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ENVIRONMENTAL MICROBIOLOGY

Tobacco, Microbes, and Carcinogens: Correlation Between Tobacco Cure Conditions, Tobacco-Specific Nitrosamine Content, and Cured Leaf Microbial Community

Audrey D. Law[1] · Colin Fisher[2] · Anne Jack[2] · Luke A. Moe[1]

Abstract Tobacco-specific nitrosamines are carcinogenic N-nitrosamine compounds present at very low levels in freshly harvested tobacco leaves that accumulate during leaf curing. Formation of N-nitrosamine compounds is associated with high nitrate levels in the leaf at harvest, and nitrate is presumed to be the source from which the N-nitrosation species originates. More specifically, nitrite is considered to be a direct precursor, and nitrite is linked with N-nitrosation in many environmental matrices where it occurs via microbial nitrate reduction. Here, we initiate work exploring the role of leaf microbial communities in formation of tobacco-specific nitrosamines. Leaves from burley tobacco line TN90H were air cured under various temperature and relative humidity levels, and 22 cured tobacco samples were analyzed for their microbial communities and leaf chemistry. Analysis of nitrate, nitrite, and total tobacco-specific nitrosamine levels revealed a strong positive correlation between the three variables, as well as a strong positive correlation with increasing relative humidity during cure conditions. 16S rRNA gene amplicon sequencing was used to assess microbial communities in each of the samples. In most samples, *Proteobacteria* predominated at the phylum level, accounting for >90 % of the OTUs. However, a distinct shift was noted among members of the high tobacco-specific nitrosamine group, with increases in *Firmicutes* and *Actinobacteria*. Several OTUs were identified that correlate strongly (positive and negative) with tobacco-specific nitrosamine content. Copy number of bacterial nitrate reductase genes, obtained using quantitative PCR, did not correlate strongly with tobacco-specific nitrosamine content. Incomplete denitrification is potentially implicated in tobacco-specific nitrosamine levels.

Keywords Tobacco-specific nitrosamines · Phyllosphere microbial community · *Nicotiana tabacum* · N-nitrosation

Introduction

Tobacco-specific nitrosamines (TSNAs) are carcinogenic compounds associated with cured tobacco products. These compounds arise from N-nitrosation of tobacco secondary alkaloids (Fig. 1), with the primary TSNAs found being N-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB). TSNA content in tobacco products is affected by a variety of factors including curing and storage conditions as well as nitrate content in the leaves [1, 2]. TSNA levels in fresh leaf material are low and can increase dramatically throughout the air curing process [2, 3]. This has led to speculation that TSNA formation may be driven by microbes on or in the leaf during curing. N-nitrosation is a complex process that is not fully understood but is presumed to result from the reaction of an activated nitrogen oxide species (e.g. NO^+, N_2O_3) with a primary or secondary amine (Fig. 1, reaction 2) [4]. In this sense, N-nitrosation can be considered an abiotic process, yet the nitrosating species in air-cured tobacco is thought to be derived from nitrite, which is generated biotically. Nitrate reductase enzymes, which are commonly

✉ Luke A. Moe
luke.moe@uky.edu

[1] Department of Plant & Soil Sciences, University of Kentucky, Lexington, KY 40546-0312, USA

[2] Kentucky Tobacco Research & Development Center, University of Kentucky, Lexington, KY 40546-0236, USA
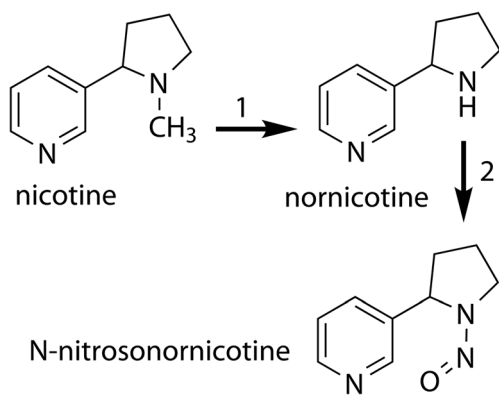
Fig. 1 Formation of NNN via N-nitrosation of nornicotine. *1* Demethylation of nicotine to nornicotine is catalyzed by the tobacco nicotine N-demethylase enzyme. *2* N-nitrosation is presumed to be an abiotic reaction between the secondary amine and an activated nitrogen oxide species

found in denitrifying bacteria, convert nitrate to nitrite as the first step in denitrification [4, 5]. Burley tobacco requires high rates of nitrogenous fertilizer to achieve an economically viable yield, and high levels of nitrate can accumulate in the leaves where it is linked to increased levels of TSNA in the cured product [3, 6].

Efforts to reduce TSNA levels in cured tobacco products have been focused largely on plant genetics and agronomic practices. One contributing factor is the level at which nicotine is converted to nornicotine via the tobacco enzyme nicotine N-demethylase (Fig. 1, reaction 1). Increased levels of levels of nornicotine leads to increased NNN production during the curing process. Plants exhibiting relatively high conversion of nicotine to nornicotine are termed "high converters," whereas varieties exhibiting low conversion are referred to as "low converters." Selection for low converter plants is currently the most effective method of limiting NNN accumulation during curing [7, 8]. However, controlling temperature and humidity during curing and storage, as well as limiting the amount of nitrate present in the plant prior to harvest has been shown to reduce TSNA levels as well [2, 9].

As a first step in assessing the role of microbes in TSNA formation, we here consider the microbial communities associated with cured tobacco from a high converter variety. Analyzing shifts in the microbial community that occur concurrent with changes in TSNA levels can help focus attempts to characterize the mechanism of TSNA formation and to better understand the circumstances in which high levels of TSNAs are formed. In this study, we correlate microbial community structure with tobacco curing conditions and levels of nitrate, nitrite, and TSNAs. Quantitative PCR is used to determine copy number of microbial nitrate reductase genes from each sample, and this data is compared against the above variables.

Materials and Methods

Tobacco Growth, Curing Conditions, and Processing of Samples

In the spring and summer of 2012, a high converter selection of burley tobacco (*Nicotiana tabacum*) variety TN 90 was grown at University of Kentucky's Spindletop farm on a plot prefertilized with 220 kg ha^{-1} of nitrogen as urea fertilizer (46 % nitrogen), following regional recommendations [10]. The plants were topped (flower heads removed) 63 days after transplanting and suckercide was applied. The plants were harvested by stalk cutting at 28 days after topping. Six plants were speared onto a stick, and 5 sticks from predetermined rows were placed into each of 22 fabricated curing chambers. The temperature and humidity in each chamber was controlled independently of each other using an air conditioning unit, a humidistat-controlled mister and a dehumidifier, so that the target temperature for each was either 15.6, 23.3, or 30.0 °C, and the target relative humidity was 60, 75, 80, or 90 %. The curing was terminated after 10 weeks, when the midribs of the leaf in all the curing units were dry. The fourth leaf from the top of each of the 30 plants in each curing unit was removed, and the lamina was separated from the midrib and air-dried in paper bags at room temperature. The lamina was ground to pass through a 1-mm sieve in a Wiley mill and the samples were subsequently stored at 4 °C. From each individual curing chamber, one composite sample was generated that was the result of the processed leaves from 30 individual plants.

Chemical Analysis of Tobacco Ground Leaf Tissue

Ground samples were analyzed for TSNAs using gas chromatography-thermal energy analysis (GC-TEA) according to the method of Morgan et al. [11]. Nitrate and nitrite content of the plant material was analyzed chromogenically using the microplate methods of Crutchfield [12, 13].

Isolation of Bacterial Cells from Ground Leaf Tissue

Bacterial cells were separated from tobacco leaf tissue in order to minimize interference from plant mitochondrial and plastid DNA in the 16S rRNA PCR amplification—the method used was adapted from Ikeda et al. [14]. Primer-based methods of excluding plant-derived sequences were not considered due to the lack of information about how this would work with the MiSeq sequencing platform we intended to use. Briefly, 20 g of ground sample was added to a 1-L blender jar (Kinematica Microtron MB 550) with 400-ml BCE buffer (50 mM Tris–HCl, pH 7.5, 1 % (*v/v*) Triton X-100, 2 mM 2-mercaptoethanol added directly before use). Samples were processed at full speed for 1 min, followed by 1-min incubation on ice for a total of three cycles. The homogenate was filtered

with a single layer of autoclaved Miracloth (EMD Millipore) using a Büchner funnel, and then centrifuged at 500×*g* for 5 min at 10 °C. The supernatant was centrifuged again at 5500×*g* for 20 min at 10 °C. The supernatant was then discarded and the pellet resuspended in 250-ml BCE and centrifuged at 10,000×*g* for 10 min at 10 °C; this step was repeated a second time. The final pellet was resuspended in 6 ml Tris Buffer (50 mM, pH 7.5) and layered over 4-ml Nycodenz (Accurate Chemical and Scientific Corp., Westbury, NY, USA) solution (8 g Nycodenz in 10 ml Tris buffer) in a 15-ml glass tube and centrifuged at 10,000×*g* for 40 min at 10 °C. The bacterial cell mass at the interface of buffer and Nycodenz was removed with a pipette, transferred to a 2-ml microcentrifuge tube with an equal volume of sterile water, and centrifuged at high speed for 5 min. The supernatant was poured off and the pellet was frozen at −20 °C.

Genomic DNA Extraction

Recent comparisons have shown the potential for bias in microbial community composition studies based on extraction methods of community DNA [15, 16]. The bacterial cell pellet obtained in the leaf extraction step contained fine solid particulates and soluble exudates from cured tobacco during the isolation process, and it was determined that detergent lysis followed by ethanol precipitation yielded more, higher quality DNA than bead-beating followed by spin column isolation. This is consistent with the work of Maropola et al., who recommended detergent lysis using SDS and/or cetyl trimethylammonium bromide (CTAB) as a suitable method for metagenomic analysis of endophytic bacterial communities [15]. In this study, the protocol for extraction and purification of genomic DNA was adapted from Wilson [17]. Both SDS and CTAB are used along with a high salt concentration for increased removal of proteins and polysaccharides. The frozen pellet obtained in the previous step was resuspended in 567 μl of TE buffer before adding 30 μl of 10 % (*w/v*) SDS and 3 μl of Proteinase K (20 mg ml^{-1}), then incubated at 37 °C for 1 h. To this mixture, 100 μl of 5 M NaOH was added and mixed thoroughly, followed by 80 μl of 10 % (*w/v*) CTAB in 0.7 M NaCl, and incubated for 10 min at 65 °C. The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1), followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1). Nucleic acids were precipitated with 0.6 vol of isopropanol, centrifuged at 12,000×*g* for 25 min at 4 °C, and rinsed twice with 1 ml of 70 % (*v/v*) ethanol. The air-dried pellet was resuspended in TE buffer and quantified with a Qubit® 3.0 Fluorometer (Life Technologies, Grand Island, NY).

qPCR of Nitrate Reductase Genes

Previously developed degenerate primers designed to amplify the bacterial *narG* (forward 5′-TCGCCSATYCCGGCSATGTC-3′,

reverse 5′-GAGTTGTACCAGTCRGCSGAYTCSG-3′) and *napA* (forward 5′-TGGACVATGGGYTTYAAYC-3′, reverse 5′–ACYTCRCGHGCVGTRCCRCA-3′) genes from diverse communities of *Proteobacteria* were used in a real-time PCR assay to determine the copy number of the genes present in each sample DNA extract [18]. Standard curves were generated using linearized plasmids containing the amplified sequence of *narG* and *napA* genes of *Psuedomonas aeruginosa* PAO1. The plasmids were the original constructs used by Bru et al. who generously shared them with us [18]. A 20-μl reaction containing 1× SYBR Select Master Mix for CFX (Applied Biosystems), a final concentration of 2 μM of each primer, and 250 pg to 8.5 ng DNA were prepared. All reactions were performed in triplicate. The reactions were run on a Bio-Rad CFX real-time PCR machine using the following conditions: for *narG*, initial carryover prevention and denaturing steps at 50 °C for 2 min, and 95 °C for 5 min, followed by 6 touchdown cycles: 15 s denaturing at 95 °C, 1 min annealing/extension at 68 °C decreasing −1 °C per cycle, followed by fluorescence reading at 81 °C for 15 s (above melting temperature for primer dimers and below melting temperature for amplicon) and finally 35 cycles with a final annealing/extension temperature of 63 °C; *napA* conditions were similar with the exception of a constant 61 °C annealing/extension temperature for 40 cycles. A melting curve was generated after the quantification run was complete, and amplification products were run on a 2 % (*w/v*) agarose gel to confirm the size of the PCR product and the absence of nonspecific products. Data was expressed as copy number per nanogram DNA.

MiSeq Amplification

The V4 region of the 16S rRNA gene was amplified using dual barcoded primers according to the protocol of Kozich et al. [19]. Briefly, 1 μl of the extracted genomic DNA (up to 10 ng) was added to 17 μl of AccuPrime Pfx Master Mix (Invitrogen, CA) with 1 μl of each primer (500 nM) in a 20-μl reaction. Amplification was verified by gel electrophoresis; the reactions were purified with Agencourt AMPure XP beads (Beckman Coulter Inc., Brea CA), quantified using a Qubit® 3.0 Fluorometer (Life Technologies, Grand Island, NY), and pooled in equimolar amounts with approximately 10 % PhiX DNA before loading on MiSeq flowcell for a 2 × 250 read run. Sequence data was uploaded to the NCBI Sequence Read Archive (SRA), with the accession number SRP055959.

Data Analysis

MiSeq community sequence data was processed using Mothur (version 1.33.0, Feburary 2014) and Mothur's MiSeq SOP, accessed June 2, 2014 [19, 20]. Briefly, paired-end reads were assembled into contigs; sequences were filtered for length, ambiguous bases, and homopolymer regions,

and then aligned to a SILVA reference alignment of the V4 region to remove sequences other than the V4 region of the 16S rRNA gene. Preclustering to merge highly similar (2 bp or less mismatch) sequences was followed by the removal of chimeras and 16S rRNA sequences derived from mitochondrial and chloroplast DNA. The data set was subsequently normalized to 21,108 sequences per sample for further analysis, as this is the number of sequences in the sample with the lowest output. Operational taxonomic units (OTUs) were defined using a cutoff of 0.05 (95 % similarity) and taxonomic classification based on Ribosomal Database Project (RDP) reference sequences (version 9, March 2012) was done.

In order to compare the microbial communities on the basis of relative abundance of OTUs, PC-ORD 6.15 (MjM Software Design, Gleneden Beach, OR) was used to perform non-metric multidimensional scaling ordination (NMDS) using Bray-Curtis distance measure. To test the null hypothesis of no difference between groups of samples, multiresponse permutation procedure using Bray-Curtis (MRPP), and perMANOVA (non-parametric MANOVA) was used [21]. Due to groups having unequal numbers of samples, perMANOVA was set up to run 1000 permutations randomly selecting three samples from each group, giving the average p value.

Mean comparison using Tukey's HSD was used for group comparison of leaf chemistry data as well as relative abundances of specific OTUs. While the leaf chemistry data was analyzed using a significance level of $\alpha = 0.05$, an increased alpha level of 0.10 was used for comparisons of relative abundances data in order to consider the trends observed despite small sample size and uneven groups.

Results

Burley tobacco was air-cured under conditions of varying heat (15.6–30 °C) and relative humidity (60–90 %) in constructed curing chambers. Each heat/humidity combination ($n = 11$) had two dedicated chambers at the beginning of the curing process, but one replicate (23.3 °C, 90 %RH) was not used due to a technical issue with the chamber, leaving the total number of samples as 21. Each sample represents a composite of 30 plants from the same curing chamber. The cured products were processed for chemical (nitrate, nitrite, TSNA) and biological (community analysis via 16S rRNA gene sequencing, qPCR of nitrate reductase genes) properties, and correlations were made to assess any relationship between microbial communities and leaf properties according to curing conditions.

Leaf Chemistry and qPCR of Nitrate Reductase Genes

For each sample, the concentrations of total TSNAs, nitrate, nitrite, and copy numbers of nitrate reductase genes *napA* and *narG* were determined (Table 1). In order to investigate

correlation between the microbial community and TSNA levels in the leaves, samples were grouped nominally according to total TSNA content in microgram TSNA per gram dry sample weight (Group 1, ≥ 98 µg g^{-1}; Group 2, 20–35 µg g^{-1}; Group 3, 10–19 µg g^{-1}; Group 4, <10 µg g^{-1} TSNA). Total TSNA levels in the leaf tissue correlated positively with relative humidity and both nitrate and nitrite levels (Fig. 2). There was no significant correlation between TSNAs and copy number of *napA* and *narG* genes. However, *napA* was positively correlated with nitrate levels (Fig. 2).

16S rRNA Gene Amplicon Sequencing

A total of 4,154,669 sequences were generated from the MiSeq run passing a quality filter score of Q30 or greater. After processing sequences through the Mothur program and normalizing the number of sequences in each sample, a response matrix consisting of 504 OTUs from 21 tobacco samples was generated from the Mothur output. OTUs that only occurred once in the dataset were removed, leaving 219 OTUs. Not all OTUs were classified to the level of genus; those listed are at the highest level of classification that was named using the RDP classifier. Where multiple taxonomic classifications are listed in the results and discussion, the order indicates relative abundance from highest to lowest. Numbers in parentheses following a taxonomic classification indicate the bootstrap confidence value generated by the RDP classifier.

Microbial Community Changes Correlated with TSNA Levels in Leaf Tissue

A total of 12 phyla were identified in the cured leaf samples. OTUs belonging to *Proteobacteria* comprised greater than 90 % of the community in groups 2–4 with *Actinobacteria*, *Firmicutes*, and *Bacteriodetes* in the range of 1–5 % of the total relative abundance. In Group 1 (highest TSNA levels), there was a statistically significant (Tukey's HSD, $\alpha = 0.05$) shift in the microbial community toward a higher percentage of *Actinobacteria* and *Firmicutes* and lower amounts of *Proteobacteria* (Fig. 3). Of the *Proteobacteria*, OTUs classified as *Alcaligenaceae*, *Methylobacterium*, and *Microvirga* were significantly more abundant in Group 1 (even though the phylum as a whole decreased in this group), while *Pseudomonas* was significantly lower. Also significantly higher in Group 1 are *Actinobacteria* OTUs classified as *Arthrobacter*, *Corynebacterium*, *Nocardioides*, and *Brachybacterium* and *Firmicutes* OTUs classified as *Jeotgalicoccus* and *Bacillaceae*.

Other phyla comprising less than 1 % of the total relative abundance in all samples included (not in order of abundance) *Acidobacteria*, *BRC1*, *Chloroflexi*, *Deinococcus-Thermus*, *Gemmatimonadetes*, *Planctomycetes*, *Verrucomicrobia*, as

Table 1 Curing conditions and tobacco leaf chemistry

TSNA group/ sample	Target temperature (°C)	Target % relative humidity	Total TSNA ($\mu g\,g^{-1}$)		NO_2^- ($\mu g\,g^{-1}$)		NO_3^- ($\mu g\,g^{-1}$)		$napA$[a]	$narG$[a]
4	15.6	60	2.7	d	2.1	b	1388	c	67.1	1975.8
4	15.6	60	3.3		2.2		1612		549.7	601.9
4	23.3	70	4.4		2.0		1604		384.8	2923.6
4	23.3	60	5.2		1.5		1440		80.5	1078.9
4	15.6	75	5.3		2.7		1332		85.1	1520.8
4	23.3	60	5.5		1.7		1403		222.8	2550.9
4	30	75	8.2		1.7		1174		89.9	1283.3
3	30	75	10.4	c	1.5	b	1714	bc	321.2	939.3
3	30	80	11.9		1.9		2003		119.6	3028.9
3	15.6	75	12.1		5.0		2218		192.5	2576.4
3	30	80	12.2		1.6		1477		320.1	3123.7
3	23.3	70	13.5		2.7		1922		3019.3	25435.9
2	30	60	19.7	b	2.3	b	1829	b	226.3	2340.2
2	23.3	75	23.7		5.1		1925		446.3	864.6
2	30	60	27.0		18.2		2093		532.8	7051.5
2	30	90	32.0		2.2		1858		234.5	3232.6
2	23.3	75	32.3		9.7		2039		1018.0	7013.4
2	23.3	90	34.8		3.7		3017		134.7	2454.2
1	15.6	90	98.2	a	334.5	a	2885	a	76.7	673.7
1	30	90	100.7		78.5		3882		2706.0	25394.8
1	15.6	90	111.3		227.1		3452		743.6	1241.9

Samples were named and separated into one of four groups based on total TSNA levels (Group 1, $\geq$98 $\mu g\,g^{-1}$; Group 2, 20–35 $\mu g\,g^{-1}$; Group 3, 10–19 $\mu g\,g^{-1}$; Group 4, <10 $\mu g\,g^{-1}$ TSNA). Different lowercase letters following the first sample in a group denotes a significant difference in a Tukey HSD means comparison, $\alpha = 0.05$

[a] $napA$ and $narG$ qPCR data listed as gene copy numbers per nanogram of DNA

well as bacteria unclassified at the phylum level. All of these phyla have been previously described in the rhizosphere, phyllosphere, or endophyte community of leafy greens or fresh tobacco leaves, and it does not appear that abundant OTUs or those correlated with TSNA levels were the result of postharvest contact [22–24].

Of the 219 OTUs, 16 were present in all samples and are considered here to be the "core microbiome" (Table 2). Of these 16 OTUs, five showed significant differences in abundance with respect to sample groups (Tukey's HSD, $\alpha = 0.10$). The genus *Pseudomonas* was significantly less abundant in Group 1 (highest TSNA levels) compared to the other three groups, while OTUs classified as *Rhizobium* (67), *Arthrobacter* (57), *Alcaligenaceae* (100), and *Rhodobacteriaceae* (100) were significantly greater in abundance in Group 1.

NMDS Ordination and Group Comparisons

A two-dimensional NMDS solution was chosen to interpret after verifying the consistency of patterns across multiple NMDS solutions (stress = 4.26, $p = 0.016$) (Fig. 4). The ordination results indicate a pattern that strongly separates samples

in Group 1 (TSNA levels $\geq$98 $\mu g\,g^{-1}$) from the others. Positively correlated leaf chemistry variables (indicated by the vectors in Fig. 4) were total TSNAs, nitrate, nitrite, and relative humidity. The MRPP result supports the rejection of the null hypothesis (i.e., no difference between groups, $A = 0.22$, $p = 0.00024$). In MRPP pair-wise comparisons (non-corrected), group 1 is statistically different from groups 2–4 (comparing Group 1 to 2, 3, and 4 respectively, $A = 0.30$, 0.30, 0.32, $\alpha = 0.005$), while groups 2–4 are not statistically different from each other. The perMANOVA results also supported group differences, significant at a higher alpha level (pseudoF = 5.18, $p = 0.010$), and pair-wise group comparisons showed a similar result as MRPP, with group 1 differing from groups 2–4 with ($t = 2.57$, 2.90, 2.37, $\alpha = 0.01$).

In order to determine which OTUs were influencing the group separation seen in the NMDS ordination, linear correlations with the first axis having an r value of 0.5 and above were visualized in an ordered matrix (Fig. 5). OTUs with high correlations to the NMDS ordination were further analyzed using ANOVA to determine significant differences in relative abundance of the OTUs between groups (Tukey's HSD, $\alpha = 0.10$). Those that showed significant difference in

Fig. 2 Log-log plots showing correlations between nitrate (NO_3^-, in µg g^{-1}), nitrite (NO_2^-, in µg g^{-1}), napA (gene copy number ng DNA^{-1}), narG (gene copy number ng DNA^{-1}), target curing temperature (°C), and curing relative humidity (%RH). *Shading* represents the 95 % confidence interval. *P* values <0.05 are listed along with *r* values
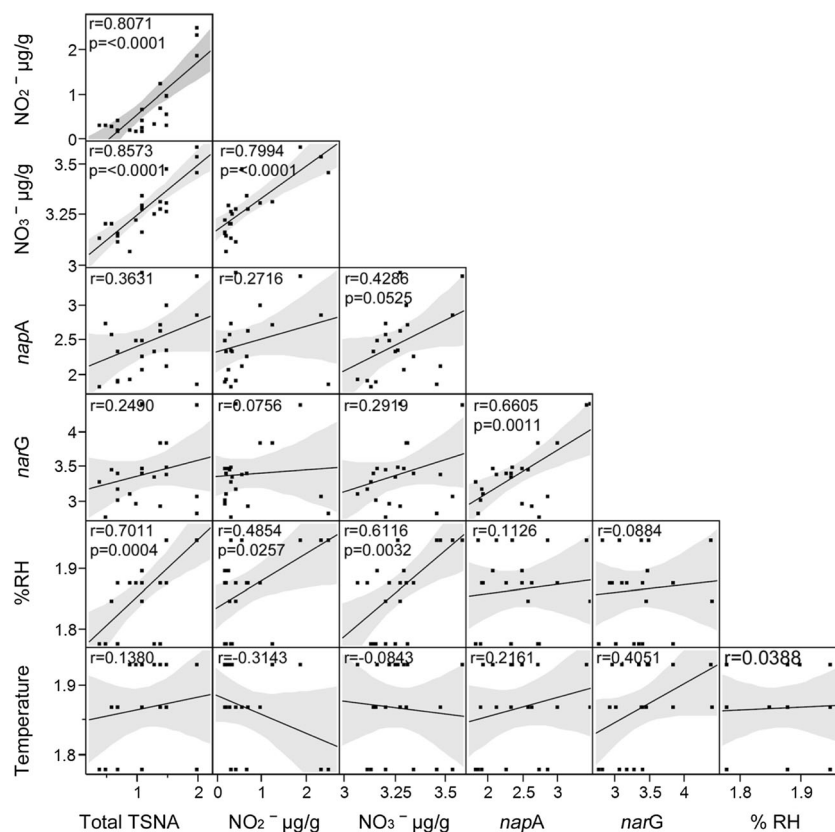
abundance were considered to be the dominant influences. Three OTUs classified as *Pantoea*, *Pseudomonadaceae*, and *Pseudomonas* were negatively correlated with TSNA levels, having the highest abundances in groups 3 and 4. The remaining OTUs in the ordered matrix were positively correlated with TSNA levels.
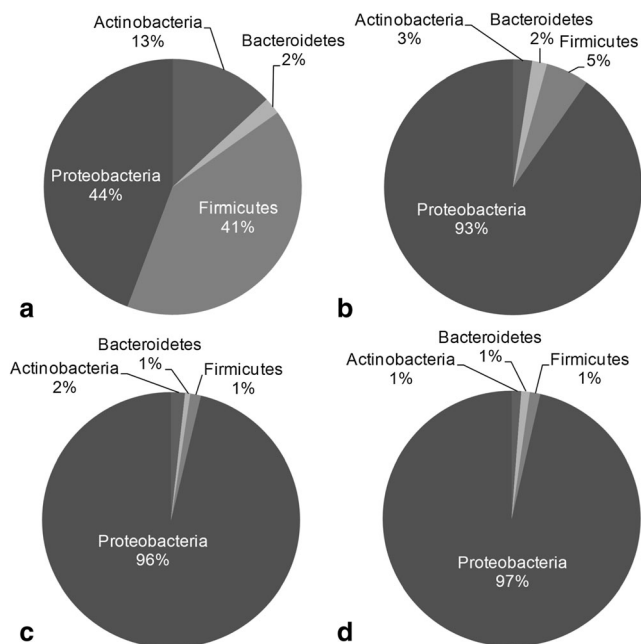
Discussion

TSNA accumulation during curing of tobacco is thought to be driven at least partly by microbial activity in or on the tobacco leaf, but to date, these microbial communities have not been thoroughly studied. Here, we have shown that burley tobacco accumulated higher levels of TSNAs, nitrate, and nitrite under curing conditions with high relative humidity (supporting previously established correlations [2, 25, 26]), and a corresponding shift in the microbial community was observed in samples with the highest levels of these variables. It should be noted here that the high converter variety grown in this study was chosen to maximize TSNA formation potential in response to curing conditions. This variety, along with selection for high heat and high humidity curing conditions, would not be used in commercially grown tobacco. The levels of nitrate in the samples with the highest TSNA content were significantly higher than the other treatments, and nitrite was unusually high even compared to the results of other tobacco curing studies [2]. The plants in this study were grown in the same

Fig. 3 *Pie charts* showing the percentages of OTUs belonging to abundant phyla within each group, according to TSNA content. Percentages are calculated from the average of each group; **a** Group 1 (highest TSNA content), **b** Group 2, **c** Group 3, **d** Group 4 (lowest TSNA content)

Table 2 Core microbiome and range of OTU relative abundance by TSNA group

OTU Classification	TSNA group			
	1	2	3	4
Pseudomonadaceae(100)	2173–7029	1075–8486	5336–11803	4032–8499
Pantoea(96)	1286–9515	332–11109	4253–13979	1075–12200
Pseudomonas(99)[a]	111–776	34–4924	728–4127	1339–6172
Rhizobium(67)[a]	1523–1089	108–465	21–659	105–899
Stenotrophomonas(94)	254–967	2–3102	15–699	58–795
Bacillus(52)	85–7692	8–10652	3–121	8–1809
Arthrobacter(57)[a]	124–3894	13–276	23–461	11–205
Sphingomonas(100)	37–137	55–308	28–610	93–253
Microbacteriaceae(93)	106–195	24–635	7–245	12–231
Massilia(95)	11–60	12–239	31–253	37–217
Rhodobacteraceae(100)[a]	116–367	6–245	1–108	5–105
Alcaligenaceae(100)[a]	165–334	32–334	12–236	5–115
Comamonadaceae(98)	8–52	3–35	1–37	4–183
Sphingobacterium(100)	1–92	1–972	3–1029	3–947
Saccharibacillus(100)	38–431	46–88	2–387	4–328
Sphingomonas(100)	10–12	8–149	9–152	7–59

Numbers in parentheses are bootstrap values for the RDP classification of OTUs

[a] Significant difference between Group 1 and groups 2–4

field and fertilized at the same rate, and we assume that nitrate levels in leaves were similar at harvest and the beginning of the curing process. Differences in nitrate levels could be explained by either variations in the rate of nitrate removal or appearance of additional nitrate during the course of cure. Unlike denitrification, nitrification is generally considered taxon-specific. Thus, if nitrate were being formed in this environment via nitrification, we would expect to observe members of genera to which this process (nitrite oxidation) has been attributed (e.g., *Nitrobacter*). This was not the case, and so we suggest that this is due to differences in nitrate removal.

Biological nitrate removal proceeds through a reductive process. Denitrification and dissimilatory nitrate reduction to ammonia (DNRA) are both initiated by nitrate reduction to nitrite. These pathways then diverge—nitrite is reduced to nitric oxide, nitrous oxide, and finally to dinitrogen in denitrification, while nitrite is directly reduced to ammonia in DNRA. Both of these processes can be uncoupled, meaning that so-called intermediates of the pathway can accumulate. Accumulation of these compounds can be due to environmental conditions, which may inhibit enzymatic reduction or expression of the cognate genes, or it can be due to the lack of the complete genetic complement required for these processes. Partial denitrification, for example, is now presumed to be quite common based on genomic analysis [27], and not all nitrate-reducing organisms encode mechanisms for further reducing nitrite.
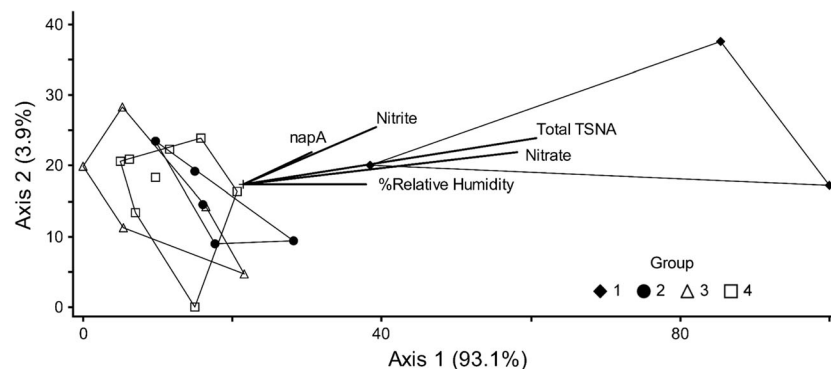

Fig. 4 NMDS ordination of microbial communities according to total TSNA levels within each group. *Vectors* correspond to variables correlated with the ordination above the cutoff $r = 0.2$, with the length of the vector proportional to its correlation value. Samples are represented according to their TSNA grouping (1 = *black diamond*, 2 = *black circle*, 3 = *white triangle*, 4 = *white square*)
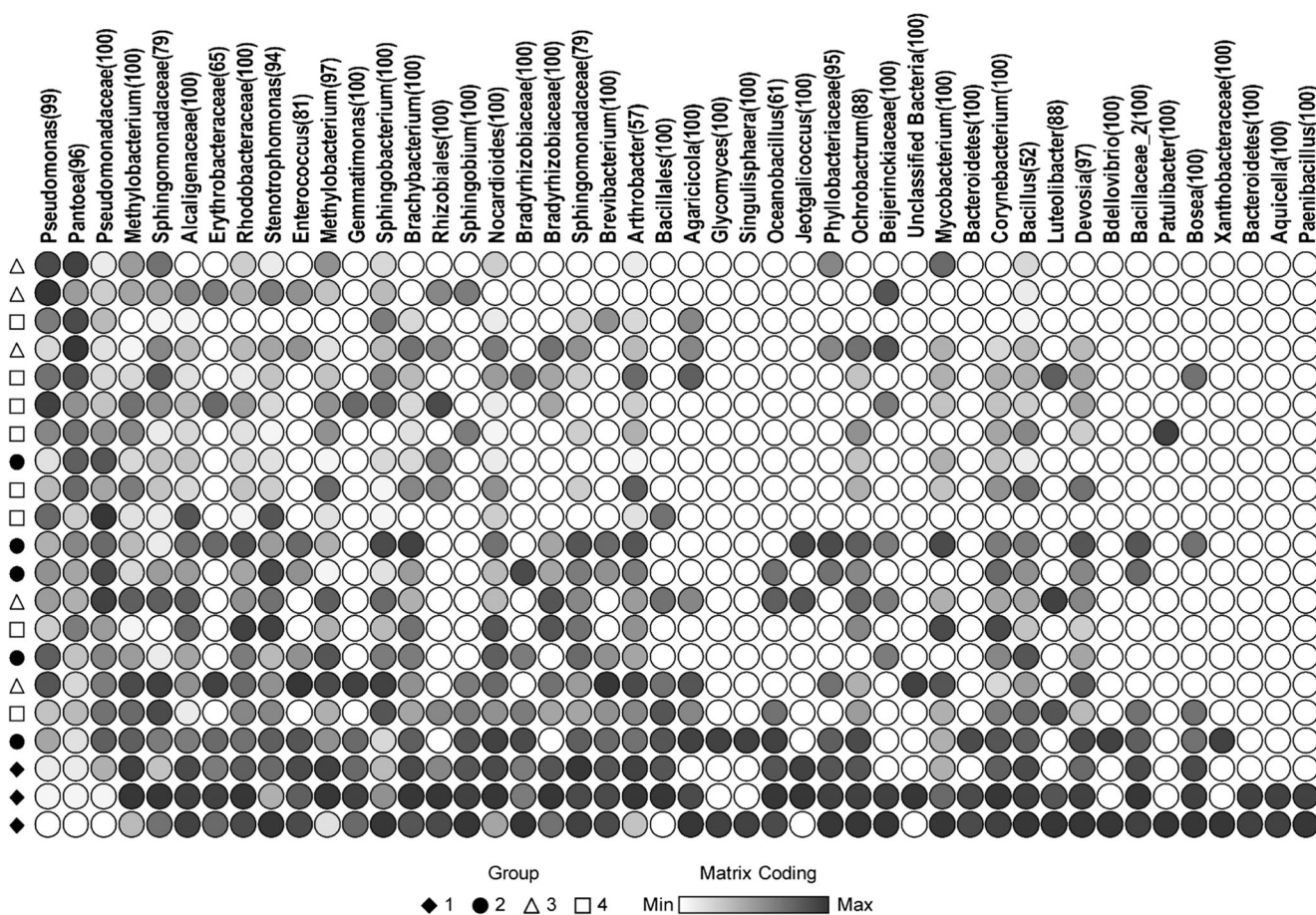
Fig. 5 Ordered matrix of OTUs showing high linear correlations within NMDS ordination. OTUs included have an *r* value of 0.5 and above. Samples and OTUs are in order according to their Bray-Curtis rank in the NMDS ordination, so that the farther apart they appear in the list, the greater the Bray-Curtis distance between them. OTUs are listed at their highest taxonomic classification, bootstrap values are in parentheses. Samples are represented according to their TSNA grouping (1 = *black diamond*, 2 = *black circle*, 3 = *white triangle*, 4 = *white sqaure*). *Shading of cells represents the proportion of the total OTU relative abundance by sample, indicated as shading from lowest (light) to highest (dark)*

In addition to the work here, nitrite accumulation has been noted in other environments (e.g., sediments, achlorhydric stomach) and has been attributed to variations in the microbial composition [28, 29]. The lack of correlation observed here between TSNA content and nitrate reductase gene copy number suggests that it is not necessarily nitrate reduction that is the most important but the further reduction of nitrite. This would explain the strong correlation between high levels of nitrite and high TSNAs seen in Table 1. Indeed, nitrite is considered to be the precursor molecule for biological N-nitrosation, and our data support a model whereby the efficiency of nitrite reduction is the key indicator for TSNA formation. Consistent with this model, researchers found that introducing a *Bacillus* species with high nitrite-reducing potential to curing tobacco leaves significantly reduces both nitrite and TSNA accumulation [30].

We speculate that the microbial communities in groups 2–4 rendered more complete denitrification, leading to lower concentrations of both nitrate and nitrite, while group 1

communities had incomplete denitrification. Recent work shows that members of the *Actinobacteria* and *Firmicutes* phyla, which were more abundant in group 1, commonly encode partial denitrification pathways [27]. In a study of non-denitrifying nitrate reducers, Smith and Zimmerman determined that 9 of 14 "nitrite accumulators" were from the *Bacillus* genus [31]. From our work, seven OTUs of the *Firmicutes* phylum positively correlated with group 1 in the NMDS ordination, and two of these were significantly more abundant in a group comparison—OTUs belonging to the order *Bacillales* and to the genus *Jeotgalicoccus*. OTUs from the genus *Bacillus* and class *Bacillaceae* also showed a strong trend toward higher abundance in groups 1 and 2, but this was not significant in a group comparison. From the *Actinobacteria*, eight OTUs positively correlated with group 1 in the NMDS ordination and four of those were significantly more abundant in the group comparison: *Arthrobacter*, *Corynebacterium*, *Nocardioides*, and *Brachybacterium*.

The *Proteobacteria*, which formed a much lower percentage in group 1, also included four OTUs that were significantly more abundant in group 1: an OTU of the family *Alcaligenaceae*, two OTUs in the genus *Methylobacterium*, and an OTU from *Microvirga*. In contrast to nitrification, it is not suitable to predict denitrification capacity based on phylogenetic classification alone. Nonetheless, denitrification is a common trait among *Proteobacteria* [27] and a member of the *Alcaligenes* genus (*Alcaligenaceae* family) has previously identified as a nitrite accumulator [32].

A single OTU from the *Pseudomonas* genus was significantly less abundant in group 1. Pseudomonads are well known for their diversity in hydrocarbon degradation, and recently, a strain of *Pseudomonas fluorescens* was found capable of degrading TSNAs [33]. As such, we cannot rule out the hypothesis that the pseudomonad here degrades TSNAs in groups 2–4. However, this would offer no explanation for the correlation between high nitrite and high TSNA levels. More likely, the *Pseudomonas* OTU contributed to efficient denitrification resulting in a decrease in both nitrate and nitrite in groups 2–4.

While this work is limited to the ecology of bacteria in these samples, the findings of this study provide some insight into the relationship between microbial community composition of air-cured burley tobacco and TSNA formation. The communities were very similar in samples with 20 $\mu g \, g^{-1}$ TSNA or less; a shift was observed when TSNA levels reached 30 $\mu g \, g^{-1}$ and became pronounced at 100 $\mu g \, g^{-1}$. Nitrite and nitrate levels were also significantly higher, and high levels of all three were correlated with curing conditions at high relative humidity. The results of this analysis support the hypothesis that humidity levels during curing have an effect on microbial community composition and may alter the nitrate and nitrite reduction activity in a way that promotes TSNA formation. From these results, it is reasonable to postulate that lower humidity levels may select for microorganisms, predominately *Proteobacteria*, that are efficient at reducing nitrite and therefore keeping nitrite levels from accumulating in the leaf tissue during curing.

Variation in relative humidity impacts colonization and survival of pathogens on produce [34, 35], as well as physiology and metabolism of bacteria on leaf surfaces [36]. Relative humidity during the cure impacts leaf water content, and leaf water content plays an important part in the complex relationship between bacteria and plants both on and in the leaf [37]. Survival under conditions of desiccation requires strategies such as cellular aggregation and exopolymer or surfactant production [37, 38]. Leaf water content can alter microbial community structure, as is shown in the interesting case of the "resurrection fern" *Polypodium polypodioides*, which can revive from near complete desiccation upon rewetting of the leaves. Jackson et al. show distinct communities exist on the dried versus wet leaves and cultured an abundance of

Actinobacteria and *Firmicutes* [39]. The elevated levels of *Actinobacteria* and *Firmicutes* that we observed in the high TSNA samples may parallel these observations. High relative humidity may select for this community composition, which we predict results in different denitrification dynamics that allows nitrite to accumulate in the leaf, resulting in conditions that promote TSNA formation. Future work will address the microbial community structure of fresh and curing tobacco leaves and will more thoroughly explore the transformations of nitrogen during this process.

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