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Microbial contamination of snuff sold in selected markets in Yenagoa, Bayelsa State, Nigeria

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Abstract
Snuff is a smokeless tobacco made from ground or pulverized tobacco leaves. It is inhaled or sniffed into the nasal cavity delivering a swift hit of nicotine and a lasting flavored scent. The microbial contamination of snuff sold in selected markets in Yenagoa, Bayelsa State, Nigeria was carried out using standard microbiological techniques. The total bacterial counts shows highest in Swali market and least in Tombia market which was 6.2 x 10^4, 5.5 x 10^4, 5.3 x 10^4 and 4.8x10^4cfu/g for Swali, Kpansia, Opolo, and Tombia markets respectively. While total fungal counts was highest in Kpansia market and least in Tombia market which was 4.2 x 10^4, 2.0 x 10^4, 1.9 x 10^4 and 1.2 x 10^4 cfu/g for Kpansia, Opolo, Swali and Tombia markets respectively. The bacteria characterization and identification shows the presence of *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Micrococcus luteus* and *Escherichia coli*. While those of fungi shows the presence of *Aspergillus niger, Aspergillus fumigatus, Geotrichum albidum, Penicillium oxalicum* and *Geotrichum candidiasis*. The snuffs sold in these markets were contaminated by potentially pathogenic microorganisms. Good manufacturing process and improvement in the sanitary conditions of the markets will help in reducing these contaminations.

Keywords: snuff, bacteria, fungi, characterization, Yenagoa

Introduction
Snuff is a smokeless tobacco made from ground or pulverized tobacco leaves. It is inhaled or sniffed into the nasal cavity delivering a swift hit of nicotine and a lasting flavored scent (especially if flavoring has been blended with the tobacco). Traditionally, it is sniffed or inhaled lightly after a pinch of snuff is either placed onto the back surface of the hand, held pinched between thumb and index finger, or held by a specially made "snuffing" device [1].
The consumption of sniffing of snuff has been a source of concern to the whole world because of its toxic effect on humans. Nigeria just like other developing and developed nations are major consumers of snuff and tobacco products. However, smokeless tobacco products are classified under foods for regulatory purposes.
Snuff is finely grinded tobacco, which comes in dry or moist forms and is sometimes packaged in ready to-use pouches. Dry snuff is usually sniffed or swallowed, whereas moist snuff is placed between the gum and the lip or cheek and slowly absorbed. Snuff is also available in wet form. Wet snuff sometimes called Snus is rubbed inside the mouth instead. Tobacco snuff is in powdered form with potash and sweeteners as the main additives [2].
There are many microorganisms that are associated with snuff mostly transferred during production. Snuff can be traditional home made and commercial or individualized snuff product. According to [3], the local way of producing snuff involves pounding or crushing of the cured leaves with locally made mortars, also depending on the scale of the operation, grinding stones may be used. People take snuff for different reasons: for medicinal purposes, cultural and traditional purposes, for smoking cessation program among others [4].
The present study aimed to investigate microbial contamination of snuff sold in selected markets in Yenagoa, Bayelsa State, Nigeria.

Materials and Methods
Samples Collection
Four (4) different samples of locally- grounded snuff were obtained from different markets in Yenagoa, Bayelsa State namely; Kpansia, Opolo, Swali and Tombia Markets. The samples were collected in sterile sample bottles and conveyed to the microbiology Laboratory of the Niger Delta University, Wilberforce Island Bayelsa State for Microbial analysis.

Isolation of Microorganisms
Culture media used were; Nutrient agar, MacConkey agar, Potato dextrose agar, Bacillus agar and Mannitol salt agar. A tenfold serial dilution of each sample was prepared by weighing 1g of each sample, using 9ml of distilled water to dissolve and serially diluted each. 0.1ml aliquots of the serially diluted samples were spread plated on the surface of nutrient agar and other agars as listed above contained in petri dishes to isolate the bacteria. The plates also had Griseofulvin to inhibit fungal growth. The plates were incubated at 37°C for 24 hours in an inverted position. The discrete colonies that developed after incubation in the incubator were noted and counted, and also sub-cultured and stored on sterile nutrient agar slants for characterization and identification.
The same procedure and culture media was followed and used

strictly for the isolation and cultural characterization of fungi, except that potato dextrose agar (PDA) was used as the growth medium with chloramphenicol at a concentration of 0.05mg/ml was used to inhibit bacterial growth. Incubation was at 37°C (room temperature) for 2-5days after which the fungal colonies that developed were counted, sub-cultured and stored in sterile PDA slants for identification tests.

Characterization and Identification of Bacterial Isolates
The bacterial colonies were characterized on the basis of their cultural and morphological characteristics. Gram staining, motility, indole, methyl-red, voges proskauer, catalase, citrate utilization, coagulase, oxidase and sugar fermentation tests were identified according to the scheme of cheesebrough [5].

Characterization and Identification of Fungal Isolates
The fungal isolates were characterized and identified based on their cultural and microscopic features. The microscopic examination was carried out using lactophenol cotton blue staining and slide culture tests.

Lactophenol Cotton Blue Staining
A fragment of the test fungus was placed on a clean microscopic slide and two or three drops of lactophenol cotton blue solution were introduced. The slide was covered with a cover ship avoiding air interference that will generate bubbles and viewed under the microscope. The features under the microscope magnification were carefully examined and recorded and were compared with the identification scheme of cheesebrough [5].

Slide Culture Test
The fungal mycelia growth was tipped up by the help of the wire loop and after incubation for 24 hours; it was stained with coverslip and viewed under the microscope.

Results

Table 1: Total Bacterial Count (Cfu/g) Sample

Kpansia market	**5.5×10^4**
Opolo market	5.3×10^4
Swali market	6.2×10^4
Tombia market	4.8×10^4

Table 2: Biochemical Reaction of Isolates (Bacteria)

Biochemical	**Test Organisms**				
Reaction	***Staph aureus hylococus***	***Bacillus cereus subtilis***	***Pseudomonas aeruginosa***	***Micrococcus luteus***	***E. coli***
Gram R×n	+ve	+ve	-ve	+ve	-ve
Catalase	+ve	+ve	+ve	+ve	+ve
Citrate	+ve	+ve	+ve	-ve	-ve
Motility	-ve	+ve	+ve	-ve	+ve
Oxidase	-ve	-ve	+ve	-ve	-ve
Methyl red	+ve	-ve	-ve	+ve	-ve
Voges Prosk.	-ve	-ve	-ve	-ve	+ve
Indole	-ve	+ve	-ve	-ve	+ve
Coagulase	+ve	+ve	-ve	-ve	-ve

⁎Abbreviation used are: +ve = positive; -ve = negative; R×n = Reaction; Prosk. = Proskauer; *E.coli =Escherichia coli*

Table 3: Total Fungal Count (Cfu/g)

Sample	**Cfu/g**
Kpansia market	4.2×10^4
Opolo market	2.0×10^4
Swali market	1.9×10^4
Tombia market	1.2×10^4

Table 4: Cultural Characteristics of Fungi Isolated from the samples

Probable organism	**Cultural appearance**	**Microscopic Features**
Aspergillus niger	Black mycelium	Conidiophores were upright, unbranched, simple and terminating, in globose.
Aspergillus fumigatus	Brown mycelium	Smooth conidiophores, walled with flasks shapes, parallel rows, of conidia.
Geotrichum albidum	White mycelia	Conidia arthrospores were hyaline J. celled, short cylindrical with truncate end.
Penicillium xalicum	Yellowish green hyphae	Septate hypha, hyphae were bome, bome and conidiophores, primary and secondary stigmata were present.
Geotrichum candidiasis	Cream white	Possess conidia.

Discussion

Bacteria and fungi were isolated from the snuff samples examined in significant numbers. The total bacterial count was highest in Swali market and least in Tombia market as shown in Table 1.The identification and characterization of bacterial isolate shows the presence of *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Micrococcus luteus and Escherichia coli* as shown in table 2. Some of these isolates have been reported by previous authors [6, 7, 8, 2]. The total bacterial count of the isolates in Swali market is highest compared to other markets could be attributed to relative differences in the sanitary conditions of the markets. Factors such as lack of proper heat treatment during the fire curing process of raw tobacco leaves can also produce contaminated products.

Staphylococcus species present in the snuff samples could be as a result of the hardy nature of the genera which enables them to withstand the low water activity and high salt content of snuff. Though *Staphylococcus* species do not grow outside the body, they are however hardy and though not spore formers, they may remain alive in a dormant state for several months when dried in dust, pus, or sputum [9]. *Staphylococcus aureus* which is pathogenic to man is the second prevalent occurrence in the snuff samples isolated which also, can cause gastroenteritis, food poisoning, skin and wound infections [10]. *Bacillus* species present in the snuff samples is a spore former and ubiquitous organism, *Bacillus* could have been introduced into the snuff from the environment during the processing of tobacco leaves to snuff. The spores could be resistant to the conditions of curing [11-15]. *Bacillus subtilis* which is the

highest prevalent occurrence in the samples has potential health implications and can cause opportunistic infections in man [8].

Micrococcus species present in the snuff samples could be attributed to their resistance to desiccation and nutrient deprivation [9, 16]. The organism, being a normal flora of the human body may have been introduced into the snuff by the handlers in the course of processing and packaging the snuff [17, 18].

The total fungi count was highest in Kpansia market and least in Tombia market as shown in table 3. The cultural characterizations of fungi shows the presence of *Aspergillus niger, Aspergillus fumigatus, Geotrichum albidum, Penicillium oxalicum, Geotrichum candidiasis* as shown in table 4. Some of these fungi have been reported as potentially pathogenic to humans [9]. The hygroscopic nature of dried tobacco leaves and snuff creates a suitable environment for the growth of microorganisms [19, 20]. Some of these fungi might however be important in the fermentation of raw tobacco leaves and hence impacting the desired flavour.

Kpansia market however recorded the highest fungal count among the fungal isolates. The sanitary conditions as well as the nature and volume of activities around the markets could be important and most of the snuff vendors were observed to also sell other items, including perishables, thus giving room for possible cross contamination. 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. The level of activities going on around the environment of the processing also tend to boast the microbial load of the resulting products and the selective effect of the process on the ecology of the surviving microbial populations [21].

Aspergillus species recorded the highest prevalent occurrence in the snuff samples. They can be found in hay, soils, and compost piles [22]. These spores and ubiquitous nature of *Aspergillus* can explain their relative high prevalent occurrence in snuff. Some *Aspergillus* species could be dangerous, having been implicated in food contamination (Aflatoxins), aspergillosis, and increased incidence of severe asthma, sinusitis and chronic bronchitis [23-25]. Most snuff users are elderly people, with lower immunity and other health conditions. This opportunistic pathogen is reported to infect immune compromised individuals with high mortality [25].

However, this may not be significant as a few spores may not be enough to establish respiratory mycosis [9].

Geotrichum species are widely distributed in the plant, soil and on dust particles, storage containers, grinding equipment and skin, because they are air-borne, they can easily contaminate snuff.

Penicillium species were present in the snuff samples which could be attributed to their resistant spores and ubiquitous nature. Snuff has low water activity and is hygroscopic in nature. *Penicillium* species have been reported to grow under such conditions [25]. As *Penicillium* species are normal microbiota of external ear, they may have been introduced into the snuff in the course of processing and handling of the snuff [25].

Conclusion

The snuff samples studied contained some bacteria and fungi which are known to be pathogenic to man. The markets where the snuffs were sold were in poor sanitary conditions and most of the snuff vendors were observed to also sell other items, including perishables, thus giving room for possible cross contamination. Snuff must therefore be processed and handled hygienically to minimize the incidence of these organisms thereby reducing the health risk they pose to its users. Good manufacturing process and improvement in the sanitary conditions of the markets will help in reducing these contaminations. The employment of the western snuff production and preservation processes adopted in developed countries by snuff producers in Nigeria is recommended.

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