

The Effect of pH and Chemical Preservatives on the Growth of Bacterial Isolates from Some Nigerian Packaged Fruit Juices

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Abstract: Bacterial pathogens were isolated from some Nigerian packaged fruit juices. The isolates were characterized and identified as *Bacillus licheniformis*, *Aeromonas hydrophila*, *Bacillus circulans*, *Proteus morgani*, *Pseudomonas cepacia*, *Bacillus alvei* and *Pseudomonas chlororaphis*. The antibiotic susceptibility profile of the seven isolates was determined and it was discovered that 65% of the microorganisms isolated were resistance to the antibiotic used while 35% were sensitive. The effect of pH, benzoic acid and sodium chloride concentration on the growth rate of isolates was investigated. It was found that as the pH of the growth medium increased from 3-9, the rate of growth of most isolates also increased except for *Pseudomonas cepacia*, which had optimum growth at neutral pH 7. As the concentration of sodium chloride increased from 2-5%, the rate of growth of all the seven isolates decreased. It was also noted that as concentration of benzoic acid increased from 250-1000 mg/l *Bacillus licheniformis* decreased from 1.330-0.167 nm, *Aeromonas hydrophila* decreased from 1.208-0.164 nm *Bacillus circulans* decreased from 1.158-0.299 nm, *Proteus morgani* decreased from 1.377-0.141 nm etc. The higher the concentration of benzoic acid the lower the rate of growth of the isolates.

Key words: *Bacillus licheniformis*, *Aeromonas hydrophila*, *Bacillus circulans*, *Proteus morgani*, *Pseudomonas cepacia*, *Bacillus alvei* and *Pseudomonas chlororaphis*, fruit juice, pH, benzoic acid, sodium chloride

INTRODUCTION

Juice is a liquid naturally contained in fruit or vegetable tissue. Juice is prepared by mechanically squeezing or macerating fresh fruits or vegetables without the application of heat or solvents. For example orange juice is a liquid extract of one fruit of orange tree. Juice may be prepared in the home from fresh fruits and vegetables using variety of hand or juice extractor. Many commercial juices are filtered to remove fiber or pulp, but high pulp fresh orange juice is a popular beverage. Juice may be marketed in concentrated form, sometimes frozen, requiring the user to add water to reconstitute the liquid back to its original state. However, concentrates generally have a noticeably different taste than their comparable fresh squeezed versions. Other juices are reconstituted before packaging for retail sale. Common methods for preservation and processing of fruit juices include canning, pasteurization, freezing, evaporation and spray drying (Fasoyiro *et al.*, 2005).

Popular juices include, but are not limited to apple, orange, grape fruit, pineapple, tomato, passion fruit mango, carrot, cranberry and pomegranate. It has become increasingly popular to combine a variety of fruit into single juice drinks. Popular blends include Cran-apple (Cranberry and apple) and apple and black currant. A demonstration of this trend is that prepackaged single fruit juices have lost market share to prepackaged fruit juice combination (Frazier and Westhoff, 1986).

However, fruit juices are nutritious which offer great taste and health benefits. The 2005 Dietary Guide lines for Americans (2005) recommended consumption of several cups per day of fruits and vegetable. Most fruit juices bought from grocery stores and supermarket shelves are pasteurized. This means that the liquid has been brought to a high temperature that kills harmful bacteria. However, a small percentage of fresh fruit juices are unpasteurized. This means that there is a chance that the product may contain bacteria harmful to our health. Most people can enjoy unpasteurized juice and drinks, however, for young children, the elderly and people with weakened Immune systems, the effect can be severe or ever deadly (Esteve *et al.*, 2005).

Unpasteurized fruit and vegetable juice have posed serious public health risk in recent years. Seventy people including a child who died-become ill in 1996 after drinking unpasteurized apple juice Contaminated by a strain of *Escherichia coli* bacteria (Amato, 1999). In 1999 and 2000, unpasteurized orange sickened hundred people in United States and three Canadian provinces. The 1999 outbreak contributed to one death (Formanek, 2001) A 2005 study in Japan found out that up to 52% of commercial fruit juices sold in Japan were contaminated with thermostable acidophilic bacteria (Hara *et al.*, 2005).

Bacteria are responsible for the contamination and spoilage of packaged fruit juices resulting in

discoloration, abnormal flavour and odour rendering it unacceptable for human consumption. The bacterial strain that spoil fruit juices includes, *Bacillus licheniformis*, *Aeromonas hydrophila*, *Bacillus circulans*, *Proteus morgani*, *Pseudomonas chlororaphis*, *Bacillus alvei*, *Pseudomonas cepacia* and soon. Their presence may pose risks to consumer's health and should not be taken for granted (Hatcher *et al.*, 1992).

Fruit juices are well recognized for their nutritive value, mineral and vitamin content. They are beverages that are consumed for their nutritional value, thirst-quenching properties, and stimulating effect or for their medicinal values (Fawole and Osho, 2002). Contamination of fruit juice by bacterial may occur when the organism enters the processing plant or on the surface of the fruit having originate from soil untreated surface water, dust and decomposing fruit. The degree of contamination varies depending upon how the fruit was handled from the field and in the processing plant. Proper handling washing and sanitizing the fruit contribute materially to good product quality. The low PH of fruit juices greatly limits the number and the type of bacteria that can survive or grow at this low PH but some bacterial that their PH is lower than that of the fruit juice can grow at this condition (Ryu and Beuchat, 1998).

Yeast and moulds are also present and can grow when the juice is held at a temperature permitting their growth. Yeasts are primarily responsible for the spoilage of chilled juice that is not sterile and some can withstand the effect of chemicals used to preserve them (Sandeep *et al.*, 2001). Most industrial juice concentrators use a high temperature evaporation (thermal accelerated short time evaporation) and microbes are generally killed during this process and the freezing process should kill many of the survivors though this process will preserve the ultimate survivor. Thus frozen concentrated fruit juice should have few if any microbes (Parish, 1997). It is possible to reduce the growth of bacterial in fruit juices by addition of some chemical preservatives, thus inhibiting abnormal flavor, odour and spoilage of fruit juices and possibly improving shelf-life and permitting preservation for longer period with maximum retention of its nutritive value.

According to the department of Zoology, Andhra University, India (2004) contamination of ready-to-eat foods and beverages sold by street vendors and hawkers rendering them unacceptable for human consumption have become a global health problem. In tropical countries, fruit juices are common man's beverages and are sold at all public places and roadside shops. However in view of their ready consumption, quick method of handling, cleaning and extraction they could often prove to be a public health threat. Improper washing of fruits add these bacteria to extract leading to contamination (Geldrich, 1974).

In addition, use of unhygienic water for dilution, dressing with ice, prolong preservation without refrigeration,

unhygienic surroundings often with swarming house flies and fruit flies and air borne dust can also act as sources of contamination such juices have shown to be potential source of bacterial pathogen notably *E. Coli*, *Salmonella*, *Shigella* and *Staphylococcus aureus* (Buchanan *et al.*, 1999). Although the infectious dose for these contaminating bacteria in fruit juices is not yet well established based on the standards provided for drinking water (APHA, 1998) the numbers required to cause illness could be low particularly with reference to faecal Coliforms and *Streptococci*.

In India, there is always a great demand for fresh vegetables and fruit juices. Being tropical in location hot weather continues for a greater part of the year (February-September) increasing the need for these commodities. While most restaurants and cafes serve juices in apparently hygienic conditions in the roadside shops and recreational areas and busy market places, their microbiological quality remains questionable (Sandeep *et al.*, 2001). In these shops juices extracted by squeezing from a variety of fresh fruits namely oranges, grape pomegranate, apple, pineapple, watermelon, papaya, carrot and soon were served after considerable dilution with water and ice (Splittstosser, 1979).

Despite periodic quality control checks and closure of shops, out breaks of gastroenteritis caused by pathogenic *E Coli*, *Salmonella* and *Shigella* are not uncommon in these areas although a specific correlation has not been shown between out break of gastroenteritis and consumption of these juices (Kobayashi *et al.*, 1963). In view of the high demand for fresh fruit juices during summer and over crowding of streets vended shop in many areas in the city rapid review of the street vended fruit juices was under taken with a view to assess their safety for human consumption and as possible sources of bacterial pathogens (Uljas and Ingram, 1998). Driven by the harm caused by the poor quality of most packaged fruit juices, this work was therefore aimed at; Isolation and characterization of bacterial pathogen from some Nigerian packaged fruit juices, determination of the antibiotic susceptibility profiles and evaluation of the effect of pH and chemical preservatives on the growth of the isolates.

MATERIALS AND METHODS

Collection of samples: A total of seven packaged fruit juices samples were purchased from different selling point in Ogbomoso South West Nigeria. The juices had at least 3 months to their expiry date from the period of analysis. The fruit juice samples were approved by the appropriate regulatory agency; which is the National Agency for Food and Drug Administration and Control (NAFDAC).

Isolation of microorganisms: 12.85 g of peptone was dissolve in 500 ml of distilled water, then a sterile pipette was used to dispense 9 ml of the prepared peptone in screw capped bottle and then sterilized for 15 min at 121°C and allowed to cool. 1 ml of each packaged fruit juice sample was serially diluted and 1 m of an appropriate dilution was inoculated on sterile MacConkey, Nutrient agar, Salmonella/shigella agar and the plate was incubated for 24 h at 37°C. After 24 h sterile wire loop was used to pick the isolate from the plate and was streaked on a freshly prepared nutrient agar then incubated for 24 h at 37°C in order to get pure culture. The routine laboratory method of Cruickshank *et al.* (1975) was used to characterize different isolates. The isolates were identified using their macroscopic, cellular, physiological and biochemical characteristics.

Antibiotics susceptibility test: Sterile nutrient agar medium was poured into sterile petri dishes and allowed to solidify. A suspension of the isolated organisms was transferred into petri-dishes accordingly and swab over the entire plate, it was then incubated for 1 h at 37°C and a forcep was used to transfer each sensitivity disc on the plate and incubated for 24 h at 37°C. The antibiotics used included amoxyllin, ampicillin, tetracycline, ampicillin-cloxacillin, gentamycin, ofloxacin, augmentin, ciprofloxacin, ciprofloxacin, erythromycin, clindamycin, nitrofurantion, chloramphenicol, cotrimazole, norfloxacin.

Growth of isolate at different pH ranges: Nutrient broth was prepared and the pH was adjusted using 0.1 M phosphate buffer of different pH to adjust the pH of the broth to 3.0, 5.0, 7.0 and 9.0. It was then dispensed into screw-capped bottles and then sterilized in the autoclave at 121°C for 15 min. After cooling, the various test isolates were inoculated into it and incubated at 30°C for 48 h. Growth was detected by increase turbidity using Cecil 2031 (automatic) spectrophotometer. Uninoculated tubes serve as control. This test was done to detect the best pH that favours growth and metabolism as indicated by the increased turbidity (Schillinger and Lucke, 1989).

Evaluation of the effect of chemical preservatives on the growth of isolates

Growth of isolates in different concentration of benzoic acid: Nutrient broth containing 250, 750 and 1000 mg/l of benzoic acid was prepared and 10 ml of the broth was dispensed into sterile screw capped bottles and then sterilized for 15 min at 121°C. After cooling, the bottles were inoculated with the test organisms and incubated for 24 h at 37°C. Growth was detected using an automatic spectrophotometer. Increase in turbidity of the medium was recorded as positive for growth while a negative result shows no turbidity. Uninnoculated tubes serve as control.

Growth of isolates in different concentration of sodium chloride: Nutrient broth containing 2, 3, 4 and 5% (w/v) NaCl was prepared and sterilized at 121°C for 15 min. 20 ml of the broth was the dispensed into sterile screw capped vials aseptically. After cooling, the tubes were inoculated with the test organisms and incubated for 24 h at 30°C increased turbidity of the medium was recorded as positive for growth while a negative result shows no turbidity. Uninoculated tubes serve as control (Schillinger and Lucke, 1989).

RESULTS

A total of 8 organisms were isolated from some packaged fruit juice samples. The isolates were subjected to physiological and biochemical tests and they were identified to be *Bacillus licheniformis*, *Aeromonas hydrophila*, *Bacillus circulans*, *Proteus morganii*, *Pseudomonas cepacia*, *Bacillus alvei*, *Pseudomonas chlororaphis* and *Bacillus licheniformis* (Table 1).

Table 1: List of sources of isolates

Sample code	Isolates
A1	<i>Bacillus licheniformis</i>
B1	<i>Aeromonas hydrophila</i>
C1	<i>Bacillus circulans</i>
D1	<i>Proteus morganii</i>
E1	<i>Pseudomonas cepacia</i>
F1	<i>Bacillus alvei</i>
R1	<i>Pseudomonas chlororaphis</i>
V1	<i>Bacillus licheniformis</i>

Antibiotic susceptibility test was also carried out on the isolates; it was observed that all the organisms were resistant to Cifloxacin (CF) and Amoxyllin (AX). Most of the organisms were also resistant to Ampicillin (AM) except *Aeromonas hydrophila* with 12.5 mm zone of inhibition. Most of the organisms were resistant to Ampicillin-cloxacillin (AP), Cephalixin (CX) and Cotrimazole (CO) except *Bacillus circulans* with zones of inhibition of 14.0 mm for Ampicillin-loxacillin (AP) 18.00 mm for Cephalixin (CX) and 19.5 mm for Cotrimozole (CO). Most of the organisms were sensitive to Ciprofloxacin (CIP) except *Aeromonas hydrophila* and *Proteus morganii*. All the organisms were sensitive to Ofloxacin (OF). All were sensitive to Gentamycin (GN) except *Aeromonas hydrophila*. Four of the organisms were resistant to Tetracycline (TE) while *Proteus morganii*, *Pseudomonas Cepacia* and *Pseudomonas chlororaphis* were sensitive with 16.0, 16.5 and 13.0 mm zones of inhibition respectively. Some of the organisms showed resistance to norfloxacin (NB) except *Aeromonas hydrophila*, *Proteus morganii* and *Pseudomonas chlororaphis* with zones of inhibition of 13.5, 11.0 and 10.5 mm respectively. All the organisms were resistant to ceftriaxone (FX) except *Bacillus circulans* and *Bacillus alvei* with zones of inhibition of

Table 2: Antibiotic susceptibility profile of the isolates

Isolates	CF	AX	AM	AP	CX	CO	CIP	OF	GN	TE	NB	FX	E	C	N	CD	AU
<i>Bacillus licheniformis</i>	-	-	-	-	-	-	19.0	16.0	18.5	-	-	-	17.5	-	-	21.0	12.5
<i>Aeromonas hydrophila</i>	-	-	12.5	-	-	-	-	15.5	-	-	13.5	-	-	15.6	-	-	-
<i>Bacillus circulans</i>	-	-	-	14.0	18.0	19.5	18.5	19.5	18.0	-	-	12.0	19.0	-	-	18.0	14.5
<i>Proteus morganii</i>	-	-	-	-	-	-	-	17.0	20.0	16.0	11.0	-	-	19.0	12.0	-	-
<i>Pseudomonas cepacia</i>	-	-	-	-	-	-	15.0	14.5	15.5	16.5	-	-	-	-	13.0	-	-
<i>Bacillus alvei</i>	-	-	-	-	-	-	13.5	15.5	16.0	-	-	-	-	-	-	-	-
<i>Pseudomonas chlororaphis</i>	-	-	-	-	-	-	13.5	15.5	16.0	-	-	13.0	-	-	-	-	-

12.0 mm and 13.0 mm respectively. Most of the organisms were resistant to Erythromycin (E) except *Bacillus licheniformis* and *Bacillus circulans* with zones of inhibition of 17.5 mm and 19.5 mm respectively. *Aeromonas hydrophila*, *Proteus morganii* and *Pseudomonas chlororaphis* were sensitive to Chloramphenicol (C) with zones of inhibition of 16.5, 19.0 and 13.0 mm respectively while all the remaining organisms were resistant. Most of the organisms were resistant to Nitrofurantion (N) except *Proteus morganii*, *Pseudomonas cepacia* and *Pseudomonas chlororaphis* with zones of inhibition of 12.0, 13.0 and 15.5 mm respectively. All the organisms were resistant to Clindamycin (CD) and Augmentin (Au) except *Bacillus licheniformis* and *Bacillus circulans* with zones of 21.0 mm and 18.00 mm for Clindamycin (CD) and 12.5 mm and 14.5 mm for Augmentin (Au) respectively (Table 2). The survival of isolates at different pH ranges was monitored using spectrophotometer at wavelength of 560nm. It was observed that as the pH of the growth medium was tending from acidic to basic the growth rate of almost all the organisms increased. As the PH increased from 3-9 the Optical Density (OD) readings for *Bacillus licheniformis* increased from 0.078-1.401 nm, *Aeromonas hydrophila* increased from 0.099-1.373 nm, *Bacillus circulans* increased from 0.182-0.833nm, *Proteus morganii* increased from 0.111-1.394 nm and soon except for *Pseudomonas cepacia* which had optimum growth at pH 7 (Table 3).

The rate of growth of isolates was also observed in different concentration of sodium chloride (NaCl), It was found that as the concentration of sodium chloride increased the growth rate of all the organisms decreased as indicated by the optical density readings. It was observed that as the concentration of sodium chloride increased from 2-5% the optical density readings for *Bacillus licheniformis* reduced from 0.683-0.072 nm, *Aeromonas hydrophila* reduced from 1.234-0.098 nm, *Bacillus circulans* reduced from 0.609-0.198 nm, *Proteus morganii* reduced from 0.484-0.010 nm, this result shows that the higher the concentration of sodium chloride the lower the growth rate of the isolates (Table 4).

The growth rate of isolates in different concentration of benzoic acid was tested. It was observed that as concentration of benzoic acid increased from 250-1000 mg/l *Bacillus licheniformis* decreased from 1.330-0.167 nm, *Aeromonas hydrophila* decreased from 1.208-0.164

Table 3: Rate of growth of isolates at different pH (OD at 560 nm)

Isolates	pH3	pH5	pH7	pH9
<i>Bacillus licheniformis</i>	0.078	0.508	1.295	1.401
<i>Aeromonas hydrophila</i>	0.099	0.880	1.110	1.373
<i>Bacillus Circulans</i>	0.182	0.772	0.781	0.833
<i>Proteus morganii</i>	0.111	0.507	1.333	1.394
<i>Pseudomonas Cepacia</i>	0.038	0.887	1.332	1.158
<i>Bacillus alvei</i>	0.091	1.115	1.341	1.377
<i>Pseudomonas chlororaphis</i>	0.088	1.087	1.388	1.451

Table 4: Rate of growth of isolates at different concentration of Sodium chloride (OD at 560 nm)

Isolates	NaCl (%)			
	2	3	4	5
<i>Bacillus licheniformis</i>	0.683	0.392	0.106	0.072
<i>Aeromonas hydrophila</i>	1.234	0.474	0.200	0.098
<i>Bacillus Circulans</i>	0.609	0.520	0.344	0.198
<i>Proteus morganii</i>	0.484	0.358	0.338	0.010
<i>Pseudomonas Cepacia</i>	1.158	1.103	1.026	0.417
<i>Bacillus alvei</i>	1.222	0.904	0.626	0.410
<i>Pseudomonas chlororaphis</i>	1.951	1.108	0.890	0.846

Table 5: Rate of growth of isolates in different concentration of Benzoic acid (OD at 560 nm)

Isolates	250 mg/l	759 mg/l	1000 mg/l
<i>Bacillus licheniformis</i>	1.330	0.550	0.167
<i>Aeromonas hydrophila</i>	1.208	1.028	0.164
<i>Bacillus Circulans</i>	1.158	0.463	0.299
<i>Proteus morganii</i>	1.377	0.498	0.141
<i>Pseudomonas Cepacia</i>	1.333	0.479	0.097
<i>Bacillus alvei</i>	1.296	0.754	0.104
<i>Pseudomonas chlororaphis</i>	1.363	0.610	0.235

nm *Bacillus circulans* decreased from 1.158-0.299 nm, *Proteus morganii* decreased from 1.377-0.141 nm and soon. The result shows that the higher the concentration of benzoic acid the lower the rate of growth of the isolates (Table 5).

DISCUSSION

The presence of different bacteria in supposedly bacteria-free commercially available fruit juice is of concern. Their presence may pose risks to consumer's health and should not be taken for granted.

The result of antibiotic susceptibility test shows that 65% of the microorganisms isolated were resistance to the antibiotic used while 35% were sensitive. The high level of resistance of the bacteria strain to the antibiotic is a reflection of misuse or abuse of these antibiotics in the environment (Malik and Ahamed, 1994). The multiple

drug resistance of these bacteria is an extremely serious public health problem and it has always been associated with outbreak of major epidemics throughout the world (Prescott *et al.*, 2002).

The high acidity of fruit juice could cause account for low number and few types of organisms isolated, although the isolates have been found to be associated with food spoilage (Prescott *et al.*, 2002; Stainer *et al.*, 1987). Results obtained from the test for survival of isolates in different pH ranges indicated that when these microorganisms are in acidic medium their growth rate was reduced but as the pH tends from acidic medium to basic medium the growth rate of all the microorganisms increases which show that acidic medium greatly reduced their growth while in basic medium their growth was favored.

From the result of the test for effect of chemical preservative on growth of isolate, it can be deduce that chemical preservative used was effective against the microorganisms. It was observed that as the concentration of benzoic acid increases from 250mg/l to 1000mg/l the growth rate of all the microorganisms' decreases. Also as the concentration of sodium chloride (NaCl) increase from 2-5% the rate of growth of all the isolates decreases. Preservative have been used to store food substances and they act by inhibiting, retarding or arresting the growth of microorganisms (Ihekoronye and Ngoddy, 1995).

Also preservative may be microbicidal and kill the target organism or they may be microbiostatic in which case they simply prevent them from growing, thus improving the self-life of the product (Fawole and Osho, 2002).

To be in accord with good manufacturing practices the use of some chemical preservatives which are Generally Regarded As Safe (GRAS) should be put into consideration and these preservatives should not permit the growth of food poisoning organism while suppressing the growth of others that would make spoilage evident.

Fruit juices are well recognized for their nutritive value, mineral and vitamin content. They are beverages that are consumed for their nutritional value, thirst-quenching properties and stimulating effect or for their medicinal values.

Contamination of fruit juices by the bacteria may occur when the organism enters the processing plant or on the surface of the fruit having originated from soil, untreated surface water, dust and decomposing fruit. The degree of contamination varies depending upon how the fruit was handled from field and in the processing plant; proper handling, washing and sanitizing the fruit can contribute materially to the product good quality. The low PH of fruit juices greatly limits the number and the type of bacteria that can survive or grow at this pH but some bacteria that their pH is lower than that of the fruit juices can grow at this condition (Ryu and Beuchat, 1998).

Overall, it is contented that contamination is mainly due to poor quality of water used for dilution prevailing unhygienic conditions related to washing of utensils, maintenance of the premises and location by the side of waste disposal system or overcrowding. The occurrence of pathogenic bacteria in fruit juices is alarming enough for an immediate action by the suitable agency. It is suggested that regular monitoring of the quality of fruit juices for human consumption must be introduced to avoid any future pathogen out breaks.

Therefore, further analysis of commercially sold fruit juices should be done and regulation in the issuance of permit to produce and sell these product should be under strict quality control to reduce and mitigate exposure to harmful microbes deleterious to consumers health.

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