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Bacteriological quality of unbranded soymilk sold in major markets in Awka, Anambra State, Nigeria

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Abstract The increased consumption of soymilk due to its low cost has led to its small-scale production under household conditions with little or no regards to quality control measures. Soymilk, though a nutritive beverage serves as a good substrate for the proliferation of microorganisms; therefore, the consumption of locally-produced soymilk, packaged in unsterile plastic containers is considered a public health risk. Samples of unbranded soymilk sold in five major markets in Awka, Nigeria was subjected to bacteriological analysis using standard methods. The heterotrophic bacterial counts ranged from 4.7 to $8.0X10^5$cfu/ml, total coliforms, $2.4 – 4.5 \times 10^5$cfu/ml and faecal coliforms, $1.0 – 1.6 \times 10^5$ cfu/ml. The bacteria were characterized and identified as *Citrobacter spp* (28.2%), *Enterobacter spp* (12.3%), *Escherichia spp* (5.0%), *Klebsiella spp* (15.7%), *Micrococcus spp* (6.8%), *Staphylococcus spp* (23.8%), *Streptococcus spp* (6.2%) and *Vibrio spp* (2.0%). However, all the bacteria were sensitive to augmentin. The detection of these bacteria in high numbers especially the coliforms reflects poor hygienic standards of production and handling. Good manufacturing practices must therefore be observed by the producers of the beverage. In addition, frequent bacteriological analysis of the product must be conducted to ensure that the end users are served with product of good quality.

Keywords Unbranded Soymilk, Bacteria, Public Health, Good Quality, Beverage, Coliforms, Antibiotics

1. Introduction

The scarcity of cow milk in developing countries has led to the development of alternative milk from vegetable source [1]. Soy bean (Glycine max) is recognized as one of the leguminous crops with huge potential the world over to produce one of the promising products, Soymilk [2].

Soybean is an excellent source of protein both in quality and quantity and contributes approximately 20% fat to the diet [3]. The fat from soybean is the unsaturated type unlike the saturated fat from animal origin, hence it is good for heart disease patients [4]. The protein of soybean contains all the essential amino acids in adequate amount except methionine and cysteine. It is also one of the best vegetarian foods and a good source of riboflavin [4].

Soymilk is a traditional oriental food beverage that is growing in popularity in the world [5]. It is watery extract of whole soya bean, rich in water-soluble protein and carbohydrate and oil [6]. It is also a white creamy emulsion which resembles cow milk in appearance and consistency [7]. In Nigeria, soymilk is known to be one of the major healthy alternatives to cow milk when hygienically prepared and is affordable and economical when compared to industrially-processed cow milk.

Soymilk can be contaminated by coliforms which usually indicate contaminated equipment, improper pasteurization of finished products, poor quality of ingredients used during the production and infected soybean varieties [8]. Other organisms implicated with soymilk contamination include *Enterobacter spp, Escherichia coli, Klebsiella spp, Salmonella spp, Alcaligenes spp, Proteus spp, Acinetobacter spp, Pseudomonas spp, Staphylococcus aureus, Bacillus spp, Streptococcus spp, Aspergillus spp, Rhizopus spp* and *Fusarium spp.*

Soymilk is consumed as a refreshing drink and for its health benefits especially its role in the prevention of health-related disorders. In Nigeria, unbranded soymilk is produced and sold under unhygienic environment thereby exposing the product to high levels of contamination by both the pathogenic and spoilage bacteria. This study was therefore aimed at isolating and identifying the bacteria in the unbranded soymilk sold in major markets in Awka, Anambra State Nigeria and their antibiotics susceptibility patterns ,with a view to determining their suitability for human consumption.

2. **Materials and Methods**

Glassware sterilization

All glassware were wrapped in aluminium foil and sterilized in the hot air oven at 160 ºC for one hour.

Media preparation

The media used namely Nutrient agar (NA), MacConkey's agar (MCA), Eosin methylene blue agar (EMB), Simmon's Citrate agar (SCA) and Urea agar (UA) were prepared and sterilized by autoclaving at 121^0C for fifteen minutes.

Samples collection

Samples of unbranded soymilk were bought from Eke-Awka, Amenyi, Ifite, Aroma and Permanent Site markets all in Awka, Anambra State, Nigeria. All the samples which were packaged in plastic containers by the vendors were conveyed in ice-packed containers to the Microbiology Laboratory of Nnamdi Azikiwe University Awka for analysis. Five samples were collected from each of the markets.

Unbranded soymilk which served as the control was prepared in the Microbiology Laboratory by soaking soybean seeds in water before grinding and straining as performed by Odu and Egbo [9].

Isolation of bacteria

Total heterotrophic bacteria

The pour plate method described by Cheesbrough [10] was adopted. The sample was serially-diluted and 1ml of the dilution (10^5) was introduced into a sterile Petri dish. Sterile nutrient agar was added and the dish was rotated gently for easy mixing of the sample and the media. The dish also contained ketoconazole at a concentration of 0.05mg/l to inhibit fungal growth. The experiment was carried out in duplicates. Incubation was in an inverted position at 37^0C for 24 hours after which the colonies that developed were counted.

Total coliforms

Total coliforms were detected from the samples using MacConkey's agar as described by Cheesbrough [10]. The medium was inoculated with 1ml of the serially-diluted sample (10^5) in a Petri dish using the pour plate method. Incubation was carried out in an inverted position at 37^0C for 48 hours after which the coliforms that developed were counted.

Faecal coliforms

This was carried out using the pour plate method described by Cheesbrough [10]. 1 ml of the serially-diluted sample (10^5) was inoculated into Eosin methylene blue agar. The plate was inverted and incubated at 37^0C for 48 hours. Colonies of faecal coliforms were thereafter counted.

Purification of the isolates

Discrete colonies were aseptically transferred to sterile nutrient agar plates using sterile wire loops and incubated at 37^0C for 24 hours, after which the colonies were stored in sterile nutrient agar slants for characterization and identification.

Characterization and identification of the isolates

The isolates were examined morphologically and biochemically as described by Cheesbrough [10]. The test carried were Gram staining, catalase test, oxidase test, citrate test, coagulase test, indole test, methyl red test, Voges proskaeur test, motility test, urease test and sugar (glucose, maltose, lactose, fructose and sucrose) fermentation test. The bacteria were identified according to the scheme of Holt et al. [11].

Antibiotics susceptibility test of the bacterial isolates

The antibiotics susceptibility test of the bacterial isolates was carried out as described by Jorgensen and Turnidge [12]. A colony of the test isolate was emulsified in a sterile saline solution and mixed thoroughly to ensure that no solid material from such colony was visible. 0.5ml of the suspension was used to streak a nutrient agar plate which was thereafter allowed to dry for five minutes. Discs of amoxicillin (10µg), amikacin (30µg), ciprofloxacin (1µg), tetracycline (10µg) and augmentin (10µg) were placed on the surface of the agar with the aid of sterile forceps. The inoculated plates were incubated in an inverted position at 37^0C for 24 hours after which the diameter of the zone of inhibition for each antibiotic used was determined in millimetre with a metric ruler. The values obtained were compared to the standard table to determine the susceptibility or resistance of the bacteria to the test antibiotics.

3. Results

The average bacteriological counts of the unbranded soymilk samples are presented in Table 1. The total heterotrophic bacterial counts were 4.7 – 8.0 x 10^5cfu/ml, total coliforms, 2.4 – 4.5 x 10^5cfu/ml while the faecal coliform counts ranged from 1.0 to 1.6x10^5cfu/ml. The control sample had no growth after incubation.

Table 1: Average bacteriological counts of the unbranded soymilk samples

Major Markets	THBC (X10^5cfu/ml)	TCC (X10^5cfu/ml)	FCC (X10^5cfu/ml)
Eke-Awka	8.0	4.5	1.2
Amaenyi	6.0	2.9	1.0
Ifite	5.9	3.5	1.6
Aroma	5.2	3.0	1.5
Permanent Site	4.7	2.4	1.1
Control	0.0	0.0	0.0

THBC = Total heterotrophic bacterial count
TC = Total coliform counts
TCC = Faecal coliform counts

The morphological and biochemical characteristics of the bacterial isolates from the unbranded soymilk samples are shown in Table 2. The isolates were identified as *Citrobacter spp, Enterobacter spp, Escherichia spp, Klebsiella spp, Micrococcus spp, Staphylococcus spp, Streptococcus spp and Vibrio spp.*

Table 2: Morphological and biochemical characteristics of the bacterial isolates from the unbranded soymilk samples

Characteristics	*Citrobacter spp*	*Enterobacter spp*	*Escherichia spp*	*Klebsiclla spp*	*Micrococcus spp*	*Staphylococcus spp*	*Streptococcus spp*	*Vibrio spp*
Morphology	Rod	Rod	Rod	Rod	Coccus	Coccus	Coccus	Rod
Gram reaction	-	-	-	-	+	+	+	-
Catalase	+	-	-	+	+	+	-	-
Citrate	+	-	+	+	-	+	+	+

Oxidase	-	-	-	-	+	-	-	+
Methyl red	+	+	+	-	+	+	+	-
Indole	-	-	+	-	-	-	+	+
Voges proskaeur	-	+	-	+	+	-	-	+
Urease	-	+	-	+	+	+	-	-
Coagulase	-	-	-	-	-	+	-	-
Motility	+	+	+	-	-	-	-	+
Fructose fermentation	+	+	+	+	+	+	+	+
Lactose fermentation	-	+	+	+	-	+	+	+
Sucrose fermentation	+	+	+	+	+	+	+	+
Glucose fermentation	+	+	+	+	+	+	+	+
Maltose fermentation	+	+	+	+	+	+	+	+

\+ = positive reaction

\- = negative reaction

Table 3 showed the distribution of the bacterial isolates in the unbranded soymilk samples. The highest number of bacteria was detected from the samples from Eke-Awka market while the samples from the permanent site market had the lowest number of bacteria.

Table 3: Distribution of the bacterial isolates in the unbranded soymilk samples

Bacterial isolates	**Samples**				
	A	B	C	D	E
Citrobacter spp	+	+	+	+	+
Enterobacter spp	+	+	+	-	-
Escherichia spp	+	+	+	+	+
Klebsiella spp	+	+	+	+	-
Micrococcus spp	+	+	-	+	-
Staphylococcus spp	+	+	+	-	+
Streptococcus spp	-	-	+	+	+
Vibrio spp	+	-	-	-	-

\+ = detected

– = not detected

The frequency of occurrence of the bacterial isolates in the unbranded soymilk samples is presented in Table 4. *Citrobacter* spp had the highest frequency of occurrence (28.2%) while *Vibrio spp* had the lowest frequency of occurrence of 2.0% in the samples.

Table 4: Frequency of occurrence of the bacterial isolates in the unbranded soymilk samples

Bacterial isolates	**Frequency of occurrence (%)**
Citrobacter spp	28.2
Enterobacter spp	12.3
Escherichia spp	5.0
Klebsiella spp	15.7
Micrococcus spp	6.8
Staphylococcus spp	23.8
Streptococcus spp	6.2
Vibrio spp	2.0
Total	**100.0**

Table 5 showed the antibiotics susceptibility test of the bacterial isolates. All the isolates were resistant to Amoxicillin, Amikacin and Tetracycline while they were sensitive to augmentin only

Table 5: Antibiotics susceptibility test of the bacterial isolates

Antibiotics	Standard values	Zone of inhibition of *Citrobacter spp*	Zone of inhibition of *Enterobacter spp*	Zone of inhibition of *Escherichia spp*	Zone of inhibition of *Klebsiella spp*	Zone of inhibition of *Micrococcus spp*	Zone of inhibition of *Staphyloccocus spp*	Zone of inhibition of *Streptococcus spp*	Zone of inhibition of *Vibrio spp*
Amoxicillin	14	R(5.3)	R(4.2)	R(5.8)	R(5.2)	R(3.6)	R(6.1)	R(6.3)	R(4.9)
Amikacin	15	R(6.5)	R(7.3)	R(6.1)	R(5.4)	R(5.7)	R(5.3)	R(8.0)	R(6.3)
Ciprofloxacin	14	S(16.6)	R(6.9)	S(17.5)	R(4.1)	R(5.8)	R(5.0)	R(3.8)	S(17.1)
Tetracycline	16	R(7.0)	R(6.4)	R(9.0)	R(9.0)	R(7.6)	R(4.7)	R(6.3)	R(8.2)
Augmentin	19	S(19.3)	S(19.1)	S(19.3)	S(19.3)	S(19.6)	S(19.0)	S(19.5)	S(19.4)

Resistance (R) = $\leq$ the standard values

Sensitive (S) = $\geq$ the standard values

4. Discussion

Soymilk is an important locally-produced beverage in parts of Africa including Nigeria. The high nutritive value of the product such as its high protein, carbohydrate, low cholesterol and fat contents which is good for diabetic patients and its low cost has made it an alternative source of protein for human consumption. It is however a good medium for the growth of microorganisms which may lead to infections if consumed.

The average bacteriological counts of the samples examined (Table 1) exceeded the acceptable limit of $<10^4$cfu/ml for both milk products and non-alcoholic beverages [13] indicating that the product was of poor quality and therefore unacceptable for human consumption. The result agreed with Ezeigbo et al. [14] that analysed soymilk samples from major markets and commercial spots in Aba, South Eastern Nigeria and reported total heterotrophic bacterial counts ranging from 0.4 to 2.0 x 10^6cfu/ml and total coliform counts from 0.2 to 1.9 x 10^6cfu/ml.

Adeleke et al. [13] assessed the microbial quality of branded and unbranded soymilk samples to ascertain their hygienic standard of production. Screening for microbial contaminants revealed high bacterial counts of 2.9 x 10^7 to 1.0x 10^8cfu/ml as well as high most probable number (>180) of coliform bacilli per 100ml of each sample. Mbajiuka et al. [15] studied the microbiological quality of locally-produced soymilk stored under ambient and refrigeration conditions and reported a bacterial count of 2.0 x10^3cfu/ml to 2.9 x 10^4 after six days of storage at ambient temperature of 27^0C while the bacterial count of the refrigerated sample ranged from 0.0 to 1.5 x 10^4cfu/ml.

Ozoh and Umeaku [16] studied the public health implication of ready-to-drink soymilk and soymilk yoghurt sold in Onitsha Urban, Anambra State, Nigeria and reported total coliforms and faecal coliform (E. coli) in the range of 1.1 x 10^3 – 8.0 x 10^3cfu/ml and 0.9 x 10^3-7.7 x 10^3cfu/ml respectively in the samples. Liamngee et al. [17] carried out the microbial analysis of soybean milk sold by women and children in Makurdi metropolis. The microbial load ranged from 6.9 X 10^7 – 7.6 X 10^7cfu/ml; 4.1 X 10^7 – 5.6 X 10^7cfu/ml; 3.0 X 10^7 – 4.7 X 10^7cfu/ml and 6.0 X 10^7 – 8.5 X 10^7cfu/ml for the samples from North Bank, Wurukum, High level and Wadata area respectively.

Asuquo and Antai [18] carried out the microbial and biochemical analysis of the soymilk produced and sold in Bogobiri, Watt, Akim barracks, Army barracks and Marian market, all in Calabar metropolis and observed that the mean bacterial counts were highest in samples from Army barracks (6.90$\pm$ 0.01x10^8cfu/ml) and lowest in Marian market (4.80$\pm$0.04x10^8cfu/ml) while Umeoduagu et al. [19] carried out the microbiology assessment of soymilk sold in Onitsha metropolis, Nigeria and reported that the total viable bacteria ranged from 6.1 x 10^6 to 9.0x10^6cfu/ml.

Citrobacter spp, Enterobacter spp, Escherichia spp, Klebsiella spp, Micrococcus spp, Staphylococcus spp, Streptococcus spp and *Vibrio spp* were detected from the samples examined (Table 2). Asuquo and Antai [18] isolated Pseudomonas, Bacillus and *Klebsiella* from the soymilk samples they examined in Calabar metropolis while Thombare et al. [20] isolated *Escherichia spp* and *Streptococcus spp* from the soymilk produced from soybean. Liamngee et al. [17] also detected *E. coli, Klebsiella spp, Salmonella typhi, Streptococcus faecalis* and *Staphylococcus* aureus from the soymilk they analysed in Makurdi metropolis, Nigeria.

Ozoh and Umeaku [16] detected *E. coli*; *Staphylococcus sp, Streptococcus sp, Klebsiella sp, Bacillus sp, Salmonella sp* and *Pseudomonas sp* from soymilk and soymilk yoghurt sold in Onitsha Urban, Anambra State Nigeria while Akinola et al. [21] studied the microbial quality of naturally-fermenting soymilk and detected *Lactobacillus fermentum, Lactobacillus acidophilus, Lactobacillus farciminis and Lactobacillus allimentarius.* Agwa and Ossai-Chidi [22] isolated *Pseudomonas, Bacillus, Staphylococcus* and *Streptococcus* from the soybean products sold within markets in Port-Harcourt metropolis, Rivers State, Nigeria.

Mbajiuka et al. [15] isolated *Bacillus spp, Enterobacter spp* and *Escherichia coli* from the soymilk stored at ambient and refrigeration conditions while Agboke et al. [23] reported that the soybean milk products consumed in Nigeria which they analysed contained pathogenic microorganisms such as *Staphylococcus aureus* and *E. coli. Adeleke et al*. [13] reported regular contamination of branded and unbranded soymilk with bacteria such as E. coli, Staphylococcus *aureus*, *Pseudomonas aeruginosa* and *Streptococci* while Ma *et al.* [24] reported the presence of *Rummeliibacillus, Acinetobacter, Enterobacter, Phyllanthus, Bergia, Zhihengliuella* and *Nesterenkonia* in commercial instant soya milk.

Ezigbo *et al*. [14] isolated *Bacillus spp, Staphylococcus spp, Lactobacillus sp, Enterobacter spp, Pseudomonas spp* and *E. coli* from the soy milk samples collected from major markets and commercial spots in Aba, Nigeria while Umeoduagu *et al*. [19] isolated *Staphylococcus aureus, Bacillus spp, E.coli, Klebsiella spp, Salmonella spp, Pseudomonas spp and Vibrio spp* from the soy milk samples sold in Onitsha metropolis. None of the samples examined was free from bacteria, with more bacteria isolated from Eke-Awka market samples (Table 3). The coliform bacterium Citrobacter had the highest frequency of occurrence of 28.2% in the samples while the gram-negative bacillus *Vibrio* exhibited the lowest frequency of occurrence of 2.0% (Table 4).

Ezeigbo *et al*. [14] however reported that *Staphylococcus spp* had the highest percentage of occurrence of 80.0% while E. coli had the least percentage of occurrence of 20.0% in the soymilk samples they examined in Aba, Nigeria while Agwa and Ossai-Chidi [22] reported that *Pseudomonas spp* (25%) and *Staphylococcus spp* (25%) were the most frequently isolated bacteria from the locally-processed soybean products examined in Port-Harcourt metropolis, Nigeria. The variation in the frequency of occurrence may be attributed to the media used, nature of the soybean used for the preparation of the soymilk, the production and handling processes, sanitary condition of the production environment, water used for the production, the packaging container and the prevailing environmental conditions.

All the bacterial isolates were resistant to amoxicillin, amikacin and tetracycline and sensitive to augmentin (Table 5). However, Agwa and Ossai-Chidi [22] reported that 70% of the bacterial isolates from the soybean products sold within markets in Port-Harcourt, Rivers State, Nigeria were sensitive to erythromycin and augmentin while 50% were resistant to ampicillin and ofloxacin. *Citrobacter spp* are found in water, wastewater and human intestine etc. They are reported to be the source of infections of the urinary tract, infant meningitis and sepsis [25].

Several strains of *Enterobacter* are pathogenic and cause opportunistic infections in immune compromised hosts and those on mechanical ventilation. The urinary and respiratory tracts are the most common sites of infection [26]. Many *Escherichia spp* are commensal members of the gut microbiota though certain strains of some species notably the serotypes of E. coli are human pathogens and cause urinary tract infections, diarrhea and dysentery-like conditions [27]. *Klebsiella* spp frequently cause human nosocomial infections such as hospital-acquired urinary tract infections, pneumonia, septicemias and soft tissue infections [28].

Micrococcus spp have been associated with various infections including bacteremia, continuous ambulatory peritoneal dialysis peritonitis and infections associated with ventricular shunts and central venous catheters, intracanial abscesses, pneumonia, septic arthritis, endocarditis, meningitis and coronary infections. [29]. *Staphylococcus* can cause a variety of diseases in humans through the production of toxin or penetration. The toxins are produced by the bacteria while growing in improperly-stored food items and are a common cause of food poisoning, boils, impetigo, cellulitis and toxic shock syndrome [30].

Certain *Streptococcus* species are responsible for many cases of pink eye, meningitis, bacterial pneumonia, endocarditis, erysipelas, necrotizing fasciitis and Streptococcal pharyngitis. [31]. *Vibrio spp* can cause cholera, vibriosis, gastroenteritis, wound infections and septicaemia in humans. [32]. The contamination of the soymilk

samples by these bacteria may be as a result of poor handling, use of contaminated equipment, raw materials and water, unhygienic production and processing environments and lack of quality control measures during production.

5. Conclusions

This study showed that the soymilk samples analysed were contaminated by bacteria and are therefore unfit for human consumption. The presence of coliform bacteria is an index of the hygienic standard of soymilk and its keeping quality. The use of potable water, sterile containers, wholesome raw materials and production in a hygienic environment must not be compromised.

6. Recommendations

Regulatory agencies such as the National Agency for Food and Drug Administration and Control, Standard Organization of Nigeria, World Health Organization and Food and Agricultural Organization should formulate and enforce policies concerning the production, distribution and handling of soymilk.

The local producers of soymilk should be educated on the negative health effects of the contamination of the product by these bacteria. In addition, soymilk should be stored and sold in refrigerated conditions.

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