THE INFLUENCE OF pH ON THE RECOVERY OF CONTAMINATING MICRO-ORGANISMS FROM HONEY.

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A project report submitted in part fulfilment for the degree of Master of Veterinary Public Health in the University of Nairobi.

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1992

DECLARATION

This project report is my original work and has not been presented for a degree in any other university.

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DEDICATION

To my mother,

Lady Juliana Wangari Kibuthu.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

ACKNOWLEDGEMENTS

The help and guidance from my supervisors, Dr. S. M. Arimi, Dr. M. Kayihura and Dr. E. K. Kang'ethe is gladly acknowledged.

My thanks also go to the Chairman of the PHPT Department, Professor J. M. Gathuma for providing an enabling environment for me to successfully complete the MVPH course in the department..

The technical staff of the Department of Public Health, Pharmacology and Toxicology especially Mr. Kaburia, a senior Technician, Mr. Macharia, Mr. Githua and Mr. Muriuki assisted in the work, and to them I say thank you.

I am grateful to Drs. Kyule and McDermott for their advice on data analysis and to Mrs. Dorcas Nduati for assisting with the typing of the work.

Family members, classmates and close friends like Dr. Ernest Njoroge prayed for, and encouraged me especially during the times when I had difficulties and for this I am grateful.

I highly appreciate the assistance of the librarians at the Norwegian college of Veterinary Medicine in getting relevant scientific journals. The help given by Messrs Kasolia and Muchiri of bee research Institute, Kenya, Dr. Timothy Wachira of the Department of PHPT, university of Nairobi and Prof. Nafstad of the Norwegian Veterinary college in this regard is also gratefully acknowledged.

Completion of this work was made possible by the generous financial assistance of the Norwegian Agency of International Development (NORAD) and the Norwegian Veterinary Association (NVA). I sincerely thank them.

Abstract

Accurate and sensitive methods for recovering contaminating micro-organisms from honey are required for an adequate control of the hygienic quality of the product. Most of these micro-organisms would be expected to be physiologically deficient (injured) due to being exposed to the antimicrobial physio-chemical components of honey such as low pH, low moisture content and high osmotic pressure .

The aim of this study was therefore to determine the pH of Kenyan honey and find out if recovery of contaminating micro-organisms in honey could be improved by using phosphate buffer to raise honey pH to about neutral. It was also intended to find out the levels of aerobic and anaerobic mesophilic total viable counts (TVC) encountered in processed, semi processed and home processed honey

The pH of 44 (17 processed, 23 semi processed and 4 home processed) honey samples was determined before and after dilution in the ratio 1:10 with distilled water of pH 5.60 and 0.1M phosphate buffers of pH 6.00, 7.00, 7.10, 7.20, 7.30, and 8.00. These same honey samples plus 4 other processed ones were diluted in sterile distilled water of pH 5.60 and O.1M phosphate buffer of pH 7.00 and cultured in Plate Count Agar (PCA, Oxoid) at 37℃ for 48 hours .Half the plates were Incubated aerobically while the other half was incubated anaerobically using the Gas Pak system. Colonies were counted according to Busta et al. (1984) and the results analysed using the paired t-test.

Undiluted honey samples examined in this study had a low average pH of 3.60 (range 2.70-4.00). The average pH of the diluted honey depended on the diluent used. The mean pH of honey diluted in distilled water of pH 5.60 and 0.1M phosphate buffer of pH 7.00 were 3.70 and 6.86 respectively.

When compared to distilled water, phosphate buffer significantly improved the recovery of mesophilic anaerobic TVC from refined honey (45.4%), semi refined honey (32%) and all the honey types combined (29.0%). Recovery of aerobic micro-organisms was also improved although the numerical increment was not statistically significant

In conclusion, 0.1M phosphate buffer of pH 7.00 was shown to be a better diluent for recovering contaminating micro-organisms from honey than distilled water of pH 5.60. Hygienic quality of fully processed honey was 2-4 times better than that of semi refined (unpasteurized) honey. It was also established that tyndallization could not achieve sterilization of honey.

CHAPTER ONE

INTRODUCTION

Honey is the sweet substance produced by honeybees from the nectar of blossoms or from secretions of or on living parts of plants which they collect, transform and combine with specific substances and store in honey combs (FAO/WHO, 1981). It is highly valued as food and has been used as a component of baby foods, pharmaceutical products and as treatment for wounds and upper respiratory tract infections. Its microbiological characteristics are less well known perhaps because it has been assumed to be sterile for a long time.

In Kenya, honey is produced by women groups, Co-operative societies and individual farmers for domestic consumption or commercial purposes. It is processed in modern refineries or at home. In the modern honey refineries, honey is extracted, strained and placed in large barrels or drums before being pasteurised and packaged. A preliminary report on the microbiological quality of fully processed (commercial) Kenyan honey is available (Kayihura *et al*, 1989) but there are no reports on the quality of semi processed or home processed honey although both are also available to some consumers. Local and international trade in honey is governed by regulations which are based on physico-chemical properties of honey; deviations from the stated ranges of values determine the honey quality (Kasolia and Mbithi, 1987; FAO/WHO, 1981). These regulations, however, lack provision for ensuring consumer safety from pathogenic micro-organisms. Thus, every time honey is used for treating wounds or is consumed as food, there is a risk of introducing dangerous micro-

organisms into the body. This is especially important with the conclusive identification of honey as a risk factor in the causation of infant botulism by Clostridium botulinum (Cl. botulinum) (Arnon *et al.*, 1979). Other human pathogens have been isolated from honeys although not from cases of food-borne poisoning. They include Clostridium perfringens (Cl. perfringens), Bacillus cereus (B. cereus) (Kokubo *et al.*, 1984), Lancefield's Group D streptococci and Escherichia coli (Tysset and Durand, 1970).

Honey is extremely hostile to the growth of micro-organisms as a result of some antimicrobial components and properties it possesses including low pH, high osmolarity, low moisture (Gojmerac, 1980), hydrogen peroxide (White *et al*, 1963), lysozyme (Mohrig and Messner, 1968) and 10-hydroxydecenoic acid (Kasolia and Mbithi, 1987). Under these conditions, some of the contaminating micro-organisms die and those which survive would be expected to be injured by the hostile environment which they are exposed to during honey processing and storage. Vegetative bacterial cells, spores, yeasts and moulds are all susceptible to injury by environmental stresses (Adams, 1978; Ray and Adams, 1984). Resuscitation is therefore necessary to enable these injured cells to be correctly enumerated especially on those media which contain selective inhibitory compounds.

Attempts to improve the culture techniques for honey have been made by several workers. Sugiyama *et al.* (1978) described a dialysis technique which lowered sugar concentration and they used it for the detection and enumeration of Cl. botulinum spores from honey using the most probable number technique. It is therefore not useful in colony count procedures because of the difficulty of estimating the amount of sugar lost from the dialysis bags. Hauschild and Hilsheimer

(1983) used membrane filtration in order to concentrate the spores of Cl. botulinum while Midura *et al.* (1979) used dilution and centrifugation to achieve the same. In none of the above methods, however, was the importance of the pH during sample dilution and incubation mentioned. In view of the acidic nature of honey it is therefore possible that the microbial recoveries stated by the above authors may not have been accurate enough.

It is well known that the pH requirement for growth by individual micro-organisms varies greatly but most of them grow optimally at about neutral pH in the range 6.5-7.5 (Jay, 1986). Consequently, better recoveries of total viable counts could be obtained by ensuring that the pH during sample dilution and incubation is about neutral. The pH of an acidic food substance can be raised by use of alkaline diluents or buffers (Straka and Stokes, 1957). According to Jayne-Williams (1963) damaged micro-organisms may be resuscitated when kept in buffer solutions. Phosphate buffers have been found to eliminate the effect of the antimicrobial components of honey (Dakic and Milosev, 1979) although they tend to remove ions from solution and may cause anomalous results (Jayne-Williams, 1963). Based on these observations, it was thought, therefore, that phosphate buffers are better suited than other diluents for improving the recovery of micro-organisms from honey during this study. The purpose of this study was therefore to find out if recovery of micro-organisms from honey could be improved by using phosphate buffer to raise honey pH to about neutral and also to asses the hygienic quality of processed, semi processed and home processed honey.

Objectives

1. To find out if raising honey pH to about neutral improves the recovery of the mixed microflora of semi processed, fully processed, and home processed, honey and to compare the microbiological quality of these groups of honey samples. Before this could be done, the following had to be assessed:-

   a) The pH range encountered in a variety of semi processed and fully processed honey samples from modern refineries and some home processed samples.

   b) The pH and strength of phosphate buffer diluent which would raise the pH of honey to about neutral.

   c) Whether raising honey pH to about neutral improves the recovery of a known number of specific micro-organisms experimentally introduced into sterile honey.

CHAPTER TWO

REVIEW OF LITERATURE

1 Micro-organisms found in honey

1.1 Sources

Most of the naturally occurring micro-organisms in the honey come from the bee, the comb and contaminated inner hive surface. El-leithy and El-sibael (1972) showed that bees are contaminated with micro-organisms on their body surfaces and that similar micro-organisms could be isolated from their internal organs. Korte (1980) showed that fungal spores were transported back to the hive with nectar and pollen while Huhtanen *et al.* (1981) demonstrated that Cl. botulinum spores experimentally fed to bees were all subsequently transfered to the honey formed. The bees' food (nectar, honeydew and honey) is therefore an important source of contamination of honey. Popa *et al*. (1965) recommended that isolation of Mycobacterium tuberculosis (M tuberculosis) from honeys should be attempted because bees are known to frequent unprotected pit latrines, restaurants and hospitals in search of water containing mineral salts and proteinaceous matter.

Commercially manufactured honey combs are increasingly more contaminated with micro-organisms depending on the number of years they have been in use (Smirnov, 1982). Consequently honey obtained from re-usable combs is of poor microbiological quality unless they are disinfected before being re-used. The inner hive surface was found by the same author to be more contaminated in spring (82 micro-organisms/100 cm^2 of hive surface) than summer (60.5 micro-

organisms/100 cm^2 of hive surface).

Sources of contamination during processing include the equipment used for extraction, the containers used for storage and the personel involved in the various operations of production processing and marketing (Tysset and Durand, 1970; Tysset and Rousseau, 1981).

1.2 Numbers and types of micro-organisms found in honey.

The number and types of micro-organisms recovered from honeys have been found to be influenced by the duration of time since harvesting of honey. Honey samples harvested a year before assay should not have gram negative micro-organisms (Tysset and Durand, 1970). Gram negative micro-organisms are therefore indicators of recent contamination. Total viable counts recovered from honey have also been influenced by the type of sample analysed. Vechi and Zambonelli (1969) examined comb honey samples and recovered total viable counts rarely exceeding 100 colony forming units (cfu) per gram of honey. Tysset and Rousseau (1981) examined 176 commercial honeys and found the mean content of bacteria to be 227 per g (range 3-9500), yeasts and fungal mycelia were 90 cfu per g (range 0-1050). Kayihura *et al.* (1989) recovered 10-8700 cfu/g of aerobic bacteria, 0-100 cfu/g of moulds and 10-100 cfu/g of Clostridium species.

1.2.1 Bee pathogens

These are important in that they reduce the bees ability to make honey and consequently lead to less food available to man. They include: Bacillus alvei , Bacillus larvae. Aspergillus flavus, Ascophaera apis , Ascophaera alvei and Nosema apis (Tysset and Durand, 1970) and Streptococcus pluton (Gojmerac, 1980). Nosema apis causes a

reduction in the lifespan of infected worker bees and and a lower brood rearing efficiency of queens while all the other pathogens cause death of bee larvae (Tysset and Durand, 1970; Gojmerac, 1980).

Antibiotics used to treat bee diseases may end up contaminating honey. Tysset and Durand (1969) have demonstrated the presence of 12.5 mg of streptomycine per g of honey and 1.2 mg of tetracycline per gram of honey. The small quantities of antibiotics in the contaminated honey, when taken by consumers, may cause problems of drug resistance.

1.2.2 Fermentation causing micro-organisms

These are important to the apiculturist because they reduce the commercial value of honey. Honey fermentation is caused by osmophilic yeasts and Brevibacterium bacteria (Poncini and Wimmer, 1986). Osmophilic yeasts, the Zygosaccharomyces and Saccharomyces species, are the only micro-organisms capable of multiplying in honey provided the moisture content is not below 17.1% (Lochead, 1933). The numbers of yeasts and corresponding moisture content required to cause fermentation are given in Table 1. Fermentation results into lowering of honey pH.

1.2.3. Human pathogens

Tysset and Durand (1970) report having isolated Group D streptococci and Escherichia coli from honeys. The isolation of these two micro-organisms is indicative of faecal contamination and hence salmonella should be investigated also. Kokubo *et al.* (1984) isolated Bacillus cereus and Clostridial species including Clostridium

perfringens and Clostridium botulinum.

1.2.3.1 Clostridium botulinum

Much of the research carried out in honey microbiology has been devoted to the isolation and enumeration of Cl. botulinum spores. Cl. botulinum is a large, gram positive, anaerobic spore forming bacterium with oval to cylindrical , terminal to subterminal spores (Jay, 1986). The bacterium is classified into seven types (A, B, C D, E, F G) based on on the serological specificity of their toxins (Kautter and Lint, 1984). The toxins cause a neuromuscular disorder of man and animals referred to as botulism.

The disease is highly fatal and it occurs in three forms. The first form is food borne botulism which occurs when preformed toxin is ingested in food. The causative agent of botulism was first isolated in 1895 in Belgium when 3 people died out of 34 who had eaten contaminated salted ham (Dolman and Murakami, 1961). The bacterium isolated from the remnants of the incriminated ham was named 'Bacillus botulinus' and is currently named Cl. botulinum. The second form is known as wound botulism. It occurs when Cl. botulinum cells infect necrotic wounds and produce toxin which is subsequently absorbed into the bloodstream (Merson and Dowell, 1973). It has been experimentally induced in horses and foals (Swerzek, 1979). The third form is called infant botulism. It results from ingestion by infants of botulinum spores which germinate, multiply and produce botulinum toxin which is absorbed into the bloodstream (Arnon *et al* , 1978).

The first case of infant botulism was reported by Midura and Arnon (1976) who also showed honey to be a source of botulinum spores

Table 1 **Moisture content-indicator of honey fermentation**

Moisture content (%)	fermentation
17.1 - 18.0	None
18.1 - 19.0	None if yeast count 1000/g
19.1 - 20.0	None if yeast count 10/g
Above 20.0	Always

Lochhead (1933) Based on 319 honeys samples.

which caused the disease. It was later established that infant botulism affects infants of an upper age limit of one year since their gut is not acidic enough and they do not have sufficient enteric microflora to prevent germination of Cl. botulinum spores (Lawrence, 1986). It was therefore recommended that honey should not be fed to infants less than one year of age (Arnon et al., 1978, Lawrence, 1986).

Since the identification of honey as a risk factor in the causation of infant botulism, numerous incidence surveys for Cl. botulinum spores in honey and other infant foods have been carried out. The incidence of Cl. botulinum spores in honey appears to depend on the geographical location of sampling area. About 10% of U.S honeys were found to be contaminated (Midura *et al* , 1979; Huhtanen *et al*, 1981 and Sugiyama *et al.*, 1978) while some European honeys were found to be free of Cl. botulinum contamination (Hartgen, 1980; Pastoni *et al* , 1986). Nakano *et al.* (1990) found contamination rates of 10% for Japanese honey, 12% for Chinese honey and 20% for Argentine honey. These authors also found 5%, 18% and 23% of commercial, drum, and apiary, samples respectively to be contaminated with Cl. botulinum. Thus the type of sample examined has an influence on the incidence values obtained. Spores of Cl. botulinum have also been isolated from other infant foods. Kautter *et al* . (1982) found 0.5% of 961 corn syrup samples to be contaminated with botulinum spores. Botulinum spores have also been isolated from vacuum cleaner dust and rain water (Murrel *et al* , 1981). Honey is therefore not the only source of botulinum spores to infants and its elimination from infant diets would therefore not eliminate infant botulism. Sakaguchi (1988) reports that only about 25% of 650 cases of infant botulism have been caused by the infant being fed contaminated honey. Other sources of

spores have accounted for the rest 75% but these have not been stated by the author. It may therefore be said that honey is not the major cause of infant botulism. However it is the only infant food which has been incriminated in outbreaks of infant botulism.

The degree of contamination has also been studied with spore concentrations of 5-80 per gram (Midura *et al.*, 1979), 0.28 spores per gram (Sugiyama *et al*, 1978) and 8 per gram (Hauschild *et al*, 1988) being obtained. Studies on the number of spores to which infants are susceptible have been carried out using animal models. Sugiyama and Mills (1978) found 50% of an infant mouse population could be intragastrically infected with 700 botulinum spores of one type of strain A and 300 spores of a different type of strain A culture. It is however not certain that human infants have the same susceptibility to infection as infant mice. Morberg and Sugiyama (1980) identified the rat as another possible animals model in pathogenicity studies of intragastrically fed Cl. botulinum spores. Knowledge of the number of spores which infants are susceptible to would be helpful in setting up microbiological guidelines for honey.

Cl. botulinum occurs in soil (Huss, 1980, and Yamakawa *et al*, 1988) and on a wide range of agricultural products such as fruits and vegetables.It has been shown to occur in the soils of Kenya (Kagiko, 1977 and Yamakawa *et al*, 1989) but there are no records of it having been isolated from Kenyan honey or of the disease infant botulism being diagnosed in Kenya.

There is as yet no known method of destroying Cl. botulinum spores in honey. The botulinum spores are extremely resistant to heat and will survive boiling at 100°C for 3-5 hours (Jawetz *et al*, 1972). Such temperatures which are high enough and for a time long enough to

destroy the spores of Cl. botulinum would caramerize the sugar (Snowdon, 1992) and also destroy honey enzymes thus leading to a product which is commercially unacceptable. However, it has been found that Cl. botulinum spores exist in two interconvertible forms; a heat resistant form referred to as the chemical or calcium-form and a heat sensitive form referred to as the hydrogen form (Alderton *et al*, 1976). The hydrogen form of type A and B spores were found to be 24 times more heat sensitive than their heat resistant chemical forms by the same authors. They made the heat-sensitive spore forms by holding clostridial spores at low pH using 0.1N Hcl at 25°C for 16 hours. These conditions can possibly be simulated by storing honey at room temperature, hence the Cl. botulinum spores present in honey would be expected to be the heat-sensitive forms.which are easier to destroy by heat.

1.3 Culture and recovery methods

1.3.1 Dilution and centrifugation technique (D-C)

This technique was used by Tysset and Durand (1970) to demonstrate total micro-organisms occurring in honey. The sample was diluted in ringers solution and centrifuged. The sediment was resuspended in physiological saline, a drop placed on a microscope slide and gram stained. A wide range of micro-organisms were displayed on microscopic viewing. Pathogenic micro-organisms such as S. aureus, Salmonella and M. tuberculosis were isolated by dilution and centrifugation of the sample coupled with standard techniques for the isolation of each organism. Midura *et al.* (1979) used a dilution and centrifugation technique for the isolation and enumeration of Cl. botulinum spores in honey by the most probable number method. In

their method samples were diluted with distilled water or 1% peptone water. The method was found capable of detecting 7-9 spores per gram honey but Hauschild and Hilsheimer (1983) found the dilution and centrifugation technique capable of detecting only 10% of experimentally added spores. Spore sedimentation rate was high (79%) when the concentration of spores in honey was high (105/ml) but the sedimentation rate was poor when spore concentration was low. A major disadvantage of the DC technique is that it cannot be used for the determination of total viable counts or the counts of particular micro-organisms by the colony count method. .

1.3.2 Dilution and Dialysis (Dialysis Bag) technique (D. D)

It was first used by Sugiyama *et al.* (1978) who diluted twenty five (25) grams of honey with 20 mls of distilled water in dialysis bags and dialysed the honey solution against unsterilized distilled water for 24 hours. The dialysate was cultured onto Trypticase Peptone Glucose Yeast Extract (TPGYE) which had been adjusted to pH 7.2. The procedure was found capable of detecting small numbers of Cl. botulinum spores, though the actual number was not stated. The authors tried various modifications of the basic procedure including collecting the precipitate formed during dialysis on millipore membrane filters (0.45 mm diameter) and culturing the membrane on cooked meat medium. This modification was not found to be superior to the basic dilution and dialysis technique. The Dilution and Dialysis procedure was used with modifications by Kautter *et al.* (1982) and Guilfoyle and Yager (1983) but this modifications were not demonstrated to be more sensitive than the basic procedure.

This Dilution and Dialysis technique has the disadvantage of being

cumbersome and it also cannot be used for the enumeration of the mixed microflora of honeys.

1.3.3 Direct Addition technique (D. A)

Huhtanen *et al* (1981) used this procedure in demonstrating the presence of Cl. botulinum. It involved adding 10 g portions of honey to 90 ml of cooked meat medium or fluid thioglycolate medium. The method has also been used by Hartgen (1980) who cultivated 1g portions of honey in liver broth. Huhtanen *et al.* (1981) obtained higher recoveries of Cl. botulinum spores than by the DC technique. The method has the advantage of simplicity but the ratio of 1:10 honey:culture medium has been found to be inhibitory to the germination or growth of certain micro-organisms (Sugiyama *et al*, 1978; Ialometzeanu *et al*, 1969).

1.3.4 Membrane Filtration Procedure (M-F)

This procedure was developed by Hauschild and Hilscheimer, (1983). They diluted honey with sterile distilled water containing 1% Tween 80, heated the sample at 65°C for 30 minutes and filtered it at this temperature. The membrane filters (MF) HAWP 04700 (0.45 um pore size, 47 mm diameter) were then cultured in Trypticase Peptone Glucose Yeast extract (TPGY) supplemented with beef extract at 10 g/litre. Rigorous efficacy tests were carried out and found it capable of detecting 1.8 spores per gram of honey 67% of the time. The major disadvantage of this M-F technique is the tendency of the M-F to get clogged before filtration is complete. It can also not be used for determining total viable counts because the heating applied would destroy the vegetative cells.

1.3.5 Colony count procedures (C C)

Kayihura *et al* (1989) used the pour plate technique to enumerate aerobic plate counts, moulds and Clostridium species. The honey samples were diluted using physiological saline as diluent and Plate Count Agar (PCA, Oxoid), Potato dextrose agar (PDA, Oxoid) and Sulfite Polymyxin Sulfadiazole agar (SPS; Difco).as culture media. Pastoni *et al.* (1986) diluted their samples with buffered peptone water and used SPS as culture medium for the isolation of Clostridium species. Suspected colonies were tested biochemically to identify the genus and species. They were also tested for pathogenicity by the mouse neutralization bioassay.

Colony count procedures have been the least effective methods for the enumeration of Cl. botulinum spores from honey. Aurreli *et al.* (1983) were unable to demonstrate the presence of botulinum spores in honey samples inoculated with 5 spores/gram by colony count procedures but were successful with dilution and dialysis (Sugiyama *et al* , 1978) and dilution and centrifugation (Midura *et al*, 1979) methods

1.4. Role of Diluent in Recovery of Micro-organisms

1.4.1 Introduction

Some of the diluents used by the authors reviewed here include Ringers solution and physiological saline (Tysset and Durand, 1970), distilled water and 1% peptone water (Midura *et al*, 1979) distilled water containing 1% tween 80 (Hauschild and Hilscheimer, 1983). Choice of diluent was apparently not a major consideration by these workers. Yet it is known that temperature, pH, oxygen tension, salt concentration, nutrient concentration and cell concentration may

influence the survival of bacteria in aqueous suspensions (Hoadley and Cheng, 1974). The nature of the diluent chosen for microbiology is especially important when damaged cells are being enumerated since they tend to die off rapidly (Jayne-Williams, 1963). Jayne-Williams (1963) described a good diluent as one which enables a true assessment of the condition of the bacterial population without death or revival of the organisms during dilution. However, it can be said that a diluent which allows the recovery of injured organisms is desirable following the discovery that freeze-injured salmonellae are just as pathogenic as normal ones (Speck and Cowman, 1971)

1.4.2 Distilled water

Distilled water is a useful diluent for robust and undamaged cells (Jayne-Williams, 1963). However, it has been found to kill various bacteria rapidly and extensively; B. cereus, Bacillus megaterium (B. megaterium) and Serratia marsenscens (S. marsenscens) die rapidly when suspended in it (Winslow and Brooke, 1927). Straka and Stokes (1957) found that sterile distilled water diminished the mixed microflora of some pies by 26-69% within 20 minutes; 41-93% of the bacterial flora were destroyed by the end of one hour. Similar bactericidal action of distilled water has been observed against Streptococcus pyogenes (S. pyogenes) and Salmonella typhimurium (S. typhimurium) but Escherichia coli (E. coli) counts were found to be stable for as long as 2 hours (King and Hurst, 1963). The Coli-aerogenes, Salmonella and Proteus groups can survive in undiminished numbers in distilled water but other bacteria like some aerobic spore formers and pseudomonads die very rapidly in distilled water (Stokes and Osborne 1955; 1956). Thus the use of distilled water

as a diluent may result into erroneous total viable counts depending on the number of sensitive species of bacteria present. The use of double distilled water as a diluent may be preferable to single distilled water as it has been found to be more effective in maintaining the viability of bacteria than single distilled water (Hoadley and Cheng, 1974).

1.4.3 Physiological saline

Physiological saline is bactericidal against B. cereus, B. megaterium and S. mersenscens (Winslow and Brooke, 1927). King and Hurst (1963) found Ringers solution and physiologic saline to cause reductions of 66 and 38% respectively on S. aureus counts when these micro-organisms were suspended in the diluents for 30 minutes. Straka and Stokes (1957) found physiological saline to be equally lethal to micro-organisms as distilled water. Salt solutions will, however, preserve the viability of many species of bacteria (Jayne-Williams,1963).

1.4.4 Peptone water

Peptone water (0.1%) is a valuable diluent which retards the death of many bacteria. Straka and Stokes (1957) found peptone water able to offer adequate protection to bacteria provided it was not brought below pH 6 by the food material. When high acid foods are being assayed for microbial content, it may be necessary to neutralize the food with alkaline phosphates or bicarbonates or to add such neutralizing agents to peptone water.

Although peptone water is the most widely used diluent in food microbiology it may permit limited metabolism and has a low surface

tension which may be harmful to damaged organisms (Jayne-Williams, 1963). The presence of nutrients in the diluent may be of an advantage in resuscitation of damaged organisms but healthy cells may multiply and thus lead to erroneous counts. Postgate and Hunter (1963) observed bacterial multiplication in 0.1% peptone water. Hoadley and Cheng (1974) found little reason to favour use of 0.1% peptone water over double distilled water or phosphate buffer.

1.4.5 Buffers

Buffers may aid revival of damaged cells (Jayne-Williams, 1963) and can be used to neutralize highly acid foods. Phosphates and citrates tend to remove ions from solution and may therefore cause anomalous results. Phosphate buffers have however been found to offer some protection to bacteria against destructive action of distilled water and physiological saline (Buttersfield, 1932), Straka and Stokes (1957) found phosphate buffer of 0.0003 M concentration and pH 7.2 to be better than distilled water but less effective than 0.1% peptone water in maintaining the viability of mixed bacterial populations. Significant bacterial destruction (20-30%) was found to occur within 20 minutes of their suspension in Phosphate buffer.

Some micro-organisms are sensitive to the concentration of buffer used which may account for the high mortalities seen by Straka and Stokes (1957). Gunter (1954) found that micro-organisms varied considerably in the range of buffer concentrations they tolerated and in the buffer concentration which was optimum for survival. The viabilities of Pseudomonas fluorescens (P. fluorescens), Azotobacter agile (A. agile) and Rhodopseudomonas spheroides (R. spheroides)

were maximum in the middle of the concentration range tested (0.001 to 0.5 M) and very low at both ends. Other organisms like E. coli and Saccharomyces cerevisiae (S. cerevisiae) were insensitive to buffer concentration.

1.5 The processing of honey

Honey processing involves the following stages:- uncapping of combs which is carried out after honey combs have been stored at 32-35°C for one day, extraction of honey from the combs and cappings at a maximum temperature of 45°C and straining to separate honey from wax and other coarse particles at 45°C (Townsed, 1975). In Kenya, the Ministry of Livestock development recommends that honey be warmed at 45 - 49°C for 5 - 10 minutes to facilitate straining. The strained honey is either pasteurised and packaged immediately or it is fed into some large storage barrels or drums awaiting pasteurization and packaging at a convenient date.

Pasteurization of honey is aimed at destroying yeasts that cause fermentation, and delaying granulation. Yeasts can be destroyed by heating honey at 63°C, 65°C, 68°C for 35, 25 or 15 minutes respectfully (Nair and Chitre, 1980). Commercially, pasteurization is carried out at 60°C for 30 minutes or by heating honey upto 71°C and cooling it to 38°C in 5-10 minutes (Gojmerac, 1980). For some tropical honey samples with high moisture contents of upto 25%, it is recommended to heat them at 70°C for 10-12 minutes then store the product in sealed containers to prevent recontamination from the air (FAO, 1986). Fully refined and packaged honey has been referred to as commercial honey (Kokubo *et al*, 1984) and as processed honey (Kayihura *et al*, 1989).

The procedure for processing of honey in the homes depends on the

knowledge of individual apiculturists. Traditionally some Kenyan tribes did not strain or pasteurise their honey (Nightingale, 1983). However, better home processing procedures are practised by those who have been trained by the extension staff of the Ministry of Livestock development.

2. Antimicrobial Components in Honey

2.1 Introduction

The antibacterial properties of honey have been known since the early civilizations when it was being used as medicine and as preservative for meat and corpses (Ialomitzeanu *et al.*, 1969). Even today, medicinal values of honey are being rediscovered and honey is therefore being utilized in the treatment of wounds and inflammatory conditions (Nderitu and Nyaga, 1989) and infections of the upper respiratory tract (Mladenov, 1975).

Experimental evidence for honey's bactericidal property has been documented (Van Ketel, 1892). Sackett (1919) showed that certain bacteria perished quickly in heat sterilized honey, diluted honeys being more effective than undiluted. This antibacterial action was attributed to the high osmolarity of honey but this author could not explain the increased bactericidal effect in diluted as compared to undiluted honeys. A discussion of the substances and factors which contribute towards the antimicrobial properties of honeys, as is currently understood, and the problems they pose to the recovery of contaminating micro-organisms is given below.

2.2 Acidity

A number of acids such as acetic, butyric, citric, formic, gluconic,

lactic, maleic, malic, oxalic, pyroglutamic, succinic, phenyl acetic, benzoic, isovaleric, valeric and propionic have been positively identified in honey (Crane, 1980). Others such as 10-hydroxydecenoic, glycollic, α-ketoglutaric, pyruvic, tartaric, 2- or 3- phosphoglyceric, α- or β- glycerophosphate, glucose-6-phosphate, phosphoric, hydrochloric and sulphuric acids are considered to be probably present in honey because although they have been reported as occurring in honey, the identification procedures used have not been rigorous enough (White, 1975). Gluconic acid is the most abundant acid in honey while 10-hydroxydecenoic acid has been shown to have antimicrobial activity against many bacteria and fungi. The effectiveness of most organic acids as antimicrobial agents is least in the pH range 5.5-6.8 (ICMSF, 1980).

The presence of these acids in honey combine to make honey one of the most acidic natural foods. White *et al.* (1962) found U.S. honey to have an average pH of 3.91 (range 3.42-6.10) while Chandler, *et al.* (1974) found a pH range of 3.54-6.32 for some 99 unifloral Australian honeys. Honeydew honeys are reported to have higher pH (> 5) than floral honeys (Dustman, 1979).

Acids exert their antimicrobial activity through their free H^+ ions, the undissociated molecules or by altering the pH of the organism's environment (Willet, 1976). Freese *et al.* (1973) determined the ratio of pH values inside the microbial cell to those outside , using weak lipophilic acids as preservatives, and concluded that it is the rate of proton leakage into the cell relative to the proton rejecting capacity of the cell which determines the extent to which an environment is inhibitory. The entry of hydrogen ions into the microbial cells acidifies their internal environment and interferes with nutrient transport.

Some weak organic acids such as propionic, sorbic, and benzoic acids enter into the microbial cells in the undissociated form and dissociate while within the cells to yield protons which lower the internal cell pH (Hunter and Segel, 1973).

The optimum pH of growth for most bacteria occurs around the neutral region of 6.5-7.5 (Jay, 1986) but they can tolerate a wider range of pH. Most micro-organisms will usually not grow or produce toxin at pH below 4.5 and may therefore be inhibited by the pH of floral honeys which is within the same range of values. Individual micro-organisms, however, show varying degrees of resistance to low pH. Prost *et al.* (1967) reported that pH values below 4.5 had bactericidal effects but did not indicate the acidulant used to adjust the pH. The lower limit of pH which would permit the growth of Salmonella was found to be influenced by the type of acidulant used (Chung and Goepfert, 1970). Tartaric, hydrochloric and citric acids permitted growth at pH values as low as 4.0±0.05. Fumaric, gluconic, glutaric, lactic, malic and succinic acids permitted growth at pH 4.20-4.70 while adipic, pimelic, acetic and propionic could only allow growth at a minimum pH of 5.10-5.50. Staphylococcus aureus (S. aureus) could be grown at minimum pH values of 4.5-4.7 depending on the acidulant used (Tatini, 1973). The above discussion suggests that the extent to which honey is inhibitory to specific micro-organisms is influenced by its organic acid composition.

2.3 Osmolarity

Honey osmolarity is a function of its high sugar and low moisture content. White *et al.* (1962) found U.S. honeys to have an average of 79.6% sugar and 17.2% moisture content. Good quality honey should

not have more than 21% moisture (FAO/WHO,1981) although heather honey (honey derived from plants commonly called heathers or heaths) and some tropical honeys could have as much as 23 and 25% moisture content respectively. FAO (1986) recommends that honey should have a moisture content of less than 18-19% as a safeguard against fermentation.

The high osmolarity of honey contributes significantly to its stability against micro-organisms. Honey has a water activity of 0.70 which is below the 0.80 and 0.91 lower limits required for the growth of spoilage moulds and bacteria respectively (Jay,1986). Pathogenic micro-organisms such as S. aureus and Cl botulinum require a minimum water activity of 0.86 and 0.94 respectively.and can therefore not grow in honey. The maximum concentration of sugar which is not inhibitory to the growth of pathogenic micro-organisms has not been documented. However, Sugiyama *et al.* (1978) found the germination of clostridial spores in honey to be inhibited by osmolarity when 25 g of the honey were inoculated into 300 ml of culture medium, a sugar concentration of approximately 8 %.

Death of micro-organisms due to honey osmolarity was first recognized by Sacket (1919). High osmolarity causes death or inhibition of growth of micro-organisms by causing a net loss of water to their environment (Jay, 1986). It is, however, possible that some of the micro-organisms classified as dead can be resuscitated under favourable cultural conditions.

2.4 Hydrogen peroxide

A major component of honey's antimicrobial property is provided by hydrogen peroxide which is produced by the action of glucose

oxidase enzyme, secreted by bees, on honey glucose (White *et al*, 1963). It had been earlier identified as inhibine and shown to be heat and light sensitive (Dold, 1937). It was found to be more light and heat sensitive at low pH (pH 3) than at about neutral pH (Crane, 1975). Dustman (1979) found that peroxide levels in honeys could be decreased by catalase, vitamin c (ascorbic acid) or other reducing substances in some honeys. The hydrogen peroxide value for any honey sample is governed by the speed of its formation and destruction. Hydrogen peroxide destroys micro-organisms by oxidizing them (Brander, 1982) and thus renders them incapable of carrying out their normal functions.

2.5 Lysozyme

Mohrig and Messner (1968) identified lysozyme as an antibacterial factor in honey and incorrectly thought that it could account for all the antibacterial activity of honey.

2.6 Other antibacterial factors

The existence of some unidentified, non-peroxide antibacterial components in honey is indicated by results of heat sensitivity experiments. Larvie (1960) found the antibacterial factors isolated from honey using ether to be labile when heated at 80°C for 30 minutes. Ialomitzeanu *et al.* (1969) found the antibacterial activity of honey to be stable when heated at 56°C for 30 minutes but labile when heated at 100°C for 30 minutes. Dakic and Milosev, (1979) found antibacterial factors stable at 100°C for 30 minutes but sensitive to phosphate buffers. Molan and Russel (1988) isolated antibacterial substances from New Zealand honeys which were heat stable at 95°C for up to 1 hour. These

antibacterial substances were associated with particular floral sources.

The differences in heat sensitivity of the non-peroxide antibacterial isolates from various honeys and their association with particular floral sources suggests that these factors could be many and variable. The contribution of these factors to the total antibacterial property of honeys has been found to be variable. Dustman (1979) found antibacterial activity in acetone extracts of floral and honeydew honeys which was comparable to 2% of the peroxide activity while Molan and Russel (1988) found non-peroxide antibacterial activity to be greater than the peroxide activity in some samples with high overall activity.

2.7.Minimum microbial inhibitory concentration of honey

The minimum concentration at which honey is inhibitory to micro-organisms has been found to vary with different micro-organisms. Ialomitzeanu (1969) found a concentration of 9% honey in the culture medium to be inhibitory to S. aureus. The other micro-organisms tested included Flexner 3 a and Flexner 3b (Shigella flexneri serotype 3 sub-serotype a, b), b), Coli 126 b_{16} (Escherichia coli) and Coli 127 b_{17} (Escherichia coli). These were only sensitive to a honey concentration of 10% and above. The floral source of honey also affects the lowest concentration at which honey inhibits microbial growth. Mladenov (1961) found lime honeys to be bactericidal even at concentrations of 1/160 (0.6%).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Samples

The honey samples used in this study were either refinery or home processed. Refinery processed samples were either taken from honey storage barrels and referred to as semi refined (semi processed) since pasteurization and packaging had not been carried out. Fully refined and packaged honey was simply referred to as refined or processed honey.

A total of 48 samples were collected of which 4 were home-processed, 23 were semi refined and 21 were refined samples. Home processed samples were collected at the Kariokor open air market, already packed into a variety of bottles. Semi refined samples were collected at Tana River Development Authority (TARDA) and Kitui Honey Products (KHP) refineries. Samples were collected in approximately 300 g quantities into sterile 250 ml sample bottles with caps. Refined samples originated from the two refineries mentioned plus a number of retail shops in Nairobi.

The samples were transported to the laboratory and stored in locked cabinets at room temperature until used.

3.2 Methods

3.2.1 Cleaning and sterilization of glasswares

All the glassware used in the experiments were well cleaned and sterilized. Pipettes and petri dishes were sterilized by the hot air oven

(Memmert) at 160°C for 2 hours while other glassware were sterilized by autoclaving at 121°C (15lb/in^2 pressure) for 15 minutes.

3.2.2 Preparation of diluents and culture media.

a) Distilled water

This was distilled in the department of Public Health, Pharmacology and Toxicology, and had a pH in the range of 5.40-6.00. It was confirmed that the pH of distilled water in other laboratories in the college of Agriculture and Veterinary Sciences was of the same pH range, hence the same was used in preparation of buffers and as a diluent for honey.

b) Phosphate Buffer

Phosphate buffers (0.1M) of pH 6.00, 7.00, 7.10, 7.20, 7.30 and 8.00 were prepared as in appendix 1.1. The pH of the buffers was confirmed using a pH meter (Kent EI 7020) after which they were sterilized.

c) Plate Count Agar (PCA)

Plate Count Agar (oxoid) was rehydrated according to the manufacturers instructions (appendix 1.2). The mixture was heated with constant shaking to dissolve and then sterilized. Sterile media were cooled to about 45° C in a water bath before pouring into petri dishes.

3.2.3 Sterilization of diluents and culture media.

All the diluents and culture media were sterilized by autoclaving at 121°C (15lb/in^2 pressure) for 15 minutes.

3.2.4 Determination of honey pH

a) Undiluted Honey

Each honey sample was well mixed after which approximately 20g of honey were aseptically transferred into a sterile universal bottle. Asepsis was maintained in order to avoid contaminating the remaining sample. Honey pH was then determined using a pH meter (Kent EIL 7020).

b) Diluted honey

One gram (1g) of each honey sample was well mixed with 9mls of distilled water (1:10 w/v) and the final pH determined. The exercise was repeated using each of the 0.1 Molar phosphate buffer solutions of pH 6.00, 7.00, 7.10, 7.20, 7.30 and 8.00 in place of distilled water.

c) Effect of diluted honey on the pH of PCA

PCA was prepared, sterilized and cooled to about 45°C after which its pH was taken. The pH was again taken after adding to 20mls of PCA medium, one ml of 1:10 dilution of honey in distilled water of pH 5.60. Similar determinations were carried out using honey which was diluted in 0.1M phosphate buffer of pH 7.00.

3.2.4 Determination of total viable mesophilic aerobic and anaerobic plate counts

Total viable mesophilic aerobic and anaerobic plate counts were determined for all the 48 samples studied. Each sample was warmed in a water bath at 45-50°C for 30 minutes to facilitate handling, mixed, and two 1g portions weighed into sterile universal bottles. Pipetting of

undiluted honey samples was not attempted because of the problem of high viscosity.

Ten-fold serial dilutions of honey were prepared in distilled water or 0.1M phosphate buffer of pH 7.00. The highest dilution prepared was 10^{-3}. One (1) ml of each dilution was then pipetted into six petri dishes after which approximately 20mls of molten PCA medium was poured into each petri dish. Mixing of the culture medium with the sample was achieved by swirling the petri dishes in a figure of 8 pattern. The agar was then allowed to solidify for about 15 minutes after which the plates were inverted and incubated at 37°C for 48 hours.

Half the plates containing each sample dilution were incubated aerobically and the other half were incubated anaerobically using the Gas-pak system. The effectiveness of the anaerobiosis was ensured by streaking cultures of Cl. perfringens on blood agar and incubating them in the anaerobic jars together with the honey cultures. Growth of Cl. perfringens was taken as evidence of the successful production of anaerobiosis.

Sterile conditions were maintained at all stages of the culture procedure. Success was assessed by pouring the PCA medium into an empty petri dish and into petri dishes containing 1ml of either distilled water or phosphate buffer. Absence of growth after incubation at 37°C for 48 hours was taken to be confirmatory of the sterility of the petri dishes, pipettes, diluents and the culture medium.

After the 48 hour incubation period, plates with countable colonies in the range 25-250 (Busta *et al*, 1984) were chosen for counting. A colony counter was used to aid in the enumeration. Mean aerobic and anaerobic counts per gram of honey were computed according to Busta

et al (1984). The results obtained were evaluated statistically using the paired t-test.

3.2.5 Sterilization of honey by tyndallization.

In order to assess correctly the culture methods and recoverable rates of contaminating micro-organisms in honey, it was decided to sterilize honey, add known numbers of known micro-organisms and assess their recoveries. Sterilization by tyndallization was considered the most suitable method in order to, as much as possible, avoid altering physico-chemical properties of honey.

After the determination of the total viable aerobic and anaerobic counts for all the 48 honey samples, two were randomly chosen and used to assess the possibility of sterilizing contaminated honey by tyndallization. One 20g and two 2g portions of each honey sample were then aseptically weighed into three universal bottles. Eighteen mls of sterile distilled water or 0.1M Phosphate buffer of pH 7.00 were added to either of the two 2g portions and mixed thoroughly to make 1:10 dilutions. The 20g portions were left undiluted.

The loosely capped universal bottles were boiled in a water bath at 95°C for 30 minutes each day for 4 days, a total heating time of 120 minutes. Between consecutive heatings, the samples were incubated at 37°C in an attempt to cause germination of bacterial spores which would then be destroyed during the subsequent period of heating. An alternative regime in which honey samples were heated only once at 95°C for 120 minutes continuosly and then cultured for TVC was carried out so as to assess the effectiveness of tyndallization as compared to continuous heating.

After each heating period, each test portion was cultured in PCA at

37°C for 48 hours.as in section 3.2.4. Those samples which were heated as 1:10 dilutions with distilled water were diluted serially with distilled water and similarly for those heated as 1:10 dilutions with 0.1M phosphate buffer of pH 7.00 were diluted in the phosphate buffer. Those samples heated as undiluted portions were diluted serially with both distilled water and 0.1M phosphate buffer of pH 7.00.as in section 3.2.4. Enumeration of the total viable micro-organisms recoverable from the honey after various periods of heating was done according to Busta *et al* .(1984).

CHAPTER FOUR

RESULTS

4.1 The pH of undiluted honey

The Mean pH of undiluted honey samples is given in Table 2. The average pH for all the honey types (44 samples) was 3.60 with a range of 2.70-4.10. The type of sample, refined, semi refined or home refined, had no observable influence on the pH of honey.

4.2 The effect of distilled water and phosphate buffer diluents on the pH of honey.

The mean pH of 1:10 honey dilutions using distilled water of pH 5.60 and 0.1M phosphate buffers of pH 6.00, 7.00, 7.10, 7.20, 7.30, and 8.00 as diluents are shown in Table 3. The average pH of honey samples diluted in the ratio 1:10 with distilled water was 3.70 which was not significantly higher than the average pH 3.60 of undiluted honey samples. However the 0.1M phosphate buffers of pH 7.00, 7.10, 7.20, and 7.30 raised the pH of all the honey samples to within 0.2 units of pH 7.00 and any of them could therefore be used for diluting honey during culturing. The average pH of honey diluted 1:10 with phosphate buffers of pH 6.0 and 8.0, 5.74 and 7.70 respectively, were outside the neutral range of 6.5-7.5 stated by Jay (1986).

Table 4 shows the pH of 20mls of Plate Count Agar mixed with 1ml of honey diluted 1:10 with distilled water or phosphate buffer. In all cases the pH of the PCA:honey mixture was 7.00

Table 2: The mean and range of the pH of undiluted honey.

Honey type	Number of samples	Mean pH	Range of pH	Standard deviation
Refined	17	3.59	2.70- 4.00	0.333
Semi refined	23	3.61	3.20- 4.00	0.196
Home refined	4	3.63	3.50- 3.80	0.126
All types combined	44	3.60	2.70- 4.00	0.245

Table 3: Mean and range of the pH of 1:10 honey dilutions using various diluents.

Diluent	Number of samples	Mean pH	Range of pH	Standard deviation
Distilled water of pH 5.60	44	3.70	3.20- 4.10	0.184
PB of pH 6.0	44	5.74	5.60- 6.00	0.100
PB of pH 7.0	44	6.86	6.80- 7.00	0.072
PB of pH 7.1	44	6.86	6.80-6.95	0.049
PB of pH 7.2	44	7.05	7.00-7.20	0.05
PB of pH 7.3	44	7.10	7.00-7.20	0.032
PB of pH 8.0	44	7.70	7.60- 7.85	0.062

Key

PB = Phosphate buffer

Table 4: Effect of the pH of honey (diluted in either distilled water of pH 5.60 or 0.1M phosphate buffer of pH 7.00) on the pH of Plate Count Agar (PCA)

Samp. No..	pH of honey diluted 1:10 in distilled water of PH 5.60	pH of a mixture of 20mls of PCA (pH 7.00) with 1ml of a 1:10 honey dilution in distilled water of PH 5.60	pH of honey diluted 1:10 in Phosphate. buffer of pH 7.00	pH of a mixture of 20mls of PCA (pH 7.00) with 1ml of a 1:10 honey dilution in phosphate buffer of pH 7.00
SR_3	3.80	7.00	6.85	7.00
SR_7	3.50	7.00	6.90	7.00
SR_8	3.60	7.00	6.85	7.00
SR_{19}	3.60	7.00	6.85	7.00
SR_{20}	4.00	7.00	6.80	7.00

Key

SR =semi refined

Samp. No. =Sample number

4.2 Recovery of total Viable Count (TVC) in refined, Semi refined and home refined samples using phosphate buffer diluent compared with distilled water.

The mesophilic aerobic and anaerobic total viable counts recovered from refined, semi refined and home refined honey samples using distilled water and 0.1M phosphate buffer of pH 7.00 as diluents are presented in appendices 1.3-1.9 and summarized in Tables 5 and 6. It was also observed that semi refined samples were the most heavily contaminated with home refined samples being the least contaminated..

4.2.1 Total viable counts recovered from refined honey

All the refined samples (100%) were contaminated with aerobic micro-organisms with an average TVC of 1460 (7-1600) and 1230 (3 - 13,000) colony forming units per gram (cfu/g) of honey for phosphate buffer and distilled water respectively. The aerobic TVC recovered using phosphate buffer as diluent were 18.7% greater than those recovered using distilled water ($p = .06$).

Anaerobic micro-organisms could only be recovered from 18 out of 21 (85.7%) honey samples. The mean anaerobic TVC were 160 (20-400) and 110 (3-240) cfu/g of honey in phosphate buffer and distilled water respectively.The count obtained when phosphate buffer was the diluent was 45.3% greater than the count when distilled water was the diluent, a difference which was statistically significant ($p = 0.01$)

4.2.2 TVC recovered from semi refined honey

The average aerobic TVC recovered from semi refined honey was 2200 (60-11,000) and 2200 (73-12000) (cfu/g) of honey for phosphate buffer and distilled water respectively. There was no difference in the

mean TVC.

The mean anaerobic TVC were 660 (17-5400) and 500 (range 17-4700) cfu/g of honey in phosphate buffer and distilled water respectively. The count obtained when phosphate buffer was the diluent was 32% greater than the count when distilled water was the diluent, and this difference was significant (p = 0.0004).

4.2.3 Total viable counts recovered from home refined honey

Aerobic TVC were 770 (100-2200) and 530 (130-1400) cfu/g of honey when the samples were diluted in phosphate buffer and distilled water respectively. Anaerobic counts were 73 (43-90) in distilled water and 71 (35-130) in phosphate buffer. Phosphate buffer did not significantly improve the recovery of either aerobic or anaerobic bacteria from home-refined honey.

4.2.4 TVC recovered from refined, semi refined and home refined honey samples combined

When TVC from all the refined, semi refined and home refined honey samples were considered together, phosphate buffer was found to improve recovery of mesophilic aerobes by 7.3% when compared to distilled water although this difference was not significant (p = 0.06). Recovery of anaerobic bacteria was 32% greater when honey was diluted with phosphate buffer as compared to distilled water. This difference was significant (p = 0.0001).

4.25 **TVC recovered from honey after sterilization by tyndallization.**

The numbers of aerobic and anaerobic survivors after heating are presented as mean colony forming units per gram (cfu/g) of honey in Tables 7 and 8 respectively. Sterilization of undiluted and diluted samples was not achieved by either tyndallization at 95°C for 30

minutes for 4 consecutive days or by heating only once at 95°C for 120 minutes continuously. This exercise was not pursued further. However, it was noted that it was relatively easier to destroy micro-organisms in diluted as compared to undiluted honey.

Table 5: The mean, mesophilic aerobic total viable counts of bacteria recovered from each of the honey sample types using distilled water of pH 5.60 and phosphate buffer of pH 7.00 as diluents

	Refined honey	Semi refined honey	Home refined honey	Refined, Semi refined and Home refined honey
number of samples	21	23	4	48
mean TVC (recovered using distilled water)	1230	2200	530	1640
mean TVC (recovered using phosphate buffer)	1460	2200	770	1760
mean difference in TVC	-230	0	-240	-120
difference as a per cent of TVC recovered using distilled water	18.7	0	45.3	7.3

Key

TVC = total viable count

Table 6: The mean mesophilic anaerobic total viable counts of bacteria recovered from each of the honey sample types using distilled water of pH 5.60 and phosphate buffer of pH 7.00 as diluents

	Refined honey	Semi refined honey	Home refined honey	Refined, semi refined and home refined honey
number of samples	18	23	4	45
mean TVC (using distilled water)	110	500	73	310
mean TVC (using phosphate buffer)	160	660	71	400
mean difference in TVC	-50	-160	2	-90
difference as a per cent of TVC recovered using distilled water	-45.4	-32.0	2.7	-29.0

Key

TVC = total viable count

Table 7: The mesophilic aerobic TVC per gram of honey recovered after samples were boiled at 95°C for 30 minutes every day for 4 days or at 95°C for 120 minutes continuosly. Incubation was done in PCA at 37°C for 48 hours.

Sample type and number	Day-0	Day-1	Day-2	Day-3	Day-4	honey heated continuosly for 120 minutes
R_{4A}	550	220	80	30	20	10
R_{4B}	480	170	95	10	15	25
R_{4C}	550	<10	5	<10	<10	<10
R_{4D}	480	5	15	<10	<10	<10
SR_{3A}	11000	1200	420	130	50	100
SR_{3B}	12000	970	470	100	50	130
SR_{3C}	11000	25	10	5	<10	<10
SR_{3D}	12000	<10	5	0	<10	<10

Key

SR = Semi refined

R = Refined

A = Honey sample heated while undiluted and later diluted with phosphate buffer during culturing.

B =Honey sample heated while undiluted and later diluted.with distilled water during culturing.

C = Honey sample heated as a 1:10 dilution with distilled water

D = Honey sample heated as a 1:10 dilution with phosphate buffer

Table 8: The mesophilic anaerobic total viable count per gram of honey recovered after honey was boiled at 95°C for 30 minutes every day for 4 days or at 95°C for 120 minutes continuosly.

Sample type & number	Day-0	Day-1	Day-2	Day-3	Day-4	honey heated continuosly for 120 minutes
$R_{4\ A}$	200	40	5	<10	<10	5
$R_{4\ B}$	110	40	5	<10	<10	10
$R_{4\ C}$	200	<10	<10	<10	<10	<10
$R_{4\ D}$	110	<10	<10	<10	<10	<10
$SR_{3\ A}$	960	240	100	30	5	10
$SR_{3\ B}$	680	210	100	15	10	40
$SR_{3\ C}$	960	40	5	5	<10	<10
$SR_{3\ D}$	680	20	5	10	<10	<10

Key

TVC = Total viable count

SR = Semi refined

R = Refined

A = Honey sample heated while undiluted and diluted with phosphate buffer during culture.

B = Honey sample heated as undiluted and diluted.with distilled water during culturing.

C = Honey sample heated as a 1:10 dilution with distilled water

D = Honey smple heated as a 1:10 dilution with phosphate buffer

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

The average pH of 3.60 (range 2.70-4.0) obtained for all the refined, semi refined and home refined honey samples (Table 2) was low when compared to the average pH (3.9) of 2 fresh honey samples from both Kitui and Baringo (Kasolia, Personal communication). The average pH was also lower and the pH range narrower than the 3.91 (range 3.42-6.10) observed by White *et al.* (1962) for 490 U.S. honeys. The pH of honey decreases with storage (White, 1975) which explains why the average pH of the fresh honey samples examined by these workers was higher than that observed in this study. Although identification of the honey samples with respect to their plant source was not carried out, the low pH obtained suggests that they were predominantly floral as opposed to honeydew in origin (Dustman, 1979).

On the basis of pH, therefore, the honey samples examined in this study would be judged stable against bacteria but not against yeasts which can grow at pH as low as 2 (Jay, 1986) if other growth parameters such as temperature, nutrient and moisture content are optimum. On site inspection of semi refined stored samples during sampling revealed that 17.4 per cent (4/23) were turbid and producing gas indicating that fermentation had already began. Fermentation may therefore be responsible for the low pH observed in some of the samples.

Distilled water is widely used as a diluent in food microbiology and is recommended together with phosphate buffer and 0.1% peptone

water for use in colony count procedures (Busta *et al*, 1984). In the microbiology of honey, distilled water has been used as a diluent especially in culture procedures designed to isolate and / or enumerate Cl. botulinum organisms by demonstrating the presence of the toxin in enrichment cultures (Sugiyama *et al*, 1978; Midura *et al*, 1979; Huhtanen *et al*, 1981). When distilled water was used to dilute the honey samples in this study (Table 3) the average pH (3.70) was hardly different from that of undiluted honey (3.60) and was well below pH 4.5 which was found to be lethal to micro-organisms (Prost *et al*, 1967). Straka and Stokes (1957) found that significant bacterial destruction occurred if the pH of sample : diluent mixture was allowed to fall below pH 6 and, on this basis therefore, distilled water was found to be unsuitable as a diluent for honey. Phosphate buffers of 0.1 molarity and pH 7.0-7.3 succeeded in raising the pH of honey to about neutral. Either of them would therefore eliminate the antibacterial effect of pH during sample dilution. The buffer of pH 7.00 was chosen and compared with distilled water on the ability to recover micro-organisms from honey.

Inspite of the considerable difference between the pH of honey diluted 1:10 in distilled water with that diluted in phosphate buffer, the pH of PCA mixed with diluted honey was about neutral irrespective of the pH of the diluted honey (Table 4). This is indicative of the the strong buffering capacity of PCA and may also be due to the high degree of dilution of honey upon addition of 20 ml of the culture medium to 1 ml of the 1:10 dilution (1:200). It can therefore be concluded that the antimicrobial effect of honey pH is likely to adversely affect microbial recovery only during the sample dilution step and not during incubation when PCA is the culture medium.

The adverse effect of any diluent on micro-organisms would depend

on the time between sample dilution and mixing with the culture medium. In this study, a time of about 30 minutes was found practical since it was necessary to plate portions of each sample diluted with both distilled water and phosphate buffer at the same time. It was, however, ensured that the exposure time in either diluent was the same. Straka and Stokes (1957) conceded that as much as 30-60 minutes can expire if a large number of samples is being plated but Busta *et al.* (1984) suggested that this time should not exceed 20 minutes.

The generally higher mesophilic aerobic TVC recovered when phosphate buffer was the diluent (Table 5). and especially the 18.7% improvement in the recovery of mesophilic aerobic TVC from refined honey is consistent with the finding by Straka and Stokes (1957) that phosphate buffer facilitates the survival of the mixed microflora of Pies better than distilled water. However the observed improvement in the recovery of aerobic micro-organisms from semi refined honey (0.01%) and from all the honey types combined (7.3%) may be due to analytical error as colony count procedures have an associated analytical error of 10% (Straka and Stokes, 1957).

More mesophilic anaerobic micro-organisms can be recovered using phosphate buffer as a diluent than distilled water (Table 6). No report on the suitability of distilled water or phosphate buffer in the recovery of anaerobic micro-organisms suspended in them was encountered for comparison. However, it appears advisable to use phosphate buffer as compared to distilled water because of the higher, statistically significant recoveries obtained from refined (45.4%), semi refined (32%) and all the honey types combined (29.0%).

Phosphate buffer, as compared to distilled water, improved the

recovery of anaerobic micro-organisms more than aerobic ones. This is indicative of the presence of a higher number of anaerobic than aerobic micro-organisms which were sensitive to destruction by distilled water but stable in phosphate buffer. Variations in the sensitivities of individual micro-organisms to destruction by distilled water have been documented (Winslow and Brooke, 1927; Gunter,1953; Stokes and Osborne, 1955, 1956). In this study, however, time constraints prevented the isolation of individual species of aerobic and anaerobic micro-organisms to verify this observation.

Siliker (1963) pointed out that total counts effectively evaluate the sanitary quality of foods that do not support microbial growth. In such foods, total counts are indicative of the type of sanitary control exercised in their production, transport and storage. The use of total counts to evaluate the sanitary quality of honey has not been sufficiently documented although bacteria do not multiply in honey. The inability of bacteria to multiply in honey may explain the generally low numbers of recoverable contaminating micro-organisms and especially those from refined honey which are, however, comparable to those recovered by Kayihura *et al.* (1989). These workers recovered 10-8700 aerobic bacteria per gram of honey but may have achieved higher recoveries had they diluted their honey samples in 0.1M phosphate buffer of pH 7.00 instead of physiological saline which is just as destructive to micro-organisms as distilled water (Straka and Stokes, 1957). Kayihura *et al.* (1989) also found the incidence of micro-organisms in honey samples to be 24 out of 26 (92.3%) which compares with 23 out of 23 (100%) incidence obtained in this study. The low TVC recovered in this study may also be partly due to the preheating of samples at 45-50°C for 30 minutes which may have killed or injured

some of the vegetative microbial cells.

Semi refined honey samples were about 2 and 4 times contaminated with aerobic and anaerobic micro-organisms respectively than the refined samples. This implies that a higher risk is taken when human infants are fed on semi refined honey than refined honey. Elliot and Michener (1961) carried out a review on the microbiological standards and handling codes for chilled and frozen foods and found out that some authors believed there was a relationship between total viable counts and food safety while others believed there was no relationship. Attempts to relate total viable counts with presence of pathogens in honey have not been documented but it is safer to adopt the view of those authors who relate high total viable counts with danger due to pathogens.

The refined honey samples were less contaminated with micro-organisms than semi refined samples possibly because of the extra heating applied in the final stage of processing. The heating regime 60°C for 30 minutes is meant to retard granulation of honey and to destroy yeasts (Gojmerac, 1980). According to Willet (1976), a similar heating regime (60°C for 30 minutes) is sufficient to destroy all vegetative bacterial cells except S. aureus and Strept. faecalis although the heating medium is not specified. Further depletion in microbial numbers of refined honey occurs due to the prolonged exposure to the antimicrobial components of honey during storage. Gram negative micro-organisms perish faster than gram positives and should not be present in honey which has been stored for more than one year (Tysset and Durand, 1970). Some of the refined samples studied were one to two years old but the duration of storage for some could not be ascertained. Home refined samples were the least contaminated of the

three honey types indicating that, contrary to expectations, sanitary conditions in the honey refineries may have been poorer than in the small-scale apiculturists' homes. However the number of samples examined (4) was small.

The destruction of bacterial populations by heat is a gradual process with exponential death kinetics (Willet, 1976). Sterilization time is directly related to the number of initial micro-organisms which explains why a higher residual contamination of 50cfu/g was observed for the undiluted honey which was initially more contaminated than for the sample which was less contaminated (20cfu/g). It was also observed that dilution of honey facilitated a faster destruction of contaminant micro-organisms. Resistance of micro-organisms to heat increases with the addition of sugars to the suspending medium (Jay,1986) . Destruction of micro-organisms by moist heat is postulated to occur through protein denaturation which is faster at higher water activities.

The failure of tyndallization as a method of sterilizing honey could be due to the inability of micro-organisms to germinate in it. Lawrence (1986) stated that Cl. botulinum could not be forced to grow in honey and the same could be true for other micro-organisms except the osmophilic ones. As honey is progressively heated there is a gradual reduction in its water activity thus making it even harder for spores to germinate in it. Tyndallization relies on germination of micro-organisms thus lowering their heat resistance and facilitating their destruction in subsequent heatings (Willet, 1976).

Due to the failure to sterilize undiluted honey by tyndallization, an attempt was made to find an alternative method of sterilization which would be suitable for honey. A search was made in Nairobi for

institutions offering facilities for sterilization of honey by irradiation but this was also unsuccessful. The idea of introducing known numbers of specific micro-organisms in sterile honey and assessing the relative recovery rates using distilled water and 0.1M phosphate buffer of pH 7.00 as diluents was therefore abandoned.

CONCLUSIONS

1) Kenyan honey is highly acidic with a mean pH of 3.60 but it can be raised to about neutral (7.0 ± 0.5) when culturing contaminanting micro-organisms in honey using 0.1M phosphate buffers of pH 7.00, 7.10, 7.20 and 7.30. It is suggested that this should always be done especially with the finding in this study that when well buffered microbiological culture media like PCA are used, low pH is likely to cause errors in the microbial numbers recovered only during the dilution step.

2) Phosphate buffer (0.1M) of pH 7.0 was demonstrated to be a better diluent than distilled water of pH 5.60 for determining the mesophilic TVC and especially the anaerobic TVC in honey. The buffer improved the recovery of anaerobic micro-organisms by 45.4 %, 32.0% and 29.0% from refined, semi refined and all the honey types combined, respectively. This finding may improve colony count procedures for the isolation of Cl. botulinum spores in honey.

3) Semi-refined samples were nearly twice as contaminated as refined samples suggesting the importance of encouraging consumers to use fully processed honey.

4) The microbial quality of honey was improved by boiling at 95°C for various durations but sterilization of honey was not achieved whether by tyndallization or by continuous heating.

CHAPTER SIX

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Appendices

1.1 Preparation of phosphate buffer

Stock solutions

A: 0.2 M solution of mono basic sodium phosphate (27.6 g in 1000 ml)

B: 0.2 M solution of dibasic sodium phosphate (35.6 g of Na_2HPO_4 $2H_2O$ in 1000 ml) 53.65 g of $Na_2HPO_4.7H_2O$ - 71.7 g of Na_2HPO_4 12 H_2O

x ml of A + y ml of B, diluted to a total of 200 ml.with distilled water

x(A)	y(B)	pH	x(A)	y(B)	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	90.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

1.2 **Preparation of plate count Agar**

	g/l
Yeast extract (Oxoid L21)	2.5
Tryptone(Oxoid L42)	5.0
Dextrose	1.0
Agar No. 1 (Oxoid L11)	9.0
pH	7.0 (approximately).
Distilled water	1000 mililitres

17.5g of the medium was suspended in 1 litre of distilled water. This was dissolved by bringing to boil with constant stirring and mixing.It was then sterilized in a pressure cooker at 121°C for 15 minutes and cooled to 45-48°C at which temperature it was ready for pouring into petri plates.

Appendix 1.3: The pH values for undiluted honey and that of 1:10 dilutions using distilled water and 0.1M phosphate buffers of various pH.

Samp. type & No.	pH of undil honey	pH of honey diluted with						
		distilled water of	Phosphate buffer (0.1M) of					
		pH 5.60	pH 6.00	pH 7.00	pH 7.10	pH 7.20	pH 7.30	pH 8.00
R_1	4.00	4.20	5.90	7.00	6.85	7.10	7.10	7.70
R_2	3.20	3.60	6.00	7.00	6.90	7.10	7.10	7.70
R_3	3.00	3.20	5.90	7.00	6.80	7.10	7.10	7.80
R_4	3.50	3.80	5.70	6.90	6.90	7.05	7.10	7.70
R_5	2.70	3.20	5.90	6.90	6.85	7.10	7.10	7.60
R_6	3.70	3.80	5.80	6.85	6.90	7.05	7.10	7.60
R_7	3.90	3.80	5.70	7.00	6.80	7.05	7.10	7.80
R_8	3.60	3.70	5.70	6.80	6.80	7.10	7.10	7.70
R_9	3.70	3.70	5.70	6.90	6.90	7.20	7.20	7.70
R_{10}	3.60	3.80	5.70	6.80	6.80	7.00	7.10	7.70
R_{11}	3.70	3.80	5.70	6.85	6.80	7.00	7.10	7.70
R_{12}	3.70	3.70	5.70	6.90	6.90	7.10	7.10	7.70
R_{13}	3.80	3.80	5.70	6.90	6.90	7.05	7.10	7.70
R_{14}	3.70	3.70	5.70	6.80	6.95	7.05	7.10	7.70
R_{15}	3.60	3.60	5.70	6.90	6.90	7.05	7.05	7.80
R_{16}	3.70	3.80	5.80	6.80	6.85	7.05	7.10	7.70
R_{17}	3.90	3.90	5.70	6.80	6.85	7.00	7.10	7.80
SR_1	3.40	3.50	5.80	7.00	6.80	7.10	7.10	7.70
SR_2	3.20	3.30	5.60	6.80	6.90	7.00	7.10	7.70
SR_3	3.90	3.80	5.80	6.85	6.90	7.00	7.10	7.60
SR_4	3.80	3.80	5.70	6.80	6.85	7.00	7.10	7.70
SR_5	3.30	3.40	5.70	6.80	6.80	7.05	7.10	7.60
SR_6	3.70	3.70	5.70	6.90	6.80	7.05	7.10	7.70
SR_7	3.40	3.50	5.80	6.90	6.90	7.10	7.10	7.70
SR_8	3.60	3.60	5.75	6.85	6.80	7.00	7.05	7.70
SR_9	3.70	3.90	5.70	6.85	6.80	7.00	7.10	7.65
SR_{10}	3.50	3.70	5.60	6.80	6.90	7.00	7.05	7.70

SR_{11}	3.80	3.80	5.70	6.80	6.85	7.05	7.10	7.65
SR_{12}	3.60	3.70	5.70	6.85	6.80	7.00	7.10	7.70
SR_{13}	3.60	3.70	5.70	6.85	6.90	7.05	7.10	7.80
SR_{14}	3.40	3.50	5.60	6.80	6.80	7.05	7.05	7.70
SR_{15}	3.70	3.70	5.60	6.80	6.80	7.00	7.05	7.65
SR_{16}	3.70	3.80	5.70	6.80	6.90	7.00	7.10	7.70
SR_{17}	3.50	3.60	5.70	6.80	6.90	7.05	7.10	7.85
SR_{18}	3.60	3.60	5.70	6.85	6.90	7.05	7.10	7.70
SR_{19}	3.50	3.70	5.70	6.90	6.90	7.10	7.05	7.70
SR_{20}	4.00	3.90	5.70	6.80	6.80	7.05	7.10	7.70
SR_{21}	3.80	4.10	5.60	6.80	6.90	7.00	7.00	7.70
SR_{22}	3.80	3.90	5.80	6.85	6.90	7.10	7.10	7.60
SR_{23}	3.60	3.80	5.70	6.80	6.90	7.05	7.10	7.70
HR_1	3.80	3.80	5.90	7.00	6.80	7.10	7.10	7.80
HR_2	3.50	3.50	5.90	7.00	6.90	7.20	7.20	7.80
HR_3	3.60	3.60	5.90	6.80	6.80	7.00	7.10	7.60
HR_4	3.60	3.60	6.00	7.00	6.90	7.10	7.10	7.70
mean	3.60	3.70	5.74	6.86	6.86	7.05	7.10	7.70
min.	2.70	3.20	5.60	6.80	6.80	7.00	7.00	7.60
max.	4.00	4.10	6.00	7.00	6.95	7.20	7.20	7.85
sd	0.245	0.184	0.10	0.072	0.049	0.05	0.032	0.062

Key

po_4	=phosphate
undil	=undiluted
samp No.	=sample number
sd	=standard deviation
R	=refined
SR	=semi refined
HR	=home refined
Max.	=maximum
min	=minimum

Appendix 1.4: The mesophilic aerobic total viable counts recovered from refined honey samples using distilled water of pH 5.60 and 0.1M phosphate buffer of pH 7.00 as diluents

Sample number	TVC_1 (using distilled water)	TVC_2 (using Phosphate buffer)	difference (TVC_1-TVC_2)	difference as a per cent of TVC_1
R1	320	360	-40	-12.5
R2	6.7	13	-6.3	-30
R3	3.3	30	-26.7	-200
R4	480	550	-70	-14.5
R5	45	110	-65	-144
R6	800	660	-140	15
R7	630	930	-300	-47.6
R8	1600	2000	-400	-25
R9	13000	16000	-3000	-23.1
R10	2400	2500	-100	-4.2
R11	540	980	-440	-81.5
R12	700	780	-80	-11.4
R13	730	1200	-470	-64.4
R14	2200	1900	300	13.6
R15	570	500	70	12.3
R_{16}	480	490	-10	-2
R17	690	1100	-410	-59.4
R18	490	440	50	10.2
R19	13	7	6	46.1
R20	17	27	-10	-58.8
R21	30	37	-7	-23.3
Mean	1226.0	1457.8	-231.9	18.9
maximum.	13000	16000		
minimum.	3.3	7		
sd	2779.9	3405.0	665.2	

Key

R = refined

sd = standard deviation

TVC = total viable count

Appendix 1.5: The mesophilic anaerobic total viable counts recovered from refined honey samples using distilled water of pH 5.60 and 0.1M phosphate buffer of pH 7.00 as diluents

Sample number	TVC_1 (using distilled water)	TVC_2 (using Phosphate buffer)	difference (TVC_1-TVC_2)	difference as a per cent of TVC_1
R1	210	77	133	63.3
R2	17	<10	-	_
R3	<10	45	-	_
R4	110	200	-90	-81.8
R5	3	20	-17	-566.7
R6	75	200	-125	-166.7
R7	77	140	-63	-81.8
R8	230	240	-10	-4.3
R9	170	390	-220	-129.4
R10	120	190	-70	-58.3
R11	130	130	0	0
R12	150	240	-90	-60
R13	240	400	-160	-66.7
R14	110	120	-10	-9.1
R15	110	110	0	0
R16	35	110	-75	-214.2
R17	75	130	-55	-73.3
R18	67	67	0	0
R19	<10	<10	-	-
R20	23	20	3	13.3
R21	20	17	3	15
mean	108.6	155.6	-47.0	43.3
maximum.	240	400		
minimum.	3	20		
sd	70.8	111.4	77.4	

Key

R = refined

sd- = standard deviation

TVC = total viable count

Appendix 1.6: The mesophilic aerobic total viable counts recovered from semi refined honey samples using distilled water of pH 5.60 and 0.1M phosphate buffer of pH 7.00 as diluents

Sample number	TVC_1 (using distilled water)	TVC_2 (Using Phosphate buffer)	difference (TVC_1-TVC_2)	difference as a per cent of TVC_1
SR1	73	93	-20	-27.4
SR2	110	60	50	45.5
SR_3	12000	11000	1000	8.3
SR_4	510	540	30	-5.9
SR_5	170	130	40	23.5
SR_6	5300	5000	300	5.7
SR_7	90	110	-20	-22.2
SR_8	4500	5600	-1100	-24.4
SR_9	3100	2500	600	19.4
SR_{10}	1130	970	160	14.2
SR_{11}	9700	9600	100	1.0
SR_{12}	560	520	40	7.1
SR_{13}	430	370	60	14.0
SR_{14}	780	720	60	7.7
SR_{15}	810	730	80	9.9
SR_{16}	2500	2800	-300	-1.2
SR_{17}	740	880	-140	-18.9
SR_{18}	2500	2500	0	0
SR_{19}	450	360	90	20
SR_{20}	2300	2800	-500	-21.7
SR_{21}	480	590	-110	-22.9
SR_{22}	660	670	-10	-1.5
SR_{23}	1700	2100	-400	-23.5
mean	2199.7	2201.9	-2.2	0.01
maximum.	12000	11000		
minimum.	73	60		
sd	3091.1	2977.5	384.7	

Key

sd = standard deviation, SR = semi refined,

TVC = total viable count

Appendix 1.7: The mesophilic anaerobic total viable counts recovered from semi refined honey samples using distilled water of pH 5.60 and 0.1M phosphate buffer of pH 7.00 as diluents

Sample number.	TVC_1 (using distilled water)	TVC_2 (Using Phosphate buffer)	difference (TVC_1-TVC_2)	difference as a per cent of TVC_1
SR_1	50	25	25	50
SR_2	17	17	0	0
SR_3	680	960	-280	-41.2
SR_4	300	370	-70	-23.3
SR_5	90	130	-40	-44.4
SR_6	720	940	-220	-30.6
SR_7	250	360	-110	-44.0
SR_8	580	1200	-620	-106.9
SR_9	37	35	2	5.4
SR_{10}	600	840	-240	-40.0
SR_{11}	4700	5400	-700	-14.9
SR_{12}	50	100	-50	-100
SR_{13}	70	110	-40	-57.1
SR_{14}	57	90	-33	-57.9
SR_{15}	170	180	-10	-5.9
SR_{16}	260	390	-130	-50
SR_{17}	75	170	-95	-126.7
SR_{18}	480	820	-340	-70.8
SR_{19}	140	120	20	14.3
SR_{20}	1100	1400	-300	-27.3
SR_{21}	110	330	-220	200
SR_{22}	410	400	10	2.4
SR_{23}	560	700	-140	-25
mean	500.3	656.0	-155.7	-31.1
maximum.	4700	5400		-200
minimum.	17	17		
sd	959.2	1110.9	193.7	

Key

sd = standard deviation, SR= semi refined

TVC = total viable count

Appendix 1.8: The mesophilic aerobic total viable counts recovered from home refined honey samples using distilled of pH 5.60 water and 0.1M phosphate buffer of pH 7.00 as diluents.

Sample No.	TVC_1 (using distilled water)	TVC_2 (Using Phosphate buffer)	Difference (TVC_1-TVC_2)	difference as a per cent of TVC_1
HR_1	360	490	-130	-36.1
HR_2	240	300	-60	-25
HR_3	130	100	30	23.1
HR_4	1400	2200	-800	-57.1
mean	532.5	772.5	-240	-45.1
maximum	1400	2200	30	23.1
minimum	130	100	-800	-57.1
sd	585.9	964.9	379.0	

Key

sd = standard deviation

HR = home refined

TVC = total viable count

Appendix 1.9: The mesophilic anaerobic total viable counts recovered from home refined honey samples using distilled water of pH 5.60 and 0.1M phosphate buffer of pH 7.00 as diluents

Sample number.	TVC_1 (using distilled water)	TVC_2 (Using Phosphate buffer)	difference (TVC_1-TVC_2)	difference as a per cent of TVC_1
HR_1	77	130	-53	-68.8
HR_2	80	50	30	37.5
HR_3	43	35	8	18.6
HR_4	90	67	23	29.9
mean	72.5	70.5	2	2.8
maximum.	90	130		
minimum	43	35		
sd	20.8	418	37.8	

Key

sd = standard deviation

HR = home refined

TVC = total viable count