Beiträge zur Tabakforschung International/*Contributions to Tobacco Research* Volume 23 · No. 6 · December 2009
DOI: 10.2478/cttr-2013-0871

Monitoring the Bacterial and Fungal Biota of Eleven Tobacco Grades Stored at Three Different Locations*

by

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SUMMARY

Tobacco as many other plants has its own microbiota. There are very few studies determining the evolution of this microbiota during tobacco storage, which may affect the quality of tobacco. Polymerase chain reaction (PCR) combined with denaturing gradient gel electrophoresis (DGGE) were used to determine changes in the microbiota of tobacco during the aging of eleven different tobacco grades stored at three different locations for twelve months. The microbial fraction of these tobacco grades was extracted, and the bacterial 16S and the fungal 18S ribosomal RNA gene (rDNA) sequences were PCR amplified before being segregated by DGGE. The bacterial complexity of the tobacco grades was represented by DGGE migrating banding profiles that varied between 20 and 30 bands. Some variations in the banding profiles were observed between the tobacco grades, but overall no substantial changes occurred in the bacterial population of the different grades during their storage at different locations. Most of the fungal DGGE profiles were identical and had only one dominating band related to the genus *Aspergillus*. Bacterial and fungal isolates were also derived from the microbial fractions of the tobacco, and part of their respective 16S and 18S rDNA sequences were determined. Bacterial isolates belonged to *Bacillales* and gamma Protobacteria. Fungal isolates belonged to the genus *Aspergillus*. Our results showed that the bacterial and fungal biota of tobacco are relatively stable throughout 12 months storage time. [Beitr. Tabakforsch. Int. 23 (2009) 368–376]

ZUSAMMENFASSUNG

Tabak besitzt wie alle Pflanzen eine eigene Mikroflora. Es gibt nur wenige Studien über die Entwicklung der Mikroflora während der Lagerung des Tabaks, die die Qualität des Tabaks beeinflussen könnte. Mit Hilfe der Polymerase-Kettenreaktion (PCR) in Kombination mit denaturierender Gradientengelelektrophorese (DGGE) wurde untersucht, ob sich die Mikroflora von 11 Tabakproben, die an drei verschiedenen Orten gelagert wurden, während der einer Lagerung über 12 Monaten ändert. Die mikrobielle Fraktion dieser Tabakproben wurde extrahiert. Die Sequenzen der bakteriellen 16S und die 18S ribosomalen RNA-Gene (rDNA) der Pilze wurden durch PCR amplifiziert und mittels DGGE getrennt. Die komplexe bakterielle Zusammensetzung der Tabakproben zeigte sich anhand der DGGE Trennprofile, die 20 – 30 Banden aufwiesen. Die gefundenen DGGE-Trennprofile zeigten einige Veränderungen; im allgemeinen ergaben sich jedoch keine wesentlichen Unterschiede in den Bakterienpopulationen während der Lagerung der verschiedenen Tabakproben an drei verschiedenen Orten. Die meisten Pilz-DGGE-Profile waren identisch und zeigten nur eine dominante Bande, die zur Gattung *Aspergillus* gehört. Es wurden auch Bakterien- und Pilz-Isolate aus den mikrobielelen Fraktionen der Tabake hergestellt und ein Teil ihrer 16S bzw. 18S rDNA-Sequenzen bestimmt. Bakterielle Isolate gehörten zu den *Bacillales* und gamma-Protobakterien, Pilzisolate gehörten zur Gattung *Aspergillus*. Unsere Ergebnisse zeigen, dass die Zusammensetzung von Bakterien und Pilzen auf Tabak während der gesamten Lagerzeit relativ unverändert bleibt. [Beitr. Tabakforsch, Int. 23 (2009) 368–376]

*Received: 14th July 2008 – accepted: 4th May 2009

RESUME

Le tabac, comme toute autre plante, possède sa propre microflore. Très peu d'études démontrent l'évolution de cette microflore durant l'entreposage du tabac. Ceci peut affecter la qualité du tabac. La réaction de polymérisation en chaîne (PCR) combinée à l'électrophorèse sur gradient de gel dénaturant (DGGE) a été utilisée pour déterminer si des changements survenaient dans la microflore du tabac durant le vieillissement de onze grades différents de tabac entreposés à trois endroits différents durant douze mois. La fraction microbienne de ces grades a été extraite. Ensuite, les séquences des gènes des ARN ribosomaux (rDNA) bactériens (16S) et fongiques (18S) ont été amplifiées par PCR avant d'être séparées par DGGE. La complexité bactérienne des grades de tabac a été représentée par des profils DGGE de migration variant de 20 à 30 bandes. Quelques variations du profil de migration furent observées entre les grades de tabac. Dans l'ensemble, aucun changement substantiel n'est apparu dans la population bactérienne des différents grades entreposés à différents endroits. La plupart des profils DGGE des moisissures étaient identiques et ne possédaient qu`une bande dominante apparentée au genre *Aspergillus*. Des souches de bactéries et de moisissures ont aussi été isolées des fractions microbiennes du tabac. Une partie de leurs séquences 16S et 18S rDNA a été déterminée. Les isolats bactériens appartiennent aux *Bacillales* et au gamma protéobactéries et les isolats de moisissures appartiennent au genre *Aspergillus*. Nos résultats démontrent que la microflore bactérienne et fongique du tabac est stable durant douze mois d'entreposage. [Beitr. Tabakforsch. Int. 23 (2009) 368–376]

INTRODUCTION

A wide variety of microorganisms are living on and in all plants and tobacco has its own microbiota which could affect the quality of tobacco during different storage conditions. Methods currently used to monitor and identify the tobacco microbiota are culture-based methods, but such assessment is incomplete as many microorganisms cannot be cultivated with existing laboratory nutrient media. Thus, in recent years, new techniques developed in molecular biology have emerged and permit analyze of microbiota that are culture-dependent and -independent (1). One of these is the Polymerase Chain Reaction coupled with the Denaturing Gradient Gel Electrophoresis (PCR-DGGE). This method uses PCR amplification of 16S or 18S ribosomal RNA gene (rDNA) sequences followed by their segregation by denaturing migration through a gel (2). This technique was investigated to define the microbial species involved in the fermentation process of Italian Toscano cigar tobacco (3) and to define the microbiota of aging flue-cured tobacco in China (1). In these two studies, mainly bacterial species were reported to take part in either fermentation (*Jeotgalicoccus, Staphylococcus, Aerococcus, Lactobacillus, Weissella, Bacillus, Corynebacterium, Yania*) or aging (sweating) (*Bacteriovorax* sp., *Bacillus megaterium*). During the early phase of cigar tobacco fermentation, a yeast population developed and was predominated by *Debaryomyces hansenii* (3). None of these two papers mentioned the occurrence of molds although *D. hansenii* which produces a lethal toxin active against molds was suggested to prevent the outgrowth of molds in the early stages of cigar tobacco fermentation. *Aspergillus* (e.g. *A. repens*) and *Penicillium* species were predominant in damaged flue-cured tobacco (4) but some species of these two genera were also isolated in undamaged tobacco. In laboratory tests, flue-cured tobacco inoculated with *Aspergillus repens*, was able to support growth of the fungus directly related to increasing moisture content (15–45%), duration (1–4 weeks) and temperature (20 and 30 ˚C) of incubation (5).

From farm curing to consumable end product, the moisture content of tobacco varies within a range of 10 to 30%. After farm curing, tobacco may be shipped to different countries where it is stored (aged) for several months or years. Aging of tobacco is usually conducted at a moisture content ranging from 10 to 13% (6). However, storage conditions may substantially vary between locations. This could induce changes in the microbiota and therefore impact the characteristics of the product. In this report, we compared the bacterial and fungal biota of eleven tobacco grades with various sugar content aged over twelve months at three locations: one in a country with a temperate climate and two in a country with a tropical climate. PCR-DGGE was used to monitor the bacterial and fungal biota. Finally, isolates of culturable bacterial and fungal fractions from one tobacco grade were identified.

MATERIALS AND METHODS

Tobacco storing conditions and sampling

Eleven grades of Canadian flue cured tobacco, varying in their sugar content, were monitored: low sugar: X2M; medium sugar: MF2C, H2M, X1C, H2C and high sugar: FS1C, X1, 01, H1, H1M, MF1M. Eleven sample cases, containing 200 kg of each tobacco grade were stored at location A and used as controls while two series of the eleven cases were sent by truck and stored in location M and T, respectively. At all locations, tobacco was stored in warehouse at ambient conditions. In all three locations, relative humidity and temperature were monitored and recorded at each sampling time (Fig. 1a). The monthly sampling of tobacco was done as follows. Five hundred grams of tobacco were taken at a minimum of 15 cm below the top surface of each tobacco case. Sterile gloves were used to sample tobacco to prevent contamination. Initial samples (first month) were taken from cases while still at location A, then two series of the eleven cases were sent to the other two locations (M, T) by ground transportation in unrefrigerated trucks and lasted approximately two weeks. M and T locations have both tropical climate, the first one being more humid while the second one being more dry. Afterwards, samples were taken as close as possible to the 15th of each month in all locations and sent to Imperial Tobacco Canada in Montreal for analysis. Samples were wrapped in well identified plastic bags and shipped by truck in carton boxes. During the travel, samples were exposed to ambient temperature.

Samples taken at location A took one day to reach Montreal while samples taken at locations M and T took in average three days to reach Montreal.

Physico-chemical parameters of stored tobacco

Upon receipt, tobacco samples were stored at 4 ˚C and processed as follows. Samples of each grade were analyzed for total sugars (7, CV: 4%) and moisture. The moisture was measured by weighing the tobacco before and after being dried in an oven at 110 ˚C for 3 h. For the total sugar content, the tobacco samples were prepared for analysis by grinding until the samples totally passed through a 1 mm sieve. Five hundred mg ± 1 mg of each ground tobacco sample was weighed in a 125 mL Erlenmeyer and extracted in 50 mL of distilled water by shaking for 15 min. The extract was filtered through a Fisherbrand filter paper P8 and the filtrate was collected in a sample cup. The samples were analyzed for total sugars using an Auto Analyzer 3 equipped with a High Resolution Digital Colorimeter 3 from Bran & Luebbe. To determine the amount of total sugars, the extract was first hydrolyzed to reducing sugars with HCl (0.1 N) and by heating the sample with the acid up to 90 ˚C. Then, the reducing sugars were determined by the reduction of orange hexacyanoferrate III to yellow hexacyanoferrate II and the decrease in color was measured at 420 nm. Working standards of glucose were made from a standard stock solution at 10 mg of glucose per mL. Eight working solutions of glucose were prepared at concentrations covering the range expected to be found in the samples (0.25 to 3.0 mg glucose per mL). A graph plotting the peak height against the equivalent glucose concentrations was made for all the standard working solutions and the percentage of total sugar (on a dry weight basis) was calculated as follow:

$$\%\text{Total sugar (dwb)} = \frac{c \times V \times 100}{m} \times \frac{100}{(100 - M)}$$

- c is the sugar concentration, expressed in mg per mL, obtained from the calibration curve
- V is the volume in mL, of the extract prepared (normally 25 mL)
- m is the mass in mg of the sample (0.50 g ± 0.01 g)
- M is the moisture content, expressed as percentage by mass

Analysis of the tobacco microbial biota by cultures

Twenty grams of each tobacco grade were weighed in Whirl-Pak filter stomacher bags. They were mixed with 250 mL of sterile Ringer's solution for 10 min at room temperature. Under aseptic conditions, an aliquot of 50 mL of the uniformly mixed macerate was transferred into a 50 mL sterile tube. Using the 50 mL macerate solution, decimal dilutions ranging from 10^{-1} to 10^{-4} were prepared into sterile Ringer's solution. Aliquots of 0.5 mL of the diluted macerates were inoculated in duplicate to Brain Heart Infusion agar (BHI) for bacteria, and Littman Oxgall agar (LOA) and Malt Salt agar (MSA) for fungi. The petri dishes were incubated at room temperature for seven days for fungi and at 30 ˚C for 48 h for bacteria. Colonies with different visual characteristics were picked. Biomass from one to two mL-culture from ten bacterial isolates and seven fungal isolates was centrifuged and used for DNA extraction.

DNA extraction

The macerate was first centrifuged at 1500 g for 10 min to remove foliar debris. The supernatant was centrifuged at 16 000 g for 10 min. The supernatant was discarded and the pellet was washed in 30 mL, 15 mL and 4 mL of saline solution (NaCl, 0,9% wt/vol) by successive centrifugations at 16 000 g for 10 min. each. Supernatants were discarded and the pellets were frozen at −80 ˚C. The frozen pellets were dispersed in 500 µL of TEN buffer (50 mM Tris-HCl pH 8.0; 100 mM EDTA pH 8.0, 150 mM NaCl). To extract DNA from the fungal and bacterial isolates, the pellet from the one to two-mL culture was dispersed in 500 µL of TEN. To all samples, 25 µL SDS 20% (w/v), 250 mg glass bead 0.40–0.50 mm and 500 µL phenol/chloroform/isoamyl alcohol (25:24:1) were then added. The cells were broken with FastPrep homogenizer (Qbiogene, CA, USA) (twice 20 sec at speed 4.0) and put on ice. The homogenate was centrifuged 5 min at 13 000 g. The aqueous phase was extracted with 500 µL chloroform/isoamyl alcohol (50:1) and centrifuged 2 min at 13 000 g. The DNA was treated with RNase (10 µg/mL) for 15 min at room temperature, and precipitated for 15 min at 20 ˚C with ammonium acetate (2 M final concentration) and two volumes of ethanol 100%. After centrifugation at 13 000 g for 15 min, the DNA pellet was washed with 70% ethanol and dissolved in 50 µL water.

PCR amplification and sequencing

16S or 18S rDNA sequences were amplified by PCR in a DNA thermal Cycler (Geneamp® PCR System 2700; Applied Biosystems Streetsville, ON, Canada). PCR amplifications were performed in a 50-µL reaction volume with 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 20 µg bovine serum albumin, 200 µM dNTP, 10 pmol of each primer (see Table 1), DNA (tobacco DNA, 100–300 ng; isolate DNA, 5–10 ng) and 2.5 U of Taq DNA polymerase (Amersham-Pharmacia Biotech, Baie d'Urfé, QC, Canada). Amplifications were done at 94 ˚C for 3 min, 55 ˚C for 3 min, then 30 cycles at 72 ˚C for 45 sec, 94 ˚C for 45 sec, 55 ˚C for 45 sec, and finally an extension period of 10 min at 72 ˚C.
One extremity of the PCR products corresponding to the 5' extremity of the rDNA sequences was sequenced by the "Centre d'innovation Génome Québec et Université McGill" (Montreal, QC, Canada). Sequences were compared in gene databases with BLASTN (National Center for Biotechnology information, http://www.ncbi.nlm.nih.gov) to determine the most probable affiliation.

DGGE

Denaturing gradient gel electrophoresis (DGGE) was done using a D-code system (Bio-Rad Laboratories Ltd., Mississauga, ON) with PCR products (between 100 to 300

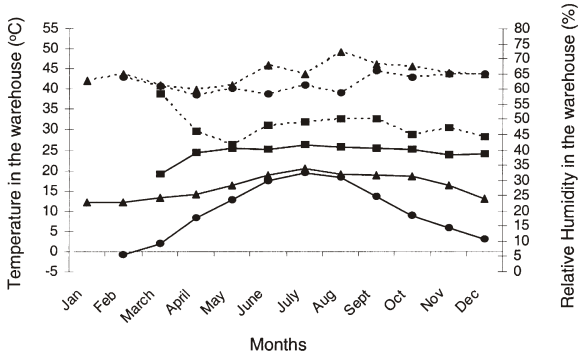

Figure 1a. Temperature (full lines) and relative humidity (dashed lines) in the warehouses from site A (circles), M (squares) and T (triangles)

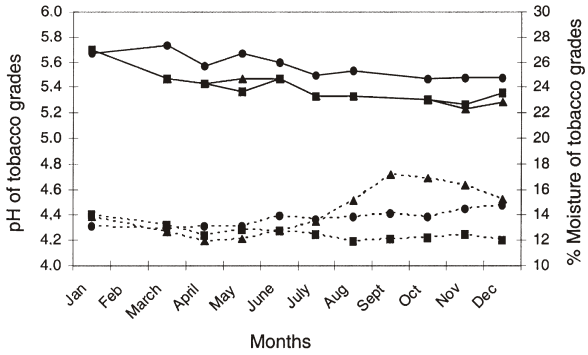

Figure 1b. pH (full lines) and moisture (dashed lines) average of the eleven tobacco grades from site A (circles), M (squares) and T (triangles)

ng for tobacco samples and 10–50 ng for isolate DNA) electrophoresed onto 8% polyacrylamide gels (acrylamide: N-N'-methylenebisacrylamide 37.5:1) containing a linear denaturing gradient (100% denaturing gel contained 7 M urea and 40% deionized formamide). Electrophoresis was carried out using a Tris-acetate (TAE) running buffer (40 mM Tris-HCl, 40 mM acetic acid, 1 mM EDTA, pH 8.0) for 16 h at 100 V and 60 ˚C. The gels were stained with ethidium bromide (10 µg/mL). A multistate discrete-characters parsimony method was used to determine relationship between DGGE profiles. For each profile, all bands were converted in 0–1 values (absence – presence). The SEQBOOT program (PHYLIP package (version 3.65; http://evolution.genetics.washington.edu/phylip.html) was used to resample 1000 times the 0–1 matrix (bootstrap and discrete morphology) and the PARS – Discrete character parsimony – program of the PHYLIP package was run. Only branched with more than 50% bootstrap were considered as significant.

The DGGE bands of interest were cut from the gel and the DNA was eluted in 500 µL of 0.5 M $AcNH_4$, 10 mM, EDTA pH 8.0, 1 mM and SDS 1% for 2–4 hours at 37 ˚C. The suspension was centrifuged and the polyacrylamide was washed with 250 µL of the elution buffer. DNA was precipitated with two volumes of ethanol. The DNA pellet was dissolved in 100 µL water and precipitated again with

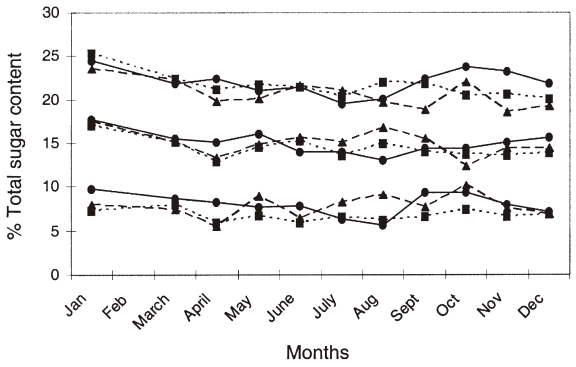

Figure 1c. Percentage of total sugar content of low (full lines), medium (small dashed lines) and high (dashed lines) sugar content samples from site A (circles), M (squares) and T (triangles)

1/10 vol. sodium acetate 3 M and two volumes of ethanol. This DNA was amplified as before with the 341F (with no GC clamp) and 534r primers before sending to the sequencing service.

RESULTS

Changes in physico-chemical parameters during tobacco storage

The eleven tobacco grades were stored in three different locations – A, M and T – and were sampled for 12 months. Location A was in a country with a temperate climate, and the locations M and T in countries with a tropical climate. During the storage, the temperature at site A fluctuated more than at the two other locations ranging from 0 ˚C in winter to 18 ˚C in summer (Fig. 1a). At site M, the temperature stayed around 25 ˚C for 9 months, and the tobacco was exposed to a lower degree of relative humidity compared with the two other locations. In the site T, the temperature stayed between 15 ˚C and 20 ˚C but the tobacco was exposed to a higher degree of relative humidity (Fig. 1a).

The percentage of moisture in the A and M samples stayed relatively constant between 12 to 15% over the twelve months period (Fig. 1b). However the percentage of moisture in T samples increased from month 5 (12%) to up to month 9 (17%) and then decreased to 15% (Fig. 1b). The pH value of tobacco varied from 5.7 to 5.2 over the twelve months period for samples stored at all three locations (Fig. 1b).

The percentage of total sugars of the X2M sample (low sugar sample) remained under 10% at all three locations. On average, the percentage of total sugars of the H2M, X1C, MF2C and H2C samples (medium sugar samples) ranged from 13 to 17% whereas, from 20 to 25% for the 01, FS1C, X1C, MF1M, H1M and H1 samples (high sugar samples) (Fig. 1c).

Bacterial and fungal counts

Bacterial counts remained stable and higher than fungal counts over the twelve months period of storage at all three locations (Figure 1d). Fungal counts at sites M and T

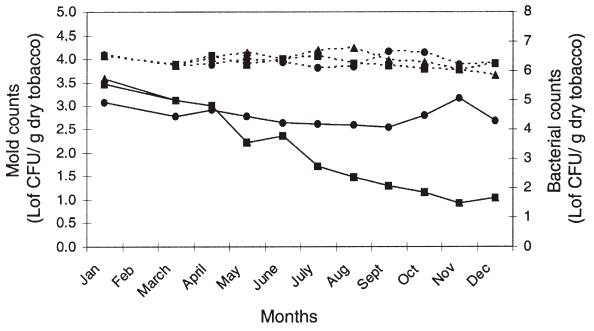

Figure 1d. Mold (full lines) and bacterial (dashed lines) counts average on a logarithmic scale for the eleven tobacco grades from site A (circles), M (squares) and T (empty triangles)

decreased by two logs over the twelve months period of storage and remained stable at site A over the same period of time (Fig. 1d).

Effect of storage duration and location on the bacterial biota of tobacco grade

Changes in the microbial population of the eleven tobacco grades aged for twelve months at the three different locations were monitored by PCR-DGGE. More than 400 samples were processed and analyzed both for bacterial and fungal populations. Representative results are illustrated in Figures 2, 3 and 4. Figure 2 shows the results of the bacterial DGGE profiles of the eleven tobacco grades stored at site A after nine months storage. All samples had between 20 and 30 apparent bands. Changes were noticeable in the banding profiles. However these changes were limited to one or two bands per profiles as indicated in Figure 2. Although we noticed changes in the intensity of several specific bands, these variations were mostly minor, and were not taken into account as several factors related to experimental handlings could have produced such variations (see Discussion). All these bands were recorded and the resulted profiles were compared with a discrete character parsimony method combined with a bootstrap analysis for relationship significance. High relationships were found between all DGGE profiles (data not shown because bootstrap results were below 50% [from 1000 bootstrap resamplings] for all samples) suggesting that the bacterial biota did not change substantially between tobacco grades. Similar results were observed with tobacco grades stored at sites M and T (data not shown).

We compared the DGGE profiles between the three locations after twelve months storage for three different grades (low, medium, high sugar content) (Fig. 3). The DGGE profiles were either identical or differed by only one band between the three locations. Only one noticeable band was prominent in the low sugar sample in the three locations (arrow *a* in Fig. 3), and a different one in the medium and high sugar samples in the three locations (arrow *b* in Fig. 3). As before, the discrete character parsimony method showed high relationships between DGGE profiles suggesting that the bacterial biota did not change substantially between the three locations.

We compared the DGGE profiles of grade MF2C located at site A and M, and sampled after zero, eight, nine, eleven and twelve months storage (Fig. 4). There were very few changes in the different profiles that occurred during the twelve month storage in both locations. Similar DGGE profiles were also observed with samples from site T (data not shown). One band (arrow in Fig. 4) present in the samples aged for zero to nine months was less intense in tobacco aged for eleven and twelve months.

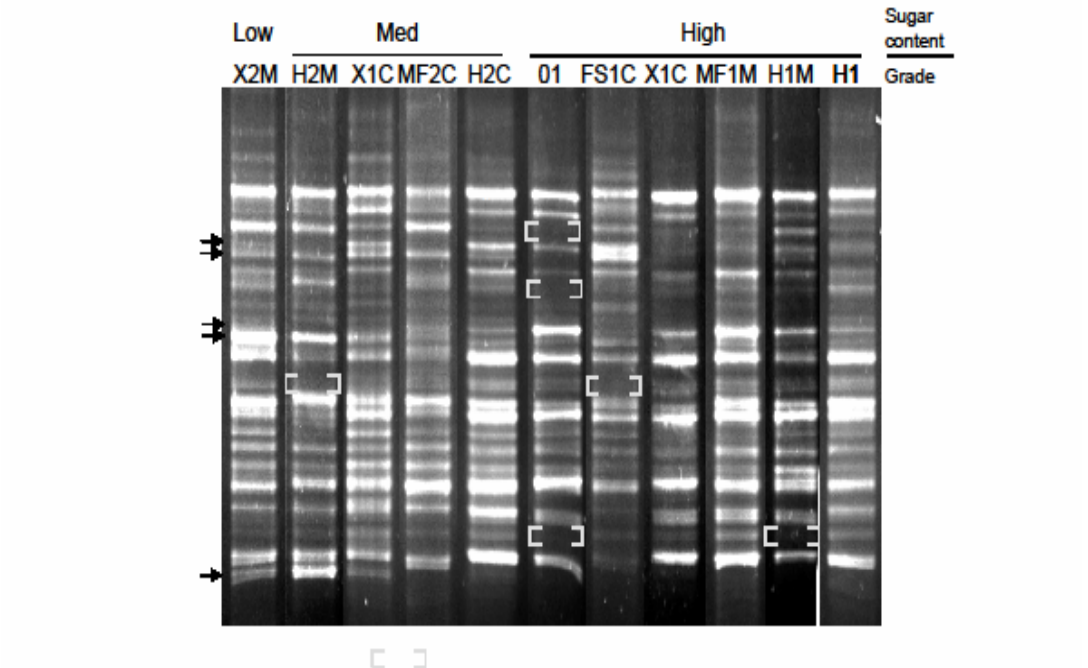

Figure 2. Bacterial profiles of the eleven tobacco grades stored at site A for nine months. Total DNA was extracted from the tobacco samples and the V3 region of the 16S rDNA was PCR-amplified. PCR products were separated by DGGE with a 20% to 70% denaturant gradient. Arrows indicate significant differences in the migration profiles of more than 2 samples. Other differences in the profiles are indicated by brackets.

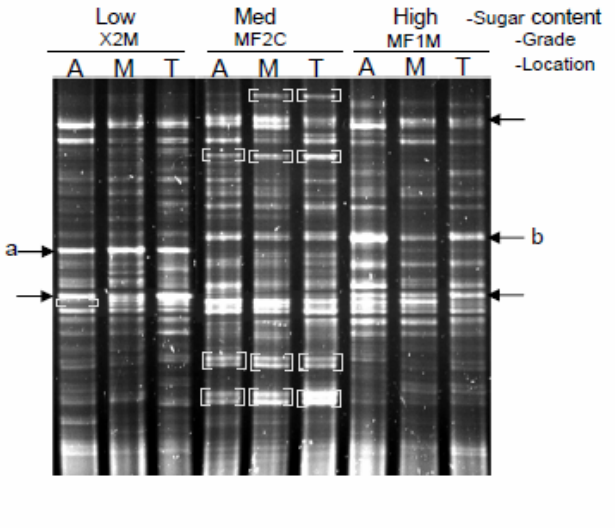

Figure 3. Effect of storage location on the bacterial biota of three tobacco grades stored for twelve months. PCR products were derived as described in legend of Figure 2 and were separated by DGGE with a 30% to 65% denaturant gradient. Arrows and brackets indicate significant differences in the migration profiles between samples.

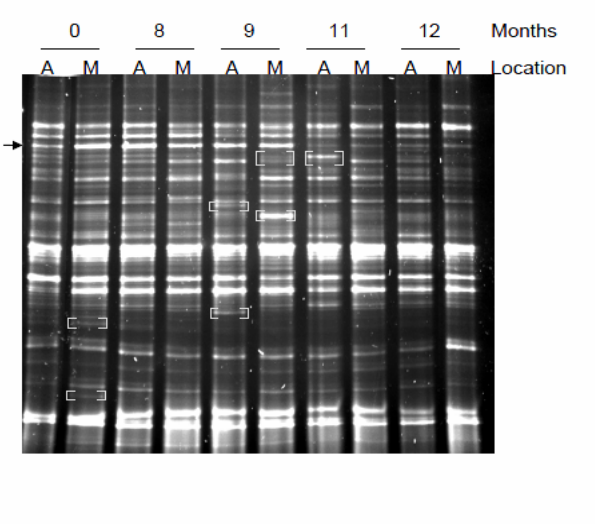

Figure 4. Effect of storage location over time on tobacco grade MF2C stored at A and M. PCR products were derived as described in legend of Figure 2 and were separated by DGGE with a 30% to 65% denaturant gradient. Arrow and brackets indicate significant differences in the migration profiles between samples.

Identification of bacterial isolates

Ten bacterial isolates were derived from grade MF2C stored at site T after twelve months. Total DNAs from the isolates and from the MF2C sample were extracted, and their 16S rDNA sequences were PCR amplified for DGGE experiments. The PCR products from the isolates were comigrated in DGGE with the PCR products from MF2C (Fig. 5). For most of the isolates, only one band was obtained and comigrated with one band of the MF2C DGGE migration profile. Longer 16S rDNA sequences were derived from each isolate and their sequence determined. Comparisons with gene databases revealed

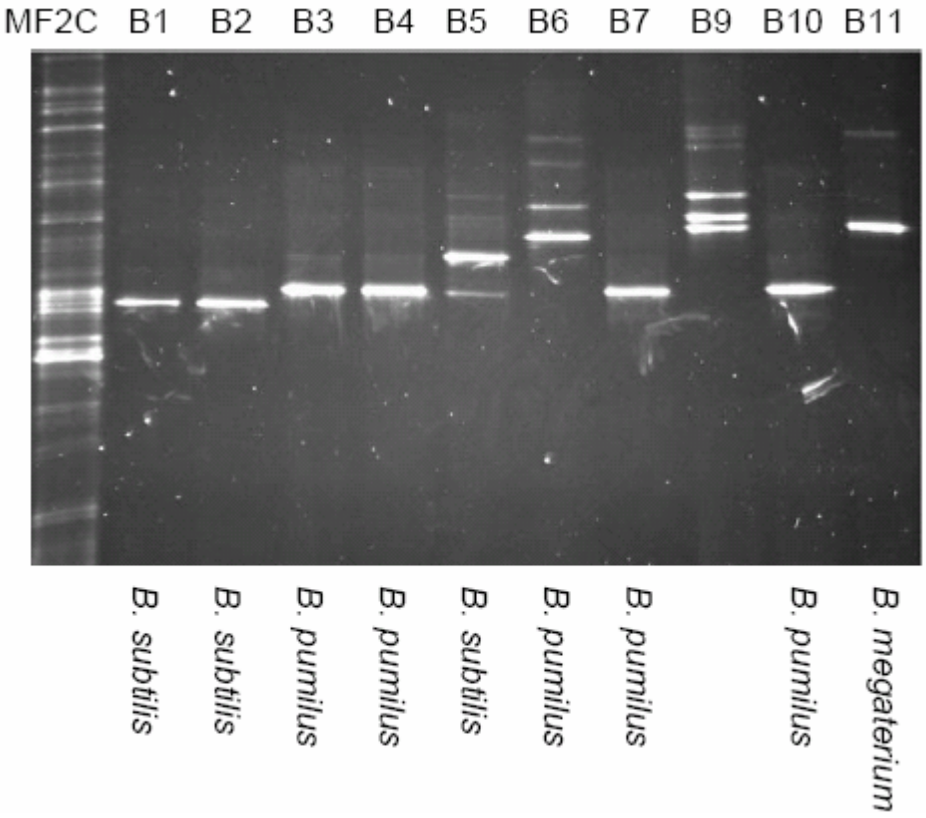

Figure 5. DGGE comigration profile between bacterial isolates and the tobacco grade MF2C. Isolates were derived from the tobacco grade MF2C aged for twelve months at the T location. PCR products were derived as described in legend of figure 2 and were separated by DGGE with a 30% to 65% denaturant gradient. MF2C; B1 to B11: Bacterial isolates.

Table 1. List of PCR primers [a]

Primer	Sequence (5'-3')	References
16S rDNA sequences		
27f	AGAGTTTGATCMTGCTCAG	(10)
1525r	AAGGAGGTGATCCARCCGCA	(10, 11)
341f-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGCC-TACGGGAGGCAGCAG	(2)
534r	ATTACCGCGGCTGCTGG	(2)
18S rDNA sequences		
NU-ssu-1196-5'	GGAAACTCACCAGGTCCAGA	(8)
NU-ssu-1536-3'-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGG-ATTGCAATGCYCTATCCCCA	(8)
Euk-82F	GAAACTGCGAATGGCTC	(12)
Uni-1392r	ACGGGCGGTGTGTRC	(10)

[a] The 341f-GC/534r and NU-ssu-1196-5'/ NU-ssu-1536-3'-GC sets of primers were used for PCR-DGGE experiments, and the 27f/1525r and Euk-82F/Uni1390r sets of primers to generate longer rDNA PCR products. 341f-GC/534r and 27f/1525r are universal bacterial sets of primers. NU-ssu-1196-5'/ NU-ssu-1536-3' target most fungi, and Euk-82F/Uni1390r most of Eukarya.

Table 2. Most probable affiliation of bacterial isolates based on 16S rDNA gene sequences

Isolate	Species	GenBank accession number	% identity [a]
B1	*Bacillus subtilis*	EU500931	99.7%
B2	*Bacillus subtilis*	DQ676957	100%
B3	*Bacillus pumilus*	EF173329	100%
B4	*Bacillus pumilus*	EF173329	99.9%
B5	*Bacillus subtilis*	EF423592	100%
B6	*Bacillus pumilus*	EF465530	100%
B7	*Bacillus pumilus*	EF173329	99.7%
B9	*not determined*		
B10	*Bacillus pumilus*	EF488975	100%
B11	*Bacillus megaterium*	EF114346	99.8%

[a]Identity is based on approximately the first 1000 nt of the 16S rDNA genes with the respective species

that all isolates (except isolate B9 which was composed of more than one species) were related to *Bacillus subtilis*, *B. pumilus* or *B. megaterium* with >99% sequence identity (Table 2).

PCR-amplified 16S rDNA sequences of the microbial fractions of nine tobacco grades were separated by DGGE. DNA from seventeen bands was retrieved from the gel, reamplified and sequenced. Nine of the seventeen DNA generated readable sequences of 150–200 nucleotides. Comparison of these readable sequences with gene databases provided affiliation at least to the genus level: *Erwinia, Pseudomonas, Enterobacter, Xanthomonas, Pantoea, Klebsiella* and *Rheinheimera* (gamma Proteobacteria); *Bradyrhizobiacea* (alpha Proteobacteria); and *Bacillus* (*Bacillales*).

Effect of storage duration and location on the fungal biota of tobacco grade

18S rDNA sequences were PCR amplified with primers specific for the main fungal lineages such as the *Ascomycota* and the *Basidiomycota*. All the DGGE migrating profiles were very simple with one dominating band that was observed mostly in all samples (Fig. 6), with some profiles having also low intensity bands. The PCR products representative of the dominating band were all related to *Aspergillus niger* at more than 99% on 324 nucleotides.

Fungal isolates were derived from the tobacco microbial fractions. Total DNAs from seven of them and from tobacco samples were extracted, and their 18S rDNA sequences were PCR amplified for DGGE experiments. All the isolates generated one DGGE band that co-migrated with the dominating DGGE band of the tobacco samples. Longer 18S rDNA sequences (app. 900 nt) were derived from each isolate and their sequence determined. Sequences of isolates m5, m7 and m8 from sample MF1M were identical, and were related to the genus *Aspergillus* with the closest species being *niger*. Sequences of isolate m6 from sample MF1M and isolates m13 and m15 from sample H1M had two nucleotides differences with isolate m5, and were also related to the genus *Aspergillus* with the closest species being *oryzae.* Finally, sequence of isolate m14 from sample H1M had a 5-nucleotide difference with isolate m5 and a 3-nucleotide difference with isolate m6. It was also related to the genus *Aspergillus* with the closest species being *unguis*.

DISCUSSION

Tobacco processing involves curing, drying the tobacco leaves up to 60–70 ˚C, and aging, i.e. leaf storage for

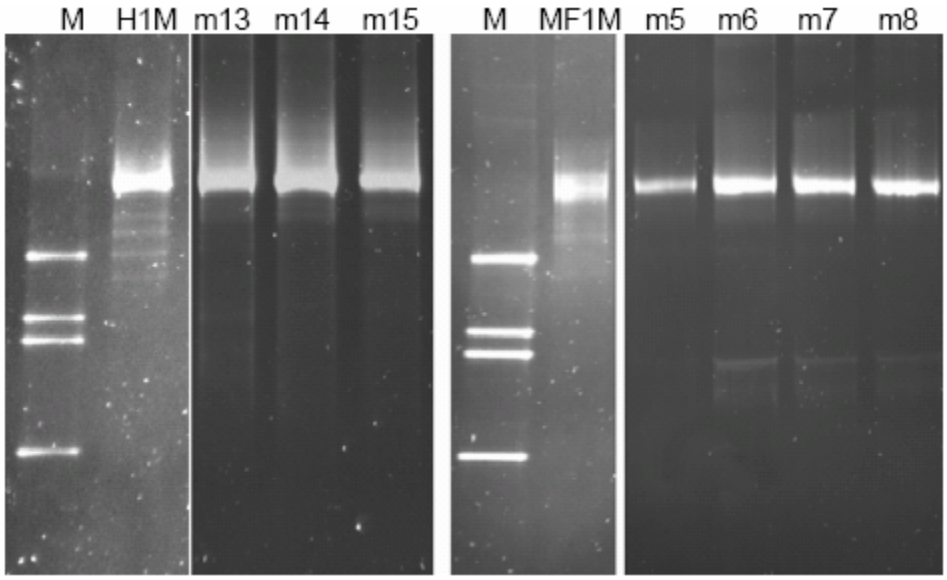

Figure 6. DGGE comigration profile between fungal isolates and tobacco grades H1M and MF1M. Isolates m13, m14 and m15 were derived from the tobacco grades H1M and m5 to m8 MF1M, both stored at site A. Total DNA was extracted from tobacco samples or fungal isolates, and part of the 18S rDNA was PCR-amplified. PCR products were separated by DGGE with 30% to 65% denaturant gradient. M: DGGE migration marker. The marker was composed of PCR products derived from 16S rDNA sequences of (top to button) *Staphylococcus aureus, Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli.*

several months. In our study, we investigated if changes of microbial population occurred during aging of tobacco at three different locations with culture-independent methods. We used PCR-DGGE to monitor changes of the bacterial and fungal biota of eleven tobacco grades aged for twelve months at the three different locations. One of the key steps in succeeding at amplifying PCR products from bacterial and fungal DNA was the maceration of the tobacco plant to release the associated microorganisms to avoid interference of microbial DNA with tobacco DNA. In the two reports on monitoring microbial populations from tobacco by PCR-DGGE, DI GIACOMO *et al.* (3) and ZHAO *et al.* (1) successfully used similar approach to separate the microbial DNA fraction from the tobacco DNA.

In all samples examined (around 400), between 20 and 30 bacterial DGGE migrating bands were observed for each sample. The number of bands that we observed was similar to the one observed in the DGGE profiles reported by ZHAO *et al.* (1) but higher than the number of bands reported by DI GIACOMO *et al.* (3) who found approximately 15 to 20 DGGE bands for some of their samples. Changes in the bacterial DGGE profiles were observed in several samples (as illustrated by representative DGGE in Figures 2, 3 and 4) but were limited to the presence/absence of one to two bands, which suggests that small changes occurred in the bacterial population. However, these changes could have been caused by the heterogeneity of the tobacco sampled. Indeed, variation in the DGGE profiles involving 2–3 bands were observed from three samplings of the original tobacco before storing (data not shown). Using a discrete character parsimony method, high relationships were found between all DGGE profiles, which suggest that no substantial changes occurred in the bacterial population of the different tobacco grades during their storage. Furthermore, neither of the location of storage, moisture content, pH, sugar content nor the bacterial counts of the tobacco grades caused any changes on the bacterial biota even though some of these parameters varied (Figure 1a, 1b, 1c and 1d). These results support the idea that tobacco storage is not a biologically dynamic process. For instance, the moisture of the tobacco was relatively low (varying between 12–17%), which did not favor bacterial metabolism. This contrasts with the work of ZHAO *et al.* (1) who observed significant changes in the DGGE profiles between three tobacco varieties. Each variety showed no changes in their first six months of aging, but presented different patterns after nine months. DI GIACOMO *et al.* (3) monitored the evolution of the microbial population of a cigar tobacco during its fermentation. They also measured significant changes in the DGGE profiles during the fermentation process. However, these changes were expected as the tobacco went through a process more biologically dynamic than tobacco aging.

In our DGGE profiles, the only variations noticed were in intensity of certain bands from one profile to the other. These could be attributed to variation in the cell concentration of the respective bacterial species present at the time of sampling. However, we did not consider the intensity of the bands as a criterion in the discrete character parsimony method because PCR-DGGE is at most a semi-quantitative method, and several factors could have influenced the intensity of these bands. First, the extracted DNA is from a mixture of microbial genomes, each may have had different number of copies of rDNA genes. Second, the primer hybridization and the PCR amplification can be influenced by the corresponding rDNA sequence. In our study, there were several low intensity bands that may have been influenced by the above factors.

In parallel to culture-independent methods, we cultured on BHI media several bacterial isolates from the microbial fractions of some tobacco grades. Ten of them were analyzed by PCR-DGGE and a particular DGGE band was associated to *Bacillus subtilis*, *B. pumilus* or *B. megaterium.* Affiliation of other bacterial isolates revealed the presence of gamma-

Proteobacteria associated to the genera of *Klebsiella, Enterobacter, Erwinia* and *Cronobacter*. Other species such as *Bacillus licheniformis* and *Staphylococcus pasteuri* were identified (data not shown). Sequences from DNA extracted from DGGE bands were also affiliated to *Klebsiella, Enterobacter, Erwinia* and *Bacillus*. These results confirmed the potential of PCR-DGGE for monitoring specific bacterial species not isolated by culture-dependent methods.

ZHAO *et al.* (1) sequenced the five most dominant DGGE bands that were related to *Bacillus megaterium* (*Bacillales*), *Bacteriovorax* sp. (delta-Proteobacteria) and three uncultured bacteria; one being affiliated to alpha-Proteobacteria. DI GIACOMO *et al.* (3) sequenced the most dominant DGGE bands but also DNA from single-strand conformational polymorphism bands. They were affiliated to *Lactobacillus*, *Bacillales* (such as *B. pumilus, B. subtilis, B. licheniformis*), *Actinobacteria* and to *Marinilactibacillus*. The same authors also derived bacterial isolates with high prevalence to *Bacillaceae*; the most numerous ones were related to *B. pumilus, B. subtilis* and *B. licheniformis*. Combined with our results, the three reports showed the constant occurrence of *Bacillus* species on flue-cured tobacco. This is not surprising as these bacteria can form thermotolerant spores, and consequently can survive in harsh environments such as those encountered in tobacco curing (high temperature) and aging (low humidity).

We used a set of primers designed to amplify rDNA from all four major phyla of fungi: *Ascomycota, Basidiomycota, Chytridomycota* and *Zygomycota* (8). Despite this broad specificity, only one dominate band was found in the DGGE profiles of most of the tobacco samples. This suggests that a high proportion of fungi related to *Aspergillus* were present. This was confirmed by the 18S rDNA sequences of isolates derived from our tobacco and all belonged to the genus *Aspergillus*.

No conclusion can be drawn on the origin of the microorganisms found on tobacco and whether they are biologically active or not. In other words, the analyzed nucleic acids were derived from microorganisms that were active in situ or from spores present with the tobacco from the high temperature curing and subsequent low moisture environment. Functionality of *Bacillus* species during tobacco fermentation has however been proposed by DI GIACOMO *et al.* (3).

In conclusion, the PCR-DGGE method allowed us to monitor a broad spectrum of microbial species as one set of primers targeted most bacteria and the other most fungi. Even if the environmental conditions of storage (Figure 1a), the microbial counts and the physico-chemical parameters varied between the three sites (Figure 1b to 1d), only minor differences in the microbiota were detected between storage location and duration. Identifying which microorganisms correspond to the DGGE bands will allow monitoring only specific species during further tobacco processing (e.g. curing, aging, fermentation and manufacturing). Finally, our work confirmed that *Bacillus* and *Aspergillus* species are the predominant bacteria and mold, respectively found in flue-cured aging tobacco, as previously reported (9). These two genera may be present in the stored tobacco environment in the sporulated forms that have no relation to a functioning microbial community.

ACKNOWLEDGEMENTS

We thank Ron Bandur, Georges Howard, Joanne Joly and the Laboratory Services Group of ITCAN for their professional advice and help to collect, process and analyze tobacco samples

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