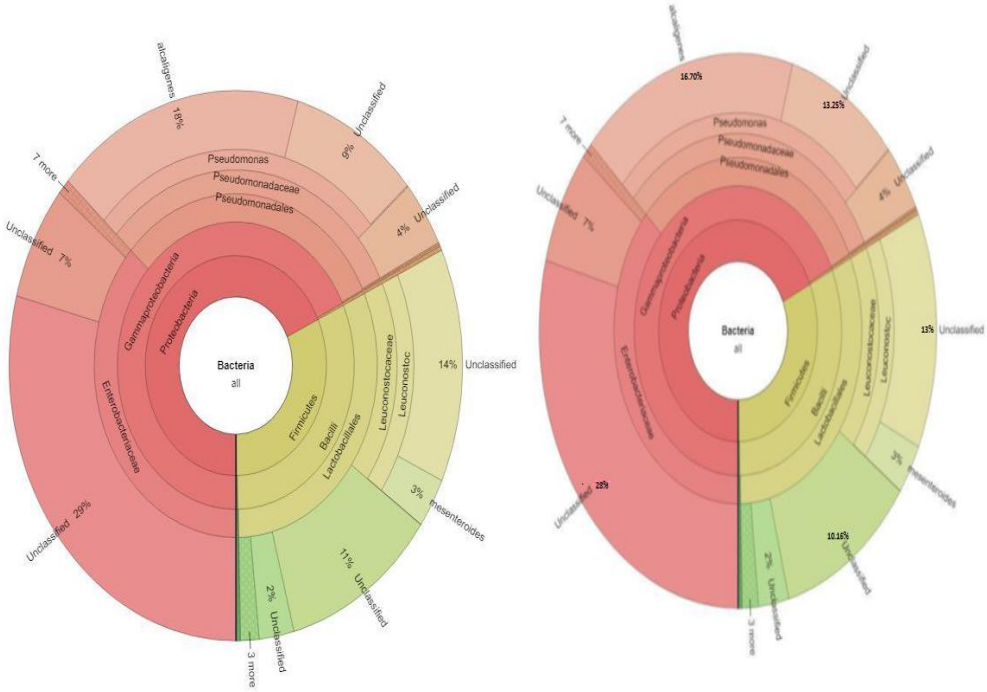
Table1.Data Statistics of 16s Amplicon analysis of probiotic bacteria present in the selected Palmyra samples.

Palmyra Sap Sample	Kanyakumari District	Tirunelveli District
Raw Reads	129,128	127,120
Raw Total Bases	77,595,585	76,583,582
Raw Data in MB	77.6	76.6
HQ Reads	88,613	87.632
HQ Total Bases	39,015,757	38.023,768
HQ Data in mb	39.02	38.02

Krona Craft

The Krona chart was a multilayered pie chart that represented the hierarchical classification of probiotic bacteria present in the Palmyra sap samples collected from Kanyakumari and Tirunelveli Districts based on the phylum level, class level, order level, family level, genus level, and species level distribution. Krona Craft displayed the multilevel distribution of probiotic bacteria present in the Palmyra sap sample collected from Kanyakumari and Tirunelveli District. The comparative results of the Krona craft revealed a slight variation in the percentage of probiotic bacteria distribution in the selected Palmyra sap samples. Figure 2 of Krona Craft displayed the multilevel distribution of probiotic bacteria present in the Palmyra sap sample collected from Kanyakumari District.

Fig. 2. Krona Craft displayed the multilevel distribution of the Probiotic bacteria isolated from Palmyra sap sample collected from Kanyakumari and Tirunelveli District



Finally, the metagenomic analysis based on the 16-s rRNA sequencing of the selected

palmyra sap sample reported the presence of different probiotic bacteria in the samples collected from Kanyakumari and Tirunelveli Districts at different hierarchical levels. Especially the phylum level, order level, class level, family level, genus level, and species level distribution. The overall metagenomics result of the Palmyra sap sample collected from Kanyakumari district distributed 233 OTUs and the most predominant taxonomy represented in the analysis was in the phylum level Proteobacteria, which was higher at 68.11%, followed by the class level Gammaproteobacteria at 67.83%, Enterobacteriales at 36.72% in the order level of 36.72% of Enterobacteriaceae at the family level, *Klebsiella* at 29.08% in the genus level, and unclassified species from the genus *Klebsiella* at 29.08%. The metagenomics result of the Palmyra sap sample collected from Tirunelveli district distributed 229 OTUs and the most predominant taxonomy represented in the analysis was at the phylum level. Proteobacteria was higher at 65.14%, followed by the class level Gammaproteobacteria at 65.23%, Enterobacteriales at 34.52% in the order level, 34.52% of Enterobacteriaceae at the family level, *Klebsiella* at 28.28% in the genus level, and unclassified species from the genus *Klebsiella* at 28.28%. The overall results of metagenomic analysis revealed that the probiotic bacteria belongs to the genus *Klebsiella* were predominant in the selected Palmyra sap samples compared to other probiotic bacteria distributed. The overall distribution of probiotic bacteria in the Palmyra sap sample of Kanyakumari and Tirunelveli District was described in Table 2.

Table 2. The Overall Hierarchical Level Distribution of Probiotic Bacteria isolated from the Palmyra sap sample of Tirunelveli District

	KANYAKUMARI DISTRICT	TIRUNELVELI DISTRICT
NUMBER OF OTUs	229	233
PHYLUM	Proteobacteria (65.14%)	Proteo bacteria (68.11%)
CLASS	Gammaproteobacteria (65.23%)	Gamma proteobacteria (67.83%)
ORDER	Enterobacteriales (34.52%)	Enterobacteriales (36.72%)
FAMILY	Enterobacteriaceae (34.52%)	Enterobacteriaceae (36.72%)
GENUS	*Klebsiella* (28.28%)	*Klebsiella* (29.08%)
SPECIES	Unclassified (28.28%)	Unclassified (29.08%)

The comparative metagenomics study of probiotic bacteria in the Palmyra sap samples of Kanyakumari and Tirunelveli districts through 16S rDNA-based metagenome analysis revealed that the distribution percentage of probiotic bacteria was predominant in the Palmyra sap samples collected from Kanyakumari District compared to the Palmyra sap samples collected from Tirunelveli District.

Conclusion

Palmyra sap is a low-cost plant-based mild alcoholic drink for rural Indian people, which is traditionally prepared by natural fermentation of fresh palm saps. Our study revealed the co-existence of bacteria in different environmental conditions in natural fermentation of fresh palm saps. The present study has authenticated the ethnic knowledge of traditional people for preserving the essential microbial resources during natural fermentation of fresh saps to mild-alcoholic drink. The predictive functional profiles of both bacterial communities collected from Tirunelveli and Kanyakumari District in Palmyra sap fermentation showed significant differences in the microbial synthesis of some secondary metabolites, vitamins and aromatic amino acids between the fresh palm saps and fermented Palmyra sap

(toddy), which predict the health-promoting benefits in the product.

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