Microbial diversity of molasses containing tobacco (Maassel) unveils contamination with many human pathogens

M.A.A. ALQUMBER

Department of Laboratory Medicine, Faculty of Applied Medical Sciences, Albaha University, Saudi Arabia

Abstract. – OBJECTIVE: Tobacco smoking remains a worldwide health issue, and the use of flavored varieties (maassel) embedded in glycerine, molasses, and fruit essence via shisha paraphernalia (waterpipe) is growing globally. 16S rRNA gene pyrosequencing was conducted on 18 different varieties representing 16 flavors and three brands in order to study the microbiota of maassel and find whether it contains pathogenic bacteria.

MATERIALS AND METHODS: The samples were selected randomly from the most utilized brands within Albaha, Saudi Arabia as determined through a questionnaire of 253 smokers. In addition, ten-fold serially diluted samples were inoculated on blood agar, MacConkey agar, half-strength trypticase soy agar and malt agar for the enumeration of mesophilic microorganisms, coliforms, Bacillus, thermophilic bacteria, and fungi.

RESULTS: A core microbiota was recognized consisting of three phyla (*Firmicutes, Proteobacteria* and *Actinobacteria*) and a total of 571 different species were identified including many pathogens, such as *Mycobacterium riyadhense, M. chelonae, Shigella sonnei, S. flesneri, Klebsiella pneumoniae, Salmonella bongori, Coxiella burnetii, Acinetobacter* spp., *Staphylococcus haemolyticus, Streptococcus pseudopneumoniae,* and *Streptococcus sanguinis*, showing the contamination of maassel.

CONCLUSIONS: The present study suggests that flavored tobaccos are potentially infectious. However, further risk assessment is required to determine transmission occurrence.

Key Words:
Microbial diversity, Molasses containing tobacco, Human pathogens, 16S rRNA.

Introduction

Tobacco is an economically important nonfood product and one of the legal harmful addictive drugs in today's modern world. Different methods are currently used to consume tobacco, including cigarettes, cigars and waterpipes[1]. Waterpipe (shisha) smoking continues to rise globally[2]. Smoking flavored tobacco (maassel), through the shisha, is becoming a global preventable cause of morbidity and mortality[3,4].

Scientists studied the chemical composition of tobacco for many years and illustrated the total number of chemicals identified in tobacco during the years from 1954 to 2005[5]. In addition, a comprehensive review of these chemicals' classification, concentration and changes with time due to changes in the shape, design and composition of cigarettes was reported almost a decade ago[6]. On the other hand, the microbiology of tobacco has not been studied in the same depth, nor have methods and standards been available for the microbiological analyses of tobacco[7]. Nonetheless, for more than a century, tobacco was known to be associated with microbial interactions that were seen to be an integral part of the development of its aroma and other desirable smoking qualities[8-10]. Moreover, in 1953, Tamayo and Cancho[11] inoculated tobacco leaves with microbes to improve the aroma of the end product. Consequently, proper fermentation with appropriate microbes is essential for good quality tobacco[12-14]. However, the microbiology of the molasses-containing tobacco product called maassel was not studied using culture-independent techniques.

Difficulty tracing the initial introduction of maassel –flavored tobacco embedded in glycerine, molasses and fruit essence– was previously reported[15]. Nonetheless, maassel was known to be in use since the 1990s[15]. Before maassel become widely used with waterpipes, another fermented type of tobacco was used raw, unflavored and produced harsher smoke[16]. Maassel, on the other hand, produced smooth aromatic smoke. Thus,

Corresponding Author: Mohammed A.A. Alqumber, Ph.D; e-mail: dr.alqumber@gmail.com maali@bu.edu.sa

maassel increased the marketability of waterpipe tobacco smoking by making it appealing to many individuals through its many flavor varieties and its smooth aromatic smoke[2,3]. Consequently, the consumption of maassel through the shisha paraphernalia (waterpipe) has been recently recognized as an increasing worldwide phenomenon[17-19]. Ill effects of smoking include significant increase in heart rate, blood pressure and carbon monoxide levels[20]. Understanding the microbial composition of maassel can add information about its origins and health effects.

Today new molecular technologies can allow for accurate determination of microbial compositions[21,22]. Determining the microbiology of maassel can provide information about its source and pathogenicity. Thus, this study reports the entire microbial composition of maassel as determined via 16S rDNA pyrosequencing. Eighteen different samples were analyzed. In total, 571 different species were identified, including many pathogens, plant, and soil microbes. Identified pathogens include *Mycobacterium riyadhense*, *M. chelonae*, *Shigella sonnei*, *S. flesneri*, *Klebsiella pneumoniae*, *Salmonella bongori, Coxiella* spp., *Acinetobacter* spp., *Staphylococcus haemolyticus, Streptococcus pseudopneumoniae* and *Streptococcus sanguinis.*

Materials and Methods

Literature Search

PubMed (Medline) and EMBASE online databases titles and abstracts were searched using the keywords ‘maassel’, ‘shisha’, ‘waterpipe’, ‘tobacco bacteria’ and ‘tobacco micr*’. The returned articles were retrieved and evaluated. Relevant articles were then discussed as far as applicable.

Sampling

Maassel packets (n = 18) representing 16 flavors (plum, grape, lemon, lemon with mint, mint, orange, watermelon, grapefruit, two apples, cappuccino, gum, grape with mint and gum mastic) from three different companies: 1) Alfakher Tobacco Trading, Ajman, United Arab Emirates; 2) Nakhla, Matco, Amman, Jordan and 3) Elbasha, Shebin El-Kom, Al-Minufiyah, Egypt were sampled. Samples were collected during the year 2014. The packets, purchased new and sealed, were delivered to the laboratory unopened. In the laboratory, specimens were collected aseptically from each packet for DNA extraction and microbial colony forming units (cfu) determinations. The packets were selected randomly from the most utilized brands within Albaha, as determined through a questionnaire of 253 smokers conducted during the three months preceding 2014. The smokers surveyed were recruited by flyers and posters and through the website: www.drqumber.wordpress.com. Surveyed participants aged 18-55 years (average age 25 ± 12.6) were all maassel waterpipe (shisha) smokers for a minimum of 2 years. The questionnaire was designed to capture anonymous demographic data (sex, age, level of education, address, career) and two questions: (1) what is your favorite maassel? and (2) what is the table of contents of your favorite masseel? For each tested brand of maassel, the pH value was measured, and the water activity (a_w) determined (Dacagon Pawkitt, Decagon Devices Inc., 2365 NE Hopkins Ct., Pullman, WA 99163, USA). Then samples were subjected to DNA extraction and 16S rDNA pyrosequencing and the resulting sequences were analyzed through established bioinformatics pipelines. DNA extraction utilized the Mobio Powersoil kit per manufacturer’s protocol (Mo Bio Laboratories, Carlsbad, CA, USA).

Determination of Colony Forming Units

One gram from each maassel packet was transferred aseptically into 100 ml of normal saline with 0.05 Tween 80 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and placed in a shaking water bath for 20 minutes at 37°C. After shaking, 10-fold serial dilutions were made and immediately spread on duplicate agar media. Blood agar, MacConkey agar (Merck, Darmstadt, Germany), half-strength trypticase soy agar (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and malt agar (Becton Dickinson and Company, Riyadh, Saudi Arabia) were used for the isolation of mesophilic (Gram-positive and Gram-negative) bacteria, coliforms (e.g., Gram-negative enterobacteria), thermophilic bacteria (e.g., actinomycetes), and fungi, respectively. The blood agar and MacConkey agar plates were incubated for 24 hours at 37°C, inspected for colony count, and then immediately incubated again at 22°C for 3 days, inspected for colony count again and lastly incubated at 4°C for 3 days. The half-strength trypticase soy agar plates were incubated at 55°C for 5 days. The malt agar plates were incubated at 30°C and then 22°C for 4 days at each temperature. The long incubations periods and the use of low temperatures were carried as previously reported for the isolation of a wide range of microbes[23]. Isolated bacteria were identified using the API 20E (BioMerieux, Marcy, France) while fungi were identified microscopically[24]. Moreover, the BIOLOG (Biolog, Inc., California, CA, USA) was used for identifying the isolated microbes. Results were reported as cfu/g of maassel.

Sequencing

16S rRNA gene sequence was targeted using the primer pair 799F (5′-GGTAGTCCACGCCGTAAACGATG-3′) and illbac1193R (5′- CRTCCMCACCTTCCTC-3′), per previously prescribed protocols[25,26]. Thirty cycles of PCR with HotStarTaq Plus Master Mix Kit (Qiagen, CA, USA) were per the following order: an initial step of 94°C for 3 minutes, then 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final expansion cycle at 72°C was carried for 5 minutes. After the PCR, the obtained reaction products were visualized using agarose gel for amplification and bands' relative intensity under ultraviolet light. Subsequently, samples were pooled (e.g., 100 samples) in equal quantities as determined from their DNA concentrations and molecular weight. The pooled samples were then purified via calibrated Ampure XP beads per manufacturer's instructions (New England BioLabs, Ipswich, MA, USA). The resulting products were made into DNA libraries and sequenced on a MiSeq platform following manufacturer's instructions at MR DNA, Shallowater, TX, USA.

Statistical Analysis

Analysis pipelines were used to process sequence data at Molecular Research MR DNA LP (Shallowater, Texas, TX, USA) by initially joining sequences together, then the barcodes were removed. Then sequences < 150 bp, chimeras or sequences with ambiguous base calls were eradicated. Next, the remaining sequences were denoised and used to generate operational taxonomic units (OTUs) by clustering at 3 divergence (≥ 97 similarity). Finally, the OTUs were classified taxonomically through BLASTn against curated databases derived from GreenGenes, RDPII and NCBI databases (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu) using previously described methods[27]. The analysis compiled results for each taxonomic level (kingdom, phylum, class, order, family, genus and species). Obtained sequence counts were presented as percentages. To determine richness, alpha (α) diversity, rarefaction curves were generated; and to know how microbial community compositions differed between maassel samples, beta (β) diversity, weighted UniFrac profiles using principal coordinates analysis (PCoA) were generated, using the workflow QIIME 1.7.0[28,29]. Here, initially sequences were denoised, primers and barcodes trimmed, and resulting sequences truncated to 300 base pairs and entered into the standard QIIME pipeline to generate the α and β diversity measures.

Taxa Presentation

Finally, the results from the different samples were inserted into Microsoft Excel (Microsoft® Excel® for Mac 2011, Version 14.0.0 (100825), Microsoft Cooperation, USA) as classified taxonomic units' relative abundance (percentages). These were ultimately reported as mean ± standard deviation (SD). Similarly, results obtained across tested samples (averages) were also presented as mean ± SD. Moreover, graphical charts were used to present these results. SPSS (version 14; 2005 SPSS inc. USA) program was used for other statistical analyses and results declared significant at $p < 0.05$.

Results

None of the articles retrieved (440 articles) from PubMed and EMBASE databases via the queries 'maassel', 'shisha' or 'waterpipe' included studies on the microbiota of maassel. Nonetheless, one study[30] found microbial compounds in waterpipe smoke and another study[31] determined the contamination levels of the waterpipe paraphernalia. Other tobacco products, however, were investigated for microbial composition[7,11,32].

The microbial community of the 18 maassel packets was exposed by high-throughput sequencings (Miseq, Illumina platform) of the variable region of 16S rRNA genes. After denoising the generated sequences, a total of 977,496 reads were obtained from the 18 sampled maassel packets (median, 51,361; mean, 54,305.33; standard deviation, 26,703.65). The mean read length was 384 (range, 314 - 504 bp; standard deviation, 34 base pairs). The reads clustered into > 751 distinct assignable OTUs with > 2 reads per cluster against the Greengenes, RDPII and NCBI databases at ≥ 97% similarity. The number of distinct OTUs per sample appeared at an average of 1.01 per 100 reads. That is, on average 405.89 species

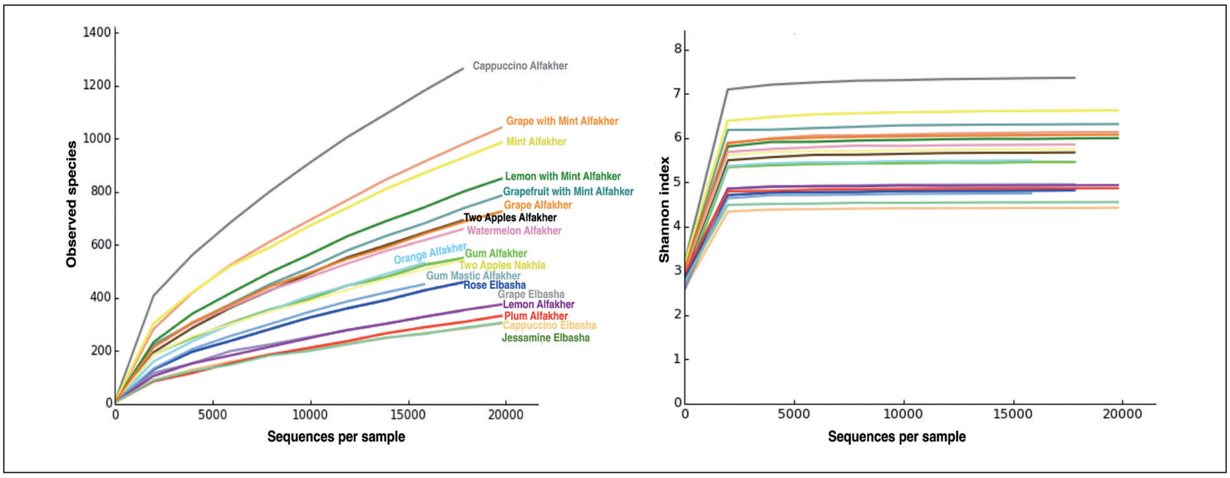

Figure 1. Beta diversity measures. Each line represents one sample; left side chart, observed species rarefaction curves; right side chart, Shannon index using the same color code used in the rarefaction curves on the left side.

or phylotypes were identified per maassel sample (standard deviation, 123.25; median, 410.5). The rarefaction curves of observed species metrics (species richness) (Figure 1) and the inter-sample β diversity weighted UniFrac profiles (PCoA) (Figure 2) indicated significant variation between samples, nonetheless, Elbasha brand samples clustered together (Figure 2). Although the rarefaction curves for all samples were a short distance from their observed species curve plateau, indicating that the obtained sequences covered most of the possible dominant bacterial species within the maassel samples, the Shannon diversity index, which reflects both richness and evenness (α diversity), plateaued (Figure 1), indicating that although other rare phylotypes could have been detected by more deep sequencing, the current sequencing depth had already captured most of the microbial diversity existing in the tested samples.

The results identified bacterial phyla (14), families (148), genera (334) and species (571). The number of prokaryotic phyla was the highest in Alfakher cappuccino maassel (11 phyla), Alfakher grape with mint (9 phyla) and Alfakher mint (8 phyla). Whereas Alfakher lemon, Al-

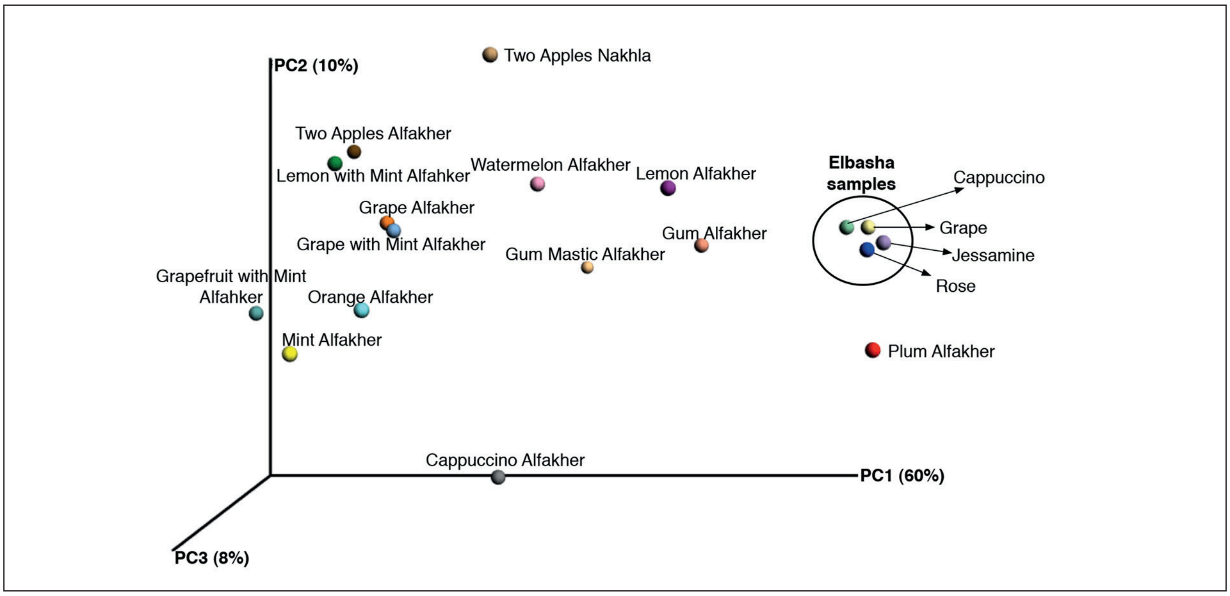

Figure 2. Weighted UniFrac-based PCoA analysis of maassel samples. Principle coordinates (PC) are indicated on the axes.

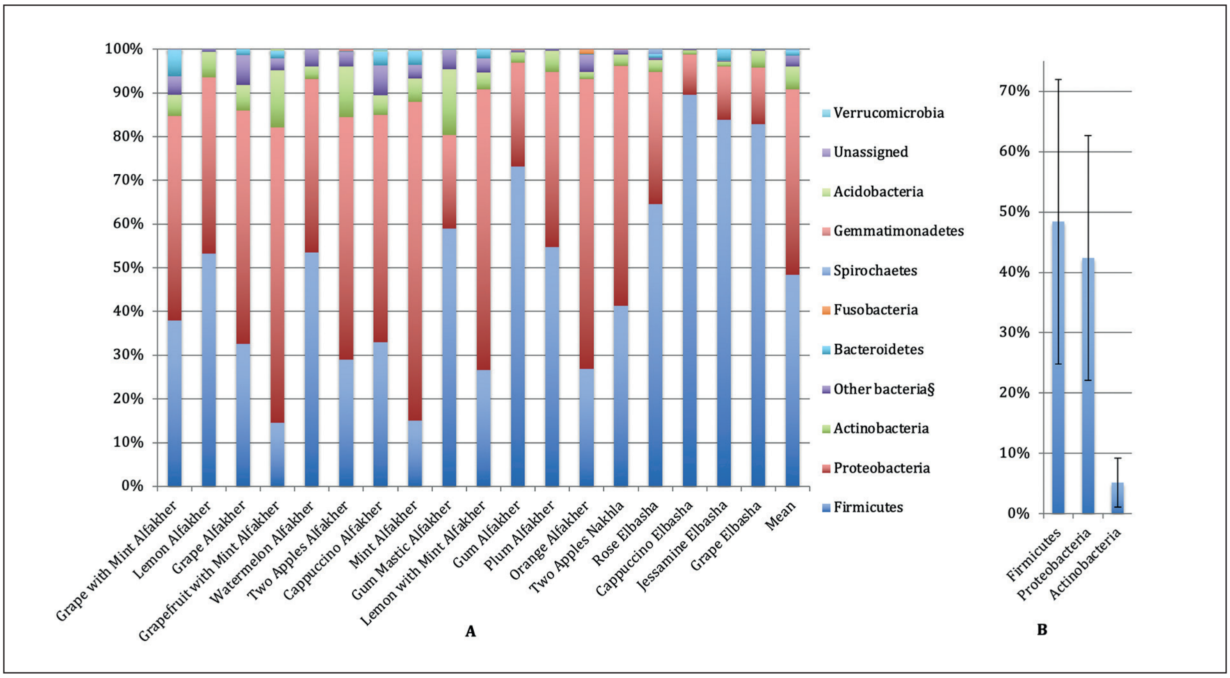

Figure 3. Phylum-level microbial composition of maassel. Legend: **(A)** phylum level comparison of maassel samples; Mean, average relative abundance of phyla; §, TM7 (candidate division), OP1 (candidate division), WS3 (candidate division), Chloroflexi, and Synergistetes; **(B)** core phylas' average relative abundance and standard deviation values.

fakher watermelon and Elbasha Jessamine had the lowest diversity of phyla, with a microbiota consisting of 4 phyla each. The average number of phyla detected per packet was 6.11 (standard deviation, 1.9; median, 5).

Phylum-level inter-packet individual variation of the maassels' microbiota is visualized in Figure 3. Three bacterial phyla, out of the identified 14, were present in all 18 tested packets while all the other detected phyla were not present in all packets. That is, a core microbiota consisting of three bacterial phyla, the *Firmicutes* (48.41 ± 23.56), *Proteobacteria* (42.43 ± 20.29) and *Actinobacteria* (5.19 ± 4.06) were identified in all tested packets (Figure 1 and Table I). Moreover, on average, the *Firmicutes* and *Proteobacteria* represented 90.84 ± 21.88 of assignable sequences across all 18 maassel packets. The relative abundance and prevalence rate of the main detected phyla are reported in Table I.

The *Firmicutes*, the most abundant phylum, was significantly more abundant in Elbasha brand packets (n = 4) 80.22 ± 10.82, than in other brands (Alfakher and Nakhla) (n = 14) 40.27 ± 17.2 (p =). Whereas Proteobacteria, the second most abundant phylum, was less abundant (16.16 ± 9.5) in Elbasha samples than the other two brands (Alfakher and Nakhla) (52.21 ± 15.65) (p =). *Actinobacteria* was more abundant in Alfakher samples (n = 13) (6.31 ± 4.24) than the other brands (n = 5) (2.39 ± 1.24).

At the family level, 19 out of 148 bacterial families were found across all maassel packets (Table II) and represented ≥ 75 of the obtained sequences in any tested maassel packet. Detected family-level inter-packet variations are represented in Figure 4.

Table I. Prevalence of major detected phyla and relative abundance.

Phylum	Prevalence*	Mean relative abundance†
Firmicutes	100	48.41 ± 23.56
Proteobacteria	100	42.43 ± 20.29
Actinobacteria	100	5.19 ± 4.06
Bacteroidetes	88.89	1.20 ± 1.70
Fusobacteria	16.67	0.06 ± 0.26
Spirochaetes	16.67	0.06 ± 0.24
Gemmatimonadetes	27.78	0.04 ± 0.10
Acidobacteria	33.33	0.03 ± 0.07
Verrucomicrobia	5.56	0.01 ± 0.06

*Percentage of positive packets (n = 18); †Average relative abundance ± standard deviation.

Table II. Bacterial families presented in all tested maassel samples.

Phylum	Family	Relative abundance	Standard deviation
Firmicutes	Bacillaceae	25.44543382	14.89846436
	Alicyclobacillaceae	4.5430342	3.978335145
	Staphylococcaceae	1.245383366	1.882452686
	Clostridiaceae	1.158002462	1.377657747
	Thermoactinomycetaceae	1.03500849	0.824003025
	Peptostreptococcaceae	1.02551553	2.528768135
Proteobacteria	Enterobacteriaceae	15.5576518	12.19257728
	Comamonadaceae	6.332932732	4.828167894
	Pseudomonadaceae	4.623575059	3.646224306
	Paenibacillaceae	4.536124528	3.715611724
	Sphingomonadaceae	3.079350307	2.39673397
	Oxalobacteraceae	2.717058596	3.105173495
	Methylobacteriaceae	1.671595464	1.90578342
	Xanthomonadaceae	1.123940696	1.681327767
	Rhizobiaceae	0.939010093	1.37697886
	Moraxellaceae	0.909776445	1.04758767
Actinobacteria	Micrococcaceae	1.04391671	1.283474437
	Microbacteriaceae	0.766204943	1.425897853
	Propionibacteriaceae	0.670706457	1.040942042

In general, *Bacillaceae* was the dominant subgroup of *Firmicutes* and commanded an average of 25.45 ± 14.9 of all assigned sequences. Other major *Firmicutes* identified include *Alicyclobacillaceae*, *Staphylococcaceae* and *Clostridiaceae* (Table II). The major subpopulations of *Proteobacteria* were the *Enterobacteriaceae*, *Comamonadaceae*, *Pseudomonadaceae*, *Paenibacillaceae* and *Sphingomonadaceae* (Table II). Three *Actinobacteria* families, dominated by *Micrococcaceae*, appeared in all packets (Table II).

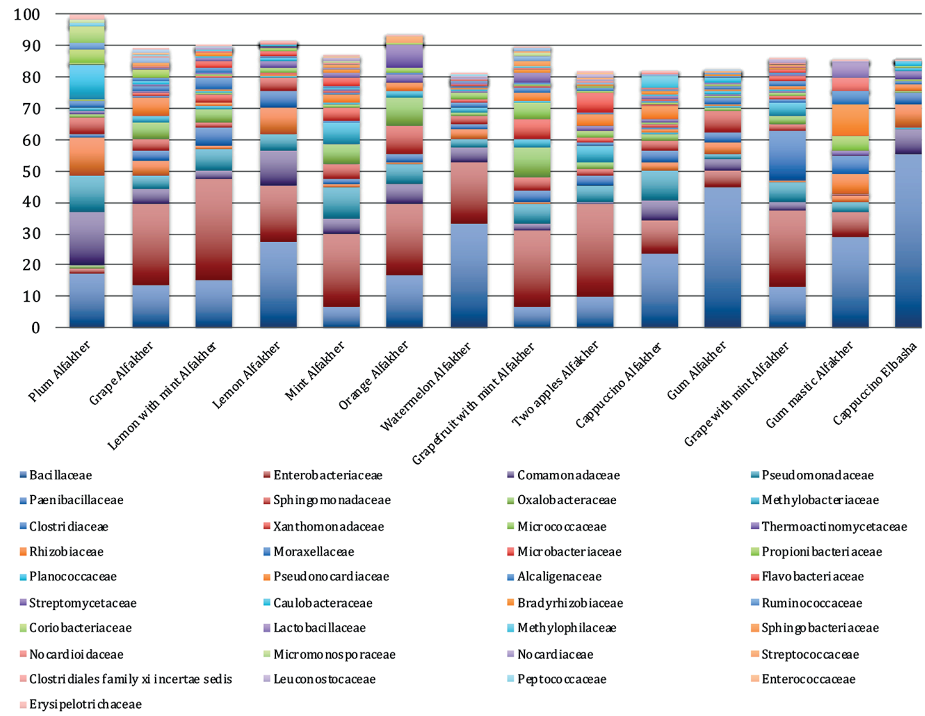

Figure 4. Family*-level comparison of maassel samples. *, Only families with average relative abundance > 1% where depicted.

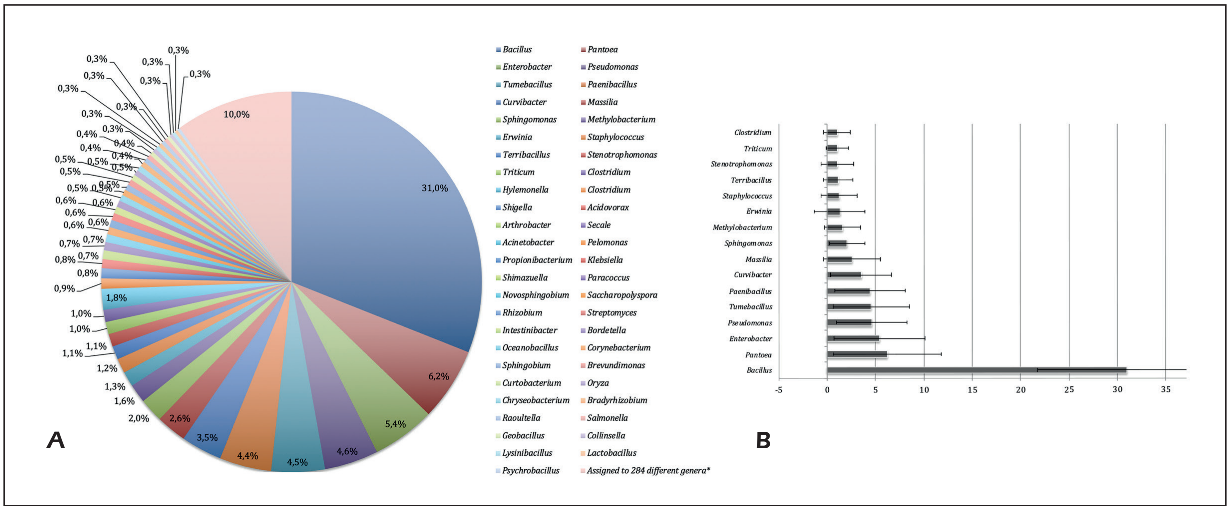

Figure 5. A, Microbial composition of maassel at the genus-level. *Represent sequences assigned to either of 284 different genera each having relative abundance < 0.24. **B,** The most abundant 33 microbial genera average relative abundance and standard deviation.

At the genus level, notwithstanding the high detected diversity (334 genera), fifty genera represented the majority of the bacterial flora (90 ± 5.21) across tested packets (Figure 5).

The *Bacillus* genus, in particular, was the most abundant and corresponded almost third of all assigned sequences (31.01 ± 9.25), followed by *Pantoea*, *Enterobacter*, *Pseudomonas* and *Tumebacillus* at a relative abundance of 6.2 ± 5.56, 5.4 ± 4.7, 4.6 ± 3.62 and 4.4 ± 3.9, respectively. Genera comprising serious pathogenic species that were found in every tested packet include *Staphylococcus*, *Clostridium, Shigella*, *Pseudomonas*, *Acinetobacter* and *Klebsiella*. *Salmonella* and *Bordetella* were found in all samples except the Elbasha Jessamine flavor and Alfakher plum flavor maassel, respectively. Other less prevalent genera include *Streptococcus, Enterococcus, Mycobacterium, Neisseria* and *Coxiella*. The rate of occurrence of these medically important genera and their relative abundance are presented in Table III.

The overall averages for each identified species indicated that the top 33 most abundant species represented 70.76 ± 3.2 of maassel's microbiota (Figure 6).

These top 33 species included human pathogens such as *Shigella sonnei*[33] (1.13 ± 2.54) and *Massilia timonae*[34,35] (1.41 ± 2); phytopathogenic bacteria such as *Erwinia persicina*[36] (1.15 ± 2.1); insect pathogens such as *Bacillus thuringiensis*[37] (1.61 ± 2); major soil bacteria such as *Bacillus subtilis*[38] (11.54 ± 3.5) and *Tumebacillus ginsengisoli*[39] (5.45 ± 3.9); aquatic bacteria such as *Methylobacterium adhaesivum*[40] (1.15 ± 1.5) and *Hylemonella gracilis*[41] (0.92 ± 0.54); rhizosphere bacteria such as *Acidovorax delafieldii*[42] (1.1 ± 1.41); several *Pseudomonas* species (Figure 4) and food associated bacteria such as *Bacillus pumilus*[43,44], which was also found to be associated with cows' udders[45] (1.33 ± 2.76). Other notable species identified include nontuberculous

Table III. Prevalence of medically important genera and their relative abundance in tested maassel samples.

Genus	Prevalence*	Mean relative abundance†
Staphylococcus	100	1.22 ± 1.88
Clostridium	100	1.03 ± 1.37
Shigella	100	0.88 ± 2.60
Acinetobacter	100	0.74 ± 0.93
Klebsiella	100	0.62 ± 0.63
Bordetella	94.44	0.48 ± 0.94
Corynebacterium	100	0.47 ± 1.16
Salmonella	94.44	0.32 ± 0.36
Lactobacillus	100	0.29 ± 1.15
Serratia	100	0.20 ± 0.33
Streptococcus	83.33	0.17 ± 0.50
Enterococcus	77.78	0.10 ± 0.19
Providencia	66.67	0.09 ± 0.39
Mycobacterium	66.67	0.07 ± 0.17
Neisseria	33.33	0.03 ± 0.14
Burkholderia	43.75	0.02 ± 0.08
Bacteroides	33.33	0.02 ± 0.10
Coxiella	5.55	0.01 ± 0.04

*Percentage of positive packets (n = 18); †Average percentage ± standard deviation.

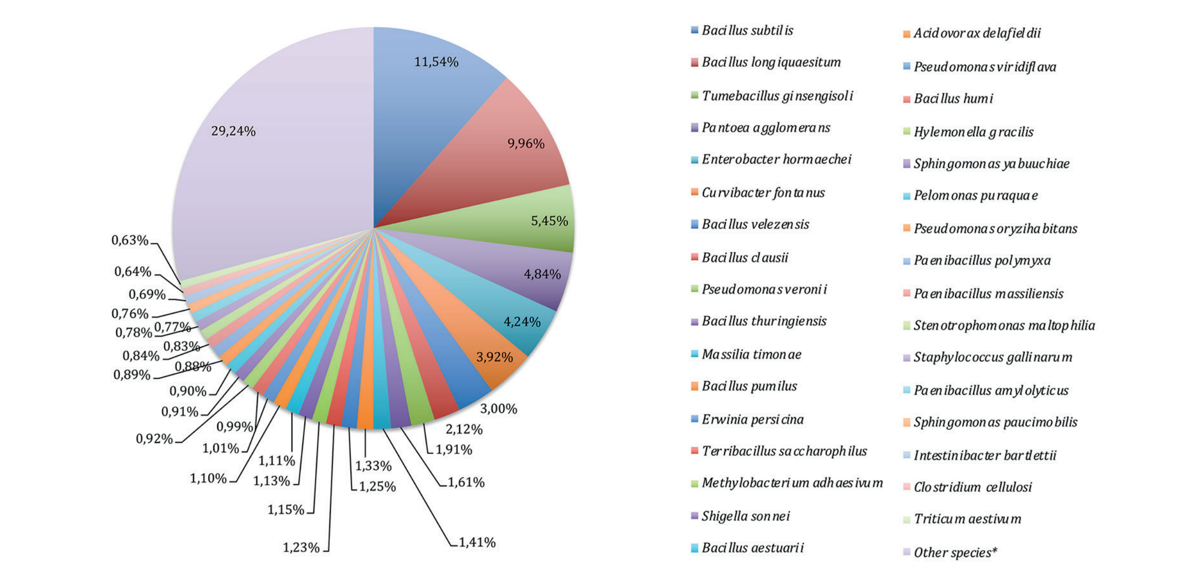

Figure 6. Average relative abundance of the most common bacteria. *Represent sequences that assigned with one of 538 species at a relative abundance < 0.5%.

mycobacteria (i.e., *M. riyadhense, M. chelonae* and *M. isoniacini*) (relative abundance, 0.072 ± 0.028; prevalence, 66.67%).

Discussion

Tobacco smoking can induce inflammation of the respiratory system[46,47]. Even though studies[7,48] established that the chemical composition of tobacco is associated with health risks, they only indicated that microbes on cured tobacco might be associated with health problems. Tobaccos' microbiology, as reviewed three years ago, showed that most culture based studies identified > 1 million colony-forming units (CFU) per gram of tobacco[7]. Nonetheless, culture based techniques are known to fail to capture the complete diversity of the microbial community, since many microorganisms are difficult, or impossible, to culture in conventional broth- or agar-based media[49]. Moreover, PubMed (Medline) and EMBASE literature searches did not return any DNA-based microbial composition analysis of maassel, and only few results were found for other types of tobacco[11,32].

In this study, the bacterial community of maassel, flavored tobacco embedded in glycerine, molasses and fruit essence, was analyzed through 16S rRNA pyrosequencing. The main phyla detected, *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were similarly found in flue-cured tobacco leaves (FCTL) K326, Zimbabwe FCTL and Italian Toscano cigar. Nonetheless, *Bacillus* and *Pseudomonas* were the two most dominant genera in FCTL K326[32], *Pantoea* and *Pseudomonas* in Zimbabwe FCTL[11], *Staphylococcaceae*, *Lactobacillales*, *Bacillus* and *Actinomycetales* in Italian Toscano cigar[14], while the most abundant genera in the maassel studied here were *Bacillus*, *Pantoea*, *Enterobacter*, *Pseudomonas* and *Tumebacillus*. That is, significant inter-tobacco samples microbial diversity (β diversity) was noted. Therefore, this study reiterates what was previously concluded: "that the dominant microbial populations might be closely related to the tobacco varieties"[11]. This may also explain the significant inter-maassel sample β diversity (PCoA) results. The additional fruit essences (grape, mint, lemon, grapefruit, apple..etc.), added to create maassel, might also contribute to the observed higher inter-sample microbial diversity seen in Figure 2. Nonetheless, several notable bacteria were commonly found in all aforementioned tobacco products. These include *Bacillus* and *Pseudomonas* species that are known to have tobacco-associated functions: *Bacillus* species improve aroma and increase smoke mildness[50]; *B. thuringiensis,* a usual isolate from stored-tobacco, might play a role in best control during storage[51]; and *Pseudomonas* species can breakdown nicotine and increase the quality of tobacco[52].

Shigella sonnei[33] (1.13 ± 2.54) and *Massilia timonae*[34,35] (1.41 ± 2); phytopathogenic bacteria such as *Erwinia persicina*[36] (1.15 ± 2.1); insect pathogens such as *Bacillus thuringiensis*[37] (1.61 ± 2); major soil bacteria such as *Bacillus subtilis*[38] (11.54 ± 3.5) and *Tumebacillus ginsengisoli*[39] (5.45 ± 3.9); aquatic bacteria such as *Methylobacterium adhaesivum*[40] (1.15 ± 1.5) and *Hylemonella gracilis*[41] (0.92 ± 0.54); rhizosphere bacteria such as *Acidovorax delafieldii*[42] (1.1 ± 1.41); several *Pseudomonas* species (Figure 4) and food associated bacteria such as *Bacillus pumilus*[43,44], which was also found to be associated with cows' udders[45] (1.33 ± 2.76). Other notable species identified include nontuberculous mycobacteria (i.e., *M. riyadhense, M. chelonae* and *M. isoniacini*) (relative abundance, 0.072 ± 0.028; prevalence, 66.67%). *Mycobacterium*, which was recovered from 79 of Albaha water reservoirs samples[53], appeared in 66.67 of Albaha tested maassel samples in this study. In addition, the recovered *Mycobacterium* species included one that was *M. riyadhense* (results not shown).

Conclusions

Flavors such as strawberry, vanilla, and cinnamon have been banned in many countries reducing the appeal of cigarettes to children. The current study elucidates the presence of human pathogens in flavored tobacco product maassel and provides more information and evidence on the harm associated with these products.

Conflict of Interest

The Author declares no conflict of interests.

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