Antibiotics susceptibility profile of biofilm-forming bacteria isolated from soybean milk drinks sold in Abakaliki Metropolis, Nigeria

ABSTRACT: This study was carried out to determine the antibiotic susceptibility profile of biofilm-forming bacteria isolated from locally produced soybean milk drinks sold within the Abakaliki metropolis. A total of 150 soybean milk samples comprising 15 from each location were collected using random sampling techniques from 10 different locations namely Nkaliki, Presco Junction, Azugwu, Kpirikpiri, Ahiaofu, Mile 50, Rice mill, Mechanic site, international market and Abofia area of Abakaliki town in Ebonyi State metropolis. The collected soybean milk samples were analyzed for the presence of bacteria using standard microbiology techniques which include; culturing, Gram staining and biochemical tests. The screening for biofilm formation on isolated bacteria species was done using the tube method. The antibiotic susceptibility profile of the isolated biofilm-forming bacteria was determined using the disc diffusion method. The result showed that a total of 100 (66.6%) bacteria were isolated from the locally produced soybean milk comprising of 5 bacteria genera namely: *Staphylococcus aureus* 25 (25%), *Escherichia coli*, 27 (27%), *Pseudomonas aeruginosa* 10 (10%), *Klebsiella* species 15 (15%) and *Salmonella* species 23 (23%). The biofilm production screening test revealed that *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* are biofilm-forming bacteria (are all the isolated bacteria of *S. aureus*, *K. pneumoniae* and *P. aeruginosa* all biofilm producers. The antibiotic susceptibility profile of biofilm-forming bacteria varies between antibiotics. The result showed that all *Staphylococcus aureus* isolates were (100%) susceptible to ceftazidime, 80 % resistant to imipenem and 72% resistant to ciprofloxacin, *Pseudomonas aeruginosa*, isolates were 100% and 80% resistant to cefoxitin and ofloxacin while *E. coli* isolates were 70.3% and 63% resistant to ofloxacin and cefotaxime respectively. The study showed the poor hygienic quality of locally produced soybean milk marketed within the Abakaliki metropolis and this call for public awareness as it could be the source of disease outbreak/spread within this community.

Keywords: Antibiotics Susceptibility, Biofilm-Forming Bacteria, Soybean Milk.

I. INTRODUCTION

Soybean milk is a plant-based drink produced by soaking and grinding soybeans and filtering out remaining particulates. It is an important food diet of the vast population in the world, due to its high nutritional value as they are excellent source of protein both in quality and quantity [1]. They are naturally valuable sources of vitamins and minerals such as thiamin, riboflavin, niacin, calcium, phosphorus, magnesium, zinc and potassium [2]. There is continued debate as to whether purified proteins that are added to food confer the same health benefits as whole foods [3]. There is increasing evidence to suggest that consuming whole foods has additive and synergistic health benefits. In addition to the essential proteins it contains, other health benefits of consuming milk are widely recognized [4]. Foodborne diseases are of great concern around the world as such, this is an important issue in developing countries like Nigeria where poor sanitation is a problem during food processing [5, 2]. *Salmonella* spp., *Klebsiella* spp., *Clostridium botulism*, *Staphylococcus* spp., *Escherichia coli* and *Shigella* spp. are pathogens that are usually found in soybean milk causing inflammatory reactions. Biofilm are extracellular matrix formed by some species of bacteria. It is a thin membranous layer covering the surface of any substance thereby causing opacity [6, 3]. Biofilm formation occurs when free-floating microorganisms attach themselves to a surface. Even though biofilm-forming bacteria have side effects, they are still beneficial to humans as they are relevant in the improvement of metal dissolution in the bioleaching industry and are also used in biodegradation [7]. The side effect of biofilm concerning antibiotics is that the biofilms protect the bacteria from antimicrobial agent attack thereby making them immune to antimicrobial attack, [8]**.** This study was designed to determine the antibiotic profile of biofilm-forming bacteria species isolated from locally prepared soybean milk sold within the Abakaliki metropolis.

MATERIALS AND METHODS

Sample collection

A total of 150 samples of locally prepared soybean milk drinks were randomly collected from ten different locations within the Abakaliki metropolis namely Nkaliki, Presco Junction, Azugwu, Kpirikpiri, Ahiaofu, Mile 50, Rice mill, Mechanic site, international market and Abofia. They were properly labelled and transported to the Microbiology Laboratory Unit of Ebonyi State University, Abakaliki for bacteriological analysis using standard microbiology techniques.

Preparation of media and Inoculation of soybean milk on media

All media were prepared according to the manufacturer's specifications. The pour Plate method [9], was used in plating all the samples. Exactly one (1mm) millilitre of the soybean milk from dilution 10^3 was placed into a sterile petri dish with the aid of a sterile pipette. A molten nutrient agar, McConkey agar and eosin-methylene blue agar were poured into several Petri dishes containing 1mm of diluted sample. The plates were swirling for easy mix-up of the sample and the media. All plates were prepared in triplicate and allowed to solidify on the bench. After solidifying the plates were incubated at 37 ^{O}C for 18 – 24 hours. After incubation, the bacteria colony growing on the surface of the agar plate was counted using a colony counter machine. The estimation of the viable number of microorganisms (total viable counts) in each sample was made in colony-forming units (CFU) as described by [9].

Identification and characterization of bacteria isolates from soybean milk

Identification and characterization of bacteria from soybean milk were done as described by [10].

Screening for Biofilm Production on isolated bacteria from soybean milk by tube method

The tube method (TM) is a qualitative assay for the detection of biofilm production in microorganisms by the presence of visible film, as described by [11]. The isolates were inoculated in a polystyrene test tube which contained TSB and incubated for 24 h at 37°C. The sessile isolates of which biofilms formed on the walls of the polystyrene test tube were stained with safranine for 1hr after planktonic cells were discharged by rinsing twice with phosphate-buffered saline (PBS). Then, the safranine-stained polystyrene test tube was rinsed twice with PBS to discharge the stain. After air drying of the test tube process, it was observed for the occurrence of visible film lines in the walls, and the bottom of the tube indicating biofilm production [12].

Antibiotic susceptibility testing

Antibiotic susceptibility testing of isolates was done by using the Kirby-Bauer disc diffusion Method according to the Clinical and Laboratory Standards Institute [13, 9]. Bacteria inoculum equivalent to 0.5 McFarland standard of the isolate was streaked on the Mueller-Hinton agar plate and allowed for 5 minutes pre-diffusion into the agar. The following antibiotic disc were placed on the surface of inoculated agar plate ceftazidime (30µg), trimethoprim-sulfamethoxazole (30µg), imipenem (10µg), cefoxitin (30µg), cefotaxime (30µg), colistin sulphate (10µg), ofloxacin (10µg), ertapenem (10µg), Erythromycin (15µg), amikacin (30 µg), amoxicillin-clavulanic acid (30µg), ciprofloxacin (5µg), Tetracycline (30µg), penicillin (10µg), vancomycin (30µg), oxacillin (1µg). This was incubated at 37^{o}C for 24 hrs and the Inhibition zone diameter was measured and interpretative using the Clinical and Laboratory Standards Institute break point [14, 9].

Statistical analysis

Prevalence data were analyzed using the generalized model of Statistical Package central tendency (percentile) [15, 14].

RESULTS

3.1 Total bacterial count of the soybean samples

Table 1: Summary of total bacteria colony isolated from soybean milk

Table 1 shows the microbial colony count from the soybean milk samples collected from Nkaliki in Abakaliki Metropolis. Out of the 15 soybean milk samples analyzed, two samples with codes NK_6 and NK_5 had the lowest and highest microbial colony count of 1.12×10^3 and 1.94×10^3 respectively.

The microbial colony count from the soybeans milk samples collected from Rice Mill in Abakaliki Metropolis showed that 13 samples had viable microbial growth with the lowest and highest microbial colony count found in RM_{17} and RM_{27}.

At mile 50 (M50), 8 samples had visible microbial growth as $M50_{44}$ and $M50_{42}$ had the lowest and highest bacteria colony count.

Nine (9) samples from the international Market in Abakaliki Metropolis had visible bacteria. growth. The sample code IM_{53} had the highest microbial growth with the colony forming unit as 1.96×10^3 and the lowest was with the sample code IM_{47} with the colony forming unit as 1.00×10^3 .

Nine (9) samples had viable growth in Abofia. The sample codes AB_{65} and AB_{73} had an insignificant ($p > 0.05$) growth while growth that was too numerous to count was recorded in sample codes AB_{68} and AB_{71}. The lowest and highest bacteria colony was from samples AB_{64} and AB_{70} respectively.

Nine (9) Soybean samples collected from the Presco junction had viable bacteria growth. The highest Significant ($p < 0.05$) microbial growth was recorded in sample PJ_{87} with the colony-forming unit as 1.90×10^3 and sample code PJ_{84} had the least number of colony-forming units as 1.00×10^3.

A total of 6 samples out of 15 soybean samples obtained from Azugwu had viable growth. The lowest microbial growth rate was seen in the sample code AZ_{101} with the colony-forming unit as 1.10×10^3 and the highest colony-forming unit as $1.92 \times 10^{3.}$

Seven samples from the mechanic site had viable growth. The lowest microbial growth rate was seen in the sample code MS_{116} with an insignificant colony-forming unit.

Ten (10) samples out of the 15 samples collected from Kpirikpiri had viable growth, with

the lowest and highest bacteria colony-forming unit in the KP_{131} and KP_{126} samples.

From Ahiaofu nine (9) samples were observed with some levels of growth but the lowest and highest bacteria colony-forming unit was 6.9×10^3 and 1.78×10^3

Table 2 below shows that out of 150 samples analyzed, 100 samples recorded bacteria growth. It revealed that an equal number of Escherichia *coli* was observed from the samples collected from Nkaliki and Ahiaofu in the Abakaliki Metropolis. The samples from Rice Mill, International Market and Presco Junction showed the highest growth of *Escherichia coli.* The samples from Ahiaofu showed an equal number of *Escherichia coli, Klebsiella pneumoniae* and *Salmonella* spp growth and an equal amount of bacteria growth of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella* *pneumoniae* and *Salmonella* spp.

Table 3 below shows the biofilm and non-biofilm-forming bacteria isolated from different soybean samples collected within Abakaliki Metropolis. It showed that *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* are biofilm formers while *Klebsiella pneumoniae* and *Salmonella* spp. *are* non-biofilm formers.

The result of antibiotic resistance and susceptibility of Biofilm forming *Staphylococcus aureus* showed that the bacteria were 100 % resistant to penicillin, cefoxitin, vancomycin and oxacillin. The level of susceptibility was recorded as imipenem (80.0 %), ceftazidime (72.0 %), ciprofloxacin (60.0 %) and erythromycin (60.0 %) respectively in Table 4.

The result of the percentage of antibiotic resistance and susceptibility of *Pseudomonas aeruginosa* to the antibiotics used showed that the bacterium was 100 % resistant to Trimethoprim-sulfamethoxazole, 90 % colistin sulphate and ertapenem respectively. The microorganism was 100 % susceptible to ceftazidime and cefoxitin and 90 % to ofloxacin as shown below respectively (Table 5).

The result of the percentage of antibiotic resistance and susceptibility of *Escherichia coli* isolated from soy milk showed that the microorganism was 100 % resistant to amoxicillin-clavulanic acid and 88.8 % to cefoxitin but was susceptible to cefotaxime and ofloxacin at 100 % and 70.3 % (Table 6).

Table 1: Minimum and maximum microbial colony count of soybean milk samples collected from ten different sites in the Abakaliki Metropolis.

S/N	Sample Code	CFU/ml	P-value
1	NK_6	1.12×10^3	1.59615
2	NK_5	1.94×10^3	1.18373
3	RM_{17}	1.30×10^3	0.20152
4	RM_{27}	1.90×10^3	0.04408
5	$M50_{42}$	1.10×10^3	0.49074
6	$M50_{44}$	1.68×10^3	1.28819
7	IM_{47}	1.00×10^3	0.03067
8	IM_{53}	1.96×10^3	1.35053
9	AB_{64}	1.28×10^{-3}	0.90019
10	AB_{70}	1.82×10^{-3}	1.59615
11	PJ_{84}	1.00×10^3	0.04034
12	PJ_{87}	1.90×10^3	1.28241
13	AZ_{101}	1.10×10^3	0.74444
14	AZ_{100}	1.92×10^3	1.84969
15	MS_{108}	9.0×10^4	3.18630
16	MS_{118}	1.80×10^3	0.26355
17	KP_{131}	1.00×10^3	0.16625
18	KP_{126}	1.90×10^3	0.23071
19	AO_{141}	6.9×10^3	1.23564
20	AO_{145}	1.78×10^3	0.22961

Keys: NK = Nkaliki, RM = Rice Mill, M50 =Mile 50, IM = International Market, AB = Abofia, PJ = Presco Junction, AZ = Azugwu, MS = Mechanic Site, KP = Kpirikpiri, AO = Ahiaofu.

Table 2: Number of samples with bacteria growth and the organisms isolated from each location within Abakaliki Metropolis.

Sample Units	**No. Sampled**	**No. of Samples with growth**	***Pseud. aeruginosa* (%)**	***E. coli* (%)**	***Kleb. pneu.* (%)**	***Salm.* spp. (%)**	***Staph. aureus* (%)**
NK	15	12	2(20)	4(14.8)	2(13.3)	2(8.6)	2(8)
RM	15	14	1(10)	3(11.1)	1(6.6)	5(21.7)	4(16)
M50	15	13	2(20)	2(7.4)	2(13.3)	4(17.3)	3(12)
IM	15	10	1(10)	2(7.4)	1(6.6)	3(13.0)	3(12)
AB	15	7	1(10)	3(11.1)	1(6.6)	2(8.6)	-
PJ	15	7	-	3(11.1)	1(6.6)	1(4.3)	2(8)
AZ	15	8	1(10)	2(7.4)	1(6.6)	2(8.6)	2(8)
MS	15	8	1(10)	2(7.4)	1(6.6)	1(4.3)	3(12)
KP	15	12	1(10)	2(7.4)	3(20)	2(8.6)	4(16)
AO	15	9	-	4(14.8)	2(13.3)	1(4.3)	2(8)
Total	150	100	10	27	15	23	25

Keys: Staph. aureus = Staphylococcus aureus, Pseud. aeruginosa = Pseudomonas aeruginosa, E. coli = Escherichia coli, Kleb. pneu.= Klebsiella pneumoniae, Salm. typhi. = Salmonella typhi, NK = Nkaliki , RM = Rice Mill, M50 =Mile 50, IM = International Market, AB = Abofia, PJ = Presco Junction, AZ = Azugwu, MS = Mechanic Site, KP = Kpirikpiri, AO = Ahiaofu.

Table 3: Biofilm forming potentials of bacteria isolated from soybean milk sold in the Abakaliki Metropolis

S/N	Test Result	Bacteria
1	+	*Staphylococcus aureus*
2	+	*Pseudomonas aeruginosa*
3	+	*Escherichia coli*
4	-	*Klebsiella pneumoniae*
5	-	*Salmonella spp.*

KEY: + = positive, - = negative

Table 4: Percentage of antibiotics resistant and susceptibility of *Staphylococcus aureus* isolated from soybean milk.

S/N	Antibiotics (Concentration)	Resistance (%)	Susceptible (%)
1	Ceftazidime	7(28.0)	18(72.0)
2	Cefotaxime	16(64.0)	9(36.0)
3	Ciprofloxacin	10(40.0)	15(60.0)
4	Erythromycin	10(40.0)	15(60.0)
5	Tetracycline	24(96.0)	1(4.0)
6	Penicillin	25(100)	0(0)
7	Cefoxitin	25(100)	0(0)
8	Vancomycin	25(100)	0(0)
9	Oxacillin	25(100)	0 (0)
10	Trimethoprim-sulfamethoxazole	14 (56.0)	11 (44.0)
11	Imipenem	5(20.0)	20(80.0)

Table 5: Percentage of antibiotic-resistant and susceptibility of *Pseudomonas aeruginosa* isolated from soybean milk

S/N	Antibiotics (Concentration)	Resistance (%)	Susceptible (%)
1	Ceftazidime	0(0)	10(100)
2	Cefotaxime	5(50)	5(50)
3	Imipenem	7(70)	3(30)
4	Cefoxitin	0(0)	10(100)
5	Colistin sulphate	9(90)	1(10)
6	Ofloxacin	2(20)	8(80)
7	Ertapenem	9(90)	1(10)
8	Amikacin	5(50)	5(50)
9	Amoxicillin-clavulanic acid	8(80)	2(20)
10	Trimethoprim-sulfamethoxazole	10(100)	0(0)

Table 6: **Antibiotics percentage of resistance and susceptibility pattern of *Escherichia coli***

S/N	Antibiotics (Concentration)	Resistance (%)	Susceptible (%)
1	Ceftazidime	10(37)	17(63)
2	Cefotaxime	0(0)	27(100)
3	Imipenem	14(51.9)	13(48.1)
4	Cefoxitin	24(88.8)	3(11.1)
5	Colistin sulphate	21(77.8)	6(22.2)
6	Ofloxacin	8(29.6)	19(70.3)
7	Ertapenem	18(66.7)	9(33.3)
8	Amikacin	7(25.9)	1(3.7)
9	Amoxicillin-clavulanic acid	27(100)	0(0)
10	Trimethoprim-sulfamethoxazole	26(96.3)	3(3.7)

DISCUSSION

In the present study, a total of 150 samples of locally prepared soybean milk drinks were collected at random from 10 different locations within the Abakaliki metropolis namely Nkaliki, Presco Junction, Azugwu, Kpirikpiri, Ahiaofu, Mile 50, Rice Mill, Mechanic site, International market and Abofia. Table 1 shows the microbial colony count from the soybean milk samples collected from the ten different sites. From the analysis, the lowest and highest microbial growth rate of each site was recorded and it was observed that of all the sites, samples collected from International Market in Abakaliki Metropolis with the sample code IM_{53} had the highest microbial growth rate and the least was recorded on AO_{141} respectively. In this study, the observation of microbial growth in the soybean milk samples has received considerable attention which is in support from several scientists [26, 28, 29], as all the soybean milk samples used in the study contained one form of bacteria or another and each sample contained different contaminants leading to the observable growth of microorganisms which is following earlier report [16].

The dusty, unhygienic environment coupled with poor handling by vendors are factors contributing to the high microbial load as seen in the samples examined from the International market and Abofia [17]. The presence of these large numbers of bacterial growths might be a result of unsanitary conditions or practices during the production, processing, distribution or storage of soybean milk [18, 1, 6].

A total of 100 bacteria isolates were isolated from the soybean milk in the order of 10 *Pseudomonas aeruginosa*, 15 *Klebsiella* species, 23 *Salmonella* species, 25 *Staphylococcus aureus* and 27 *Escherichia coli* respectively. The high presence of *E. coli* is an indicator of faecal contamination of the water that was used in the preparation and the unhygienic activities of the handlers [19, 2]. *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella* organisms are widely distributed in water, soil and sewage too as reported by [20]. According to [21], the presence of *Salmonella* species in the soybean milk sample is an indicator of post-processing contamination which could cause typhoid fever and other food poisoning if such soybean milk is consumed. Also, milk is a rich source of various nutrients. [22, 19], as it provides all the nutrients to sustain health, grow, protect and heal. The nutrients in milk serve as an ideal medium for microbial growth [23].

After cultural and biochemical characterization using standard microbiology techniques *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, *Klebsiella* and *Salmonella* species were isolated from different soybean samples [24].

Out of the 5 bacteria isolates obtained, 3 of them are biofilm-forming bacteria namely; *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. This implies that they were able to form a thin layer or membranous covering on the surface of the test tube thereby causing opacity during the biofilm test which is in line with the result of [25, 16]. The antibiotics resistance and susceptibility test of the 25 *Staphylococcus aureus* determined against 11 antimicrobial agents is shown in (Table 4). Biofilm-forming *Staphylococcus aureus* was resistant to penicillin 25 (100 %), cefoxitin 25 (100%), vancomycin 25 (100 %), oxacillin 25 (100 %), tetracycline 24 (96%) and Cefotaxime 16 (64.0 %) was relatively high compared to the other antibiotics which are reported by

Johnson and Snyder [26]. Its high level of resistance can be attributed to the ability of *Staphylococcus aureus* to form biofilm which confers its resistance to antibiotics [27, 5].

Table 5 shows the susceptibility and resistance profile of *Pseudomonas aeruginosa.* The susceptibility level was 10 (100 %) to ceftazidime and cefoxitin respectively but 10 (100 %) resistance to trimethoprim - sulfamethoxazole. This shows that ceftazidime and cefoxitin are the best antibiotics that can be used to treat an infection caused by *Pseudomonas aeruginosa*. This was in agreement with the report of Osundahunsi *et al* [28], conducted on the antibiotics resistance and susceptibility test on bacteria isolates obtained from soybean milk sold in Kwara state Nigeria. On the other hand, the development of antimicrobial resistance among pathogenic bacteria poses a problem of high concern [29, 6]. The present study showed that *E. coli* isolates (Table 5) as a biofilm-forming bacterium were highly sensitive to cefotaxine 27 (100 %) followed by ofloxacin 19 (70.3 %) and ceftazidime 17 (63%) respectively, which is in line with the report of [30, 4]. The biofilm-forming bacterium was resistant to amoxicillin-clavulanic acid 27 (100%), trimethoprim-sulfamethoxazole 26 (96.3%), cefoxitin 24 (88.8%) and ertapenem 18 (66.7%) relatively which can be depicted as the capacity of *E. coli* isolate to form a thick layer and film called biofilm as well as acquire resistant genes which offer it the resistance ability to antibiotics [31, 12] (Table 6). From the results of this study, the highest level of contamination of soybean milk by biofilm-forming bacteria was observed in the samples collected from Rice Mill and Nkaliki. The large number of biofilm-forming bacteria in these products from the colony counts observed, confirms that the soybean milk sold within the Rice mill and Nkaliki were prepared in a poor hygienic environment and may pose serious health hazard to consumers while the ones sold at Azugwu and mechanic site were fairly better [31, 11]. The consumption of large amounts of these biofilm-forming bacteria in soybean milk could change the normal flora which may lead to food-borne diseases like dysentery, diarrhoea and stomach ache [31, 8].

Sequel to the antibiotics susceptibility profile of biofilm-forming bacteria isolated, the antibiotics that should be considered as the first drugs of choice in the treatment of infections caused by these biofilm-forming bacteria include; imipenem, ceftazidime and ciprofloxacin for *Staphylococcus aureus*, ceftazidime, cefoxitin and ofloxacin for *Pseudomonas aeruginosa* and cefotaxime, ofloxacin and ceftazidime for *E. coli.* due to their high-level susceptibility to those antibiotics. The high level of resistance to the other antibiotics can be attributed to their ability to form biofilms as well as acquire resistant genes which act as resistant factors.

Conclusion

This study revealed that locally produced soybean milk sold in the Abakaliki metropolis is contaminated with biofilm-forming bacteria that are multi-drug resistant. This shows the poor hygienic state of locally produced soybean milk and it calls for worry because this will be a threat to human health when consumed. We suggested that the National Agency for Food and Drug Administration and Control (NAFDAC) and other regulatory agencies of concern should monitor the quality of locally produced soybeans and other products sold to ensure they meet the standard regulations and that they are microbiological -safe for human consumption. Further exhaustive studies from various laboratories to confirm and monitor the quality of locally produced soybean milk and other such products should not be contaminated before they are sold in the market and used as milk additive

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