

EVALUATING THE BACTERIAL DIVERSITY OF SMOKELESS TOBACCO PRODUCT USING SHOTGUN METAGENOMIC ANALYSIS

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Abstract. Naswar is a smokeless tobacco product, commonly consumed in Pashtun population of Pakistan and Afghanistan. Despite being an established risk factor for oral cancer, not much is known about microbiological constituents of Naswar. Objectives: The objective of the study was to explore bacterial diversity in Naswar using shotgun metagenomic sequencing. Methodology: DNA was extracted from twelve (n = 12) famous brands of Naswar samples using an enzymatic and mechanical lysis method followed by next generation sequencing on Illumina Miseq platform. Results: Naswar samples exhibits differential abundance profile at all taxonomic levels with Khamar brand having the highest species richness and diversity indices. Of the total 22 phyla identified, phylum Proteobacteria was the most abundant (88.4%) followed by Bacteroidetes (4.71%) and Firmicutes (3.73%). At genus level, 234 genera (range: 32 – 132) were identified. Metagenomic sequencing also revealed presence of 455 (range: 44 – 210) different bacterial species in Naswar samples. The most common species were *Pseudomonas aurgenosa*, *Yersinia enterocolitica*, *Salmonella enterica*, *Xanthomonas campestris*, and *E. coli*. Some of the species are of concern due their ability to cause infections in humans. Conclusions: Naswar products harbor diverse bacterial microbiota with differential abundance at phylum, genus and species level. These findings may have several implications in terms of further research to explore microbiological associated health risks and provide scientific evidence to inform potential regulations regarding manufacturing and testing of Naswar in Pakistan.

Keywords: metagenome, shotgun sequencing, bacteriome, snuff, pathogens, tobacco control

Introduction

Smokeless tobacco are tobacco containing products that are used without burning or combustion of tobacco (Cogliano et al., 2004). The majority of smokeless tobacco products are used orally, while a few are used through the nasal route. An estimated 40 different varieties of smokeless tobacco exist across the globe, with more than 350

million users worldwide (Mehrotra et al., 2019). More than 90% of the global smokeless tobacco users live in South Asian countries and its prevalence is gradually increasing (Suliankatchi et al., 2019). An estimated 8% of the population in Pakistan use smokeless tobacco in some form (Saqib et al., 2018), with Naswar, Paan and Gutkha being the most common smokeless tobacco products (Khan et al., 2019). Naswar, is a smokeless tobacco product that is predominantly consumed by the ethnic “Pashtun” population native to the Khyber Pakhtunkhwa province in Pakistan (Ahmad et al., 2020). Naswar is locally prepared by the cottage industry from ground tobacco leaves (*Nicotina rustica*), slaked-lime, wood ash and different flavorings and contain nicotine, toxic heavy metals and carcinogenic substances (Zakiullah et al., 2012). Naswar constitutes approximately 60% of the total tobacco consumed in Peshawar, the capital city of Khyber Pakhtunkhwa, warranting public health action (Ali et al., 2017). Naswar use is associated with an increased risk of oral cancer, the second most common cancer in Pakistan, with pooled estimates suggesting a more than 10-fold increase in the risk of oral cancer among Naswar users compared to non-users (Khan et al., 2019). Mechanistically, the risk of Naswar related carcinogenesis can be attributed to the presence of toxic chemical substances in the product (Stanfill et al., 2018). However, the role of microbial agents in Naswar associated cancer development has often been overlooked. Smokeless tobacco associated bacterial species have been shown to reduce nitrate to nitrite and potentially contribute to the production of Tobacco Specific Nitrosamines (TSNAs) (Wei et al., 2014). TSNA’s are the most powerful carcinogens present in smokeless tobacco products and their concentration varies considerably among different smokeless tobacco products (Warnakulasuriya and Straif, 2018). Moreover, there is experimental evidence suggesting the presence of certain bacterial species (mainly bacilli) in smokeless tobacco products that can induce plasma exudation from oral mucosal cells (Rubinstein and Pedersen, 2002) resulting in localized injury, chronic inflammation and malignant transformation (Pauly and Paszkiewicz, 2011). Traditionally, culture based methods relying on the detection of culturable microorganisms in smokeless tobacco, have been used to study microbial presence in smokeless tobacco products (Mehra et al., 2020; Odangowei et al., 2018; Saleem et al., 2018). However, these methods have limited applicability, allowing only a fraction of microorganisms present in a sample to be cultured and identified (Ferone et al., 2020). A few contemporary studies have used advanced, culture-independent methods based on DNA markers sequences for profiling microbial communities in smokeless tobacco products (Al-Hebshi et al., 2017; Smyth et al., 2017; Tyx et al., 2016a). These methods target one or more hypervariable regions of 16S rRNA gene using next generation DNA sequencing technology to characterize bacterial population, providing a deeper insight into microbial communities present in smokeless tobacco products. This approach, however, has its own limitations. Targeted sequencing of 16SrRNA gene require PCR amplification which has chances of inherent errors (nucleotide substitution, insertion and deletion) and biases (limited primer coverage, differential amplification) resulting in under or over-estimation of microbial diversity (Al-Hebshi et al., 2018). To overcome these issues, shotgun metagenomics sequencing is the preferred method that does not require PCR amplification (Sharpton, 2014). Additionally, shotgun metagenomics sequencing also allows identification of bacterial communities at higher resolution than 16S rRNA, characterization of other microbes (fungi, viruses) and dissection of functional potential (gene and pathways analysis) (Ranjan et al., 2016). The high prevalence of Naswar use in Khyber Pakhtunkhwa combined with the lack of sound

evidence on the presence of potentially carcinogenic microbes in Naswar warrants an investigation to characterize microbial contents of Naswar products available in the local market. The aim of the present study was to characterize microbial communities in a smokeless tobacco product (Naswar) available in Pakistan using shotgun metagenomics sequencing technology. This might broaden our knowledge of any role played by microbes in the process of carcinogenesis by using ST.

Materials and methods

Sample collection and storage

A single investigator collected twelve, ready to use, commercially available and most popular Naswar brands from retail locations in Peshawar district, Pakistan in October 2019. Information about brand, manufacture date, seller location and date of purchase were also recorded. Although different varieties of Naswar such as black or green and moist or dry are being available, we have collected only the black moist variety as it is the commonest variety used by the local population. The commonest brands available and used in the study consisted of brands namely Imtiaz Wazir, Bannu tor, Shehenshah, Karamar, Special gandavi, Peshawar local, Khamar, Dubian, B-52, Chamnay, Ghulam Mohiuddin, Bara. The samples were purchased, placed in un-opened Ziploc bags and hand carried to the microbiology laboratory at Khyber Medical University Peshawar on the same day. The samples were stored at room temperature until analysis. Prior to analysis, the packages were placed un-opened under ultraviolet light for 30 min in a biological safety cabinet to decontaminate exterior packaging.

DNA extraction

DNA extraction was done using enzymatic lysis procedure following Smyth et al. method (Smyth et al., 2017) with only slight modifications. Briefly, 1 gram of the samples were dissolved in 5 mL PBS, vortexed at full speed to recover bacterial cell and then centrifuged at 1000 rpm to remove particulate matter. The supernatant was transferred to another tube and centrifuged at 13000 rpm to obtain a pellet. The pellet was re-suspended in 1 mL of ice-cold PBS and transferred to DNA lysing tubes (MP Biomedicals) containing 1 mL of ice-cold phosphate buffer saline followed by addition of 5 μ L of lysozyme (10 mg/mL) and lysostaphin (5 mg/mL) and 15 μ L of mutanolysine (5 mg/mL) (Sigma Aldrich, Pool, UK). After half an hour incubation at 37°C, 10 μ L of 20 mg/mL proteinase K and 50 μ L of 10% w/v SDS were added into the samples. The samples were again incubated at 55°C for 45 min followed by mechanical lysis Bioprep-24 instrument (Hangzhou, China). Lysate was centrifuged and DNA purified using commercially available kits (Qiagen, CA) following manufacturer instructions. Quality of DNA samples was assessed by agarose gel electrophoresis and quantified using NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Genomic DNA samples were sent to Rehman Medical Institute Peshawar, Pakistan for NGS analysis. Only one sample (Dubian) does not yield quality DNA and hence excluded from further analysis.

Library preparation and shotgun metagenomic sequencing

Genomic DNA libraries were prepared using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, USA) following manufacturer instructions. Libraries were

prepared using 1 ng of input DNA followed by tagmentation and PCR amplification. The Pooled libraries were cleaned, normalized and loaded onto Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) and paired end sequencing (2 x 150 bp) performed using the V2 sequencing reagent kit.

Bioinformatics analysis

Sequencing data was pre-processed by KneadData (version 0.6.1); a computational tool utilizing FastQC (version 0.11.5) (Andrews, 2021), Trimmomatic (version 0.33) (Bolger et al., 2014), and Bowtie2 (version 2.2) (Langmead and Salzberg, 2012) to perform quality check, quality filtering, and host sequences decontamination, respectively. Filtered reads from the different metagenomes were then assembled de novo using SPAdes v3.10.1 (Bankevich et al., 2012) followed by alignment of merged against NCBI's non-redundant protein database using DIAMOND aligner v.0.9.25 (Buchfink et al., 2015). The file produced by DIAMOND (.daa) was "meganized" and subsequently imported into MEGAN 6 to perform taxonomic and functional binning of the quality filtered reads (Huson et al., 2007). Unassigned reads were removed and the remaining reads were normalized to those with lowest read count. Diversity indices were calculated for each sample using MEGAN. Taxonomic heatmaps at species level were generated using hclust2 tool available at <https://bitbucket.org/nsegata/hclust2>.

Results

Taxonomic diversity in Naswar samples

Taxonomic diversity in Naswar samples were calculated by resolving next generation sequencing reads at phylum, genus and species levels. Microbial diversity within the samples also known as α -diversity was assessed using Shannon-Weaver and Simpson's reciprocal indices (Table 1). All the samples exhibit variable diversity at all taxonomic levels with Peshawar local, Bannu tor and Khamar brands have highest diversity indices at phylum, genus and species levels respectively.

Table 1. Alpha diversity indices calculated as mean $\pm$ standard deviation for all ST samples at phylum, genus and species levels

Alpha diversity indices	Taxonomy		
	Phylum	Genus	Species
Shannon-Weaver Index	6.522 $\pm$ 0.43	62.705 $\pm$ 0.48	53.316 $\pm$ 0.73
Simpson-Reciprocal Index	15.346 $\pm$ 0.40	141.598 $\pm$ 3.26	226.191 $\pm$ 6.5

Differences in microbial diversity between the samples (β -diversity) was assessed by principal coordinate analysis (PCoA) plot using the Bray–Curtis dissimilarity matrix (Bray and Curtis, 1957) at species level (Fig. 1). Greater the distance between the samples, the higher is the differences in diversity between the samples. As shown in the figure, majority of the Naswar brands clustered together in four different core microbiomes, each representing somewhat similar microbial composition. However, Imtiaz wazir brand form a separate cluster and thus different from all other core clusters. Similar trend was also observed at genus and phylum level (Figs. 1 and 2).

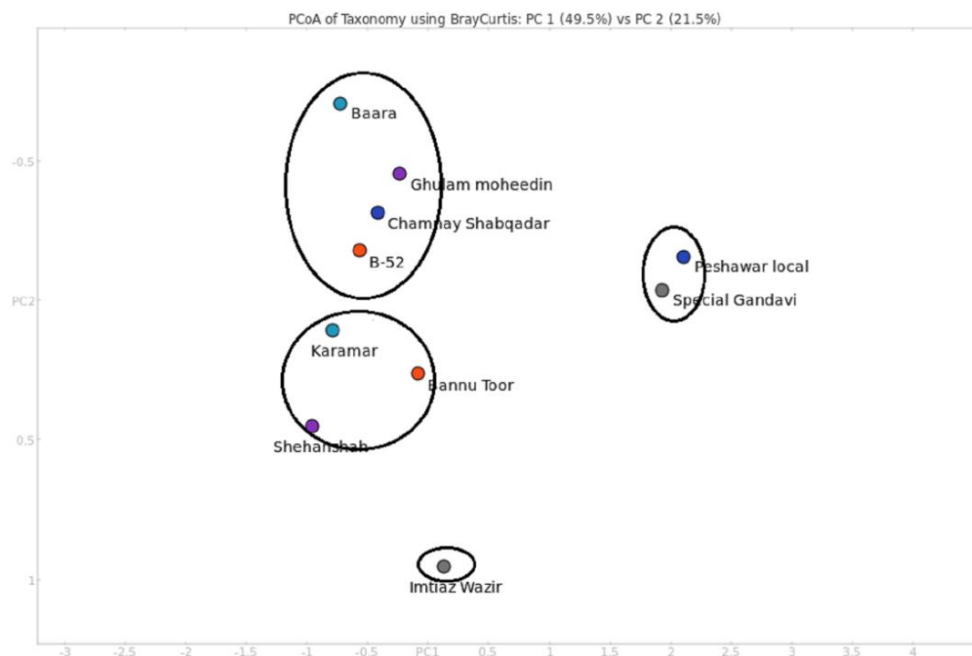


Figure 1. Clustering of Naswar samples microbiome by Principal coordinate analysis (PCoA) plot based on Bray–Curtis dissimilarity

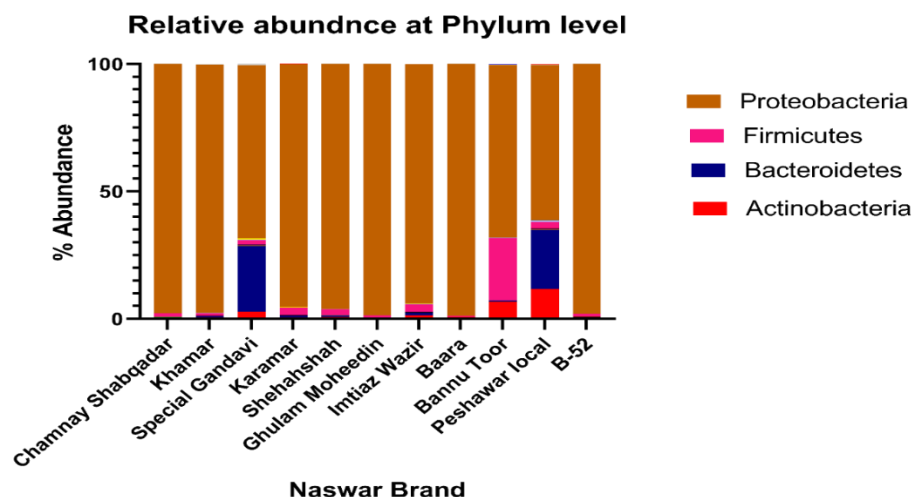


Figure 2. Relative abundance at phylum level: Stack bar showing relative abundance of different phyla identified in each Naswar brand. Labels are only shown for phyla with relative abundance more than 1%

Relative abundance of microbial communities in Naswar samples

Naswar samples overall exhibited a wide range of taxonomic diversity at phylum, genus and specie level. A total of 22 different phyla were identified in all Naswar brands (Fig. 2). The number of phyla ranges from 3 (Bara brand) to 12 (Peshawar local brand). *Proteobacteria* were the most abundant phyla that accounted for $\geq 90\%$ of sequences in all Naswar brands except Special Gandavi, Bannu tor and Peshawar local

where it constitutes more than 60% of the sequences. *Firmicutes*, *Bacteroidetes* and *Actinobacteria* were also common in all Naswar brands.

The relative abundance of bacteria at genus level is shown in Figure 3. Overall, 234 genera identified, 43 genera have relative abundance of $\geq 1\%$. The number of genera range from 32 – 132 per sample. On genus level, Khamar brand was the most diverse with 132 genera identified followed by Bannu tor (108 genera) and Imtiaz Wazir (106 genera). The lowest number of genera were identified in Bara brand of Naswar. *Pseudomonas* was the most abundant genus with a relative abundance of 8%–36% in all brands except Shehenshah in which it was totally absent. *Yersinia* was the second most abundant genus found in all brands except Specail gandavi. Of all Naswar brands, Shehenshah has significantly different genus profile with *Thermosynechococcus* (20%) and *Thiomonas* (10%) were the most abundant genera.

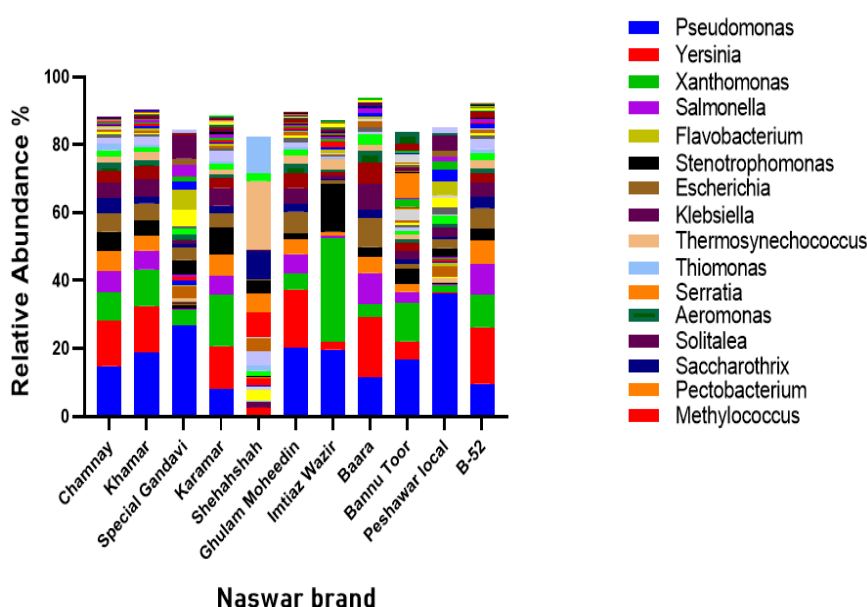


Figure 3. Relative abundance at genus level. Stack bar showing relative abundance of different genera identified in each Naswar brand. Labels are only shown for genera with relative abundance of $\geq 5\%$

Overall, 455 different bacterial species were identified of which 56 had abundance of $\geq 1\%$. Figure 4 shows heat maps showing relative abundance of top 30 bacterial species found in all Naswar samples. Overall, *Pseudomonas aurgenosa* was the most abundant bacterial specie found in all samples and the relative abundance range from as low as 1.9 in Shehenshah brand to as high as 25.5 in Peshawar local brand. *Yersinia enterocolitica* was the second most abundant bacterial species which was found in all Naswar brands except Special Gandavi bran where it was not found. Other commonly identified bacterial species were *Salmonella enterica*, *Xanthomonas campestris*, and *E. coli*.

Discussion

Scientific studies on chemical composition of smokeless tobacco products has revealed abundance of toxic constituents; the commonest being tobacco specific

nitrosamines mainly contributing to tobacco associated carcinogenicity (“Harmful Chemicals in Tobacco Products | American Cancer Society,” n.d.). There is, however, little scientific evidence on the microbial colonization of smokeless tobacco products that are not only directly harmful for human health (potential pathogens) but can also contribute in the production of toxic nitrosamines, endotoxins and mycotoxins. Such studies can help us profoundly to understand the harms related with the product with regards to toxicity and carcinogenicity. This study describes the microbial profile of Naswar, a commonly consumed smokeless tobacco product in Pakistan through robust laboratory technique. The product is used by placing a pellet in the mouth that may potentially introduce microbial pathogens and toxic chemicals directly into the oral cavity of the users.

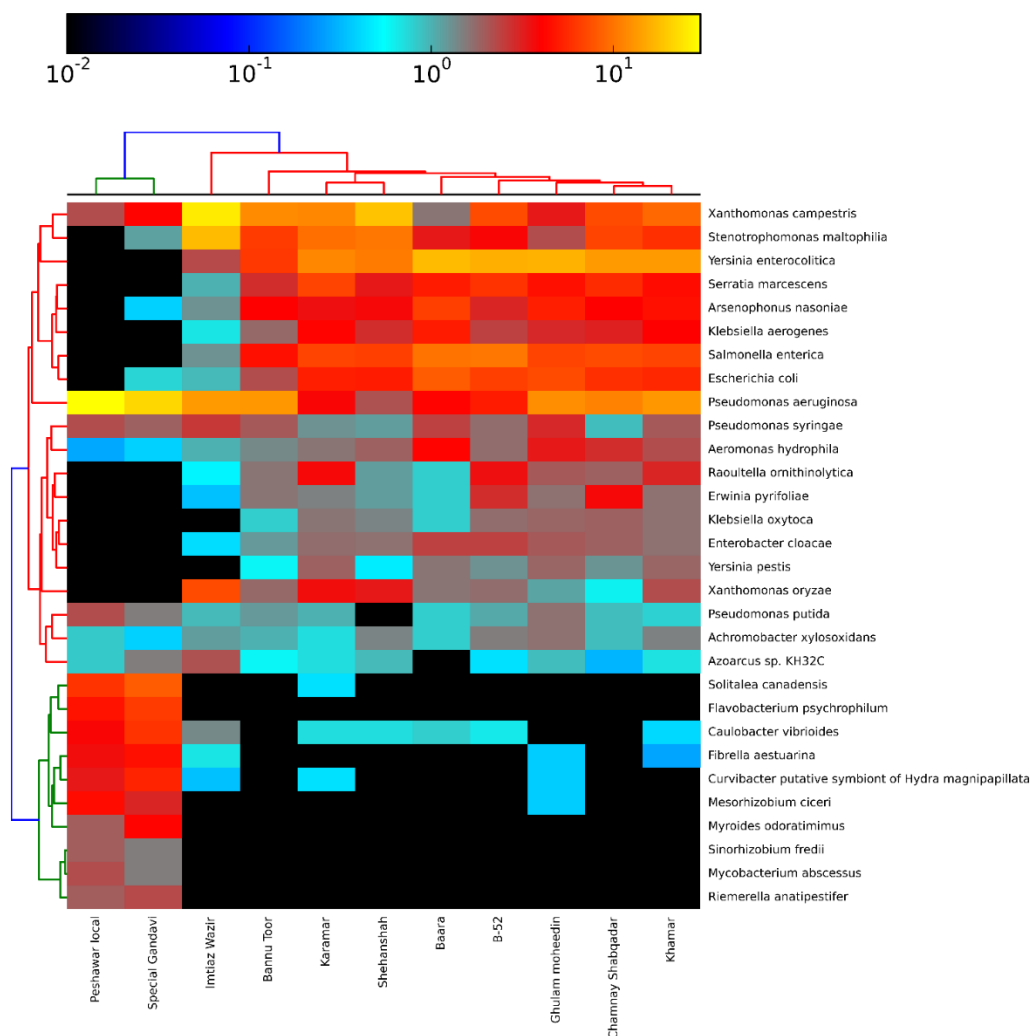


Figure 4. Heatmap of the relative abundance of top 30 bacterial species found in Naswar samples

In the current study, using advanced next generation sequencing technology, we were able to identify differentially abundant bacterial communities in Naswar samples thus confirming previous reports that ST products harbor diverse microbiota (Rivera and

Tyx, 2021). While there were some variations in different brands in terms of bacterial composition, we were able to identify 22 different bacterial phyla with *Proteobacteria* being the most abundant bacterial phyla in Naswar samples followed by *Firmicutes*, *Bacterioidetes* and *Actinobacteria*. Our results are similar to a study by Sapkota et al. who also reported highest abundance of two bacterial phyla; *Proteobacteria* and *Firmicutes* in cigarette metagenome (Sapkota et al., 2010). Recent studies on metagenome analysis of different smokeless tobacco products has also revealed high abundance of the above four bacterial phyla (Aj et al., 2020; Al-hebshi et al., 2017; Smyth et al., 2017; Tyx et al., 2016b). However, contrary to our study findings, *Firmicutes* were the most abundant bacterial phyla in majority of the published reports. *Proteobacteria* were predominant in only few brands of smokeless tobacco products including Swedish Snus and commercially available dry snuff products (Al-hebshi et al., 2017). All this evidence points towards existence of specific bacterial communities in smokeless tobacco products, the composition of which primarily depends on the type of product and method of preparation.

On genus level, *Pseudomonas* and *Yersinia* were commonly found in Naswar samples. These findings are markedly different from the previous studies who reported genus *Bacillus* to the most dominant bacterial genera in smokeless tobacco products (Al-hebshi et al., 2017; Smyth et al., 2017; Tyx et al., 2016b). Again, genus *Pseudomonas* was only reported in Swedish suns (Al-hebshi et al., 2017). On the species level, bacteria such as *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Salmonella enterica*, *Xanthomonas campestris* and *E. coli* dominate the bacteriome in Naswar samples. Highest abundance of *Pseudomonas aeruginosa* in these samples is probably due to high nicotine contents in Naswar products which these bacteria use as nutrients. The presence of nicotine degradation pathways in *Pseudomonas aeruginosa* and other bacterial species has already been confirmed (Brandsch, 2006). Some of the species isolated from Naswar samples are known or opportunistic human pathogens. For examples, *Pseudomonas aeruginosa*, is a Gram negative, anaerobic bacilli commonly found in environmental samples such as water and soil and on the skin, throat and feces of healthy human (Iglewski, 1996). It is a known opportunistic pathogen that can infect virtually any tissue in human body due to the presence of several different virulence factors and antibiotic resistance potential (Caskey et al., 2018). The bacterium is also responsible around 10% of the hospital acquired infections with significant effect on patient morbidity and mortality in respiratory infections (Labovská, 2021). The vast presence of *Pseudomonas aeruginosa* in Naswar samples is alarming and may pose a significant threat to human health. High risk group such as older people and patients with chronic respiratory infections especially cystic fibrosis should consider quitting the Naswar habit to avoid possible complications. Similarly, *Yersinia enterocolitica* is the causative organism of Yersiniosis in humans. Recently, the organism has increasingly been associated with small scale outbreaks of gastrointestinal infections in different countries (Karlsson et al., 2021).

Tobacco use is among the leading cause of morbidity and mortality across the globe. According to WHO, in 2017, approximately 7 million people lost their lives due to diseases and complications resulting from the use of tobacco products (smoking and smokeless) (WHO, 2017). Smokeless tobacco products, irrespective of how it is produced and utilized, contain over 4000 chemicals including nicotine, carcinogens and toxic element (Kaur et al., 2019). Of these, 93 chemicals have been identified as harmful and potentially harmful constituents (HPHC) that need to be reported

quantitatively in all tobacco products as per US Food and Drug Administration (USFDA) regulations (FDA, 2020). However, these regulations failed to recognize microorganisms, an important constituent of tobacco products from public health point of view. The findings of our study further add into the growing research of microbial contamination in smokeless tobacco products. The understanding of Naswar's microbial contamination has implications for both tobacco control policies and manufacturing practices in Pakistan.

While our study is the first to report microbial contamination of Naswar using next generation sequencing technology, it has some limitations. First, we have used only one Naswar sample per brand and thus could not explore variations across different batches of the same brand. Second, the DNA was extracted from the supernatant instead of solid Naswar samples. As a result, we might have not captured all the microbial contents. However, this step was necessary to the presence significant solid matter including plant material. Third, we have only analyzed a single smokeless tobacco product (Naswar) in our study and therefore, missed the opportunity to compare microbial diversity of Naswar with other commonly consumed smokeless tobacco products in Pakistan.

Conclusions

Summing up, our findings shows that Naswar products harbor diverse bacterial microbiota with differential abundance at phylum, genu and species level, across different brands of the same product. Further research is required to assess the role of Naswar bacterial microbiota that could play in introducing potentially harmful bacteria into users' oral cavities, as well as the effects that this might have on the oral microbiome and users' health.

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Conflicts of interest. The authors declare no conflict of interests.

Data availability statement. For access to the data, please contact corresponding author via email at shahzad.ibms@kmu.edu.pk.

Consent of publication. All authors have read and agreed to the published version of the manuscript.

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