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FIRST REPORT OF THE MYCOLOGICAL QUALITY AND PUBLIC HEALTH IMPLICATIONS OF LOCALLY-MADE SNUFFS SOLD IN EBONYI STATE, SOUTHEASTERN NIGERIA.

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ABSTRACT

The microbial quality of tobacco leaves, personal hygiene of handlers as well as various activities that go on around the processing and marketing environments of locally made snuffs sold in Nigeria can impact heavily on the microbial quality of the end products. This study examined the mycological quality of locally-made snuff sold in Ebonyi State, Nigeria. Sixty-four (64) samples of locally-made tobacco products (32 fermented dried tobacco leaves and 32 snuff samples) were purchased from different tobacco shops in two senatorial zones of the state and processed using standard microbiological techniques. The results revealed mean total fungi counts of snuff from Ebonyi Central and Ebonyi South senatorial zones as $8.5x10^5$ and $6.5x10^5$ cfu/g respectively, with mean total aerobic fungi counts of $5.75x10^5$ and $8.94x10^5$ cfu/g for fermented dried tobacco leaves from Ebonyi Central and Ebonyi South respectively. Out of the hundred fungal isolates, *Aspergillus fumigatus* occurred highest (n=45, 45.0%), followed by *Penicillium marneffei* (n=34, 34.0%) and *Rhizopus azygosporus* (n=21, 21.0%). For fungal isolates, *Penicillium marneffei* had the highest MARI of 0.42 followed by *Rhizopus azygosporus* (0.3) and *Aspergillus fumigatus* (0.1). The study showed that the locally-made snuff sold in the zones were highly burdened with potential fungal pathogens and unfit for human consumption.

KEYWORDS: Tobacco, Snuff, Fungi, Contamination, MARI, Ebonyi.

INTRODUCTION

Snuff, a pulverized form of smokeless tobacco widely consumed by inhalation and/or orally, is considered a less harmful form of tobacco when compared with cigarettes.[1-3] This can be in dry or moist form. The use of snuffs is worldwide and transcends several centuries ago. In Nigeria, snuffs are mostly used by southeasterners among the Igbos for several reasons ranging from health, tradition and culture.[4] The demand for snuff among the Igbo users and the cost of foreign medicated snuffs, have resulted to a massive local production, which often lack quality microbiologically.[3] The high sugar content and hygroscopic nature of snuff provide conducive environment for growth and survival of many contaminating microorganisms including fungi especially moulds.[5,6] Most often, the local markets where snuffs are made and sold are surrounded by unsanitary conditions that promote contamination by these microbes.[7] In addition, handlers often lack personal hygiene and processing materials in unhygienic states, thereby further increasing the potential for microbial contamination.[8] As most research emphases are laid on the chemical analysis of snuff, only few studies have reported the presence of bacteria and fungi in snuff.[9] We therefore report the first assessment of the mycological quality of locally made snuffs sold in Ebonyi State with the aim of enumerating the public health consequences.

MATERIALS AND METHODS

Sample Collection and Processing

A total of sixty-four (64) tobacco samples (32 fermented dried tobacco leaves and 32 powdered tobacco - snuff) were aseptically collected from local markets in two Local Government Areas in Ebonyi central senatorial zone and two Local Government Areas in Ebonyi south senatorial zone. All samples collected were transported within one hour to the laboratory unit of Applied

Microbiology Department, Ebonyi State University, Abakaliki, in clean polythene bags for the analysis. One gram (1 g) of each powdered tobacco (snuff) sample was aseptically weighed into sterile tube containing 4 ml of peptone water after which the mixture was shaken vigorously and then serially diluted up to the 10^{th} tube. 1 ml aliquot of each dilution was inoculated on Sabouraud dextrose agar by pour plate techniques and incubated at 28°C for 72 hours. The number of estimated colony forming units (CFU) for each sample was counted using the Quebee colony counter (Rechert, USA). Discrete colonies of aerobic fungi were subcultured for purification by streaking on fresh sabouraud dextrose agar and potato dextrose agar.[10]

Microscopic Identification of Fungi

One drop of Lactophenol Cotton Blue (LPCB) stain was placed on a clean glass slide. With a flamed and cooled wire loop, a small portion of the fungal colony was picked up, cutting through the aerial and vegetative mycelium. A cover slip was used to cover the smear by carefully pressing it down to spread out the fungus. The preparation was examined under the microscope for reproductive structures.[10]

Susceptibility Pattern of Isolates

The susceptibility and resistance patterns of the isolates were determined by disk diffusion technique on Mueller-Hinton agar after standardizing to 0.5 McFarland turbidity level. The antifungal discs were aseptically placed on inoculated Mueller-Hinton agar using sterilized forceps. The plates were incubated at 28^{o}C for 24 hrs. The following antifungal agents were used: Amphotericine B (5 µg), Itraconazole (10 µg), Ketoconazole (10 µg), Fluconazole (10 µg), Voriconazole (15 µg). The degree of susceptibility of the tested isolates to each antibiotic was interpreted according to the principles established by Clinical and Laboratory Standard Institute (CLSI) as susceptible (S) or resistance (R) by measuring the zone diameter of inhibition in millimeter using ruler and interpreted according to the guideline.[11] Multi drug resistant index (MDRI) was determined to ascertain the number of antibiotics/antifungal the isolates were resistance to. Mathematical expression of MDRI is given as thus: n/b; Where n=number of antibiotics to which the isolate resist; while b=total number of antibiotics to which the isolates were subjected to.[12]

Statistical Analysis

The values obtained were analyzed using Analysis of Variance (ANOVA), Windows SPSS (Statistical Package for the Social Sciences) and version 20.0.

RESULTS

The results of the assessment of the mycological quality of locally-made snuffs sold in Ebonyi State revealed mean total fungi counts of snuff from Ebonyi Central and Ebonyi South senatorial zones as $8.5x10^5$ and $6.5x10^5$ cfu/g respectively, with mean total aerobic fungi counts of $5.75x10^5$ and $8.94x10^5$ cfu/g for fermented dried tobacco leaves from Ebonyi Central and Ebonyi South respectively (Table 1). Hundred (100) fungal isolates falling within three (3) genera and three (3) species were encountered; these included *Aspergillus fumigates, Penicillium marneffei* and *Rhizopus azygosporus*. Out of the hundred fungal isolates, *Aspergillus fumigatus* occurred highest (n=45, 45.0%), followed by *Penicillium marneffei* (n=34, 34.0%) and *Rhizopus azygosporus* (n=21, 21.0%). *Aspergillus fumigatus* was the most predominant fungi in both powdered tobacco (n=26, 43.3%) and tobacco leaves (n=19, 47.5%) followed by *Penicillium marneffei* which occurrences of 21(35%) and 13(32.5%) for powdered tobacco and for tobacco leaves respectively. *Rhizopus azygosporus* recorded the least in both powdered tobacco (n=13, 21.7%) and tobacco leaves (n=8, 20%) from Ebonyi Central and Ebonyi South Zones (Table 2).

Aspergillus fumigatus had the highest percentage frequency of 14(40.0%) and 12(48.0%) for powdered tobacco (snuff) from Ebonyi central zone and Ebonyi south zone respectively, and 10(47.6%) and 9(47.4) for fermented dried tobacco leaves from Ebonyi central zone and Ebonyi south zone respectively. While *Rhizopus azygosporus* had the least percentage occurrence in both powdered tobacco (snuff) and fermented dried tobacco leaves from Ebonyi central zone and Ebonyi south zone (Figure 1). No statistical difference was observed among bacterial counts from different zones for powdered tobacco and tobacco leaves at P=0.05.

Table 1: Total Aerobic Fungi Counts (cfu/g) of Powdered Tobacco (Snuff) and Fermented Dried Tobacco Leaves From Ebonyi State, Southeastern Nigeria.

Organism Type	Powdered Tobcco (Snuff)		Fermented Tobacco Leaves	
	Ebonyi Central	Ebonyi South	Ebonyi Central	Ebonyi South
Minimum Count (cfu/g)	$4.0X10^5$	$2.0X10^5$	$2.0X10^5$	$2.0X10^5$
Maximum Count (cfu/g)	$2.6X10^6$	$1.6X10^6$	$2.1X10^6$	$2.6X10^6$
Total Count (cfu/g)	$1.36X10^7$	$1.04X10^7$	$9.2X10^6$	$1.43X10^7$
Mean Count (cfu/g)	$8.5X10^5$	$6.5X10^5$	$5.75X10^5$	$8.94X10^5$
Standard Deviation (cfu/g)	$6.0X10^5$	$4.0X10^5$	$4.94X10^5$	$7.0X10^5$
Variance (cfu/g)	$3.6X10^{11}$	$1.6X10^{11}$	$2.4X10^{11}$	$4.9X10^{11}$
Control (Medicated Snuff) (Orji *et al.*, 2017)				
Sample Code	**Total Microbial Count (cfu/g)**			

MS1	$1.0x10^2$
MS2	0.0
MS3	$3.0x10^2$
MS4	0.0
MS5	$2.0x10^2$

KEY: MS=Medicated snuff.

Table 2: Fungal Isolates Obtained from Powdered Tobacco (Snuff) and Fermented Dried Tobacco Leaves from Ebonyi State, Southeastern Nigeria.

Isolate	Powdered Tobacco (Snuff)		Fermented Tobacco Leaves		Total (%)
	Frequency	Percentage (%)	Frequency	Percentage (%)	
Aspergillus fumigatus	26	43.3	19	47.5	45(45.0)
Penicillium marneffei	21	35	13	32.5	34(34.0)
Rhizopus azygosporus	13	21.7	8	20	21(21.0)
Total	**60**	**100**	**40**	**100**	**100(100)**

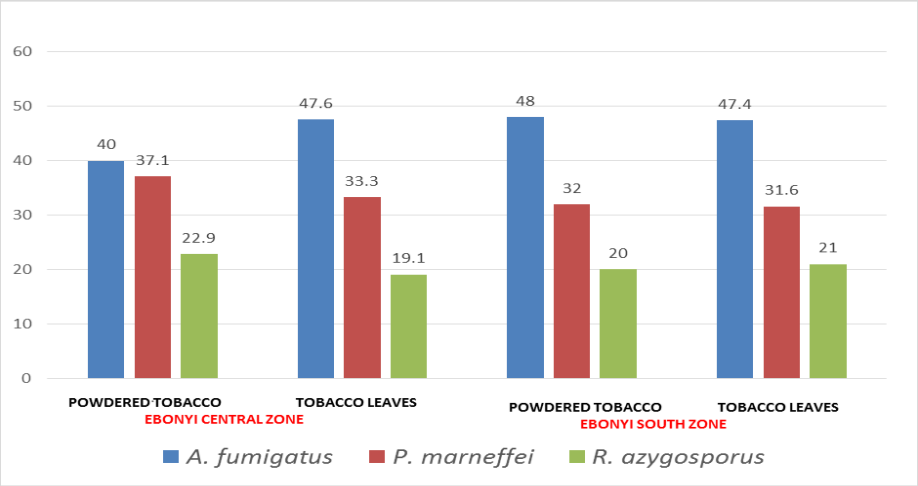

Fig. 1: Percentage frequency of occurrence of fungal isolates from powdered tobacco (snuff) and fermented dried tobacco leaves with reference to specific zones.

The result from Table 3 revealed that *Aspergillus fumigatus* showed 100% susceptibility to Amphotericine B, Itraconazole, and Ketoconazole. While *Penicillium marneffei* showed 100% susceptibility to Itraconazole and Ketoconazole. *Rhizopus azygosporus* showed 100% susceptibility to Amphotericine B, and Itraconazole. Table 4 revealed that *Penicillium marneffei* isolates were resistant to more class of antifungal agents used followed by *Rhizopus azygosporus* and the least *Aspergillus fumigatus* (0.1).

Table 3. Antifungal Sensitivity Pattern of Isolated Fungi Species

Fungal Isolates	No. Tested	Percentage Sensitivity Pattern (%)				
		AMB	ITC	KET	FLU	VCZ
Aspergillus fumigatus	44	44(100)	44(100)	44(100)	30(68.2)	28(63.6)
Penicillium marneffei	36	18(50)	36(100)	36(100)	10(27.8)	5(13.9)
Rhizopus azygosporus	21	21(100)	21(100)	16(76.2)	17(81.0)	0(0.0)

KEY: AMB= Amphotericin B, ITC= Itraconazole, KET= Ketoconazole, FLU= Fluconazole, VCZ= Variconazole.

Table 4: Multiple Anti-fungal Resistance Index of fungal Isolates from Tobacco Samples in Ebonyi State, Southeastern Nigeria.

Isolate	Total MARI	Average
Aspergillus fumigates	5.0	0.1
Pencillium marneffei	15.0	0.42
Rhizopus azygosporus	6.0	0.3

DISCUSSION

Over the years, fungal isolates have been implicated and associated with a number of disease conditions and deaths especially due to the production mycotoxins. However, in screening for microbial quality and safety of food and other products greater attention is given more to bacteria than fungi. The assessment of the mycological quality of locally-made snuff sold in Ebonyi State, Southeastern Nigeria undertaken in this study, revealed that the mean total fungi counts from powdered tobacco (snuff) were $8.5x10^5$ cfu/g and $6.5x10^5$ cfu/g for Ebonyi

Central and Ebonyi South respectively, while the fermented dried tobacco leaves, had mean total aerobic fungi counts of $5.75x10^5$ and $8.94x10^5$ cfu/g for Ebonyi central and Ebonyi south senatorial zones respectively. These values were exceptionally high when compared with the control (Table 1) and greatly surpassed values previously reported by Okechi *et al.*[13] who recorded fungi counts ranging from 1.0×10^3 cfu/g to 2.4×10^3 cfu/g of snuff and Onuorah and Orji[7] who observed fungi counts in the range of 1.0×10^2 cfu/g to 4.2×10^2 cfu/g of snuff. Differences in the hygienic conditions of the study areas is hereby suggested to have explained the variations in the observed results between this study and the previous ones. The local market environments in Ebonyi State where snuffs are prepared and sold are often in unsanitary states and would contributed to the overall fungi burden of the sampled snuffs. Also, processing materials such as containers in which the dry tobacco leaves and processed snuffs are put as well as the mortar and pestle used to pulverize the leaves could have been pre-contaminated with moulds before being used to pound the leaves.[3]

The array of fungal isolates (*Penicillium marneffei, Aspergillus fumigatus* and *Rhizopus azygosporus*) recovered corroborates with the reports of some previous authors.[1,7,13-16] In addition, some of these fungi have been reported as potential pathogens to humans.[17] Factors such as lack of proper heat treatment during the curing process of the raw tobacco leaves can also produce contaminated products. Szedljak[18] has previously reported infection of tobacco leaves by aerobic fungi before fermentation to be due to its high sugar content and this further explains the extent of microbial contamination. The hygroscopic nature of dried tobacco leaves and snuff creates a suitable environment for the growth of microorganisms.[3,5,6] Some of these fungi might however be important in the fermentation of raw tobacco leaves and hence impacting the desired flavour.

Ebonyi central zone recorded highest among the fungal isolates (56%) compared to Ebonyi south zone (44%). The source of their contamination could be through exposure to soil, dust, during growth, processing, storage, marketing and consumption. The sanitary conditions as well as the nature and volume of activities around the markets could be important.

Among the fungal isolates, *Aspergillus fumigatus* recorded the highest percentage occurrence of 47.5% and 43.3% in fermented dried tobacco leaves and powdered tobacco (snuff) respectively. The result is in line with the result obtained by Onuorah and Orji[7], who isolated the organism at high percentage frequency. Eicker[19] reported that *Aspergillus* species can be found in hay, soils and compost piles. The spores and ubiquitous nature of the genus, *Aspergillus* can explain their relative high percentage occurrence in snuff. Some *Aspergillus* species could be dangerous, having been implicated in food poisoning (aspergillosis) due to the production of aflatoxins, increased incidence of severe asthma, sinusitis and chronic bronchitis.[20-22] Varman[23] reported the isolation of nine species of *Aspergillus* in stored leaves of chewing tobacco and approximately eighteen of the *Aspergillus* species were found to be mycotoxigenic. In Ebonyi State, most snuff users are elderly people with low immunity, which could make them vulnerable to infections due to contaminated snuffs. This opportunistic pathogen is reported to infect immunocompromised individuals with high mortality.[22]

Penicillium species recorded high percentage occurrence of 32.5% and 35% in fermented dried tobacco leaves and powdered tobacco (snuff) respectively. This could equally be attributed to their resistant spores and ubiquitous nature. Snuff has low water activity and is hygroscopic in nature and *Penicillium* species have been reported to grow well under such conditions.[22] Okechi *et al.*[13] reported high percentage frequency of the organism in snuff samples. Also, *Penicillium* species are normal microbiota of external ear and they may have been introduced into the snuff in the course of processing and handling of the snuff.[22]

Rhizopus species recorded the least percentage occurrence of 20% and 21.7% in fermented dried tobacco leaves and powdered tobacco (snuff) respectively. Okechi *et al.*[13] reported low percentage frequency of the organism in snuff samples. *Rhizopus* species are spore formers and these spores could elicit allergic reactions in humans.

Furthermore, fungi and actinomycetes in tobacco and its products and their health implications have been previously reported.[24,25] Aspergillus species, Penicillium species and Rhizopus species were isolated by Szedljak[18] from tobacco leaves before fermentation. Moreover, this fact was attributed to the high sugar content of tobacco leaves before fermentation. Contamination by these fungi and their spores could have resulted from processing with contaminated materials such as dirty pestles, mortars, stones, machines, and even cellophane papers.

Interestingly, the result of the anti-fungal susceptibility testing revealed all the fungal isolates recovered from this study showed reasonable degree of susceptibility to standard anti-fungal agents (Table 3). This is in agreement with the reports of Pfaller *et al.*[26] and Jain *et al.*[27] who independently stated that Aspergillus species were susceptible to Triazoles and Amphotericine B. Also, Pfaller *et al.*[26] reported that Voriconazole and Itraconazole were more active against isolates of *Penicillium* species than *Rhizopus* species. However, there was disparity between this present result and the report of Vitale *et al.*[28], who stated that Amphotericine B was the most active drug against fungal species, though somewhat less active against *Rhizopus* species.

CONCLUSION

The snuff samples studied showed high fungal burden contained array of fungal isolates that have pathogenic potentials. As at the time of this study, the snuffs sold at the sampled markets were unfit for human consumption. The microorganisms may have entered the snuff from the air, soil, dust, processing and storage materials, suggesting that unhygienic and unsanitary conditions around the processing and marketing lines were responsible for the contamination of locally-made snuffs sold in Ebonyi State. Therefore, proper sanitary conditions around the processing, packaging and marketing of snuffs should be strictly ensured to avoid and/or reduce contamination. Snuff vendors are also advised to imbibe the culture of personal hygiene and avoid open display of snuffs in the market to avoid post processing contamination. Employment of the western snuff production and preservation techniques, which reduce microbial contamination, is hereby recommended to snuff producers in Nigeria is recommended.

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