ANTIBIOTIC SUSCEPTIBILITY SCREENING OF PATHOGENIC BACTERIA ISOLATED FROM LOCALLY PRODUCED SOYBEAN *(Glycine max)* MLK SOLD IN PORT HARCOURT, NIGERIA

ABSTRACT

Antibiotic susceptibility of pathogenic bacteria isolated from locally produced soymilk hawked in Rumuolumeni, Port Harcourt was carried out. Three samples of soymilk were obtained from Rumuokoro Market, Rumuolumeni Market and Choba Markets. Standard microbiological techniques including the pour plate method and relevant biochemical tests were used to isolate, characterize and identify the bacteria cultures. The result of the bacteria count was high in Rumuokoro market $4.2x10^3$ cfu/ml and lowest in Rumuolumeni market $3.4x10^3$cfu/ml. The probable bacteria isolates identified are *Escherichia coli, Staphylococcus aureus, Klebseilla pneumonia, Salmonella spp* and *Streptococcus faecalis. E. coll* had the highest percentage occurrence (38.18%) and *klebsiella pneumonia* the lowest (7.27%). The antibiotic screening showed that the gram-positive isolates *S. aureus* and *Stretococcus faecalisare* sensitive to Ampiclox (80%), (100%). Among the gram-negative isolates *E. coli* was most sensitive to Augumentin (71%), *Klebsiella pneumonia* was sensitive to Ampicilin (15%) *Salmonella spp* most sensitive to Streptomycin (70%). The result of this research work shows that all soymilk samples are contaminated with different bacterial species. The implication is that the quality of locally produced soymilk must be closely regulated to avoid transmission of infections to customers. It is also critical that local producers are educated on good manufacturing practices.

Keywords: antibiotic susceptibility, pathogenic bacteria, soybean milk

INTRODUCTION

Soymilk (also known as soybean milk, soy juice, and soy drink/beverage) is a beverage prepared from soybeans. Soymilk is a stable emulsion that is a staple in Asian cuisine. To make soymilk, overnight soaked soybeans are crushed with water and filtered. The pale liquid, which is similar to milk, soymilk, or simple soybean beverage, is high in protein and other nutrients and can be made with or without optional additives or components. Soy milk has a benefit over cow/buffalo milk in that it does not include lactose or cholesterol, and the fibres present may be eliminated from the finished product (FAO, 2018). Some people may eat soymilk for environmental or health reasons, while others may do so if they are vegan or lactose intolerant and need to replace dairy milk (Guibourg & Briggs, 2019). According to Jimoh and Kolapo (2007), “soymilk is a traditional food beverage from Asia that is increasingly popular in the United States and other countries”. “Since soymilk has many health advantages, including low lactose and cholesterol levels, the capacity to lessen menopausal symptoms like bone loss, and the prevention and reduction of heart disease and

some malignancies, it is becoming more and more popular as a beverage around the world" (Iwe 2003; Kolapo & Oladirneji, 2008). Traditional methods for making soymilk include soaking the beans in clean water first, then grinding and filtering them. The milk produced resembles a white or creamy emulsion with a viscosity and texture similar to cow milk (traditional milk) (Iwe 2003; Kolapo & Oladimeji 2008). It is a thin extract made from soybean seeds that is high in oil, carbs, and water-soluble proteins (Adebayo et al., 2008). As is the case with the majority of yoghurt products, lactic acid fermentation can enhance the soy flavour (Jimoh & Kolapo, 2007).

For close to 5000 years, millions of oriental people have relied on soybeans and items made from them as a significant source of protein in their diet. It is a possible food source that has a high concentration of lysine compared to other plant proteins and is made up of all the essential amino acids that are crucial for the body's healthy growth. All of the essential amino acids—aside from methionine and cystine—are present in sufficient proportions in the protein of soybeans. Regarding the amount of protein it contains, it is among the greatest vegetarian foods. For riboflavin, it is a reliable source. According to Noroz *et al.* (2011), soya beans account for 20% of the diet's total fat. Since soya bean fat is unsaturated, as opposed to saturated fats from animal sources, it is advantageous for people with heart disease (Anderson & Bush, 2011). It also has a healthy quantity of fat and calories, with 19.5 grammes of fat, 21 grammes of carbohydrate, and 432 calories per 100 grammes (Ayo, 2011). When soy beans are properly processed, they produce soy bean milk, which may then be turned into yoghurt, which can be used as a real protein supplement or replacement for both adults and young children. Since soy milk does not contain lactose, it can be used as a milk substitute by those who are lactose intolerant. According to Anderson and Bush (2011), trypsin, a digestive enzyme that is destroyed by heat, can be inhibited by a component found in soybeans.

Due to microbial activity, soymilk's most significant issues continue to be its beamy flavour and extremely limited shelf life. However, because soymilk is nutrient-rich, it is vulnerable to microbial attack if it is not properly processed and kept. "This is because the nutrients in soymilk are also necessary for the growth of most spoiling organisms. Numerous microorganisms, including mesophilic aerobic bacteria, coliforms, and fungi, are known to cause milk to spoil and cause unfavourable alterations in soymilk" (Momoh et al., 2011). Unhygienically cooked soymilk can harbour bacterial infections linked to food poisoning, gastroenteritis, and enteric fever (Adebayo et al., 2008). Some potentially pathogenic dairy food pollutants have been linked to mycetomas in humans (Cheesbrough, 2005). According

to Nawal et al. (2013), “diarrheal diseases are a primary cause of illness and death in underdeveloped nations, killing an estimated 1.9 million people annually worldwide. Diarrheal diseases are caused by foodborne microbial pathogens”. According to Prescott *et al*. (2005), *Aspergillus flavus* causes allergy or pulmonary Aspergillosis and also generates the extremely carcinogenic aflatoxin.

Antibiotics were once thought to be the most efficient therapeutic agents to treat microbial illnesses, however due to antibiotic abuse, multidrug resistant strains of bacteria have emerged and spread throughout many groups of germs. Worldwide, infection-resistant bacteria pose a growing hazard, both as community- and hospital-acquired pathogens. One of the major issues in both clinical and public health is antibiotic resistance. The usage of more expensive antibiotics to treat infections as well as the threat of resistance to them are both caused by the antimicrobial resistance, which is also increasing morbidity and mortality (Subedi et al., 2016). Therefore, research on area-specific illnesses and their patterns of resistance is required in order to produce information that will aid doctors in selecting the best course of treatment. Treatment of infections is made more challenging by an increase in antibiotic-resistant bacteria (Gautam et al., 2013). WHO cautioned the global population that multi-drug resistant bacteria are growing, posing a significant threat to healthcare, and that if quick action is not taken, antibiotics may lose their ability to cure diseases. The primary goal of this study was to evaluate the microbiological quality and antibiotic susceptibility of pathogenic bacteria isolated from locally made soymilk sold in different areas of Port Harcourt. The study's findings will expose the bacteria contaminants in soymilk and raise awareness about how to minimise microbial deterioration of soymilk through proper handling during production and storage.

MATERIALS AND METHODS

2.1 The Study Area

The research area is located in Rivers State's Obio/Akpor Local Government Area, which includes Rumuolumeni, Choba, and Rumuokoro. Rumuolumeni lies between latitude 4°48'59.238'N and longitude 6°57'16,866'E, Choba lies between latitude 4°53^{S}20,31'N and longitude 6'54'3,438'E and Rumuokoro lies between latitude 4°52'9,762'N and longitude 6°59'56,094'E.

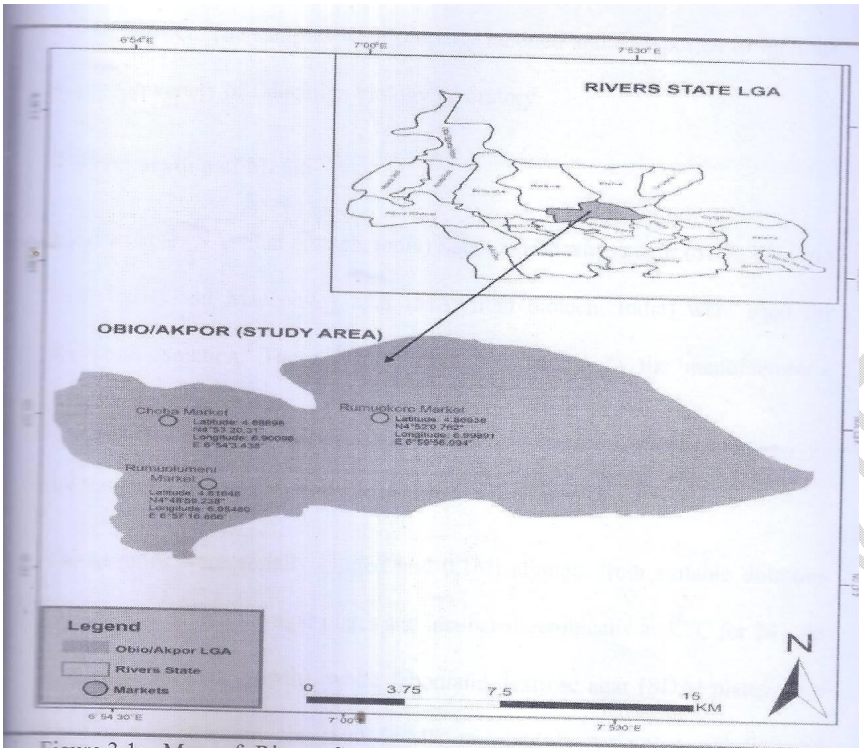

Fig.1: Map of Rivers State showing sampling station in Obio/Akpor Local Government Area.

2.2 Sample Collection

At Rumuolumeni market, Rumuokoro market, and Choba market, three samples of soymilk that were previously packaged in plastic bottles were acquired from street vendors. The samples were delivered to the Ignatius Ajuru University of Education Biology Laboratory for analysis after being appropriately labelled.

2.3 Media Preparation

Nutrient agar, Sabourand Dextrose Agar and MacConkey agar were used for culture and isolation. The media preparation was as prescribed by the manufacturers.

2.4 Preparation of Sample and Inoculation

The samples were serially diluted and 0.1 ml aliquots from 10^4 dilutions was inoculated into the sterile prepared plates and incubated aerobically at 37°C for 24 - 48 hours (for bacteria growth), while Sabourand Dextrose Agar (SDA) plates were fortified with Streptomycin (500mg/ml) to inhibit bacteria contamination and incubated at 22 C for 72 - 120 hours (for fungal growth), MacConkey agar (MA) was inoculated with serial dilutions of the sample by

the pour plate method and incubated at 37^0C for 24-48 hours for coliform growth. Developed microbial growths were counted and recorded according to Dhawale & Lamaster, (2003).

2.5 Colony Counting

The overnight culture that was acquired through serial dilution was placed in petri dishes, and readings were taken after all of the plates had been incubated. Colony forming units (cfu/ml) were calculated using the number of colonies counted on the plates after taking the dilution factor into account.

$$\text{cfu/ml} = \frac{\text{number of colonies x dilution factor}}{\text{volume of culture plate}}$$

2.6 Purification

Every single individual colony on every medium was counted, sub-cultured to nutrient agar plate, and then incubated for 24 hours at 37°C before being streaked in agar slant medium as stock for biochemical assays. To obtain pure bacterial and fungal cultures, this was done.

2.7 Identification of Fungal species

According to their culture morphology and microscopy, such as colour, structure, consistency, and mycelial growth pattern, fungi isolates were described and identified. Before observing the isolates using the microscope's X40 objective, a wet mount procedure (Robinson *et al.*, 2020; 2021). The morphological structures include sporangiospores, fruiting bodies, septate and non-septate structures, as well as unique organisms like rhizoids.

2.8 Identification of Bacteria species

Each pure culture of bacteria was microscopically evaluated for colony morphology before being gram stained to ascertain its gram status. Through various common biochemical tests, the bacterial isolates were characterised.

2.9 Biochemical Test

Gram stain was used to identify the bacteria. A heat-fixed smear of the isolated organism was made on a clean, grease-free slide and flooded with crystal violet solution for one minute. This slide was then rinsed under slow running tap water and flooded with lugols iodine solution for one minute. The slide was then rinsed under slow running tap water and then decolored dropwise with acetone for two minutes. Finally, the slide was rinsed under slow

running tap water and then counter stained with safranin for 30 seconds. The slide was rinsed, air dried and viewed with a magnification of xI00 using oil immersion.

Catalase test:

Cells from the centre of a well-isolated colony were transferred to a glass slide with a wire loop for the catalase test, and 1-2 drops of 3% hydrogen peroxide were applied to the slide. Rapidly forming persistent gas bubbles are a sign of a successful outcome. No bubble production yields undesirable outcomes.

Indole test

"In this test, the test organism was inoculated into test tube containing 5ml of sterile tryptone broth and incubated at 37^0C for up to 48 hours. Indole was then tested by adding 0.5ml of Kovac's reagent to the culture which was then shaken gently and examined for red color on the surface layer within 10 seconds. The production of red ring layer indicated a positive result" (Prescott *et al.*, 2011).

Oxidase test

This test was carried out to check for the presence of the enzyme, cytochrome oxidase in the isolates. A piece of filter paper was placed in a clean petri dish and three drops of freshly prepared oxidase reagent was added. A glass rod was used to remove a colony of the 24hrs old test organisms and smeared on the filter paper and the development of purple color within 10 seconds indicated the positive result while non-purple color formation indicated negative result

Methyl red test

Methyl red test (MR) is used to determine the ability of an organism to produce *2nd* maintain stable acid and products from glucose fermentation. If the bacteria has the ability to utilize glucose, with production of a stable acid, the color of the methyl red changes from yellow to red, when added into the broth culture, Two drops of methyl red solution was added to the test organism, shaken and examined for color change, red color indicate a positive reaction and yellow a negative reaction.

Sugar fermentation test

Sucrose sugar was used in the experiment. The test detects organisms that can use the sugar in question. The medium contained 1% sugar, peptone, and two drops of indicator solution. After fully mixing, it was distributed into test tubes, and Durham tubes were dropped into the test tubes inverted. For 156 minutes, the test tubes were cooked and sterilised in an autoclave set to 121°C. After chilling, the test organisms were inoculated into various tubes, and ions in the Durham tube indicated positive gas and acid production, respectively.

Motility Test

A thin layer of Vaseline was applied around the well's edge in a concave slide. In the centre of the cover slip, a loop of the bacteria suspension was placed. The concave slide was inverted and pressed on top of the cover slip, entirely covering the well. The preparation was swiftly turned back around. The edges of the cover slip were checked to confirm that the Vaseline had properly sealed the edges. Under the microscope, bacterial cells in motion were spotted in suspension on the bottom of the cover slip.

2.10 Antibiotic sensitivity testing

"The antibiotic sensitivity testing was carried out using the Kirby-Bauer disc diffusion technique" (Wemedo and Robinson, 2018). Pure colonies of 24 hours old isolates were adjusted to 0.5 MC Farland standards in 0.9% saline. Swab sticks were dipped into the solution of the isolate and normal saline and streaked uniformly on Muller-Hinton agar plates and multi-antibiotics sensitivity discs were placed on the surface of the media, using a pair of forceps. Little force was applied to ensure firm contact with the agar plant. The plates were then inverted and inculcated aerobically at 37°C for 18-24hrs.

RESULTS

The results of microbial counts obtained in the study are represented in table 1 while the percentage occurrence of bacterial isolates is presented in Fig. 2. The cultural characteristic and biochemical characteristics are presented in Table 2 and 3, respectively while the antibiotic susceptibility test is presented in Table 4 and 5.

Table 1: Total viable count for bacterial and fungal isolates in cfu/ml

Location	Total Heterotrophic Bacteria count	Fungal count	Total coliform count
	cfu/ml	cfu/ml	cfu/ml
Rumuolumini	3.4×10^3	3.1×10^3	3.2×10^3
Rumuokoro	4.2×10^3	4.0×10^3	4.4×10^3
Choba	3.8×10^3	3.4×10^3	3.5×10^3

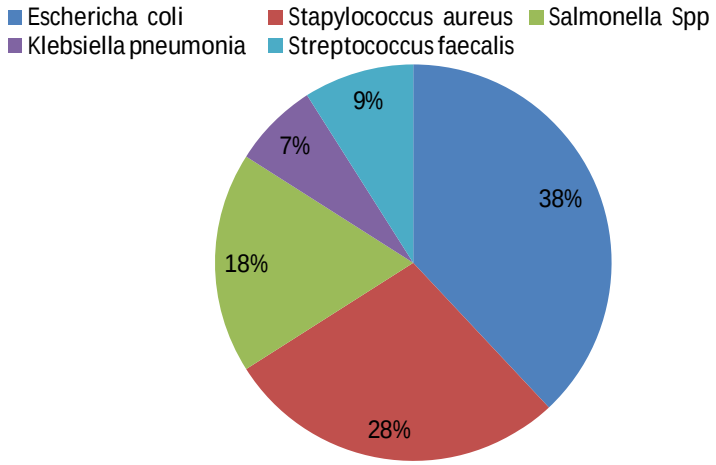

Fig.2: Percentage occurrence of bacteria isolates in soymilk sample.

Table 2: Cultural characteristics and results of test for coliforms

Cultural growth on Macconkey agar	Microscopic appearance	Probable organism
Red, opaque, flat colonies with undulate edges about 1.75mm diameter	Single, fat, short rods	*E. coli*
Red, opaque, flat colonises with undulate edges about 1.75mm diameter	Single, fat, short rods	*Klebsilla* spp
Red flat opaque colonies with rhizoids edges about 0.75mm in diameter	Single fat short rods	*Salmonella* spp

Raised, red, opaque colonies with undulated edges about 0.85mm diameter	Single chains	*Streptococcus faecalis*
Deep yellow, convex papillate, opaque colonies with entire edges about 0.75mm in diameter	Spherical clusters	*Staphylococcus aureus*

Table 3: Biochemical characteristics of bacteria isolates

Gram Stain	Catalase test	Oxidase test	MR test	Indole test	Motility test	Sugar fermentation test	Probable organism
-	+	-	+	+	-	+	*E. coli*
-	+	-	-	-	-	+	*Klebsiella spp*
-	+	-	-	-	+	+	*Salmonella spp*
+	-	-	-	-	-	+	*Staphylococcus aureus*
+	+	+	-	-	-	+	*Streptococcus faecalis*

Table 4: Antibiotic sensitivity profile of gram positive isolates.

Isolate	N	AML (10NG) n%	APX (30NG) n%	CN (30ug) n%	LEV (lOug) n%
S. aureus	15	9, 60%	12, 80%	3, 20%	9, 60%
S. faecalis	5	2, 40%	5, 100%	4, 80%	3, 60%

KEY: N = total number of isolates, N = number of sensitive organisms, % = percentage of sensitivity (n/N x 100), AML = Amoxil, APX = Ampiclox, CN = Gentamycin, LEV = Levofloxacin,

Table 5: Antibiotic sensitivity profile of gram-negative isolate

Isolate	N	PN (10ug) n%	CN (30ug) n%	S (10ng) n%	AU (30ug) n%

E. coli	21	9,43%	12,53%	6,29%	15,71%
Klebsiella spp	4	3,75%	2, 50%	2,39%	1,25%
Salmonella spp	10	5,50%	3, 30%	7,70%	2,20%

KEY: N = total number of isolates

N = number of sensitive organisms

% = percentage of sensitivity (n/N x 100)

PN = Ampicillin

CN = Gentamycin

S = Streptomycin

AU = Augmentin

DISCUSSION

This study recorded the highest bacterial count of 4.2 x $l0^3$cfu/ml was observed at Rumuokoro market, while the least count of 3.4 x 10^3 cfu/ml was recorded at Rumuolumini market. The organisms identified were *E. coli, Klebsiella spp, Salmonella spp, Streptococcus faecalis, Staphylococcus aureus., E. coli* was the most predominant having the highest occurrence (38.18%), while *Klebsiella pneumonia* had the lowest occurrence (7.27%). The antibiotic screening profile of the gram-positive bacteria isolates from this study indicated that *Staphylococcus aureus* was sensitive to all the antibiotics, especially Ampiclox (80%). The same pattern was observed for *Streptococcus faecalis* which was also most sensitive to Ampiclox (100%). For the gram-negative bacteria isolates from the soymilk samples, the antibiotic sensitivity pattern showed that *E. coli* was most sensitive to Augmentrin (71%), *Klebsiella spp* most sensitive to ampicillin (75%) and *Salmonella spp* most sensitive to streptomycin 70%. The gram-negative isolates were not completely resistant to any of the antibiotics used in the study.

In an investigation of the microbiological and biochemical quality of soymilk produced and sold within Calabar metropolis, Asuquo & Antai, (2017) reported that mean bacteria counts were highest in samples from Army barracks 6,9 ± 0.01 *X* 10^8 cfu/ml and lowest in Marian market 4.80 ± 0.04 x 10^8 cfu/ml, while the mean fungal count was highest in Army barracks 650 + 0.17 x 10^8 cfu/ml and lowest in Akim barracks 3,50 ± 0.22 x10^8cfu/ml. Liamngee et *al*, (2013) studied the microbial quality of soyabean milk sold in Makurdi metropolis and noted a microbial load ranging from 6.9 x 10^7 - 7.6 X 10^7 cfu/ml for North bank, 4.1 x 10^7- 5.6x 10^7cfu/ml for Wokurum, 3.0x 10^7- 4.7x 10^7 cfu/ml for high level and 6.0x 10^7- 5x $l0^7$ for Wadata respectively. All samples were contaminated with members of the

Enterobacteriaceae, including *Escherichia coli, klebsiella spp, Salmonella typhi, Streptococcus faecalis and Staphylococcus aureas.*

Agwa and Ossai-Chidi (2016) looked at the microbiological quality of locally produced and industrially produced soybean products sold in Port Harcourt. They found that rural samples included considerably more bacteria and fungus than industrial samples (p 0.05). *Pseudomonas* sp. (S7.5%), *Bacillus* sp., *Staphylococcus* sp. (25% each), and *Streptococcus* sp. (12%) were among the microorganisms identified from industrial samples. *Pseudomonas* sp. and *Staphylococcus* sp. were isolated from locally processed materials (25%). *Rhizopus* sp., *Aspergillus* sp., and *Saccharomyces* sp. make up 33.3% of the fungi that were isolated. *Rhizopus* (40%) is the most prevalent fungus in samples made locally, followed by *Saccharomyces* sp. and *Aspergillus* sp. (35%).

Goli *et al,* (2016) studied "the antibiotic susceptibility patterns of the gram negative bacteria in Iran. The sensitivity rates of isolated gram negative bacteria were for Amikacin(95.7%), Nitrofurantion (91.5%), Gentamicin (64.1%) Ceftizoxim (56 . 8%) , Ciprofloxacin (37 . 6%), cotrimoxazole (31.4%) and Nalidvcic *add* (56.8%)".

Report of work done by Pradip *et al.,* (2016) on the antimicrobial sensitivity of *Staphylococcus aureus* showed highest sensitivity towards linezolid (92.3%), *Streptococcus pyrogens was* 100% sensitive towards *Vancomydn, Cefuoxzime, Cetazoline, Teicoplanin and 100%* resistance towards *Erythromycin, Ofloxacin*, *£iprofloxacin, Cefadroxil, Gentamycine, Ampicillin + Sulbactam, Cloxacillin/Oxacillin, Levoftoxacin, cefdinirand* linezolid. On the other hand, *Entrococcus* showed 100% sensitivity towards Ampicillin + Sulbactam, and *moxifloxacin*. *Escherichia coli was* 90% sensitive to most of the antibiotics. *Pseudomonas* showed 100% sensitivity towards *ceftriaxone, Gentamycin, piperacillin + Tazobactam. Klebsiella spp* showed highest sensitivity (89.5%) towards ciprofloxacin.

Obi, (2014) investigated the microbiological quality of Home and Industrial made soymilk samples consumed in Umuahia metropolis, Abia State, Nigeria and reported that the two soymilk types were contaminated. The bacterial count for the home made soymilk was in the range 6.2 X 10^6 - 4.0 x 10^5*cfu/ml,* while that of the industry ranged from 2.0 x 10^2 to 1.10^2*cfu/ml,* and identified *Escherichia coll, Klebsiella pneumoniae, Staphylococcus aureus and Pseudomonas aeruginosa,* with *Escherichia coli* havmg the highest occurrence level (42%) and *Klebsiella pneumonia* being the lowest (8%). The total fungal count for the home made soymilk was in the range 4.1 x 10^6 - 3.0 x 10^5cfu/ml, while the industry sample was in the range of 2 x 10^2 -1.10^2cfu/ml. The Fungi, *Aspergillus niger* and *Penicillium notatum* were detected from the soymilk samples with *Aspergillus niger* (66.7%) occurring

more than *Penicillium notatum* (33.3%). Antibiotic sensitivity screening results showed that the only gram positive isolate *Staphylococcus aureus* was sensitive to all the antibiotics tested but was most sensitive (80%) to Ampiclox. Among the gram negative isolates, *Escherichia coli* was sensitive to all the antibiotics tested, but was most sensitive to Augmentin (71%). *Klebsiella pneumonia was* also sensitive to all the antibiotics but most sensitive to *Ampicillin* (75%). *Pseudomanas aeruginosa was* completely sensitive (100%) Nalidixic acid and Septrin respectively and completely resistant (100%) to *Tarivid, Augmentin, Streptomycin, Ceporex* and Ampicillin respectively.

Mbajiuka *et al.* (2014) reported on the isolation and identification of microorganisms responsible for spoiling locally manufactured soymilk and shown that the bacterial count increased from $2x10^3$ cfu/ml to $2.9X10^4$ cfu/ml. *Escherichia coli, Bacillus* species, *Enterobacter* species, and three strains of fungi. In the soymilk, *Aspergillus* spp., *Rhizopus* spp., and *Penicillium* spp. were found.

Conclusion

A variety of bacteria, including *Escherichia coli, Staphylococcus aureus, Klebsiella* spp., *Salmonella* spp., and *Streptococcus faecalis*, were found in the soymilk sold in the Obio/Akpor neighbourhood of Port Harcourt. The faecal origin of the bacteria found in the soymilk can be attributed to the producers' poor personal hygiene, as well as the unhygienic conditions of the processing machinery and raw materials.

Recommendations

The National Agency for Food and Drug Administration Control (NAFDAC) in Nigeria needs to monitor the production process of locally made soymilk as well as organize awareness programs to inform the local producers of the microbiological challenges associated with local production of soymilk. The milk should be properly processed with clean treated municipal water in order to avoid contamination with enteropathogenic bacteria. Also, a health education training should be organized for the local people on the importance of cleanliness of their environment and the use of sterilized packaging materials for processing and packaging of these products.

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