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Full Length Research Paper

Occurrence and implications of resistance to antibiotics and organic acids in *Enterococcus faecalis* isolated from fruit juices marketed in Ado-Ekiti

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The study investigated the growth of *Enterococcus faecalis* in various fruit juice products, their resistance to antibiotics and their ability to be grown in the presence of most organic acids commonly employed as preservatives. *E. faecalis* strains were isolated from seven out of nine fruit juices using serial dilution and pour plate method. The total bacterial counts observed in the juice samples ranged between 20 to 460 colony forming unit per 100 ml (CFU/100 ml) with the highest and least values detected in fruit juice brands EXT and MNN respectively. The total coliform count in brand EXT exceeded those in all the other samples (275 CFU/100 ml) but enterococcal load in the fruit juices was within the range of 10 CFU/100 ml to 65 CFU/100 ml in MNN and LCS brands respectively. The isolates were all susceptible to vancomycin while resistance was observed to other antibiotics evaluated. Resistance was highest for erythromycin (93%); while penicillin and tetracycline exhibited less resistance (90%). *E. faecalis* strains isolated from brand TTT (EFTT) FAL (EFFA) and BST (EFBT) were all inhibited at higher concentrations of acetic acid (1.25 and 5.0% w/v) while lower concentrations (0.31% and below) could not effectively inhibit the growth of the isolates. The inhibitory effect of citric acid was pronounced in EFFA. At 0.31% w/v all the isolates were inhibited except EFFA. Benzoic acid had inhibitory effect on just two of the five strains, while formic acid showed no inhibition to all the isolates. EFFA and EFXT were also resistant to ethanoic acid at the different concentrations. Excellent manufacturing practices must be adopted during the production of commercial juice, as appropriate precautions will prevent contamination. Since most organic acids used for the preservation of most juice allow the survival of *E. faecalis*, there is a need to provide rational basis for designing interventions that are needed to assure the microbiological safety of final products.

Key words: Antimicrobial resistance, *Enterococcus faecalis,* fruit juice, bacterial count, organic acids.

INTRODUCTION

Juices are much appreciated for their nutritive values. With the help of modern technologies juices are now more similar to the raw fruits and vegetables from which they are produced (Calloway and Carpenter, 1981; Conway, 2006). Juices in general, are a good source of sugars, vitamins, and minerals; being all valuable components to human health (Arthey and Ashurst, 1996; Conway, 2006). The current trend towards healthier diets makes juice consumption an important natural food alternative (Parish, 1997), and this improves the availability of its nutritive compounds (Kumpulainen et al., 1999).

Contamination of juice could arise from the field through fecal contact, but other causes like the use of dropped, unwashed fruits have been implicated as the source of pathogens in some disease outbreaks associated with fruit juice (Besser et al., 1993; CDC, 1996). However, vectors such as birds could potentially deposit pathogens on tree-bound fruit (Wallace et al., 1997). In an outbreak of salmonellosis in 1995 from unpasterurized orange juice, *Salmonella* spp. was isolated from amphibians around the processing facility (Parish, 1997).

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Some of the contaminated pathogens have been demonstrated to survive for periods of 18 days or more in fruit juice (Linton et al., 1999). Prior to exposure, the pathogens incur sub-lethal pH adaptive mechanisms, which greatly enhance their ability to withstand acidic conditions (Ryu et al., 1999; Brudzinski and Harrison, 1998). Such adaptation could potentially be induced while inhabiting the digestive system of some mammals, thereby increasing resistance to juice acidity, as well as stomach acidity upon ingestion (Lin et al., 1996). Most of the time, organic acids are used to prevent fruit spoilage and elongate the shelf life. They have been reported to have growth inhibitory effect on enteric pathogens (Buchanan et al., 1999).

Enterococci are widely distributed, they are found mostly in water, sewage, soil, and vegetation but their primary source is the intestine of humans and warm-blooded animals. Enterococci survive environmental conditions that destroy most microorganisms of sanitary significance (Ksoll et al., 2007). Due to their resistance to freezing, low pH, and moderate heat treatment, enterococci have been suggested as indicators of faecal contamination in food products (Hartz et al., 2008).

Organic acids occur throughout nature and are used in food industry. Apart from being used as antimicrobial, they serve as anti-foaming agents and emulsifiers, aid in setting of pectin gels, and have a strong effect on the taste of a food (Fennema, 1996). With a characteristically sour taste, organic acids have an important role in the flavor of fruits and their juices by balancing the sugar/acid ratio (Arthey and Ashurst, 1996).

The objective of this study is to determine isolation of strains of *Enterococcus faecalis* from juice samples, their resistance to commonly used antibiotics and organic acids and to evaluate the implications of such resistance.

MATERIALS AND METHODS

Source of sample

Different brands of juice drinks (CVT, FAL, BST, MNN, BMM, LCS, TTT and GLT) were purchased from different super markets in Ado-Ekiti, Nigeria. The juices were examined for any sign of spoilage; expiry date and National Agency for Food and Drug Administration and Control (NAFDAC) approved numbers. The method of Fawole and Oso (2001) was used to determine the microbial load of the samples. Plate count agar was used for bacterial enumeration. The plates were incubated at 37°C for 24 h. Nutrient agar (Oxoid), MacConkey agar (Oxoid) and Bile aesculin agar (Oxoid) were used to determine the total bacterial, coliform and enterococcal loads respectively.

The characterization of enterococcal isolates was carried out on overnight culture using standard methods of Olutiola et al. (2000) and Fawole and Oso (2001) by the observation of cultural, morphological and biochemical properties.

Antibiotic sensitivity test

The isolates were grown at 37°C in Mueller-Hilton broth (Oxoid) for16 to 18 h and diluted to an optical density of 0.1 (0.5 McFarland Standard) at a wavelength of 625 nm and stored at 4°C according to the method of Bauer et al. (1998). The disc diffusion method was used for susceptibility testing as described by Clinical and Laboratory Standard Institute (2008). The isolates were tested against 15 different commercial antibiotic disks (Abtek Biologicals and Oxoid Limited) with their concentrations (in μg): amoxicillin (25), gentamicin (10), cotrimoxazole (25), augmentin (30) and tetracycline (30), erythromycin (5), chloramphenicol (30), cloxacillin (5), and vancomycin (30).

Determination of susceptibility of *E. faecalis* strains to organic acids

Five strains of *E. faecalis* were selected, based on their antibiotic resistant pattern, for the organic acids susceptibility. The organic acids: acetic, citric, benzoic, formic and ethanoic acids (analytical grades, BDH) were serially diluted to obtain concentrations ranging from 5.0 to 0.08% w/v. Agar diffusion method was used to assess the antimicrobial effect of the organic acids using Mueller-Hilton agar (Oxoid). The plates were incubated at 37°C for 24 h and the zones of inhibitions were measured to the nearest mm with a graduated ruler.

RESULTS AND DISCUSSION

The total bacterial count observed in the juice samples ranged between 20 and 460 CFU/100 ml. The highest and the least values were detected in EXT and MNN respectively as shown in Table 1. The prevalence of the bacteria in the fruit juice could be as a result of the suitable environmental conditions provided them by the fruit juice (Parish, 1997).

The total coliform count in EXT exceeded those for all the other samples (275 CFU/100 ml). MNN and TTT brands had the same number of coliform (10 CFU/100 ml). Enterococcal load in the fruit juices ranged from 10 CFU/100 ml to 65 CFU/100 ml in MNN and LCS respectively. *E. faecalis* like *Salmonella* can be more readily isolated from decaying fruits and vegetables as reported by Wells and Butterfield (1997). This study shows that *E. faecalis* was present in most of the juice products examined except in MNN and LCS.

The pattern of resistance for the enterococci isolated from the fruit juices is shown in Table 2. The isolates were all susceptible to Vancomycin. The highest resistance was observed against Erythromicin 93%, followed by Penicillin and Tetracycline that exhibited 90% resistance respectively. Gentamicin was very effective against the isolates with 23%. All the isolates were sensitive to Vancomycin (30 µg). They are all resistant to tetracycline (30 µg) and erythromycin (15 µg) as shown in Table 3. EFTT was resistant to all the antibiotics except vancomycin. EFFA showed intermediate resistance to gentamicin, and streptomycin. Comparatively, EFBT has the least resistance. It was susceptible to four out of the nine antibiotics.

This study suggests that *E. faecalis* isolated from fruit juices was resistant to most antibiotics used in clinical practice. This is similar to the report of Ruiz-Garbajosa et

Table 1. Total bacterial load, coliform and enterococcal load of fruit juice samples (colony forming unit/100 ml).

Sample	Load		
	Total bacteria	Coliform	Enterococci
CVT	110	26	4
FAL	225	50	19
BST	110	45	15
MNN	20	10	0
BMM	150	115	29
LCS	65	17	0
TTT	130	20	12
GLT	200	115	19
EXT	460	275	65

Data is the modal value of three determinations.

Table 2. Antibiotic resistance pattern of *E. faecalis* isolated from fruit juice.

Antibiotics	Resistant pattern of strains of *Enterococcus faecalis*		
	Resistance n (%)	Intermediate n (%)	Susceptible n (%)
Gentamicin (10 µg)	7 (23)	4 (13)	21 (70)
Ampicillin (10 µg)	22 (73)	3 (10)	6 (20)
Chloramphenicol (30 µg)	23 (77)	2 (7)	5 (17)
Cloxacillin (1 µg)	19 (63)	3 (10)	8 (26)
Erythromycin (15 µg)	28 (93)	2 (7)	0 (0)
Penicillin (10 µg)	27 (90)	0 (0)	3 (10)
Streptomycin (10 µg)	23 (76)	1 (3)	6 (10)
Tetracycline (30 µg)	27 (90)	2 (7)	1 (3)
Vancomycin (30 µg)	0 (0)	0 (0)	30 (100)

Table 3. The zone of inhibition (mm) of selected *E. faecalis* strains used for organic acid susceptibility test.

Antibiotics	Strains of *Enterococcus faecalis*				
	EFTT	EFFA	EFBM	EFBT	EFXT
Gentamicin (10 µg)	8.0 (R)	13.0 (I)	17.0 (S)	6.0 (R)	15.0 (I)
Ampicillin (10 µg)	12.0 (R)	9.0 (R)	9.0 (R)	20.0 (S)	18.0 (S)
Chloramphenicol (30 µg)	10.0 (R)	8.0 (R)	7.0 (R)	4.0 (R)	19.0 (S)
Cloxacillin (1 µg)	10.0 (R)	7.0 (R)	12.0 (I)	13.0 (S)	6.0 (R)
Erythromycin (15 µg)	10.0 (R)	3.0 (R)	8.0 (R)	9.0 (R)	8.0 (R)
Penicillin (10 µg)	12.0 (R)	10.0 (R)	14.0 (R)	12.0 (R)	8.0 (R)
Streptomycin (10 µg)	10.0 (R)	14.0 (I)	5.0 (R)	15.0 (S)	10.0 (R)
Tetracycline (30 µg)	11.0 (R)	8.0 (R)	13.0 (R)	6.0 (R)	10.0 (R)
Vancomycin (30 µg)	18.0 (S)	20.0 (S)	24.0 (S)	21.0 (S)	22.0 (S)

R = resistance, I = intermediate and S = susceptible.

al. (2009) and Calva et al. (1996) that reported the resistance of enterococci to tetracyclines and erythromycin. It follows therefore that the isolates recovered from the juice samples were resistant to the common (first hand) antibiotics. This is not surprising since they are often used as handy antimicrobials in disease prevention, and their widespread use may have contributed to their high rates of resistance. For instance the frequency of tetracycline resistance in the *E. faecalis* earlier inestigated was 63% (Calva et al., 1996). The fact that most

Table 4. Susceptibility pattern of selected *E. faecalis* to acetic acid (zone of inhibition in mm).

Strain	Concentration (% w/v)							
	5.00	**2.50**	**1.25**	**0.63**	**0.31**	**0.16**	**0.08**	**Control**
EFTT	21.0	16.0	16.0	0	0	0	0	0
EFFA	33.0	28.0	17.0	9.0	0	0	0	0
EFBM	40.0	38.0	38.0	20.0	0	0	0	0
EFBT	0	0	0	0	0	0	0	0
EFXT	0	0	0	0	0	0	0	0

Values are mean of three determinations.

Table 5. Susceptibility pattern of selected *E. faecalis* to citric acid (zone of inhibition in mm).

Strain	Concentration (% w/v)							
	5.00	**2.50**	**1.25**	**0.63**	**0.31**	**0.16**	**0.08**	**Control**
EFTT	38.0	28.0	0	0	0	0	0	0
EFFA	40.0	40.0	38.0	20.0	10.0	0	0	0
EFBM	45.0	38.0	19.0	0	0	0	0	0
EFBT	18.0	16.0	0	0	0	0	0	0
EFXT	0	0	0	0	0	0	0	0

Values are mean of three determinations.

Table 6. Susceptibility pattern of selected *E. faecalis* to benzoic acid (zone of inhibition in mm).

Strain	Concentration (% w/v)							
	5.00	**2.50**	**1.25**	**0.63**	**0.31**	**0.16**	**0.08**	**Control**
EFTT	40.0	38.0	25.0	19.0	0	0	0	0
EFFA	0	0	0	0	0	0	0	0
EFBM	0	0	0	0	0	0	0	0
EFBT	10.0	10.0	7.0	4.0	0	0	0	0
EFXT	0	0	0	0	0	0	0	0

Values are mean of three determinations.

resistance genes of large number of handy antimicrobials are located on mobile genetic elements makes them easily transmissible between bacteria (Marcinek et al., 1998; Philippon et al., 2002).

Five representative isolates with different resistant patterns were selected for acid tolerance. Tables 4 to 8 show the resistant pattern of different enterococcal isolates to different organic acids commonly employ in the food industries for preservatives. Acetic acid, the oldest organic acids has been employed as food antimicrobial (Eklund, 1989). *E. faecalis* strains isolated from TTT (EFTT) FAL (EFFA) and BST (EFBT) were all inhibited at higher concentrations (1.25 and 5.0% w/v) of acetic acid. Lower concentrations (0.31% and below) were not effective in controlling the growth of the isolates. Eklund (1989) and Levine and Feller (1940) similarly, reported that 0.5% acetic acid is not effective in controlling Enterobactericeae. The inhibitory effect of acetic acid depends on the un-dissociated form, as well as its ability to donate hydrogen ions in an aqueous system (Uljas and Ingham, 1998). This report supports the findings of Lillard et al. (1987) which stated that acetic acid inhibits the growth of *Bacillus* spp., *Clostridium* spp., *Listeria* spp., *Salmonella* spp., *Staphylococcus* spp. and *Escherichia coli*.

The inhibitory effect of citric acid was pronounced in EFFA. At 0.31% w/v all the isolates were inhibited except EFFA. Isolate EFXT was not inhibited by the acid even at 5.0% w/v. Citric acid prevents toxin production by most bacterial pathogens, inhibits the growth of *Listeria monocytogenes* and own its inhibitory action due to chelation by the anion (Doores, 1993). At lower pH values in

Table 7. Susceptibility pattern of selected *E. faecalis* to formic acid (zone of inhibition in mm).

Strain	Concentration (% w/v)							
	5.00	2.50	1.25	0.63	0.31	0.16	0.08	Control
EFTT	0	0	0	0	0	0	0	0
EFFA	0	0	0	0	0	0	0	0
EFBM	0	0	0	0	0	0	0	0
EFBT	0	0	0	0	0	0	0	0
EFXT	0	0	0	0	0	0	0	0

Values are mean of three determinations.

Table 8. Susceptibility pattern of selected *E. faecalis* to ethanoic acid (zone of inhibition in mm).

Strain	Concentration (% w/v)							
	5.00	2.50	1.25	0.63	0.31	0.16	0.08	Control
EFTT	17.0	13.2	8.0	7.0	0	0	0	0
EFFA	0	0	0	0	0	0	0	0
EFBM	28	14.0	10.0	0	0	0	0	0
EFBT	40	38	30.0	30.0	19.0	0	0	0
EFXT	0	0	0	0	0	0	0	0

Values are mean of three determinations.

this form, the cell membrane is more permeable to the acid, allowing it to enter the cell (Bruice, 1995). The type of microorganism, inoculum load, and environmental conditions influences conditions for growth of pathogens in juice. Other factors are pH of the juice, temperature of storage and water activity (a_w).

Benzoic acid had inhibitory effect on just two out of the five strains. The inhibition of the susceptible isolates was 0.63% w/v. Formic acid was not inhibitory to any of the isolates at the tested concentrations. In addition to affecting enzymes, excess protons in the cytoplasm upset the membrane potential necessary for energy production and transport across the cell membrane (White, 1999). Thus, organic acids can act on a cell by affecting both the external and internal pH.

Ethanoic acid was not effective in controlling isolates EFFA and EFXT; they were all resistant to the organic acid at the test concentrations. EFBT showed the highest susceptibility to the organic acid.

When the acid dissociates, it thus lowered the internal pH of the cell and disrupting cellular functions (that is, enzyme stability) (Lück and Jager, 1997). Most of the organic acids are not effective against bacterial pathogens. This confirmed the findings of Brudzinski and Harrison (1998) and Subramanian and Marth (1968) that reported that organic acids are better acidulants and flavouring rather than preservatives. Chung and Goepfert (1970) showed that various organic acids are bacteriostatic to some pathogens at different pH levels. Citric, malic, and tartaric acids were inactivated in acidic medium (Conner and Katrola, 1995; Ryu et al., 1999; Tsai and Ingham, 1997).

In conclusion, since fruit juices are a good media for most microorganisms, contamination can occur starting from the orchard to the packaging process. This mandates appropriate measures to be adopted to prevent contamination. Good manufacturing practices must be practiced in handling and processing. Since most organic acids used for the preservation of most juice are not effective in inhibiting the pathogen, there is a need to provide rational basis for designing intervention that are needed to assure the microbiological safety of juice. Therefore, to achieve microbiological safety of juice, good manufacturing practices must be followed for raw material purchase, processing, product handling, storage and distribution.

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