Microbiological screening of tobacco raw materials for rolling of cigarettes

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Abstract. In the modern market of tobacco products worldwide, there is a trend towards increased consumption and trade in cut tobacco for manual rolling of cigarettes (RYO tobacco), including and from unregulated producers. This hides additional risks for the health of the consumers due to the possibility to use low-quality and uncontrolled raw materials, including and with increased presence of pathogenic and conditionally pathogenic microorganisms.

The aim of the study was to determine the total amount of heterotrophic bacteria (microbial count), the amount of microscopic fungi and the most common species in the raw material for tobacco RYO, distributed on the Bulgarian market.

The microbiological analyzes were performed by classical methods accepted in sanitary microbiology. In all samples, spores of the genus Bacillus showed mass development, and according to the macro-morphological characteristics of the colonies, the predominant species can be attributed to B. cereus. The detected amounts of microscopic fungi in the tested tobacco mixtures were significant at strong presence of species of the genus Aspergillus, over 85.48%. Macroscopically, species were defined as A. flavus (41.94%), A. niger (27.42%) and A. fumigatum (16.13%). The relative share of species of the genus Penicillium was 9.68% and of species of the genus Mucor - 4.84%. The studied tobacco raw material for manual rolling of cigarettes had significant contamination by microorganisms that had the rank of pathogens.

Quantitative microbial assessment suggests a potential health risk for consumers.

Key words: RYO tobacco, heterotrophic bacteria, microscopic fungi.

1. Introduction

Although the research on the tobacco microbiome dates back to the 1970s, it focused on the raw material after harvest from the field, before it was used in tobacco products, mainly on the causes by mold and its storage conditions. Studies on the microbiome of tobacco blends have been done mainly by large cigarette companies and the results have been confidential until now [7; 9; 15; 28; 29]. In a relation with the discussions about the harm of smoking, in recent years research on the microbiological composition of various tobacco products and their additives has expanded. There were growing evidences of a direct relationship between the amounts of microorganisms (microscopic fungi, bacteria, spore), microbial toxins released by them (mycotoxins and endotoxins) and the increased risk of chronic inflammations, malignant transformations, allergic reactions, liver diseases, etc., especially in immunosuppressed individuals [2; 3; 5; 11; 20]. In the modern market of tobacco products worldwide, there is a trend towards increased consumption and trade in cut tobacco for manual rolling of cigarettes (RYO tobacco), including and from unregulated producers. Additional risks for the health of the consumers due to the
possibility to use low-quality and uncontrolled raw materials, including and with increased presence of pathogenic and conditionally pathogenic microorganisms it hides. The scope of existing standards for tobacco and related production chemicals substances with a proven health risk was focused, mainly. The opinion on the need to create standards for the safest levels of pathogenic microorganisms and their toxins have been confirmed [3; 13; 21; 23; 24; 25].

The aim of the study was to determine the total amount of heterotrophic bacteria (microbial count), the amount of microscopic fungi and the most common species in the raw material for tobacco RYO, distributed on the Bulgarian market.

2. Materials and methods

The objects of the study were industrial tobacco mixtures designed for manual rolling of cigarettes stored under different conditions (acclimatized depots). The microbiological analyzes were performed by classical methods accepted in sanitary microbiology [15]. Ten-fold dilutions with 0.9% saline and inoculation of the diluted suspensions on solid growth media were prepared from the substrates. The following indicators were determined: Quantity of heterotrophic bacteria (microbial count) - on meat-peptone agar (MPA), the reading was performed after 48 h of incubation at 30° C; Quantity of microscopic fungi - on Czapek-Dox agar (CDA), after 72 h of incubation at 28° C. The density was calculated as the number of colonies formed units per 1g of dry mass (CFU/g d.m.). Analyzes were performed in triplicate. Mean population density at microbial communities formed into the different tobacco mixtures was calculated. Pure cultures from the developed colonies of microscopic fungi were isolated. The determination to genus and species was made on the basis of macro-morphological characteristics of the colonies and micro-morphological of the spore-bearing hyphae, according to the procedures described by Barnett & Hunter, 1998 [6].

Moisture content (%) in three replications, according to BDS 8025-84; dry matter content (%) in 1g tobacco mass and width of tobacco fibers (mm), measurements of 50 pieces, according to BDS 12973-75, as concomitant technological indicators of tobacco mixtures were defined [8].

All data were presented as mean value ± standard deviation. A correlation analysis was made between the microbiological and technological indicators.

3. Results and discussion

3.1. Technological indicators

The conditions of the storage are essential for preserving the quality and specific properties (physical, chemical and smoking) of tobacco mixtures as a raw material for taste-aromatic products such as cigarettes. In the period before their use in the final product, a number of oxidation and reduction processes take place in them, the speed of which depends mainly on the relative humidity and air temperature in the landfills in which they are stored. Tobacco is a hygroscopic material and high above 80-85% or low below 60% humidity in the premises, as well as temperature above 30° C changes the moisture content in the tobacco mixtures themselves, which in turn negatively affects their properties and physiological effects. These parameters of the environment create favourable conditions for the development and multiplication of the saprophytic microorganisms, which is inevitably present in them. The visible manifestation of intensives microbiological processes are a change in color, odor and the appearance of molds. As a result, not only the physical but also the chemical characteristics, respectively the flammability, the taste and the smoking qualities change. There was a strong reduction in the content of soluble carbohydrates (about 4 - 5 times) and dry matter, as well as an increase in the content of protein substances due to the accumulation of microbial protein, mainly from the fungal mycelium [14]. The intensive metabolic processes of the activity of microorganisms destroy tobacco mixtures and can lead to their complete degradation.
The tested samples, depending on the degree of their contamination with molds were conditionally grouped into six groups: I. Very-clean - no changes in the tobacco material observed; II. Clean - without visible traces of molds, but with a slight change in the color of the material; III. Slightly contaminated - traces of molds, with a visible change in color; IV. Moderately contaminated - presence of mold, discoloration and sticking of tobacco fibers; V. Heavily contaminated - visible mold, discoloration and initial degree of conglomerate formation; VI. Very-heavily contaminated - presence of mold, conglomerates, unpleasant odor of defective material and the occurrence of putrefactive processes (Figure 1).

According to BDS 866-82 the moisture content in tobacco products is in the range from 11.00% to 14.00%. Higher values are a prerequisite for the development of mold and low flammability, and lower below 10-11%, accelerate combustion and form irritating products in the smoke. The moisture content in the tested samples only in those of group I. "Very clean" were within the standard - 11.42%. The samples from group VI "Very heavily contaminated" were higher values than the standard - 16.72%. The other samples were low moisture content - from 8.41% to 10.43%.

A decrease in the dry matter content was reported depending on the degree of mold on the tobacco substrates. The width of the tobacco fibers affects the quality of cigarettes - the filling capacity, the mass of tobacco used in the cigarette, the burning rate, the number of suctions and other technological indicators. The higher density in the cigarette product could also affect of the quantitative development of microorganisms. The standard width varies from 0.7 mm to 1.2 mm [22]. The width of the tobacco fibers in the tested samples did not deviated from these parameters (Table 1).
Table 1. Values at some technological parameters of the studied tobacco mixtures: moisture content (%), dry matter content (%) and width of tobacco fibers (mm).

| Samples group          | Moisture content (%) mean value ± standard deviation (n = 3) | Dry matter content (%) mean value ± standard deviation (n = 3) | Width of tobacco fibers (mm) mean value ± standard deviation (n = 50) |
|------------------------|-------------------------------------------------------------|----------------------------------------------------------------|---------------------------------------------------------------|
| I. Very-clean          | 11.42 ± 0.14                                               | 91.91 ± 6.10                                                   | 1.062 ± 0.23                                                    |
| II. Clean              | 10.43 ± 0.03                                               | 90.42 ± 0.05                                                   | 0.976 ± 0.25                                                    |
| III. Slightly contaminated | 8.41 ± 0.17                                               | 91.59 ± 5.85                                                   | 1.016 ± 0.16                                                    |
| IV. Moderately contaminated | 9.62 ± 0.26                                | 89.57 ± 0.59                                                   | 0.968 ± 0.17                                                    |
| V. Heavily contaminated  | 9.58 ± 0.21                                               | 88.58 ± 0.59                                                   | 0.994 ± 0.19                                                    |
| VI. Very-heavily contaminated | 16.72 ± 0.30                               | 83.28 ± 5.12                                                   | 1.226 ± 0.36                                                    |

3.2. Microbiological indicators
The results of the performed microbiological analyses showed significant differences in the quantitative development of the populations of the tobacco microbiotas at the different groups of tobacco mixtures. The average population densities of tobacco blends conditionally classified as "very pure" and "pure" were in the range of 1.36x10^2 and 2.34x10^2 CFU / g d.m. In the other samples the quantities of microorganisms were drastically increased and reach values of the order of thousands. The samples from the group of "very heavily contaminated" had the highest density - 31.62 x10^3 CFU / g d.m. In the other samples the quantities of microorganisms were drastically increased and reached values of the order of thousands. When comparing the quantities of microorganisms registered at the defined as "very pure" tobacco mixtures, which in this study can be accepted as controls, with the quantities reported in the other groups of tobacco substrates, the increase was the times. Logically, it was weakest at group II - 1.73 times; at group III - 4.16; at group IV - 11.27, at group V - 13.93 and at group VI it was reached 23.30 times (figure 2).

Figure 2. Population densities average (CFU/g d.m.) at microbial communities formed into the different tobacco mixtures.
The microbial number is a universal sanitary-hygienic indicator and unites mainly heterotrophic mesophilic bacteria with the same type of metabolism. Their quantities in the tested samples were vary from $2.71 \times 10^2$ in the conditionally pure samples (groups I and II); in slightly contaminated $9.73 \times 10^2$ (group III); in moderately and heavily contaminated they were in the range of $2.88 - 5.82 \times 10^3$ CFU / g d. m. (groups IV, V and VI) (figure 3). In all samples, spores of the genus Bacillus showed mass development, and according to the macro-morphological characteristics of the colonies, the predominant species can be attributed to the group of B. cereus, with a high probability of var. B. mycoides.

**Figure 3.** Quantity of heterotrophic bacteria /Microbial count/ (CFU/g d.m.) at microbial communities formed into the different tobacco mixtures.

*Bacillus cereus* is an aerobic spore-forming, rod-shaped bacterium, widespread in the environment, including and in food products. Its amount can reach to $10^6$/g, even after processing products. Consumption of products highly incriminated with bacteria from the group of *B. cereus* causes various food diseases [12; 13; 17; 19]. Endospores of species in this group are resistant to adverse environmental conditions [23; 24; 27]. The group of *B. cereus* includes species with similar main characteristics - *B. cereus, B. thuringiensis, B. anthracis, B. weihenstephanensis* and rhizoid varieties - *B. mycoides*, to which probably included most of the bacterial isolates obtained in the present study. The reason for this was the specific morphology of the colonies [26]. The Gram staining and rapid test for catalase activity with hydrogen peroxide H$_2$O$_2$ on bacterial smears showed that the cultures were G (+) and with a positive reaction for catalase production (figure 4).

**Figure 4.** Isolated bacterial colonies from tested samples tobacco.
Tobacco mold is caused by microscopic fungi that are widespread in the environment and part of the epiphytic microflora of tobacco leaves. After harvesting the raw material, they remain on the surface of the tobacco leaf or in the epidermis in the form of spores or hyphae, multiply and continue their life cycle [7; 11]. Conditions favourable under, their quantity can reach sizes that make the tobacco raw material unusable, and the smoking articles - unfit for consumption [10; 21]. The most common causes of mold the species of the genus Mucor, Cladosporium, Penicillium, Alternaria and Aspergillus were reported [11; 15; 17]. Some of the causative agents can be pathogenic or conditionally pathogenic to humans by releasing mycotoxins [7; 27; 28; 29]. Microscopic fungi are active producers of various biologically active substances and actively participate in the transformations of organic matter in nature.

Tobacco blends are a suitable source of nutrients and energy needed for their metabolism.

The detected amounts of microscopic fungi in the tested tobacco mixtures were significant, except for the samples grouped as visually pure (groups I and II). The density was insignificant from 0.004 x 10^2 to 0.33 x 10^2 CFU / g d.m. For the others - slightly, moderately and heavily polluted, the numbers were in the order from hundreds to thousands in grams of dry material - from 1.54 x 10^2 to 5.22 x 10^2 CFU / g d. m (figure 5).

![Figure 5](image-url)  
**Figure 5.** Quantity of microscopic fungi (CFU/g d.m.) at microbial communities formed into the different tobacco mixtures.

Although standards are lacking, according to some authors, the recommended acceptable quantitative levels of microscopic fungi in substrates for cigarette products (dry tobacco or medical marijuana) should not exceed 200 CFU/g [30]. At the studied tobacco samples they are significantly more.

Significant correlations between the amounts of microorganisms, the moisture content of tobacco blends and the width of tobacco fibres were reported. The correlation coefficients were: with the moisture content at heterotrophic bacteria r = 0.70, at microscopic fungi r = 0.73; with the width of the tobacco fibres in heterotrophic bacteria r = 0.64, at microscopic fungi r = 0.69.

The macro and micro-morphological characteristics of the pure cultures from the developed microscopic fungi showed a very strong presence of species of the genus Aspergillus, over 85.48%. Macroscopically, species were defined as A. flavus (41.94%), A. niger (27.42%) and A. fumigatum (16.13%). The relative share of species of the genus Penicillium was 9.68% and of species of the genus Mucor - 4.84% (figure 6 and figure 7).
Aspergillus flavus
Aspergillus niger
Aspergillus fumigatum

Figure 6. Isolated colonies of species of genus Aspergillus from tested samples tobacco.

Figure 7. Relative share (%) of the species of the microscopic fungi isolated from the different tobacco mixtures.

In addition to direct damage to the tobacco substrate, by deteriorating its quality characteristics, some of the causative agents may be pathogenic or conditionally pathogenic to humans through the release of mycotoxins. Mycotoxins are chemicals, relatively low molecular weight, products of the secondary metabolism of the microscopic fungi [5]. They are characterized by a huge structural diversity, more often with an aromatic molecular structure. Mycotoxins are stable compounds and are significantly resistant to environmental factors. Their heat resistance and stability make it almost impossible to detoxify contaminated products [1; 6; 17; 24]. The mycotoxins fall into various categories: coumarin derivatives, sesquiterpenoids, anthraquinols, piperazines, piranhas, butenolides, phenolic macrolides, alkaloids, steroids and others. These fungal metabolites have been shown to have a destructive effect on many systems of the animal and human body: hepatotoxic, nephrotoxic, cardiotoxic, dermonecrotic, carcinogenic, immunosuppressive. Their small molecule prevents their recognition by the body's immune system and the formation of antibodies [1; 6; 12; 13].

In this aspect some of the species of microscopic fungi of the genus Aspergillus, isolated in the present study were indicated as highly pathogenic. These were the species - A. fumigatus and A. flavus, causing severe pulmonary and allergic aspergillosis [23; 24]. The species A. niger has been reported very rarely as a cause of infection [1; 17; 24].
4. Conclusions
The studied tobacco raw material for manual rolling of cigarettes had significant contamination by microorganisms that had the rank of pathogens. The identified species of the genus Aspergillus are producers of alpha toxins, the spore bacterium B. cereus of exotoxins - lecithinase, which have been shown to play a role in the development of a number of diseases. Quantitative microbial assessment suggests a potential health risk for consumers.

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