MICROBIOLOGICAL ANALYSIS OF PACKAGED ORANGE DRINK MARKETED IN KADUNA METROPOLIS

BY

OLUTIMAYIN , GBONJUBOLA OLUSESAN

A THESIS SUBMITTED TO POSTGRADUATE SCHOOL
AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL
FULFILMENT OF THE RQUIREMENT FOR THE AWARD OF
THE DEGREE OF MASTER OF SCIENCE IN PHARMACEUTICAL

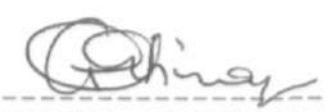
MICROBIOLOGY

DEPARTMENT OF PHAMACEUTICS & PHAMACEUTICAL MICROBIOLOGY

FACULTY OF PHAMACEUTICAL SCIENCES
AHMADU BELLO UNIVERSITY
ZARIA, NIGERIA
MARCH 1995

DECLARATION

I hereby declare that the work reported in this thesis was carried out by me under the supervision of Dr. J.A. Onaolapo and Dr. P.F. Olurinola, both of the Department of Pharmaceutics and Pharmaceutical Microbiology. It has not been presented in any previous application for higher degree. The work of other investigators are acknowledged and referred to accordingly.



OLUTIMAYIN, GBONJUBOLA OLUSESAN [MRS]
Dept. of Pharmaceutics & Pharm. Microbiology
Faculty of Pharmaceutical Sciences
Ahmadu Bello University
Zaria - Nigeria

CERTIFICATION

This thesis, entitled "Microbiological Analysis of Packaged Orange Drinks Marketed in Kaduna Metropolis" by OLUTIMAYIN, Gbonjubola Olusesan (Mrs), meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

DIGITIZED

SUPERVISOR
DR. J.A. ONAOLAPO, B.SC. PHARM (ABU),
M.SC. (ABU), PH.D (ASTON),
DEPARTMENT OF PHARMACEUTICS &
PHARMACEUTICAL MICROBIOLOGY,
FACULTY OF PHARMACEUTICAL SCIENCES,
AHMADU BELLO UNIVERSITY, ZARIA.

20/5/96
D A T E

CO-SUPERVISOR
DR. P.F. OLURINOLA, B.SC. PHARM (ABU),
PH.D (BRADFORD),
DEPARTMENT OF PHARMACEUTICS &
PHARMACEUTICAL MICROBIOLOGY,
FACULTY OF PHARMACEUTICAL SCIENCES,
AHMADU BELLO UNIVERSITY, ZARIA.

21/05/96
D A T E

EXTERNAL EXAMINER
PROFESSOR ODETOLA
B.PHARM, MS.C, PH.D (PROFESSOR OF
PHARMACEUTICAL MICROBIOLOGY,
DEPT. OF PHARMACEUTICAL MICROBIOLOGY,
UNIVERSITY OF IBADAN,
IBADAN.

D A T E

DIGITIZED

HEAD OF DEPARTMENT
DR. J.A. ONAOLAPO, B.SC. PHARM (ABU),
M.SC (ABU), PH.D (ASTON),
DEPARTMENT OF PHARMACEUTICS &
PHARMACEUTICAL MICROBIOLOGY,
FACULTY OF PHARMACEUTICAL SCIENCES,
AHMADU BELLO UNIVERSITY, ZARIA.

28/5/97
D A T E

DEAN, POSTGRADUATE SCHOOL,
AHMADU BELLO UNIVERSITY, ZARIA.

23/6/97
D A T E

TO

GOD BE

THE GLORY FOR

GREAT THINGS HE

HAS DONE IN

MY LIFE.

"Let not the wise man glory in his wisdom... But let him that glories glory in this: that he understands and knows God..." through the Lord Jesus Christ.

DEDICATION

This work is dedicated to:

MY DARLING HUSBAND
Engr. Rotimi Olutimayin

for his encouragement, perseverance
and constant love.

ACKNOWLEDGEMENTS

I wish to express my profound gratitude to my Supervisor, Dr. J.A. Onaolapo for his immeasurable efforts, fatherly care, commitment and useful suggestions given during the course of this work.

My thanks go to Dr. P.F. Olurinola, my co-supervisor, for his care and dedication.

My sincere appreciation goes to the technical staff and the entire staff of the Department. I am also grateful to Dr. J.O. Ehinmidu for his useful contribution.

My great appreciation goes to Mr. and Mrs. Moses Adepoju and the entire family for their rare love during the course of my programme. I remain grateful to the Babatundes and the entire members of the Zaria Family Fellowship for their constant prayer and encouragement.

My gratitude must also go to Rev. and Mrs. D.A. Bakare for their encouragement and prayers. The encouragement of Bro. Tunde Alabi cannot be forgotten.

To my mother, for giving me the solid beginning in education, I am grateful.

Above all, I give the Lord God of Host, the everlasting, the I AM that I AM, all the glory and honour due to Him.

ABU, ZARIA — OLUTIMAYIN, G.O. (Mrs)

March 1995

ABSTRACT

The microbiological quality of seven types of packaged orange drinks marketted in Kaduna metropolis were analysed by microbiological routine methods. The analysis involved the determination of the presence and the level of bacteria and fungi contaminants. The analysis was carried out under aseptic condition.

All the orange drinks analysed had high level of microbial contamination. The bacteria count observed was between 3.0 x l(r to 1.43 x 10^6 cfu/ml, also the mould/yeast count was between 8.0 x 10^3 to 1.31 x 10^5 spu/ml. The bacteria species detected consisted of Escherichia coli, Pseudomonas aeruginosa. Micrococcus spp and the spore-forming Bacillus subtilis. The mould/yeast consisted mainly of Aspergillus niger, Penicillium sp, Rhizopus oryza, Candida guillermondi and Candida tropicalis.

Orange drinks packaged in plastic bottles showed the highest bacteria count followed by those in glass bottles while those in foil paperboard showed the lowest bacteria count. However, drinks packaged in foiled paperboard showed the highest fungi count followed by those in glass bottle.

E. coli and Ps. aeruginosa were less sensitive to sodium benzoate and benzoic acid used in the drinks as preservatives than B. subtilis and Micrococcus spp. Ps. aeruginosa appeared to be

less sensitive to the preservatives than all the other isolates. Also, the isolates from the samples were found to be more sensitive than the standard cultures.

The result of the Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC) of the preservatives against the isolates and the standard cultures showed that the official recommendation of 0.1-0.5 ug/ml for their use looks relatively low.

The measurement of surface charge of the isolates and the standard cultures showed that E. coli and Ps. aeruginosa isolated from the packaged orange drinks showed more absorption to the anion and cation resins than the standard NCTC cultures while the B. subtilis standard NCTC 8236 however showed more absorption than the isolated B. subtilis from the orange drinks. These differences in the surface properties may have direct effect on the sensitivity of these organisms to the preservatives.

The result of this work has shown that the recommended concentration of the anti-microbial preservatives in the packaged orange drinks were not adequate. Also, there is need to adhere strictly to the Good Manufacturing Practice in the manufacturing companies.

TABLE OF CONTENTS

		Page
	Declaration	ii
	Certification	iii
	Dedication	v
	Acknowledgements	vi
	Abstract	vii
	List of Figures	xii
	List of Tables	xiii
	CHAPTER ONE - INTRODUCTION	
1.1	ORIGIN OF CITRUS FRUITS	2
1.1.1	COMPOSITION OF CITRUS FRUITS	2
1.2	THE SWEET ORANGES (CITRUS SINESIS) ..	4
1.2.1	THE SWEET ORANGE JUICE	5
1.3	PROCESSING OF PACKAGED SWEET ORANGE JUICE	7
1.3.1	INDUSTRIAL PROCESSING OF PACKAGED SWEET ORANGE JUICE	7
1.3.2	HOME-MADE PROCESSING OF PACKAGED SWEET ORANGE JUICE	8
1.3.3	THE PACKAGING MATERIALS	9
1.3.4	STORAGE OF PACKAGED ORANGE DRINK ..	10
1.4	SOURCES OF MICROBIAL CONTAMINATION OF PACKAGED ORANGE DRINK	10
1.4.1	RAW MATERIAL (ORANGE FRUIT)	10
1.4.2	WATER	12
1.4.3	PACKAGING MATERIALS	14

1.4.4	PERSONNEL	15
1.4.5	PROCESSING EQUIPMENT..	16
1.4.6.	PROCESSING ENVIRONMENT	17
1.4.7.	PROCESSING	18
1.4.8.	ADDITIVES	19
1.4.9.	STORAGE	20
1.5.	COMMON MICROBIAL CONTAMINANTS OF PACKAGED ORANGE DRINK	20
1.6.	PRESERVATION OF PACKAGED ORANGE DRINK ..	21
1.7.	PRESERVATIVES	22
1.8.	VARIOUS WAYS OF PRESERVATION OF ORANGE DRINK	23
1.8.1.	PRESERVATION BY HEAT OR PASTEURIZATION ..	23
1.8.2.	PRESERVATION WITH CHEMICALS	23
1.8.3.	PRESERVATION BY LOW TEMPERATURE ..	25
1.9.	JUSTIFICATION OF RESEARCH	25

CHAPTER TWO - MATERIALS AND METHODS

2.1.	MATERIALS	28
2.1.1.	ORANGE FRUIT DRINKS	28
2.1.2.	CULTURE MEDIA	28
2.1.3.	CHEMICAL REAGENTS	29
2.1.4.	GLASS WARE	29
2.2.	METHODS	30
2.2.1.	ISOLATION PROCEDURES..	31
2.2.2.	IDENTIFICATION OF MICROBIAL ISOLATES ..	31

2.2.2.1.	BACTERIA ISOLATES	32
2.2.2.2.	FUNGI ISOLATES	32
2.2.3.	SENSITIVITY TEST	37
2.2.4.	MINIMUM INHIBITORY CONCENTRATION ..	38
2.2.5.	MINIMUM BACTERIOCIDAL CONCENTRATION ..	39
2.2.6.	MEASUREMENT OF SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY	39
CHAPTER THREE - RESULT		
3.1.	BACTERIA CELL COUNT	41
3.2.	FUNGI COUNT	42
3.3.	IDENTIFICATION OF THE MICROBIAL ISOLATES FROM THE ORANGE DRINKS	43
3.3.1.	BACTERIA ISOLATES	43
3.3.2.	FUNGI ISOLATES	45
3.4.	PACKAGING MATERIALS VARIATION	47
3.5.	SENSITIVITY OFTHE ISOLATES TO BENZOIC ACID AND SODIUM BENZOATE	50
3.6.	DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERIOCIDAL CONCENTRATION (MBC) OF BENZOIC ACID AND SODIUM BENZOATE AGAINST THE ISOLATES FROM THE ORANGE DRINKS AND STANDARD NCTC CULTURES	51
3.7.	SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY (EIC)..	52
CHAPTER FOUR - DISCUSSION	.. .. ..	58
REFERENCES	.. .. ..	71

LIST OF FIGURES

FIGURE 1: SENSITIVITY OF THE BACTERIA ISOLATES FROM ORANGE DRINKS AND STANDARD NCTC CULTURES TO BENZOIC ACID .. 49

FIGURE 2: SENSITIVITY OF THE BACTERIA ISOLATES FROM ORANGE DRINKS AND STANDARD NCTC CULTURES TO SODIUM BENZOAT 50

FIGURE 3: SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY OF ISOLATED E. COLI FROM ORANGE DRINKS AND STANDARD E. COLI NCTC 10418 54

FIGURE 4: SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY OF ISOLATED B. SUBTILIS FROM ORANGE DRINKS AND STANDARD B. SUBTILIS NCTC 8236 55

FIGURE 5: SURFACE CHARGE BY ELECTROSTATIC INTERATION CHROMATOGRAPHY OF ISOLATED PS. AERUGINOSA FROM ORANGE DRINKS AND STANDARD B. SUBTILIS NCTC 6750 56

FIGURE 6: SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY OF ISOLATED MICROCOCCUS SP. FROM ORANGE DRINK 57

LIST OF TABLES

TABLE 1: NUTRIENT COMPOSITION OF SWEET ORANGE FRUIT .. 5

TABLE 2: AMOUNT OF VITAMIN B COMPLEX IN SWEET ORANGE JUICE .. 6

TABLE 3: TYPES OF SPOILAGE OF ORANGE FRUIT WITH EXAMPLES OF CAUSATIVE ORGANISMS 11

TABLE 4: COLONY COUNTS OF THE BACTERIA ISOLATED FROM THE ORANGE DRINKS 42

TABLE 5: FUNGI COLONY COUNT FROM THE ORANGE DRINK 43

TABLE 6: MICROSCOPIC AND COLONIAL MORPHOLOGY OF THE BACTERIA ISOLATES 44

TABLE 7: BIOCHEMICAL CHARACTERS OF THE BACTERIA ISOLATES .. 45

TABLE 8: CULTURAL MORPHOLOGY AND BIOCHEMICAL CHARACTERISTICS OF THE FUNGI ISOLATES 46

TABLE 9: MIC AND MBC OF BENZOIC ACID AND SODIUM BENZOATE AGAINST THE ISOLATED BACTERIA FROM ORANGE DRINK AND THE STANDARD NCTC BACTERIA SPECIES 52

CHAPTER ONE

INTRODUCTION

Fruits offer the most rapid and relatively cheapest means of providing adequate nutritional supplies of vitamins, minerals and fibres. They play a very prominent role in the socio-cultural, nutritional and economic development of Nigeria. There is an increasing awareness of the benefits of fruits over the last ten years in the domestic and industrial consumption habits of the people. The increase in demand could be attributed to increase supplies, higher standard of living, growing health and nutritional awareness and the increasing search for locally available raw materials to feed our growing agro-allied industries (Adeyemi, 1988).

Fruits are excellent sources of raw materials for our fresh, processed domestic food and for industrial markets. Fruits are highly perishable and, therefore, it is difficult to guarantee adequate and regular supply of fruits for local consumption. With the perishable nature of fruits, compounded by the on-going rapid urbanization and the demand for higher standard of living, their preservation becomes inevitable. Increased use of processing techniques appear to be one of the major solutions. Examples of fruits commonly found in Nigeria are citrus fruits, plantain, banana, pawpaw, pineapple, mango, carrot, guava and cashew.

The fruit of interest in this research is orange, which is a citrus fruit.

1.1 ORIGIN OF CITRUS FRUITS

Citrus species is believed to have originated from Southern Asia, especially South Vietnam and the southern regions of nearby China as far back as 2400 BC. Today, citrus trees are grown in many parts of the world wherever climatic conditions permit cultivation. Citrus fruit trees are found between latitude 40° N and 40° C where conditions are not too dry (i.e. not desert or on steppes), not too moist (i.e. not in tropical virgin forests) and not too cold (i.e. not at high altitudes) (Delfs-fritz, 1970).

There are many types of citrus fruits, e.g.

i] Citrus cinesis (Sweet orange)

ii] Citrus reticulata (Tangelo, Mandarin)

iii] Citrus limon (Lemon)

iv] Citrus aurantifolia (Lime)

v] Citrus aurantium (Sour orange)

1.1.1 Composition of Citrus Fruits

[a] Citric acid: This is obtained from citrus fruits, especially from limes and lemons. Other citrus fruits contain citric acid but the highest percentage of the acid is found in limes and lemons.

[a] Citronade: This is made from the peel of C. medica fruit.

[c] Pectin: Is also extracted from citrus peel. The peel may contain about 20-40% pectic substances; lemon peel also contain abundant source of pectin.

[d] Alcohol: May also be obtained from fruit skin and juice.

[e] Oil: Citrus fruit seed contains oil which can be extracted. Shoots, leaves, flowers and fruit, especially fruit peel, contain a high percentage of essential oils, mostly of terpenes (limoene).

[f] Other substances: Citrus fruit also contain scent and flavour-producing substances of alcohols, aldehydes, esters and sequiterpenes (Delfs-fritz, 1970).

The approximate characteristics of citrus juices are reported as follows:

pH	2.00- 6.00
Brix	9.20-15.00
Total sugar	0.9 - 11 %
Citric acid	0.50-8.20% of weight
Brix: acid ratio	3.54 - 12.24
Carotenoids	0.56-4.00 mg/litre
Xanthophyll	0.1-1.5 mg/litre
Ascorbic acid	28.00-93.00 mg/litre
Ash substances	0.23-0.41 %

1.2 THE SWEET ORANGES (CITRUS CINESIS)

This is a native of China or Cochin China from where it has spread to other tropical and sub-tropical regions of the world.

The tree grows to about 8-13 m with enclosed conical top. The branches are mostly covered with spines; leaves are smooth, glossy, light on the underside, slightly winged petioles, white fragrant flowers. The orange fruit is light orange or yellowish with smooth peel. It has a juicy pulp and sour sweet taste. The trees reach a respectable age between 30-35 years. The group of sweet oranges occupies a position of dominance in the citrus industry of the world, and deserved so, for no other citrus fruit is so universally liked and used. Sweet oranges are cultivated in all sections of the world where climatic conditions are favourable, and are grown on a much larger acreage than other citrus species (Webber and Batchelor, 1948).

The popularity of the sweet orange is probably due to its suitability for eating 'out of hand' as a fresh fruit or for serving as a desert fruit. Oranges were consumed in these ways, but in recent years, since the practice of using orange juice became popular, much of the crops have been utilized for juicing (Adeyemi, 1987).

TABLE 1: NUTRIENT COMPOSITION OF SWEET ORANGE FRUIT

NUTRIENT	CONTENT [%]
Water	87.2
Carbohydrate	11.2
Protein	0.9
Fat	0.2
Vitamin	1.4

Source: Jay (1978).

Industrial uses of sweet orange fruit are:

[a] Single strength orange juice;

[b] Concentrated juice for squashes;

[c] Dehydrated juice;

[d] Citrus juices;

[e] Essence oil from the peel;

[f] Peel products for marmalade;

[g] Animal feed meals.

1.2.1 The Sweet Orange Juice

The orange juice has a sweet or sour sweet taste.

The general composition of orange juice is as shown below:

Extract	9.57-12.71 %
Invert sugar	6.70- 8.30 mg
Cane sugar	1.46- 3.30 mg
Sugar free extract	2.20- 2.24 mg

Total acid	0.76- 2.24 mg
Pectin	0.08- 0.21 mg
Ash	0.32- 0.50 mg
Essential oil	2.60-14 mg
Vitamin C	29.60-54 mg
Ph	2.8 - 4.0 mg
Citric acid	0.95-3.41% of weight
Carotenoids	0.68-3.77 mg/litre
Xanthophyll	0.16-1.43 mg/litre
Ascorbic acid	28.00-92.20 mg

It was also found out that the sweet orange juice contains various types of vitamin B complex in various amount as shown in Table 2.

TABLE 2: AMOUNT OF VITAMIN B COMPLEX IN SWEET ORANGE JUICE

VITAMIN	AMOUNT [mg/100g]
B1	0.9
B2	0.03
B5	0.4
B6	0.06
B12	-

Source: Banwart (1987).

1.3 PROCESSING OF PACKAGED SWEET ORANGE JUICE

Due to the perishable nature of the orange fruit, the nutritional value of the fruit and the fact that large quantity of the sweet orange juice is consumed fresh at juice stands and at home, the phenomenal of processing the juice into various packaging materials becomes necessary for easy consumption and in order to be close to nature. The orange fruit juice was first packaged into cans in 1930 in the United States of America (Cruess, 1952). But in recent years, it has become an international drink being packaged all over the world.

1.3.1 Industrial Processing of Packaged Sweet Orange Juice

The fruit to be used for juice production should be orchard run, freshly picked and should not consist of packing house culls that have stood too long in a packing shed. If the fruits had stayed for too long in storage, it is apt to give juice with poor flavour and aroma. It should be well ripened. Also, if the oranges are delivered in bulk by truck and stored in ventilated slant-bottomed bins, it may also cause damage to the fruit, particularly if it is allowed to stand in the bins several days before use. The best quality is obtained by using the fruit as soon as delivered and with as little rough handling as possible from the grove.

The orange fruit is sorted on a belt to remove rots and unfit oranges. The fruit is then washed by passing it through a tank of water or soapy water, then to a brush washer where rapidly revolving brushes and sprays wash the oranges very vigorously and thoroughly. In order to reduce the number of micro-organism, a strong antiseptic solution may be used in the tank of water before the brush washing. Sorting is then repeated with brush washed fruit and then graded for size in order that each juice-extracting machine will operate on fruit of fairly uniform size. The oranges are then automatically cut in half by circular knives and are then reamed on 'Brown' juice extractors, which gives a pulpy juice. The juice is then sieved to give a fine-grained cloudy juice. The juice is de-aerated under high vacuum in a stainless steel chamber through which the juice travels in a thin layer or film or into which it is sprayed. The juice is then ready to be packaged into various packaging materials. The canned juice is heated to about 91° C and rapidly cooled under sprays of cold water with vigorous agitation in order to inactivate enzyme, particularly the enzyme responsible for curdling of the juice after canning (Pekzar and Reid, 1965).

1.3.2 Home-made Processing of Packaged Sweet Orange Juice

Fresh orange fruit is also preferred for high quality in flavour and to avoid spoilage. The orange fruit is washed

thoroughly with water or soapy water. The fruit is peeled to avoid bitterness of the juice. The fruit is squeezed into a bowl and the seeds removed through local sieve. Quality amount of sugar is dissolved in warm water and then allowed to cool. The sugar solution is then added to the orange fruit juice. The final drink can be taken immediately or stored in a refrigerator.

1.3.3 The Packaging Materials

There are various types of packaging materials used in packaging the final product, such as glass bottles, plastic containers, paper-board and aluminum foil paper. The use of cans in packaging is fading out. This might be due to the fact that metals react with the acidic contents of the drink.

Glass bottles can also react with the chemicals but is still being used. It has an advantage of transparency so that the content is clearly seen.

Paperboards, foil paper and plastic containers are the most utilized recently. The advantages of use of paperboard, foil paper and plastic containers include:

[a] unbreakable, therefore, easy for transportation, and

[b] do not react with chemicals.

1.3.4 Storage of Packaged Orange Drink

Packaged orange juice stored at room temperature deteriorates rather rapidly in flavour and aroma. Therefore, packaged orange drink is better stored under refrigeration between 1-4°C. This makes it to retain its flavour for several months with very little deterioration.

1.4 SOURCES OF MICROBIAL CONTAMINATION OF PACKAGED ORANGE DRINK

When there is an undesirable presence of high proportion of microorganisms, usually of bacteria and moulds, in a product, exceeding the levels acceptable for safety when used, the product is said to be contaminated by microorganisms.

Sources of microbial contamination in a product are related to the raw material used in the processing of the final product, the processing equipment, the operators, the processing environment, the processing, the packaging material and storage of the final product.

1.4.1 Raw Material (Orange Fruit)

Certain microorganisms are found in the orange fruit. The fruit can be attacked by microbes either on the tree, during

harvesting or on storage. The microorganisms may produce changes in the appearance, flavour, odour and other qualities of the fruit. Although most of the bacteria in or on fruit neither cause spoilage nor health hazards. The common type of spoilage and their causative bacteria are shown in Table 3.

TABLE 3: TYPES OF SPOILAGE OF ORANGE FRUIT WITH EXAMPLES OF CAUSATIVE ORGANISMS

TYPE OF SPOILAGE	MICROORGANISMS INVOLVED
Soft Rot	Rhizopus, erwinia
Gray Mould Rot	Botrytis cinerea
Black Rot	Aspergillus niger
Blue Rot	Penicillium group

Several genera of yeast are important in the spoilage of fruits. Untreated orange fruit usually support an extensive and varied microflora in the final product.

The orange juice during processing, may be affected by microorganisms. Some microflora that have been found to cause spoilage of orange juice are Lactobacillus sp, Gluconobacter oxydan and Acetobacter which cause off flavour and several genera of yeast (Pekzar and Reid, 1965).

Therefore, the raw material (orange fruit) should be properly treated and handled before and during processing.

1.4.2 Water

Water has multiple uses in the processing of orange drink. It is used as constituent, in washing and cooling processes. The larger part of the packaged orange drink is water, therefore the aqueous nature of the orange drink makes it a more suitable environment for the growth and survival of both bacteria and moulds. It was found out that preparations containing water present more microbiological problems than the dry preparations (Baird, 1987).

Micro-organisms which are usually found in fresh water include Pseudomonas spp, Alkaligenes spp, Flavobacterium spp, Chromobacter spp, and Seratia spp. These microflora are usually nutritionally undemanding and often have a relatively low optimum growth temperature. Some bacteria are introduced as a result of soil erosion, heavy rainfall and decaying plant matters, these microorganisms are Bacillus subtilis, B. megaterium, Klebsiella aerogenes and Enterobacter cloacae contamination by sewage results in the presence of Proteus spp; E. coli and other enterobacteria, Streptococcus faecalis and Clostridium spp. Due to unfavourable conditions, bacteria which are introduced as a result of animal or plant debris usually die (Colling, 1964).

An examination of stored industrial water suppliers showed that 98% of the contaminants were Gram negative bacteria; other organisms isolated were Micrococcus spp, Cytophaga spp; yeast-like fungi and actinomycetes (Chambers and Clark, 1968). If

microorganisms colonize a storage vessel, it then acts as a microbial reservoir and contaminates all water passing through it. Water from surface has abundant microflora than that of supplies from fast moving or deep water sources such as well or spring water.

Salmonella, Shigella, Leptospira, enteropathogenic Escherichia coli, Vibro as well as protozoa such as Entamoeba hystolylica have been found to be present in untreated water (Mitchell, 1972).

Portable water is considered to be unsuitable for use in food industry because it is a source of many contaminating organisms and it acts as a suitable medium for microbial proliferation.

Contamination of freshly distilled water has been known to occur through collection material or during storage (Baird, 1987).

Counts of the order of 10^5 bacteria per ml (all Gram negative bacilli) in distilled water fresh from the still was reported by Sykes (1972); but on replacing the plastic tubing daily the level of contamination dropped to less than one organism per ml.

Contamination of deionized water usually occur as a result of the ion exchange column which can retain organic debris and other nutrient alike. The ion exchange column make a good breeding place for bacteria if it is not easily accessible to continuous cleaning and sterilization (Underwood, 1982).

Due to all these microbial contamination of water sources, the water used in the processing of the orange drink should be treated properly by chemicals such as sodium hypochlorite and chlorine or by boiling, filtering or passing through ultra-violet sterilizer.

1.4.3 Packaging Materials

The roles of packaging material are to contain the product and to prevent the entry of microorganisms or moisture which may result in spoilage. Therefore, it is important that the sources of contamination is not the packaging material itself.

Glass bottles are sterile on leaving the furnace, but are often stored in dusty conditions and packed for transport in boxes. They may as a result of this contain mould spores of Penicillium spp; Aspergillus spp; and bacteria such as Bacillus spp.

Plastic bottles have a low microbial count and may not require disinfection but in a case where they are also transported in a non-sanitary packaging material, they may become contaminated into mould spores (Hugo and Russell, 1987).

Paperboard unless treated, carry mould spores of Cladosporium spp; Aspergillus spp; and Penicillium spp and bacteria such as Bacillus spp and Micrococcus spp.

The bottle covers or closures can contribute to contamination of the final product, for example Erwinia and Enterobacteriaceae were isolated from Capliners (Smart and Spooner, 1972).

Laboratory service 1971, found that contaminants arose from improperly washed containers.

Moist heat, chemicals, ethylene oxide, or formaldehyde gas, or irradiation may be used in sterilizing containers and closures before use.

1.4.4 Personnel

The natural skin flora organisms Staphylococcus aureus is common on the hands and face. It can not be eliminated by washing because it resides in the deep layers of the skin. This microflora can be transferred from the operating personnel to the final product of the orange drink. Other bacteria which can be found in the bodies of the operator are Sarcina spp; diphtheriods; occasionally Gram negative rods such as Mima spp and Alcaligenes spp; Lipophilic yeast found in fatty and waxy sections of the skin, Pityrosporum ovale found on the scalp, P. orbicular on glabrous skin, dermatophytic fungi such as Epidermophyton spp; Microsporon spp; Trichophyton spp; and Saprophytic bacteria found in the ear secretions (Denyer, 1987).

Microflora apart from the normal flora may be transferred from the operator as a result of poor personal hygiene e.g. faecal organisms from the anal region or bacteria from a wound. Micrococci, enterococci and -haemolytic and non-haemolytic streptococci, Clostridium spp; Bacillus spp and Gram negative intestinal bacteria with Staphylococcus aureus in 20% can be supported by open wounds with clinical manifestation of bacteria growth. Staphylococcus aureus, Streptococcus pyogenes, enterococci, coliforms, Proteus spp and Pseudomonas aeruginosa are supported in growth by wound showing signs of infection (Skinner and Carr, 1974).

Larger number of Staphylococcus aureus, few of Staphylococcus albus may be found in nasal passages. Streptococcus salivarius or Neisseria pharyngis, pathogens such as Haemophilus influenzae and Klebsiella pneumonia may be present in the nasopharynx and saprophytic streptococci of the viridans group are secreted during normal respiratory function (Underwood, 1987).

During normal activities scales of skin are continuously shed into environment at a rate of 1000 scales per minute and an individual looses 6g scales everyday, which contain microorganisms (Denyer, 1987).

Avis, (1975) found out that even clothing are known to shed debris which may be loaded with contaminants.

Comprehensive training in personal hygiene coupled with regular medical check-ups may reduce contamination of the final packaged orange drink by the processing personnel.

1.4.5 Processing Equipment

The equipment used in the processing of the orange drink can support the growth of microorganisms. When the previously processed drink is not properly cleaned from the equipment, it can serve as nutrient for microbes. Salmonella has been found to be present in the processing equipment (Packer & Litchfield, 1972).

Therefore, the processing equipment should be easy to dismantle, clean and sterilized appropriately to avoid making it reservoir for micro-organisms.

1.4.6 Processing Environment

The microorganisms which have been isolated from air include non-spore forming bacteria Streptococcus spp; Staphylococcus spp; and Corynebacterium spp; the spore forming bacteria Bacillus spp; Clostridium spp; the moulds Penicillium spp; Cladosporium spp; Aspergillus spp; and Mucor spp as well as the yeast Rhodotorula spp (Gregory, 1973).

The amount of microorganisms in the atmosphere depends on the activity in the environment and the amount of dust which is disturbed. Therefore, an area containing working machinery and active personnel will have a higher microbial count than one with a still atmosphere and the air count of a dirty, untidy room will be greater than that of a clean room. Humidity also influence the microbial growth, that is, a damp area will have higher amount of microbes than a dry area. Particles of dust, skin or clothing, or droplets of moisture or sputum following talking, coughing or sneezing are factors which contribute to the microbial flora of the air.

The walls and the ceilings surrounding the processing equipment can also be source of microbial contamination. Moulds

are the most common flora of the walls and ceilings, for example Cladosporium spp; Aspergillus niger, A. flavus, Penicillium spp; and Aurebasidium spp. Painted wall and poor ventilation can also lead to the growth of these microorganisms (Nishannan, 1977).

Floor, drain pipes, doors, windows and fitting for electricals and water can contribute to microbial contamination of the environment of the processing plant. Crackings on the wall and floor can also contribute to microbial contamination of the processing environment.

Dust should be prevented from collecting on the surfaces to prevent microbial contamination.

The processing environment should be kept as much as possible clean and dry. It can be disinfected as often as possible before processing begins.

1.4.7 Processing

The processing of the orange drink can contribute to the growth or contamination of the product. Microbial contaminations can occur at the various stages of processing the orange drink especially the drinks that are processed locally or home made orange drinks. In the established industries, sorting, extraction and sieving can lead to microbial contamination. Bacillus

subtilis, Staphylococcus aureus, Salmonella, Aspergillus spp; Penicillium spp and some yeast spp have been found in the orange drink during processing (Jackson, 1990).

1.4.8 Additives

[a] Preservatives:

Most organism preservatives are metabolized readily by many bacteria and fungi. They serve as growth substrates and concentrate well below 'use' level.

Some preservatives react with other components of the product and are thereby decreased in concentration, this may lead to increase in the microbial growth in the final product.

[b] Colourings and Sweetness:

Colourings and sweetness have been shown to support bacterial and fungal growths.

Sugars used in the packaged orange drink are ready substrate for microbial attack. Several genera of yeast can be isolated from sugar solution. Fermentation of the sugar by these organisms causes foul flavours due to the production of

alcohol, lactic acid and other organic acids while the production of carbon dioxide leads to gassing and troublesome pressure increase (Margalith and Schwartz, 1970).

1.4.9 Storage

Packaged orange drink stored in a hot humid temperature can support the growth of microorganisms depending on the length of storage.

1.5 COMMON MICROBIAL CONTAMINANTS OF PACKAGED ORANGE DRINK

Microorganisms involved in contamination of packaged orange drink are mostly the spore forming bacteria species such as Clostridium and Bacillus. Lactobacillus, Leuconostoc, Acetobacter, non-spore formers (mostly lactic acid types of bacteria), yeasts and moulds have been found in packaged orange drink (Pekzar and Reid, 1965).

The common microbial contaminants of the packaged orange drink are also of the pathogenic types such as Salmonella, E. coli which can be as a result of faecal material from the personnel.

1.6 PRESERVATION OF PACKAGED ORANGE DRINK

Orange juice are liable to support growth of bacteria and moulds unless it is adequately preserved. To prevent microbiological deterioration of the packaged orange drink, the product must be preserved. Deterioration through microorganisms is prevented by the addition of antimicrobial preservatives into the orange drink. The antimicrobial preservatives are agents which prevent the growth and proliferation of microorganisms in a product. Antimicrobial preservatives cannot be used in place of hygienic procedures while processing or the use of clean, treated raw materials.The reason for the above is that if the microbial load of a product is small then the preservative will be more effective but the preservative may totally fail, if the microbial load is high.

The need for preservation arise due to the various steps of processing the orange drink. The sweet orange juice serve as good media for the growth of many different microorganisms. Given a chance to grow, the microorganisms will produce changes in appearance, flavour, odour and other qualities of the orange juice. For instance, microorganisms such as Clostridium sp; Bacillus spp; non-spore forming yeast and moulds have been found to cause spoilage of packaged orange drink.

1.7 PRESERVATIVES

An ideal or satisfactory preservative is a substance which remains effective in a product where it is used until the product is consumed. This quality depends on a number of factors such as:

[a] Ability to kill all microorganisms likely to enter the final product, that is, the preservative must be microbiocidal. If the preservative is not microbiocidal, it can at least be microbiostatic but if microbiostatic, then the microorganisms can adhere to the product during storage.

[b] Should be effective at low concentration. Concentration of preservative should not be more than 1%.

[c] The ideal preservative should not be toxic to human being and should be non-irritant.

[d] It should have sufficient stability to withstand manufacturing procedures and therefore remain active throughout the intended life of the product.

[e] It should be effective over a wide range of pH.

[f] Selective reactivity with microbes and minimum attraction for ingredient of the product.

[g] The ideal preservative must be free from objectionable odour and flavour.

1.8 VARIOUS WAYS OF PRESERVATION OF ORANGE DRINK

There are various ways of preserving packaged orange drink due to its perishable nature.

1.8.1 Preservation By Heat or Pasteurization

This method is mostly used in the canned fruit juice. This is done by heating the canned juice to a temperature of 91°C and then rapidly cooled under sprays of cold water with vigorous agitation in order to inactivate enzyme, particularly the enzyme responsible for cuddling of the juice after canning.

1.8.2 Preservation With Chemicals

Addition of chemicals to foods for the purpose of preservation is subject to the provision of the Federal Food, Drug, Cosmetic Act of 1938. According to this act, a food is adulterated if any poisonous or deleterious substance has been added which may render it injurious to health (Pekzar and Reid, 1965).

There are various types of chemical preservatives which are used in preservation of fruit juice:

[a] Benzoate of Soda (Sodium Benzoate):

Fruit juices can be preserved satisfactorily by 0.1 - 0.5% of

the benzoate. The salts of benzoic acid are more readily soluble than the acid, therefore the sodium salt is employed in preference to the acid. The benzoic acid exerts a selective action upon the organisms, often preventing the growth of yeasts and moulds, but they permit the development of vinegar and lactic acid bacteria. The pH value of the juice often affect the preservative action of sodium benzoate, the preservative action being much greater at low pH than at higher pH values. The sodium benzoate possesses a disagreeable 'burning' taste, that readily perceptible in juice containing 0.1% of the benzoate.

The benzoic acid would uncouple substrate transport and oxidative phosphorylation from the electron transport system by making the cytoplasmic membrane freely permeable to protons. Benzoates interfere with the utilization of acetate required for the formation of energy rich compounds which results in blockage of cell metabolism.

[b] Sulfurous Acid:

Fruit juice can be preserved for more than a year by the addition of 0.1% of sulfurous acid, provided the juice is made from sound fruit and stored in clean containers at a temperature not above 16°C. It can not be used in juice that is to be stored in tin or other metal containers, due to the fact that in contact with metal, the sulfur dioxide is reduced to hydrogen sulphide with the development of a disagreeable

flavour. The sulfurous acid is not commonly used.

[c] Sugar:

All fruit juices may be preserved by the addition of sugar or by increasing the natural sugar content of the juice by concentration.

1.8.3 Preservation By Low Temperature

Fruit juices can be stored at very low temperature in order to prevent the growth of microorganisms. Temperature of about -20°C can keep successfully the packaged orange drink. It can be kept in the refrigerator for preservation.

1.9 JUSTIFICATION OF RESEARCH

Certain microorganisms such as Salmonella spp (WHO Expert Committee, 1988; WHO, 1989; D'Aoust, 1989), E. coli (Starvric and Speirs, 1989; Doyle and Padhye, 1989), and certain other microbial agents of enteric infection (Schwartz et al, 1988; D'Aoust, 1989) can contaminate processed drink at every stage of production, from collection of the raw materials through processing, transport and storage to final purchase and consumption (Jackson et al, 1991).

The microorganisms of concern may be in the soils and waters

that support the plant. They may even survive on surfaces in transport vehicle, on the equipment or in the environment of processing plants, in or on the bodies of the handlers; on the packaging material. Water used in the processing of the fruit drink can also contribute to the growth or contamination of the product. These microorganisms are frequently able to grow in the product not only because of temperature abuse and poor quality control but also because certain microorganisms (psychrotolerant or psychrotrophic bacteria) can reproduce to some extent even under refrigeration.

Increasing volume of product from a single manufacturing source through wider distribution networks may lead to more massive contamination and bigger food-borne disease outbreaks.

Most organic preservatives are metabolized readily by certain microorganism and may serve as growth substrates at concentration well below 'use' level.

The proliferation of local companies who are involved in the manufacture of orange drinks with little or no emphasis on quality control, where bottles and other packaging materials are reused may lead to more microbial contamination of the orange drinks.

Some of these drinks are even manufactured at homes or at the backyards where there is no knowledge of quality control or laid down standard of manufacturing or processing of the orange drink.

Therefore, it is necessary for an independent microbiological analysis of some of the packaged orange drinks.

This research will attempt the analysis of selected orange drinks marketed in Kaduna metropolis with the aim of determining the presence of bacteria and fungi contamination, level of microbial contamination and types of contaminants. It is anticipated that the degree of type of contamination would give an idea on the effectiveness of the recommended preservatives in the packaged orange drink.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Orange Fruit Drinks:

Seven different types of orange fruit drinks were analysed. They were all marketed in Kaduna metropolis. The different orange drinks are as follows:-

(a)	Royal Crown Orange	-	Sample A
(b)	Paddy Natural Fruit	-	Sample B
(c)	Caprisone Orange Drink	-	Sample C
(d)	Vegfru Orange Drink	-	Sample D
(e)	Treetop Orange Drink	-	Sample E
(f)	Suntop Orange Drink	-	Sample F
(g)	Fruitmaid Orange Drink	-	Sample G

Four samples of each type of the drinks were analysed.

2.1.2 Culture Media:

The culture media used were:-

(a) Nutrient Agar (NA) (Oxoid)

(b) Sabouraud's Dextrose Agar (SDA) (Oxoid)

(c) Nutrient Broth (NB) (Oxoid)

(d) MacConkey Agar (MA) (Gibco Brl)

(e) 0.03% Centrimide Agar

(f) Mannitol Salt Agar (MSA) (Gibco Brl)

All media were prepared according to manufacturers direction and autoclaved at 121°C for 15 mins.

2.1.3 Chemical Reagents:

(i) Crystal Violet

(ii) Lugol's Iodine

(iii) 95% Alcohol

(iv) Dilute Carbol Fuchsine

(v) Methylene Blue

(vi) Peptone Water

(vii) Andrade's Solution

(viii) Lactose

(ix) Glucose

(x) Sucrose

(xi) Paradimethyl Amino - Benzaldehyde

(xii) Hydrochloric Acid

(xiii) Saturated Aqueous Potassium Phosphate

(xiv) Koser's Citrate Medium

(xv) Methyl Red Solution

(xvi) Creatinine and 5ml of 40% Potassium Hydroxide Solution

(xvii) Hydrogen Peroxide

(xviii) Aqueous Solution of Tetramethyle-Phenylenediamine

(xix) Benzoic Acid

(xx) Sodium Benzoate

(xxi) QAE Sephadex

(xxii) CM Sephadex

(xxiii) Tween 80

2.1.4 Glass Ware:

(a) Petri Dishes

(b) MacCartney Bottles

(c) Bijou Bottles

(d) Test-Tubes

(e) Durham Tubes

(f) Pipettes

(g) Pasteur Pipettes

(i) Glass Slides

Glass ware were sterilized at 160°C for 1 hour.

2.2 METHODS

2.2.1 Isolation Procedures:

Each sample of orange drinks was diluted between 1:10 to $1:10^5$ in sterile normal saline. For bacteria count, 1.0ml of each dilution was added to each MacCartney bottle containing the sterile NA and 3% Tween 80 and mixed thoroughly. The contents of the MacCartney bottles were poured into sterile petridishes and allowed to set. They were incubated for 24 - 36 hours at 37°C. After which the colonies which developed were counted using colony counter.

For fungi count, the same procedure as for bacteria was carried out but the sample was diluted between 1:10 to $1:10^3$ and sterile SDA was used as the growth medium. The incubation was at 25°C for 7 days.

All experiments were carried out in an aseptic cupboard and repeated three times.

2.2.2 Identification of Microbial Isolates:

2.2.2.1 Bacteria Isolates:

The bacteria isolates were identified using their colonial morphology, cellular morphology and biochemical characteristics as described below:-

[a] Colonial Morphology:

The following parameters were used in the colonial identification: shape, colour, elevation, transparency, edge and surface texture.

[b] Cellular Morphology:

The cellular morphology of the isolates was determined using the following methods:-

Simple staining:

A smear of the culture was made on a clean glass slide and heat fixed After cooling, methylene blue solution was dropped on the fixed smear and allowed to stay for 3 minutes, it was washed off and dried. The slide was then examined under the oil immersion lens.

Gram's staining:

A smear of the culture was made on a clean glass slide and heat fixed. The slide was flooded with crystal violet after cooling, the crystal violet was left for one minute after which it was washed off with Lugol's iodine solution and then Lugol's iodine solution was dropped on the slide for one minute after which it was washed off with Lugol's iodine solution and then Lugol's iodine solution was dropped on the slide for one minute and drained off. It was decolorized with 95% alcohol until no colour was observed coming out from the slide, it was then washed off with distilled water. The slide was counterstained with dilute carbol fuchsine for 30 seconds, drained, and washed off with distilled water and dried with blotting paper. The slide was then examined under the oil immersion lens (Cowan, 1974).

Other Identification:

For further identification selective media, i.e. 0.03% centrimide agar for Pseudomonas aeruginosa, Mannitol salt agar for Staphylococcus aureus and MacConkey agar for E. coli were used.

Sterile 0.03% centrimide agar was dispensed at 15 ml in MacCartney bottles. Colonies of the bacteria cells harvested from the isolation procedures were grown on nutrient agar slope in many MacCartney bottles and further subcultural in nutrient broth at 37° C for 24 hours. The sterile 0.03% centrimide agar was poured into

sterile petridishes after melting in a water bath. The agar plates were allowed to cool and set. A loopful of the nutrient broth culture was used to streak the surface of the centrimide agar plate and incubated at 37° C for 24 hours.

The same process as above was carried out for mannitol salt agar and MacConkey agar. All the experiments were carried out in duplicates. Characteristic colonies were further Gram stained and tests carried out according to Cowan (1974) for identification of bacteria species.

[a] Biochemical Tests:

Various biochemical tests were carried out as confirmatory tests for the different bacteria isolates according to Sharby (1978).

[b] Sugar Fermentation Test:

The ability of the isolates to ferment sucrose, lactose and glucose was tested. 1% of each sugar was prepared in peptone water, using Andrade's solution as indicator. The solution was dispensed into Bijou bottles and a durham tube was placed in each bottle in an inverted position. The bottles were autoclaved at 115°C for 30 minutes to avoid destruction of the sugars. Two drops of 24 hour-broth culture of each isolate was inoculated into each bottle. Some bottles which were not inoculated with the organisms, served as controls. Both test

and control bottles were then incubated at 37°C for 5 days. A red colour indicated acid production while collection of gas in the durham tubes indicated gas production.

[c] Eijkman's Test:

Same process as for lactose fermentation test was carried out but the bottles were incubated in a water bath at 44.5°C for 24 hours to confirm the presence of E. coli.

[d] Indole Formation Test:

10ml of xylene was added to 5 day-broth culture and was shaken thoroughly and allowed to stand until the xylene collected on the surface. 1.0ml of solution A containing Paradimethylaminobenzaldehyde (1.0gm), 95.0ml of 95% and 20.0ml of Hydrochloric acid was added down the side of the tube. Colour reaction was noted. When no colour appeared within a minute, 1.0ml of solution B containing saturated aqueous potassium phosphate was added. A pink colouration indicated production of indole.

CITRATE UTILIZATION

The isolates were streaked on sterile koser's citrate medium slopes in test-tubes. The test-tubes were then incubated at 37°C for 7 days. The changing of the colour of medium from light green to blue indicates the utilization of citrate.

METHYL RED TEST (MR):

Five drops of 0.04% methyl red solution was added to 3 day-MRVP medium culture. A red colour indicates methyl red positive while a yellow colour indicates methyl-red negative.

VOGAS PROSKAVER TEST (VP):

A trace of creatinine and 5.0ml of 40% KOH solution were added to 3-day MRVP culture, the tube was shaken properly. A pink colour indicates VP positive while no colour is VP negative.

CATALASE TEST:

Some of the culture was emulsified on slide and a drop of 10 volume of hydrogen peroxide was added to it. The presence of catalase was shown by rapid evolution of gas.

OXIDASE TEST:

Two-three drops of a 1.0% aqueous solution of tetramethyl-Phenylenediamine was placed on a one-inch square of filter paper laid on a clean porcelain tile. The culture was smeared on the paper in a line about 5.0mm long. Oxidase positive indicated by the colour of the culture which turned dark purple in 5-10 seconds.

2.2.2.2 FUNGAL ISOLATES:

The fungal isolates were identified using conventional methods i.e. cultural and microscopic morphology (Bryan, 1977).

(i) Cultural Method:

The appearance on the culture medium.

(ii) Microscopic Morphology:

A portion of the isolate was put on a glass slide and lactophenol blue was dropped on it. It was then observed under X10 objective lens.

For the yeast species, both simple staining and Gram's methods were used for their identification.

2.2.3 SENSITIVITY TEST:

The sensitivities of the isolated organisms to the used preservatives were tested.

The cup plate method was employed for this test. A 24 hour broth culture of each isolate was used throughout this experiment

15ml of sterile NA was inoculated with the 24 hour broth culture, shaken properly and poured into a sterile petridish and allowed to set. A sterile cork borer was used to bore five evenly distributed cups into the agar. Different concentrations of the preservatives, benzoic acid and sodium benzoate used in the orange

drinks were tested, a 1 hour predifusion time was allowed, after which the plates were incubated at 37°C for 24 hours. The zones of inhibition were then measured. The sensitivities of standard organisms to the preservatives were also tested as control (Waterworth, 1978).

2.2.4 MINIMUM INHIBITORY CONCENTRATION (MIC):

Serial dilution method was employed for the MIC test. 10 test-tubes containing 2.5mls NB were set. The first test-tube contained double strength NB. 2.5mls of the preservative was added to the first tube and shaken properly. 2.5mls was withdrawn out of this tube and added to the second tube, this dilution was continued until the 10th tube, where 2.5mls were withdrawn and discarded. The tubes were then inoculated with the test organism and incubated at 37% of 24 - 72 hours. All the test were carried out in duplicates. The tubes were compared with the control which contained no organisms.

The above experiment was carried out to test the MIC of the preservatives against the standard organisms. The least concentration where there was no growth is the MIC (Waterworth, 1978).

2.2.5 MINIMUM BACTERIOCIDAL CONCENTRATION (MBC):

Sterile NA plates were streaked with the contents of the tubes which contained the MIC to the tube with the highest concentration where there was no growth. The plates were then incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration of the agent that showed no colony on subculturing (Waterworth, 1978).

2.2.6 MEASUREMENT OF SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY (EIC):

The relative surface charge was measured by the attachment of cells to ion exchange resins. The method employed was according to Onaolapo (1986). Pasteur pipettes about 85mm long and 5mm internal diameter were plugged with glass wool and filed to a length of 40mm. The pasteur pipettes were packed with QAE sephadex (anion exchanger) which has quartenary ammonium as the functional group. Other set of Pasteur pipettes were packed with CM sephadex (which has sulfuric acid as functional group).

To dampen down the charges on the gel, the columns were washed with the eluent after packing.

Cell suspensions to be tested were washed at least twice with the appropriate eluent using centrifuge at maximum speed of 10 r.p.m for 10 minutes. Cells were re-suspended to $OD_{470\ nm}$5.0 0.1ml of the suspension was then applied to the top of the column.

Elution was immediately performed with 2.0ml of the eluent, the eluate collected and the OD_{470} taken.

CHAPTER THREE

RESULT

3.1 BACTERIA CELL COUNT

The total counts of the bacteria isolates from the various packaged orange drinks are as shown in Table 4.

Sample B orange drink had the highest number of bacteria colonies of about 1.43 x 10^6 cfu/ml. Samples A and G orange drinks had colony count of about 1.11 x 10^6 and 1.01 x 10^6 cfu/ml respectively. Sample C orange drink had the lowest bacteria colony count of about 3.0 x 10^5 cfu/ml. The control plates showed no growth of bacteria.

TABLE 4: COLONY COUNTS OF THE BACTERIA ISOLATED FROM THE ORANGE DRINKS

ANALYSED ORANGE DRINKS	BACTERIA COLONY COUNTS (cfu/ml)
SAMPLE B	1.43×10^6
SAMPLE A	1.11×10^6
SAMPLE G	1.01×10^6
SAMPLE F	9.3×10^5
SAMPLE E	9.1×10^5
SAMPLE D	8.1×10^5
SAMPLE C	3.0×10^5

3.2 FUNGI COUNT

The total counts of the fungal isolates from the various orange drinks are shown in Table 5. Sample D orange drink had the highest number of fungi colonies of about 1.31×10^5 spu/ml followed by Sample E orange drink with about 9.8×10^4 spu/ml The control plates showed no fungi growth.

TABLE 5: FUNGI COLONY COUNT FROM THE ORANGE DRINK

ANALYSED ORANGE DRINKS	FUNGI COUNT (Spu/ml)
SAMPLE D	1.31×10^5
SAMPLE E	9.8×10^4
SAMPLE C	8.5×10^4
SAMPLE A	1.4×10^4
SAMPLE F	8.0×10^3
SAMPLE B	----
SAMPLE G	----

--- = absent.

3.3 IDENTIFICATION OF THE MICROBIAL ISOLATES FROM THE ORANGE DRINKS

3.3.1 BACTERIA ISOLATES

The bacteria species which are persistently isolated from all the packaged orange drinks are cream coloured colonies which on staining showed rod-like shaped cells which were Gram positive that are likely to be Bacillus species. This organism forms about 60% of the bacteria colony isolated.

A few rod shaped cells that stained Gram negative likely to be E. coli were isolated from Samples B, A, G, D and C orange drinks. These cells appeared pink on MacConkey Agar and forms about 25% of the bacteria colony isolated.

Very few whitish cream coloured colonies which on staining showed single spherically shaped cells which were Gram positive

that are likely to be Micrococcus spp. were isolated from Samples B, F, E and D orange drinks. These bacteria cells showed no growth on Mannitol Salt Agar and forms about 10% of the bacteria colony isolated.

Samples B, A and G contained few white coloured colonies which on staining showed short rod cells which were Gram negative and on centrimide agar showed greenish colour which are likely to be Pseudomonas aeruginosa. This organism forms about 5% of the bacteria colony isolated.

The microscopic and colonial morphology of the bacteria isolated from the various orange drinks are shown in Table 6.

TABLE 6: MICROSCOPIC AND COLONIAL MORPHOLOGY OF THE BACTERIA ISOLATES

ORANGE DRINK SAMPLE	COLONIAL MORPHOLOGY	MICROSPICAL CHARACTER (SIMPLE & GRAM'S STAINING)	PROBABLE ORGANISM
A,B,C,D, E,F & G	Creamy white, Spreading, Convex entire, dull, Opaque colony with serrated edge.	Gram positive rods in diplo and singles	Bacillus subtilis
A,B,C,D, & G	White, circular, flat, smooth, shining translucent colony with smooth edge. Faecal odour.	Gram negative short rods	Escherichia coli
A,B, & G	Faint green, circular, flat, smooth, shining translucent colony with smooth edge.	Gram negative short rods	Pseudomonas aeurginosa
B,D,E & F	Creamy white, circular, convex entire, smooth, glistening opaque colony.	Gram positive cocci in singles	Micrococcus sp

The biochemical characteristics of the bacteria cells are as shown in Table 7.

TABLE 7: BIOCHEMICAL CHARACTERS OF THE BACTERIA ISOLATES

SUGAR FARMENTATION										
A	S	L	EIJKMANS TEST	INDOLE TEST	CITRATE TEST	METHYL RED TEST (MR)	VOGAS PROSKAVER TEST (VP)	CATALASE TEST	OXIDASE TEST	PROBABLE ORGANISM
A	A	NAG	A	-	+	+	-	+	-	B. subtilis
AG	AG	AG	AG	+	-	+	-	+	-	E. coli
AG	AG	NAG	NAG	-	-	-	-	+	+	Ps. aeruginosa
A	A	A	A	-	+	+	-	+	-	Micrococcus sp.

A = Acid Production
AG = Acid and Gas Production
NAG = No Acid and Gas Production
\+ = Positive
\- = Negative
G = Glucose
S = Sucrose
L = Lactose

3.3.2 FUNGI ISOLATES

The fungi/mould identified in samples D and F orange drinks were very few white large colony likely to be Aspergillus niger and Penicillium sp. Rhizopus oryza was isolated from sample F orange drink.

The yeast identified in samples A, C, D, E and F orange drinks were Candida guillermondi and Candida tropicalis.

The cultural morphology and biochemical characteristics of fungi isolates are as shown in Table 8.

TABLE 8: CULTURAL MORPHOLOGY AND BIOCHEMICAL CHARACTERISTICS OF THE FUNGI ISOLATES

ORANGE DRINK	CULTURAL MORPHOLOGY	BIOCHEMICAL CHARACTERISTICS SUGAR FERMENTATION: GLUCOSE	SUCROSE	LACTOSE	PROBABLE ORGANISM
D & F	White large colony with powdery, rough surface which turned black with age of incubation	-	-	-	Aspergillus niger
D & F	White large colony with yellow centre with a rough surface	-	-	-	Penicillium sp.
F	White coarse colony with wooly mycelium	-	-	-	Rhizopus cryza
A,C,D,E & F	Pink circular convex entire smooth and shining opaque colony	NAG	A	NAG	Candida guillermondi
A,C,D,E & F	White circular, convex entire smooth and moist opaque colony	NAG	AG	NAG	Candida tropicalis

- = Not Determined
NAG = No Acid and Gas production
A = Acid Production
AG = Acid and Gas Production.

3.4 PACKAGING MATERIALS VARIATION

The packaging materials used for the analysed orange drinks were plastic bottles, glass bottles and foil paper boards.

Sample B which was packaged in plastic bottles had the highest bacteria colony count of about 1.43 x 10^6 cfu/ml. E. coli, B. subtilis, Ps. aeruginosa and Micrococcus sp were isolated from Sample B, but no fungi growth was observed.

Samples A and G were packaged in glass bottles. They contained high bacteria cell counts of 1.11 x 10^6 and 1.01 x 10^6 cfu/ml. B. subtilis, E. coli and Ps aeruginosa were isolated from these orange drinks. Sample G had no fungi growth. Many yeasts cells were isolated from Sample A.

Samples C, D, E and F were packaged in foil paper boards. They contained the lowest bacteria count of about 9.3 x 10^5 - 3.0 x 10^5 cfu/ml. Moulds, mucor and yeasts species were isolated from these drinks.

The samples packaged in plastics bottles showed the highest bacteria colony followed by samples packaged in glass bottles while samples packaged in foil paper boards showed the lowest bacteria count. Foil paper board showed the highest fungi colony followed by samples packaged in glass bottle and non in the plastic bottle.

3.5 SENSITIVITY OF THE ISOLATES TO BENZOIC ACID & SODIUM BENZOATE

The efficacy of the specified preservatives used for the preservation of these orange drinks was tested. The results are as shown in figures I and II. B. subtilis was the most sensitive organism to benzoic acid at the test concentration of 0.1 mg/ml.

Micrococcus sp was the most sensitive organism to benzoic acid at the test concentrations of 0.2 and 0.4 µg/ml, while Ps. aeruginosa was the least sensitive organism to benzoic acid at the test concentration of 0.1, 0.2 and 0.4 µg/ml.

B. subtilis was the most sensitive organism to sodium benzoate at the test concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 µg/ml while Ps. aeruginosa was the least sensitive.

The isolates from the orange drinks were more sensitive to the preservatives than the standard bacteria species, except E. coli where the isolates have about the same sensitivity like the standard strain against benzoic acid.

3.4 PACKAGING MATERIALS VARIATION

The packaging materials used for the analysed orange drinks were plastic bottles, glass bottles and foil paper boards.

Sample B which was packaged in plastic bottles had the highest bacteria colony count of about 1.43 x 10^6 cfu/ml. E. coli, B. subtilis, Ps. aeruginosa and Micrococcus sp were isolated from Sample B, but no fungi growth was observed.

Samples A and G were packaged in glass bottles. They contained high bacteria cell counts of 1.11 x 10^6 and 1.01 x 10^6 cfu/ml. B. subtilis, E. coli and Ps aeruginosa were isolated from these orange drinks. Sample G had no fungi growth. Many yeasts cells were isolated from Sample A.

Samples C, D, E and F were packaged in foil paper boards. They contained the lowest bacteria count of about 9.3 x 10^5 - 3.0 x 10^5 cfu/ml. Moulds, mucor and yeasts species were isolated from these drinks.

The samples packaged in plastics bottles showed the highest bacteria colony followed by samples packaged in glass bottles while samples packaged in foil paper boards showed the lowest bacteria count. Foil paper board showed the highest fungi colony followed by samples packaged in glass bottle and non in the plastic bottle.

3.5 SENSITIVITY OF THE ISOLATES TO BENZOIC ACID & SODIUM BENZOATE

The efficacy of the specified preservatives used for the preservation of these orange drinks was tested. The results are as shown in figures I and II. B. subtilis was the most sensitive organism to benzoic acid at the test concentration of 0.1 mg/ml.

Micrococcus sp was the most sensitive organism to benzoic acid at the test concentrations of 0.2 and 0.4 µg/ml, while Ps. aeruginosa was the least sensitive organism to benzoic acid at the test concentration of 0.1, 0.2 and 0.4 µg/ml.

B. subtilis was the most sensitive organism to sodium benzoate at the test concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 µg/ml while Ps. aeruginosa was the least sensitive.

The isolates from the orange drinks were more sensitive to the preservatives than the standard bacteria species, except E. coli where the isolates have about the same sensitivity like the standard strain against benzoic acid.

FIGURE 1

SENSITIVITY OF THE BACTERIA ISOLATES

AND

STANDARD NCTC CULTURE TO BENZOIC ACID

KEY:

A = B. subtilis isolate

A' = B. subtilis NCTC 8236

B = E. coli isolate

B' = E. coli NCTC 10418

C = Ps. aeruginosa isolate

C' = Ps. aeruginosa NCTC 6750

D = Micrococcus sp isolate

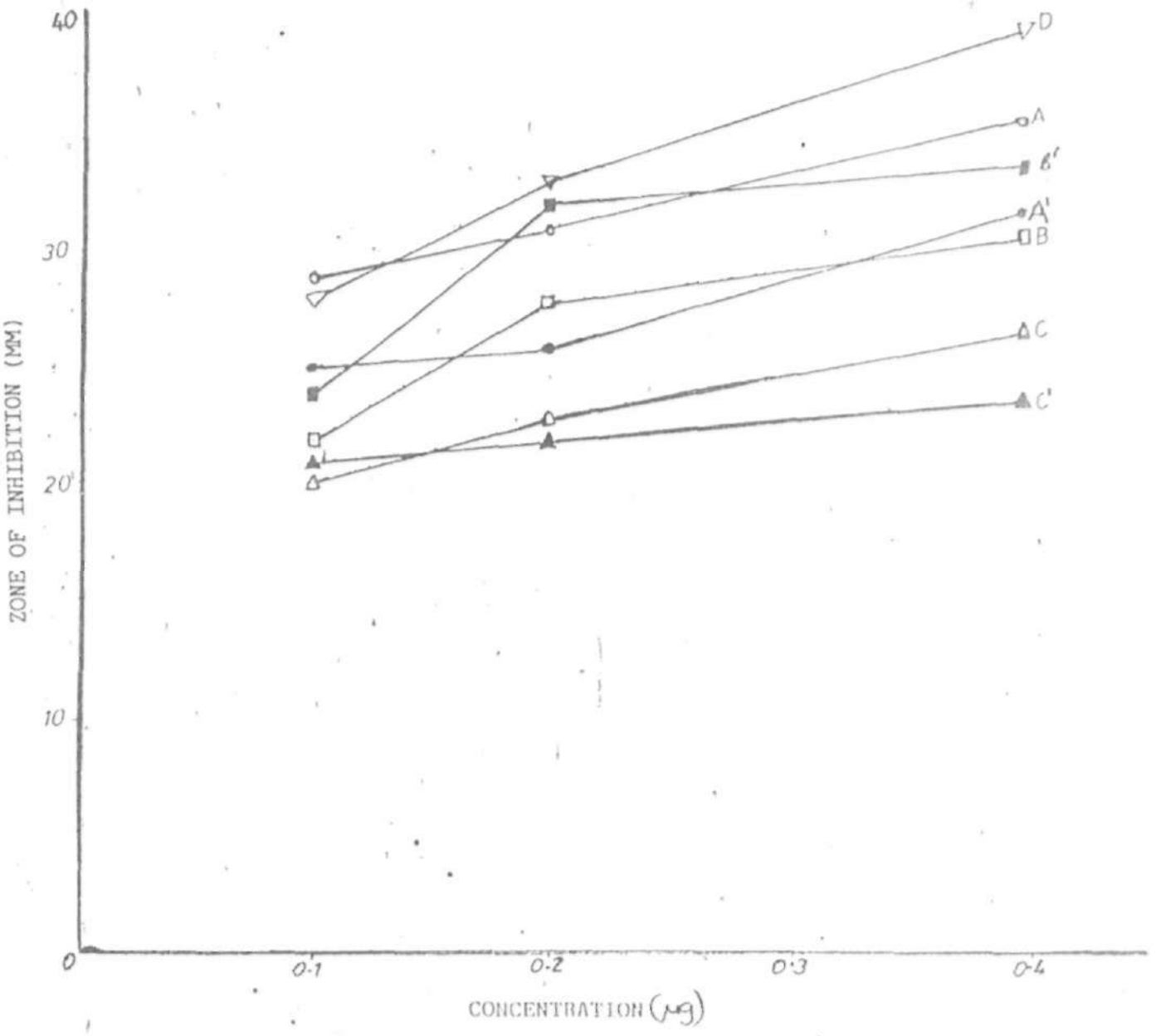
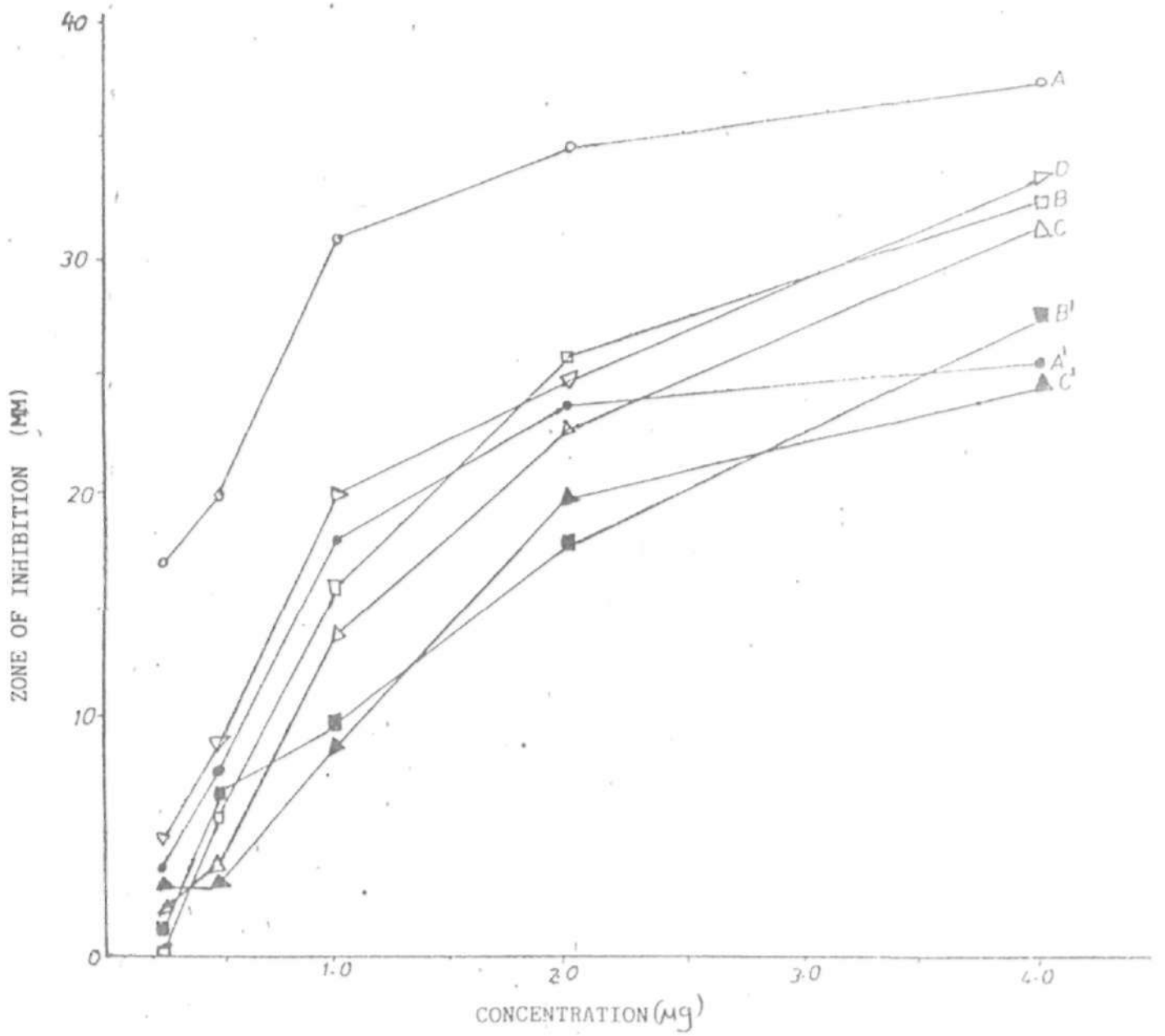

40
30
20
10
0
ZONE OF INHIBITION (MM)
0·1
0·2
0·3
0·4
CONCENTRATION (µg)
D
A
B'
A'
B
C
C'

FIGURE 2

SENSITIVITY OF THE BACTERIA ISOLATES
AND
STANDARD NCTC CULTURE TO SODIUM
BENZOATE

KEY: A = B. subtilis isolate
A' = B. subtilis NCTC 8236
B = E. coli isolate
B' = E. coli NCTC 10418
C = Ps. aeruginosa isolate
C' = Ps. aeruginosa NCTC 6750
D = Micrococcus sp isolate


ZONE OF INHIBITION (MM)
40
30
20
10
0
1.0
2.0
3.0
4.0
CONCENTRATION (µg)
A
D
B
C
B'
A'
C'

3.6 DETERMINATION OF THE MINIMUM INHIBITION CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF BENZOIC ACID AND SODIUM BENZOATE AGAINST THE ISOLATES FROM THE ORANGE DRINKS AND THE STANDARD NCTC CULTURES

The MIC of benzoic acid and sodium benzoate on the test organisms are as shown in Table 9. The MICs of benzoic acid against the organisms were found to be the same except against Gram negative bacteria, E. coli and Ps. aeruginosa, which were found to be 0.3125-0.625 µg/ml and 0.625 µg/ml respectively. The MICs of sodium benzoate against the organisms were about the same concentration except against Ps. aeruginosa which was between 0.3125-0.625 µg/ml.

The MBC of benzoic acid and sodium benzoate on the test organisms are also as shown in Table 9. The MBC of benzoic acid against the NCTC standard organisms were relatively higher than that against the isolated organisms from the orange drinks while the MBC of sodium benzoate against the isolates were also relatively higher than that against the standard organisms.

TABLE 9: MIC AND MBC OF BENZOIC ACID AND SODIUM BENZOATE AGAINST THE ISOLATED AND STANDARD (NCTC) BACTERIA SPECIES

	BENZOIC ACID		SODIUM BENZOATE	
ORGANISM	MIC(μg/ml)	MBC(μg/ml)	MIC(μg/ml)	MBC(μg/ml)
B. subtilis	0.3125	0.3125	0.625	1.25-2.50
B. subtilis NCTC 8236	0.15625	1.25	0.3125-0.625	0.625
E. coli	0.3125-0.625	0.625	0.625	2.50
E. coli NCTC 10418	0.15625	1.25	0.625	2.50
Ps. aeruginosa	0.625	0.625	0.3125-0.625	2.50-5.00
Ps. aeruginosa NCTC 6750	0.15625	0.625-1.25	0.625	2.50
Micrococcus sp	0.3125	0.625-1.25	0.625	1.25

3.7 SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY (EIC)

The result of the surface charge of the bacteria isolated from the packaged orange drinks and the standard NCTC bacteria species by EIC are as shown in figures 3 - 6.

The E. coli and Ps. aeruginosa isolated from the orange drinks adhere more to both the sephadex CM and QAE than the standard NCTC cultures while the B. subtilis standard NCTC culture adhere more to than the B. subtilis isolated from the orange drinks. Micrococcus sp showed the least adherence to the CM sephadex.

E. coli and Ps. aeruginosa showed more adherence to the resins than B. subtilis and Micrococcus sp. The Gram negative organisms adhere more to QAE sephadex (anion) than to CM sephadex (cation)

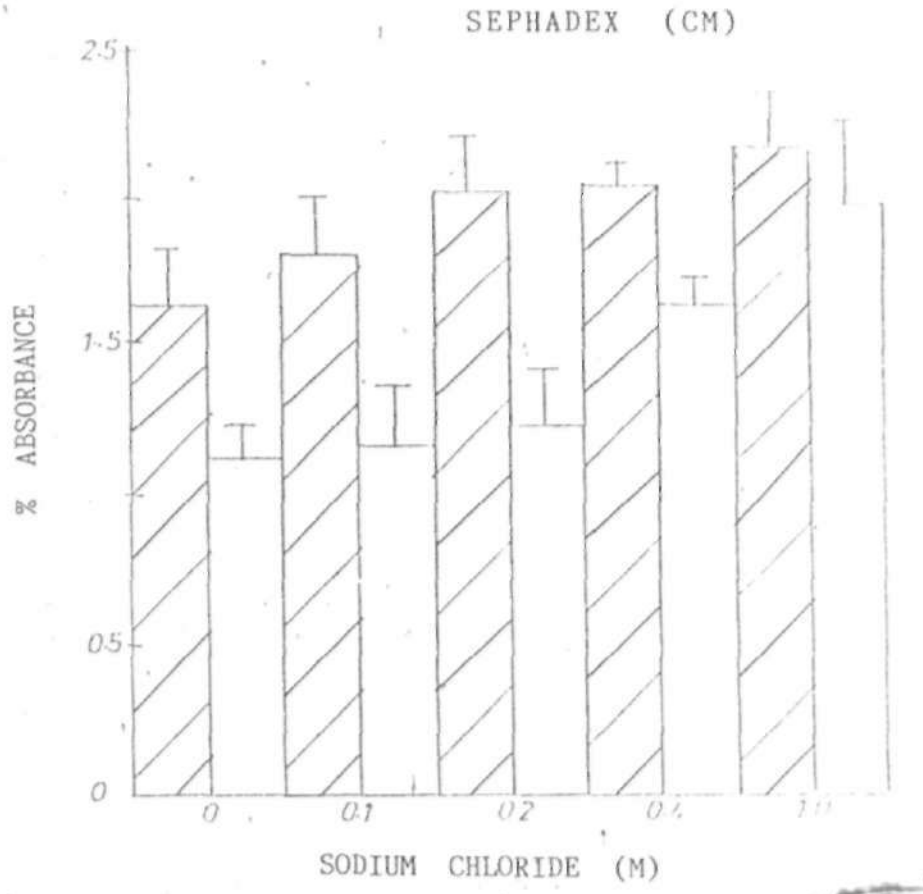
while B. subtilis adhere more to CM sephadex (cation) than the QAE sephadex. Ps.aeruginosa adhere more to the resins than any of the bacteria spp.

FIGURE 3

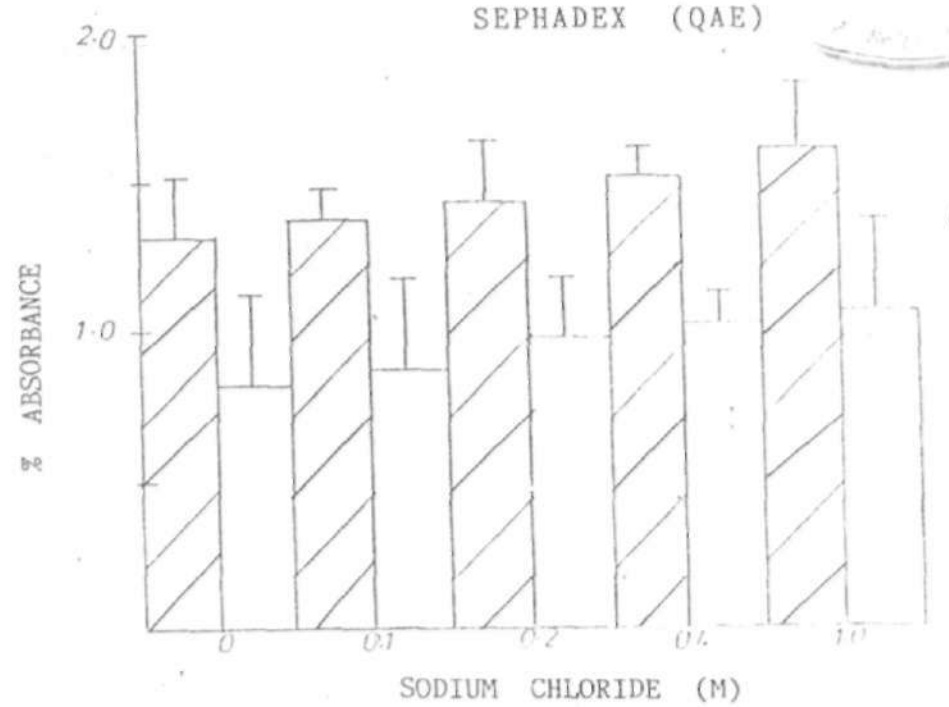
SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY OF E. coli ISOLATED FROM THE ORANGE DRINKS AND STANDARD E. coli NCTC 10418

KEY: ▨ = E. coli NCTC 10418

□ = E. coli isolate


SEPHADEX (CM)
% ABSORBANCE
2·5
1·5
0·5
0
0
0·1
0·2
0·4
1·0
SODIUM CHLORIDE (M)




SEPHADEX (QAE)
% ABSORBANCE
2·0
1·0
0
0·1
0·2
0·4
1·0
SODIUM CHLORIDE (M)

457606

FIGURE 4

SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY OF *B. subtilis* ISOLATED FROM THE ORANGE DRINKS AND STANDARD *B. subtilis* NCTC 8236

KEY:  = *B. subtilis* NCTC 8236

 = *B. subtilis* isolate

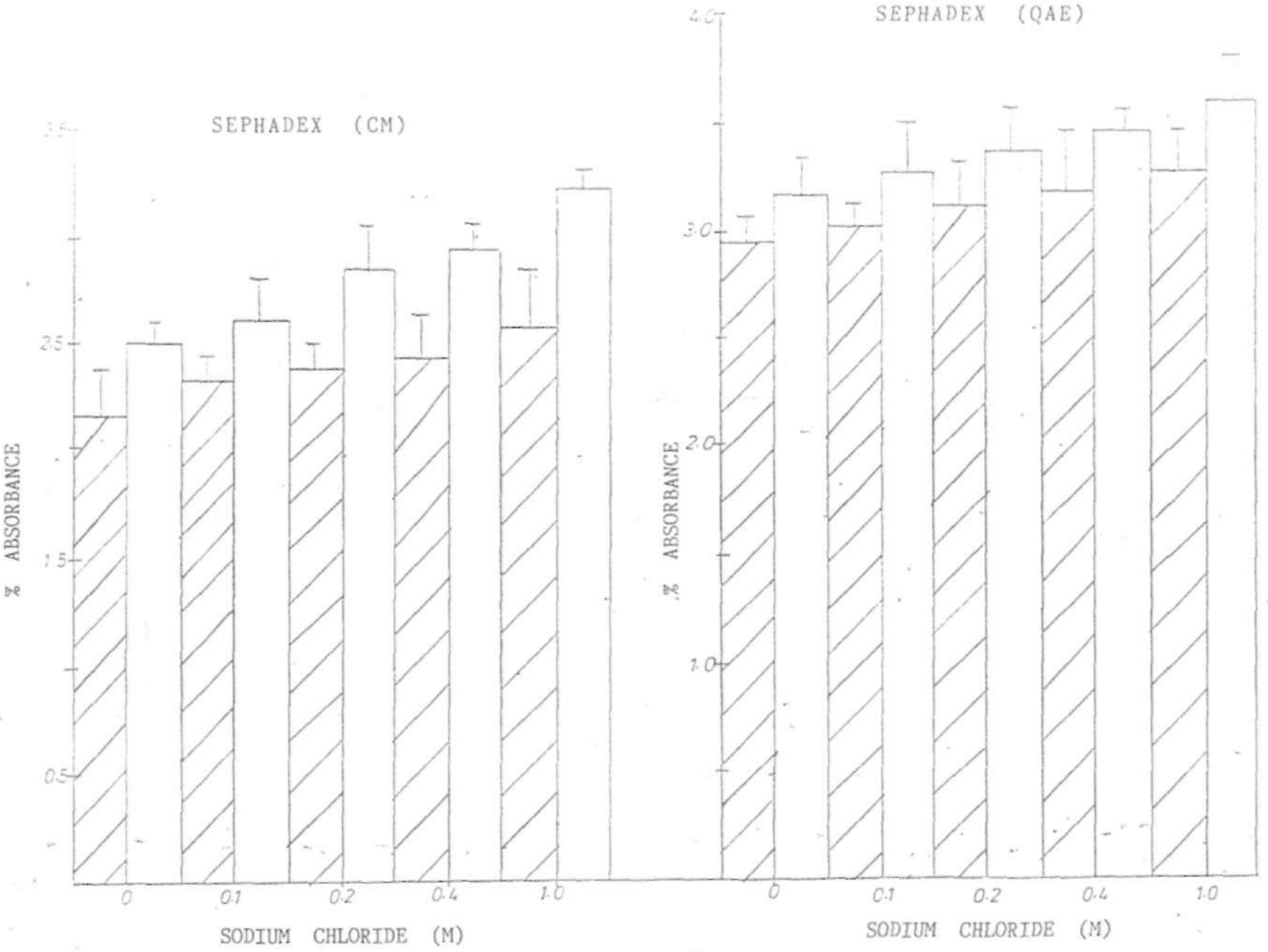

SEPHADEX (CM)
SEPHADEX (QAE)
% ABSORBANCE
SODIUM CHLORIDE (M)
0
0.1
0.2
0.4
1.0
0.5
1.5
2.5
3.5
1.0
2.0
3.0
4.0

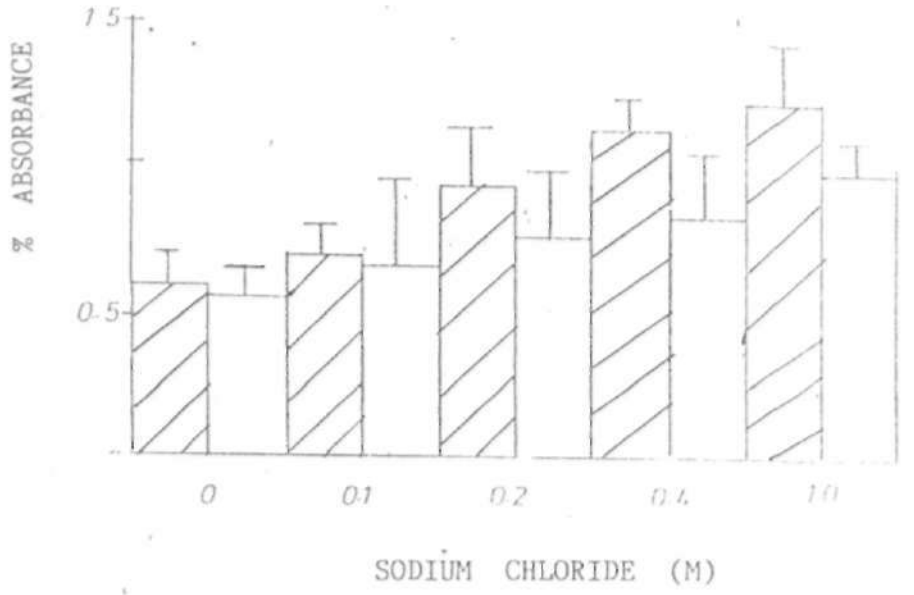
FIGURE 5

SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY OF *Ps. aeruginosa* ISOLATED FROM THE ORANGE DRINKS AND STANDARD *Ps. aeruginosa* NCTC 6750

KEY: ▨ = *Ps. aeruginosa* NCTC 6750

□ = *Ps. aeruginosa* isolate

SEPHADEX (CM)



SEPHADEX (QAE)

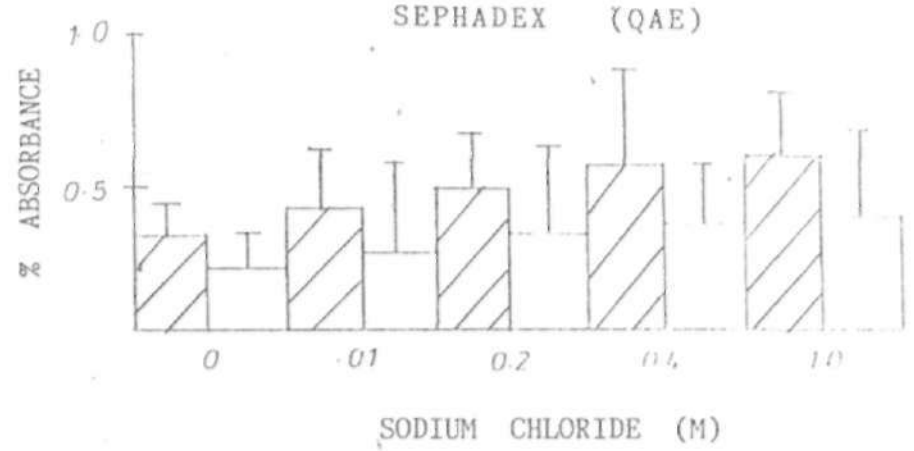
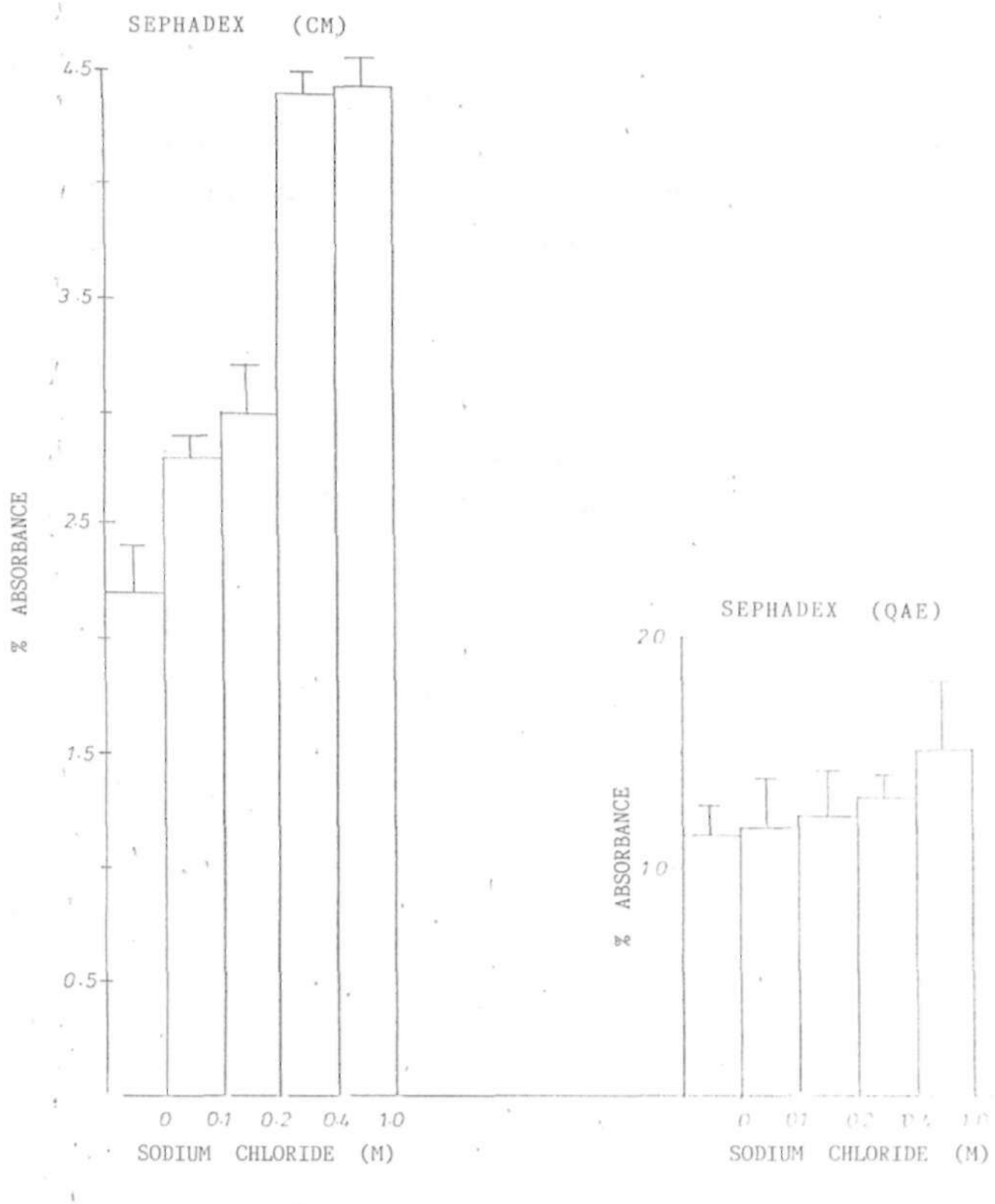


FIGURE 6

SURFACE CHARGE BY ELECTROSTATIC INTERACTION CHROMATOGRAPHY OF *Micrococcus* sp ISOLATED FROM THE ORANGE DRINKS

KEY: ☐ = *Micrococcus* sp isolate.


SEPHADEX (CM)
% ABSORBANCE
4.5
3.5
2.5
1.5
0.5
0
0.1
0.2
0.4
1.0
SODIUM CHLORIDE (M)
SEPHADEX (QAE)
% ABSORBANCE
2.0
1.0
SODIUM CHLORIDE (M)

CHAPTER FOUR

DISCUSSION

The analysis of the various packaged orange drinks showed extensive microbial contamination in the packaged orange drinks. The official limit recommended for microbial contamination requires the complete absence of some enterobacteriaceae like E. Coli, Salmonella, Shigella and Pseudomonas aeruginosa and some other organisms like Staphylococcus aureus. Other revivable microorganisms should not be more than 10^3 cells/ml/g for aerobic bacteria and 10^2 cells/ml/g for mould/yeast (FIP, 1972).

The analysed orange drinks did not comply with this official limit as the bacteria count observed was between 3.0×10^5 to 1.43×10^6 cfu/ml, also the mould/yeast count was between 8.0×10^3 to 1.31×10^5 spu/ml. Some of the orange drinks also showed presence of some indicator organisms like E. coli and Ps. aeruginosa which are meant to be completely absent.

Generally, the microorganisms found in the analysed orange drinks include Bacillus subtilis, E. coli, Ps. aeruginosa, Micrococcus spp, Aspergillus niger, Penicillium sp, Rhizopus oryza, Candida guillermondi and Candida tropicalis.

The presence of E. coli in some of these samples indicated that the water used for the manufacture of these drinks was not of

good quality, probably contaminated with faecal matter. This becomes serious since a large percentage of the orange drink is water and water is also used in cleaning and washing of equipments and packaging materials.

Antai and Anozie (1987), reported the incidence of infant diarrhoea due to E. coli in some parts of Nigeria which resulted from the quality of water supply. Also endotoxins, produced by Gram negative bacteria such as E. coli are intimately bound to the cell, they are lipopolysaccharide in nature and are not necessarily inactivated by sterilization as they are heat stable (Barnes, 1970), these are very dangerous on consumption.

Pseudomonas aeruginosa which was found in three of the analysed packaged orange drinks, samples A, B and G, could have resulted from contamination of the water and the processing personnel. Ps. aeruginosa is supported in growth by wounds showing signs of infection (Skinner and Carr, 1974). Indeed contaminated deionized water has often been incriminated as the original source of spoilage in a product and often the responsible organisms are Pseudomonads which are not only highly resistant to preservatives but are also able to use the widest range of organic compounds as substrates (Smart and Spooner, 1972). Ps. aeruginosa was also isolated from stored industrial water (Chambers and Clarke, 1968). Ps. aeruginosa is an opportunist pathogen, and is known to cause complications in medically compromised individuals. The occurrence of Ps. aeruginosa in orange drinks give cause for concern. Due to the ready-to-drink nature of the packaged orange drinks, they are

consumed by different groups of people throughout the country and this could constitute a health risk particularly in persons who may have low resistance to infection.

Other bacteria species isolated from the tested samples such as Bacillus subtilis and Micrococcus sp could have been as a result of contamination of water used in the manufacture of the orange drinks, the processing personnel and the processing environment. The persistent isolation of B. subtilis in all the analysed samples might be due to the poor environment of manufacture and the sporing nature of the organism. Bacillis spp is one of the micro-organisms commonly isolated from air and the number of organisms in the atmosphere depends on the activity in the environment and the amount of dust which is disturbed (Anderson and Cox, 1967).

The presence of moulds; Aspergillus niger, Penicillium sp and Mucor; Rhizopus oryza might be as a result of the packaging material, moist processing environment or during storage. Thenmoulds commonly isolated from air are Penicillium spp, Aspergillus spp and Mucor spp. An area containing working machinery and can ative personnel will have a higher microbial count than one with a still atmosphere. Microorganism are carried into the atmosphere. Microorganisms are carried into the atmosphere suspended on particles of dust, skin or clothing, or in droplets of moisture or sputum following talking, coughing or sneezing (Anderson and Cox, 1967). The yeast, Candida guillermondi and C. tropicalis isolated from samples A, C, D, E and F might have been due to the presence of preservatives and the sugar used in the

packaged orange drinks. Additives such as preservatives and sugars have been implicated in acting as substrates for microbes especially yeasts (Hugo and Russel, 1987).

The aim of preservation is to satisfactorily keep a product aginst microbial challenge while it is in trade channel and in the hands of consumers, but despite the preservation, the microbial counts were higher in the tested samples. For instance, it was specifically indicated on the packaging of sample A that sodium benzoate was used in the preservation of the drink but the bacteria viable count observed was as high as 1.11×10^5 cfu/ml while the mould/yeast count was as high as 1.4×10^4 spu/ml. For the other samples, though the preservatives used were not specified, they have not met the objectives of their inclusion.

The packaged orange drinks are supposed to be stored under low temperature. However, there are some microorganisms that can survive in low temperature such as the psychrophilic organisms. The low temperature will inhibit the multiplication of the micro-organisms. It was found however that most of the samples analysed were not stored under low temperature as at the time of purchase and many of the drinks are being hawked around while those in shops were not under refridgeration, this might have encouraged the high microbial load. Since it has been found that increase in temperature increase the microbial load of the orange drinks. The number and type of viable and non-viable micro-organisms present in orange drinks kept at low temperature reflect the degree of contaimination of the raw material, the sanitation in the

processing plant and the speed and care with which the product has been processed (Pekzar and Ried, 1965). Under the tropical climate, characterized by ambient temperature frequently above 30°C, rapid increase of micro-organisms are likely to occur (Ogan, 1992). The storage effects on the microbial quality of the orange drinks are related to factors such as; assimilable organis nutrients, organisms competing for dominance in the microbial flora, pH characteristics and storage temperature of the drink.

The preservation and storage of these orange drinks were not adequate in view of the high microbial load in the drinks. However, preservation alone cannot actually be blamed for the high microbial load in the orange drinks because the microorganisms might have been in the drinks at the time of processing before the addition of preservatives and packaging. Preservation cannot take the place of Good Manufacturing Practice (GMP). Preservation can only slow down the rate of growth of the microorgamism or prevent the product against microbial challenge, but not eliminate the already existing ones. The elimination of microorganisms by disinfection or sterilization is geenrally not permitted in drinks (Anon, 1982). Therefore, "extra hygiene" with good manufacturing practice cannot be overemphasized especially in view of the vitamins and possible growth-promoting constituents of such orange drinks.

The type of containers used may also affect the bacterial load. Sample B packaged in a plastic bottle contained the highest bacteria load of 1.43×10^6 cfu/ml. This might be due to the fact

that plastic containers increase the bacteria load of product as a result of the filler they are made of (Dempsey, 1988). Plastics are also porous to varing degrees and some allow the diffusion of oxygen and carbondioxide and may thus facilitate microbial growth in the packaged orange drink. They also encourage condensation of water, therefore can facilitate microbial spoilage. Sample A and G packaged in glass bottle had the second and third highest bacteria load of 1.11×10^6 cfu/ml and 1.01×10^6 respectively, this might be due to the fact that glass bottles support the growth of microorganisms such as B. subtilis, Penicillium spp and Aspergillus niger due to contamination of these glass bottles during transportation (Dempsey, 1988). Glass bottles are also reused in some local manufacturing companies which may lead to high microbial load of the orange drinks due to lack of proper washing and sterilization of the glass bottles leading to carryover of contaminants. All other analysed samples packaged in foil paper boards also had the least bacteria load of 9.3×10^5 to 3.0×10^5 cfu/ml. According to Hugo and Russell (1987), paper boards unless treated carry mould spores of Aspergillus spp, Penicillium spp. and bacteria such as Bacillus spp and Micrococcus species. Samples D, E and F packaged in paper boards contained all the bacteria and mould species mentioned above. Paper board, being absorbent soak up liquids thus providing a substrate for microorganisms.

Most preservatives are able to interact with containers, plastic containers play a role in the degredation of preservatives, this may contribute to the microbial load of 1.43×10^6 cfu/ml

observed in sample B packaged in plastic bottle. Preservatives, if not compactible with the packaging materials can cause loss of preservative potency as a result of interactions with the packaging materials (Croshaw, 1977).

Preservatives are used to reduce the likelihood of microbial growth in aqueous products and to reduce the chance of microbial survival in an hydrous products that may be contaminated. Packaged orange drinks must have a preservative system that is capable of self-sterilizing the drinks should contamination occur. The commonly used preservatives in the processing of packaged orange drinks are sodium benzoate and benzoic acid due to their stability at low pH. The salts of benzoic acid are more readily soluble than the acid, therefore the sodium salt is employed in preference to the acid. The recommended concentration for their use is 0.1-0.5%. The result of the Minimum Inhibitory Concentration (MIC) of the preservatives against the isolates from the analysed samples and the standard NCTC culture showed that the MIC of benzoic acid is between 0.15625-0.625 µg/ml while that of sodium benoate is between 0.3125-0.625 µg/ml. From these results, it can be concluded that the recommended concentration of 0.1-0.5% of these preservaties is low and may not be effective aginst the isolated bacteria species and this may account for the high bacteria load in the samples. The MIC of sodium benzoate is higher than that of benzoic acid, signifying that benzoic acid is more active than sodium benzoate but the disadvantage of benzoic acid is the development of resistance to it by some organisms involving in some cases of

metabolism of the acid resulting in complete loss of activity (Russell et al, 1982). The result of the Minimum Bacteriocidal Concentration (MBC) showed that the MBC of sodium benzoate is higher than that of benzoic acid. The MBC of sodium benzoate against the bacteria species is between 0.625-5.00 µg/ml while that of benzoic acid is 0.3125-1.25 µg/ml. The above result also showed that the in-use concentration of sodium benzoate is low if the orange drinks are to remain safe for consumption. From the result of the MIC and MBC, it is therefore suggested that the in-use concentration of the preservatives should be looked into and adjusted.

The result of sensitivity of the isolates to benzoic acid and sodium benzoate showed that E. coli and Ps. aeruginosa were less sensitive to both preservatives than B. subtilis and Micrococcus spp, however, Ps. aeruginosa appeared to be less sensitive to the preservatives than all the other isolates. Also the isolates from the orange drinks were found to be more sensitive than the standard culture strains of Ps. spp have been shown to be less sensitive to many antimicrobial agents (Mavridou et at, 1994). It has been recorded that benzoic acid and sodium benzoate are less active against Gram negative bacteria (Chapman, 1987). Benzoic acid and sodium benzoate had also been found to permit the development of some bacteria (Block, 1983). Among other factors, the lack of sensitivity to antimicrobial agent by bacteria may be due to inability of the agent to diffuse into the cell (Jones et al, 1986). The presence of bacteria which are less sensitive to

antimicrobial agents in the orange drinks is of great importance to public health as there is the risk of transferring this character to other bacteria present in human body and to some pathogenic ones. Proda (1979) found that Pseudomonas spp. transferred resistance plasmids to E. coli strains inside the enteric tube.

The result of the surface charge by Electrostatic Intraction Chromatography (EIC) showed that the Gram negative bacteria E. coli and Ps. aeruginosa adhere more to QAE sephadex (anion exchange resin) than B. subtilis and Micrococcus spp which are Gram positive organisms. Wood (1979) found that the surface protein of E. coli mediated absorption to anion exchange resin. Surface structures of Gram negative bacteria may be involved in absorption to ion exchange resins which include flagella, fimbrae or pili and lipopolysaccharide (Onaolapo, 1986).

The percentage absorption of both the isolated and standard organisms on the resins increase with increase in the concentration of sodium chloride. Beachey et al (1981) found that there is decrease of charge on the surface of the resin and on bacteria which lead to increase absorption of the bacteria species with increase concentration of salt.

The Gram negative bacteria, E. coli and Ps. aeruginosa, isolated from the samples showed more absorption to the anion and cation resins than the standard NCTC cultures while the B. subtilis standard NCTC 8236 however showed more absorption than the isolated B. subtilis from the orange drinks. The differences in the charges exposed on the surface of the test organisms suggest differences in

the surfaces of the isolates from orange drinks and the standard cultures. Changes in the cell envelope with nutrient availability and environment may affect the quality of bacteria that adhere to resins (Brown and Williams, 1985). Most anionic groups could be exposed on the surface of bacteria which could promote absorption to ion exchange resins. Ionisation of Carboxyl group (-CooH) are involved in absorption of both Gram positive and Gram negative species of bacteria, also the ionisation of N-acetylneuramic acid (siali acid), a major ionogenic component of glycoprotein (Zuaginstev and Guser, 1971).

The differences in the surface properties may have a direct effect on the sensitivity of these organisms to the preservatives. E. coli and Ps. aeruginosa are less sensitive to the preservatives used in the orange drinks and also showed more absorption to the resins than the standard NCTC cultures. This can be explained that these organisms may have the ability to adhere to the surfaces of the packaging material, thus preventing the effect of the preservatives on them. Therefore, these organisms are able to multiply and become less sensitive to the preservation.

This study has shown that the various packaged orange drinks were not adequately protected in all likelihood due to disregard to the principles of Good Manufacturing Practice (GMP) in the factories. Some local companies get involved in the manufacturing of orange drinks with little or no emphasis on quality control and some marketers store large number of these packaged orange drinks in hot humid temperature not realising that as the temperature

increases, the microbial load of the orange drink will also increase to a limit. The above factors are not only contributing factors, also the source and quality of orange fruit, poor personnel hygenic conditions, the condition of the processing equipment, processing environment, the packaging materials and source of water can also contribute to microbial contamination. Some manufacturing companies practice the re-use of plastic and glass bottles. If these are not corrected, it may lead to serious health hazards to consumers who depend on these packaged orange drinks because of their easy to drink attribute.

Public concern has been caused in many countries due to the presence of high amount of floride, chloride, sodium and offensive odours and tastes in municipal water supply, as well as possible contamination of underground sources by toxic industrial wastes and micro-organisms (Geldreich, et al, 1975; Tobin, 1984). Therefore, care should be taken more on the water used for the preparation of the orange drinks, since large percentage of the orange drink is water. The water should be taken from right source and adequately treated. The microbiological quality standards for drinking water in Nigeria are essentially those established by the World Health Organisation (WHO) (Anon., 1971). Drinking water must be free from faecal indicator bacteria, Ps. aeruginosa and pathogens according to the WHO standards. Faecal pollution indicator bacteria (Geldreich et al, 1975) and Ps. aeruginosa (Rivilla and Gonzalez, 1988) are isolated in practice.

Preservatives cannot always be relied upon to prevent the

multiplication of micro-oragnisms because the ideal preservatives does not exist and therefore the choice for each product is an individual, and often not an entirely successful matter. In any case, preservatives are not substitute for good hygiene during production. The proper solution to the problem of microbial adaptation does not rest with increasing the potency of preservative systems in all products, because this may lead to the use of preservative levels well in excess of those normally required. Instead, alternative types of packaging or product reformulation may be indicated to eliminate condition which assist the adaptation process (Denyer, et al, 1982).

Yablonski (1978), noted the importance of equipment cleaning and sanitization in preventing microbial contamination in manufacturing plants. Improperly cleaned and sanitized equipment provides dilute product residues that enable microoorganisms to adapt to the product (Orth, 1981).

Microbial contamination of the orange drink is significant both from health and economic view points and obviously should be prevented. It is clear that disease-causing organisms must be excluded because even if they occur in small number can sometimes be dangerous, although sometimes it may be difficult to decide if an opportunist pathogen will be troublesome in the product. If all microorganisms are excluded from the product, contamination will be avoided, but it is not practicable to eliminate microbial contamination completely because the types of bacteria present are far more important than the total number. This raises the question

of how many organisms of what sort are acceptable in a given product. It can be concluded from this study tthat the selected or analysed orange drinks contained various microbial species ranging from Gram negative bacteria, Gram positive bacteria to mould and yeasts in high cell counts.

Therefore, it is suggested that:

(1) Fresh, spoilage free orange fruit should be used in processing the packaged orange drinks.

(2) Water used in these manufacturing companies should be properly and thoroughly treated and changed constantly. Good protection of the water source is important.

(3) The packaging materials should be properly washed and treated before use and the re-use of the packaging materials especially plastic and glass bottle should be discouraged.

(4) The processing environment and processing equipment should be cleaned, disinfected and kept in the highest hygenic condition always during and after processing.

(5) The inclusion of preservatives should not be taken for granted.

(6) The personnel involved in the processing of the orange drinks should be educated on good personnel hygiene and be in the highest hygenic condition during processing.

(7) These products be stored in adequate temperature of -20°C.

(8) The product should be of the highest quality from the very first stage of the production through adoption of quality assurance principles.

(9) Only products which pass the official recommended quality control tests should be allowed to go out for public consumption.

(10) The suppliers of the orange drinks can supply in small quantities to avoid poor storage of the drinks.

REFERENCES

ADEYEMI, S.A.O. (1987): Available Fruits and Vegetables in Nigeria and their contributon to national economy: Key note address presented at the opening ceremony of the First National Home Economics Workshop held at Agricultural Extension and Reasearch Liason Service, Ahmadu Bello University, Zaria.

ADEYEMI, S.A.O. (1988): The Current Status of Research and Development in Promoting the Processing and Utilization of Fruits and Vegetables in Nigerian Industries. In Processing of the Technical Seminar on Raw Materials for the Food, Beverage and Tobacco Industrial Sector. Raw Materials Research and Development Council, Nigeria, pp. 40-52.

ANDERSON, J.D. & COX, C.S. (1967): Microbial Survival. In Airbone Microbes, Gregory, P.H. and Monteith, J.L. (Eds), Seventeenth Symposium of the Society for General Microbiology, Cambridge University Press, pp. 203-226.

ANONYMOUS, (1982): The Bacteriological Examination of Drinking Water Supplies: Report on Public Health and Medical Subjects, No. 71, London HMSO.

ANONYMOUS, (1971): International Standards for Drinking Water: 3rd Edition, Geneva: World Health Organisation.

ANTAI, S.P. & ANOZIE, S.O. (1987): Incidence of Infantile Diarrhoea Due to Enteropathogenic Escherichia coli in Nigeria: Journal of Applied Bacteiology 62, 227-229.

AVIS, K.E. (1975): Parental Preparation. In Remington's Pharmaceutical Sciences: 15th Edition, Mack Publishing Company, Easton, Pennyslavania; pp. 1461-1487.

BAIRD, R.M. (1987): Contamination of Non-Sterile Pharmaceuticals in Hospitals and in Community Environment. In Pharmaceutical Microbiology, Hugo, W.B. & Russell, A.D. (eds), 4th Edition, Blackwell Scientific Publications, Oxford, pp. 381-391.

BANWART, E. (1987): Analysis of the Content of Fruits: Journal of Food Sciences 35, 30-33.

BARNES, J.M. (1970): Aflatoxin as a Health Hazard: Journal of Applied Bacteriology 33, 285.

BEACHEY, E.H., EISENSTEN, B.I. & OFEK, I. (1981): In Adhension and Microorganisms Pathogenity: Ciba Foundation Symposium. C.J. Elliot et al (eds). Vol. 80, pp. 288-304. Pitman Medical, London.

BLOCK, S.S. (1983): Chemical Disinfectants, Antiseptics and Preservatives: In Pharmaceutical Microbiology, Hugo, W.B. & Russell, A.D. (eds), 4th Edition. Blackwell Scientific Publications, Oxford, pp. 226-252.

BROWN, M.R.W. & WILLIAM, P. (1985): Influence of Substrate Limitation And Growth Phase On Sensitivity To Antimicrobial Agents: Journal of Antimicrobial Chem. 15, Suppl A 7-14.

BRYAN, C. STRATFORD (1977): Mycology: In An Atlas of Medical Microbiology. De Szors, J. and Wilson, J.V. (eds) Scientific Publications, London, pp. 117-129.

CHAMBERS, C.W. & CLARKE, N.A. (1968): Control of Bacteria in Non-Domestic Water: Advances in Applied Microbiology 8, 105-143.

CHAPMAN, D.G. (1987): Preservatives Available For Use: In Preservatives in The Food, Pharmaceuticals & Environmental Industries: Board, R.G., Allwood, W.C. & Banks, J.G. (eds) SAB Technical Series, 22, Blackwell Scientific Publications, Oxford, pp. 177-195.

COLLING, V.G. (1964): Microorganism Ecology and The Pharmaceutical Industry: In Pharmaceutical Microbiology, Hugo, W.B. & Russell, A.D. (eds), 4th Edition, Blackwell Scientific Publications, Oxford, pp. 343-359.

COWAN, S.T. (1974): Cowan and Steel's Manual For The Indentification of Medical Bacteria: 2nd Edition, Cambridge University Press, Cambridge.

CROSHAN, B. (1977): Preservatives for Cosmetics and Toiletries: Journal for SOC Cosmetics Chem. 28, 3-16.

CRUESS, W.V. (1952): Unfermented Fruit Beverages: In Commercial Fruit & Vegetable Products, Wismenski, T. (ed), Maple Press Company, pp. 304-365.

D'AVOUST, J.Y. (1989): Salmonella: In Foodborne Bacteria Pathogens, Doyle, M.P. (ed), Marcel Dekker, New York, pp. 327-445.

DELFS-FRITZ, W. (1970): Origin and Contents of Citrus Fruits: In Citrus Fruits, Perlman, D. & Peruzzotti, G.P. (eds), The Marple Press company, 2nd Edition, pp. 167-169.

DEMPSEY, G. (1988): The Effect of Container Materials & Multiple Phase Formulation Components on The Activity of Antimicrobial Agents: In Microbial Quality Assurance in Pharmaceuticals, Cosmetics & Toiletries, Bloomfield, S.F., Baird, R., Leak, R.E. & Leach, R. (eds), Ellis Horwood, Chichester, pp. 94-103.

DENYER, S.P., RUSSELL, A.D. & HUGO, W.B. (1982): Factory And Hospital Hygiene And Good Manufacturing Practice: In Pharmaceutical Microbiology, Hugo, W.B. & Russell, A.D. (eds), 4th Edition, Blackwell Scientific Publications, Oxford, pp. 433-445.

DENYER, S.P. (1989): Hospital Hygiene And Good Manufacturing Practice: In Pharmaceutical Microbiology, Hugo, W.B. & Russell, A.D. (eds), 4th Edition, Blackwell Scientific Publications, Oxford, pp. 433-445.

DOYLE, M. & PADHYE, V. (1989): Escherichia coli.: In Foodborne Bacterial Pathogens, Doyle, M.P. (ed), Marcel Dekker, New York, pp. 235-281.

FIP Working Party (1972): Report on Permisible Contaminant. Journal Mond. Pharmacy 15, 88.

GELDREICH, E.E., NASH, H.D., REASONER, D.J. & TAYLOR, R.H. (1975): The Necessity of Controlling Bacterial Populations In Potable Water: Bottle And Emergency Water Supplies: Journal of The American Water Works Association 67, 117-124.

GREGORY, P.H. (1973): Microbiology of The Atmosphere: Kumar, P. et al (eds), 2nd Edition, London, Edward Arnold, pp. 380-390.

HUGO, W.B. & RUSSELL, A.D. (1987): Microorganism Ecology and the Pharmaceutical Industires: In Pharmaceutical Microbiology, Hugo, W.B. & Russell, A.D. (eds), 4th Edition, Blackwell Scientific Publications, Oxford, pp. 343-359.

JACKSON, G.J. (1990): Public Health And Research Perspectives On The Microbial Contamination of Foods: Journal of Animal Science 68, 884-891.

JACKSON, G.J., LANGFORD, C.F. & ARCHER, D.L. (1991): Control of Salmonellosis And Similar Foodborne Infections: Journal of Food Control 14, 26-33.

JAY, C. (1978): Nutrient Composition of Sweet orange Fruits: In Fruits & Vegetables: Ayres, J.C. (ed), 1st Edition, Scientific Publications, London, pp. 20-22.

JONES, J.G., GARDENER, S., SIMON, B.M. & PICKUP, R.W. (1986): Antibiotics Resistant bacteria In Windermere And Two Remote

Upland Bacteriology 60, 443-453.

LABORATORY SERVIES (1971): Report of The Public Health Laboratory Service Working Party: Microbial Contamination of Medicines Administered To Hospital Patients: Pharmaceutical Journal 207, 96-99.

MARGALITH, P. & SCHWARTZ, Y. (1970): Flavour And Microorganisms: In Advances In Applied Microbiology: Shooter, R.A. et al, (eds) Academic Press, New York and London 12, 35.

MAVRIDOU, A., PAPAPETROPOULOU, M., BOUFA, P., LAMBIRI, M. & PAPADAKIS, J.A. (1994): Microbiological Quality of Bottled Water In Greece: Letters In Applied microbiology 19, 213-216.

MITCHELL, R. (1972): Water Pollution Microbiology: 1st Edition: John Wiley & Sons, New York, Elsevier Publishing Co., Amsterdam, London & New York.

NISHANNON, A. (1977): Comparative Studies of Microbial Contamination of Surfaces by the Contact Plate and Swab Methods: Journal of Applied Bacteriology 42, 53-63.

OGAN, M.T. (1992): Microbiological Quality of Bottled Water Sold In Retail Outlets In Nigeria: Journal of Applied Bacteriology 73, 175-181.

ONAOLAPO, J.A. (1986): The Measurement of Surface Charge By Electrostatic Interaction Chromatography (ESIC): In The Effect of R-Plasmid RPI On The Properties of Proteus Mirabilis: Ph.D Thesis, Aston University, Birmingham, pp. 109-190.

ORTH, B.F. (1981): The Clinical Significance of Microbial Contamination in Pharmaceutical And Applied Products: Advances in Pharmaceuticals Sciences 5, 201-226.

PACKER, M.E. & LITCHFIELD, J.H. (1972): Microorganisms Ecology And ThePharmaceutical Industries: In Pharmaceutical Microbiology, Hugo, W.B. & Russell, A.D. (eds), 4th Edition, Blackwell Scientific publication, Oxford, pp. 343-359.

PEKZAR, A. & REID, B. (1965): Microbiology of Food: In Microbiology, Geosmin, H. & Lechevalier, A. (eds), McGraw-Hill Inc. Book Company, New York, pp. 550.

PRODA, P. (1979): Plasmids, Oxford: W.A. Freeman & Company, pp. 40-51.

RIVILLA, R. & GONZALEZ, C. (1988): Simplified Methods For The microbiological Evaluation of Bottle Natural Mineral Waters: Journal of Applied Bacteriology 64, 273-278.

RUSSELL, A.D., HUGO, W.B. & AYLLIFE, G.A.J. (1982): Principles And Practice of Disinfection, Preservation And Sterilization: Russell, A.D. et al, (eds), Oxford: Blackwell Scientific Publications, pp. 306-320.

SHWARTZ, B., BROOME, C., BROWN, G., HIGHTOWER, A., CIESIELSKI, C., GEVENTA, S., et al (1988): Association of Sporadic Listerosis With Consumption of Food: Lancetii, 779-782.

SHATBY, S.W. (1978): Biochemical Tests Of Importance In The Identification Of Bacteria: In Basic Bicrobiology: Issued By The Department of Pharmaceutics And Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria, pp. 32-39.

SKINNER, F.A. & CARR, F.G. (1974): The Normal microbial Flora of Man: Society For Applied bacteriology Symposium No. 5, London Academy Press, pp. 30-38.

SMART, R. & SPOONER, D.F. (1972): Contamination of Non-Sterile Pharmaceuticals: In Pharmaceutical Microbiology: Hugo, W.B. & Russell, A.D. (eds), 4th Edition, Blackwell Scientific Publications, Oxford, pp. 380-391.

STAVRK,S. & SPEIRS, J.I. (1989): Escheirchia coli Associated With Haemorrhagic Colitis: Canada Institute Of Food Science And Technology Journal 22, 3.

SYKES, G. (1972): The Control Of Microbial Contamination : In Pharmaceutical Products For Oral And Topical Use-Raw Materials: Indian Jorunal of Pharmacy 34, 25-34.

TOBIN, R.S. (1984): Water Treatment For Home And Cottage: Canadian Journal of Public Health 75, 79-82.

UNDERWOOD, E. (1982): Good Manufacturing Practice: In Principles And Practice of Disinfection, Preservation And Sterilization: Editors A.D. Russell, W.B. Hugo & G.A.J. Ayllife: Blackwell Scientific Publication, Oxford, pp. 221-243.

UNDERWOOD, R.S. (1987): Ecology of Microorganisms As It Affects The Pharmaceutical Industry: In Pharmaceutical Microbiology: Hugo, W.B. & Russell, A.D. (eds), 4th Edition, Blackwell Scientific Publications, Oxford, pp. 343-359.

WATERWORTH, P.W. (1978): Qualitative Methods For Bacteria Sensitivity Testing: In Laboratory Methods In Antimicrobial

Chemotheraphy: Waterworth, P.E. (ed), 1st Edition, Churchill Livingstone, Edinburgh, pp. 35-38.

WATERWORTH, P.W. (1978): Determination of Minimum Inhibitory Concentrations Reported in Clinical Bacteriology: Stokes, E.J. (ed), 4th Edition, Arnold, Britain, pp. 226-238.

WEBBER, H.J. & BATCHELLOR, L.D. (1948): Cultivated Varieties of Citrus: In Citrus Industry: Worrel, Celia S. (ed), 1st Edition, Maple Press Company, pp. 501-502.

WHO (1989): Guidelines For The Safe use of Waste Water And Excreta in Agriculture And Aquaculture: World Health Organisation, Geneva.

WHO Expert Committee (1988): Salmonellosis Control: In Control of Salmonellosis And Similar Foodborne Infections: WHO Technical Report Series 774, Geneva.

WOOD, J.M. (1979): The Interation of Microorganism With Ion Excange Resins: Ph.D Thesis, University of Surrey.

YABLONSKI, W. (1978): Environmental And Equipment Cleaning in Manufacturing Plants: Journal of Food Technology 21, 44-48.

ZUAGINSTEV, D.G. & GUSER, V.S. (1971): Concentration And Separation of Bacteria on Dowex Anionite: Microbiologiya 40, 123-126.