

Full Length Research Paper

# Antimicrobial resistance of bacterial strains isolated from orange juice products

LATEEF, A. \*, OLOKE, J.K., AND GUEGUIM-KANA, E.B.

Department of Pure and Applied Biology, Ladoko Akintola University of Technology, PMB 4000, Ogbomoso, Nigeria.

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Forty samples of twenty brands of sachet orange juice products were examined microbiologically. All the products were contaminated with bacteria and yeasts. The organisms encountered include *Saccharomyces cerevisiae*, *Saccharomyces sp*, *Rhodotorula sp*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Micrococcus sp*. The resistances of thirty bacterial strains isolated from orange juice products to the commonly used antibiotics were studied. About 66.67% of the isolates were resistant to augmentin and amoxycillin; 63.33% to cotrimoxazole, 56% to cloxacillin, and 23.33% to tetracycline. Resistances of 10, 6.67, and 3.33% were obtained for gentamicin, erythromycin and chloramphenicol respectively. Among the eight antibiotics tested, seven patterns of drug resistance were obtained. Six out of these are multiple-drug resistance with number of antibiotics ranging between 2 to 8. While MIC of amoxycillin ranged between 10-25mg/ml for the strains of *E. coli*, MIC of 10-20mg/ml was obtained for the strains of *S. aureus*. The MIC for cloxacillin was 0.1-1.0mg/ml for *E. coli* strains, and 0.01-1.0mg/ml for *S. aureus* strains. In all, ten strains of the bacterial isolates had evidence for the production of  $\beta$ -lactamases.

**Keywords:** Orange juice, antibiotics, resistance pattern,  $\beta$ -lactamase, microbiological standard.

## INTRODUCTION

The southern part of Nigeria is the principal orange-producing region in the country, from where it is exported to various markets in other parts of the country and even abroad. It accounts for more than 90% of total fruit production in the region. Because of the perishable nature of oranges, several small-scale industries in Nigeria have gone in to the processing of orange juice, such that the juice would be available in the market throughout the year. This is important in view of the fact that orange is usually available in the dry season period (October/February).

The inherent advantages of local processing of orange are enormous, reduction of waste through spoilage, and

wealth generation. However, the presence of a high microbial load detected in some of the products frequently renders them unfit for human consumption, and therefore unacceptable to the quality conscious markets. In order to assess the magnitude of the problem, a study was conducted to test randomly samples of locally processed pasteurized orange juices from the southwest Nigeria through conventional culturing techniques. The bacterial isolates were evaluated to determine their resistances to the commonly recommended antibiotics in Nigeria.

The incidence of resistant bacteria in foodstuff is a worldwide phenomenon. It is a major public health threat (Rahman Khan and Malik, 2001) as these organisms have been isolated from wide range foodstuffs consumed by man. The relevance of information obtained on the resistance of bacteria to antibiotics is to appreciate the magnitude of the problem and establish baselines for taking action (Caprioli et al, 2000).

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\*Corresponding author. Tel.: +234-0803-740-0520. E-mail: [agbaje72@yahoo.com](mailto:agbaje72@yahoo.com).

In several studies that have been conducted on the resistance of food-borne pathogens, emphasis had been on *E. coli* and *S. aureus* (Umoh et al., 1990; Grewal and Tiwari, 1990; Abbar and Kaddar, 1991; Singh et al., 1995; Desai and Kamat, 1998; Leegar et al., 1999; Silva et al., 2000; Rahman Khan and Malik, 2001). However, in the present study, an attempt was made to evaluate the resistance of selected strains of all the bacterial strains that were isolated from the orange fruit products. The resistance of the bacterial strains to  $\beta$ -lactam antibiotics was also undertaken with special reference to the production of  $\beta$ -lactamases. It is believed that the results of this finding will not only add to the existing world data on bacterial resistance of food origin, but will sensitize the operators in this industry, policy makers and the regulatory agencies on the need to improve the quality of these products.

## MATERIALS AND METHODS

### Collection of food samples

A total of forty sachets of twenty brands of locally processed pasteurized orange fruit juices were purchased from different selling points in Ogbomoso, Southwest Nigeria. The juices were produced by different small-scale industries in the southwestern part of Nigeria, and had at least 3 months to the expiry date from the period of analysis. None of the juices was approved by the appropriate regulatory agency, which is the National Agency for Food and Drug Administration and Control (NAFDAC), because of the absence of NAFDAC number on the products. Two units of each orange sample were transported to the laboratory and analyzed within 2 h.

### Total colony count and isolation of microorganisms

The total colony count of bacteria was performed using the pour plate method using nutrient agar (Oxoid). The juices were serially diluted and 0.2ml of an appropriate dilution was used to inoculate the plate in duplicate. The plates were incubated at 37°C for 24-48 h, after which the total colony count was determined as previously described (Nwachukwu, 2000). To isolate the fungi, dilutions of the juices were surface-spread on potato dextrose agar in duplicate and then incubated at  $28 \pm 2^\circ\text{C}$  for 48-96 h. At the end of incubation, the colonies were screened and identified based on the taxonomic schemes and descriptions by Buchanan and Gibbons (1974) for bacteria and Mislivec et al. (1992) for yeasts.

### Faecal coliform test and isolation of *E. coli*

The three-tube procedure using lactose broth (Hammad and Dirar, 1982; Fawole et al, 2002; Bakare et al., 2003) was used to detect the coliform and determine the most probable number (MPN) of coliform bacilli. A 0.1 ml, 1 ml, and 10 ml of each sample was used to inoculate the lactose broth in five replicates. Tubes were incubated at 37°C for 48 h and the MPN was determined in accordance with standard method (APHA, 1985). For the detection of faecal coliform bacteria, production of acid and gas was taken as positive indication (D'Auriac et al., 2000). Tubes showing positive results were cultured into MacConkey broth and incubated at 37°C for 48 h. These tubes were plated on eosin methylene blue (EMB)

agar and incubated as before. Colonies grown on EMB plates were selected and finally identified on the basis of morphological, cultural and biochemical characteristics for the isolation of *E. coli* (APHA, 1992).

### Antibiotic Sensitivity Test

All the bacterial isolates were tested for their sensitivity to antibiotics by means of a disc diffusion method (Bauer et al., 1966). It was investigated using commercial discs (Abtek Biologicals Ltd) containing the following: augmentin (aug), 30  $\mu\text{g}$ ; amoxycillin (amx), 25  $\mu\text{g}$ ; erythromycin (ery), 5  $\mu\text{g}$ ; tetracycline (tet), 10  $\mu\text{g}$ ; cloxacillin (cxc), 5  $\mu\text{g}$ ; gentamicin (gen), 10  $\mu\text{g}$ ; cotrimoxazole (cot), 25  $\mu\text{g}$ ; and chloramphenicol (chl), 30  $\mu\text{g}$ . The commercial antibiotic discs were placed on nutrient agar plates previously seeded with 18 h-broth culture of the test organisms. The plates were incubated at 37°C for 48 h, after which zones of inhibition were examined and interpreted accordingly (Chortyk et al., 1993). Earlier, the potencies of all the antibiotics used in the study were confirmed using susceptible *E. coli* strains.

### Determination of minimum inhibitory concentrations (MIC)

The MIC of two commonly recommended  $\beta$ -lactam antibiotics in Nigeria, cloxacillin and amoxycillin were determined using the paper disc method as described by Oloke (2000). Sterile paper discs were dipped into different concentrations of cloxacillin and amoxycillin. Each soaked disc was then aseptically layered on each nutrient agar plate already seeded with a 18 h-broth culture each of the *S. aureus* and *E. coli* strains in duplicate. Each plate was incubated at 37°C for 24 h and then examined for zones of inhibition. The lowest concentration of each antibiotic, which inhibited growth, was taken as the MIC.

### Assay for $\beta$ -lactamase Production

$\beta$ -lactamase production was assayed using the method of Ahmad and Yadava (1979). Broth culture of the test organism was spot inoculated on to starch agar and then incubated overnight at 37°C. The plates were then flooded with freshly prepared phosphate buffered saline containing potassium iodide, iodine and penicillin. The presence of clear colourless zones around the bacterial growth is an indication of  $\beta$ -lactamase production.  $\beta$ -lactamase converts penicillin to penicilloic acid, which reduces iodine to iodide monitored via decolourisation of the starch iodine complex. All the bacterial isolates were tested for the production of  $\beta$ -lactamases.

## RESULTS AND DISCUSSION

The orange juices contained large amounts of bacteria ( $3.5 \times 10^4 - 2.15 \times 10^5$  cfu/ml) and yeasts ( $7.5 \times 10^4 - 1.25 \times 10^5$  cfu/ml). The values obtained in this study are within the range of  $10^2 - 10^5$  cfu/ml reported for microbial populations in fruit juices (Hatcher et al., 1992). However, the colony counts, which are within the high end of this range, could be an indication of spoilage. Also, high bacterial and mould counts may be indicative of improper hygiene and may perhaps be a result of poor quality fruit being used.

The juices were acidic (pH 3.0-3.65), thereby creating a good condition for the growth of yeasts. In this study,

**Table 1.** Resistance of bacterial isolates against individual antibiotics.

Antibiotics	Number of resistant Isolates (%)						*Cumulative resistance
	Ec	St	Sp	Bs	Bc	Mc	
aug	5(100)	5(100)	ND	5(100)	ND	5(100)	20(66.67)
amx	5(100)	5(100)	ND	5(100)	ND	5(100)	20(66.67)
ery	2(40)	ND	ND	ND	ND	ND	2(6.67)
tet	3(60)	1(20)	1(20)	1(20)	ND	1(20)	7(23.33)
cxc	3(60)	2(40)	1(20)	5(100)	1(20)	5(100)	17(56.67)
gen	1(20)	ND	ND	ND	2(40)	ND	3(10)
cot	2(40)	ND	2(40)	5(100)	5(100)	5(100)	19(63.66)
chl	1(20)	ND	ND	ND	ND	ND	1(3.33)

ND, not detected; a total of thirty isolates used, five for each bacterium; \*, resistance of all the bacterial isolate to each antibiotic; Ec (*E. coli*); St (*S. aureus*); Sp (*S. pyogenes*); Bs (*B. subtilis*); Bc (*B. cereus*); Mc (*Micrococcus sp.*)

strains of *Saccharomyces sp*, *Saccharomyces cerevisiae* and *Rhodotorula sp* were isolated from the fruit juices. The isolation of these yeasts from orange juice has been previously reported (Deak and Beuchat, 1993). The pH of fruit juices is usually too low for the growth of pathogenic bacteria (Hatcher et al., 1992), but the incidence of such bacteria in the juices used in this study might not be unconnected with the nature of the juices. All the juices are ready-to-serve fruit juices and the water activity ( $a_w$ ) values are sufficiently high to allow microbial growth (Harley et al., 1996).

The isolation of *Saccharomyces* can cause unusual flavour in the orange juices. Studies have shown that *Saccharomyces* can metabolize ferulic acid found in fruit juices to form 4-Vinylguaiacol, thereby producing the off flavours in the juices (Sutherland et al., 1995). However, since many species of *Saccharomyces* are used in the production of food commodities (Ray, 1996), the genus is generally not considered a safety concern. In contrast, the genus *Rhodotorula* is implicated in the spoilage of dairy products, sea foods, fresh and processed products and juices. It has the ability to grow within a broad temperature range (0.5-35°C), and a pH as low as 2.2 in the presence of HCl and organic acids (Jay, 1996). Although, members of the genus can be useful as sources of lipids, proteins and beta-carotene, the presence of *Rhodotorula* in the juices in large numbers seems to have marred such importance, because of the extensive spoilage that can be done to these products.

The presence of notable bacterial pathogens such as *E. coli*, *Micrococcus sp*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Bacillus cereus* and *Staphylococcus aureus* in the orange juices is considered a safety concern. *Bacillus cereus*, an aerobic spore former is associated with food borne illnesses (Peng et al., 2001), while *B. subtilis* has also been associated with food borne diseases. Some strains of *E. coli* synthesize heat stable enterotoxins and are responsible for diarrhoeal disease in humans and domestic animals. There is no justification for processed ready-to-eat food being contaminated with

these organisms, and their presence even in small numbers results in such foods being of unacceptable quality or potentially hazardous (PHLS, 2000). The processing units of the juices are likely primary causes of high bacterial and fungal load. The maintenance of proper hygienic conditions and use of good quality oranges and water will certainly improve the microbiological quality of these juices, and make them acceptable to quality conscious markets both locally and abroad. In this line, the establishment of quality control unit/laboratory becomes imperative to detect contamination of either the raw materials or the products early enough. In addition, operators in this sector should utilize the technical assistance of NAFDAC towards attaining acceptable quality standard. Recent findings have shown that some NAFDAC approved sachet water produced by small-scale industries in Nigeria attained an acceptable microbiological standard, whereby only one sample out of twenty two brands that were analyzed was defective (Lateef and Yusuff, 2002).

The antibiotic sensitivity test and assay for the production of  $\beta$ -lactamase were conducted on all the bacterial strains to ascertain the level of resistance. The results of the antibiotic sensitivity test were interpreted and are presented as the resistance of bacterial isolates to the antibiotics (Table 1), and the antibiotic resistance pattern among the bacterial isolates (Table 2). In several earlier works on similar studies carried out on foodstuffs including orange juices, researches have been focused mainly on the strains of *E. coli* and *S. aureus* (Umoh et al., 1990; Grewal and Tiwari, 1990; Abbar and Kaddar, 1991; Singh et al., 1995; Desai and Kamat, 1998; Leegar et al., 1999; Silva et al., 2000; Rahman Khan and Malik, 2001). However, in this work, a broad overview of the study was conducted and this is why the resistance pattern and the mechanisms in selected strains of all the bacteria isolated from the orange juices were studied. This is important in view of the necessity for the worldwide surveillance of antibacterial resistance (Singleton, 1997) to appreciate and understand the

**Table 2.** Antibiotic resistance pattern among the bacterial isolates.

No of Antibiotics	Resistance Pattern	No of Isolates	Designation	%
1	Cot	4	1Sp; 3Bc	13.33
2	gen cot	1	1Bc	3.33
	aug amx	5	3St; 2Ec	16.67
3	tet cxc cot	1	1Sp	3.33
	cxc gen cot	1	1Bc	3.33
	aug amx cxc	1	1St	3.33
4	aug amx tet cxc	2	1St; 1Ec	6.67
	aug amx cxc cot	8	4Bs; 4Mc	26.67
5	aug amx tet cxc cot	2	1Bs; 1Mc	6.67
6	aug amx ery tet cxc cot	1	1Ec	3.33
8	aug amx ery tet cxc gen cot chl	1	1Ec	3.33

Antibiotics, see abbreviation under materials and methods; Ec (*E. coli*); St (*S. aureus*); Sp (*S. pyogenes*); Bs (*B. subtilis*); Bc (*B. cereus*); Mc (*Micrococcus sp.*)

magnitude of the bacterial resistance to antibiotics and to establish baselines for taking action (Caprioli et al., 2000).

A high level of resistance was obtained among the five *E. coli* strains. Both augmentin and amoxycillin were not active against the strains of *E. coli*. The level of resistance is similar to that reported by Rahman Khan and Malik (2001). All the strains of *S. aureus* were sensitive to erythromycin, gentamicin, cotrimoxazole and chloramphenicol.

The levels of resistance of other bacterial strains also ranged between 20 to 100%. The resistance to augmentin, amoxycillin, cloxacillin and cotrimoxazole may reflect the widespread use of these antibiotics. The trio of amoxycillin, cloxacillin and cotrimoxazole are commonly recommended in the hospitals in Nigeria.

The relatively high level of resistance to antimicrobial agents is a reflection of misuse or abuse of these agents in the environment (Umoh et al., 1990, Abbar and Kaddar, 1991; Silva and Hoffer, 1993; Malik and Ahmad, 1994). Antibiotic prescriptions in some hospitals are given without clear evidence of infection or adequate medical indication. Toxic broad-spectrum antibiotics are sometimes given in place of narrow-spectrum drugs as substitute for culture and sensitivity testing, with the consequent risk of dangerous side effects, super infections, and the selection of drug-resistant mutants (Prescott et al., 1999).

Seven patterns of drug resistance in the bacterial isolates were obtained, out of which six were multiple drug resistance (Table 2). The number of such antibiotics ranged from two to eight, and falls within the range obtained by earlier workers (Grewal and Tiwari, 1990; Singh et al., 1995; Rahman Khan and Malik, 2001). Multiple drug-resistance is an extremely serious public health problem (Prescott et al., 1999) and it has always been associated with outbreak of major epidemics throughout the world.

**Table 3.** MIC values of amoxycillin and cloxacillin against strains of *E. coli* and *S. aureus*.

Bacterial strains	Amoxycillin	cloxacillin	$\beta$ -lactamase activity
EC1	25	1.0	+
2	20	0.5	+
3	10	0.1	-
4	10	1.0	-
5	10	1.0	-
St 1	20	1.0	+
2	10	0.10	+
3	10	0.01	-
4	10	0.01	-
5	10	0.10	-

Ec, *E. coli*; St, *S. aureus*; +, positive; -, absent.

The MIC of cloxacillin and amoxycillin against the strains of *E. coli* and *S. aureus* were determined (Table 3). The MIC (mg/ml) of amoxycillin ranged between 10-25 for *E. coli* and 10-20 for *S. aureus*. The MIC (mg/ml) of cloxacillin was lower, 0.1-1.0 for *E. coli* and 0.01-1.0 for *S. aureus*. Out of the 10 strains, two each of *S. aureus* and *E. coli* with high MIC values for amoxycillin and cloxacillin showed evidence for the production of  $\beta$ -lactamase (Table 3). This indicates that the high MIC values against the two  $\beta$ -lactam antibiotics may directly relate to the production of  $\beta$ -lactamases.  $\beta$ -lactamase production was also detected in three strains of *Micrococcus sp.*, two strains of *B. subtilis* and one strain of *B. cereus*. Several workers have reported the production of  $\beta$ -lactamase by bacteria with high resistance to  $\beta$ -lactam antibiotics (Nandivada and Amyes, 1990; Esperson, 1998; Rahman Khan and Malik, 2001). There have also been reports of

the spread of  $\beta$ -lactamase genes into species that previously were not known to possess them (Mascaretti et al., 1999; Kawakami et al., 2000; Silva et al., 2000).

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