Microbiome status of unregulated raw tobacco blends for hand-rolling cigarettes (RYO tobaccos)

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Abstract. The research was conducted on 18 samples of RYO tobacco. The density (CFU/g a.d.s.) of three heterotrophic groups of microorganisms and some physical-chemical indicators were determined. The analyses were performed according to classic methods. Quantitative and qualitative changes at the microbiome as a result of disruption of homeostasis in microbial communities and development of secondary succession in the direction of intensive mineralization processes were found. Statistically significant correlation dependences with the physical-chemical parameters were registered. In addition to a potential risk in term of sanitary-hygiene and health aspects, the changes also have a negative impact on the consumer qualities of tobacco blends.

1 Introduction

The growth of unregulated tobacco products, including fine-cut tobacco blends for hand-rolling cigarettes (roll-your-own, RYO), is increasing worldwide. This trend is particularly strong in countries with an underdeveloped economy and a low socio-economic status of the population [1 - 3].

Although statistical analyses of the unregulated world tobacco market are almost entirely focused on cigarettes, an increase in the relative share of bulk or cut tobacco is progressively reported [4, 5]. Active illegal trade in this type of tobacco products is also a fact in Bulgaria. It is estimated that more than 86% of the unregulated bulk tobacco distributed in our country is of local origin [6].

The analysis of these processes is primarily focused on the causes and factors determining the flourishing of illegal trade and on the economic damages [7]. The research on the qualitative composition and on the changes in the physico-chemical characteristics of these products is limited [8, 9], with just a few studies on their microbiome [10]. Moreover, microbiological analysis is limited to the legal RYO tobaccos [11].

Discussions about the harm of smoking in terms of health have also caused an increase in interest in the microbial composition of various tobacco products, but the emphasis is still on the content of chemical substances harmful to human health [12, 13].

There were data showing a direct relationship between the microbial composition and density with the accumulation of microbial toxins in tobacco products and accordingly with an increase in the risk of a number of diseases [14, 17].

More recently, different response models of the separate resident microbiotes in the human body, a decrease in bacterial diversity and a greater abundance of opportunistic pathogens under the influence of smoking have been reported [18-21]. Standards for threshold safe levels of pathogenic microorganisms and the toxins produced by them in tobacco products are missing.

The illegally distributed RYO tobaccos are usually not professionally prepared for consumption, which poses additional risks in terms of sanitation and health. Therefore, the purpose of the conducted research was to analyze the state of the microbiome in illegally distributed RYO tobaccos by building quantitative microbiological characteristics.

2 Materials and methods

The research was carried out on 18 samples of illegally distributed RYO tobacco, provided at the Tobacco and Tobacco Products Institute, Markovo (Agricultural Academy), Bulgaria.

Depending on the visible degree of mold growth, the samples were grouped as: I. Very clean - no changes in the tobacco material were observed (5 samples); II. Clean - without visible traces of mold, but with a slight change in the color of the material and a faint smell of mold (2 samples); III. Slightly to moderately contaminated - with traces of mold, visible change in color and weak adhesion of tobacco fibers (2 samples); IV. Heavily contaminated - visible mold growth, discoloration and initial degree of conglomeration (4 samples); V. Very heavily contaminated - with abundance of mold, conglomerates and unpleasant odor (5 samples). The samples from each group were prepared and analyzed.

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2.1 Microbiological analyses

Microbiological analyses were performed according to classical methods accepted in sanitary microbiology [22]. From the medium samples of the substrates, 10-fold dilutions were prepared with 0.9% NaCl solution and the diluted suspensions were inoculated on solid nutrient media. The following indicators were defined:

- *Quantity of micromycetes* - on Chapek-Dox agar, after 72 h of incubation at 28°C. The determination to genus was made on the basis of macro-morphological characteristics of the colonies and micro-morphological characteristics of the spore-bearing hyphae [23].

- *Total amount of heterotrophic bacteria* on two nutrient media. The reading was performed after 48 h of incubation at 30°C. Average population density was calculated.

1) A group of bacteria mineralizing organic nitrogen compounds (ammonifying bacteria) - on meat-peptone agar (MPA).

2) A group of bacteria immobilizing mineral nitrogen - on starch-ammonium agar.

Analyses were carried out in three replications.

The quantities of groups of microorganisms were calculated as colony forming units per g absolutely dry substrate (cfu/g a.d.s.), with a confidence level 0.05 using the following formula:

$$cfu = \frac{(\bar{\chi} \pm t\sigma_{\chi}).\frac{K}{V}}{D_m} \quad (1)$$

where:
$\bar{\chi}$ - the average number of colonies of all repetitions;
t = 2 at $P_{0.95}$;
σ_{χ} - mean square deviation;
K - dilution; V - the volume of the inoculum in ml;
D_m - amount of dry matter in g substrate.

- *Mineralization index* - was calculated as the ratio between the amounts of immobilizing mineral nitrogen and ammonifying bacteria.

2.2 Accompanying indicators

Accompanying indicators of the tobacco blends were defined: moisture content (%) - according to BDS 8025:1984 [24]; dry matter (g) and pH in H_2O.

Statistical analysis: All data were presented as means ± mean square deviation. Correlation between the microbiological and accompanying indicators was made *(r - Pearson's coefficient).* The hierarchical cluster analysis was performed of the microbial communities in the different groups of tobacco blends, using Ward's method. Statistical analyses were carried out using the program package SPSS 13.

3 Results and discussion

The studied groups of microorganisms are part of the naturally present epiphytic microflora of tobacco leaves. However, their quantities are minimal and with proper processing, do not impair the quality of the commercial product. The unprofessional preparation of illegally distributed RYO tobaccos, their improper storage, etc. lead to additional contamination and are a prerequisite for the active multiplication and development of microorganisms [25].

3.1 Microbiological analyses

3.1.1 Micromycetes

The amount of micromycetes found in the investigated tobacco blends were high - from about 10^3 cfu/g a.d.s. in the blends classified as very clean and clean to over 10^4 - 10^5 cfu/g a.d.s. in the heavily and very heavily polluted blends (Fig. 1). In the first two groups of tobacco blends the species of the genera *Mucor, Fusarium* and *Penicillium* predominated (Fig. 2). The contaminated tobacco blends were dominated by species of genus *Aspergillus* and genus *Penicillium*.

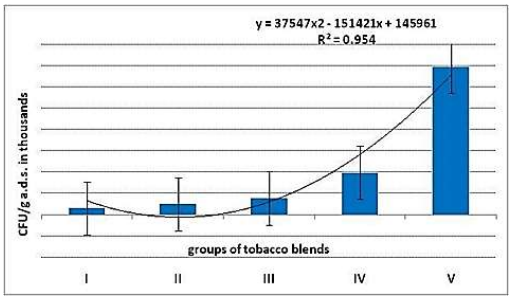

Fig. 1. Amounts of micromycetes (cfu/g a.d.s.) in microbial communities formed in the different tobacco mixtures

Fig. 2. Colonies of *Aspergillus flavus, Fusarium spp., Mucor spp.* isolated in microbial cultures of tobacco blends (author's photos)

3.1.2 Total amount of heterotrophic bacteria

The total population density of heterotrophic bacteria in the microbial communities formed in the various tobacco blends was of the order of hundreds of millions, inclusive of the samples which were classified as very clean. As the degree of mold growth their quantity increases to a certain stage. In the samples determined as very highly contaminated, their amount decreases to tens of millions - 67.43 cfu/g a.d.s. (Fig. 3 and Fig. 4).

The representatives of the heterotrophic microbiota, depending on their structural needs, perform catabolic and metabolic processes that are inherently different. In the present study, we determined the densities of two trophic groups of bacteria: bacteria carrying out the mineralization of nitrogen-containing organic compounds - ammonifying bacteria and bacteria assimilating the nitrogen released during the mineralization, i.e., they immobilize mineral nitrogen in their cells.

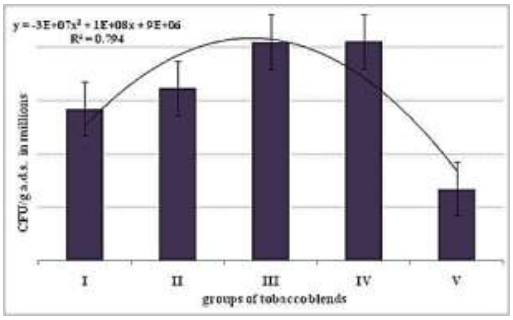

Fig. 3. Total amount of heterotrophic bacteria (cfu/g a.d.s.) - average population density in microbial communities formed in the different tobacco blends

Fig. 4. A view of population density of heterotrophic bacteria in microbial communities in moderately and heavily contaminated tobacco blends

The recorded changes in the densities of both trophic groups of bacteria were analogous to the changes of the total density of heterotrophic bacteria. The amount of ammonifying bacteria increased up to the stage of heavy pollution, after which it decreased more than 2.5 times compared to that in medium and heavily polluted. This trend was also observed but to a much lesser extent in the density of the group of assimilating mineral nitrogen bacteria. Their quantities in the various tobacco blends from I to IV were approximately the same (150 - 200 $\times 10^6$ cfu/g a.d.s.) and decreased to values of 20 - 21 $\times 10^6$ cfu/g a.d.s. for the very heavily polluted (Fig. 5).

The representatives of both groups of microorganisms are part of the epiphytic microflora. Their density and the metabolic processes they carry out change the microbial communities. Their state of homeostasis was disturbed. This was evident from the percentage ratio between them in the different tobacco blends (Fig. 6).

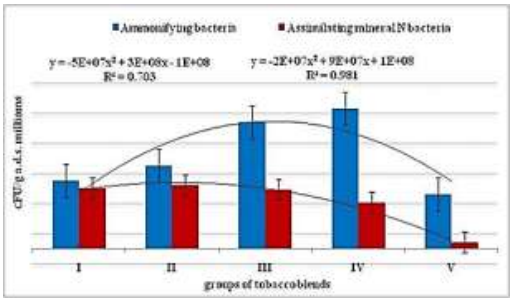

Fig. 5. Amount of the ammonifying and the mineral nitrogen assimilating bacteria (cfu/g a.d.s.) in the microbial communities formed in the different groups of tobacco mixtures

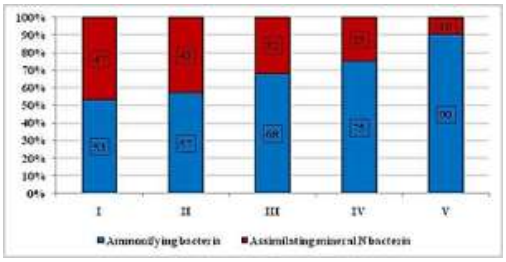

Fig. 6. Percentage ratio between ammonifying and assimilating mineral nitrogen bacteria in the microbial communities of the different tobacco blends

At tobacco blends defined as very clean, the equilibrium state in the microbial communities was preserved. In the case of the other groups tobacco blends this ratio was disturbed and the relative share of ammonifying bacteria increased, reaching up to 90% in highly contaminated ones.

The obtained results showed that in heavily and very heavily contaminated blends, enhanced processes of mineralization of organic matter occurred. The mineralization index being about 1.1 in the clean and very clean groups of tobacco blends reaches over 2.0 and 3.0 in the medium and highly polluted, and over 8.7 in the very heavily contaminated blends (Fig. 7).

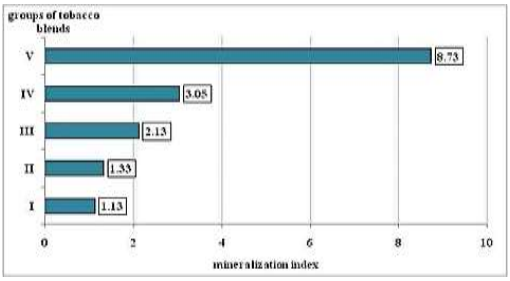

Fig. 7. Mineralization index values in the microbial communities of different tobacco blends

3.2 Accompanying indicators

Some of the specified co-indicators of RYO tobacco blends are indicators of physical properties, others of

chemical properties. They are all related to the quality of the product. The average values of the studied indicators were presented in Table 1.

Moisture content in tobacco blends is a factor related to proper storage and product quality. The moisture content requirements for cigarettes according to BDS 866:1982 [26] should be within the range from 11.00% to 14.00 %. There is no standard for moisture content limits in legally distributed RYO tobaccos. The established values of the moisture content in the investigated tobacco blends exceed the determined norms for cigarettes and reach over 20-30%, with the exception of the samples defined as very clean.

Table 1. An average value of the studied co-indicators

Groups tobacco blends	Moisture content, %	Dry matter content of tobacco mass, mg/g	pH in H_2O
I	12.21 ± 0.14	0.161 ± 1.12	5.26 ± 0.20
II	19.63 ± 0.22	0.239 ± 0.93	5.29 ± 0.25
III	23.29 ± 0.17	0.304 ± 0.78	6.59 ± 0.18
IV	24.73 ± 0.15	0.374 ± 0.90	7.86 ± 0.14
V	31.17 ± 0.19	0.405 ± 0.65	8.11 ± 0.23

Note: all data presented as means ± standard deviation

A change in the alkaline-acidic reaction (pH) of the investigated blends and a tendency towards an increase in alkalinity with an increase in the degree of mold growth was found. The pH values ranged from 5.2 in the very pure and clean to 7.9 - 8.1 in the heavily polluted blends. In the samples classified as slightly and moderately countermined with molds, the reaction was neutral. The content of dry matter (g of tobacco material) also changes depending on the degree of moldiness of the tobacco substrates, in the direction of increase.

Correlations were established between the amounts of microorganisms in tobacco blends and the reported values of physico-chemical co-indicators (Table 2).

In the case of micromycetes, the correlation coefficients with all three physico-chemical indicators of tobacco blends were high and positive. For the total amount of heterotrophic bacteria, a moderate, negative relationship was reported only with the moisture content. In the case of ammonifying bacteria, the correlations were weak, and no dependence was reported with the moisture content. In the case of immobilizing bacteria, the correlation dependences with all three co-indicators were high and negative. The dependences were also high and positive for the mineralization index in tobacco blends.

Very strong dependencies were registered between individual microbiological indicators. The reason was the intense mineralization processes taking place in the microbial communities RYO tobacco blends.

The cluster analysis clearly showed the separate grouping of RYO tobacco based on the values of the microbiological and physico-chemical indicators (Fig. 8).

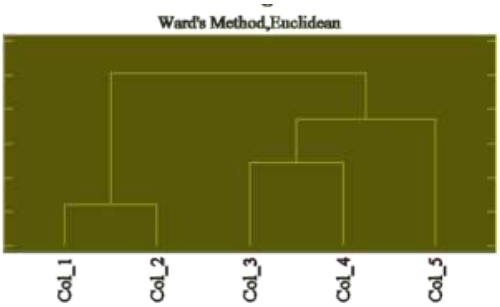

Fig. 8. Hierarchical cluster analysis of the investigated RYO tobacco blends.

Abbreviations: *Groups of tobacco blends - col_1 – (I) very clean; col_2 – (II) clean; col_3 – (III) slightly to moderately contaminated; col_4 – (IV) heavily contaminated; col_5 – (V) very heavily contaminated*

Four separate similarity clusters were formed. At the first level, three clusters were configured. The samples from groups I and II determined depending on the visible degree of moldiness as very clean and clean were grouped into a separate cluster, the samples from group III determined as slightly to moderately polluted together with part of the samples from group IV (highly polluted) formed a second cluster. The rest together with the samples from group V (very heavily contaminated) formed a third cluster. The distance of the contaminated RYO tobacco blends (groups III, IV and V) from the groups I and II defined as clean was significant (between 2 and 4 times), which is an indicator of the remoteness of the similarities. All groups of tobacco blends on the second level formed one common cluster.

4 Conclusions

The obtained results showed significant quantitative changes in the microbiome of unregulated RYO tobacco blends. A part of the observed microorganisms were pathogenic for humans. Such were the established presence of *A. flavus* from the group of micromycetes, of bacteria of the genus *Bacillus*. Quantitative microbiological changes have caused qualitative changes in products. Apart from a potential risk in terms of sanitary-hygienic and health aspects from the presence of opportunistic and pathogenic microorganisms, highly contaminated tobacco blends were defective and should not be used by consumers. As a result of the disturbed homeostasis in the microbial communities and the development of secondary succession in the direction of intense mineralization, the contaminated tobacco substrates were practically unsuitable for use as a smoking product. The results support the opinion about the pressing need for the development and introduction of sanitary-hygienic microbiological standards not only for tobacco blends for hand-rolling cigarettes (legally distributed), but also for the other tobacco products.

Table 2. Correlation dependences between the amounts of microorganisms in tobacco blends and physico-chemical co-parameters

Indicators	MC	DMC	pH	MS	THB	AmB	MNAsB	MI
MC	1							
DMC	0.967***	1						
pH	0.884***	0.960***	1					
MS	0.807***	0.759***	0.771***	1				
THB	-0.411**	-0.171nd	-0.197nd	-0.766***	1			
AmB	-0.073nd	0.248*	0.234*	-0.427**	0.907***	1		
MNAsB	-0.779***	-0.760***	-0.794***	-0.996***	0.751***	0.403*	1	
MI	0.804***	0.776***	0.785***	0.998***	-0.743***	-0.397*	-0.994***	1

$r \leq 0.2$ - 0.4 weak and moderate*; $r \geq 0.4$ - 0.6 considerable**; $r \geq 0.6$ - 0.8-1.0 high and very high***

Abbreviations: *MC - moisture content (%); DMC - dry matter content of tobacco mass (mg/g); pH in H_2O; MS - amount of micromycetes (cfu/g a.d.s.); THB - total amount of heterotrophic bacteria (cfu/g a.d.s.); AmB - amount of ammonifying bacteria (cfu/g a.d.s.); MNAsB - amount of mineral nitrogen assimilating bacteria (cfu/g a.d.s.); MI - mineralization index*

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