ANTIBIOTIC SUSCEPTIBILITY SCREENING OF PATHOGENIC BACTERIA SPECIES ISOLATED FROM LOCALLY PRODUCED SOYBEAN *(Glycine max)* MILK HAWKED IN PORT HARCOURT, RIVERS STATE.

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 needs to be critically monitored to avoid transmission of infection to consumers. It is also very necessary that local producers be enlightened about good manufacturing practices.

Keywords: antibiotic susceptibility, pathogenic bacteria, soybean milk

INTRODUCTION

Soymilk (also called soybean milk, soy juice and sometimes referred to as soy drink/beverage) is a beverage made from soybeans. Soymilk is a stable emulsion, which is considered as Staple *Asian Cuisine.* Overnight Soaked Soybeans are crushed with water and filtered to get the soymilk. Similar to milk, soymilk or plain soybean beverage is the pale liquid, which is rich in protein and other nutrients with or without adding optional additives or ingredients. One advantage of soy milk over cow/buffalo milk is, this milk does not contain Lactose or Cholesterol and the fibers present can be removed from the final products (FAO, 2018). Soymilk maybe used as a substitute for dairy milk by individuals who are vegan or lactose intolerant, while others may consume it for environmental or health reasons (Guibourg & Briggs, 2019). Soymilk is a traditional oriental food beverage that is becoming well accepted in the United States of America and other parts of world (Jimoh & Kolapo, 2007). The growing acceptance of soymilk as a beverage worldwide is due its health benefits

such as low cholesterol and lactose, ability to reduce bone loss and menopausal symptoms, prevention and reduction of heart diseases and certain cancers, (Iwe 2003; Kolapo & Oladirneji, 2008). The Soymilk can be produced traditionally by soaking soybeans in clean water before grinding and straining. The milk that is obtained appears like a white or creamy emulsion resembling cow milk (conventional milk) in both texture and consistency (Iwe 2003; Kolapo & Oladimeji 2008). It is a watery extract derived from soybean seed known to be rich in water soluble proteins, carbohydrates and oil (Adebayo *et* a/., 2008). The soy flavor can be improved by lactic acid fermentation, as is the case with most yoghurt products, (Jimoh & Kolapo, 2007).

Soybeans and products derived from it have served as an important source of protein in the diet of millions of oriental people for nearly 5000 years. It is a potential food material that contains all essential amino acids that are very important for the proper development of the body, consisting of high content of lysine compared to other plant proteins. The protein of soybean contains all the essential amino acids in adequate amounts except methionine and cystine. It is one of the best vegetarian food items as far as protein content is concerned. It is a good source of riboflavin. Soya bean contributes approximately 20% fat to diet (Noroz *et al*, 2011). The fat from the soya bean is the unsaturated type unlike saturated fats from animal origin, hence its good for heart disease patients (Anderson & Bush, 2011). It also contains a good amount of calories and fat; 19.5gms of fat, 21gms carbohydrate and provides 432 kcal per 100gms (Ayo, 2011). Soya bean when well processed results in Soya beans milk which can be converted to yoghurt that serves as a veritable protein supplement or substitute for adults and infant feeding. Soya milk is lactose-free and can be consumed by the lactose-intolerant people as a substitute to milk. Anderson & Bush, (2011) reported that Soybeans contains a factor that can inhibit the activity of trypsin, a digestive enzyme which is destroyed by heat.

The greatest problem associated with soymilk remains its beamy flavor and very short shelf life due to microbial activity. The nutritious nature of soymilk however, makes it prone to microbial attack if not properly processed and stored as the nutrients it contains are also required for the growth of most spoilage organism s. a large number of microorganisms such as mesophilic aerobic bacterial, coliforms and fungi are known to be responsible for the spoilage of soymilk and producing undesirable changes in milk, (Momoh *et al,* 2011). Bacteria pathogens identified with food poisoning, gastroenteritis and enteric fever can be harbored in un-hygienically prepared soymilk (Adebayo *et* a/,2008). Mycetomas in

humanshave been caused by some potential pathogenic: contaminants of dairy food (Cheesbrough, 2005). Foodborne microbial pathogens cause diarrheal diseases, which is a leading cause of illness and death in less developed countries, killing an estimated 1.9 million people annually at the global level (Nawal *et al,* 2013). *Aspergillus flavits* is involved in allergic or pulmonary Aspergillosis and also produces aflatoxinthat is highly carcinogenic (Prescott *et al.,* 2005).

Antibiotics were considered to be the most effective therapeutic agents to combat microbial infections but due to the overuse of antibiotics, emergence and spread of multidrug resistant strains among different groups of microorganisms have taken place. Infections resistant bacteria are an emerging threat all over the world both as hospital acquired as well as community acquired microorganisms. Antimicrobial resistance is one of the main problems in clinical as well as in public health. The antimicrobial resistance is not only increasing morbidity and mortality but also great economic loss encompassing use of more expensive antibiotics to treat infection as well as threat to resistance to them (Subedi *et al.*, 2016). Hence, there is a need to conduct area specific infections and their resistance patterns, so as to generate data that would help clinicians to choose the correct therapy. A rise in bacteria resistance to antibiotics complicates treatment of infections (Gautam *et al.*, 2013). WHO warned the community that multi-drug resistant bacteria are emerging worldwide, which is a big challenge to healthcare and if immediate action is not taken, then antibiotics may lose their power to cure diseases. The main aim of this research work was to assess the microbiological quality and antibiotic susceptibility of pathogenic bacteria species isolated from locally prepared soymilk hawked in some parts of Port Harcourt. The finding of the study will reveal the bacteria contaminates of the soymilk and create awareness on how to prevent the microbial deterioration of soymilk through proper handling during production and storage.

MATERIALS AND METHODS

2.1 The Study Area

The study area falls within Rumuolumeni, Choba and Rumuokoro, all in Obio/Akpor Local Government Area of Rivers State. 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.

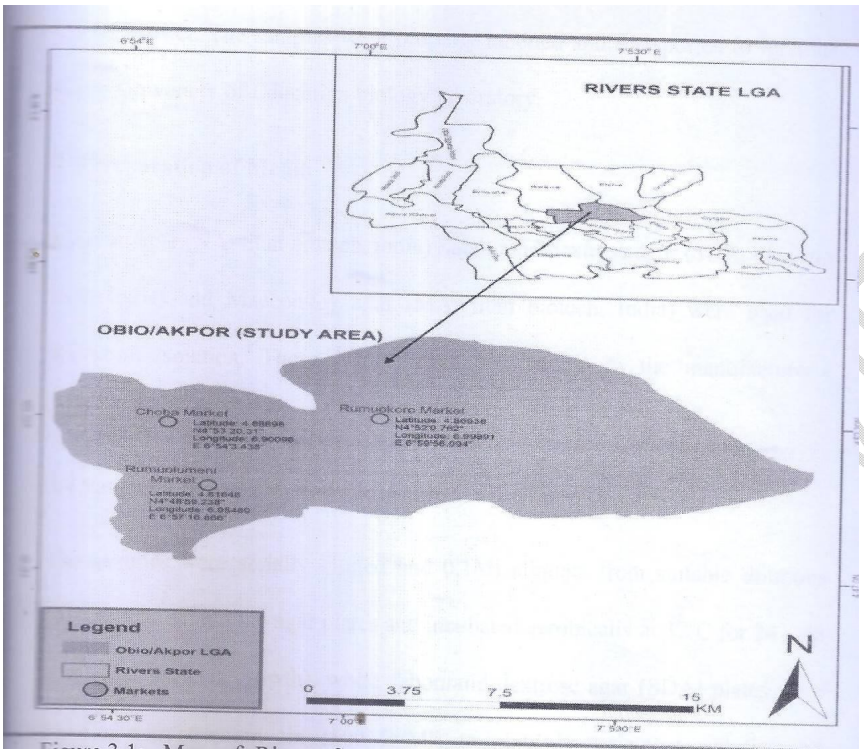

Fig.1: Map of Rivers State showing sampling station in Obio/Akpor Local Government Area.

2.2 Sample Collection

Three soymilk samples which were already packaged in plastic bottles were purchased from hawkers at Rumuolumeni market, Rumuokoro market and Choba market. The samples were properly labeled and transported to Ignatius Ajuru University of Education Biology Laboratory analysis.

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.

2.4Preparation 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

After incubation of all the plates, the petri dishes containing the overnight culture that was obtained from serial dilution was placed on a colony counter and the readings were taken. The number of colonies counted on the plates was recorded taking into consideration the dilution factor and used to calculate colony forming units(cfu/ml)..

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

2.6 Purification

All individual colonies on each medium were counted and sub-cultured to nutrient agar plate and incubated for 24 hours at 37°C and subsequently streaked in agar slant medium as stock for biochemical tests. This was done to obtain pure bacterial and fungal cultures,

2.7 Identification of Fungal species

Fungal isolates were characterized and identified according to their cultural morphology and microscopy such as color, structure, and consistency and growth pattern of mycelia. A wet mount method (Robinson *et al.,* 2020; 2021) was done before viewing the isolates under the X40 objective of the microscope. The morphological structures include septate, non-septate, presence of sporangiospores, fruiting bodies and special organisms like rhizoids.

2.8 Identification of Bacteria species

Each pure culture of bacteria was examined microscopically for colony morphology, microscopically and then gram stained to determine the gram status. Characterisation of the bacterial isolates was done through some standard biochemical test.

2.9 Biochemical Test

Gram stain was carried out to identify bacteria isolated from the microscope. 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 was rinsed under slow running tap water and then flooded with lugols iodine solution for one minute. The slide was rinsed under slow running tap water and then decolourised drop wise with acetone for two minutes. After which 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:

The catalase test was done using a glass slide with a wire loop, cells were transferred from the center of a well isolated colony, 1-2 drops of 3% hydrogen peroxide was added to the slide. A positive result is indicated by the rapid appearance of sustained gas bubbles. No bubble production shows negative results.

Indole test

Using Kovac's reagent, Tryton water was added to the test organism in a test tube and incubated at 37°C for 48 hours. 0.5M1 of Kovac's reagent was added and shaken properly, then examined after one minute. A red color ring in the rube indicated the presence of indole (positive reaction). No red color indicated negative results.

Oxidase test

This test is used to assist in the identification of pseudomonas, vibrio and some other species which produce the enzyme cytochrome oxidase. This test was performed by placing a piece of filter paper in a clean petri dish and 2 drops of freshly prepared oxidase reagent was added. An inoculating loop was used to remove colonies of the test organism and smear on the filter paper. The development of a blue purple color within 10 seconds signified a positive result. No blue purple color signified a 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 fromglucose 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

The test was carried out using sucrose sugar. The test identifies organisms capable of utilizing the sugar concerned. The medium was prepared using 1 % sugar, peptone and two drops of indicator solution. After mixing thoroughly it was dispensed into test tubes and Durham tubes were dropped into the test tubes in an inverted position. The test tubes were cooked and sterilized in an autoclave at 121°C for 156 minutes. After cooling, the test organisms were inoculated into different tubes and ions inthe Durham tube and color change from yellow to pink indicated a positive gas and acid production respectively.

Mortality Test

Thin layer of Vaseline was supplied around the edge of the well in a concave slide. A loopful ofthebacteria suspension was put in the centre of the cover slip. The concave slide was inverted and pressed on top of the cover slip so that the cover slip completely covered the well. The preparation was quickly turned right side up again. The edges of the coverslip were checked to ensure that the Vaseline sealed the edges properly. Bacterial suspension on the underside of the cover slip was observed under the microscope for bacterial cells in motion.

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 isolates were obtained from agar slants and sub-culture plates. After growth within 24hrs, isolates from each of the plates 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 to obtain confluent growth. 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 x10^3	3.1 x10^3	3.2 x10^3
Rumuokoro	4.2 x10^3	4.0 x10^3	4.4 x 10^3
Choba	3.8 x10^3	3.4 x10^3	3.5 x 10^3

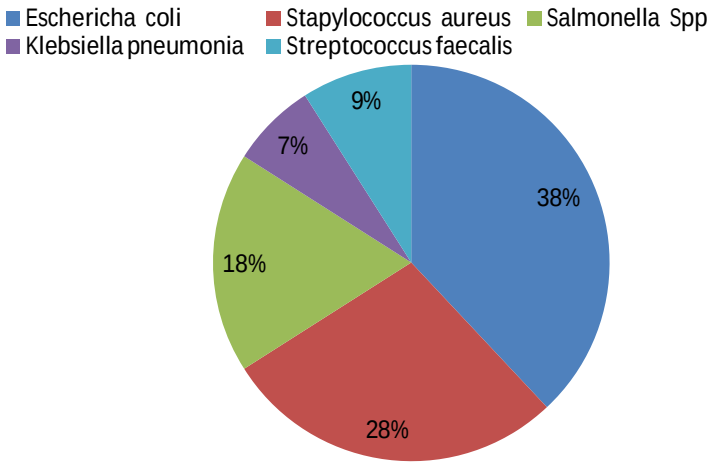

Fig.2: Percentage occurrence of bacteria isolates in soymilk sample.

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	CN	S	AU

		(10ug) n%	(30ug) n%	(10ng) n%	(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/mland 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 10^8cfu/mlandlowest 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/mlfor Wokurum, 3.0x 10^7- 4.7x 10^7cfu/mlfor high level and 6.0x 10^7- 5x 10^7 for Wadata respectively. All samples were contaminated with members of the *Enterobacteriaceae,* including *Escherichia coli, klebsiella spp, Salmonella typhi, Streptococcus faecalis and Staphylococcus aureas.*

Accordingly, Agwa & Ossai - Chidi (2016) investigated the microbial quality of locally and industrially soybean products sold within Port Harcourt. They observed significantly higher bacteria and fungi counts ($p<0.05$) in local samples than the industrial samples. bacteria isolated from industrial samples include: *Pseudomonas sp.(S7.5%), Bacillus sp, Staphylococcus sp. (25% each)* and *Streptococcus sp,* (12%), while *Pseudomonas sp. and Staphylococcus sp,* were isolated from locally processed samples (25%). the fungi isolated include: *Rhizopus sp., Aspergillus sp. Saccharomyces sp. '33.3%).* The most frequent fungus in locally produced samples is *Rhizopus (40%)* 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* showedhighest 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* showedhighest 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,* whilethat 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* havmgthe 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 the isolation and identification of microorganisms involved in the spoilage of locally-produced soymilk and showed that bacterial count increased from *2x!0*^{3}cfu/mlto *2.9X10*^{4}cfu/ml. *Bacillus spp, Enterobacter spp, and Escherichia coli,* and three fungal isolates. *Aspergillus spp, Rhizopus spp,* and *Penicillium spp* were detected in the soymilk

Conclusion

Soymilk on sale in Obio/Akpor area of Port Harcourt was contaminated by various microorganisms such as *Escherichia coli, Staphylococcus aureus, Klebsiella spp, Salmonella spp, Streptococcus faecalis.* The microorganisms identified in the soymilk were of fecal origin which may be traced to poor hygiene of producers, unsanitary conditions of processing equipment 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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