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BACTERIAL BURDEN AND POTENTIAL PUBLIC HEALTH RISKS OF LOCALLY MADE SNUFFS SOLD IN EBONYI STATE, NIGERIA.

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ABSTRACT

The unsanitary conditions often associated with the preparation and marketing of locally-made snuff in southeastern Nigeria suggest a potential for contamination with a microbial population that can endanger the health of snuff consumers. This study examined the bacteriological 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 the mean total aerobic bacterial counts of locally-made snuff from Ebonyi Central and Ebonyi South as $3.14x10^6$ and $2.08x10^6$ cfu/g respectively, while fermented dried tobacco leaves recorded mean total aerobic bacterial counts of $1.17x10^6$ and $1.83x10^6$ cfu/g for Ebonyi Central and Ebonyi South respectively. Seven (7) bacterial genera totaling 247 isolates were recovered including: *Escherichia coli* (49/247, 33.1%), *Salmonella* spp. (41/247, 16.6%), *Staphylococcus aureus* (40/247, 16.2%), *Shigella* spp. (38/247, 15.4%), *Streptococcus pyogenes* (29/247, 11.74%), *Klebsiella* spp. (29/247, 11.74%) and *Pseudomonas aeruginosa* (21/247, 8.5%). The result of multiple antibiotic resistant index (MARI) revealed that *Shigella* spp. had the highest MARI of 0.8 and *Staphylococcus aureus* recorded the least (0.02). The study showed that the locally-made snuff sold in the zones were highly burdened with potential bacterial pathogens with high multi-antibiotic resistance indexes..

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

INTRODUCTION

Snuff is a dry or moist powdery form of smokeless tobacco widely consumed by sniffing or inhalation through the nasal cavity and orally by placement between the gum and the lips.[1] It contains the chemical stimulant, nicotine that is medically considered harmful to human health.[2] The consumption of powdery tobacco (snuff) is worldwide and dates back to several centuries ago. Although smokeless tobacco has been associated with such medical conditions as cancer, cardiovascular diseases, diabetes and others[3], it is estimated that more than 300 million people use smokeless tobacco worldwide.[4] It has been noted that users of smokeless tobacco products in the form of snuff, do not face any known cancer risk in the oral region than smokers; however, they have a greater cancer risk than people who do not use any tobacco products.[5] Philips and Heavner submitted that snuff is recommended as a way of reducing harm from tobacco, as the primary harm of smoking tobacco comes from the smoke itself.[6] Previous studies on tobacco including snuff have centred on the chemical, carcinogenic and addictive effects on health, undermining the microbiological implications. In the southeastern part of Nigeria (predominated by the Igbos), snuff is almost a household name due to its wide consumption among the populace for cultural and traditional reasons. Most users in the process have become addicted with ever increasing desire to sniff it.[7,8] This addiction has further increased the demand and fueled local production of snuff widely retailed in almost every local market within the area. Due to the sanitary condition of the processing and marketing environments, snuff have be reportedly contaminated by potential pathogenic bacteria communities, which further endanger the health of snuff users. This gross addiction and contamination by potential bacterial pathogens call

for public health concern. Therefore, this study assessed bacteriologically, the quality of locally-made snuff retailed in the local markets of Ebonyi State, southeastern Nigeria.

MATERIALS AND METHODS

Study Area

This study was carried out in the two senatorial zones (Ebonyi Central, and Ebonyi South) of Ebonyi State, located in the South East geo-political zone of Nigeria within Coordinates: 6°15′N 8°05′E. The state occupies an area of about 5,935 sq. km with an estimated population of 4,339,136 based on the 2005 census. The people of the state are predominantly farmers and traders.

Sample Collection/Analysis

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 onto Nutrient agar by pour plate technique and incubated at 37°C for 24 hrs as described by Cheesbrough.[9] The number of estimated colony forming units (CFU) for each sample was counted using the Quebee colony counter (Rechert USA). Discrete colonies of aerobic bacteria were subcultured for purification by streaking in fresh solid media (Blood agar, MacConkey agar, Salmonella-Shigella agar and Manitol salt agar).[9] The culture plates were incubated at 37°C for 24 hours. All pure isolates were identified using cultural/morphological characteristics, Gram staining and biochemical characteristics based on catalase, oxidase, methyl red, indole, Voges proskauer, citrate, coagulase, urease, motility and sugar fermentation tests.[9]

Antibiotic Susceptibility Pattern of Bacterial Isolates

The antibiotic susceptibility and resistance patterns of the isolates were determined by disk diffusion technique on Mueller-Hinton agar after adjusting to 0.5 McFarland standard against the following antibiotics: Erythromycin (30 µg), Ciprofloxacin, (10 µg), Gentamycin (10 µg), Chloramphenicol (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), Amoxicillin (20 µg), Streptomycin (20 µg), Rifampicin (20 µg), Ampicillin/Cloxacillin (20 µg), levofloxacin (20 µg), Ofloxacin (10 µg), Reflacine (10 µg), Augumentin (30 µg), Cephalexin (10 µg), respectively (Figure 1). No statistical difference was observed among bacterial counts from different zones for powdered tobacco and tobacco leaves at P=0.05.

Sulphamethoxazole/Trimethoprin (30 µg), Ampicillin (30 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.[10] Multi drug resistant index (MDRI) was determined to ascertain the number of antibiotics/antifungal the isolates were resistant to. Mathematical expression of MDRI is given as: 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.[11]

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 bacteriological examination of locally-made snuff sold in Ebonyi State revealed high microbial burden with mean total aerobic bacterial counts of $3.14x10^6$ and $2.08x10^6$ cfu/g for locally-made snuff from Ebonyi Central and Ebonyi South respectively. The range of the bacterial load was $8.0 \times 10^5 – 6.0 \times 10^6$ cfu/g for snuff (powdered tobacco) from Ebonyi Central and $9.0 \times 10^5 – 4.0 \times 10^6$ cfu/g for Ebonyi South. On the other hands, the fermented dried tobacco leaves recorded mean total aerobic bacterial counts of $1.17x10^6$ and $1.83x10^6$ cfu/g for Ebonyi Central and Ebonyi South respectively with ranges of $4.0 \times 10^5 – 2.6 \times 10^6$ cfu/g (Ebonyi Central) and $3.0 \times 10^5 – 3.9 \times 10^6$ cfu/g (Ebonyi South). Other details are shown in Table 1.

Table 2 reveals that a total of 148 bacteria were isolated from powdered tobacco (snuff) while a total of 99 bacteria were isolated from fermented dried tobacco leaves, making a total of 247 bacterial isolates. The variety of bacterial isolates recovered included: Escherichia coli (49/247, 33.1%), Salmonella spp. (41/247, 16.6%), Staphylococcus aureus (40/247, 16.2%), Shigella spp. (38/247, 15.4%), Streptococcus pyogenes (29/247, 11.74%), Klebsiella spp. (29/247, 11.74%) and Pseudomonas aeruginosa (21/247, 8.5%). Based on the tobacco products the occurrences of bacteria with other details are shown in Table 2 while the geographical distribution of the isolates are shown in Figure 1. For fermented dried tobacco leaves, Escherichia coli had the highest percentage frequency of 12(23.5%) in Ebonyi central zone while Shigella had the highest percentage frequency of 10(20.8%) in Ebonyi south zone. Moreover, Pseudomonas aeruginosa had the least percentage frequency of 4(7.8%) and 4(8.3%) in Ebonyi Central zone and Ebonyi South zone

Table 3 shows that all the Gram negative bacterial isolates were not susceptible to Ampicillin and Sulphamethoxazole. *Escherichia coli* showed 100%

susceptibility to Streptomycin, Cephalexin, and Gentamycin. *Pseudomonas aeruginosa* showed 100% susceptibility to Streptomycin and Ciprofloxacin. *Salmonella* spp. and *Shigella* spp. showed 100% susceptibility to Streptomycin and Gentamycin. *Klebsiella* spp. showed 100% susceptibility to Cephalexin, Nalidixic acid, Peflacine, Gentamycin, Augumentin and Ciprofloxacin. On the other hands, *Staphylococcus aureus* showed 100% susceptibility to Streptomycin, Chloramphenicol, Ciprofloxacin, Erythromycin, Levofloxacin, Gentamycin, Rifampicin and Amoxicillin. While *Streptococcus pyogenes* showed 100% susceptibility to Ciprofloxacin, Levofloxacin and Gentamycin (Table 4).

Table 5 reveals that among the Gram negative isolates, *Salmonella* spp. and *E. coli* were resistant to more classes of antibiotics, followed by *Pseudomonas aeruginosa* (0.49) while *Shigella* species had the least resistance rate of 0.8. *Streptococcus pyogenes* were resistant to more classes of antibiotics than *Staphylococcus aureus* (0.02) among the Gram positive isolates.

Table 1: Total Aerobic Bacterial Counts (cfu/g) of Powdered Tobacco (Snuff) and Fermented Dried Tobacco Leaves From Ebonyi Central Zone and Ebonyi South Zone

Organism Type	**Powdered Tobcco (Snuff)**		**Fermented Tobacco Leaves**	
	Ebonyi Central	**Ebonyi South**	**Ebonyi Central**	**Ebonyi South**
Bacteria (P=0.05)				
Minimum Count (cfu/g)	$8.0X10^5$	$9.0X10^5$	$4.0X10^5$	$3.0X10^5$
Maximum Count (cfu/g)	$6.0X10^6$	$4.0X10^6$	$2.6X10^6$	$3.9X10^6$
Total Count (cfu/g)	$5.03X10^7$	$3.33X10^7$	$1.87X10^7$	$2.93X10^7$
Mean Count (cfu/g)	$3.1X10^6$	$2.1X10^6$	$1.2X10^6$	$1.8X10^6$
Standard Deviation (cfu/g)	$1.4X10^6$	$9.0X10^5$	$7.6X10^5$	$1.04X10^6$
Variance (cfu/g)	$1.9X10^{12}$	$8.1X10^{11}$	$5.8X10^{11}$	$1.1X10^{12}$
Control (Medicated Snuff)				
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. Bacterial Isolates Obtained from Powdered Tobacco (Snuff) and Fermented Dried Tobacco Leaves from Ebonyi Central and Ebonyi South Zones

Isolate	**Powdered Tobacco (Snuff)**		**Fermented Tobacco Leaves**		**Total (%)**
	Frequency	**Percentage (%)**	**Frequency**	**Percentage (%)**	
Bacteria					
Escherichia coli	29	19.6	20	20.2	49(19.84)
Pseudomonas aeruginosa	13	8.8	8	8	21(8.50)
Staphylococcus aureus	25	16.9	15	15.2	40(16.20)
Streptococcus pyogenes	17	11.4	12	12.1	29(11.74)
Salmonella spp.	25	16.9	16	16.2	41(16.59)
Shigella spp.	21	14.2	17	17.2	38(15.40)
Klebsiella spp.	18	12.2	11	11.1	29(11.74)
Total	**148**	**100**	**99**	**100**	**247(100)**

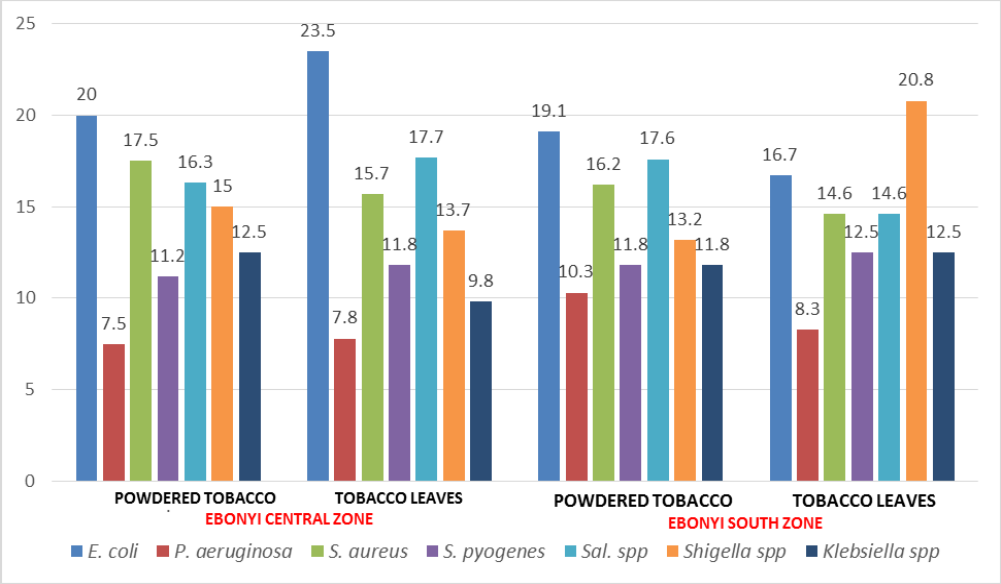

Figure 1: Geographical Distribution of Bacterial Isolates from Powdered Tobacco (Snuff) and Fermented Dried Tobacco Leaves locally marketed in Ebonyi State.

Table 3: Antibiotic Sensitivity Pattern of Isolated Gram Negative Bacteria.

Bacterial Isolate	No. Tested	Percentage Sensitivity Pattern (%)									
		S	PN	CEP	OFX	NA	PEF	CN	AU	CPX	SXT
Escherichia coli	49	49(100)	0(0.00)	49(100)	20(40.8)	0(0.00)	10(20.4)	49(100)	30(61.2)	28(57.1)	0(0.00)
Pseudomonas aeruginosa	17	17(100)	0(0.00)	8(47.1)	10(58.8)	0(0.00)	9(52.9)	16(94.1)	10(58.8)	17(100)	0(0.00)
Salmonella spp.	40	40(100)	0(0.00)	36(90.0)	15(37.5)	0(0.00)	0(0.00)	40(100)	20(50.0)	16(40.0)	0(0.00)
Shigella spp.	38	38(100)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	38(100)	0(0.00)	0(0.00)	0(0.00)
Klebsiella spp.	29	14(48.3)	0(0.00)	29(100)	26(89.7)	29(100)	29(100)	29(100)	29(100)	29(100)	0(0.00)

KEY: S= Streptomycin, PN= Ampicillin, CEP= Cephalexin, OFX= Ofloxacin, NA= Nalidixic acid, PEF= Peflacine, CN= Gentamycin, AU= Augumentin, CPX= Ciprofloxacin, SXT= Sulphamethoxazole/Trimethoprim.

Table 4: Antibiotic Sensitivity Pattern of Isolated Gram Positive Bacteria.

Bacterial Isolate	No. Tested	Percentage Sensitivity Pattern (%)									
		S	NB	CH	CPX	E	LEV	CN	APX	RD	AMI
Staphylococcus aureus	40	40(100)	36(90.0)	40(100)	40(100)	40(100)	40(100)	40(100)	35(87.5)	40(100)	40(100)
Streptococcus pyogenes	26	20(76.9)	14(53.8)	0(0.00)	26(100)	0(0.00)	26(100)	26(100)	20(76.9)	0(0.00)	22(84.6)

KEY: S= Streptomycin, NB= Norfloxacin, CH= Chloramphenicol, CPX= Ciprofloxacin, E= Erythromycin, LEV= Levofloxacin, CN= Gentamycin, APX= Ampicillin/Cloxacillin, RD= Rifampicin, AMl= Amoxicillin.

Table 5: Multiple Antibiotic Resistance Index of Gram Negative Bacterial Isolates from Tobacco Samples from Ebonyi South and Ebonyi Central Zones.

Isolate	Total MARI	Average
Bacteria		
Escherichia coli	25.4	0.52
Pseudomonas aeruginosa	8.3	0.49
Salmonella species	23.3	0.59
Klebsiella species	7.6	0.26
Shigella species	30.4	0.8
Staphylococcus aureus	0.9	0.02
Streptococcus pyogenes	10.41	0.41

DISCUSSION

The microbial populations that contaminate snuff across the pre-processing, processing and post-processing lines may impact negatively on the health of snuff users due to their pathogenic potentials. The bacteriological examination of locally-made snuff sold in Ebonyi State revealed an incredibly high and unacceptable microbial burden when compared with medicated snuffs (Table 1). This was much higher than other previous studies by Onuorah and Orji[2] whose bacterial counts ranged from 3.0 x 10^2 cfu/g to 6.7 x 10^2 cfu/g, and Okechi *et al.*[8] who recorded a microbial burden in the range of 2.85 x 10^4 cfu/g - 5.67 x 10^4 cfu/g. The variation in results could be attributed to the differences in the sanitary conditions of the processing and marketing environment as well as the personal hygiene of snuff

handlers/vendors. The snuff could have been cross-contaminated from unclean processing materials, handlers themselves and dust particles due to open display of retail snuff in the local markets.

Seven (7) bacterial genera totaling 247 isolates were recovered from the samples. These included: *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Salmonella* species, *Shigella* species and *Klebsiella* species. This agrees with the reports of Rubinstein and Pederson[12], Ayo-Yusuf *et al*.[1], Alsaimary *et al.*[13], Okechi *et al.*[8], Onuorah and Orji[2] and Tyx *et al.*[4] who reported the isolation of some of these isolates. However, it does not corroborate with the report of Ahamed[14] who mainly described *Bacillus* species. The health risks associated with majority of these bacterial isolates have been reported by Hardy *et al*.[15] Factors such as lack of proper heat treatment during the fire curing process of raw tobacco leaves can also produce contaminated products especially if the leaves were infected as reported by Szedljak.[16] The hygroscopic nature of dried tobacco leaves and snuff was previously suggested to create a suitable environment for the growth of contaminating microorganisms.[17,18] Contrastingly, the varieties of bacteria in this current study are not normal flora of tobacco but mainly of human origin and could be thought to have resulted from human contamination.

The high percentage occurrence of bacterial isolates in Ebonyi central zone (53%) compared to Ebonyi south zone (47%) could be attributed to relative differences in the sanitary conditions of the markets in these zones. The source of their contamination can be through exposure to the soil, dust, during growth, processing, storage and consumption. The sanitary conditions as well as the nature and volume of activities around the markets could be important culprits.

The high occurrence of *Escherichia coli* in powdered tobacco and fermented dried tobacco leaves disagreed with some previous results obtained by Onuorah and Orji[2] and Okechi *et al*.[8] However, it supports the work of Tyx *et al*.[4], where high frequency of *Escherichia coli* was isolated from dry snuff. This high percentage occurrence of *Escherichia coli* in snuff samples could be attributed to their resistance to desiccation and nutrient deprivation.[19,20] 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.[21,22] *Salmonella species* showed second highest prevalence 25(16.9%) in powdered tobacco (snuff) and third in fermented dried tobacco leaves 16(16.2%). This is in conformity with the report Tyx *et al*.[4], who isolated enteric bacteria from dry snuff in high frequency. Other enteric bacteria isolated in this study were *Shigella* species, *Klebsiella* species and *Escherichia coli.* The isolation of the above-mentioned enteric bacteria from tobacco samples reflects faecal contamination of snuff from handlers and the market environment.

Staphylococcus aureus showed the second highest prevalence 25(16.9%) in powdered tobacco (snuff) and fourth in fermented dried tobacco leaves 12(12.1%). This agrees with the results obtained by Okechi *et al*.[8], Onuorah and Orji[2] and Tyx *et al*.[4], where *Staphylococcus aureus* showed the highest prevalence. But the result disagrees with the result obtained by Alsaimary *et al*.[13], who isolated the organism at low percentage frequency (2.8%). The percentage occurrence of *Staphylococcus aureus* could be due to the hardy nature of the genera, which enables them 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.[19] The low prevalence of *Pseudomonas aeruginosa* recorded in this study, contradicts the work of Alsaimary *et al*.[13], who isolated the organism in very high prevalence rate from smokeless tobacco products. The isolation of *Pseudomonas aeruginosa* from tobacco samples may be as a result of the addition of contaminated water to snuff during grinding and the use of water to moisten the tobacco leaves during dry season.

Besides, the antibiotic susceptibility profile of Gram negative bacterial isolates revealed that all the Gram negative bacterial isolates were not susceptible to Ampicillin and Sulphamethoxazole. This supports the report of Sahm *et al.*[23] that Ampicillin is not effective in treating infections caused by the bacterial isolates. *Escherichia coli* showed 100% susceptibility to Streptomycin, Cephalexin and Gentamycin. *Pseudomonas aeruginosa* showed 100% susceptibility to Streptomycin and Ciprofloxacin. *Salmonella* spp. and *Shigella* spp. showed 100% susceptibility to Streptomycin and Gentamycin. *Klebsiella* spp. showed 100% susceptibility to Cephalexin, Nalidixic acid, Peflacine, Gentamycin, Augumentin and Ciprofloxacin. This is contrary to the report of Tula and Iyoha[24] where most of their isolates were resistant to the same range of antibiotics. In addition, Mamkandan *et al.*[25] reported that 83.3% and 80.6% of their bacterial isolates were resistant to sulphamethoxazole/trimethopim and Nalidixic acid respectively.

This study also revealed that S*taphylococcus aureus* isolates were 100% susceptible to Streptomycin, Chloramphenicol, Ciprofloxacin, Erythromycin, Levofloxacin, Gentamycin, Rifampicin and Amoxil; 90% susceptible to Norfloxacin and 87.5% susceptible to Ampicillin. On the other hand, *Streptococcus pyogenes* isolates were 100% susceptible to Ciprofloxacin, Levofloxacin and Gentamycin; 76.9% susceptible to Streptomycin and Ampicillin; 53.8% susceptible to Norfloxacin. This is similar to the report of Tula and Iyoha,[24], where all the Gram positive bacterial isolates

were highly susceptible to all the antibiotics. However, all the *Streptococcus pyogenes* isolated in this study were resistant to Erythromycin. This may be due to variation in geographical locations, environmental conditions, genetic background of the organisms and improper use of the antibiotic in the region.[26]

The multi drug resistance indexes of bacterial isolates were also studied. The result revealed that *Shigella* species had the highest MARI of 0.8, followed by *Salmonella species* (0.6), *Escherichia coli* (0.52), *Pseudomonas aeruginosa* (0.5) and the least *Klebsiella species* (0.30). For Gram positive bacteria, *Streptococcus pyogenes* had the highest MARI of 0.41 followed by *Staphylococcus aureus* which recorded (0.02).

CONCLUSION

Bacteriological analysis of the snuff samples revealed high bacterial burden, which could be associated with unhygienic environmental conditions surrounding the local processing of snuff, sold in the local markets of Ebonyi State. Also, the snuff samples were grossly contaminated with potential human pathogens some of which showed resistance to conventional antibiotics. The presence of this range of bacteria in snuff with their high multi-antibiotic resistance indexes is of alarming public health concern. Since these organisms could have entered through the processing and handling chain, handlers of locally-made snuff should be enlightened on the need for personal hygiene to curb the transmission of these pathogens via retail snuff. All bacterial infected individuals should be promptly treated before being involved in the processing of snuff. All snuff-processing materials should be regularly kept clean. The public health agencies should imbibe and enforce continuous microbial monitoring and evaluation of retail snuff sold in Nigeria.

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