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Antibiotic Resistance in Non-enterococcal Lactic Acid Bacteria Isolated from Yoghurt

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Authors' contributions

This work was carried out in collaboration between all authors. Author UNJ designed the study, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Author ODK performed the statistical analysis and literature searches. Author ONC managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the antibiotic resistance in non- enterococcal lactic acid bacteria.
Study Design: Examination of commercially available yoghurts.
Place and Duration of Study: Department of Applied microbiology and Brewing, Nnamdi Azikiwe University, Awka, from June 2011 and May, 2012.
Methodology: Commercially available yoghurt (25 brands in all) purchased in Awka and Onitsha towns, Anambra State, Nigeria were evaluated for the lactic acid bacteria (LAB) strains present, their probiotic potentials and their antibiotic resistance/susceptibility. Out of this total, 8 isolates (6 *Lactobacillus* sp and 2 *Streptococcus* sp) were obtained for further studies. The probiotic characteristics of the 8 isolates were evaluated *in vitro*. The *in vitro* tests used to evaluate probiotic potentials were: tolerance to low pH and bovine bile, cell surface hydrophobicity, and antimicrobial activities.
Results: Generally, *Lactobacillus* E5 showed the best probiotic characteristics among the strains

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tested. This strain was able to survive at pH of 2.5 for 3 hours of exposure to fresh bovine bile and had low cell surface hydrophobicity. It was also resistant to some of the commonly used antibiotics and inhibited test pathogenic bacteria (*Escherichia coli 1* and *Staphylococcus aureus*). The antimicrobial resistance patterns of the 8 isolates were tested against 12 antibiotic agents. All the isolates obtained in this study were found to be resistant to Gentamycin but sensitive to Clindamycin and Lincomycin.

Conclusion: Although the use of LAB has a long and safe history and has acquired the "generally regarded as safe" (GRAS) status, the safety of selected strains should be evaluated before use, not only for virulence factors and other disease-causing traits, but also for the presence of antibiotic resistance determinants and their capability of disseminating these determinants.

Keywords: Lactic acid bacteria; antibiotic resistance; yoghurt; virulence.

1. INTRODUCTION

Dairy yoghurt is produced using a culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* bacteria. In addition, *Lactobacillus acidophilus*, *Lactobacillus bifidus* and *Lactobacillus casei* are also sometimes used in culturing yoghurt [1]. Non-enterococcal Lactic Acid Bacteria (LAB) are those species of enterococci which are not commensal organisms in the intestine of humans. They are termed non-enterococcal because they are not resident in, but gain access into the gastrointestinal tract (GIT) by ingestion of food or other matter that may contain them. Some of these non enterococcal LAB are known to be pathogenic e.g. *Enterococcus gallinarum* and *E. raffinosus* [2].

From a medical stand point, an important feature of the non-enterococcal LAB is their high level of intrinsic antibiotic resistance. Some of them are intrinsically resistant to β-lactam-based antibiotics (penicillins, cephalosporins, carbapenems) as well as many aminoglycosides and clindamycin. Some other lactobacilli have a high natural resistance to bacitracin, cefoxitin, gentamycin, kanamycin and metronidazole [3].

Recently many investigators have speculated that commensal bacteria may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens [4] and are thus very important in our understanding of how antibiotic resistance genes are maintained and spread through bacterial populations.

This study was therefore conducted to find out whether the locally made yoghurt purchased in Onitsha and Awka towns, Anambra State, Nigeria, contain starter cultures, isolate and identify the particular strain used in the particular product (yoghurt), determine the resistance/ susceptibility of the identified strains to known concentrations of known antibiotics and determine the possibility of transfer of antibiotics resistance genes.

2. MATERIALS AND METHODS

2.1 Sample Collection

Samples of yoghurt (15 brands) were purchased from vendors on the highways (Awka-Onitsha Expressway) where they were sold to a large number of people on transit. These samples were quickly transported to the laboratory and were stored in the refrigerator.

2.2 Isolation and Identification of LAB

The samples were serially diluted up to 10^{-2} factor and cultured on de Mann Rogosa Sharpe (MRS) and M17 agar medium, incubated at 37°C under microaerophilic condition for 48 hours. Cultured plates were observed at the end of 48 hours with the aid of a hand lens. Individual colonies were selected and transferred into sterile MRS and M17 broth. The isolates were purified by streak plate technique. The isolates were selected based on their colonial morphology and biochemical tests. Gram positive and catalase negative rods were inoculated in MRS and M17 agar stab and stored at refrigeration temperature for biochemical tests [5,6].

2.3 *In vitro* Studies of the Probiotic Properties

The isolated Lactobacillus species were selected for *in vitro* studies.

2.3.1 Tolerance to acidity

Ten ml of MRS broth and M17 was dispensed into test tubes and adjusted to pH values of 2.5, 3.0 and 4.0, with HCl. The test tubes were inoculated with 0.1 ml of overnight MRS and M17 broth culture of the eight isolates and incubated microaerophilically at 37°C for 3 hours. The absorbance values of the cultures were checked, before and after 3 hours of incubation, spectrophotometrically at wavelength of 600 nm [7].

2.3.2 Bile tolerance

The agar well-diffusion assay was used for bile tolerance test. MRS and M17 agar (20 ml), melted and tempered to about 45°C, was mixed with 0.2 ml of overnight cultures of each isolate. Wells of 6 mm in diameter were made in each agar plate, and approximately 0.2 ml of fresh bovine bile was placed in each well. The plates were incubated microaerophilically at 37°C for 36 hours. Diameters of zones of inhibition around the wells were observed and recorded [8].

2.3.3 Cell surface hydrophobicity assay

This assay was carried out to measure the ability of the isolates to adhere to intestinal mucosa. Fresh cultures of the isolates were centrifuged at 8,000 x g for 10 minutes at 50^{o}C. The cells were washed three times with phosphate buffer saline (PBS) and suspended in 1.2 ml of PBS. The absorbances of the bacterial cells were adjusted to 1.0 at 560 nm in the spectrophotometer (Jenway, Essex, UK). 0.6 ml of xylene (Avondale, Oxon, England) was added to 3 ml of the cell suspension. The mixture was thoroughly vortexed for 2 minutes and allowed for the xylene to separate completely (approximately 1 hour at 37°C). The aqueous phase was carefully removed, and the remnant transferred to a cuvette. The absorbance values were measured spectrophotometrically at 560 nm. Percentage hydrophobicity was calculated:

$$\% \text{ hydrophobicity} = \frac{A^0 - A}{A_0} \times \frac{100}{1}$$

A_0 = Absorbance values of the mixture before addition of xylene

A = Absorbance values of the mixture after addition and removal of xylene [9].

2.3.4 Antibiotic susceptibility test

The antibiotic disk susceptibility test was done according to Kirby-Bauer method. The strains were screened for possible resistance against 12 commonly used antibiotics which included: amoxicillin, ampicillin, pefloxacin, rocephin, zinacef, gentamycin, septrin, lincomycin, streptomycin, ciprofloxacin, clindamycin, and tetracycline. The assay was carried out using multiple discs on the same plate to eliminate differential effects from growth time and temperature [10].

2.3.5 Antimicrobial activity of the isolates

The agar well-diffusion assay was used. 10 ml of MRS and M17 broth was inoculated with of the strains and incubated microaerophilically at 37°C for 36 hours. After incubation, the culture was subjected to centrifugation (6000 x g for 15 minutes), followed by decantation of the supernatant to obtain the cell-free supernatant (CFS). The test pathogens, *E. coli* 1 and *E. coli* 2, Pseudomonas, and Staphylococcus species were cultured using Nutrient agar while Sabouraud Dextrose Agar was used for Candida sp. Wells of about 10 mm in diameter were made in the agar layer, and the CFS (0.2 ml) from each test strain was placed in each well. Plates were incubated aerobically at 37°C for 24 hours and the diameters of the inhibition zones around the wells were observed and recorded [11,12]

2.4 Transfer of Antibiotic Resistant Plasmid

The resistant strain (donor strain) was incubated with non resistant strains of *E. coli* and Salmonella species for 3 hours at 37°C. The mixture was plated out on Mac Conkey agar plates and incubation was carried out for 24 hours at 37°C. The isolates were separated based on their Gram reaction and the sensitivity of the Gram negative organisms to the various antibiotics used in the study was evaluated [13].

3. RESULTS AND DISCUSSION

Out of the 15 samples of yoghurt evaluated only eight isolates were obtained and critically studied. Table 1 shows the list of isolates, 2 of the isolates were found to be Streptococcus (cocci) while 6 were found to be Lactobacillus (rods). All of them were Gram-positive and catalase negative. Figs. 1 — 3 show initial and

final absorbance of the isolates grown in MRS and M17 broth at pH of 4.0, 3.0, and 2.5 for 3 hours. All isolates survived pH of 4.0 since their final absorbance/culture turbidity value appreciated significantly. However, this survival was reduced at pH 3.0 (Fig. 2) with Lactobacillus E5 surviving the most. At pH of 2.5, not all test strains survived with significant change in culture turbidity as evidenced in Fig. 3. Lactobacillus E5 was the most acid tolerant compared to other isolates, as supported by the increase in the final culture turbidity value relative to the initial value. Generally, the survival of the isolates appreciated with increase in pH.

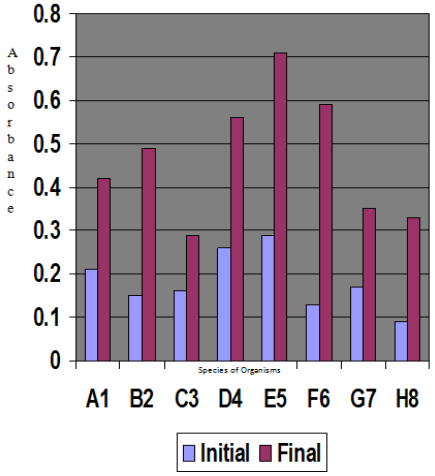

Fig. 1. Absorbance of the eight strains exposed to pH of 4.0 for 3 hours

At pH of 4.0 all isolates showed increasing viability but decline in viability was noticed as the pH dropped to 2.5. This is in accordance with the fact that several notable LAB have been found to retain viability when exposed to pH values of 2.5–4.0, but displayed loss of viability at lower values [14].

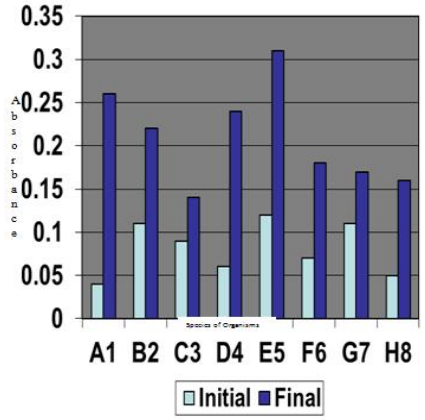

Fig. 2. Absorbance of the eight strains exposed to pH of 3.0 for 3 hours

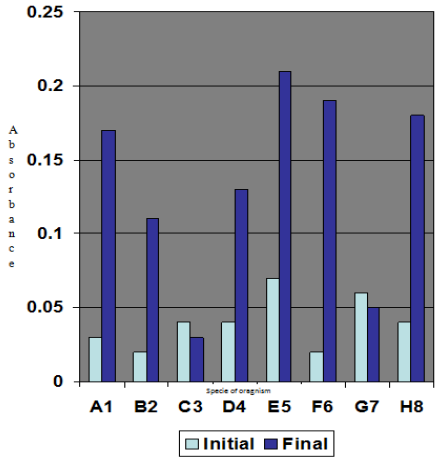

Fig. 3. Absorbance of the eight strains exposed to pH of 2.5 for 3 hours

Table 1. Morphological and metabolic characteristics of the eight isolates

Isolates	Cell Morphology	Gram Reactiom	Catalase	Citrate Utilization	Nitrate Reduction	Glucose	Sucrose	Fructose	Maltose	Lactose	Galactose	Arabinose	Ribose	Presumptive isolates
A	Rods in chains	+	-	+	-	+	+	+	+	+	+	+	+	*Lactobacillus sp*
B	Rods in chains	+	-	+	-	+	+	+	+	+	+	+	+	*Lactobacillus sp*
C	CB in clusters	+	-	+	-	+	+	+	+	-	+	-	+	*Streptococcus sp*
D	Rods in chains	+	-	+	-	+	+	+	+	+	+	+	+	*Lactobacillus sp*
E	Rods in chains	+	-	+	-	+	+	+	+	+	+	+	+	*Lactobacillus sp*
F	Rods in chains	+	-	+	-	+	+	+	+	+	+	+	+	*Lactobacillus sp*
G	CB in clusters	+	-	+	-	+	+	+	-	+	+	-	+	*Streptococcus sp*
H	Rods in chains	+	-	+	-	+	+	+	+	+	+	+	+	*Lactobacillus sp*

Table 2 shows results obtained on the study of resistance to fresh bovine bile among the isolates. Isolates A1, B2, D4 and H8 were resistant to the bile used in this study. The same cannot be said of Isolates C3, E5, F6 and G7.

Table 2. Survival of the strains in fresh bovine bile

Isolates	Zone of Inhibition(mm)
Lactobacillus A1	0.0
Lactobacillus B2	0.0
Streptococcus C3	12.0
Lactobacillus D4	20.0
Lactobacillus E5	0.0
Lactobacillus F6	15.0
Streptococcus G7	22.0
Lactobacillus H8	0.0

The strains at Table 3 showed variable degree of hydrophobicity with an average of 18.2%. This aligns with the result of the study by [15,16] which revealed that most of the isolated Lactobacillus strains exhibited hydrophobicity values of less than 40%. One of the explanations for the variation in hydrophobicity among strains is that adhesion depends on the surface properties of the organism.

In Table 4, all the eight isolates were found to be resistant to Gentamycin. This is in line with the study of Ashraf and Shah, 2011 which stated that most LAB are resistant to Gentamycin. Resistance to Gentamycin is also in line with the work of [17] which stated that S. thermophilus showed moderate to high resistance to Gentamycin, Kanamycin and Streptomycin.

All strains were sensitive to clindamycin and lincomycin which are lincosamide antibiotics as shown in Table 4. Nevertheless, *Lactobacillus* A1, B2, D4 and *Streptococcus* G7 were resistant to most of the antibiotics used in this study. The zones of inhibition ranged from 3.0 mm to 40 mm. *Lactobacillus* A1, B2 and D4 were resistant to 77% of the antibiotics used, while *Lactobacillus* E5 was susceptible to 69% of the antibiotics. The antimicrobial activities of the eight isolates were evaluated against known pathogens. The results showed weak to moderate antimicrobial activity against the pathogens. This agrees with the work [18][19] of which found that strains of *Lactobacillus casei* and *Lactobacillus bulgaricus* isolates showed weak (<12 mm zone of inhibition) antibacterial activity against *E. coli, S. aureus, P. aeroginosa, B. subtilis, K. pneumonia, S. typhimurium*, and *E. cloacae*.

Table 3. Cell surface hydrophobicity of the strains

Isolates	Hydrophobicity(%)
Lactobacillus A1	37.9
Lactobacillus B2	51.5
Streptococcus C3	0.0
Lactobacillus D4	0.0
Lactobacillus E5	24.1
Lactobacillus F6	0.0
Streptococcus G7	0.0
Lactobacillus H8	32.1

Table 5 shows that the cell free supernatants (CFS) of the isolates generally had poor antimicrobial activity towards pathogenic bacteria. *Staphylococcus* sp was moderately inhibited by *Lactobacillus* E5 as shown in Table 5. *Lactobacillus* F6 failed to inhibit any of the bacteria tested. *Candida* was only inhibited by *Lactobacillus* D4. No inhibitory activity was detected against *Pseudomonas* species.

Table 4. Antibiotic susceptibility profiles of the LAB strains as obtained by disk diffusion method

Antibiotics	A1	B2	C3	D4	E5	F6	G7	H8
Amoxicillin	0.0	0.0	25.0	0.0	25.0	28.0	0.0	30.0
septrin	0.0	25.0	0.0	0.0	0.0	0.0	0.0	0.0
Ciprofloxacin	0.0	0.0	0.0	30.0	0.0	0.0	0.0	25.0
Gentamycin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Streptomycin	20.0	30.0	0.0	26.0	0.0	0.0	0.0	0.0
Pefloxacin	0.0	0.0	30.0	0.0	20.0	0.0	40.0	0.0
Ampiclox	0.0	0.0	0.0	0.0	25.0	30.0	30.0	25.0
Zinacef	0.0	0.0	0.0	0.0	0.0	25.0	28.0	25.0
Rocephin	0.0	0.0	20.0	0.0	0.0	25.0	0.0	20.0
Clindamycin	24.0	19.0	20.0	12.0	25.0	9.0	10.0	14.0
Lincomycin	13.0	18.0	17.0	20.0	20.0	6.0	9.0	13.0
Tetracycline	0.0	0.0	35.0	0.0	6.0	9.0	0.0	0.0

**Measured in millimeter*

Table 5. Antimicrobial activity of the CFS obtained from the isolates

Test Oragnisms	A1	B2	C3	D4	E5	F6	G7	H8
Candida sp	0.0	0.0	0.0	7.0	0.0	0.0	0.0	0.0
Escherichia Coli 1	2.0	5.0	5.0	3.0	13.0	0.0	0.0	0.0
Escherichia Coli 2	0.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0
Staphylococcus aureus	0.0	0.0	4.0	0.0	17.0	0.0	0.0	6.0
Pseudomonas	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

**Measured in millimeter*

4. CONCLUSION

Although the use of LAB has a long and safe history and has acquired the 'generally regarded as safe' (GRAS) status, the safety of selected strains should be evaluated before use, not only for virulence factors and other potential disease-causing traits, but also for their capability of acquiring and disseminating resistance determinants especially to pathogens. Therefore, the results of this work cannot support any suggestions about the transfer of resistance determinants in the brands of yoghurt studied. The brands of yoghurt are declared safe, at least for the present period, for consumption and the emanating fears associated with this can be dispelled.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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