

CHARACTERIZATION OF BACTERIAL DIVERSITY OF SMOKELESS TOBACCO OF INDIA

SUMMARY OF THE THESIS

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Summary of the thesis

The study of the thesis discusses the bacterial diversity of smokeless tobacco (ST) products collected from north Indian regions through culture-independent (16S rRNA analysis of ST metagenomes) approach and culture-dependent approaches and identify the associated health risk attributes. **CHAPTER ONE** of the thesis consists the introduction part where global burden of smokeless tobacco users, chemical constituents of smokeless tobacco history of smokeless tobacco associated microorganism described. Besides, this section also describes the lacunae in previous investigations on bacterial diversity of smokeless tobacco samples through cultivable and metagenomic approach of Indian smokeless tobacco samples. These laid down the idea to further research on the bacterial diversity of smokeless tobacco products and explore the functional attributes such as nitrogen metabolism, biofilm formation, proinflammatory biomolecules, and antibiotic resistance genes of inhabitant bacteria. A part of the work presented in this chapter has been published as book chapter (Vishwakarma and Verma, 2020 doi.org/10.1016/B978-0-12-819001-2.00008-5). This section also includes the significance of the study, hypothesis, major scientific problems addressed in this study, approaches adopted, scope and applications of the study. To address the above problems, three major objectives were defined as follows (**CHAPETER TWO**)

- I. Isolation of metagenomic DNA from smokeless tobacco products.
- II. Characterization of bacterial diversity of smokeless tobacco using cultivable and metagenomic approaches.
- III. Assessment of the risk factors associated with the inhabitant bacteria of smokeless tobacco.

CHAPTER THREE of the thesis describes the review of literature. It provides the information about the smokeless tobacco consumption, their types and bacterial diversity of smokeless tobacco samples through cultivable and metagenomic approach and their associated risk factors. As India is second largest consumer of the world, despite, only one or two reports discusses the bacterial aspects of smokeless tobacco products. A part of the work presented in this chapter has been published as review article (Vishwakarma and Verma, 2021; doi.org/10.1007/s00253-021-11460-2). This section also discusses the unresolved issues in the line of smokeless tobacco microbiology of the present investigation. **CHAPTER FOUR** of the thesis outlines

the detailed methodology used to achieve each objective of this investigation. Objective I describe the tedious isolation of metagenomic DNA from thirty different ST products. Objective two and three were divided into two parts (I) Culture-independent approach and (II) Culture-dependent approach for analysing the bacterial diversity as well as exploring their functional attributes. **CHAPTER FIVE** describes the results section. According to the methodology, this section also divided into two parts i.e., culture-independent approach (I) and culture-dependent approach (II). Part-I divided in two sections (A) results of taxonomy assignment of twenty smokeless tobacco samples (Vishwakarma et al., 2023a; doi.org/10.1007/s11274-022-03461-8). and (B) describes the results of taxonomy assignment of ten commercial snuff samples (Vishwakarma and Verma, 2024, doi.org/10.1007/s12010-024-04857-y). Part II of this chapter describes the findings and observations using culture-dependent approach and discusses few parameters associated with bacterial derived health risk factors (Vishwakarma and Verma, 2023b; doi.org/10.1007/s12010-023-04689-2). **CHAPTER SIX** consists of a discussion of the findings and conclusion of the thesis. This chapter discusses the detailed finding of each objective of this investigation. And this section also includes the future prospects of this study. **CHAPTER SEVEN** of the thesis describes the summary of the thesis. **CHAPTER EIGHT** provides the complete list of the references cited in this thesis in a standard format. Besides, the links of other relevant references cited in this thesis have also been included in the reference list. **CHAPTER NINE** provides a compiled information of various results in Annexure (I, II, and III) files along with, published and communicated papers and other scientific outcomes of the work to date. The prints of full-length papers, review and chapters published in various international journals and book have been enclosed. Certificate of conferences and workshops during the Ph.D. work are also included. In this study, 30 smokeless tobacco samples were purchased from various places of north region of India. Physicochemical parameters such as nitrate and nitrite reduction, pH, moisture content was analysed from the samples. Thereafter, metagenomic DNA of the ST products were extracted to explore the bacterial diversity of smokeless tobacco samples. Extracted metagenomic DNA was processed for next generation sequencing (NGS) targeting V3-V4 bacterial specific 16 rRNA hypervariable regions only. Taxonomy assignment and diversity indices analysis was done by using QIIME2 pipeline. Pathways involved in nitrogen metabolism, proinflammation, biofilm formation and antibiotic resistance

and their related genes were analysed through PICRUSt2 pipeline using KEGG and BRITE databases. Correlation and network analysis were performed between existing bacterial communities and their functional attributes.

Cultivable approach was also used to isolate the inhabitant bacterial community of smokeless tobacco samples. Samples were pooled in 5 groups (2 from CT group, 2 from LT group, and one from snuff samples) which were used in 16S rRNA analysis of respective ST metagenomes. Further morphological and biochemical characterization were carried out on 74 isolated bacterial isolates. Tobacco aqueous extract experiment was performed to check the stimulatory effect on these bacterial isolates. Molecular level characterization was done on those bacterial isolates who showed strong biofilm forming ability along with stimulatory effect of tobacco extract. The antibiotic susceptibility test and minimum inhibitory concentration (MIC) of salt resistance, heavy metal resistance, and effect of nicotine were also investigated on selected bacterial isolates. The major findings of the present study are summarized below.

Part I: Characterization of the bacterial diversity through culture-independent approach

Part IA: CT and LT group of smokeless tobacco samples

This study relies on 16S amplicon-based bacteriome analysis of Indian smokeless tobacco (ST) products by using their metagenomic DNA. A total of 59,15,143 high-quality reads were assigned to 34 phyla, 82 classes, 176 orders, 256 families, 356 genera, and, 154 species using the SILVA database. Of the phyla (>1%), Firmicutes dominate among the Indian smokeless tobacco followed by Proteobacteria, Bacteroidetes, and, Actinobacteria (>1%). Whereas, at the genera level (>1%), *Lysinibacillus*, *Dickeya*, *Terribacillus*, and, *Bacillus* dominate. The comparative analysis between the loose tobacco (LT) and commercial tobacco (CT) groups showed no significant difference at the phyla level, however, only three genera (*Bacillus*, *Aerococcus*, and *Halomonas*) were identified as significantly different between the groups. It indicates that CT and LT tobacco share similar bacterial diversity and poses equal health risks to human oral health. The phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt 2.0) based analysis uncovered several genes involved in nitrate/nitrite reduction, biofilm formation, and pro-inflammation that find roles in oral pathogenesis including oral

cancer. The strong correlation analysis of these genes with several pathogenic bacteria suggests that tobacco products pose a high bacterial-derived risk to human health. The study paves the way to understand the bacterial diversity of Indian smokeless tobacco products and their putative functions with respect to human oral health. The study grabs attention to bacterial diversity of the smokeless tobacco products from a country where tobacco consumers are rampantly prevalent however oral health is of least concern (Vishwakarma et al., 2023a).

Part IB: Snuff samples

In this investigation, 10 snuff tobacco products were assessed for their bacterial diversity to understand the correlation between the inhabitant bacteria and predicted ARGs using a metagenomic approach. Firmicutes (43.07%) and Proteobacteria (8.13%) were the two most abundant phyla observed which were dominated with *Bacillus* (9.76%), *Terribacillus* (8.06%), *Lysinibacillus* (5.8%), *Alkalibacterium* (5.6%), *Oceanobacillus* (3.52%), and, *Dickeya* (3.1%) like genera. The PICRUSt-based analysis revealed 217 antibiotic resistance genes (ARGs) of nine types. Cationic antimicrobial polypeptides (CAMP; 33.8%), vancomycin (23.4%), penicillin-binding protein (13.8%), multidrug resistance MDR (10%), and β -lactam (9.3%) were the top 5 contributors to ARGs. *Staphylococcus*, *Dickeya*, *Bacillus*, *Aerococcus*, and, *Alkalibacterium*, showed their strong and significant correlation with various antibiotic resistance mechanisms. Some signature sequences of ARGs of different classes (*blaTEM*, *blaSHV*, *blaCTX*, *tetX*, *vanA*, *aac3-II*, *mcr-1*, *intI-1*, and, *intI2*) were frequently detected in the metagenome of SMT samples. The prevalence of ARGs in inhabitant bacteria of smokeless tobacco products suggests making firm policies to maintain the hygiene of commercial tobacco products (Vishwakarma and Verma, 2024).

Part II: Characterization of the bacterial diversity through culture-dependent approach

This investigation was carried out to analyze the inhabitant bacterial population of the Indian ST products for assessing the health-associated risk attributes using cultivable approach. Traditional cultivation approach recovered several bacterial isolates from commercial ST products on different culture media (Nutrient agar, MacConkey agar, Trypticase soy agar, Sheep blood agar, and, EMB agar). A high colony formation unit (CFU) count was observed that ranged from 173×10^4 to 630.4×10^5 per gram of ST products. Morphological and biochemical characterization has been performed on the

74 randomly selected and distinct bacterial isolates. 17 isolates showed a significantly enhanced growth ($p\text{-value} < 0.05$) in the presence of the aqueous tobacco extract. Most of these bacteria also exhibited biofilm-forming activity, where eight bacterial isolates were identified for strong biofilm-forming action. 16S rRNA-based molecular characterization of these eight bacteria identified them as *Bacillus subtilis* (TS3-NA1, TS3-NA3, and TS3-TSA2), *Bacillus paralicheniformis* (TS3-NA4), *Siccibacter colletis* (TS1-NA3), *Serratia marcescens* (TS4-NA2), *Pantoea anthophila* (TS4-MA2), and, *Enterobacter cloacae* (TS5-MA2). Moreover, these bacteria also exhibited the potential to withstand high salt and heavy metal concentrations. The findings demonstrate that Indian ST products are heavily populated with wide bacterial species exhibiting potential in biofilm formation, heavy metal resistance, and salt tolerance (Vishwakarma and Verma, 2023b).

Conclusion of the study

Overall bacteriome analysis concluded that Indian smokeless tobacco products are populated with Firmicutes, followed by Proteobacteria, Actinobacteria, Bacteroidota, and, Cyanobacteria. Where, *Bacillus* spp. dominate among the samples followed by *Dickeya*, *Halomonas*, and, *Aerococcus*. Therefore, chewing tobacco exhibits potential for oral dysbiosis. The functional prediction identified several bacterial-associated risk attributes, which include the prevalence of genes involved in nitrate reduction, biofilm formation, and, pro-inflammation that may led to the onset of several oral diseases including oral cancer.

The comparative analysis revealed that the CT and LT groups exhibits no significant difference at the phyla level, whereas, at the general level only three bacteria (*Aerococcus*, *Halomonas*, and, *Bacillus*) were identified as significantly different. Therefore, commercial tobacco products are at equal risk as loose tobacco products in the context of their bacterial-derived health risks. It further indicates the ignorance of quality checks of tobacco products while processing them at the commercial level.

A deep insight is required in the line to understand the transition of resident bacteria of tobacco products into the oral cavity of tobacco chewers having oral cancer. Though several potential bacterial markers of oral cancer such as *Prevotella*, *Fusobacterium*, and, *Porphyromonas* have not been identified as dominant genera among smokeless tobacco products during this investigation. Therefore, it may be hypothesized that these bacteria were not transferred from ST products, however,

chewing tobacco may support the proliferation of these oral pathogens, which needs to be further confirmed by laboratory experiments.

Despite of highest tobacco consumers in India, we severely lack the information on the bacterial diversity of indigenous tobacco products. The present investigation paves a way to understand the bacterial diversity and its correlation with associated health risk factors. In addition, the findings will assist the companies involved in tobacco processing in their monitoring to check the microbial status.

The study of ten commercial snuff types of smokeless tobacco samples corroborates the finding of CT and LT group smokeless tobacco products at the bacterial diversity level, where *Bacillus* type Firmicutes were also prevalent. ARG profiling of these snuff samples showed the supremacy of CAMP resistance genes, followed by vancomycin, penicillin-binding protein, and multidrug-resistant MDR, among the top 10% of ARGs. While correlating, the top dominant genera showed a strong positive correlation with several ARGs.

The introduction of antibiotic-resistant bacteria along with smokeless tobacco products enhances the possibility of disseminating antibiotic-resistance genes in the bacteria of the oral cavity to enhance the health risks. Therefore, extensive research is required in line with culture-dependent approaches to investigate the ARG threat on commercial SMT products.

The high bacterial biomass, tobacco-stimulated growth of the bacterial isolates, and their biofilm-forming characteristic make the smokeless tobacco products nastier to consume in any form. Besides, these bacteria further exhibited the potential to tolerate high concentrations of heavy metals and salt. The findings pave the way to assess the bacterial population for various health-associated risk factors; it also includes the antibiotic resistance profile of inhabitant bacteria of ST products. With these findings, the bacterial population on ST products cannot be ignored and needs a sincere concern while making cessation policies on ST products. Especially, in South and South-East Asian countries, where ST product consumers predominate as compared to the other parts and oral hygiene is almost ignored. It should also be noted that India harbors the second largest consumer and tobacco producer.

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