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RESEARCH PAPER

PREVALENCE OF MULTI-DRUG RESISTANT BACTERIA ASSOCIATED WITH VARIOUS YOGHURT PRODUCTS COMMONLY SOLD IN MINNA, NORTH CENTRAL, NIGERIA

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ABSTRACT

The predominance of Multidrug resistant (MDR) bacteria among dairy products especially yoghurt is fast becoming a major concern in most communities. Five (5) industrially prepared yoghurts were aseptically collected and transported to the Microbiology Laboratory of Federal University of Technology, Minna. Samples were serially diluted and inoculated on various media through the pour plate method. The bacterial isolates were identified through their Gram reaction and other biochemical tests. The antibiotic susceptibility tests were carried out using the disc diffusion method on Muller Hinton agar. The result revealed that, sample C (1.7x103) had the highest microbial count while Sample D (8x102) had the lowest microbial count respectively. Various bacterial pathogens were isolated and identified with Klebsiella sp, Staphylococcus sp and Escherichia coli having the highest frequency of occurrence (20%), followed by Pseudomonas sp and Lactobaacillus sp which had their frequency of occurrence to be 13.3% while the least frequency of occurrence (6.7%) was observed in Salmonella sp and Streptococcus sp. The antibiotic susceptibility tests revealed that all bacterial isolates were multidrug resistant and as such are a great threat to the general public especially the consumers of yoghurt drinks. Hence, there is a need for adequate and continuous surveillance by food regulatory bodies in Nigeria, to curtail the infections associated with Multidrug resistant bacteria.

Keywords: Yoghurt, Multi-drug Resistant Bacteria, Antibiotic Susceptibility Test

INTRODUCTION

Yoghurt is considered by most regulatory agencies around the world as a by-product of milk fermentation that supplies the body with lower lactose and well-defined strains of bacterial cells (Fisberg and Machado, 2015), typically *Pediococcus*, *Lactobacillus, Streptococcus* and *Leuconostoc*. These organisms often associated with the production of yoghurt are generally known as lactic acid bacteria (LAB) (Fisberg and Machado, 2015; Erginkaya *et al*., 2018). These organisms are Gram-positive, and their shapes are either bacilli (i.e. *Lactobacillus*) or cocci (i.e. *Streptococcus*) (Erginkaya *et al*., 2018; Kisanthini and Kavitha, 2021). LAB is a group of bacteria that produces lactic acid in their fermentation medium (which is said to be the milk). LAB fermentation of milk improves the characteristics of yoghurt such as consistency, appearance, digestibility, flavor and taste (Rani *et al*., 2012).

However apart from LAB, most pathogenic bacteria have over time been observed to be associated with the contamination of various yoghurt products and as such are considered as vectors for the horizontal or vertical transfer of antibiotics resistance genes (Erginkaya *et al*., 2018).

Emergence of antibiotic resistance (AR) is now a global threat; it diminishes the efficacy of antibiotic therapy, causing increased hospital stay, waste of time and resources and could lead to increased morbidity and mortality (Pérez-Rodríguez and Mercanoglu, 2019; Serra-Burriel *et al*., 2020). Yoghurts are important vehicle for movement of large number of bacteria into the body of humans. These bacteria may possess antibiotic resistance (AR) determinants, which could be transferred to other bacterial cells that constitute the human gut microbiota (Pérez-Rodríguez and Mercanoglu, 2019).

Similarly, when yoghurts containing bacteria multiple resistant genes are consumed, such resistant genes can easily be transferred to the gut microbiota, thereby causing adverse health effects, which could lead to treatment failure (Magiorakos *et al*., 2011). Various studies such as Zhou *et al*. (2012), Ayeni *et al*. (2018), Erginkaya *et al*. (2018), Shahriar *et al*. (2019) and Wang *et al*. (2019) have all reported the occurrence of *Lactobacillus* sp., and *Streptococcus* sp., to be abundant in various yoghurt samples with most of them showing resistance to two or more antibiotics tested, thus, raising great concern on the use of LAB as probiotics, since they can also serve as vehicle for transmitting resistance gene to other gut microbiota. Based on the intake of resistant bacteria during the consumption of various products of yoghurts, there is an immediate need to access and determine the prevalence of MDR- bacteria associated with various yoghurt products sold in Minna Niger state, Nigeria.

MATERIALS AND METHODS

Sample Collection

Five (5) different products of yoghurt were collected randomly from different vendors located in different streets in Minna between March and April, 2021. The samples were placed in an ice pack and transported to the Microbiology Laboratory in Federal University of Technology Minna, and they were immediately stored inside the refrigerator (at 4 °C).

Inoculation of Samples

Each yoghurt sample was analysed microbiologically to determine the presence of bacteria, using deMann Rogosa Sharpe (MRS) agar, Nutrient agar, MacConkey agar and *Salmonella Shigella* agar as explained by Erginkaya *et al*. (2018) and Wang *et al*. (2019) with little modifications. Five test

tubes containing 9 mL of distilled water were sterilized and autoclaved at 121 °C for 15 minutes. The test tubes were labeled from the first one (10-1) through to the 5th one (10-5). A 1 mL yoghurt sample was taken and aseptically transferred to the first test tube labeled 10-1; the cap was closed and the tube was vigorously shaken. From it, another 1 mL was taken and introduced to the second test tube labeled 10-2, the cap was closed and the tube was shaken vigorously before taking 1 mL from it and introducing to the third tube labeled 10-3, same procedure was carried out through to the 5th test tube. A 0.1 mL aliquot of each yoghurt sample was withdrawn aseptically from the second test tube and introduced into four (4) different Petri dishes (containing deMann Rogosa Sharpe (MRS) agar, Nutrient agar, MacConkey agar and *Salmonella Shigella* agar) using pour plate method. The Petri dishes were incubated at 37 °C for 24 hours.

Isolation of Bacteria

Colonies that are distinctly separated from one another on MRS agar and nutrient agar were aseptically sub-cultured on to newly prepared plates of MacConkey and *Salmonella-Shigella* agar (Sharma, 2014).

Bacterial Identification and Biochemical Characterization

The discrete colonies from these sub-cultured plates were identified by Gram staining techniques (Sherman, 2005; Presscott, 2002; Holt *et al*., 1994) and other types of biochemical tests namely: Indole test, Catalase test, Coagulase test, Urease test and Oxidase test.

Gram staining

A smear was made on a clean grease-free glass slide, the smear was heat-fixed and then flooded with crystal violet for 60seconds. The smear was then diluted with Gram's iodine for 60seconds. The smear was decolorized with 95% ethyl alcohol for 1 minute. After decolorization, the smear was counter stain with safranin for 60seconds. The slide was finally washed with distilled water and was allowed to air dry. A drop of oil immersion was placed on the slide and it was viewed at x100 (objective lens) using binocular microscope. The Gram-positive bacteria retained the colour of the dye, crystal violet (dark purple) whereas the Gram-negative retained the colour of the dye, safranin (reddish).

Indole test

This test is used to determine the ability of the organisms to breakdown amino acids such as tryptophan to indole. 5 mls of peptone water containing tryptone was dispensed in test tubes, autoclaved and inoculated with test organism. It was incubated at 37 °C overnight. Few drops of Kovac's reagent were added into the test tubes and the tubes were shaken. Observation of red colouration in the alcohol interphase depicts a positive result while a yellow colouration depicts a negative result (Cheesbrough, 2010).

Catalase test

A colony of the test bacteria was picked and emulsified with 3% hydrogen peroxide on a clean glass slide. Observation of bubble formation, indicates a positive (+ve) result while no bubble formation indicates a negative (-ve) result.

Coagulase test

The test is used to differentiate coagulase-positive *Staphylococcus aureus* from coagulase-negative *Staphylococcus* (CONS). The test bacteria were picked and emulsified with a drop of blood serum on the slide. Observation of coagulation indicates a positive (+ve) result whereas no coagulation indicates a negative (-ve) result.

Urease test

The test isolate was inoculated on urea agar base medium aseptically and incubated in the incubator at 37 °C for 24 hours. Observation for color change in the culture medium to pink, indicates a positive result of urea hydrolysis while the unchanged color of the culture medium indicates a negative (Cheesbrough, 2010).

Oxidase test

A piece of filter paper was placed in a sterile Petri dish and two drops of freshly prepared oxidase reagent (referred to as tetra-p-diaminechloride) was applied onto the piece of filter paper. A colony of the test organism was introduced onto the soaked filtered paper. Observation for blue- purple colour within few seconds was done and result recorded (Cheesbrough, 2010).

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was done using Kirby-Bauer method on Mueller Hinton Agar. The prepared Mueller Hinton Agar was autoclaved and poured into Petri dishes. On gelling, a sterile swap stick was used to spread the standardized inoculum from a test tube, onto a Petri dish. After the inoculum have been spread, the antibiotic susceptibility discs such as: Penicillin G (10 µg), Augmentin (30 µg), Streptomycin(10 µg), Erythromycin(10 µg), Pefloxacin(5 µg), Ciprofloxacin(5 µg), Sparfloxacin(5µg), Gentamicin (10µg), Septrin (Cotrimoxazole)(10 µg), Ofloxacin(5 µg), Chloramphenicol(10 µg), Rifampicin(10 µg), Ampicillin(5µg) and Amplicox(5µg) were introduced using sterile forceps and then the plates were incubated for 24 hours at 37 ^{0}C.

RESULTS AND DISCUSSION

Results

Out of all the yoghurt samples analysed, samples A and C had the highest microbial count (0.04**×10**^{2} cfu/mL) while samples D and E had the least microbial count (0.02**×10**^{2} cfu/mL) (as seen in Table 1)

Table 1: Microbial count of the various yoghurt samples

Sample	Cfu/mL	Shape	Elevation	Colour	Edge
A	0.04×10^{2}	Irregular raised	Circular convex	White	Entire
B	0.03×10^{2}	Circular, Irregular	Flat	Colour-less,	Entire, Undulate
C	0.04×10^{2}	Irregular	Raised	Colour-less,white	Undulate
D	0.02×10^{2}	Circular	flat, Raised	Creamy	Undulate
E	0.02×10^{2}	Irregular	Flat	White	Entire

The biochemical tests revealed seven (7) bacterial isolates from the yoghurt samples. *E. coli, Staphylococcus* sp and *Klebsiella* sp had the highest frequency of occurrence (20%) and *Salmonella sp* and *Streptococcus* sp had the least frequency of occurrence (6.7%) as seen in Table 2.

Table 2: Frequency of occurrence of bacteria isolated from various yoghurt samples

Isolates	Frequency of occurrence	Frequency of occurrence (%)
Escherichia coli	3	20
Staphylococcus sp	3	20
Klebsiella sp	3	20
Salmonella sp	1	6.7
Pseudomonas sp	2	13.3
Lactobacillus sp	2	13.3
Streptococcus sp	1	6.7
TOTAL	15	100

Out of the five (5) yoghurt samples, sample C and A had the highest bacterial isolates compared to other yoghurt samples as seen in Table 3.

Table 3: Sources of bacterial isolates associated with the yoghurt samples

Bacterial isolate	Number of colonies (cfu/mL)	Source of isolates
Escherichia coli	3	SAMPLE A (2 **colonies**) SAMPLE C (1 **colony**)
Klebsiella sp	3	SAMPLE B (1 **colony**) SAMPLE C (1 **colony**) SAMPLE D (1 **colony**)
Staphylococcus sp	3	SAMPLE A (1 **colony**) SAMPLE E (1 **colony**) SAMPLE B (1 **colony**)
Pseudomonas sp	2	SAMPLE E (1 **colony**) SAMPLE D (1 **colony**)
Lactobacillus sp	2	SAMPLE A (1 **colony**) SAMPLE C (1 **colony**)
Salmonella sp	1	SAMPLE C (1 **colony**)
Streptococcus sp	1	SAMPLE B (1 **colony**)

The antibiogram revealed that 100% of most isolated Gram - negative bacteria were resistant to multiple classes of antibiotics. Most of the isolates exhibited total resistance to Streptomycin, Augmentin, Ciprofloxacin, Rifampicin and Ampicillin as seen in Table 4.1 and 4.2.

Table 4.1: Susceptibility pattern for Gram negative multidrug resistant isolates

Bacteria Isolate	NI	Pattern	OFX (%)	S (%)	AU (%)	PEF (%)	SP (%)	SXT (%)	CN (%)	AM (%)	CH (%)	CPX (%)
Escherichia coli	3	S	3(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(33.3)	0(0)
		I	0(0)	0(0)	0(0)	3(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
		R	0(0)	3(100)	3(100)	0(0)	3(100)	3(100)	3(100)	3(100)	2(66.7)	3(100)
Klebsiella sp	3	S	1(33.3)	0(0)	0(0)	0(0)	0(0)	0(0)	1(33.3)	0(0)	0(0)	0(0)
		I	0(0)	0(0)	0(0)	0(0)	0(0)	1(33.3)	0(0)	0(0)	1(33.3)	0(0)
		R	2(66.6)	3(100)	3(100)	3(100)	3(100)	2(66.7)	2(66.6)	3(100)	2(66.7)	3(100)
Salmonella sp.	1	S	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)
		I	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)
		R	0(0)	1(100)	1(100)	1(100)	0(0)	1(100)	1(100)	1(100)	0(0)	1(100)
Pseudomonas sp.	2	S	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(50)	2(100)
		I	0(0)	0(0)	0(0)	0(0)	0(0)	1(50)	0(0)	0(0)	0(0)	0(0)
		R	2(100)	2(100)	2(100)	2(100)	2(100)	1(50)	2(100)	2(100)	1(50)	0(0)

KEY: S=Susceptibility, I=Intermediate, R=Resistance, OFX=Ofloxacin, S=Streptomycin, SP=Sparfloxacin, CH=Chloramphenicol, SXT= Septrin, PEF= Pefloxacin, CPX=Ciprofloxacin, AU=Augumentin, CN=Gentamicin AM=Amplicin, NI=Number of Isolates

Table 4.2: **Susceptibility pattern for Gram- positive multidrug resistant isolates**

Bacteria Isolate	NI	Pattern	PN (%)	R (%)	ST (%)	CPX (%)	AM (%)	SXT (%)	CN (%)	PEF (%)	APX (%)	E (%)
Staphylococcus sp	3	S	0(0)	0(0)	0(0)	0(0)	0(0)	1(33.3)	0(0)	1(33.3)	0(0)	0(0)
		I	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(33.3)	0(0)	0(0)	0(0)
		R	3(100)	3(100)	3(100)	3(100)	3(100)	2(66.6)	2(66.6)	2(66.6)	3(100)	3(100)
Lactobacillus sp	2	S	0(0)	0(0)	0(0)	0(0)	0(0)	1(50)	0(0)	2(100)	0(0)	0(0)
		I	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(50)	0(0)	0(0)	0(0)
		R	2(100)	2(100)	2(100)	2(100)	2(100)	1(50)	1(50)	0(0)	2(100)	2(100)
Streptococcus sp	1	S	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)
		I	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)
		R	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	0(0)	1(100)	1(100)	1(100)

KEY: S=Susceptibility, I=Intermediate, R=Resistance, CN=Gentamicin, CFX-Ciprofloxacin, APX=Ampliclox, SXT=Septrin, PEF=Pefloxacin, R=Rifampicin, E=Erythromycin, ST= Streptomycin, PN=Penicillin, AM=Amplicin, NI=Number of Isolates

DISCUSSION

The high microbial count observed in samples A and C (0.04 ×102 cfu/mL) could be attributed to the fact that adequate hygiene procedures are not employed during the collection of raw materials (such as milk) from milk producing animals, hence the introduction of high microbial contaminants from the udder of these animals into the milk samples collected. Similarly, due to poor packaging system employed by most yoghurt industries, most yoghurt are said to be easily prone to heavy microbial contamination. This result agrees with the finding of Aryna, (2017); Ayeni *et al.*, (2018) and Kisanthini and Kavitha, (2021) in India.

High occurrence of *E. coli, Staphylococcus* sp and *Klebsiella* sp in this study could be attributed to the fact that most water added to the various yoghurts samples during their production were contaminated with both feacal and environmental wastes. This result agrees with the findings with Allen-Mcfarlane *et al.,* (2019) in Europe.

Similarly, the high bacterial isolates obtained from both samples A and C could be attributed to the fact the production room of these yoghurts are not completely kept void of microbial contaminants, hence microbial contamination is said to occur during the production of these yoghurts.

This study also revealed that all bacterial isolates were multi-drug resistant (MDR) (as seen in Table 4.1 and 4.2). This could be due to the fact that most of these bacteria were from either animal or human sources that were exposed to or engaged in antibiotics misuse, and this in-turn encouraged the development and high dissemination of resistant genes, especially multi-drug resistant genes, among these bacterial isolates through genetic transfer materials such as plasmid. The result is in agreement with the findings of Perovic *et al.,* (2018) who carried a study in Serbia.

CONCLUSION AND RECOMMENDATION

This study revealed that all yoghurt samples analysed were contaminated with bacteria such as *E. coli, Klebsiella* sp*, Staphylococcus* sp*, Pseudomonas* sp and *Salmonella* sp. However, most of these isolated bacterial contaminants are highly resistant to most antibiotics commonly used in the study area, hence there is a need for Government to encourage yoghurt producers to employ adequate precautions in the, collection of raw materials for yoghurt, processing of raw materials into yoghurt and packaging of yoghurt to ensure that the consumption of bacterial contaminants, particularly resistant organisms are curtailed and controlled.

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