PREVALENCE AND CHARACTERISTICS OF COLIFORMS AND STAPHYLOCOCCI IN A LOCAL CEREAL DRINK (KUNUN ZAKI) IN SAMARU ZARIA

BY

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DECLARATION

I hereby declare the originality of this piece of work which was carried out by me in the laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria under the supervision of Drs. A. A. Adesiyun, J. O. Adekeye and Professor E. D. Belino.

The work of other investigators is duly acknowledged and referred to. No part of this thesis has previously been submitted for a degree or diploma.

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Ahmadu Bello University
November 1984

CERTIFICATION

This thesis entitled "Prevalence and Characteristics of Coliforms and Staphylococci in A Local Cereal Drink (Kunun Zaki) in Samaru Zaria" meets the regulation governing the degree of Master of Science of Ahmadu Bello University Zaria, and is approved for its scientific knowledge and literary presentation.

SUPERVISOR

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Postgraduate School

Date: _____

AHMADU BELLO UNIVERSITY, ZARIA

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AHMADU BELLO UNIVERSITY, ZARIA

DEDICATION

Dedicated to my father, brothers
and sisters for their love and
encouragement.

AND

To my late mother, the best of
mothers.

ACKNOWLEDGEMENTS

I am particularly grateful to my major supervisor, Dr. A. A. Adesiyun, who not only initiated the project but played a prominent role in the supervision of every aspect of the study. I also appreciate his critical review and suggestions made while writing up this thesis.

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ABSTRACT

The prevalence of staphylococci and coliforms in kunu and utensils from points of preparation and sale in Samaru was determined. The prevalence rate for staphylococci ranged from 75.0% to 100.00% at both points of preparation and sale outlets. Coliforms were detected in 75.0% to 91.6% of samples from points of sales. For all samples of kunu, the mean staphylococcal counts ranged from 5.2×10^2 to 2.1×10^4 per ml, coliforms from 4.5×10^5 to 3.3×10^6 and total aerobic plate counts from 1.3×10^{10} to 8.4×10^{10} per ml. All water samples were contaminated with both total and faecal coliforms.

Of the spices usually added to kunu, cloves was found to be most inhibitory to S. aureus and E. coli at both 0.25% and 1.25% concentration, while Red pepper, Black pepper and Ginger at similar concentrations showed very little antimicrobial activity.

The effects of pH and temperature on survival and growth of S. aureus, E. coli and other microorganism were more pronounced (in population decline) at $25^{\circ}C$ and $37^{\circ}C$, while only minimal decline in population was detected at $4^{\circ}C$. PH's of 3 and 4 were most bactericidal while 5 and 6 allowed growth of organisms

within the first 8 hours but declined thereafter.

All staphylococcal isolates from kunu and utensils fermented glucose while 240 (98.0%) fermented mannitol. Two hundred and thirty-two (95.0%) isolates produced coagulase and 193 (79.0%) elaborated thermonuclease. Non-hemolytic staphylococcal isolates were most predominant type, 140 (57.1%) while 70 (28.7%) and 35 (14.3%) showed beta and alpha hemolytic patterns respectively.

The incidence of enterotoxin production by staphylococcal isolates was 8.1%. Staphylococcal enterotoxin A (SEA) was produced by 2 (0.8%) isolates, SEB by 10 (4.1%) isolates, SEC by 2 (0.8%) isolates, SED by 8 (3.3%) isolates while no isolates produced SEE. Only 2 isolates elaborated more than one enterotoxin, types BD. Upon inoculation of kunu with known enterotoxin producers (S. aureus strains Z88 and 418), no detectable amount was produced after 24 hours at 37°C with accompanying 9.5×10^6 to 1.3×10^3 per ml decline in staphylococcal population observed. Based on the low incidence of enterotoxin production by staphylococcal isolates from "kunun zaki" coupled with the inability of enterotoxigenic strains to elaborate toxins in kunu, it was concluded that the risk of staphylococcal intoxication to consumers of kunun zaki is low.

The sensitivity of staphylococcal isolates to eleven antimicrobial agents showed that 247 (97.6%) were resistant to one or more antimicrobial agent. Resistance to penicillin 234 (97.2%) ampicillin 233 (92.1%) and Trimethoprim 203 (80.2%) were most common while resistance was least developed to gentamycin 8 (2.8%). The relative high resistance to S. aureus from "kunun zaki" to tested antimicrobial agents suggests a misuse of these drugs in this environment.

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CHAPTER ONE

INTRODUCTION

Despite extensive research on growth requirements, epidemiology and measures for control of Staphylococcus aureus, it continues to be a prominent causative agent of foodborne disease (Troller 1976). Approximately 50% of S. aureus are known to be enterotoxigenic (Todd, 1978; Wieneke, 1974; Bergdoll, 1972). In Nigeria, where reporting of food poisoning outbreaks is virtually non-existent, the role and possible involvement of staphylococci in foodborne disease is not yet fully known. The fact that staphylococci are ubiquitous in nature especially in the air, milk, foods and sewage makes this organism a constant source of concern in Nigeria.

Enterotoxigenic staphylococci and enterotoxins in amounts capable of causing intoxication, have been found in numerous foodstuff and ready-to-eat food in Nigeria and other countries (Burbianka 1971; Genigeorgis, 1963; Payne and Wood 1974; Adesiyun 1984). It was recommended that foods should be screened for thermonuclease as an index of staphylococcal growth and possible enterotoxin production (Tatini et al., 1976; Tatini, 1981), in situations where cell death has taken place. Thermonuclease as an index of

staphylococcal growth has some limitations because there is poor correlation between thermonuclease detection and staphylococcal growth (Nambudripad 1975; Park et al., 1978).

Environmental factors like temperature, pH, water activity, nutrition have been shown to have direct effects on staphylococcal growth and enterotoxin production (Genigeorgis et al. 1969; Landoler et al., 1964). Staphylococcal growth occurred in some locally fermented milk product at a pH as low as 3.5 (Umoh et al., 1982) and the organism may also be found in kunu, a locally fermented cereal product.

The constant presence of coliforms especially Escherichia coli a normal inhabitant of human and vertebrate intestines has been used as an index of faecal contamination of water (Bissonnette et al., 1975) The presence of coliforms in foods suggests either inadequate treatment during processing or access of undesirable materials to the food after processing (Kabler and Clark 1960). Numerous workers have attempte to identify factors affecting the survival of faecal coliforms in water. This is very important as far as identification and enumeration of indicator bacteria is concerned. Factors influencing the survival include temperature, oxygen tension, pH, salt

concentration, nutrient and cell concentration (Hoadley and Cheng, 1974; Bissonnette, et al., 1975, Smith 1975).

Kunu is traditionally prepared with the addition of spices. In 1974, Julseth and Diebel isolated staphylococci and coliforms from spices like ginger and black pepper. These spices which add flavour to kunu may act as sources of contamination for organisms like staphylococci and E. coli. The poor sanitary practices that accompany preparation of kunu, starting with grinding of the cereal millet, guinea corn, or corn in public grinding machines and mixing with water, to bottling suggest that there may be various points at which staphylococcal and coliforms contamination might occur especially from human handlers. It is therefore pertinent to determine the prevalence of these micro-organisms in kunu.

Kunu is usually kept at room temperature for long hours when hawked about or kept in refrigerators which often do not function properly because of frequent electricity failure. These factors could allow for rapid multiplication of coliforms and staphylococci with possible toxin production. Since kunu is an uncontrolled fermented product, the pH of the product at a stage during storage may be acidic to kill off

some E. coli and staphylococci while having no effect on preformed enterotoxin. Therefore detection of low counts of viable staphylococci could give a false security as regards staphylococcal intoxication. Kunu is often prepared under insanitary conditions and this may result in high counts of staphylococci, some of which may be enterotoxigenic. It is therefore necessary to determine the incidence of enterotoxigenicity of staphylococcal isolates in kunu as well as assessing the ability of kunu to allow toxin elaboration.

The above-mentioned problems have therefore led to a study with the following specific objectives:

1. To determine the prevalence of staphylococci and coliforms in kunun zaki.
2. To determine the bacterial load of various spices added to kunun zaki and evaluate their antimicrobial effects.
3. To evaluate the effects of pH and temperature on survival and proliferation of E. coli and S. aureus in kunu.

4. Finally, to determine some characteristics such as coagulase, thermonuclease, hemolysin and enterotoxin production by staphylococcal isolates from kunu.

LITERATURE REVIEW

Staphylococcal food poisoning is a foodborne intoxication caused by the consumption of foods containing preformed enterotoxin which are produced by certain strains of Staphylococcus aureus. Under certain conditions, however, the symptoms of this common type of food poisoning may occur as a result of growth of staphylococcus in the intestinal tract (Casman 1965; Kienity 1966; Hallender and Korlof, 1967).

The role of staphylococcus as a causative agent of food poisoning was first recognised by Barber in 1914. Medical descriptions of food poisonings before the discovery of staphylococcus by Koch in 1878 and Pasteur in 1880 indicated that they were responsible for food poisoning for centuries (Dack 1956). The name staphylococcus was coined by Ogston in 1881; pure cultures of staphylococcus organism on solid media were first obtained by Rosenback in 1884. In 1930, Dack and his co-workers isolated

staphylococcus from foods and reproduced the disease by administering culture filterates to human volunteers (Genigeorgis 1974).

Staphylococcus aureus are gram-positive grape-like bacteria that grow in clusters. They are non-motile asporogenic organisms and are between 0.7 to 1.2 microns in diameter. The colonies are usually round, raised and shiny with pigmentation ranging from white to golden yellow, although some strains may be unpigmented. Staphylococci are facultative in their oxygen requirement in a complex glucose medium but grow better aerobically than anaerobically (Barber and Deibel 1972).

The disease caused by this organism is characterised by low case fatality rates and relatively short duration. The frequency of outbreak and severity of the symptoms make staphylococcal food poisoning an important food borne hazard in many types of foodstuffs (Troller, 1976). Present information indicates that up to 50% of S. aureus isolates are able to produce enterotoxin when tested in the laboratory and that a single strain may produce two or more enterotoxins (WHO Report 1976). Enterotoxigenic strains are culturally indistinct from non-enterotoxigenic strains. At least five serologically

distinct enterotoxins A, B, C, D, and E are now recognised (Casman et al, 1967; Gilbert et al, 1973 and Gilbert 1974).

The enterotoxins of staphylococci are toxic metabolites which cause food poisoning symptoms in human beings. Enterotoxin types A and D are most commonly involved in foodborne intoxication cases. These enterotoxins are produced in relatively small amounts when compared to enterotoxin B and C (Jarvis et al, 1973). Synthesis of enterotoxin occurs during the exponential, transitional and stationary growth phases of S. aureus cells (Czop and Bergdoll 1974). Enterotoxigenic S. aureus and enterotoxins have been isolated from foods involved in food poisoning outbreaks. Enterotoxigenic strains have also been isolated as well from various foods routinely sampled and not involved in any intoxication (Burbianka 1971, Simkovicova and Gilbert 1971).

Numerous foods have been implicated as sources of enterotoxin produced by S. aureus in outbreaks of food poisoning, as indicated by Angelotti (1969) and the foods that were usually involved were cooked protein containing products which were contaminated by handling after and allowed to remain at room temperature or refrigerated in large masses for

several hours frequently overnight (Bryan 1970).

It is very difficult to avoid contamination of food by this organism since approximately 50% of the human population are healthy carriers (Rayman 1976). The environment and handling of food processings contribute immensely to staphylococcus food poisoning.

Extensive studies have been done to determine the prevalence of staphylococci in the raw or processed foods. Roskéy and Hamdy (1972) surveyed the possible sources of staphylococcal infection of bruised poultry tissue and they noted that S. aureus was found in 86.6% of swab samples obtained from infected workers, in 40% of swabs from hands of workers who handled bruised birds, and in 38.7% of bruised tissues, and was absent from all samples obtained from hands of workers who do not handle bruised birds. They concluded that poultry bruises are a source of staphylococcal infection encountered among poultry workers. In Nigeria, some workers have reported the prevalence of this organism in raw-meat, ready-to-eat meat products and fermented milk and cereal (Nkanga and Uraih, 1981; Adesiyun, 1983; Umoh et al., 1982).

Staphylococcal food poisoning is caused by the ingestion of preformed enterotoxin present in the food by a susceptible individual. It has also been reported that symptoms may arise as a result of growth of enterotoxigenic strains in the intestinal tract and conceivably elsewhere in the body (Casman, 1965). There is variation in the literature with regards to the length of the incubation period. It is usually about 3 hours but may vary from less than one hour to several (rarely more than 6-8 hours) and depends on the susceptibility of the individual and dosage consumed (Frazier 1967; Angelotti 1969; Langree 1972). Benenson (1975) stated that food poisoning are usually recognised by the sudden occurrence of illness within a short period of time among individuals who have consumed one or more foods in common.

Human symptomology include most commonly salivation, then nusea, vomitting, retching, abdominal cramping of varying severity and diarrhea. Headache, muscular cramping, sweating, chills, prostration, weak pulse, shock and shallow respiration are known to occur in occasions, with subnormal temperature. Duration is brief with low mortality (Frazier 1967; Angellotti 1969; Dack 1956; Langree 1972).

Identification of S. aureus enterotoxins is a labourious, time-consuming and costly procedure, routine testing of every batch of fermented foods for the presence of enterotoxin would require more resources than are available (Emswiller-Rose et al., 1980). As a result, researchers have developed the thermonuclease test which appears to be a rapid and indirect screening method for enterotoxin (Tatini et al., 1976). Various other workers have assessed the application of thermonuclease to screen for staphylococci in foods and found it very reliable (Cords and Tatini, 1973; Batsh et al., 1978; Park et al., 1978). The thermonuclease (TNase) is useful in screening foods for the likely presence of enterotoxin (Erickson and Deibel, 1973). Since TNase is more stable to heat and low pH than S. aureus itself and TNase is as stable to heat as enterotoxin, this test would be of particular value in thermally processed and fermented finished food products, like the Nigerian Kunu. A logical means is to first determine the incidence of thermonuclease production by staphylococcal isolates from kunu prior to assessing the production of this metabolite in kunu.

It has been suggested that not all coagulase-positive are pathogenic (Jay, 1971). Coagulase

production is one of the major criteria used to differentiate between S. aureus and S. epidermidis. It has been determined that approximately 99% of S. aureus associated with pathogenic processes in man are capable of elaborating this enzyme and are then classified as S. aureus. All strains of S. aureus are by definition coagulase-positive (Baer et al., 1976). In the light of this, coagulase-positive S. aureus isolated from food should be regarded as potential food poisoning types. Langree (1972) however disagrees, for he noted that although most if not all the enterotoxigenic S. aureus are coagulase-positive, its not a full proof for pathogenicity since there are possibilities that some strains of coagulase negative strains of staphylococci have since been shown to be enterotoxigenic and even implicated in food poisoning outbreaks (Omori and Kato, 1959; Breckinridge and Bergdoll, 1971, Danielssen and Hellberg, 1977).

S. aureus produces alpha, beta and gamma toxins; human biotypes of S. aureus are known to produce alpha hemolysin while animal biotypes predominantly elaborate beta hemolysin (Elek and Levy 1950; Hajek and Marsalek, 1969). The types of hemolysins produced coupled with the type of plasmas coagulated by some

strains have been used to determine the probable sources of staphylococci (Live, 1972; Adesiyun and Usman, 1983).

It has been found that most strains of S. aureus ferment glucose and mannitol and can tolerate high salt concentration. The anaerobic fermentation of these sugars by the organism distinguishes it from other staphylococcal species. (Stiles and NG 1982). Mossel (1962) cited anaerobic dissimilation of mannitol and coagulase production as the best criteria for distinguishing S. aureus from other catalase - positive cocci, and recommended that coagulase-negative or anaerobic mannitol negative strains be classified as S. epidermidis or S. saprophyticus. It is however also known that strains of S. aureus vary in their rates or ability to utilize mannitol (Devriese and Derycke, 1979). Since the various source of staphylococci contaminating kunu are presently unknown, it is necessary to determine the relative abilities of such isolates to utilize mannitol and glucose anaerobically.

For S. aureus to grow and produce enterotoxin, environmental conditions such as, water activity (aw), pH, O_2 tension, temperature and competitive

growth of other microorganisms found in raw, semi-processed and fermented foods must be favourable (Tatini 1973). Consequently the interaction of environmental factors such as these which exist in foods at various phases of handling that results in permitting either unrestricted, restricted or no growth and/or production of enterotoxin have been studied (Tatini 1973). Kunu is a locally uncontrolled fermented cereal drink with accompanying variable pH, exposed to contamination at many points during preparation and sale and experiencing changing temperatures. All these present conditions that may affect survival and proliferation of S. aureus.

Spices are natural products of pronounced odour and taste that serve as flavour and to some extent as preservatives (Omakwu 1980). Their aromatic and pungent properties are used in adding tang to beverages, savour to dishes and zest to appetizers (Omakwu 1980). Some spices like cloves, ginger, cinnamon and pepper could be used as carminitives, stimulants and stomachics in modern medicine because of their bactericidal and fungicidal properties (Omakwu 1980). Frazier (1967) stated that spices may serve as preservatives in the food to which they are ingredients and that the essential oils of

these spices may serve as preservative in the food to which they are ingredients, and that the essential oils of these spices are more inhibitory to microorganism than the corresponding ground spices. He also noted that cinnamic aldehyde and eugenol, respectively have bacteriostatic action on some microorganisms while ginger may have weak inhibitory effects.

Resistance of staphylococci to various antibiotics have been well documented. (Noble 1977; Adekeye, 1979). Attempts have been made to control staphylococcal infection with the use of antibiotics in both man and animal and this has resulted in increased prevalence of resistance to these agents (Barber et al., 1949; Nakhla, 1973). In developed countries stringent measures have been devised in controlling the misuse of these antibiotics and these have successfully reduced the prevalence of antibiotic resistance to these agents (Bulger and Sherris; 1968). In Nigeria the situation is however different. Antibiotics are available to people in local drug stores and this has led to misuse of antibiotics and resistance amongst staphylococci (Adekeye 1979, Paul et al, 1982).

Coliforms

The science of sanitary water bacteriology began in 1880 when Von Fritsch described Klebsiella pneumoniae and K. rhinoscleromatic as organisms characteristic of human faecal contamination. A short time later Escherichia identified Bacillus coli as an indicator of faecal pollution. Both observers considered human faeces as a dangerous source of pollution while the faeces of other warm blood animals were not considered a health hazard. Later the current coliform group developed to include numerous microorganisms of diverse biochemical and serological, characteristics (Geldrieich, 1966). Standard methods define the coliform group as including all the aerobic and facultative anaerobic gram-negative, non-spore forming rod-shaped bacteria which ferment lactose with gas production within 48 hours at 35°C (American Public Health Association 1971). The use of the membrane filter technique has led to the establishment of another definition of coliforms which is "all organism which produce a dark colony (generally purplish-green) with a metallic sheen within 24 hours of incubation in a specified medium and at a specified temperature (Wolf, 1972). Smith

(1895) stated that the coliform group represented faecal pollution and danger to health, regardless of the environment in which they were detected. He believed all coliform bacteria come from the gut of warm blooded animals and their presence elsewhere to be the result of faecal pollution. The increasing reports of recoveries of coliform organism from non-feacally contaminated environment together with reports of the multiplication of coliforms in some natural environment stimulated researchers to separate faecal from non-faecal coliform, (Wolf 1972). Sanitary bacteriologist, therefore invented the elevated temperature tests as a means of differentiating faecal from non-faecal coliforms, a test originally reposed by Eijkman in 1904. Eijkman had observed that coliform bacteria derived from the gut of warm-blooded animals produced gas from glucose at 47°C while coliform strains from non-faecal source failed to grow at this temperature. E. coli is therefore frequently referred to as faecal coliform (Wolf, 1972).

The American Public Health Association (1980) specified the methods for the microbiological analysis of potable water, the most-probable - number and the membrane filter (MF) method. The MF technique

has gained wide acceptance because the procedure is simple, rapid, precise, and gives definitive results (Lechevallier et al., 1983). However factors such as turbidity (Fryt, 1979, Lechevallier et al., 1981), high number of non-coliform bacteria (Clark et al., 1977; Clark 1980, Lechevallier 1980) and membrane filter types (Mcfeters et al., 1972, Sladek et al., 1975) may severely influence the sensitivity of the procedure. The use of an elevated temperature of 44.5°C for enumerating faecal coliform organism has been specified by MF technique.

Numerous workers have attempted to identify factors affecting the survival of faecal bacteria in surface waters (Allen et al., 1952; Allen, 1953; Hanes et al., 1964; Hanes et al., 1965; Hoadley et al., 1968; Geldreich and Kenner, 1969). Other workers have attempted to evaluate and improve diluents used to count bacteria in water, sewage and food products in addition to enumerating E. coli in some foods (Postgate and Hunter, 1962, 1963; Vanderzant and Kruegur, 1968; Geoffert, 1975). Environmental factors such as pH, temperature, oxygen tension, salt concentration, nutrient and cell concentration may influence survival of coliforms in aqueous suspension (Hoadley and Cheng 1974). With the possibility of

coliforms gaining access to kunu through either water used in the preparation under insanitary conditions by handlers, it is imperative that all conditions that may affect survival and proliferation of coliforms, especially E. coli, in kunun zaki need to be studied.

CHAPTER TWO

PREVALENCE OF STAPHYLOCOCCI AND COLIFORMS IN KUNU AND UTENSILS USED IN ITS PREPARATION

SUMMARY

The prevalence of staphylococci and coliforms in kunu and on utensils used in it's preparation were determined. Samples were obtained at weekly intervals from randomly selected points of preparation and sale outlets in Samaru over a 12-week period. Baird-Parker agar (BPA), MacConkey and nutrient agars were use to enumerate staphylococci, coliforms and total aerobic plate counts, respectively.

For kunu from all sources, the range of mean counts per ml were 5.8×10^2 to 2.1×10^4 for staphylooocci, 4.5×10^5 to 3.3×10^6 for coliforms and 1.3×10^{10} to 8.4×10^{10} for total aerobic plate counts. On utensils, mean staphylococcal counts ranged from 2.0×10^1 to 3.0×10^2 per sq. cm while for aerobic plate count it was 1.0×10^3 to 2.5×10^5 per sq. cm.

Water samples used in kunu preparation at all sources were grossly contaminated with mean total coliforms and faecal coliforms per 100 ml ranging from 598 to 1383 and 393 to 818 respectively.

The relatively high degree of contamination of kunu make it a possible health hazard to consumers.

INTRODUCTION

Staphylococci are known worldwide to cause foodborne intoxications (Bergdoll, 1979). In Canada and U.S.A. nearly 30% of all reported foodborne disease outbreaks of bacterial origin are attributed to staphylococci (Bergdoll, 1979). In developed countries of the world, the frequency of involvement of staphylococci in foodborne intoxications are documented due to the existence of effective reporting systems (Todd, 1978). However, in Nigeria there is virtually no information on the involvement of Staphylococcus aureus in foodborne disease primarily due to poor or virtually non-existing reporting system. This has resulted in the lack of statistical data which Nnochiri (1975) attributed to the absence of trained man-power especially in rural areas where the most pitiable environmental sanitation and personal hygiene exist.

Food items such as meat and meat products, dairy product, bakery products, custard and cream-filled pastries have been found to contain enterotoxigenic staphylococci or enterotoxins and some implicated in foodborne disease outbreaks (Baer, et al. 1976;

Payne and Wood 1974; Wieneke, 1974). Little information is available on Nigerian foods.

Nkanga and Uraih (1981) reported a high degree of contamination of raw meat sold at a Benin City market by S. aureus. Adesiyun (1984) recently isolated enterotoxigenic staphylococci from five commonly consumed Nigerian ready-to-eat products and high-lighted the health hazard involved. A locally fermented milk product called "Nono" was also reported to be highly contaminated by S. aureus (Umoh et al., 1982). To date little information is available on the role kunu, a popular drink in both rural and urban areas, can play in staphylococcal intoxication in its consumers.

Coliforms have been isolated in varying degrees from foods like ground beef (Geoffert, 1975), sausage, chicken pot pie, mechanically deboned poultry meat (Mercur and Cox 1979) and pumkin pies (Wyatt and Guy, 1981). Some of these coliforms isolated have also been shown to be of faecal origin.

Makinde (1979) detected a mean coliform count of 5.8×10^6 per millilitre of "Nono" samples studied. Omakwu (1980) also found flour used in preparing kunun tsamiya to be grossly contaminated by coliforms.

Recently, Igene (1983) reported that grilled beef "suya" sold around Maiduguri were highly contaminated by coliforms, predominantly faecal Escherichia coli, indicating poor sanitary practices during preparation of the product.

Presently, the degree of coliform contamination of kunu by the time it gets to the consumer is unknown.

Kunu, being predominantly water could be contaminated from the water sources. Well water samples have been reported to be grossly contaminated by coliforms as reflected by Most Probable Number indices with demonstration of faecal coliforms in Katsina (Adesiyun et al., 1983) and Samaru (Bello, 1971) where 42% of wells sampled contained coliforms. Umoh (1976) further identified approximately 50% of coliforms from Samaru wells to be faecal coliforms.

In view of these points, this study was undertaken to determine the prevalence of staphylococci and coliforms in kunu and utensils used to prepare it as well as to determine the quality of water used in its preparation.

MATERIALS AND METHOD

Preparation of kunun zaki

Guinea corn, millet or maize could be used in the preparation. The grains, when purchased, are usually soaked in water for 1 to 2 days and then ground into flour form at any mill in Samaru Village.

Kunun zaki is prepared locally by making a paste of the flour in cold water. To half of this paste, boiled water is added which results in a homogenous or semi-solid form. Cold water is added to the remaining portion of the paste and this is subsequently mixed with the homogenous form. At this stage various spices (ginger, black pepper, cloves and red pepper) already mixed in water are passed through a nylon cloth or metallic seive to the solution of kunu. The approximate proportion of the various spices are in the order, Ginger (65%), Red pepper (25%) Cloves and Black pepper (10%). Ground sugar is also seived through the nylon material, but the quantity is subject to individual taste. The kunu is then bottled and ready for consumption.

Source of Samples

Six points of preparation and six sale outlets (local kiosks and hawkers) for kunu were randomly

selected in Samaru to serve as sources of kunu and water samples.

Sample Collection

From the points of preparation, kunu and samples of water used in its preparation were taken at weekly intervals. Kunu samples only were taken from the sale outlets for 12 weeks. The samples were taken for 12 weeks.

Determination of pH

The Corning pH/ion meter 135 was used to determine the pH of kunu samples immediately after collection from the various sources.

Detection of Microorganisms in kunu

Appropriate serial dilutions (1ml to 9mls) of kunu samples were inoculated (0.1ml) onto prepoured duplicate plates of Nutrient Agar (NA), MacConkey Agar (Mac), and Baird Parker Agar (BPA) for total aerobic plate, coliform and staphylococcal counts, respectively. The plates were streaked using a sterile glass "hockey stick" or spreader and incubated at 37^{o}C for 24hrs for total aerobic plate counts and coliform counts while BPA plates were left for 48 hours. Colonies were counted and recorded. Typical and

atypical black colonies on BPA plates were picked and stored at 4°C on nutrient agar slants for further analysis.

Detection of Coliforms in water used for kunu preparation

Water samples (tap and well water) used for preparation of kunu at the six sampling sources were assayed for total and faecal coliforms. The membrane filter (MF) technique earlier described by Lechevallier et al.,(1983) was used with some modification. Water samples were filtered for both total and faecal coliforms using M endo and M.Fc broth, respectively. Pads were placed on 47mm plastic petri dishes and saturated with M Endo and M.Fc broths. A pre-determined amount of water sample was pippetted into a sterile funnel onto a 0.45um membrane filter before being drawn into a flask at the base through the action of a vacuum pump. The funnel was rinsed with a sterile phosphate buffer solution and with a sterilized forcep, the membrane filter paper (grid side up) was placed on the petri dish. At least, two different dilutions (3mls and 5mls for total coliforms; 1ml and 3mls for faecal coliforms, volumes, were used for each sample. The petri dishes were incubated upside down for 24 hrs at 37°C for total coliforms and at an elevated

temperature of 44.5°C in a water bath for faecal coliforms. Colonies with green or gold metallic sheen were regarded as total coliforms but light blue to dark green ones were regarded as faecal coliforms. They were enumerated and expressed per 100ml of water sample.

RESULTS AND DISCUSSION

Table 2.1. displays the prevalence of staphylococci and coliforms in kunu samples taken at weekly intervals over a 12-week period. Regardless of source, staphylococci and coliforms were very frequently isolated from kunu samples, as at least 75.0% of kunu samples contained staphylococci while 66.0% were contaminated by coliforms. Although the frequency of staphylococcal contamination was higher than for coliforms for most sources, the difference was not statistically significant ($P>0.05$, X^2). Kunu samples from sale outlets had similar frequency of contamination with those from the points of preparation.

Table 2.2. shows the mean values of total aerobic plate, coliform and staphylococcal counts in kunu. High mean total aerobic plate counts were recorded for all sources, ranging from 1.3×10^{10} to

7.0×10^{10} cfu/ml. The coliform counts were also relatively high with mean counts ranging from 4.5×10^{5} to 3.3×10^{6} cfu/ml while mean staphylococcal counts were from 5.8×10^{2} to 2.1×10^{4} per ml. Again there was no significant difference ($P>0.05$, X^2) in the mean aerobic plate, staphylococcal and coliform counts between the samples taken at the points of preparation and sale outlets. Data presented so far indicate that no additional contamination occurred between the points of preparation and sales based on the frequency of contamination (Table 2.1) and relative counts (Table 2.2). The mean staphylococcal counts were however significantly lower ($P<0.05$, X^2) than either the mean aerobic plate or coliform counts from all the sources. The mean pH values for kunu from all sources ranged from 3.5 to 4.1 while the range for all samples taken was from 3.1 to 5.8. Overall the samples were in the acidic pH range and this may have been responsible, in part, for the relatively low staphylococcal counts detected. Staphylococci are known to survive and grow best towards neutral pH although the pH range reported is 4-10 (Barber and Deibel, 1972).

Table 2.3. illustrates the mean aerobic plate and staphylococcal counts of utensils used in kunu

preparation. The mean total aerobic plate counts ranged from 4.6×10^4 to 1.0×10^5 per sq. cm. while that of staphylococcal counts was from 2.0×10^1 to 3.0×10^2 per sq. cm. Although the values obtained for utensils were much lower than those detected in kunu samples (Table 2.2), they may have contributed to the degree of contamination of kunu.

The mean coliform counts of water used in kunu preparation are shown in Table 2.4. The highest mean total (1383 per 100ml) and faecal (818 per 100ml) coliform counts were recorded for the only well water supply (Point of preparation F) used in kunu preparation. The faecal coliforms specifically indicate faecal contamination of water supply and the possible presence of other enteric pathogens. This finding emphasizes the need to identify the other micro-organisms other than coliforms and staphylococci responsible for the very high mean aerobic plate counts of kunu. (Table 2.2). It was also alarming to have observed that tap water from all five other points of preparation were highly contaminated by faecal coliforms. This is a health hazard in the light of the U.S. Environmental Protection Agency recommendation less than 2.2 coliforms per 100 ml for potable

water, (Rand et al., 1975). It is pertinent to observe that kunu is not subjected to much thermal processing and this was reflected in the high faecal and total coliform counts obtained. The present findings, though lower in counts, are in agreement with the report of Bako (1978), where coliform counts of well and tap water in Samaru were found to be 9.2×10^5 and 2.1×10^4 per 100 ml respectively.

The finding of similar degrees of contamination for both utensils and water used for preparation of kunu from all sources may explain, in part, the absence of significantly different mean aerobic plate, staphylococcal and coliform counts between sources. Furthermore, it confirms the similarity in the method of processing, handling and storage of kunu at the various sources from the viewpoint of sanitation.

In conclusion, kunu could be a source of human health hazard based on the relatively high coliform counts and the high number of faecal coliform in the water used in it's preparation. Although the apparent degree of staphylococcal contamination was low, it was not known if there was multiplication and

subsequent death of staphylococci due to low pH, with a potential for prior enterotoxin production, if the strain of Staphylococcus aureus contaminants were enterotoxigenic.

Table 2.1: Prevalence[a] of staphylococci and coliforms in a cereal drink (kunun zaki) obtained from various sources in Samaru

Sample Source Point of Preparation	Number of Samples	Staphylococci		Coliforms	
		No. Positive	Percent Positive	No. Positive	Percent Positive
A	12	11	91.6	9	75.0
B	12	9	75.0	9	75.0
C	12	12	100.00	9	75.0
D	12	11	91.6	9	75.0
E	12	10	83.3	10	83.3
F	12	11	91.6	11	91.6
Sale Outlet					
G	12	11	91.6	8	66.6
H	12	12	100.0	10	83.3
I	12	9	75.0	9	75.0
J	12	10	83.3	10	83.3
K	12	12	100.00	9	75.0
L	12	10	83.3	9	75.0

[a]Samples were collected at weekly intervals from each of the sources for 12 weeks

Table 2.2: Mean values of aerobic plate, coliform, staphylococcal counts and pH of kunu

Sample[a] source Point of Preparation	Mean values of: Total aerobic plate count, cfu/ml	Coliform count, cfu/ml	Staphylococcal count, cfu/ml	PH Mean	PH Range
A	7.0×10^{10}	4.5×10^{5}	1.4×10^{3}	3.5	3.1 - 3.9
B	6.0×10^{10}	2.5×10^{6}	2.1×10^{4}	3.5	3.3 - 3.9
C	4.3×10^{10}	2.1×10^{6}	2.0×10^{3}	3.7	3.2 - 3.9
D	1.9×10^{10}	3.3×10^{6}	7.8×10^{3}	3.7	3.2 - 4.1
E	6.2×10^{10}	1.1×10^{6}	3.1×10^{3}	3.9	3.7 - 4.7
F	3.7×10^{10}	2.3×10^{6}	7.8×10^{3}	4.1	3.7 - 4.5
Sale Outlets					
G	7.0×10^{10}	2.1×10^{6}	3.3×10^{3}	3.7	3.4 - 4.1
H	8.4×10^{10}	8.3×10^{5}	1.5×10^{3}	3.9	3.7 - 4.1
I	1.3×10^{10}	9.0×10^{5}	5.8×10^{2}	3.6	3.4 - 3.9
J	4.1×10^{10}	2.1×10^{6}	2.1×10^{3}	4.1	3.2 - 4.3
K	6.8×10^{10}	7.2×10^{5}	1.2×10^{3}	4.2	3.8 - 4.5
L	4.3×10^{10}	1.8×10^{6}	3.2×10^{3}	4.3	3.7 - 5.8

Table 2.3: Mean staphylococcal and total aerobic plate counts of utensils[a] obtained at the six points of kunu preparation

Point of Preparation	Total aerobic plate count, cfu/sq. cm.		Staphylococcal count cfu/sq. cm.	
	Mean	Range	Mean	Range
A	6.2×10^4	6.3×10^3-2.5×10^5	9.8×10^1	1.0×10^1-3.2×10^2
B	4.6×10^4	1.3×10^3-1.7×10^5	1.8×10^2	1.6×10^1-1.1×10^3
C	8.9×10^4	1.0×10^3-2.5×10^5	3.0×10^2	1.0×10^1-3.0×10^2
D	8.5×10^4	1.5×10^4-1.6×10^5	5.3×10^1	1.0×10^1-3.0×10^2
E	1.0×10^5	3.8×10^3-2.5×10^5	2.0×10^1	1.0×10^1-6.5×10^2
F	6.9×10^4	3.5×10^3-2.1×10^5	2.0×10^1	1.8×10^1-1.2×10^2

[a]Comprised of nylon and metallic sieves, spoons, buckets and pots.

Table 2.4: Mean coliform counts of water used in kunu preparation

Point of Preparation	Coliform counts per 100mls			
	Total		Faecal	
	Mean	Range	Mean	Range
A	1105	25-2300	715	40-1560
B	634	125-2100	393	40-1388
C	847	80-3700	593	80-1450
D	598	100-1450	396	40-988
E	974	75-2725	440	40-1750
F*	1383	200-3833	818	67-2285

*The only well water source

CHAPTER THREE

EFFECTS OF SPICES ON SURVIVAL AND MULTIPLICATION OF STAPHYLOCOCCI AND COLIFORMS IN KUNU

SUMMARY

The bacterial loads and possible antimicrobial activities of ginger, cloves, black pepper and red pepper on staphylococci and coliforms in kunun zaki were determined. Ginger was most contaminated and red pepper least, with total aerobic plate counts of 9.5×10^{10} per g. and 7.3×10^{6} per g, respectively. Of the four spices tested, cloves was most inhibitory to S. aureus and E. coli, at both 0.25% and 1.25% concentration used. Red pepper and black pepper at similar concentrations showed very little antimicrobial activity while any inhibition by ginger was hardly detected. The pH of the product may have obscured the antimicrobial effect of some of these spices.

Spices usually added to kunu contain some bacteria, cloves specifically reduces the staphylococcal and coliform counts of kunun zaki at the concentration usually added to kunu.

INTRODUCTION

Spices are products used in flavouring foods and could serve as preservatives (Omakwu,

1980). Spices have been shown to aid other antimicrobial agents in the prevention of growth of undesirable organisms in food. (Frazier, 1967). In addition, it has been observed that spices could harbour bacteria and mycotoxin producing fungi so serving as a source of contamination in foods (Christensen et al., 1967; Flannigan and Chui, 1976). Zaika et al. (1978) further reported that the growth and enterotoxin production by some microorganisms are enhanced by some of these spices.

Ginger (Chitta mai yatsu), the rhizome of Zingiber officinale contains 1-2% volatile oil predominantly terpenes (Trease and Evans, 1972). The pungency of ginger has been attributed to gingerol and it is widely used as an aromatic stimulant although also as a carminative (Nelson, 1951; Lewis and Lewis 1977). Ginger is used in some Nigerian ready-to-eat products primarily as a spice (Nkanga and Uraih, 1981; Adesiyun 1984). In a study with raw meat, ginger was found to be contaminated and to lack antimicrobial effect (Nkanga and Uraih 1981).

Cloves (Kanumfari) the dried flower of Euginia caryophyllata was reported to contain 11-24% volatile oil and a crystalline substance called caryophyllin (Lewis and Lewis, 1977). Cloves oil

contain 84-95% of phenols (eugenol with about 3% of acetyleugenol) sesquiterpenes (alpha and beta caryophyllenes and small quantities of esters, ketones and alcohols. Cloves are used as stimulants, as a spice and for the preparation of volatile oils (Makinde 1979). Huhfanen (1980) demonstrated that extract of cloves showed some inhibitory effect on Clostridium botulinum.

Red pepper (Barkono) is a dried ripe fruit of Capsicum annuum, a shrub (Makinde 1979). The main constitutent is capsaicum and is mainly used as a condiment.

Black pepper (masoro) is dried fruit of Piper guineese. The spice contains oils which are alkaloids, including peperin chaviane, piperi-dine and B-methyl pyvolline which may exhibit antimicrobial properties (Omakwu 1980). These oils constitute up to 13% w/v of black pepper and could be used for flavouring of soups and non-alcoholic beverages (Pearson, 1976; Omakwu, 1980).

Red pepper and black pepper have often been added to various Nigerian foods predominantly as spices (Nkanga and Uraih 1981).

Some spices, despite their antimicrobial activities, harbour staphylococci. The organism has been isolated from both ground black and red pepper (Christensen et al., 1967). However, Makinde (1979) while studying locally fermented milk with cereal (fura de Nono) observed that cloves and red pepper harbour no staphylococci. In the same study, negro pepper and ginger were found to contain very high counts of staphylococci. Nkanga and Uraih (1981) reported finding ginger and black pepper to be contaminated with Staphylococcus aureus. Cloves, onions and red pepper were found to be inhibitory to S. aureus on the same study. Al-Delaimy and Ali (1970) had earlier reported the bactericidal effects of filtered onion extracts on Shigella dysenteriae and S. aureus.

Coliforms, along with staphylococci have been isolated from spices like ginger and black pepper (Julseth and Deibel, 1974). Patel et al., (1976) isolated spices of coliforms including E. coli, klebsiella pneumonia, K. ozaenae, Enterobacter chloacae and E. hafniae from various spices. Masson (1979) also isolated coliforms and other bacteria of faecal origin in 44% of spices surveyed. The effect of black pepper and it's constituents on faecal bacteria was reported by Salzel et al. (1977) and

E. coli was found to be inhibited most compared to other enterics. A similar finding was reported by Omakwu (1980) with the inhibition of coliforms by black pepper and ginger.

Gal (1968) however found that capsaison, the hot principle of red pepper at a dilution of 1:1000 did not affect E. coli or S. aureus but prevented growth of Bacillus substilis and B. cereus.

In view of the variable inhibition effects of various spices depending on their concentration coupled with the fact that some of these spices may act as sources of contamination, this study was designed to determine the effects of some spices on E. coli and S. aureus in kunun zaki.

MATERIALS AND METHOD

Preparation of local spices

Four different spices (Ginger, Red pepper, Black pepper and cloves) sold locally in the market were used. Each spice was ground in the laboratory using sterile mortar and pestle and stored at room temperature in sterile containers.

Determination of microbial load

A 10% solution of the ground spice in distilled water was serially diluted (1ml to 9mls) in sterile saline and plated on nutrient agar (NA), Baird-Parker agar (BPA) and MacConkey agar for total viable count, staphylococcal and coliform counts respectively. Plates were incubated for 48 hours at 37°C and typical colonies counted. The counts were then expressed per gram of the spice.

Investigation of the inhibitory effect of spices on bacterial proliferation

Seven tubes, each containing 15mls of kunu were used. Five of the tubes and their contents were sterilized while the remaining two were left as purchased, non-sterile. Sterile kunu in two tubes were inoculated with an overnight growth of S. aureus (Strain 361) and 5mls of 1% spice was added to one making a final spice concentration of 0.25%. Five millilitres of distilled water was added to the other to serve as control. A set of two tubes was similarly treated following inoculation with E. coli (strain 669). One tube containing sterile kunu was left uninoculated but with 5mls of 1% spice added for the two tubes. With non-sterile kunu, one tube had 5mls of 1% spice added while the other was with 5mls of water to serve

as control. All tubes were incubated at room temperature with portions removed at 0, 4, 8, 12 and 24 hrs following inoculation and plated on NA, BPA and MacConkey as appropriate. A similar procedure was followed for 5% spice solution given a final concentration of 1.25% and for each spice. Two experimental trials were conducted for each spice and the mean values reported.

RESULTS AND DISCUSSION

Table 3.1 lists the spices commonly added to kunu at all the sources of preparation studied with their scientific and local names.

Tables 3.2 shows the bacterial load of each of the spice studied and the mixture. Ground ginger was most contaminated with a mean total aerobic plate count of 9.5×10^{10} cfu/g, while ground red pepper had the least mean count of 7.3×10^{6} per g. Ginger was most contaminated with coliforms, 3.2×10^{6} cfu/g while ground black pepper had less than 10^{2} cfu/g. Again, ginger contained the highest staphylococcal counts of 1.4×10^{5} per g while black pepper and cloves each had 10^{2} per g. As expected, a mixture of the four spices, as routinely used during kunu preparation contained very high counts

2.4×10^{11}, 1.1×10^{7} and 6.2×10^{3} cfu/g for total aerobic plate, coliform and staphylococcal count, respectively. Filteration with a sieve hardly made any difference. Sieves themselves appear to be sources of contamination when the filterate was found to have a higher mean aerobic plate count (6.0×10^{11} cfu/g) than the mixture of the four spices (2.4×10^{11} cfu/g). The finding that all the spices were contaminated is in agreement with the reports of others(Peppler, 1975; Omakwu, 1980; Nkanga and Uraih, 1981). A suggestion that contamination of spices may have taken place in the field was earlier made (Markus and Amilang 1973). The potential of these spices serving as sources of contamination in foods to which they are added cannot be ignored as it poses a health hazard more especially if the organisms proliferate.

The relative inhibitory effects of ginger, black pepper, red pepper and cloves on staphylococci (including S. aureus) and coliforms, (including E. coli) and other contaminants (microorganisms) are shown in figs 3.1 to 3.8. Ginger used as both 0.25% and 1.25% concentration, (figs 3.1 and 3.2) respectively, hardly displayed any detectable inhibitory effects on the test microorganisms i.e. S. aureus, E. coli and other contaminants. That ginger did not inhibit these microorganisms at the two concentration used,

as demonstrated by the lack of significant difference in counts of tubes with kunu plus water (K+W) and kunu plus spice (K+S), is hardly surprising. Ginger was earlier shown to be heavily contaminated by these organisms (Table 3.1) an indication of its inability to inhibit or kill them. These findings are in agreement with earlier reports by others (Omakwu 1980, Nkanga and Uraih 1981). The decline in population of microorganisms after 8 hours for both controls (K+W) and experimental (K+S) may be explained, in part as being due to the relatively low pH of kunu. The possibility of the pH obscuing some inhibitory effect of ginger on these microorganisms cannot therefore, be ignored.

Red pepper at 0.25% and 1.25% concentration (figs 3.3 and 3.4) had little detectable inhibitory effects on staphylococci, coliforms and other microorganisms either with sterile or non-sterile kunu. The difference in S. aureus counts of control (K+W) and Spice (K+S) in sterile kunu (Fig 3.4) was not significant ($P>0.05$, X^2).

Although black pepper at both concentrations showed little antimicrobial effects the difference between control and kunu with spices was not significant. The findings is in agreement with the findings of others

that black pepper acts more as a contaminant (Table 3.2) than an inhibitor of microorganisms (Omakwu 1980, Nkanga and Uraih, 1981). Extracts of black pepper have however been shown to be very inhibitory against Clostridium botulinum in culture media (Huhfanen, 1980).

The ihibitiory effects of cloves are shown in fig. 3.7 and 3.8. Although some inhibitory effects were detected at 0.25% concentration (fig. 3.7), the effects on E. coli, S. aureus and other microorganisms were most dramatic at 1.25% concentration (fig 3.8). Cloves have been reported to inhibit a variety of yeasts, molds and bacteria (Bullerman, 1974). The antimicrobial activity of cloves has been attributed to the 14.2% of volatile oil, it contains with eugenol making up 95% of these oils (Lewis, and Lewis 1977).

Other spieces studied but with no demonstrable inhibitory effects on E. coli, S. aureus and other contaminants, contain essential oils like terpenes which may inhibit other microorganisms. (Trease et al., 1972). The possible role of low pH in obscuring any inhibitory effect, no matter how small, cannot be ignored.

In conclusion, cloves when added to kunu at concentrations routinely used as a spice is bactericidal to staphylococci, coliforms and other contaminants

Ginger, red pepper and black pepper, despite the fact that they were heavily contaminated by bacteria coupled with little or no antimicrobial activity did not result in a high degree of contamination of kunu. The low pH of kunu apparently had some effect in survival and growth of these organisms.

Spices in conjunction with good sanitation may be useful in providing a good safety margin for kunu that could be exposed to contamination during preparation, storage and sale.

Table 3.1: Spices used in the study

Common name	Scientific name	Hausa name
Ginger	Zingiber officinale	Citta mai yatsu
Red pepper	Capsicum annuum	Barkono
Black pepper	Piper guineense	Masoro
Cloves	Eugenia caryophyllata	Kanumfari

Table 3.2: Bacterial load of various spices used in the study

Spice[a]	Bacterial load, mean count, cfu/g		
	Aerobic plate count	Coliform count	Staphylococcal count
Ground ginger	9.5×10^{10}	3.2×10^{6}	1.4×10^{5}
Ground Red Pepper	7.3×10^{6}	2.0×10^{4}	1.2×10^{3}
Ground black pepper	7.9×10^{7}	$< 10^{2}$	$< 10^{2}$
Ground cloves	2.0×10^{7}	3.5×10^{4}	$< 10^{2}$
Mixture of 4 spices	2.4×10^{11}	1.1×10^{7}	6.2×10^{3}
Filtrate	6.0×10^{11}	7.5×10^{6}	7.9×10^{3}

[a]All spices were from sources of preparation

FIG 3.1
EFFECT OF GINGER ON SURVIVAL AND GROWTH OF S. AUREUS & E. COLI AND OTHER MICROORGANISMS IN KUNU AT 25°C

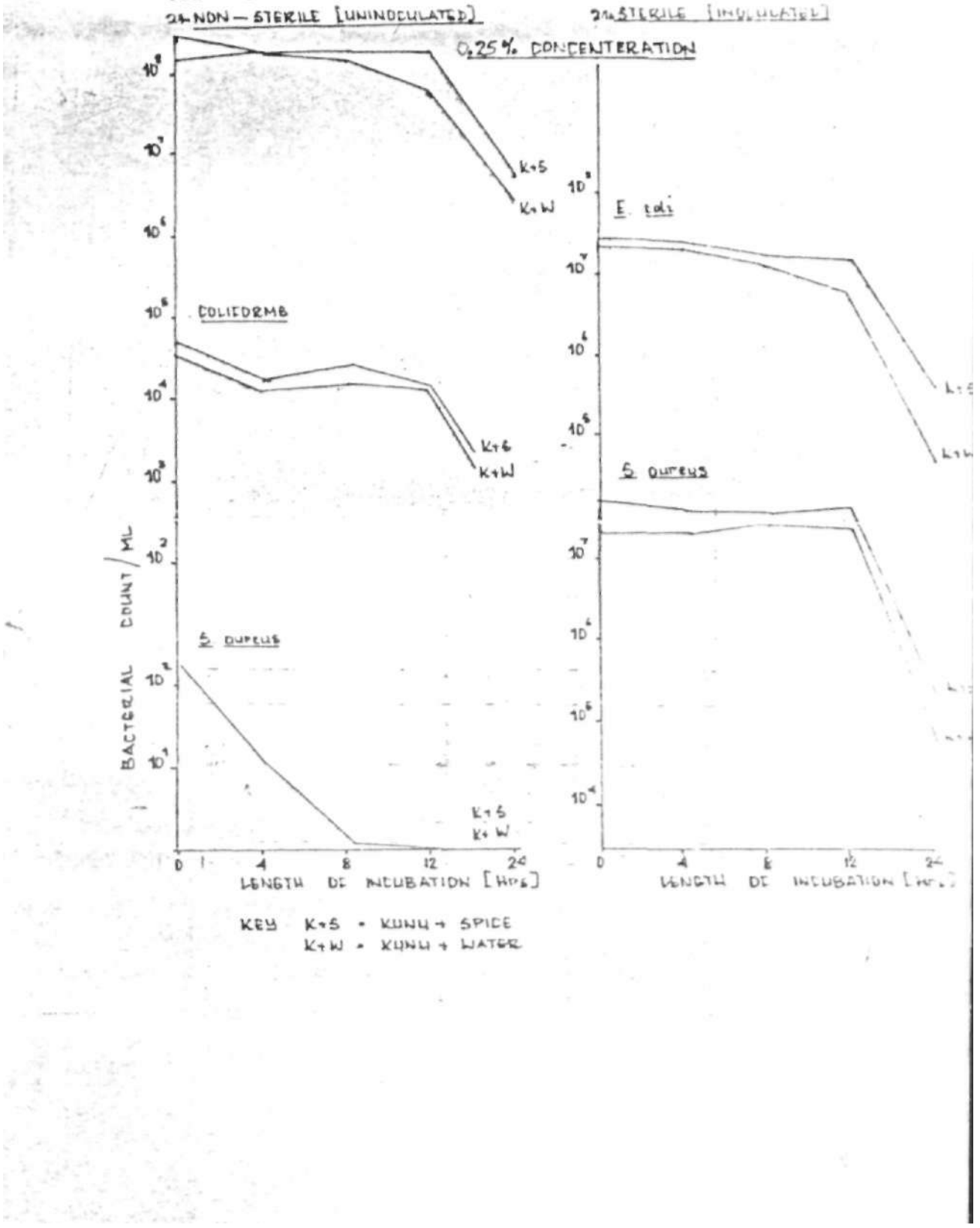
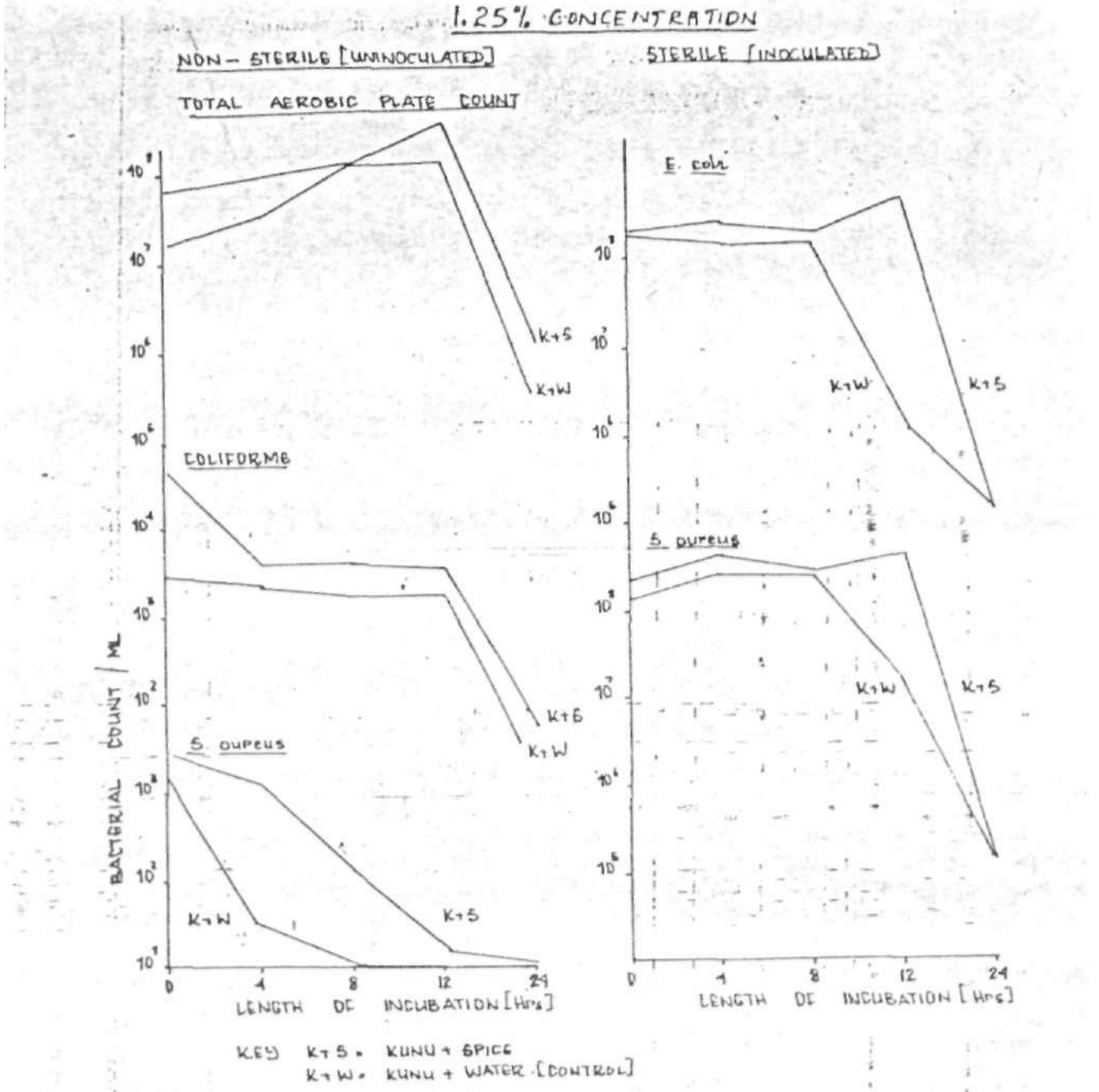


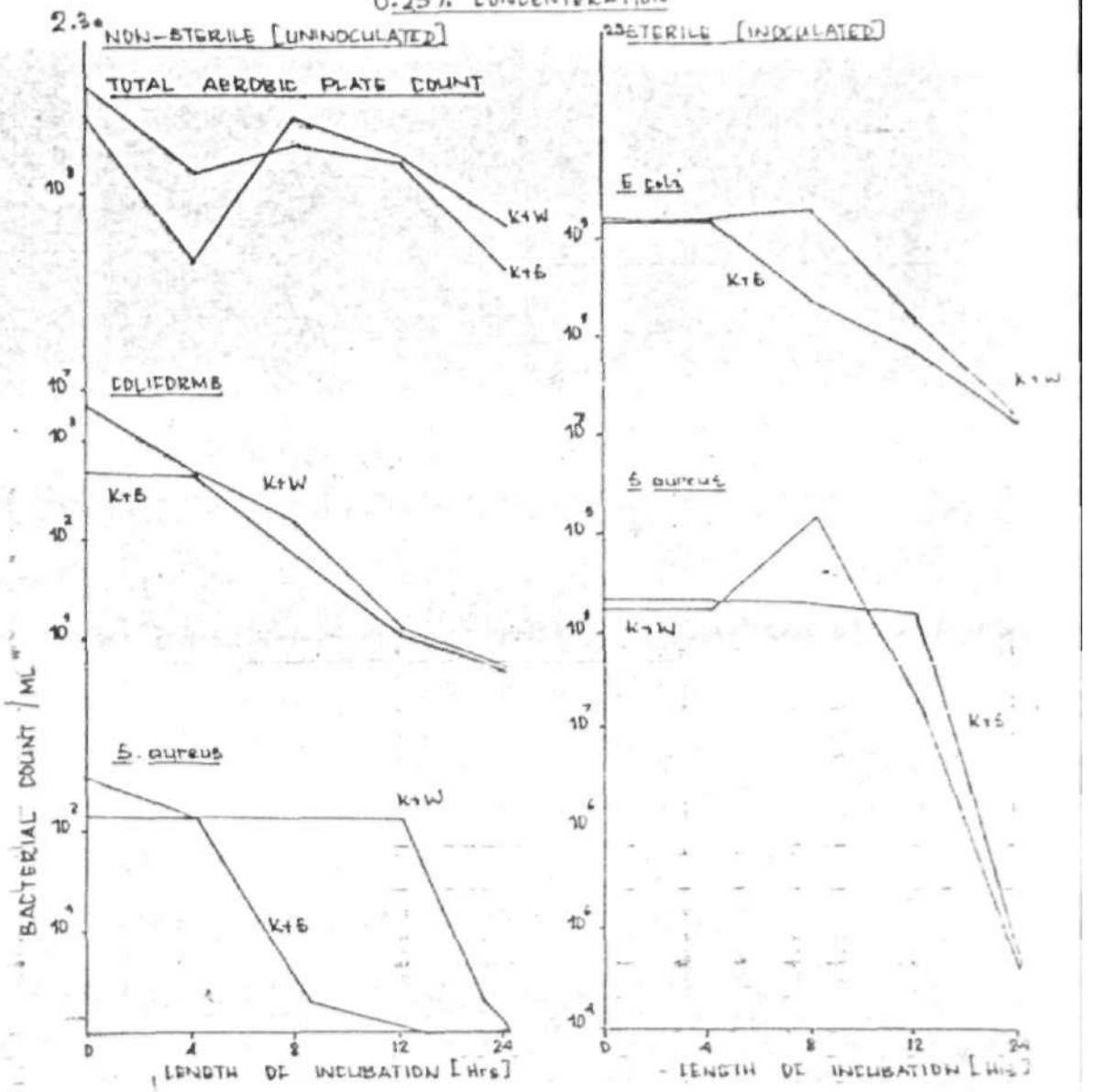
FIG 3.2 EFFECT OF GINGER ON SURVIVAL AND GROWTH OF *S. aureus* *E. coli* AND OTHER ORGANISMS IN KUNU AT 25°C

1.25% CONCENTRATION



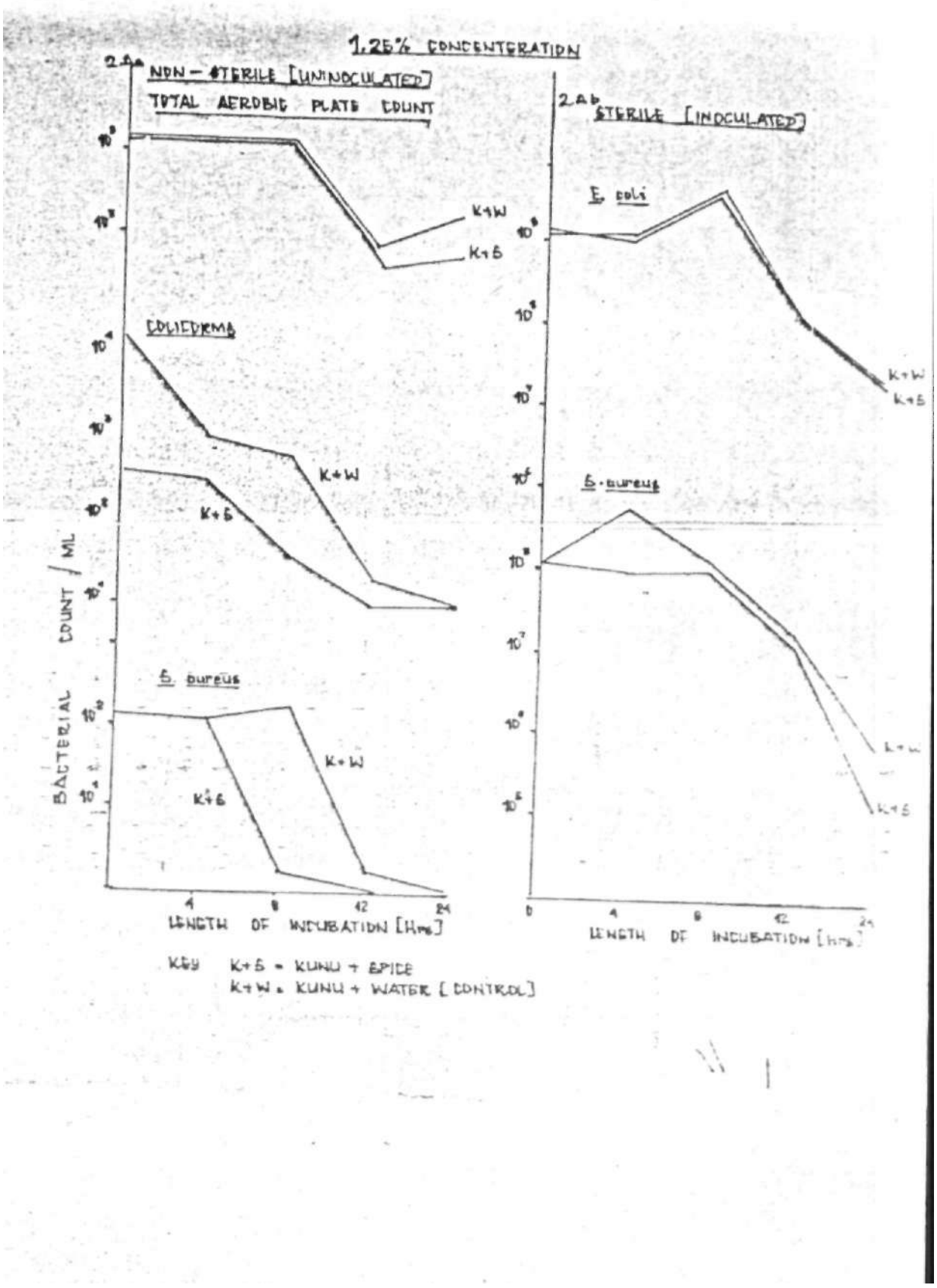
KEY K+S = KUNU + SPICE
K+W = KUNU + WATER [CONTROL]

Fig 3:
EFFECT OF RED PEPPER ON SURVIVAL AND GROWTH OF S AUREUS AND OTHER MICROORGANISMS IN KUNU AT 25°
0.25% CONCENTRATION



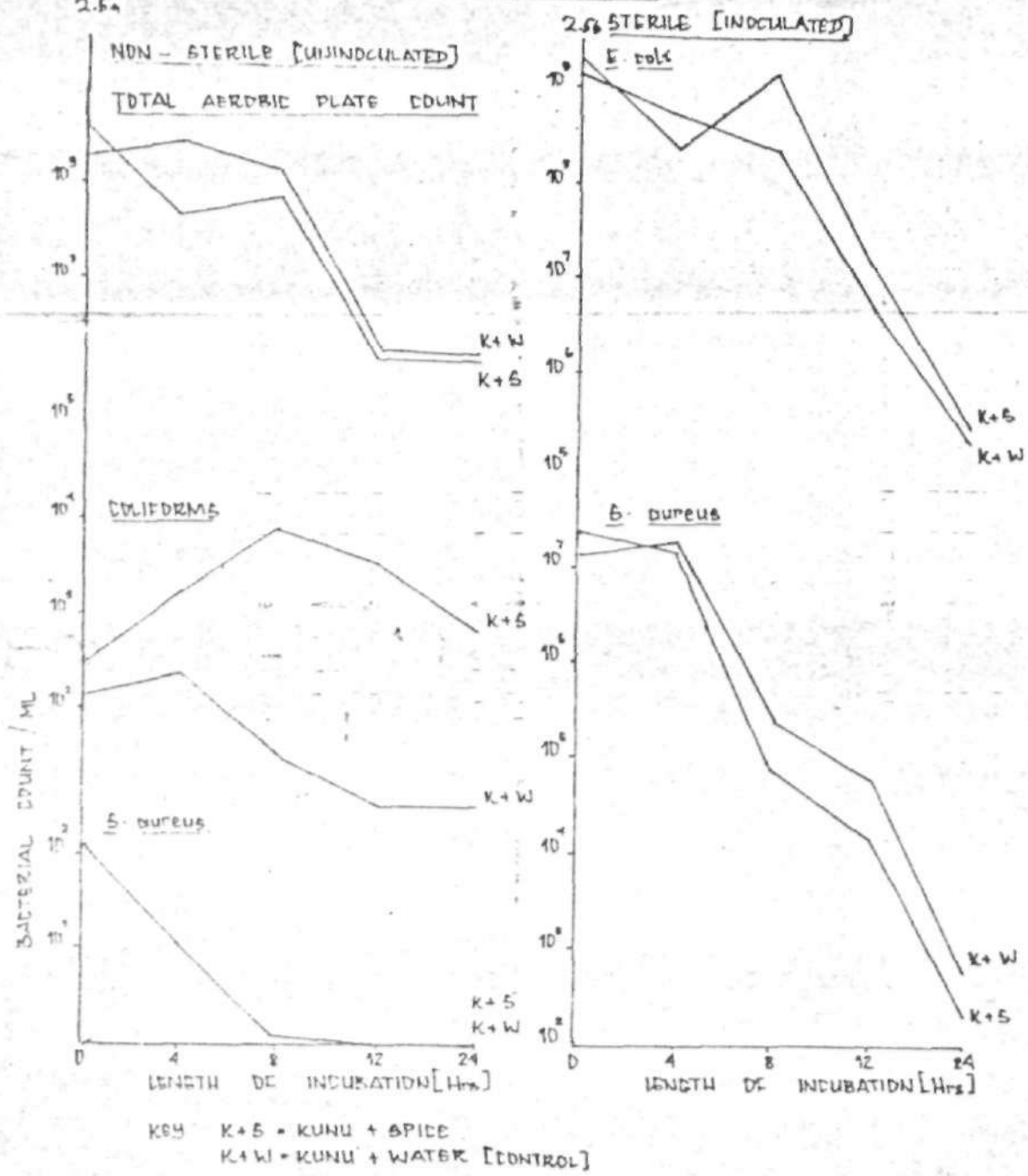
KEY K+S = KUNU + SPICE
K+W = KUNU + WATER [CONTROL]

FIG 3.4
EFFECT OF RED PEPPER ON SURVIVAL AND GROWTH OF S. aureus & E. coli AND OTHER MICROORGANISMS IN KUNU AT 25°C.



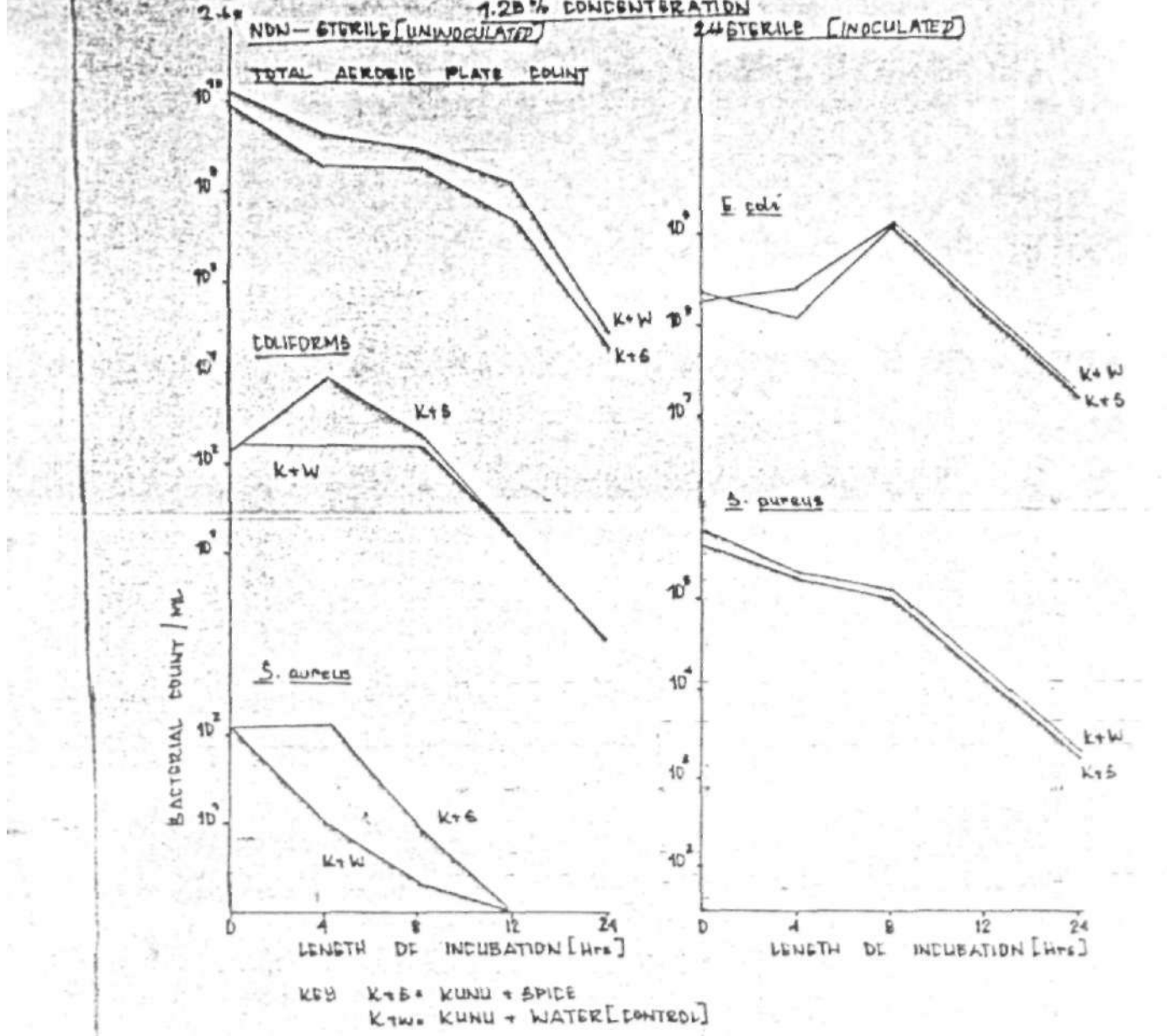
KEY K+S = KUNU + SPICE
K+W = KUNU + WATER [CONTROL]

FIG 35
EFFECT OF BLACK PEPPER ON SURVIVAL AND GROWTH OF *S. aureus*, *E. coli*, AND OTHER MICROORGANISM IN, KUNU AT 25°C.
0.25% CONCENTERATION



KEY K+S = KUNU + SPICE
K+W = KUNU + WATER [CONTROL]

FIG 3.6
EFFECT OF BLACK PEPPER ON SURVIVAL AND GROWTH OF S. aureus, E. coli AND OTHER MICRO ORGANISMS IN KUNU AT 25°C.
1.2B % CONCENTRATION



KEY K+S = KUNU + SPICE
K+W = KUNU + WATER [CONTROL]

FIG 3.7

EFFECT OF CLOVES ON SURVIVAL AND GROWTH OF *E. DUPLEX*, *E. COLI* AND OTHER MICROORGANISMS IN KUNU AT 25°C.

0.25% CONCENTERATION

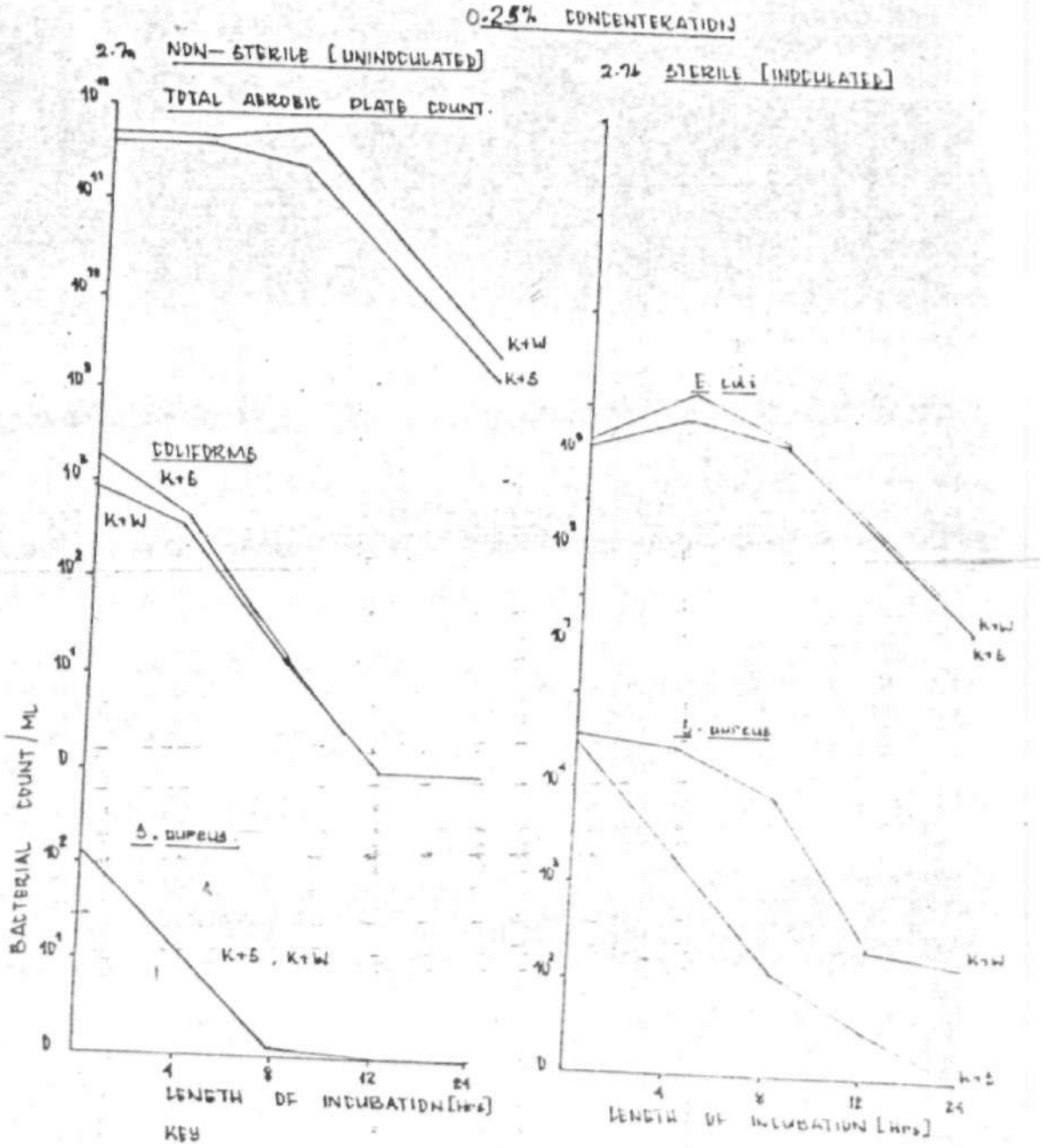
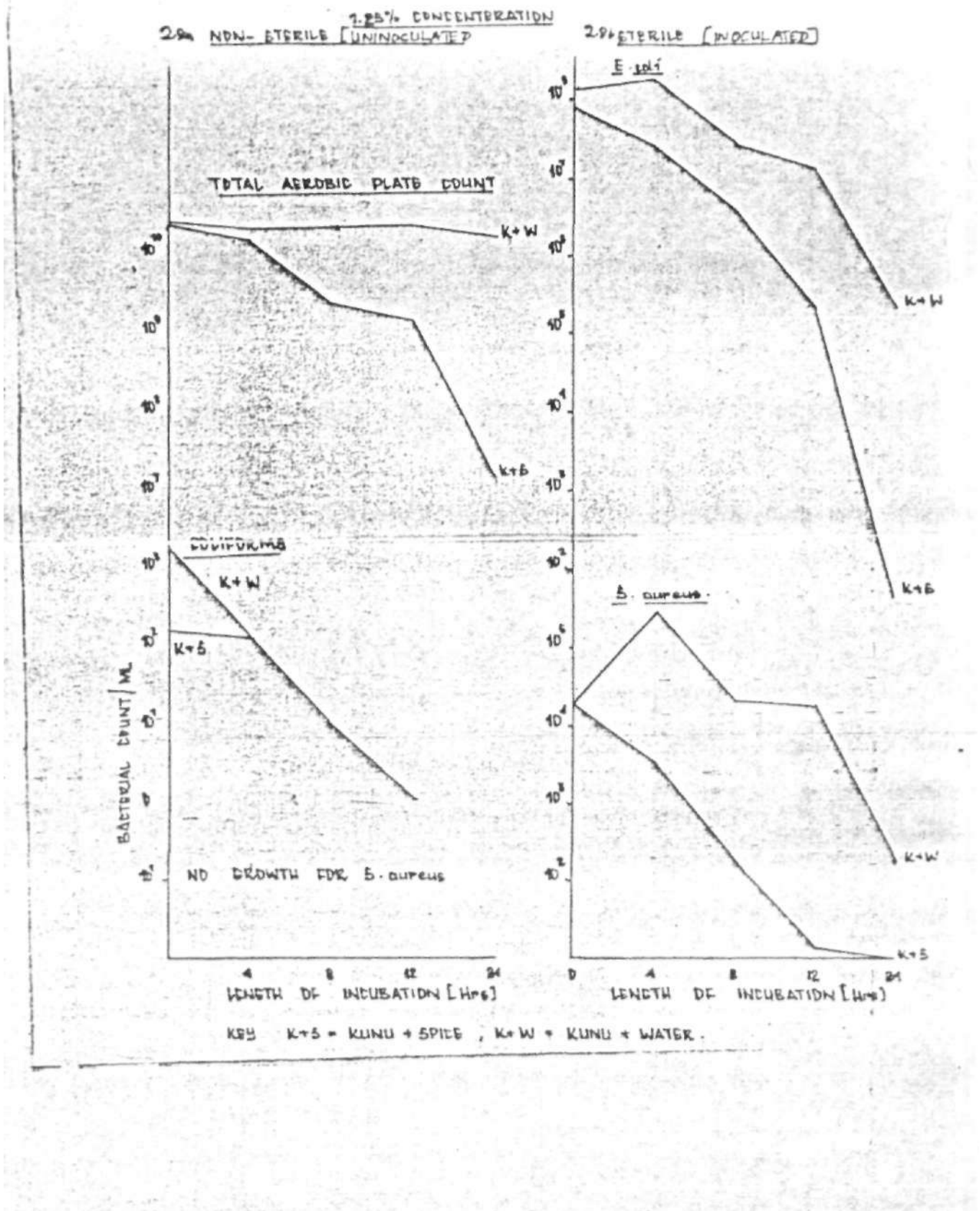


FIG 3.1

EFFECT OF CLOVES ON SURVIVAL AND GROWTH OF *S. aureus* & *E. coli* AND OTHER MICROORGANISMS IN KUNU AT 25°C



CHAPTER FOUR

INFLUENCE OF TEMPERATURE, AND pH ON SURVIVAL AND PROLIFERATION OF STAPHYLOCOCCI AND COLIFORMS IN KUNU

SUMMARY

The effect of pH, temperature and naturally occuring contaminants of kunu on survival and growth of staphylococci and coliforms were investigated. For S. aureus, E. coli and other microorganisms, survival and growth were least effected at 4°C but decline in population was more pronounced with increase in temperature, 25°C and 37°C. The pH of the product appeared to be the bactericidal factor with pH 3 and 4 most effective and pH 5 and 6 allowing growth of staphylococci and coliforms within the first 8 hrs but with a decline thereafter.

It was concluded that the pH of kunu was effective in keeping the population of microbial contaminants (S. aureus, coliforms) low, so that the risk of kunu-borne infection or intoxication is also low.

INTRODUCTION

The effects of sub-lethal adverse environmental conditions on the viability, growth and metabolism of microorganisms are now well documented (Jackson, 1974). Staphylococci have been shown to be capable of growing

between temperatures of 6.7 and 45.4°C (Angelotti et al., 1961; Angelotti, 1969), but Tatini (1973) observed a temperature growth range of 7.0 to 47.8°C. Exposure of S. aureus to 5°C was also reported to have resulted in metabolic injury, manifested as a progressive loss of ability to form colonies on mannitol salt agar while the recovery of such cells occured following incubation at 37°C for 2 hrs. This finding was further confirmed by Doyle et al. (1982) who found that refrigeration of S. aureus at 4°C was an effective means of suppressing its growth. It is however pertinent to note that such low temperature exposure of staphylococci does not really kill the microorganism. Kunu, a local cereal drink in Nigeria urban and rural areas is often contaminated by staphylococci. The effects of various temperatures during preparation, storage and sale on the survival and multiplication of S. aureus is unknown.

Investigations on the effect of pH of certain food products show that growth of S. aureus could take place at pH values as low as 4.5 to 4.7 (Tatini et al., 1971). However, strain variation in response by staphylococci to acidity have been reported (Genigeorgis, 1969; Genigeorgis et al., 1971a). The response is influenced by conditions such as size of

the inoculum (Genigeorgis et al., 1971a, Nderu and Genigeorgis, 1975), type of medium (Genigeorgis et al., 1971b; Minor and Marth, 1972; Nderu and Genigeorgis, 1975), the temperature (Minor and Marth, 1972) and the atmosphere (Genigeorgis, 1969, Genigeorgis et al., 1971b; Barber and Deibel, 1972) in addition to interaction of these factors. Barber and Deibel (1972) observed that under aerobic conditions, staphylococci were significantly more acid tolerant and believed that this characteristic accounted for the large number of staphylococci occasionally encountered in fermented sausage.

It has been shown that loss of viability of S. aureus was most pronounced at pH's 4 and 5 with little difference in viability observed at pH's 6, 7 and 8.

Kunu being an uncontrolled fermented cereal product might create changing pH's to contaminating staphylococci. The effects of various pH's of kunu on survival and multiplication of staphylococci is presently unknown.

Numerous workers have attempted to identify factors that affect survival of faecal coliforms in surface water (Allen et al., 1952; Allen, 1953; Hanes et al., 1964; Hanes et al., 1965; Geldreich and Kenner 1969).

Studies have also been conducted to evaluate and improve diluents used to count bacteria in water, sewage and food products (Vanderzant and Krueger, 1968). Both temperature and pH may influence survival of coliform, thus Bissonnette (1975) observed that a substantial proportion of the total population of coliforms may become physiologically injured following exposure to stresses of certain aquatic and food environment.

Kunu is predominantly water with the source often being contaminated like well water. The quality of well water in some parts of Nigeria have been shown to be poor (Adesiyun et al., 1983). It is not presently known whether pH of kunu or temperature of storage of the product will have any effect on contaminating coliforms. Furthermore, the effect of other microorganisms on staphylococci and coliforms in kunu is unknown. It is however known that growth of S. aureus is adversely affected by competing growth of other microorganisms (McCoy and Faber, 1966). Escherichia coli H-52 was also found to inhibit S. aureus by means of an antibiotic substance which was seitz-filterable and dialyzable at 90°C for 10 minutes (Troller and Frazier, 1963).

This study was therefore conducted to determine the effects of temperature, pH and other natural contaminants on survival and growth of staphylococci and coliforms in kunu.

MATERIALS AND METHOD

Determination of effect of pH on bacterial survival and proliferation

The effects of the following pH's 3.0, 4.0, 5.0 and 6.0 on survival and multiplication of S. aureus, coliforms and other microorganisms in kunu were investigated. Four 60ml portions of sterile kunu had their pH adjusted to 3.0, 4.0, 5.0 and 6.0 respectively with IN HCL and IN NaOH using corning pH meter 135. These were inoculated with an overnight growth culture of S. aureus (strain 361) to have an approximate initial count of 2.1×10^6 per ml of kunu. Portions were removed at 0, 4, 8, 12, and 24 hours following inoculation and incubation at $25^{\circ}C$ for enumeration on Baird-Parker agar (BPA). In addition, at each sampling time, the pH was read and appropriately adjusted to the desired pH to maintain relatively constant pH. A similar procedure was followed for non-sterile kunu. For E. coli the same procedure as above was followed using E. coli (Strain 660) but plating on MacConkey agar (MA). However to investigate

the effect of other microorganisms, four 60ml portions of kunu were left non-sterile i.e. as bought and uninoculated but with pH adjusted to 3.0, 4.0, 5.0 and 6.0 respectively. These were similarly incubated as described above and at intervals portions were plated on BPA, MA and nutrient agar (NA) for staphylococcal, coliforms and total aerobic plate counts respectively. Colonies on plates were counted using a colony counter.

Determination of effect of Temperature on bacterial Survival and proliferation in kunu

A similar procedure as described for pH was used but the pH of samples were not adjusted and inoculated kunu were incubated at 4°C, 25°C and 37°C. Portions were removed at 0, 4, 8 and 24 hrs and pH also taken, but not adjusted. Plates were subsequently incubated at 37°C for 24 - 48 hrs and colonies counted.

RESULTS AND DISCUSSION

Figures 4.1, 4.2 and 4.3 display the effects of temperature, pH and other contaminants on the survival of S. aureus, E. coli and other microorganisms respectively. Three temperatures studied, 4°C, 25°C and 37°C are those to which kunu, commonly sold could be exposed to during refrigerated storage, storage at room temperature and when being hawked about in hot

afternoons. For non-sterile kunu, with the pH left unadjusted, at 4°C, approximately a 2-logarithmic decline in staphylococcal count over a 24hr period was observed while at 25°C and 37°C over 5 log decline was detected (fig 4.1). A similar trend was observed with coliform counts (fig 4.2) in non-sterile kunu.

With sterile kunu, and with pH unadjusted at the three temperatures (4°C, 25°C and 37°C), the differences in S. aureus count over a 24 hr period were insignificant with about a 2-log decline (fig 4.1). The differences were however dramatic with E. coli as shown in fig 4.2. At 4°C, less than a log decrease in E. coli counts was detected after 24 hrs while at 25°C, over a 3 log decline and over 5 log decrease at 37°C.

It appears that with kunu kept at refrigeration temperature, as done in some kiosks, growth of microorganisms is reduce possibly due to fermentation and accompaning decrease in pH resulting from acid production. This may be detrimental to some microorganisms. Staphylococcal and E. coli counts were therefore lower for samples kept at 25°C and 37°C for 24 hrs. than those refrigerated. The role of fermentation is obvious from the dramatic decline in E. coli population in sterile, but inoculated kunu at 25°C and 37°C. (Fig. 4.2), when compared to

data on S. aureus (fig 4.1). The difference could be explained in part by the relative abilities of S. aureus and E. coli to ferment components of kunu. E. coli are known to be better fermenters of carbohydrates in kunu.

Fig 4.3 shows the effect of pH and temperature on total aerobic plate count of kunu. A similar trend was observed for the three temperatures with rate of decline in population increasing with temperature, i.e. lowest decrease at 4°C and highest decline at 37°C.

To fully elucidate the effects of pH in survival and growth of S. aureus and E. coli on kunu, data are presented in fig. 4.4 to 4.6 with the temperature kept constant at 25°C.

Overall, it appears that the pH of the product was the dominant factor on survival and growth of microorganisms in kunu and not temperature per se as might be thought. With a constant temperature, regardless of the microorganisms assayed, decline in population was directly related to decrease in pH, greatest decline occuring at pH 3.0 and least at pH 6.0. The finding that within the first 8 hrs of incubation at pH 5.0 and 6.0 there was an initial increase in population, then followed by a decline agreed with

some published work on S. aureus (Tatini, 1973). The initial increase in staphylococcal counts is in agreement with the observation that staphylococci can survive and grow at pH as low as 4.5 to 4.8 (Tatini et al., 1971). The decline in population of staphylococci after 8 hrs may be explained in part by the accompaning drop in pH. It is also known that strains of S. aureus respond to acidity differently (Genigeorgis, 1969).

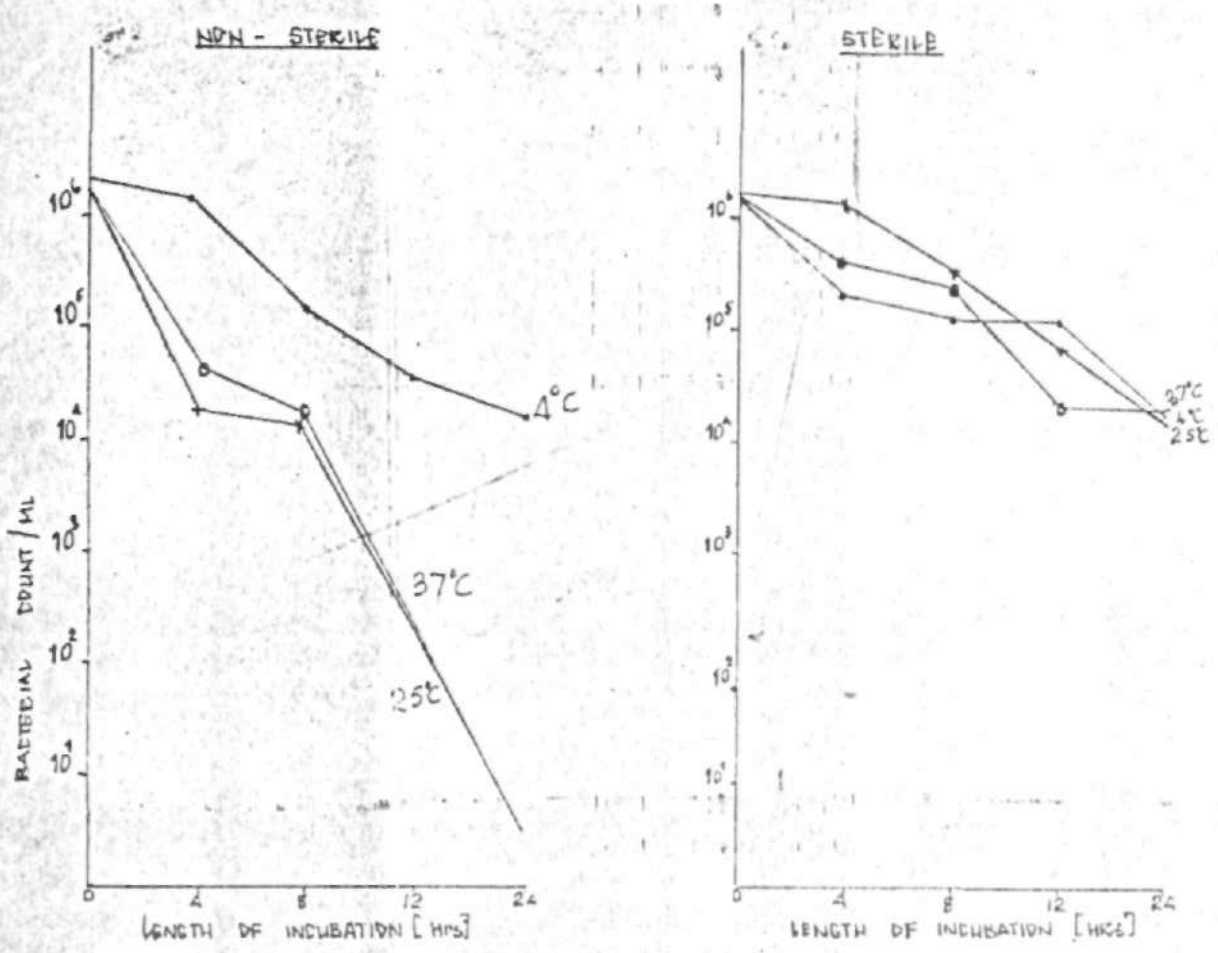
In conclusion, the pH of kunu due to fermentation, plays a major role in the loss of viability of the contaminants. Finally, although the pH of kunu as is sold appears detrimental to the survival of most microorganisms, the possibility of fermentation failure, being uncontrolled, cannot be eliminated with a resultant increase in pH which will allow microbial proliferation.

FIG. 7

EFFECT OF TEMPERATURE AND PH AND OTHER MICROORGANISM ON B. AUREUS.

[S. 361] GROWTH

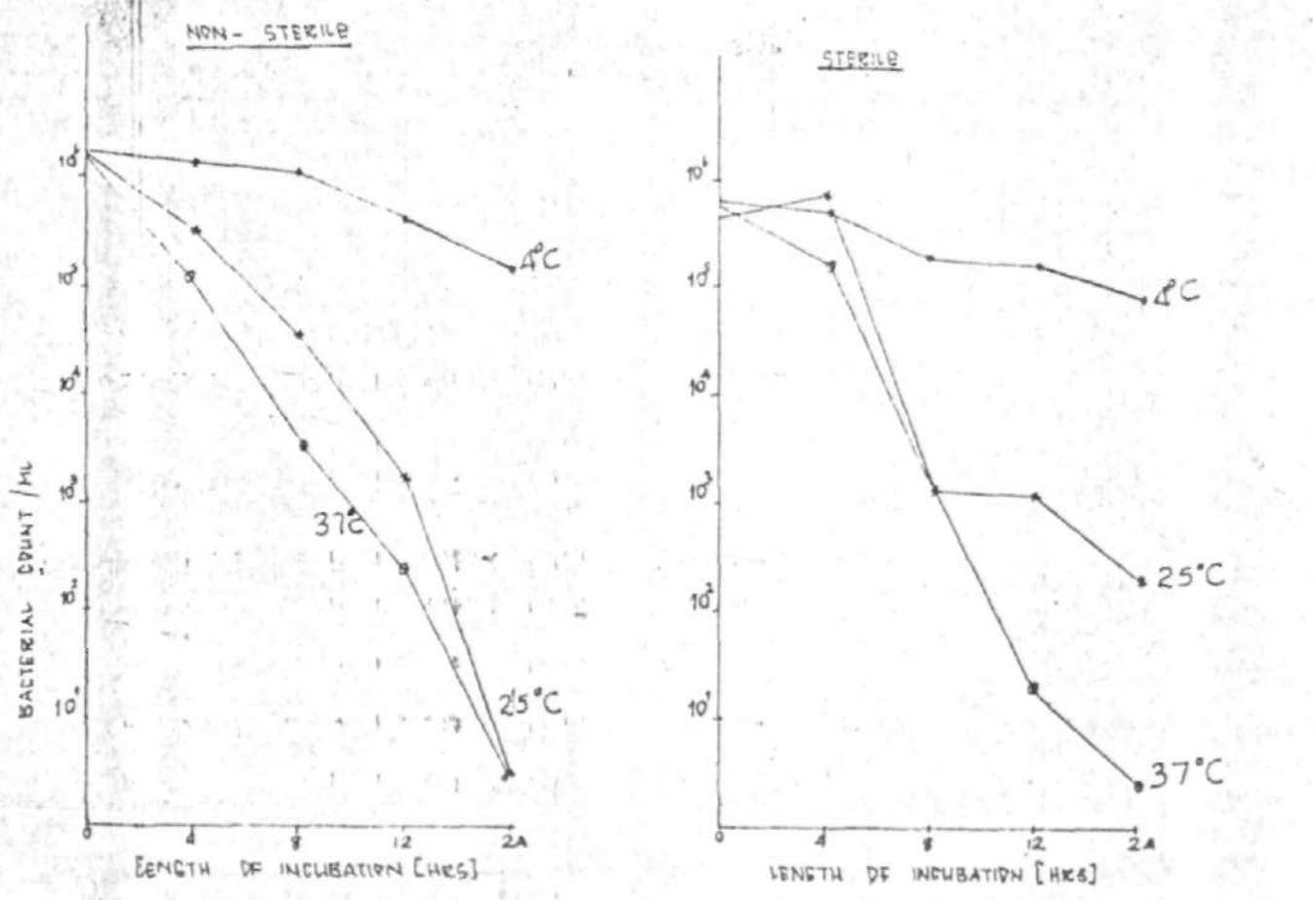
CONSTANT LIGHT



□ : VALUES WERE MEAN OF DUPLICATE TRIALS

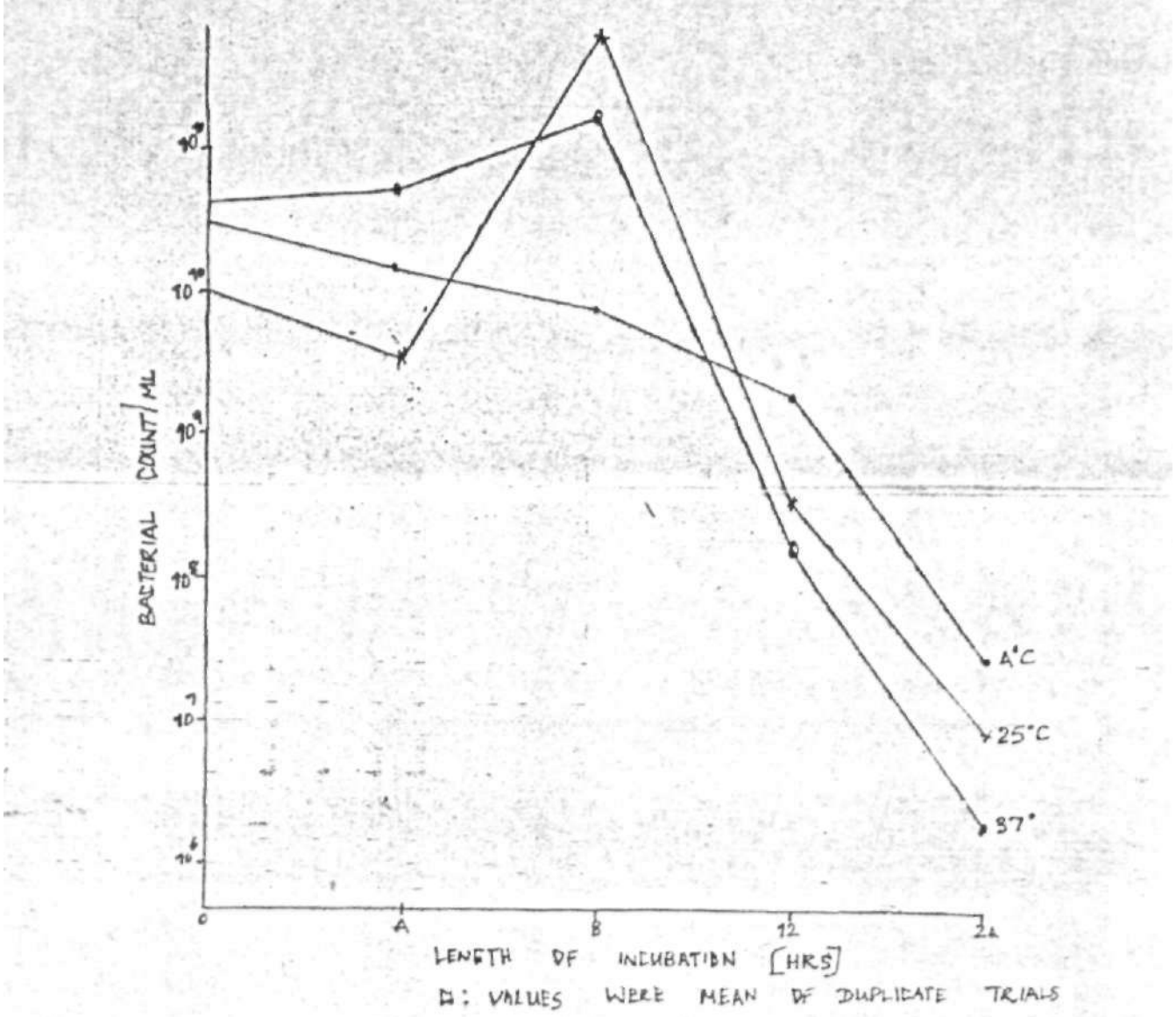
FIG 4.2

EFFECT OF TEMPERATURE, PH AND OTHER MICROORGANISMS ON SURVIVAL AND GROWTH OF E. COLI.



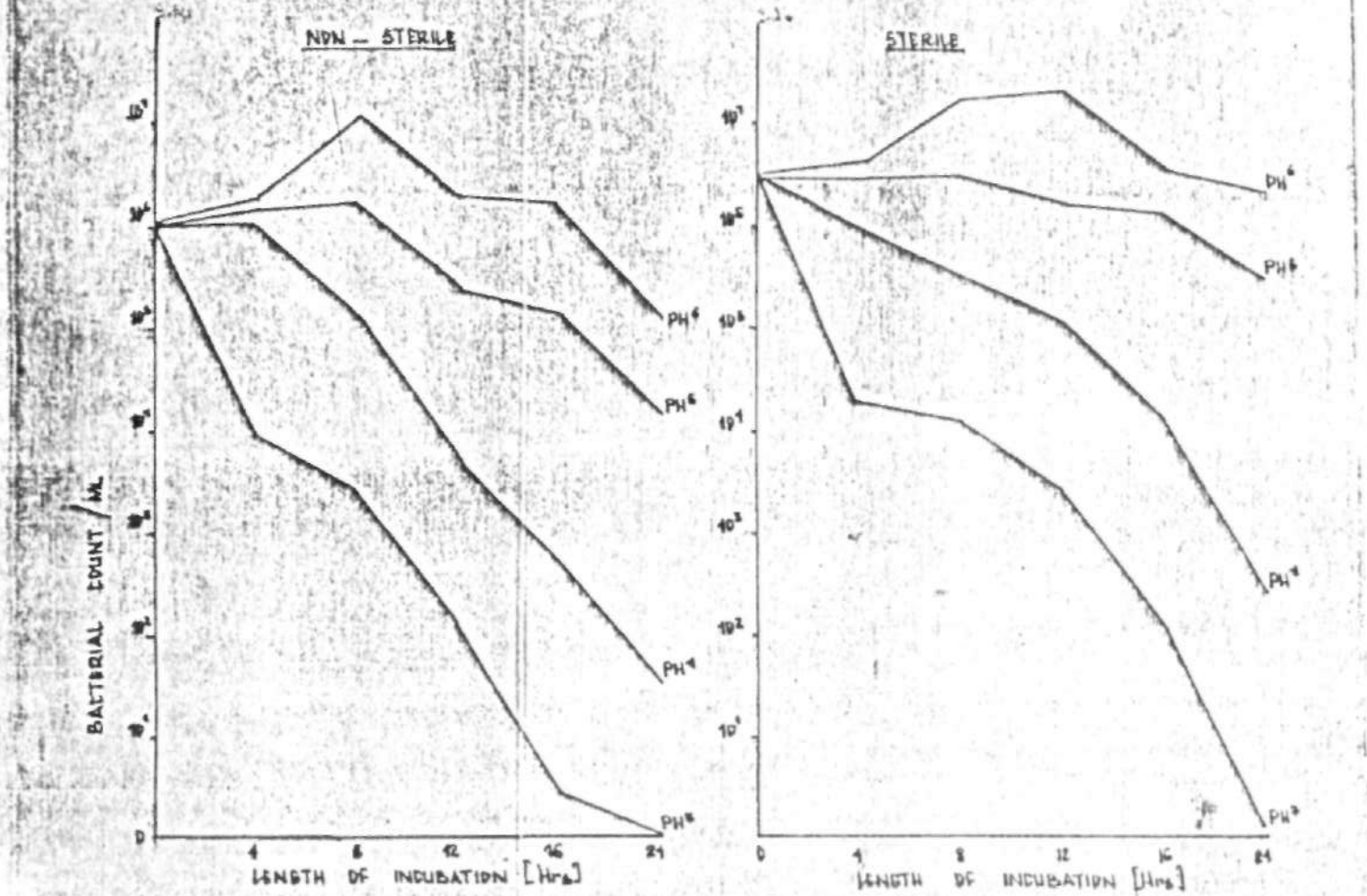
ALL VALUES WERE MEAN DUPLICATE OF TRIALS.

FIG 4.3
EFFECT OF TEMPERATURE AND PH ON TOTAL AEROBIC PLATE COUNT[a]



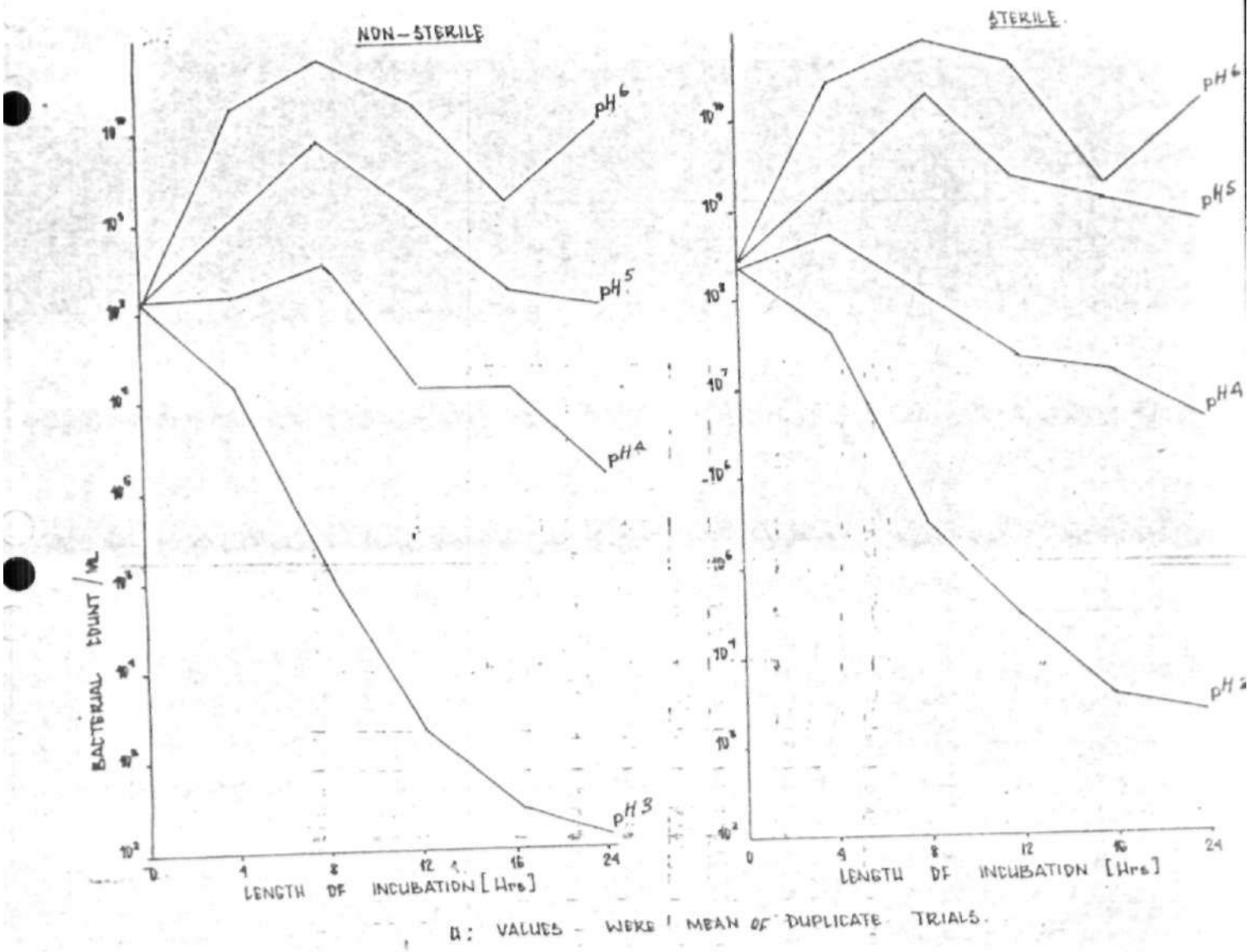
[a]: VALUES WERE MEAN OF DUPLICATE TRIALS

FIG. 7. EFFECT OF PH AND OTHER MICROORGANISMS, ON SURVIVAL AND GROWTH OF *S. BOVIS* [8 861] IN STERILE AND NON-STERILE KUNU AT 25°C



D: VALUES WERE MEAN OF DUPLICATE TRIALS.

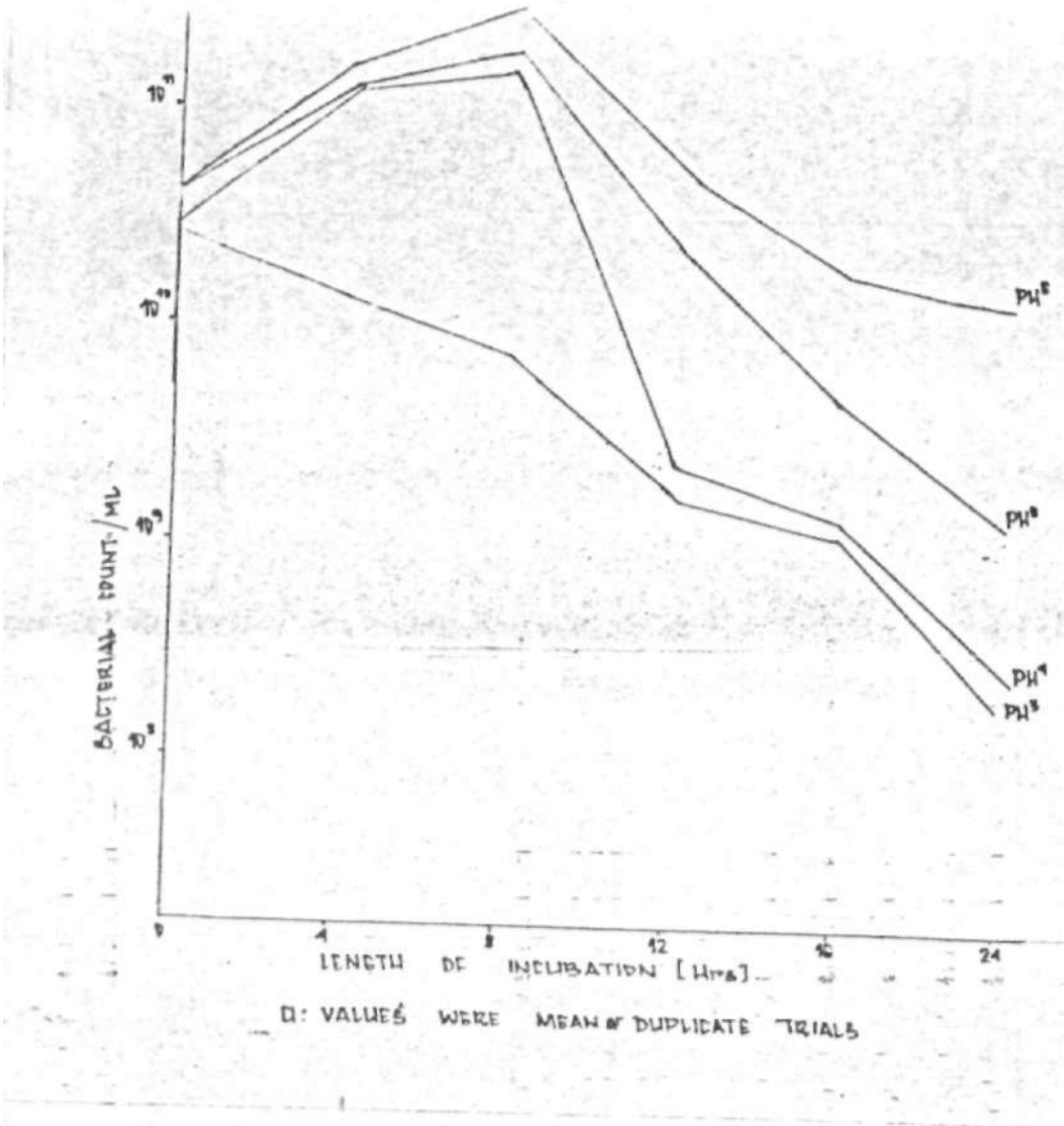
FIG 4.5 EFFECT OF PH AND OTHER MICROORGANISMS, ON SURVIVAL AND GROWTH OF E. COLI [S 600] IN STERILE AND NON STERILE KUNU AT 25°C



Δ: VALUES - WERE MEAN OF DUPLICATE TRIALS.

FIG # 6

EFFECT OF PH ON THE TOTAL AEROBIC PLATE□ COUNTS IN KUNU [NON-STERILE] AT 25°C.



□: VALUES WERE MEAN OF DUPLICATE TRIALS

CHAPTER FIVE

CHARACTERISTICS OF STAPHYLOCOCCAL ISOLATES FROM KUNU I. COAGULASE, THERMONUCLEASE AND HEMOLYSIN PRODUCTION

SUMMARY

The ability of staphylococcal isolates from kunu and the utensil used in it's preparation to ferment glucose and mannitol, produce coagulase, thermonuclease and hemolysin were determined. All 244 (100%) isolates fermented glucose while 240(98.0%) fermented mannitol. Two hundred and thirty two (95.0%) coagulated rabbit plasma; the higher incidence (97.7%) of coagulase production was amongst staphylococcal isolates from utensils. Thermonuclease was produced by 193(79.0%) isolates. Non-hemolytic isolates were the predominant type, 140(57.1%) while 70(28.7%) and 35(14.3%) showed beta and alpha hemolytic patterns, respectively. Twenty-one (60.0%) of alpha hemolysin-producing strains were obtained from utensils at the source of kunu preparation.

INTRODUCTION

Laboratories monitoring the microbiological quality of foods routinely assay for coagulase-positive staphylococci, yet there appears to be some uncertainty in the interpretation of the coagulase test (Rayman

et al., 1975). Therefore, because of the subjective nature of this semi-quantitative test, the thermostable nuclease test has been employed. The test is qualitative, specific and so far there are no reports of other bacteria possessing the enzyme. It appears that the thermostable nuclease is specific for S. aureus (Chesbro and Auborn 1967; Lachica et al., 1969; Lachica et al., 1971).

Cords and Tatini (1973) demonstrated that thermonuclease production paralleled the growth of S. aureus. Also an association has been established between thermonuclease and enterotoxin production in various cheese, beef and pork products. (Lachica et al. 1971). Tatini et al, (1975, 1976) believed that thermonuclease is produced by almost all strains of S. aureus including nearly all (98.3%) enterotoxigenic strains.

Coagulase production is one of the major criteria used to differentiate S. aureus from S. epidermidis. Despite its widespread acceptance as an index of pathogenicity the mode of action of this substance and its significance in vivo remain hypothetical, with many theories on it's mode of action (Duthie, 1955). Furthermore the interpretation given to coagulase test have varied between authors as to what should be

regarded as coagulase-positive (Rayman et al., 1975, Sperber and Tatini, 1975). The ability of various strains of S. aureus to coagulase different plasmas have been found to vary, as for example, canine biotypes of S. aureus coagulating only canine plasma (Live, 1972). This has led to the use of many plasmas by some investigators to detect coagulase production (Live, 1972; Adesiyun and Usman 1983).

Human biotypes of S. aureus are known to produce alpha hemolysin predominantly while animal biotypes predominantly elaborate beta hemolysin (Elek and Levy 1950; Hajek and Marsalek, 1969). The hemolytic patterns of staphylococcal isolates could then be a useful tool in determining their sources. Adesiyun and Usman (1983) found staphylococcal isolates from dogs to be predominantly alpha hemolysin producers and suggested that most of these isolates were from humans. A similar finding had earlier been reported for staphylocccal isolates from ovine mastitis (Lernau et al., 1961; Tamerin, 1972).

It has been shown that most strains of S. aureus ferment glucose and mannitol and can tolerate high concentration of salt (Baer et al., 1976). Anaerobic utilization of mannitol and coagulase production have been proposed by Mossel (1962) as the best criterion

for distinguishing S. aureus from other catalase-positive cocci with a recommendation that strains which are coagulase-negative or failure to ferment mannitol be classified as S. epidermidis. Stiles and Ng (1982) however found that 30% of isolates that failed to utilize mannitol anaerobically were coagulase-positive and classified as S. aureus. Strains of S. aureus are known to dissimilate mannitol anaerobically slowly or weakly (Dewaart et al., 1968, Mossel 1962).

This study was conducted to determine some of these characteristics of staphylococcal isolates from kunun zaki.

MATERIALS AND METHODS

Source of isolates

Kunu samples were collected from points of preparation sale outlets and from utensils used in the preparation of kunun zaki as earlier described.

Anaerobic utilization of glucose and mannitol

Anaerobic fermentation of glucose and mannitol was tested by the method recommended by the sub-committee of Taxonomy of Staphylococci and Micrococci (1965). Both sugars were sterilized after addition of phenol

red broth base. Isolates on nutrient agar were innoculated and incubated at 37°C for 5 days. Development of yellow coloration due to acid production was considered as a positive reaction.

Hemolysin detection

Production of alpha, beta and gamma hemolysins was investigated by the plate method of Nakagawa (1958) using tryptose blood agar base (acid) with 10% of sheep erythrocytes. The sheep blood plates were prepared by sterilizing the agar base and tempered before addition of 10% erythrocyte. Isolates stored on NA slants were streaked on plates and incubated at 37°C for 24 hrs. Complete clearing around the colony was considered as due to alpha hemolysin, incomplete clearing with a greenish tinge was interpreted as caused by beta hemolysin and the absence of any clearing considered gamma hemolysin (Elek and Levy 1950).

Coagulase detection

Coagulase activity was determined by the procedure of Baer *et al.*, (1976). Dessicated rabbit plasma were used. The test was performed by adding 0.5ml of plasma to 0.3ml of an overnight culture grown in brain heart infusion (BHI) broth. *S. aureus* (strain 361) served as a positive control while uninoculated BHI was the

negative control. The tubes were incubated at 37°C and examined after 2, 4 and 24 hrs. for clot formation. Results were interpreted as suggested by Sperber and Tatini (1975). Only 4+, 3+ and 2+ reactions were considered as positive evidence of coagulase production.

Thermonuclease detection

Production of heat-stable nucleases was detected by a plate method using toluidine blue - deoxyribonuclease acid agar as described by Lachica et al.(1971). Approximately 15mls of agar was poured into 95mm petri dishes. After cooling a Pasteur pipette was used to make 20 wells (diameter of 2mm) in each dish. Overnight culture suspension in BHI broth was boiled for 10 mins and allowed to cool. Different pasteur pipettes were then used to fill the wells. S. aureus (361) and sterile but uninoculated BHI served as positive and negative controls, respectively. Plates were incubated in a humid chamber at 37°C for 4 hrs. The appearance of a pink zone around the wells was indicative of a positive result.

RESULTS AND DISCUSSION

The anaerobic fermentation of glucose and mannitol by staphylococcal isolates from both kunu and utensils

is shown in Table 5.1. All isolates fermented glucose while 240 (98.0%) utilized mannitol anaerobically. The four isolates that failed to ferment mannitol were obtained from kunu at point of preparation.

The ability of these isolates to ferment glucose and mannitol indicated that most of them were S. aureus because majority of S. aureus ferment both sugars (Mossel, 1962; Rayman et al., 1975). Strains of S. aureus from bovine and pigeons are however known to exhibit poor ability to ferment mannitol (Devriese and Oeding, 1975).

Table 5.2 shows the relationship between coagulase and thermonuclease producing ability of staphylococcal isolates. Overall, 232 (95%) of 245 isolates were coagulase-positive while 193 (79.0%) isolates produced thermonuclease. Several workers have observed that almost all coagulase-positve strains of staphylococci are thermonuclease positive (Lachica et al., 1969; Cords and Tatini 1973, Rayman et al., 1975; Tatini, 1980). Adekeye (1982) while working with animal staphylococcal isolates however observed only a 79.7% correlation between coagulase and thermonuclease production in staphylococcal isolates from animals. The author further suggested that the incidence of thermonuclease

production amongst staphylococci of animal origin might be lower than those from human beings. This aspect needs further investigation.

The relative degrees of coagulation by staphylococcal isolates are shown in Table 5.3. Of the 244 isolates tested, 165 (67.1%) showed coagulation classified as 2+ which was the predominant type, 56 (22.5%), were 3+ and only 12 (4.9%) were 4+. A total of 12 (4.9%) of isolates showed 1+ or no coagulation at all and therefore considered as coagulase-negative. The findings of the present study are at variance with the report of Stiles and Ng (1981) that most coagulase-positive isolates had a 3+ reaction with only 7% showing the 4+ coagulation. Rayman et al. (1975) had earlier observed that most isolates of S. aureus were either 3+ or 4+ when tested for coagulase. The predominance of 2+ coagulase-positive strains may be attributed, in part to factors such as production of small amount of coagulase during growth, destruction of the fibrin by fibrinolysin before positive results are noted, persistent electricity failure and the use of plasma from rabbit only for the study. These factors have been known to affect coagulase test results (Lotter and Genigeorgis, 1977).

The hemolytic pattern of staphylococcal isolates are shown in Table 5.4. Of the 244 isolates studied, 140 (57.1%) had gamma hemolytic pattern while 70 (28.7%) produced beta hemolysin and 35 (14.3%) with alpha hemolytic pattern. Of the 35 staphylococcal isolates that elaborated alpha hemolysin, 21 (60.0%) were from utensils at the points of preparation and 10 (28.6%) were from kunu also at points of preparation. The incidence of alpha hemolysin production by staphylococcal isolates from utensils (at point of preparation) i.e. 23.6% was significantly higher ($P<0.05$, X^2) than for isolates from kunu obtained either from point of preparation (11.1%) or sale outlets (5.7%). These suggest that majority of the human biotypes of S. aureus obtained in the study were introduced through the utensils possibly through human handlers during preparation. This is because human biotype of S. aureus are known to elaborate alpha hemolysin prodominatly while animal biotypes produce mostly beta hemolysin (Elek and Levy, 1950; Hajek and Marsalek, 1969). The interchange of S. aureus between animals and human beings earlier established (Adekeye, 1981; Adesiyun and Usman, 1983) in this environment makes determination of the definite source of staphylococcal isolates difficult (Adekeye, 1981; Adesiyun and Usman, 1983).

In conclusion the incidence of coagulase and thermonuclease production by staphylococcal isolates obtained from kunu is high. Because of the relationship between coagulase, thermonuclease and enterotoxin production there is a need to determine how enterotoxigenic these isolates could be.

Table 5.1: Anaerobic fermentation of glucose and mannitol by S. **aureus** isolates from kunu and utensils

Source of Sample	Type of Sample	No. isolates tested	Glucose Fermentation		Mannitol Fermentation	
			Positive	Negative	Positive	Negative
Point of Preparation	Kunu	86	86(100)	0(0.0)	82(95.3)	4(4.7)
	Utensils	88	88(100.0)	0(0.0)	88(100.0)	0(0.0)
Point of sale	Kunu	70	70(100)	0(0.0)	70(100)	0(0.0)
Total		244	244(100.0)	0(0.0)	240(98.0)	4(1.6)

Numbers in parenthesis ( ) are the percentage of the number of tested isolates from the source

Table 5.2: Production of coagulase and thermonuclease by S. aureus isolates from kunu and utensils

Source of Sample	Type of Sample	No. isolates tested	Coagulase Production		Thermonuclease Production	
			Positive	Negative	Positive	Negative
Point of Preparation	Kunu	86	80(93.0)	6(7.0)	60(69.8)	26(30.2)
	Utensils	88	86(97.7)	2(2.3)	74(85.2)	14(15.9)
Point of sale	Kunu	70	66(94.3)	4(5.7)	59(84.3)	11(15.7)
Total		244	232(95.0)	12(4.9)	193(79.0)	51(20.3)

( ) Percent

Table 5.3: Coagulase production patterns of staphylococcal isolates from different kunu and utensils

Source of Sample	Type of Sample	No. of isolates tested	Coagulase Positive			Coagulase Negative
			2+	3+	4+	1+ and negative
Point of Preparation	Kunu	86	61(72.0)	14(16.3)	5(5.8)	6(7.0)
	Utensils	88	55(61.8)	25(28.4)	5(5.6)	2(2.3)
Point of sale	Kunu	70	48(68.6)	16(22.9)	2(2.9)	4(5.7)
Total		244	165(67.4)	55(22.5)	12(4.9)	12(4.9)

( ) Percent

Table 5.4: Production of hemolysins by staphylococcal isolates from kunu and utensils

Source of Sample	Type of Sample	No. of isolates tested	Hemolysin production		
			Alpha	Beta	Gamma
Point of Preparation	Kunu	86	10(11.1)	20(23.3)	56(65.1)
	Utensils	88	21(23.6)	23(26.1)	44(49.4)
Point of sale	Kunu	70	4(5.7)	27(38.6)	39(55.1)
Total		244	35(14.3)	70(28.7)	140(57.1)

( ) Percent

Table 5.5: Relationship between hemolysin coagulase thermonuclease production

		Hemolysin production			Thermonuclease production	
		Alpha	Beta	Gamma	Positive	Negative
No. of Coagulase	+	35(100)	69(98.6)	137(98.9)	193(100)	50(98.0)
Strains	-	0(0.0)	1(1.4)	3(1.1)	0(0.0)	1(2.0)
Total		35	70	140	193	51

( ) Percent

CHAPTER SIX

CHARACTERISTICS OF STAPHYLOCOCCAL ISOLATES FROM KUNU II. ENTEROTOXIN PRODUCTION

SUMMARY

The incidence of enterotoxin production by Staphylococcus aureus strains isolated from kunu and utensils used in its preparation was determined. Twenty (8.1%) of the 246 staphylococcal isolates tested were enterotoxigenic, elaborating staphylococcal enterotoxins A(SEA), B(SEB), C(SEC) or D(SED). No isolate produced SEE while only two strains elaborated more than one enterotoxins, types BD. SEB production was most prominent with 10(4.1%) strains being producers and it accounted for 50% of all enterotoxins produced by all isolates. There was no significant difference ($P>0.05$, X^2) between the incidence of enterotoxigenicity of S. aureus strains isolated from the points of preparation and sale outlets. The incidence of enterotoxin production by S. aureus strains was highest amongst those that were coagulase, thermonuclease and alpha hemolysin producers.

Following inoculation of kunu with known enterotoxigenic strains of S. aureus no detectable enterotoxin was produced after 24 hrs at 37°C possibly due to the low pH of kunu ranging from 2.9 to 4.0.

Based on the low incidence of enterotoxin production by staphylococcal isolates from kunu coupled with the inability of enterotoxigenic strains to elaborate toxins in kunu, it was concluded that the risk of staphylococcal intoxication to consumers of kunu is low.

INTRODUCTION

Staphylococcal foodborne intoxication has long been known to be caused by enterotoxigenic strains of staphylococci (Bergdoll, 1972). It presently believed that up to 50% of Staphylococcus aureus strains are able to produce enterotoxins and that a single strain may produce two or more enterotoxins. To date, five serologically distinct enterotoxins A, B, C. D and E are recognised (Casman et al., 1967; Gilbert and Wieneke, 1973; Gilbert, 1974).

Of the serological types of enterotoxin, SEA and SED are known to be most commonly implicated in food-poisoning outbreaks although they are produced in relatively small amount when compared to SEB and SEC (Jarvis et al., 1973). The difference has been attributed, in part, to the relative abilities to elaborate enterotoxins, during the exponential, transitional and stationary growth phases of S. aureus

(Czop and Bergdoll, 1974; Bergdoll, 1979).

In addition to isolating enterotoxigenic S. aureus and enterotoxins from foods implicated in foodpoisoning outbreaks, enterotoxigenic strains, have also been isolated from various foods routinely sampled and not involved in any intoxication (Burbianka 1971; Simkovicova and Gilbert, 1971). A difference in incidence of enterotoxigenicity of isolates have however been observed between foods that caused intoxication and those not implicated in any outbreak (Hajek and Marsalek, 1973; Wieneke, 1974). In Nigeria there are no documented reports of staphylococcal intoxication and possible types of enterotoxins involved. The only available report was that on the enterotoxigenicity of S. aureus strains from ready-to-eat products, not implicated in any outbreak, where 59.1% of the strains were found to be enterotoxigenic (Adesiyun, 1984). To date no investigation has been published on the enterotoxigenic status of S. aureus in a fermented local product like kunun zaki. It is also not known whether kunu supports S. aureus growth and enterotoxin production.

Staphylococci are unique among foodborne pathogens in that they grow and produce enterotoxins under conditions that adversely affect others;

growing in a range of water activity of 0.83 - 0.99 (Scott, 1953), of pH 4.10 and absence or presence of oxygen (Barber and Deibel, 1972), of temperature 10°C - 45°C (Genigeorgis et al. 1968; Tatini 1973; Scheusner et al., 1973). While growth of staphylococci occurs under these conditions, the level of enterotoxin produced is considerably reduced (Jarvis et al., 1973, Reiser and Weiss, 1969).

In kunu, an uncontrolled fermented cereal product, it is not known how varying pH and other contaminating microorganism will affect staphylococcal growth and toxin production.

It has long been accepted that the vast majority of enterotoxigenic staphylococci are also coagulase and thermonuclease positive although the converse is not true. Casman et al. (1967) estimated that 50% of coagulase-positive strains isolated from clinical specimens were enterotoxigenic while 96.2% of those from food products produced toxins. Tatini (1975) observed that thermonuclease was detected in samples showing detectable amounts of enterotoxins. Although Lachica et al. (1969) had demonstrated a correlation between thermonuclease, coagulase and enterotoxin production, thermonuclease and coagulase negative strains have also been found to be

enterotoxigenic (Devriese and Oeding 1975; Adesiyun and Usman 1983; Danielsson and Hellberg 1977).

This study was conducted to determine the enterotoxigenicity of staphylococcal isolates from kunu and assess the possibilities of enterotoxin production during storage or sale.

MATERIALS AND METHODS

Source of staphylococci

All staphylococcal isolates were from kunu obtained at points of preparation and sales and also from utensils used in its preparation.

Identification of staphylococci

All typical black colonies on BPA after 48 hrs were tested for anaerobic fermentation of glucose and mannitol according to the recommendation of the subcommittee of Taxonomy of Staphylococci and Micrococci (1965). They were tested for coagulase production using rabbit plasma (Baer et al.,1976) and thermonuclease production (Lachica et al., 1972). The hemolytic patterns were also determined as described by Elek and Levy (1950). All isolates were either coagulase or thermonuclease positive were regarded as staphylococci in addition to

fermenting glucose and mannitol.

Growth of staphylococci

The cellophane-over-agar, method of Robbins et al., (1974) was used to grow the cells for enterotoxin production. 0.1ml of overnight growth at 37°C of staphylococcal isolate in BHI broth was inoculated onto sterile 9cm diameter cellophane discs cut from dialysis tubing - visking size 16-2 ⅜ in C (Medicell International Ltd. London) on BHI plates. The inoculum was spread using sterile curved glass rods and then incubated at 37°C for 24 hrs. To recover enterotoxins, 25mls of 0.01 N Na_2 HPO_4 was used to suspend cell growth on the membrane and the suspension was centrifuged at 7,000 rpm for 15 minutes.

Detection of enterotoxin

The double gel diffusion (microslide) technique similar to that described by Casman and Bennett (1969) was used. Standard enterotoxins and antisera for types A, B, C, D and E. kindly supplied by Prof. M. S. Bergdoll of the University of Wisconsin, U.S.A. were used. Approximately 0.4ml of 1.2% melted agarose was put on precoated (using 0.2% agarose) slides within a taped area. Immediately,

grease-coated templates were gently placed on molten agar and allowed to solidify. Using Pasteur pipettes, standard antiserum was placed in the centre well of the templates with the standard antigen in the well above the central well. The sample supernatant being tested was put in the wells to the right and left of the central well (See fig 6.1). Triplicate slides were set up for each sample. The slides were incubated in a humid chamber for 48 hr at room temperature. The slides were read at an oblique angle against a fluorescent desk lamp, after staining briefly in thiazine red stain.

Detection of staphylococcal enterotoxins A, B and D in kunu experimentally inoculated with S. aureus strains Z88 and 418

To investigate a possible production of staphylococcal enterotoxin in kunu, S. aureus (Z88), SEA and SED producer and S. aureus (418) an. SEA, SEB and SED producer were used. Overnight culture growths of Z88 and 418 were each inoculated into 60mls of non-sterile kunu and incubated at 37°C under stationary conditions. At 4-hourly intervals for a period of 24 hrs. 3 mls were aseptically removed from the incubated flask. One millilitre was used for plating after serial dilution on BPA and NA for staphylococcal and total aerobic plate counts, respectively after serial dilutions. The remaining

2ml was centrifuged and the supernatant freeze-dried. Subsequently the freeze-dried material was reconstituted in 0.1 ml of saline resulting in a 20-fold concentration which was then tested for SEA, SEB or SED as appropriate, using the double gel diffusion (Microslide) technique of Casman and Bennett (1965).

RESULT AND DISCUSSION

Table 6.1 shows the enterotoxigenicity of staphylococcal isolates from kunu and utensils. Twenty (8.1%) of the 246 isolates tested were enterotoxigenic elaborating either SEA, SEB, SEC and SED. No isolate produced SEE while only two isolates produced multiple enterotoxins BD.

The difference in incidence of enterotoxigenicity of isolates from kunu between the point of preparation (10.5%) and sale outlets (7.1%) was not statistically significant ($P>0.05$, X^2).

Previous studies have mainly assayed for enterotoxin production by S. aureus strains associated with food-poisoning outbreaks. Simkovicova and Gilbert (1972) found 92% of such strains to be enterotoxigenic while Toshach and Thorsteinson (1972) reported that only 44% of strains associated with foodpoisoning were enterotoxigenic. Variable reports

of incidence of enterotoxigenicity of isolates of S. aureus not associated with any outbreaks ranging from 10% to 62.5% (Casman et al., 1967; Burbianka, 1971; Payne and Wood 1974; Wieneke, 1974). Recently, Adesiyun (1984) determined the ability of S. aureus strains from some ready-to-eat products in Nigeria to produce enterotoxins and found 39.1% to be producers. Although the 8.1% found in the present study is much lower than that found in the same environment, ready-to-eat foods are exposed to more frequent human handlings. Human biotypes of S. aureus are known to be more enterotoxigenic than animal biotypes (Bergdoll, 1979; Hajek and Marsalek, 1973). The interchange of human and animal biotypes of S. aureus in Nigeria have however been documented (Adekeye, 1981; Adesiyun and Usman, 1983).

The finding that SEB was the predominant type produced is at variance with previous reports where SEA and SED have been shown to be more frequently encountered in food products (Bergdoll, 1979; Adesiyun, 1984). Although the possibility of this being the true pattern in S. aureus strains in kunu, the few enterotoxigenic strains (20) encountered in the present study cannot be easily explained.

The relationship between hemolysin, coagulase, thermonuclease and enterotoxin by isolates is shown in Table 6.2. Alpha, beta and gamma hemolysins were produced by 35 (14.2%), 71 (28.9%) and 140 (56.9%) strains, respectively. The incidence of enterotoxin producers amongst isolates with ∝-hemolytic pattern (25.7%) was significantly higher ($P<0.05$, X^2) than either the Beta (7.0%) or gamma (4.3%) hemolysin producers. Human biotypes of S. aureus are known to produce predominantly alpha hemolysin while animal biotypes produce more of beta hemolysin (Elek and Levy, 1950; Hajek and Marsalek, 1969). It is also known that human biotype of S. aureus are usually more enterotoxigenic than animal biotypes (Bergdoll, 1970; Hajek and Marsalek, 1973). These, to some extent explain the higher degree of enterotoxigenicity detected amongst alpha hemolysin producing strains in this study.

It was observed that the incidence of enterotoxin production amongst coagulase-positive strains (8.2%) was only slightly higher than that found for the coagulase-negative strains (7.7%). It was of interest because enterotoxin production has previously been reported to be most common amongst coagulase-positive strains (Bergdoll, 1972, 1979). It is however known

that the type of plasma used in the coagulase test affects coagulation by various biotypes of S. aureus (Live, 1972; Adesiyun and Usman, 1983).

The ability of isolates to produce thermonuclease correlated with coagulase production with 78.9% and 94.7% of isolates being thermonuclease-and coagulase-positive, respectively. This does not however totally agree with the study by Tatini (1980) where all coagulase-positive (100.0%) strains of staphylococci were thermonuclease-positive. A close association was observed between enterotoxin production by coagulase-positive and thermonuclease-positive strains. In the study, 8.2% of isolates that were coagulase-positive or thermonuclease-positive were enterotoxigenic. A good correlation had earlier been established between coagulase, thermonuclease and enterotoxin production (Lachica et al., 1969).

Fig 6.2 shows the growth patterns of S. aureus (Z88) and (418) in non-sterile kunu with the pH changes during incubation. The changes in staphylococcal counts per millilitre observed were from 9.5×10^6 at 0 hr to 1.3×10^3 after 24 hrs at $37^{\circ}C$, representing approximately a 4-logarithmic decline for strain Z88, an SEA and SED producer.

However, for strain 418 an SEA, SEB and SED producer, approximately a 5-log decline in population was observed within 24 hrs. at 37°C i.e. 1.3×10^7 to 4.0×10^2 cfu/ml. For both strains of S. aureus the pH changes of kunu were similar starting with 4.0 (at 0 hr.) to 2.9 (after 24 hr.).

Although both strains (Z88 and 418) had counts over a million (10^6) per ml of kunu from 0 hr up to about 6 hr at 37°C, no enterotoxin was detected even after concentrating the supernatant 20-fold. A growth of up to a million organisms per ml or gm of a food by an enterotoxigenic strain of S. aureus is needed before enterotoxin can be detected (Bergdoll, 1970). It is, however, known that parameters such as oxygen tension, water activity, pH and temperature affect enterotoxin production and factors that affect enterotoxin production do not necessarily affect growth (Bergdoll, 1970; Genigeorgis et al., 1971; Tatini, 1973). The pH appears to be important in this study with a range of 2.8-4.0 throughout incubation in view of the fact that Troller (1976) had observed that enterotoxin production by S. aureus normally will not occur below pH of 5.0.

In conclusion, there appears to be a low risk of staphylococcal intoxication from consumption of contaminated kunun zaki because of a relatively low incidence (8.1%) of enterotoxigenicity amongst staphylococcal isolates from it coupled with it's rather low pH that does not allow enterotoxin production by enterotoxigenic strains.

Table 6.1: Incidence of enterotoxin production by Staphylococcus aureus strains from kunu and utensils

Source of Sample	Type of Source	No. of isolates tested	No. of strains enterotoxigenic	No. of strains[a] which produce enterotoxins				
				A	B	C	D	E
Point of Preparation	Kunu	86	9(10.5)	1(1.2)	4(4.7)	-	5(5.9)	-
	Utensil	89	6(6.7)	-	2(2.3)	2(2.3)	2(2.3)	-
Point of Sale	Kunu	70	5(7.1)	1(1.4)	4(5.7)	-	-	-
Total		246	20(8.1)	2(0.8)	10(4.1)	2(0.8)	8(3.3)	-

[a] Two strains produced multiple (BD) enterotoxins with all others single enterotoxin producers.

( ) Percent

- No enterotoxin detected

Table 6.2: Relationship between hemolysin, coagulase, thermonuclease and enterotoxin production by staphylococcal isolates

	Hemolysin production			Coagulase production[a]		Thermonuclease production	
	Alpha	Beta	Gamma	Positive	Negative	Positive	Negative
No. of strains	35(14.2)	71(28.9)	140(56.9)	233(94.7)	13(5.3)	194(78.9)	52(21.1)
No. of enterotoxigenic strains	9	5	6	19	1	16	4
Percent of enterotoxigenic strain	25.7	7.0	4.3	8.2	7.7	8.2	7.7

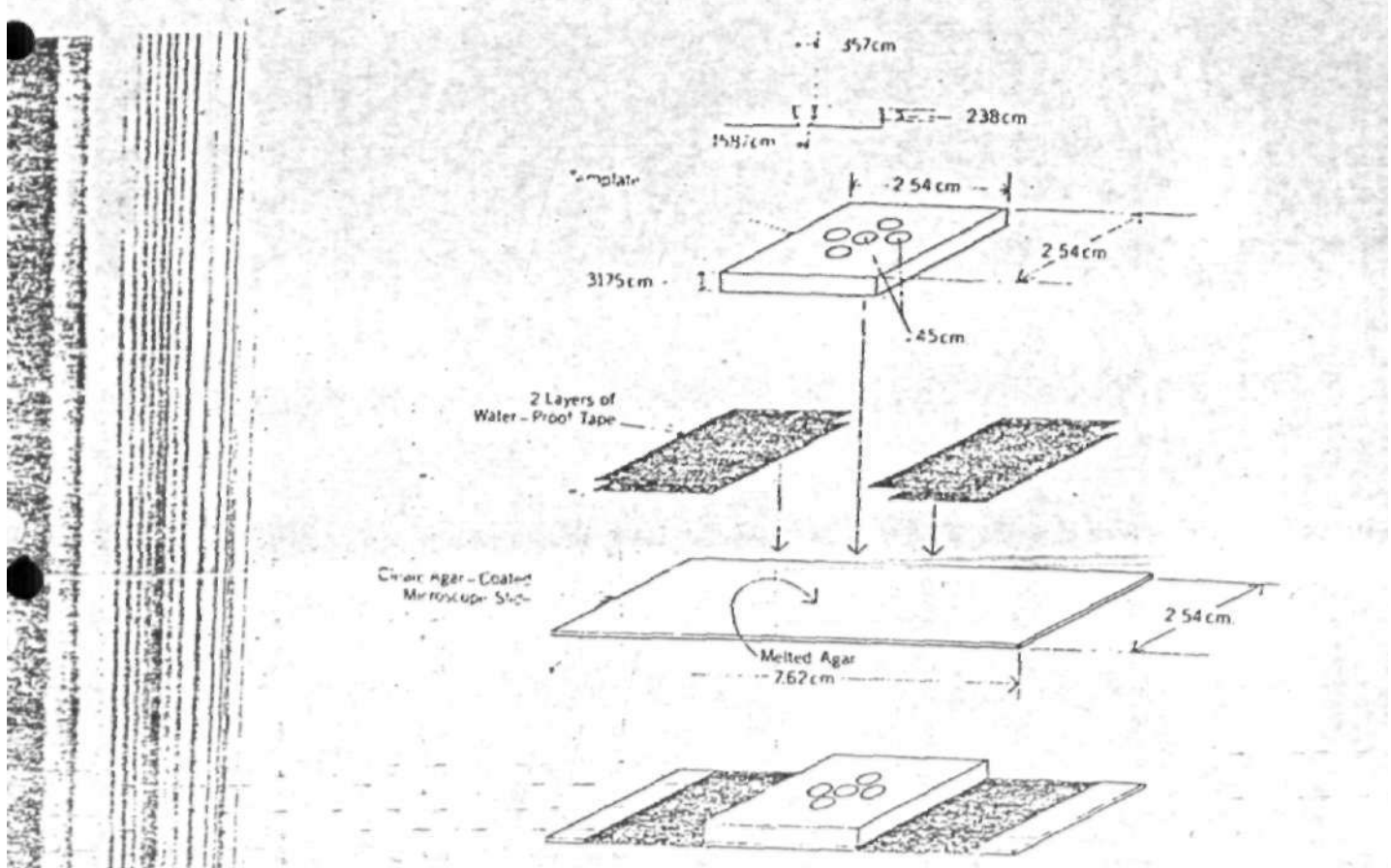
[a]Using rabbit plasma

Table 6.3: Type of enterotoxin produced by staphylococcal isolates from kunu and utensils

Source of Sample	Type of source	No. of entero-toxogenic strains	Enterotoxin produced					
			A	B	C	D	BD	E
Point of	Kunu	9	1	2	-	6	2	-
Preparation	Utensil	6	-	2	2	2	-	-
Point of Sale	Kunu	5	1	4	-	-	-	-
Total		20	2	8	2	8	2	-

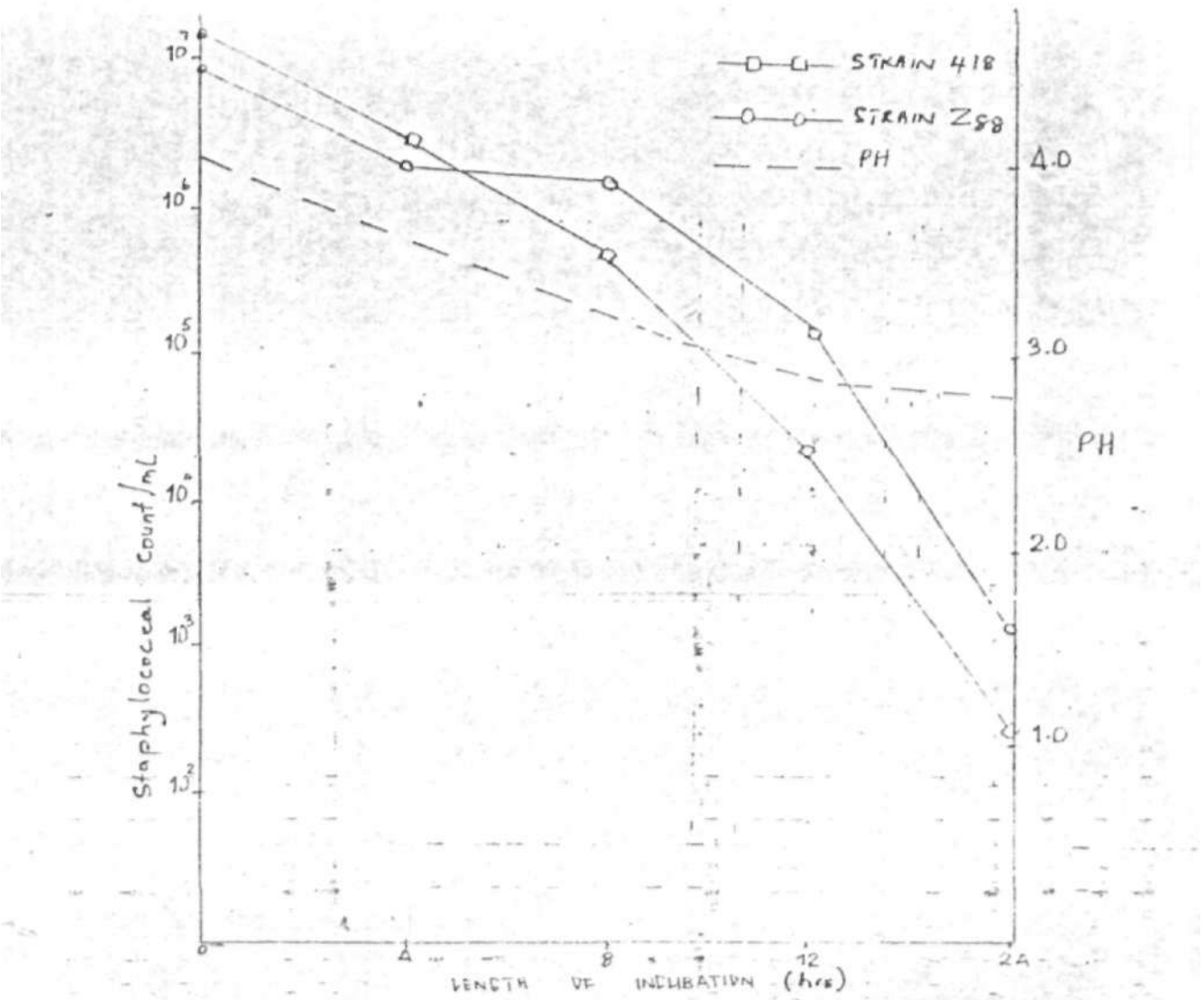
- Not detected

FIG. 6.1 MICROSLIDE GEL DIFFUSION ASSEMBLY WITH SPECIFICATIONS FOR PLEXIGLAS TEMPLATE.



Adapted after Bergdoll and Casman (1976) in "Staphylococcal enterotoxins", chapter 32 page 390, Compendium of methods for microbiological examination of foods. Editor, M. L. Speck.

FIG 6:- GROWTH IN TWO ENTEROTOXIGENIC STRAINS (Z88 AND 418) OF *S. aureus* IN KUNU AT 37°C



CHAPTER SEVEN

CHARACTERISTICS OF STAPHYLOCOCCAL ISOLATES KUNU III ANTIBIOGRAM

SUMMARY

The sensitivity of 253 Staphylococcus aureus strains isolated from a locally fermented cereal drink, kunun zaki, to 11 antimicrobial agents was determined. Two hundred and forty seven (97.6%) strains were resistant to one or more of the antimicrobial agents. About ninety-eight percent (97.8%) of isolates from source of kunu preparation were resistant while 98.6% of those from kunu bought at sale outlet and 96.7% of those isolated from utensils were resistant. There was no significant difference in resistance of isolates from the sources ($P>0.05$, X^2). Resistance to penicillin (97.2%), ampicillin (92.1%) and trimethoprim (80.2%) were most frequently encountered while resistance was least developed to neomycin (5.5%) and gentamycin (2.8%). The most common antimicrobial resistance pattern was ampicillin - penicillin - trimethoprim (17.0%).

The relatively high resistance of S. aureus from kunu to tested antimicrobial agents suggests a misuse of these drugs in this environment.

INTRODUCTION

Staphylococci being ubiquitous in nature, are usually present in raw and ready-to-eat foods and are responsible for one of the most common food-poisoning syndromes and a number of human and animal diseases (Tatini et al., 1976, Pattee et al., 1980, Wentworth et al., 1958). Efforts to control these diseases by chemoprophylaxis and chemotherapy through the use of antimicrobial agents, particularly antibiotics, have resulted in increased prevalence of resistance to these agents (Barber et al., 1949; Nakhla, 1973). Relatively high prevalence of resistance to various antibiotics have been reported in a developing country, like Nigeria where control of these drugs are presently absent or inadequate (Adekoye, 1979; Paul et al., 1982; Kwaga and Adesiyun, 1984).

Resistance of S. aureus to penicillin, chloramphenicol and tetracycline usually develops slowly in multiple small steps and many successive subcultures may be required before a high level of resistance is obtained. For penicillin, for example, resistance by S. aureus is due to the production of penicillinase which is controlled by a plasmid-based gene (laycey, 1975). Bulger and Sherris (1968) suggested that only

a rational utilization policy for antibiotics can increase their effectiveness.

A widespread interchange of staphylococci between animals and humans have been observed in Nigeria (Adekeye, 1979; Adesiyun and Usman, 1983). The antibiograms of S. aureus strains isolated from ready-to-eat products exposed to human handling were earlier determined (Kwaga and Adesiyun, 1984). To date however, no information is available on the antibiotic sensitivity patterns of S. aureus in any Nigerian fermented product with a pH not particularly favourable for staphylococcal growth with a possibility of selecting out certain strains of S. aureus.

The present study was therefore conducted to determined the prevalence of antimicrobial resistance of S. aureus strains isolated from a locally fermented cereal drink, kunun zaki, to various antimicrobial agents used in Nigeria.

MATERIALS AND METHODS

Source of Isolates

Staphylococcal isolates obtained from kunu and utensils used in it's preparation were used in the

study.

Identification of staphylococci

Typical black colonies of staphylococci on Baird Parker agar (BPA) which utilized glucose and mannitol anaerobically within 5 days (Subcommittee on Taxonomy of Staphylococci and Micrococci, 1965), coagulated rabbit plasma (Baer et al., 1976) or produced thermonuclease (Lachica et al., 1972).

Susceptibility of isolates to antibiotics

Antibiotics

The antibiotic discs (Difco) and concentrations used are as follows: Penicillin (10 units), erythromycin (15 ug), Streptomycin (10 ug), tetracycline (30 ug) methicillin (5 ug), neomycin (30 ug), trimethoprim (5 ug), chloramphenicol (30 ug), gentamycin (10 ug), ampicillin (10 ug) and bacitracin (10 units).

Antibiotic susceptibility testing

A modification of the disc diffusion method of Bauer et al. (1966) was used. Isolates to be tested were grown for 2-8hrs on tryptic soy broth at 37°C and spread using sterile cotton swabs. Turbidity testing

was determined using $BaSO_4$ as standard. Approximately 5 to 6 antibiotic discs were placed on each petri dish and the plates were incubated at 37°C for 16-18hrs. Zone of inhibition, to the nearest millimeter were measured with a ruler and the results recorded as sensitive, intermediate or resistant depending on the interpretative table recommended by the disc manufacturer.

RESULTS AND DISCUSSION

Table 7.1 shows that 247 (97.6%) of 255 isolates tested were resistant to one or more of the 11 antimicrobial agents used. The resistance of staphylococcal isolates from kunu at it's points of preparation was 97.8% but 98.6% for those from it's points of sale and 96.7% for those from utensils used in its preparation. The high prevalence of resistance (97.6%) to the antimicrobial agents studied should be of concern as they might reflect resistance patterns of the same organisms in medical and Veterinary practice in Nigeria (Adekeye, 1979; Kwaga and Adesiyun, 1984). Kwaga and Adesiyun (1984) reported that 60.3% of staphylococcal isolates from some ready-to-eat products sold around Samaru were resistant to 10 antimicrobial agents tested, a resistance prevalence much lower than that

found in the present study. It is pertinent to mention that similar antimicrobial concentrations were used in both studies, except that methicillin and bacitracin were not tested for in the study while kanamycin used by them was not tested for in this study.

The distribution of antimicrobial resistance of S. aureus isolates in relation to the source of kunu is displayed in Table 7.2, while regardless of source, it is shown in Table 7.3. Resistance to penicillin, ampicillin, trimethoprim and methicillin were most frequently encoutered while resistance was least developed to neomycin and gentamycin.

With regards to source (Table 7.2), no distinct pattern was established in resistance to the various antimicrobial agents tested between the points of preparation or sales and utensils. It was however observed that with kunu from the points of preparation, resistance by staphylococcal isolates to methicillin (39.3%), erythromycin (18.0%) and streptomycin (16.9%) were significantly lower ($P < 0.05$, X^2), than those from the sale outlets, 97.3%, 52.1% and 84.9%, respectively. The reason for this difference is not clear.

The rather similar prevalence of resistance to penicillin (92.4%) and ampicillin (92.1%) displayed in Table 7.3 is in agreement with an earlier study at Ile-Ife by Paul et al., (1982) where 97.2% and 92.1% were obtained for penicillin and ampicillin, respectively. This is despite the fact that different antibiotic concentrations were used in the two studies. Kirby (1944) had earlier suggested that resistance to both antibiotics was mainly due to production of B-lactamase enzymes. Kwaga and Adesiyun (1984) had earlier attributed a higher prevalence of reistance to penicillin (41.0%) than the 28.4% found to ampicillin amongst staphylococcal isolates from some ready-to-eat products to the more frequent and widespread use and misuse of penicillin in the environment.

Of interest was the finding that 56.5% of staphylococcal isolates from all sources (Table 7.3) were resistant to methicillin. This is because methicillin is not commonly used in medical and veterinary practice in Nigeria. Ojo (1972) in a study at Ibadan first reported the resistance of two strains of S. aureus to methicillin may not be unconnected with the free use of penicillin in Nigeria. The rather high prevalence of resistance to penicillin

(92.4%) found in the present study supports this thinking.

Parker and Hewitt (1970) found that strains resistant to methicillin were also resistant to at least one antibiotic other than penicillin and methicillin. This is confirmed in the present study (Table 7.4) where no staphylococcal isolates was resistant to methicillin alone and in the 21 resistance patterns involving methicillin, 3 to 8 other antibiotics were involved. Twenty of these patterns contained penicillin and ampicillin while 14 contained ampicillin, penicillin and trimetoprim.

The predominant antimicrobial resistance patterns were ampicillin - penicillin - trimethoprim (17.0%), ampicillin-bactracin-erythromycin-methicillin-penicillin-streptomycin-tetracycline-trimethoprim-chloramphenicol (6.3%) and ampicillin-methicillin penicillin-streptomycin-trimethoprim (5.9%) regardless of source (Table 7.4). Ampicillin and penicillin were most frequently encountered in all the patterns. Four (1.6%) isolates of S. aureus were sensitive to all antimicrobial agents tested. The high occurrence of multiresistance to antibiotics by isolates from all sources should be of concern as it indicates that little or no effort has been made to control the use

of these antimicrobial agents which unfortunately are readily available without prescriptions at the local drug stores. Bulger and Sherris (1968) had advocated the use of penicillinase-resistant penicillins to reduce emergence of resistant variants which may help the alarming situation in Nigeria.

In conclusion the high prevalence of resistance to various antibiotics should be of great health concern to necessitate enactments of laws to regulate the use of antibiotics in Nigeria. This is because consumers of kunu are exposed to S. aureus strains which are resistant to some antibiotics used in the treatment of various diseases in this environment. The risk of treatment failures that may follow infection by these strains of S. aureus cannot be ignored.

Table 7.1: Resistance of Staphylococcus aureus from kunu and utensils to one or more of the antimicrobial agents tested

Source of Sample	Type of Sample	No. of isolates tested	No. resistant (Percent)
Point of Preparation	Kunu	90	88(97.8)
	Utensils	91	88(96.7)
Point of Sale	Kunu	72	71(98.6)
Total		253	247(97.6)

Table 7.2: Distribution of antimicrobial resistance by S. aureus isolates from various sources

Antimicrobial tested	Concentration ug	No. and (%) of S. aureus from sources resistant to selected antimicrobials		
		Point of preparation		Point of sale
		kunu	utensils	kunu
Ampicillin	10 ug	87(97.8)	98(84.8)	70(95.9)
Bacitracin	10 units	25(28.0)	43(46.7)	32(43.8)
Erythromycin	15 ug	16(18.0)	33(35.9)	38(52.1)
Gentamycin	10 ug	1(1.1)	2(2.2)	3(4.1)
Methicillin	5 ug	35(39.3)	39(40.2)	71(97.3)
Neomycin	30 units	2(2.2)	2(2.2)	5(6.8)
Penicillin	10 units	88(98.8)	79(85.9)	68(93.2)
Streptomycin	10 ug	15(16.9)	34(38.2)	62(84.9)
Tetracycline	5 ug	34(38.2)	36(39.1)	25(34.2)
Trimethoprim	5 ug	73(82.0)	68(73.9)	62(84.9)
Chloramphenicol	30 ug	9(10.1)	22(23.9)	29(39.7)
Total number of isolates tested		89	92	73

Table 7.3: Percentage resistance of 253 S. aureus isolates to 11 antimicrobial agents

Antimicrobial agents	Resistance
Penicillin	234(92.4)
Ampicillin	233(92.1)
Trimethoprim	203(80.2)
Methicillin	143(56.5)
Streptomycin	107(42.3)
Bacitracin	102(40.3)
Tetracycline	95(37.5)
Erythromycin	87(33.4)
Chloramphenicol	60(23.7)
Neomycin	14(5.5)
Gentamicin	7(2.8)

( ) Percent resistance

Table 7.4: Predominant Patterns of antimicrobial resistance[a] 253 isolates of S. aureus from kunu and utensils

	Resistant Pattern[a,b]	No. of isolates (Percent)
1.	Amp, P, Trim	43(17.0)
2.	Amp, Ba, Ery, Me, P, S, Te, Trim, C	16(6.3)
3.	Amp, Me, P, S, Trim	15(5.9)
4.	Amp, P, S, Trim	11(4.3)
5.	Amp, P, S, Te, Trim	9(3.6)
6.	Amp, Ba, Ery, Me, P, S, Te, C	8(3.2)
7.	Amp, Ba, Me, P, Te, Trim	8(3.2)
8.	Amp, Ba, Me, P, S, Trim	7(2.8)
9.	Amp, Ba, P, Trim	7(2.8)
10.	Amp, Me, P, Trim	6(2.4)
11.	Amp, Ba, Me, P, Trim	6(2.4)
12.	Amp, Ba, Ery, P, Trim	6(2.4)
13.	Amp, Ba, Ery, Me, P, Te	5(2.0)
14.	Amp, Ba, Ery, Me, P, Trim	5(2.0)
15.	Amp, Ery, P, Trim	5(2.0)
16.	Amp, P	4(1.6)
17.	Amp, Me, P, S, Te, Trim	4(1.6)
18.	Amp, Me, P	3(1.2)
19.	Amp, Me, Te, Trim	3(1.2)
20.	Amp, Ba, Me, P, S, Te, Trim, C	3(1.2)
21.	Amp, Ba, Ery, Me, P, S, Te, Trim	3(1.2)
22.	Amp, P, Trim, C	3(1.2)
23.	Amp, Ba, Ery, Me, P, Te, Trim	3(1.2)
24.	Amp, Ba, Ery, Me, P, S, Te	3(1.2)
25.	Amp, Ba, Ery, Me, P	2(0.8)
26.	Amp, Ba, Ery, Me, P, Te, Trim, C	2(0.8)
27.	Amp, Ery, P, S, Te, Trim	2(0.8)
28.	Amp, Ery, Me, P, S, Trim, C	2(0.8)
29.	Ery, Me, P, S, Trim, C	2(0.8)

Table 7.4 (cont)

	Resistant Pattern[a,b]	No. of isolates (Percent)
30.	Amp, Ery, Me, P, Trim	2(0.8)
31.	Am, P, Te	2(0.8)
32.	Amp, Ba, Ery, Me, P, S, Trim	2(0.8)
33.	Amp, Ba, Me, P, S, Te, Trim	2(0.8)
34.	Amp, Ba, Me, P	2(0.8)
35.	None of the above[c] (1-34)	43(17.0)
36.	Sensitive to all antimicrobials tested	4(1.6)

[a]Overall, 78 antimicrobial resistance patterns were observed

[b]Amp = Ampicillin, Ba = Bacitracin,
Ery = Erythromicin, Me = Methicillin
P = Penicillin, S = Streptomycin,
Te = Tetracycline, Trim = Trimethoprim,
C = Chloromphenicol

[c]Each of the other 44 patterns not shown had only one isolate

GENERAL DISCUSSION AND SUMMARY

Studies on the prevalence of staphylococci and coliforms in kunu zaki at points of preparation and sale outlets demonstrated a high prevalence of contamination by coliforms and staphylococci at all sources sampled. The difference in degrees of staphylococcal and coliform contamination between the sources were not significant ($P>0.05$, X^2). The degree of contamination by staphylococci were relatively low with mean count per millilitre ranging from 5.8×10^2 to 2.1×10^4 while for coliforms the range was 4.5×10^5 to 3.3×10^6 per ml. All water samples used in preparation of kunu tested were grossly contaminated by faecal coliforms.

All four spices namely ginger, red pepper, black pepper and cloves routinely added to kunu were grossly contaminated with staphylococci, coliforms and other microorganism. Only cloves at 0.25% and 1.25% concentration demonstrated bactericidal effect against E. coli, S. aureus and other contaminants. The acidic pH of kunu, a mean range of 3.5 to 4.5 possibly resulted in the observed general decline in microbial population and may have obscured any inhibitory or bactericidal effects of other spices.

The study established that cloves usually added to kunu primarily for flavouring may play a vital role in reducing the microbial population of contaminants, thereby reducing the hazard of kunu-borne infection or intoxication when consumed.

Storage of kunu at 4°C for 24 hrs. resulted in approximately a log decline in coliform and staphylococcal population but at 25°C or 37°C a 5-6 log decrease was observed. The implication is that with kunu stored in the refrigerator (4°C) was done at some kiosks, further fermentation is checked, thereby keeping the taste unchanged and thus retains consumer acceptability. On the other hand, kunu hawked around at ambient temperature (25°C and over) undergoes further fermentation with resultant pH drop which is detrimental to microbial contaminants. This further pH drop may however adversely affect the consumers' acceptability of the product.

All staphylococcal isolates from kunu, regardless of source fermented glucose and only 1.6% failed to utilize mannitol anaerobically. Similarly coagulase and thermonuclease production by isolates was high and there was a good correlation between the production of these two metabolities. The isolates were predominantly gamma hemolytic (57.1%) while only 14.3%

of them elaborated alpha hemolysin. Of significance was the finding that 60.0% of the alpha hemolysin producers were staphylococcal isolates from utensils exposed to human handling. This indicates that the isolates may be human biotypes of S. aureus since alpha hemolysin is predominantly elaborated by human biotypes.

The incidence of enterotoxin production by isolates of S. aureus from kunu was 8.1% with SEB and SED predominatly produced by 10 (4.1%) and 8 (3.5%) isolates respectively. Of interest was the finding that known enterotoxigenic strains of S. aureus, Z88 (SEA and SED) and 418 (SEA, SEB and SED) when inoculated into kunu failed to elaborate any enterotoxin(s) after 24 hrs. at 37°C. In addition a four-log decline in count was observed.

Based on the low staphylococcal counts found in all kunu sampled (mean range, 5.8×10^2 to 7.8×10^3 per ml) and the low incidence of enterotoxigencity of the staphylococcal isolates, coupled with the failure of known enterotoxigenic strains to produce enterotoxins in kunu, it is concluded that the risk of staphylococcal intoxication from consumption of kunu is low. The risk posed by other pathogens (not studied here) in kunu can, however, not be ignored.

The isolates were mostly resistant to penicillin, (92.4%), ampicillin (92.1%) and trimethoprim (80.2%). They were least resistant to gentamycin (2.8%). These findings may be related to the degree of use and misuse of the antimicrobial agents in the environment.

The frequency of resistance to various antimicrobial agents and the resistance patterns of S. aureus strains isolated from kunu in this study may reflect the antibiograms of S. aureus involved in human and animal infections around Zaria.

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