

**Antibacterial effect of lemon juice-extract on bacteria isolated from  
Traditional African sausages (*Mutura*) sold in Nairobi County and  
pathogenicity of one isolate**

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This thesis is my original work and has never been presented for a degree in any other University.

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## **DEDICATION**

*I dedicate this work to my parent, my wife-Leah and my daughters-Maryanne and Esther*

## **ACKNOWLEDGEMENT**

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## LIST OF ACRONYMS AND ABBREVIATIONS

AM	-	Ante meridiem
ANOVA	-	Analysis of Variance
BKC	-	Benzalkonium chloride
BUN	-	Blood Urea Nitrogen
CFU	-	Colony Forming Units
CM	-	Centimetres
CPE	-	Cytopathic Effect
EDTA	-	Ethylene Diamine Tetra acetic acid
FDA	-	Food and Drug Administration
GIT	-	Gastro intestinal Tract
GIT	-	Gastro-Intestinal Tract
Hb	-	Haemoglobin
HC	-	Hemorrhagic colitis
HEPA	-	high-efficiency particulate air
HRS	-	Hours
HUS	-	Hemolytic Uremic Syndrome
KEMRI	-	Kenya Medical Research Institute
KPHC	-	Kenya population and Housing Census
KSH	-	Kenya Shillings
LAB	-	Lactic Acid Bacteria
LB	-	Luria Bertani
MCH	-	Mean Corpuscular Haemoglobin
MCHC	-	Mean Corpuscular Haemoglobin Concentration

MCV	-	Mean Corpuscular Volume
MIC	-	Minimum Inhibitory Concentration
ML	-	Millilitres
Mm	-	Millimetres
PBS	-	Phosphate Buffer Saline
PCV	-	Packed cell volume
PM	-	Post meridiem
RDW	-	Red Cell Distribution Width
Sp.	-	Species
SPSS	-	Statistical Package for the Social Sciences
TBC	-	Total Bacteria Concentration
TLC	-	Total Leucocyte Count
UK	-	United Kingdom
uL	-	Microliter
VTEC	-	Vero Cytotoxigenic <i>Escherichia coli</i>
v/v	-	Volume per Volume
WHO	-	World Health Organization

## ABSTRACT

Demand for African sausages has resulted in this delicacy's vendors being on the increase. However, risk of humans to food-borne bacterial disease from these sausages arising from intestinal bacteria or external contamination as a result of unhygienic food preparation is largely unknown. In addition, lemons which are common food additives are known to possess antibacterial activity, but data on their efficacy on pathogens isolated from African sausages remains scanty. The objectives of this study were: to isolate bacteria from African sausages sold in Nairobi County, to quantify the bacterial load, to determine susceptibility of the three most prevalent bacterial isolates to lemon juice extract and to determine pathogenicity of one of the bacteria using a mouse model. A descriptive study design was employed where one hundred (100) non-roasted and roasted African sausages were conveniently collected from three meat eatery points of Westlands, Kangemi slum and Pangani estates. Total viable bacterial counts were calculated through preparation of ten-fold dilutions of aseptically homogenised African sausages. The lemon juice extract was tested for antibacterial activity using minimum inhibitory concentration and agar diffusion methods. Thirty Balb /C mice were inoculated intraperitoneally with  $4.36 \times 10^4$  –  $4.36 \times 10^8$  of *Escherichia coli* (*E.coli*) isolated from the sausages and pathogenicity monitored through expression of clinical disease and clinico-anatomical pathology. Five genera of bacteria namely; *Staphylococcus* sp., *Bacillus* sp., *Streptococcus* sp., *Proteus* sp. and *E. coli* were isolated from 78 African sausage samples. Their prevalences were 50.4%, 19.5%, 9.8%, 2.4% and 1.6 %, respectively. There was no significant difference in distribution of isolates between different sites  $p \geq 0.05$ . The bioload range was between  $1.0 - 9.9 \times 10^1$  -  $1.0 - 9.9 \times 10^7$  cfu/g with 37/100 (37%) samples having bioload of between  $1.0 - 9.9 \times 10^4$  to  $1.0 - 9.9 \times 10^7$  cfu/g.

The *lemon* juice extract had antibacterial activity on all three bacterial isolates tested. Undiluted lemon juice extract had the highest activity at 20mm for *Staphylococcus*, 18mm for *Bacillus* and 20mm for *Streptococcus*. All mice inoculated with test organism survived to end of experiment with no expression of clinical disease. Clinical pathology revealed no significant variation ( $p>0.05$ ) between infected and non-infected animals. The peritoneum was white, smooth and shiny. All other organs retained normal colour, size and shape. Organs examined were normal except for accumulation of blood in the venous side of circulation and prominence of lymphoid follicles in the spleen, Peyer's patches and peri-bronchial lymphoid areas. This study has demonstrated presence of bacteria which are potentially zoonotic to humans in the roasted and non-roasted African sausages. *E.coli* load of between  $10^6$ - $10^8$  is potentially infectious to sausage consumers although similar dosage proved non-pathogenic to mice, experimentally. Diluted lemon extract juice has antibacterial activity at 25%. There was no significance difference ( $p\geq 0.05$ ) in distribution of isolates and bacterial load across geographical sites studies among the roasted and non-roasted African sausages. Comprehensive study is needed to sample more eatery meat points in Nairobi and other areas in order to isolate, establish the bacterial load and the pathogenicity of isolated organisms to humans. There is also need to establish the sources of bacteria due to high bacterial load determined in the current study. Further studies are needed to establish the mode of action of lemon juice extract whether bacteriostatic or bactericidal and how heat stable it is to determine if it can be incorporated as one of the ingredients in preparation of African sausages.



## **CHAPTER 1: INTRODUCTION**

Traditional African sausages, popularly known as Kenyan sausages locally known as 'Mutura' in Kikuyu dialect is a local delicacy for low, middle income earners and beer drinkers. It is a protein rich meat snack comprising goat or cow cleaned intestines stuffed with cooked small pieces of meat and formed into long coils; sometimes blood is added. The preparation involves cutting the intestines into a length of about 150-1200cm. One end of the intestinal segment is tied using a sisal rope. Small pieces of meat, and sometimes mixed with same or other animal's blood is stashed into the intestines from untied end. When full, the other end is also tied. Processed African sausages are then placed in boiling water or soup for 30 to 40 minutes and then roasted over coals on outdoor grills using low to medium heat and turning frequently to dehydrate the meat and give it the sensational smoky taste. The internal temperature should be at least 160 degrees Fahrenheit (Wiens, 2011).

African sausages roasts are common cuisine among low to middle income earners not to forget beer drinkers, as they can be cut into different sizes, each pegged on a different price - from as little as Ksh. 5/- for a 5-10 cm size depending on the locality (Ojode, 2001). African sausage provides a ready-to-eat meat which Kenyans of all walks of life have at one time or another had a pleasure of enjoying it; whether on one's way from school, work (at a road side butchery) or during festivities that include meat roasting; however, this is dependent on one's background and preference. Musa and Akande (2002) noted that the increase in demand for these finger-licking "sausages" has resulted in this delicacy's vendors being on the increase, especially in slum areas. The responsibility for good sanitary measures and proper food handling is left at the hands of these vendors, majority of whom are semi-literate.

According to Yousuf *et al.* (2008), meat is considered a high-risk perishable commodity in respect to micro-organism content, natural toxins and other possible contaminants. Food borne illnesses are a major world-wide health risk with subsequent economic impact which prompted World Health Organization (WHO) to develop a global strategy for food safety (Adak *et al.*, 2005). Food borne infections result from ingestion of microorganisms and toxins present in food. According to Torok *et al.* (1997), food-borne disease from African sausages arises from intestinal bacteria or external contamination, which is a result of unhygienic food preparation - if the vendor fails to adhere to hygiene practices during processing, preparation, handling and/or storage.

Some of the unhygienic practices noted to contribute to food borne illness arise from failure of the vendors to maintain cleanliness. This involves dirty or improper dressing, chopping boards/surfaces and knives that are not regularly cleaned, handling sausages and money without proper hand-washing practices, poor storage of unsold sausages such as failure to refrigerate or storage in dirty polythene bags (Torok *et al.* 1997). Many diseases are caused by eating food that has been contaminated with an infectious agent, usually from faeces. If these diseases lead to diarrhoea or vomiting they are easily spread further if sanitation provision such as toilets is poor or lacking. This could be due to the handlers' ignorance and/or carelessness. Meat and blood contain enough nutrients needed for the growth of microorganisms. Among the bacterial isolates from animal products documented in recent studies are; *Staphylococcus aureus*, *Streptococcus sp.*, *Escherichia coli*, *Clostridium perfringens*, *Shigella sp.*, *Salmonella sp.* and *Campylobacter jejuni* (Kazuaki *et al.*, 1999).

Globally, World Health Organisation (2007) estimates approximately 1.5 billion episodes of diarrhoea and over 3 million deaths recorded annually results from consumption of microbial agents in food. This has posed a very serious health threat to humans, especially to meat lovers. Unfortunately, various chemical agents and additives that can be used to preserve African sausages, through prevention of microbial growth, are considered to be potentially harmful to human health (Sharma, 2015). Thus, this study investigated the possible use of lemon juice-extract, a biological preservative/antibacterial compound.

The main reason for using lemon juice-extract is because lemons are relatively inexpensive due to their easy availability, universal acceptability, palatability and their being well tolerated by most people; lemon juice-extract is therefore, regarded as being safe. The antibacterial efficacy of lemon juice-extract was tested against the isolated bacteria from fresh African sausages. This study was necessary since there has been a public health concern over production and sale of these African sausages especially if cooking and roasting is not sufficient and hence the need for natural antimicrobials (Mastromatteo *et al.*, 2011).

### **1.1 Justification**

Unhygienic practices in food preparation and consumption of undercooked meat and meat products has often been associated with many human infections (Sang *et al.*, 1996). Although reported food borne outbreaks by pathogenic bacteria in Africa have been few to date, available information indicates that these pathogens have wide geographic distribution. Meat-borne microbial hazards vary in different geographical and socio-economic settings (Codex Alimentarius Commission, 2005).

Despite numerous researches on pathogens isolated from meat products, knowledge on bacterial isolates, the antibacterial effect of lemon juice-extract on the bacterial contaminants from African sausages sold at food outlets in Nairobi County and their pathogenicity is limited. There are many sources of food contamination. Contamination of food by food-borne microbes found in healthy intestines of animals can occur during preparation. Meat carcasses can be contaminated during slaughter or during cleaning with contaminated water. Kenya's food industry is regulated by public health and safety regulations such as public health act, food, drugs and chemical substances acts (Kenya Law, 2015) which enforce policies on food hygiene and sanitation. Concerns have been raised about food safety due to numerous occurrences of food-borne disease outbreaks. African sausage business, which is part of food production practice, remains largely unregulated while the said policies are poorly enforced. The increased informal trade of African sausages, mostly operating in unhygienic makeshift and road side meat points of Nairobi poses a potential serious health risk to unsuspecting consumers through outbreaks of food borne illnesses.

The general environmental and personal hygiene of African sausage sellers in this area is poor. The vendors are just beside the road and major streets which exposes the African sausages to dust and other environmental contaminants. Their dressing (white coat) is dirty with no provisions for themselves or clients to hand wash before consuming the African sausage. The sellers rely on commercial water vendors for their source of water which exposes the would-be buyers to more infection since the source of water is not well established. The presence of these organisms in ready to eat African sausages is a pointer that these African sausages were either processed under poor hygienic and sanitary conditions or could have been from the animal intestines.

Results of this study will help the relevant regulatory body in laying down food safety measures for the African sausages; lemon-extract with antimicrobial activity being exploited as both a nutritional agent and a potent preservative.

### **1.3 Hypothesis**

African sausages sold in Nairobi County contain zoonotic, pathogenic bacteria, which are sensitive to lemon juice-extract.

### **1.4 Objectives**

#### **1.4.1 Broad Objective**

To determine the effect of lemon juice-extract on bacteria isolated from African sausages sold in Nairobi County and pathogenicity of one of the isolates.

#### **1.4.2 Specific Objectives**

- i) To isolate bacteria from African sausages sold in Nairobi County.
- ii) To quantify the bacterial load of the African sausages
- iii) To determine susceptibility of the three most prevalent bacterial isolates to lemon juice extract.
- iv) To determine pathogenicity of one of the bacterial isolates using a mouse model

## CHAPTER 2: LITERATURE REVIEW

### 2.1 Overview of bacteria associated with fresh and stored sausages

Bacterial growth and lipid oxidation in meat products are the main causes of food becoming unacceptable for consumption. There are different factors that affect the quality of meat and meat products, which include temperature, water activity, pH and microbial composition (Romans *et al.*, 2001). All these factors, therefore, have an influence on the spoilage potential of food. Spoilage of food involves a complex process and excessive amounts of food are lost due to microbial spoilage. This results in high economic losses and may even pose health hazards (Yang & Li, 2006). High water content and nutrients have rendered fresh pork sausages highly perishable since they serve as substrates for microorganisms (Cocolin, 2004). Spoilage micro-organisms in meat leads to the development of bad odor and off-flavours, oxidative rancidity, discoloration, gas production and, often, slime formation. *Escherichia coli* and *Staphylococcus aureus* are commonly isolated from meat since they occur as normal flora in animals and humans; therefore, their presence in such sausages indicates mishandling or improper cooking.

Some enzymes and toxins produced by these organisms are heat stable and render foods dangerous even after cooking or roasting (Torok *et al.*, 1997). Lactobacilli in meat products are considered a major micro-organism found on various types of packaged or stored cooked beef sausages. Great diversity of lactic acid bacteria can be found in different types of spoiled cooked beef sausages (Von *et al.*, 1991). Among the bacteria isolated in animal products documented in recent studies are; *Staphylococcus aureus*, *Streptococcus* sp., *Escherichia coli*, *Campylobacter jejuni*, *Clostridium perfringens*, *Shigella* sp., *Salmonella* sp. (Oluwafemi and Simisaye, 2006).

Risk of infection or disease arising from consuming micro-organisms found in raw meat is significantly higher than cooked meat. Meat can be incorrectly or insufficiently cooked, allowing disease-carrying pathogens to be ingested (Newell *et al.*, 2010). Contamination may arise during meat processing or from slicing of ready meat product or from cross-contamination during refrigeration. Meat can be contaminated during the production process at any time, from the slicing of prepared meats to cross-contamination of food in a refrigerator. All of these situations lead to a greater risk of disease.

## **2.2 Commonly isolated bacteria in animal products**

### **2.2.1 *Escherichia coli***

According to Cheesbrough (2006), *E.coli* is a Gram negative, motile, aerobic and facultative anaerobic rods. These fecal coliforms are considered indicators of fecal contamination from warm blooded animals (Yousef *et al.*, 2008). Some strains of this organism are pathogenic and can cause serious food poisoning in humans. *Escherichia coli* is particularly abundant in gastro intestinal tract (GIT) especially bowels or intestines of mammals and birds. Some strains are normal flora while others, such as *E. coli* 0157:H7, are pathogenic. Shiga toxins produced by these strains are heat stable and render foods poisonous even after cooking and roasting because they cause hemorrhagic colitis (Enabulele and Uraih 2009). Bebora *et al.* (2005) isolated enterotoxigenic *E. coli* from food handlers working in selected tourist-class hotels in Nairobi.

### **2.2.2 *Staphylococcus***

According to Kloos and Schleifer (1986), *Staphylococci* Sp. are found to be normal flora on mucous membrane of warm-blood animals, in foodstuffs such as meat and meat products and from environments source such as soil, dust, air or natural water.

The large numbers of these organisms tend to be found in the nasal passages, axillae and perineal areas (Kloos and Bennerman, 1994). Staphylococci are able to multiply readily in many foods including meat and dairy products. Contamination of food products by *Staphylococcus* species may occur during the phase of manufacturing and handling of final products (Rosec *et al.*, 1997; Letertre *et al.*, 2003). Presence of competing micro-organisms constrains staphylococcal growth. *Staphylococci* sp. grows well in a less competitive environment with other organisms such as in food products with high concentration of salt or sugar that impede the growth of other organisms. According to Prescott *et al.* (2005), the presence of *Staphylococcus aureus* could be attributed to raw ingredients such as raw tomato sauce and raw sliced onion among others, dirty utensils and environment in which the food is prepared. Enterotoxigenic strains of *Staphylococcus aureus* are known to cause serious foodborne illness.

### **2.2.3 *Proteus***

*Proteus spp.* in the meat product samples usually detected due to unhygienic food processing. Al-Mutairi (2011) reported presence of *Proteus* sp. from raw meat and its products in studies in Egypt. A study by Gwida *et al.* (2014) reported a 78% and 58% *Proteus* isolates from raw chicken meat and raw beef meat samples, respectively. Unhygienic practices in meat and meat products processing and handling is associated with presence of *Escherichia coli*, *Klebsiella* sp., *proteus* sp. and *Enterobacter* sp. leading food-borne infection (Rajashekara *et al.*, 2009). The source of water, status of the environment, failure to hand wash and dirty utensils are major hazards associated with roasted meat.



### **2.2.5 *Bacillus***

*Bacillus* species are gram positive bacterial rods. Certain *Bacillus sp.* are common food contaminants. They contaminate raw food and food materials, particularly foods in contact with soil. Spores of some certain species are heat stable and can subsequently re-germinate and grow under favourable conditions, particularly those in warm kitchens. Consumption of meat or meat products with certain species of *Bacillus* organisms causes gastrointestinal illness, especially due to pre-formed toxin or by toxins produced by these bacteria in the gut (Cheesbrough, 2006). According to Götz (2002), various food stuff that may contain *Bacillus subtilis* includes; meat, vegetable pastry products, poultry products and occasionally bakery products, including bread, crumpets, sandwiches and ethnic meat or seafood dishes.

### **2.2.6 Other pathogens associated with meat and meat products**

*Salmonella, Shigella, Campylobacter, faecal streptococci* and *Listeria monocytogenes* are also major bacteria associated with foodborne illness (Le-Loir *et al.*, 2003). However, no data is available, with respect to presence of these microorganisms in African Sausages.

### **2.3 Source of contamination**

According to Borch and Arinder (2002), presence of microorganisms in meat and intestines of carcasses used for preparation of African sausages is due to contamination occurring immediately before, during and after slaughter. The microbial contamination of carcasses occurs mainly at abattoir and retailer establishments during skinning and processing (Gill, 1998; Abdalla *et al.* 2009). The main sources of meat contamination include: animal / carcasses source, on farm factors, transport factors, abattoir and butcher facilities, parasites and wild animals, meat vans, abattoir and retail meat outlet workers.

Meat contamination in abattoirs and retail meat outlets result from the use of contaminated water, unhygienic practices like poor handling, use of contaminated tables to display meat intended for sale and the use of contaminated knives and other equipments in cutting operations (Fasanmi *et al.*, 2010). Similar finding by Adzitey *et al.* (2011) reported that cutting knives, intestinal contents, chopping boards, hides, meat handlers, containers, vehicle for transporting carcasses and the meat selling environment are possible sources of contaminations. Notably handling the food without washing hands after handling currency has been demonstrated to carry *E.coli* 0157:H7 and *Salmonella enteritidis* (Kuria *et al.*, 2009).

#### **2.4 Bacterial loads observed on beef sausages**

Oluwafemi and Simisaye (2006) researched on contamination on and in beef sausages and reported mean total viable counts of between  $1.3-1.47 \times 10^6$  colony forming units per gram (cfu/g) for *Staphylococcus aureus*, between  $2.13-2.33 \times 10^6$  cfu/g for lactic acid bacteria (LAB) count and between  $1.7-2.17 \times 10^6$  cfu/g for Enterobacteriaceae. According to Ukut *et al.* (2010), the mean microbial load on fresh meat from Watt and Marian markets were between  $2.62-4.84 \times 10^4$  and  $2.24 - 5.01 \times 10^4$  cfu/g, respectively, while total coliform counts were between  $1.05-3.72 \times 10^3$  and  $1.23-3.42 \times 10^3$  cfu/g, respectively. With respect to the general principles of meat hygiene according to Codex Alimentarius Standards (2005). The government, local authorizes and the general public are tasked with ensuring that meat and meat products are safe and suitable for human consumption. Hygiene practices in meat processing and preparation for live animal production up to the point of retail sale need to be observed (Codex Alimentarius Standards, 2005).

#### **2.5 Antimicrobial activity testing**

Antimicrobial susceptibility testing is commonly applied for epidemiological studies, drug discovery and/or prediction of therapeutic outcome.

The *in vitro* test has been used successfully to study effects of antibiotics (Cheesbrough, 2006). This study focussed on effect of lemon juice-extract on three bacterial types isolated from African sausages, which has not been done in Kenya.

### **2.5.1 Agar disk-diffusion method**

Microbiologists routinely uses agar disk-diffusion testing method developed in 1940 for routine antimicrobial testing work. Clinical and Laboratory Standards Institute (CLSI) has published approved standards for bacteria testing (CLSI, 2012). Agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6 mm in diameter), containing the testing components at specific concentrations, are placed on the agar surface (Cheesbrough, 2006). The culture plates containing testing organisms and antimicrobial components are incubated under suitable conditions for the particular microorganism. Generally, antimicrobial component diffuses through the agar plate and inhibits the growth of the test organism and then the diameters of inhibition growth zones are measured (Cheesbrough 2006).

### **2.5.2 Agar well-diffusion method**

Agar well-diffusion method aids in evaluating antimicrobial activity of plant extracts (Valgas *et al.*, 2007). The culture plate surface is inoculated with the test organisms, a well with a diameter of 6 to 8 mm is punched aseptically with a sterile agar puncture and a volume (20–100 $\mu$ L) of the antimicrobial plant extract solution at specific concentration is introduced into the well. Then, agar plates are then incubated under suitable conditions. The antimicrobial plant extract diffuses through the agar medium and inhibits the growth of the microbial strain tested. The growth of the test organism and then the diameters of inhibition growth zones are measured (Valgas *et al.*, 2007).

### **2.5.3 Agar plug-diffusion method**

According to Elleuch *et al.* (2010), this technique involves streaking agar with organism of interest, after an overnight growth, certain molecules produced by the organism diffuse into the agar. An agar plug is removed with help of an agar puncture. The cut Agar plug is put on another culture plate inoculated with test micro-organism. The molecules diffuse from the plug to the second culture plate. The diameter of inhibition zone around the agar plug indicates antimicrobial activity.

### **2.5.4 Agar contact method**

According to Marston (2014), antimicrobial component is transferred from a thin layer chromatogram to culture plate inoculated with test micro-organism. Diffusion takes place after few hours. The culture place is then incubated and the diameter of inhibition zone around the area of contact with the chromatogram indicates antimicrobial activity.

### **2.5.5 Dilution methods**

According to Pfaller *et al.*, (2004) and Valgas *et al.* (2007), this technique presents one of the most common techniques for MIC determination either in agar or broth medium. The method for performing broth technique involves dilution preparation of antimicrobial component in a liquid media in tubes. All tubes are adjusted according to 0.5 McFarland scale. Endpoint will be determined by the highest dilution that inhibits microbial growth after incubation.

### **2.5.6 Time-kill test (time-kill curve)**

This is an effective method for antimicrobial activity determination. It shows the interaction between a microbial agent and antimicrobial component. According to Pfaller *et al.* (2004), microbial suspension and broth culture media in 3 tubes are needed in performing test. The antibacterial effect is tested in relation to time of kill.

## **2.6 Bacterial inhibitory effect of natural products including lemon**

Lemon (*Citrus limon*) is plant with antimicrobial effect belonging to family *Rutaceae*. It is grown mainly for food, that is, its fruit is used in beverages and cooking. Its alkaloids, citric acids and other compounds have anticancer and antibacterial potential. It is also used as a preservative due to its anti-oxidant properties (Conte *et al.*, 2009). According to Kawaii *et al.* (2000), all parts of the plant such as leaves, stem, fruit, roots and flower are useful; they have demonstrated activity against various microbial agents. Study done by Maruti *et al.* (2011) showed that the peel of lemon is a good antimicrobial agent against specific bacteria such as *Pseudomonas* and *Micrococcus*. The respective study involved incubation of different concentrations of crude solvent extracts of the lemon peel (1:20, 1:40, 1:60, 1:80 and 1:100), prepared as discs, with different microbial cultures for 24 hrs at 37°C. The diameter of zones of inhibition are measured, recorded and interpreted.

## **2.7 Testing for pathogenicity of bacterial isolates**

Animal models and In-Vitro epithelial culture tissue culture have been exploited extensively in the study of human enteric pathogens and their effect on their host. Virulence study have made use of several in *in vivo* models such as mice, rabbits, rats and even humans among other models. According to Vallance *et al.* (2004), inoculation of pathogens through various routes in animal models requires care consideration and expertise to lower the effect arising from the procedure. The nature of effect anticipated from inoculation of suspected pathogenic agent determines the route of inoculation effect (Vallance *et al.*, 2004). Parenteral route offers the best method of administration since GIT and hepatic degradation of the microbial agent is bypassed. The oral administration offers easy, convenient and safe route though bioavailability is much slower compared with other methods due to degradation and metabolic effect of hepatic and GIT effects (FDA, 2010).

According to Ngo and Maibach (2010), intramuscular and intra-dermal administration offers another route of though absorption of injected component is affected by variety of factors among them; integrity of skin surface, size of skin thickness among others. Intraperitoneal route is among common methods of administration of substances in small animals (Turner *et al.*, 2011). According to Turner *et al.* (2011), absorption of microbial components through intravenous route is faster than intraperitoneal route. Intranasal method of administration is also exploited for delivery of sprays (Illum, 2002). Nasal mucosal and the lungs are highly vascularized thereby maximizing absorption of administered substances (Illum, 2002). Necropsy, haematological and biochemical processes are essential in monitoring and evaluating pathogenicity (Porter *et al.*, 2012). In a similar study, Firoz *et al.* (2010), after intravenous inoculation, isolated *Salmonella enterica* serovar Typhi (BKC 3233) from the liver, spleen, blood and bone marrow samples collected at the end of the experiment on day 9 post-infection.

## **CHAPTER 3: MATERIALS AND METHODS**

### **3.1 Study design**

A descriptive study design was employed whereby a convenience sampling of retail meat outlets from Westlands, Pangani and Kangemi was carried out. Non-roasted and roasted African sausage samples were collected and homogenates of inner contents prepared using sterile peptone water were used to determine total bacterial concentration (TBC) and identification of the isolates. Three most prevalent bacterial isolates were then tested for susceptibility to lemon juice-extract, while one of them was further tested for pathogenicity using mouse experimental model.

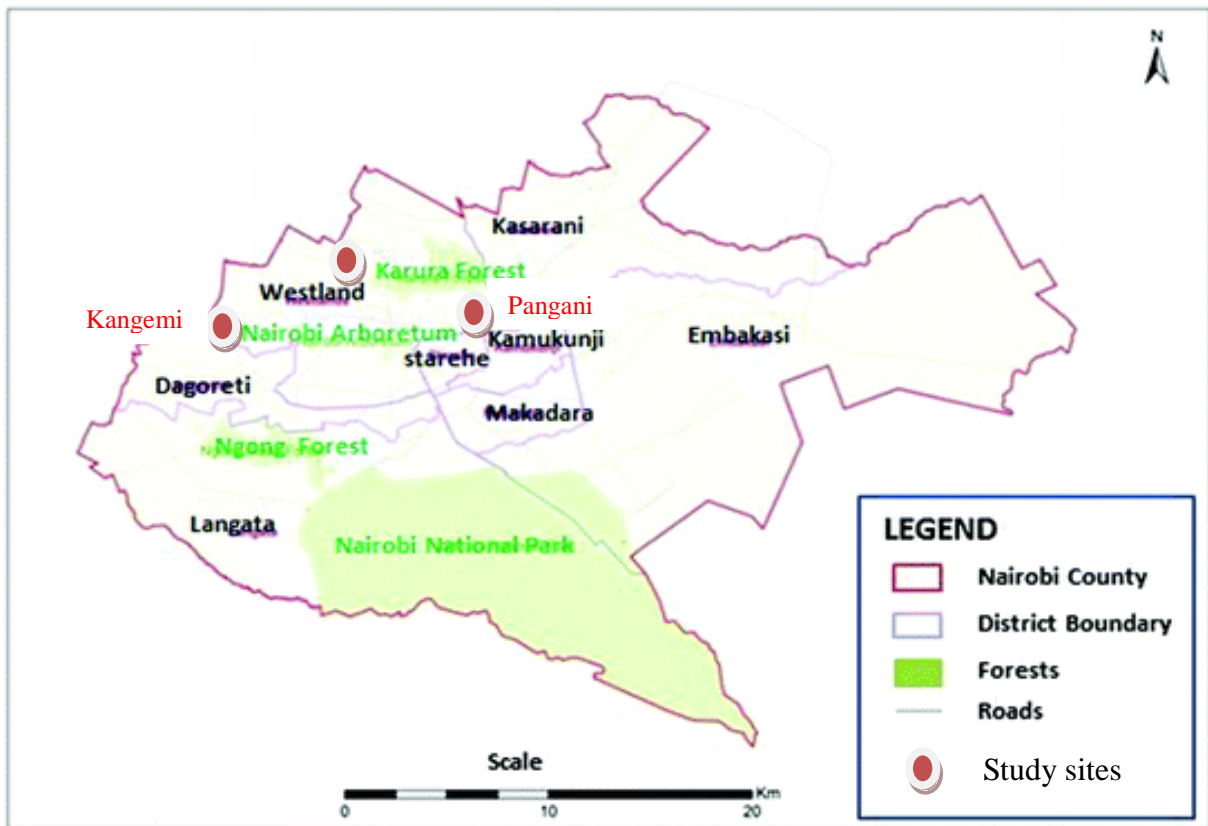
### **3.2 Study area**

The study was carried out in Nairobi County, Kenya (Figures 1 and 2). It is the smallest, yet most populous county; the capital and largest city of Kenya, which has experienced one of the most rapid growths in urban centres with a population of 3,375,000 as at year 2009 census (Kenya population and Housing Census, 2009). It has a total area of 696 km<sup>2</sup> with 17 parliamentary constituencies. Nairobi is a cosmopolitan and a multicultural city. Economically, it can be subdivided into three main categories; (1) the high end or leafy suburbs or upper-class estates, the likes of Muthaiga, Karen, Westlands among others, (2) the Middle-class estates of Pangani, Buruburu among others and (3) low class estates of Mukuru, Mathare, Kangemi slums among others (Nairobi Mitaa, 2011). Three ready-to-eat vending sites and meat eatery points of Westlands market, Kangemi market and Pangani estate (Figure 2) were conveniently selected on the basis of easy access, the limited budget that the researcher was operating on, perceived sanitation and relative hygiene levels. The number of vendors in these areas is not known; but they tend to converge around the shopping areas.



**Figure 1:** Map of Kenya, showing position of Nairobi County (in red). (Source: [nairobimetro.go.ke](http://nairobimetro.go.ke))





**Figure 2:** Map of Nairobi County, indicating the three study sites of Pangani, Westlands and Kangemi. (Source: nairobimetro.go.ke)

### 3.3 Sample size calculation

Prevalence of common meat contaminants in previous studies was used to determine the sample size required to detect the presence of the bacteria. An expected prevalence rate of 7% was used to estimate the sample size in this study since similar studies (Miyoko, *et al.*, 2004; Magwira *et al.*, 2005; Weese *et al.*, 2009; Kabwanga *et al.*, 2013) reported a prevalence rate of between 3 - 14%. Using the above information, the sample size was calculated using the formula given by Fisher *et al.*, (1998), as follows:

$$n = \frac{z^2 p(1-p)}{d^2}$$

Where:

n = sample size

Z = Standard normal value for 95% (1.96)

p = Estimated prevalence set at 7%

q= 1 – p

d= precision level of the study set at 5% ( $\pm 0.05$ ).

The calculated sample size (n) was 100 samples.

### 3.4 Sample collection and handling

A total of hundred (100) African sausage (63 roasted and 37 non-roasted) samples were conveniently acquired from three locations (33 samples from Westland market, 33 samples from Pangani and 34 samples from Kangemi shopping center). The samples were obtained aseptically from the vendors, picked separately as they are sold using sterile glass bottles, labelled and stored in a cool box before processing in the laboratory within 24 hours of collection. Samples were transported to the Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi, Kabete where they were homogenized in readiness for bacteriological analysis which was carried-out within 2 hours of collection.

Figure 3 shows the how the African sausage is roasted and served.



**Figure 3:** The African sausage – roasting and serving.

### **3.4.1 Homogenate preparation**

At the laboratory, one-gram portions of the African sausages (roasted and non-roasted) were obtained aseptically and cut into small pieces on a sterile chopping board using a sterile knife, and blended (homogenized) in 4ml of 0.1% peptone water to obtain 1:5 initial dilution. (*Health Protection Agency, 2005*).

### **3.5 Bacterial isolation**

Since the researcher suspected presence of coliforms and other fastidious organisms, the homogenates of the African sausages were streaked on general purpose enriched medium (blood agar) and selective and differential medium for members of family Enterobacteriaceae (MacConkey agar) (Oxoid Ltd. Termo Scientific, UK) and incubated aerobically at 37°C for 24 hours. The isolated bacteria were identified based on colony morphology, Gram staining reaction, and biochemical characteristics using established standardized methods according to Bergey's Manual of determinative bacteriology (Holt *et al.*, 1994).

### **3.6 Quantification of bacterial load of the African sausages**

For the determination of bacterial load (total bacterial count), method given by Miles and Misra (1938) was used. Serial dilutions of  $10^{-1}$  to  $10^{-10}$  were prepared from the African sausage homogenate stock solution that was prepared earlier. Nutrient agar plate was divided into four quadrants, and each quadrant served as one plate. Using a 25ul calibrated dropper (equivalent to  $1/40^{\text{th}}$  of an ml), one drop from each dilution tube was placed per quadrant; each dilution was done in quadruplicate. The drop was then allowed to dry and the plate incubated aerobically at 37°C for 24 hours (Jersek, 2017), after which the number of colonies that grew per drop was counted using Quebec Dark Field colony counter taking the average count for the quadruplicate drops of each dilution.

The concentration of the original bacterial suspension was then calculated and expressed as colony forming units per millilitre (cfu/ml), using the formula,  $a \times 40 \times 10^y$ , where  $a$  is the average number of colonies in the 4 drops of one dilution tube/diluted suspension, 40 is the number of drops that make one millilitre (the drop being equivalent to  $1.40^{\text{th}}$  of a ml), and  $10^y$  is the dilution factor of the respective dilution tube/diluted suspension. This is then multiplied by 5, the initial dilution at homogenization stage

### **3.7 Susceptibility of the three most prevalent isolates to lemon juice-extract**

#### **3.7.1 Preparation of lemon juice extract**

Ten (10) large, fresh lemons (*Citrus Limon*) were purchased from a local supermarket. Within 2 hours of purchase, lemon juice-extract was prepared in readiness for carrying out of respective bacterial susceptibility testing. The lemons were cleaned with sterile water and surface-sterilized by immersing them into 70% (v/v) ethanol for one minute. Fifty (50) grams of lemon was sliced and homogenized aseptically using mortar and pestle. The homogenized mixture was filtered through sterile cheesecloth. The lemon juice-extract was considered as the 100% concentration. The juice-extract was used while fresh; however, in case of some delay, it was maintained at 4<sup>0</sup>C. The concentrated juice-extract was then diluted with sterile distilled water to give other concentrations (75%, 50% and 25%) for the assay.

#### **3.7.2 Lemon susceptibility testing**

Minimum inhibitory concentration (MIC) using broth inhibitory testing and Agar well diffusion methods were used to test for bacterial lemon susceptibility. They were carried-out on each of the three most prevalent bacterial isolates separately and in triplicate, using varying concentrations of the lemon juice-extract prepared in section 3.7.1 above.

### **3.7.2.1 Preparation of bacterial inocula.**

*Escherichia coli* was chosen for pathogenicity studies since some strains of this organism are pathogenic and can cause serious food poisoning in humans. Inocula for antibacterial activity screening were prepared from 24 hour culture of each of the test isolate, suspended in physiological saline to match a turbidity of 0.5 McFarland *Barium sulfate* standards (Valgas *et al.*, 2007). The harvest was then stored in sterile bijou bottles and used as the stock suspension of organisms.

### **3.7.2.2 Screening for antibacterial activity**

Broth inhibitory testing was done using the method of Valgas *et al.* (2007), One millilitre of each of the prepared concentrations of the juice-extract i.e. 25%, 50%, 75% and 100% was separately mixed with 1 ml of nutrient broth in a test tube. A loopful of the test organism was then introduced to each of the tubes; a tube containing nutrient broth only, seeded with the test organism, served as control. All the tubes were then incubated at 37°C for 24 hrs and then examined for growth by observing turbidity (Ajaiyeoba *et al.*, 2003). If the organisms were susceptible to the lemon juice-extract, there would be no bacterial growth in the particular broth/test tube (CLSI, 2012)

Agar diffusion method was carried-out using the method of Valgas *et al.* (2007). Streaking of bacteria from the stock culture prepared in 3.7.2.1 above was done on Mueller Hinton agar (Oxoid Ltd. Thermo Scientific, UK) so as to produce confluent growth; care was taken to have prepared agar of the same thickness. Wells (6 mm diameter and depth of 4 mm) were then dug on the streaked agar using sterile agar puncture. Respective concentrations of the lemon juice extract were poured into the wells; the plates were then incubated at 37°C for 24 hours. The diameter of clear region around an antimicrobial agent on the agar surface (inhibitory zone) was measured in millimeters. All tests were done in triplicate.

### **3.8 Determination of pathogenicity of one of the bacterial isolates using mouse model**

*Escherichia coli*: some strains of this organism are pathogenic and can cause serious food poisoning in humans (Cheesbrough, 2006).

#### **3.8.1 Experimental animals**

Thirty (30) Balb /C mice aged three weeks, equal numbers of males and females were used for pathogenicity testing. The mice were obtained from Kenya Medical Research Institute (KEMRI), Nairobi, Kenya. They were transported in small numbers in shoe-box cages measuring 18x25cm and housed in groups of 5 in polypropylene plastic base cages, with lids made of straight stainless-steel wire measuring 450 cm<sup>2</sup> floor area x 32cm<sup>2</sup> height. They were kept at small animal isolation unit in the Department of Veterinary Pathology, Microbiology and Parasitology, Kabete Campus, University of Nairobi, where they were allowed to acclimatize for 3 days before start of the experiment (Obernier and Baldwin, 2006). They were provided with mice pellets and clean water *ad libitum* while wood shavings, provided as beddings, were changed after every two days until the end of the experiment.

#### **3.8.2 Ethical clearance**

Prior to the commencement of the study, ethical clearance was obtained from Animal Use and Ethics committee, Faculty of Veterinary Medicine, University of Nairobi (appendix I).

#### **3.8.3 Biosafety and Occupational Safety**

As per the Animal Biosafety Manual (2012), mice were handled in a single sided animal containment work station that protected the operator, the animal and the assistant against allergens during animal handling. All manipulations performed on mice using suspected pathogenic strains were conducted in a class II Biosafety Cabinet equipped with High Efficiency Particulate Air (HEPA) filters. Appropriate mice restrainers were used.

Aseptic techniques were observed to avoid cross contamination. The suspected infectious pathogen that was inoculated into the mice was handled in safety cabinet using protective clothing, latex gloves, nose masks and laboratory coats and sharps that were used in the experiment were disposed into well labeled sharps container. Reference biological and non-biological materials used in the experiments were stored in a refrigerator while others, to be discarded, were transported in tightly sealed polythene bags for disposal at Kabete Campus incinerator, University of Nairobi,

### **3.8.4 Inoculation of mice for pathogenicity testing**

#### **3.8.4.1 Preparation of inocula**

*E. coli* was chosen for pathogenicity studies since some strains of this organism are pathogenic and can cause serious food poisoning in humans. Sterile swabs, moistened in sterile peptone water, were used to pick and streak the organism on prepared MacConkey agar plates; multiple streaks were made in order to obtain confluent growth. Harvesting of the bacteria was done by flooding the entire plate with 4 ml sterile physiological saline and agitating it so as to dislodge the bacteria. The harvest was then placed in sterile tubes, from where bacterial viable count was carried-out following the method of Miles and Misra (1938), as given in section 3.6 above.

#### **3.8.4.2 Mice inoculation with test bacterium and monitoring for clinical disease**

The mice were divided into six groups of five each; caged separately. Different groups were identified using different coloured dyes. Dilutions  $10^{-1}$ -  $10^{-5}$  were selected for pathogenicity testing after viable count. Each mouse was inoculated 0.4 ml intra-peritoneally with respective bacterial suspension ( $10^{-1}$ - $10^{-5}$ ). The control group was inoculated with sterile normal saline.

During the experimental period, the inoculated mice were observed twice daily; morning at 8 am and evening at 5 pm, for manifestation of clinical signs, namely activity, alertness, presence of discharge in the eyes/nose, appearance of the coat and state of their droppings (Burkholder, 2013).

### **3.8.5 Clinical pathology**

Two sets of blood samples were collected at once from each mouse on the 7<sup>th</sup> day of the experiment: one with anticoagulant, Ethylenediaminetetraacetic acid (EDTA) and one without anticoagulant, in vacutainer tubes. All blood samples were used for haematology and clinical chemistry analysis, which included blood cell counts, packed cell volume, haemoglobin concentration and red cell indices, using IDEXX ProCytel DX automated haematology analyser (IDEXX Laboratories Inc.). In addition, thin blood smears were prepared, stained with 1:5 Giemsa and examined under light microscope for cellular morphology and differential leukocyte counts. Serum samples obtained by centrifugation of coagulated blood sample were analysed for serum proteins and blood urea nitrogen (BUN) using IDEXX Catalyst DX automated chemistry analyser (IDEXX Laboratories Inc). All blood samples were analysed at Pathologist Lancet veterinary laboratories, Kenya. Figure 4 shows Veterinary pathologist collecting blood sample for analysis.





**Figure 4:** Investigator being assisted by a veterinary pathologist collecting blood sample from a mouse

### **3.8.6 Necropsy findings**

Necropsy on all euthanized mice was carried out according to standard procedures (Fieldman and Seely, 1988). The mice were euthanized all at once on the 7<sup>th</sup> day of the experiment. Any gross lesion observed was recorded while major organs namely liver, spleen, intestines, lungs, heart, brain were collected, fixed in 10% formal saline, processed, stained with Hematoxylin and eosin and examined under a light microscope for microscopic lesions. These were observed and recorded for each experimental group. Figure 5 shows Veterinary pathologist collecting tissue samples for histological examinations on euthanized mice.



**Figure 5 :** Veterinary pathologist collecting tissue samples from a mouse

### **3.9 Quality control check**

Two randomly-selected control mice were sacrificed before and after the experiment; their livers and spleens were cultured to ascertain that the un-inoculated-mouse population was free from the study organism and other bacterial pathogens.

### **3.10 Statistical analysis**

The findings of the study were entered, cleaned and stored into Microsoft Excel program (Ms Excel). Data on bacteria isolated, bacterial load quantified from African sausages sold in Nairobi County were analysed and presented in tables and figures. Susceptibility data of the three most prevalent isolates to lemon juice-extract were subjected to the analysis of variance (ANOVA) tests (Snedecor and Cochran, 1976). The pathogenicity data of one of the isolates using a mouse model was imported into Statistical package for social scientists (SPSS version 14).

The determination of the different zones of inhibition from varied concentrations of lemon extracts, the values for total viable bacterial counts and haematological and biochemical parameters were subjected to the analysis of variance (ANOVA) tests (Snedecor and Cochran, 1976). Statistical significant differences between haematological and biochemical qualities of various samples from different locations were evaluated at  $p < 0.05$  and confidence limits at 95%.

## CHAPTER 4: RESULTS

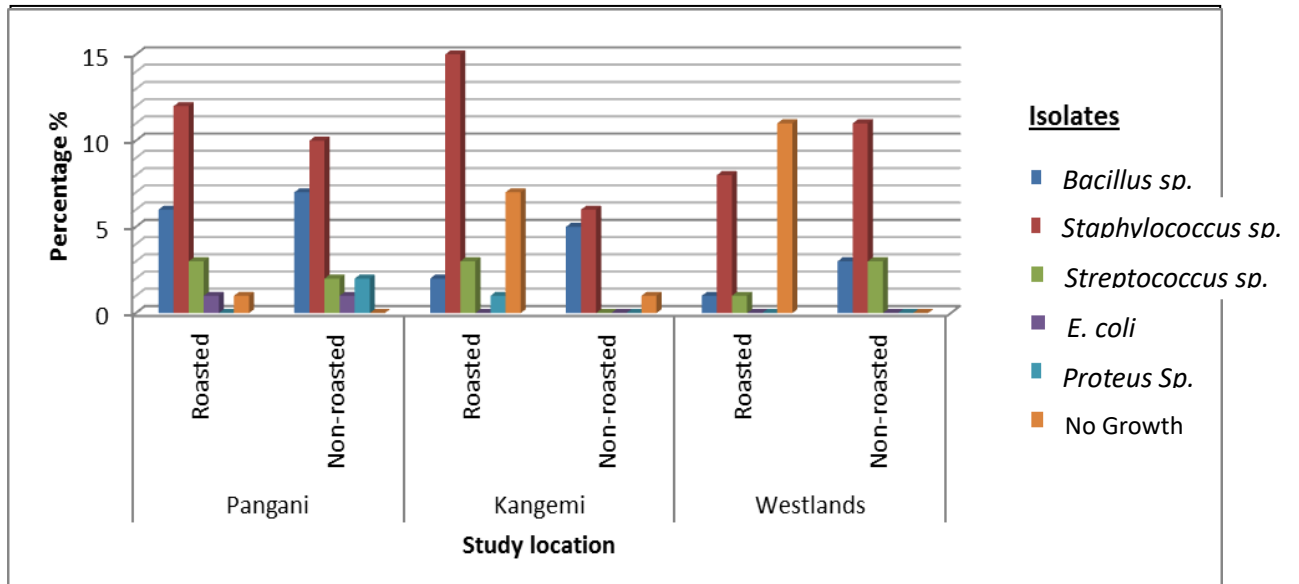
### 4.1 Isolation of bacteria from African sausages in Nairobi County

A total of one hundred (100) African sausage samples were collected and analysed - 33 samples from Westlands market, 33 samples from Pangani and 34 samples from Kangemi. In Figure 5, five genera of bacteria (123 isolates) were isolated from 80/100 (80%) roasted and non-roasted African sausages. They were: *Staphylococcus*, *Bacillus*, *Streptococcus*, *Proteus* and *Escherichia*. *Staphylococci* were the most predominant bacteria in all the sausage samples collected with a prevalence of 50.4% (62/123), *Bacillus* at 19.5% (24/123), *Streptococcus* 9.8% (12/123), *Proteus* 2.4% (3/123) while *E.coli* was isolated at 1.6% (2/123).

With respect to roasted African sausages, *Staphylococcus* accounted for 53.6% (15/28) of the isolates in Kangemi, 52.2% (12/23) in Pangani and 38.1% (8/21) in Westlands. *Bacillus* organisms were isolated at 7.1% (2/28) in Kangemi, 26.1% (6/23) in Pangani and 4.8% (1/21) in Westlands; *Streptococcus* 10.7% (3/28) in Kangemi, 13% (3/23) in Pangani and 4.8% (1/21) in Westlands; *Proteus* 3.6% (1/28) in Kangemi, 0% both in Pangani and in Westlands; *E.coli*, 4.3% (1/23) in Pangani, 0% both in Kangemi and in Westlands .

With respect to non- roasted African sausages, *Staphylococcus* accounted for 50% (6/12) of the isolates in Kangemi, 64.7% (11/17) in Westlands and 45.5% (10/22) in Pangani. *Bacillus* organisms were isolated at 41.7% (5/12) in Kangemi, 31.8% (7/22) in Pangani and 17.6% (3/17) in Westlands; *Streptococcus* 17.6% (3/17) in Westlands, 9.1% (2/22) in Pangani and 0% in Kangemi; *Proteus*; 9.1% (2/22) in Pangani, 0% both in Kangemi, and in Westlands areas; *E.coli*; 4.5% (1/22) in Pangani 0% both in Kangemi and in Westlands areas. The results are as given in Figure 6. Table 1 gives the mean distribution of bacterial isolates.

There was no significant difference ( $p>0.05$ ) in distribution of isolates across the geographical areas under study. (appendix II).



**Figure 6 :** Prevalence of the five genera of bacteria isolated from African sausages sampled from Pangani, Kangemi and Westlands, Nairobi County

**Table 1:** Evaluation of the mean distribution of bacterial isolates from African Sausages across the three geographical areas using paired sample t-test ( $P$  values evaluated at 95% confidence limits).

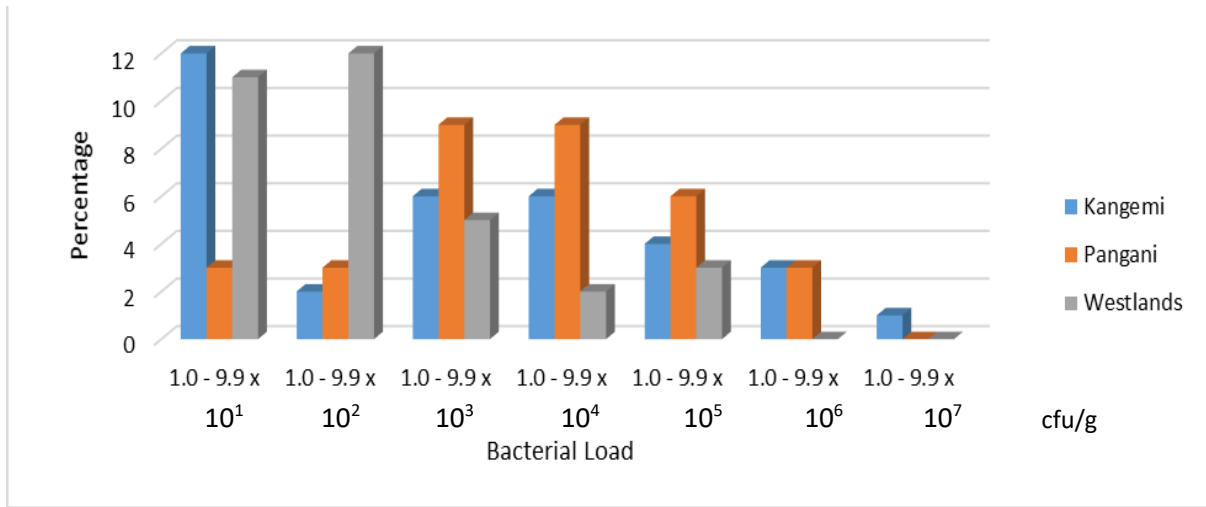
		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Kangemi - Pangani	-1.333	4.590	1.874	-6.150	3.483	-.712	5	0.509
Pair 2	Kangemi - Westlands	0.333	2.160	0.882	-1.934	2.600	.378	5	0.721
Pair 3	Pangani - Westlands	1.667	6.282	2.565	-4.926	8.259	.650	5	0.544

## **4.2 Quantification of bacterial load of the African sausages**

### **4.2.1 Bacterial load of both roasted and non-roasted African sausages**

Overall, 26/100 (26%) African sausage samples had a bacterial load of between 1.0- 9.9 x10<sup>1</sup> cfu/g , 17/100 (17%) samples had a bacterial load of between 1.0- 9.9 x10<sup>2</sup> cfu/g, 20/100 (20%) samples had a bacterial load of between 1.0- 9.9 x10<sup>3</sup> cfu/g, 17/100 (17%) samples had a bacterial load of between 1.0- 9.9 x10<sup>4</sup> cfu/g, 13/100 (13%) samples had a bacterial load of between 1.0- 9.9 x10<sup>5</sup> cfu/g, 6/100 (6%) samples had a bacterial load of between 1.0- 9.9 x10<sup>6</sup> cfu/g and 1/100 (1%) sample had a bacterial load of between 1.0- 9.9 x10<sup>7</sup> cfu/g. With respect to individual study sites, 12/34 (35.3%) of samples from Kangemi, 3/33 (9%) from Pangani and 11/33 (33.3%) from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>1</sup> cfu/g. Two of the thirty four (5.9%) samples from Kangemi, 3/33 (9.1%) from Pangani and 12/33 (36.36%) from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>2</sup> cfu/g. Six of the thirty four (17.6%) samples from Kangemi, 9/33 (27.27%) from Pangani and 5/33 (15%) from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>3</sup> cfu/g.

Six of the thirty four (17.6%) samples from Kangemi, 9/33 (27.3%) from Pangani and 2/33 (6%) from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>4</sup> cfu/g. 4/34 (11.76%) of samples from Kangemi, 6/33 (18%) from Pangani and 3/33 (9%) from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>5</sup> cfu/g. Three of the thirty four (8.8%) of samples from Kangemi, 3/33 (9%) from Pangani and 0% from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>6</sup> cfu/g. One of the thirty four (2.9%) of samples from Kangemi, 0% from Pangani and Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>7</sup> cfu/g (Appendix III and IV). The results are as given in Figure 7.



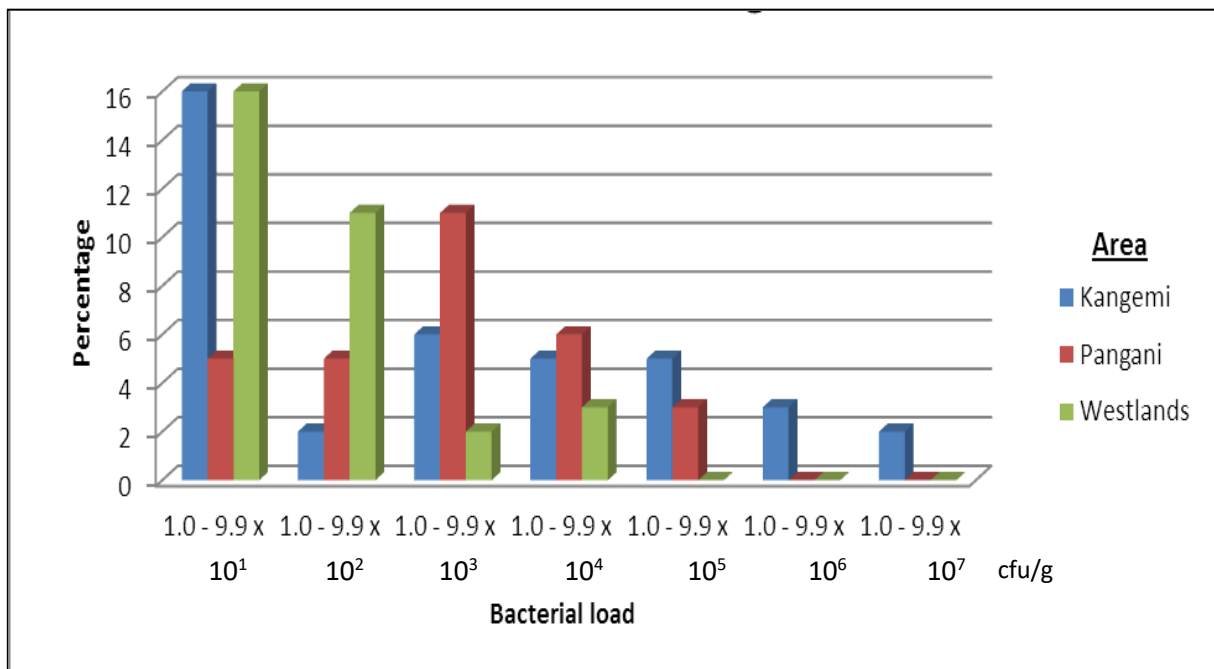
**Figure 7 :** Percent bacterial loads of the sampled African sausages (roasted and non-roasted) sold in the three study sites

#### 4.2.2 Bacterial load from roasted African sausages

Twenty two of sixty two (35.5%) roasted African sausage samples had a bacterial load of between 1.0- 9.9 x10<sup>1</sup> cfu/g , 11/62 (17.7%) samples had a bacterial load of between 1.0- 9.9 x10<sup>2</sup> cfu/g, 12/62 (19%) samples had a bacterial load of between 1.0- 9.9 x10<sup>3</sup> cfu/g, 9/62 (14.5%) samples had a bacterial load of between 1.0- 9.9 x10<sup>4</sup> cfu/g, 5/62 (8%) samples had a bacterial load of between 1.0- 9.9 x10<sup>5</sup> cfu/g, 2/62 (3%) samples had a bacterial load of between 1.0- 9.9 x10<sup>6</sup> cfu/g and 1/62 (1.6%) sample had a bacterial load of between 1.0- 9.9 x10<sup>7</sup> cfu/g. With respect to individual study sites, 10/24 (41.7%) of samples from Kangemi, 3/19 (15.8%) from Pangani and 9/20 (45%) from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>1</sup> cfu/g.

One of twenty four (4.17%) of samples from Kangemi, 3/19 (15.8%) from Pangani and 7/20 (35%) from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>2</sup> cfu/g. 4/24 (16.7%) of samples from Kangemi, 7/19 (36.8%) from Pangani and 1/20 (5%) from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>3</sup> cfu/g.

Three of the twenty four (12.5%) samples from Kangemi, 4/19 (21%) from Pangani and 2/20 (10%) from Westlands area had a bacterial load of between  $1.0- 9.9 \times 10^4$  cfu/g. 3/24 (12.5%) of samples from Kangemi, 2/19 (10.5%) from Pangani and 0% from Westlands area had a bacterial load of between  $1.0- 9.9 \times 10^5$  cfu/g. Two of the twenty four (8.3%) of samples from Kangemi, 0% from Pangani and Westlands area had a bacterial load of between  $1.0- 9.9 \times 10^6$  cfu/g. 1/24 (4.17%) of samples from Kangemi, 0% from Pangani and Westlands area had a bacterial load of between  $1.0- 9.9 \times 10^7$  cfu/g. The results are as given in Figure 8. Table 2 gives the mean total aerobic bacterial count from roasted African sausages across the three geographical areas. There was no significant difference ( $p \geq 0.05$ ) in mean total aerobic bacterial count across the areas under study.



**Figure 8 :** Percent bacterial loads of the sampled roasted African sausages sold in the three study sites



**Table 2:** Evaluation of the mean total aerobic bacterial count from roasted African sausages across the three geographical areas using paired sample t-test (*P* values evaluated at 95% confidence limits).

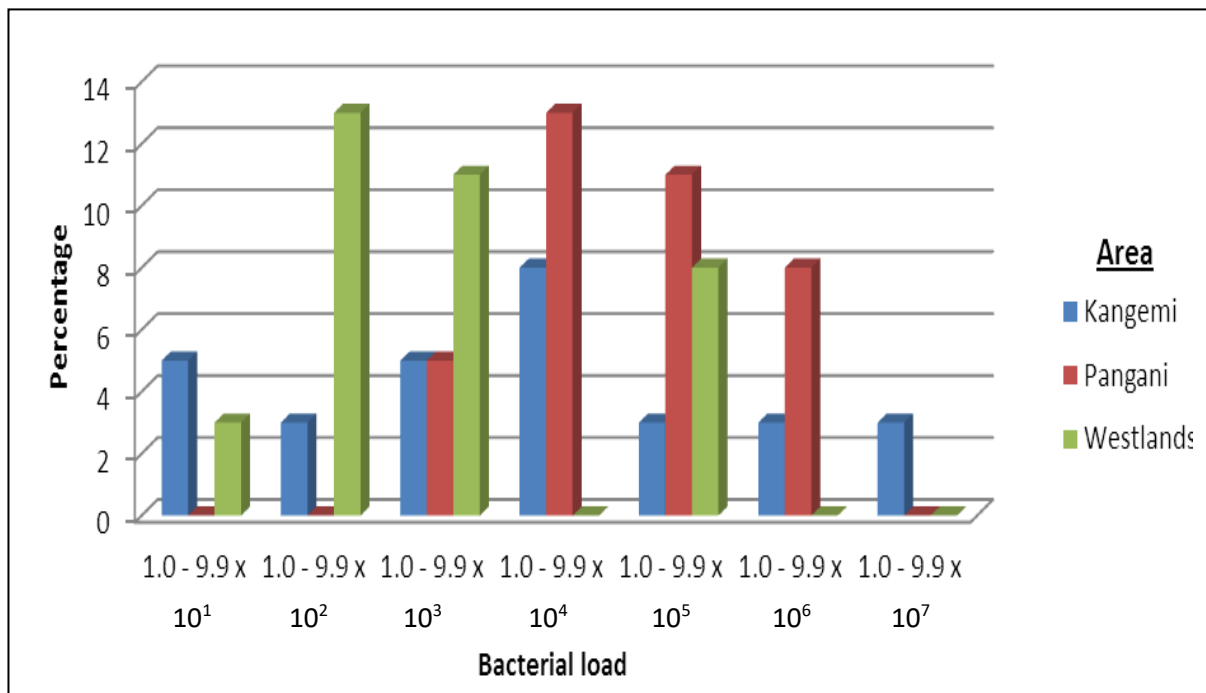
		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Kangemi - Pangani	147885.000	694148.169	159248.512	-186683.708	482453.708	.929	18	.365
Pair 2	Kangemi - Westlands	175895.500	658535.387	147252.989	-132308.548	484099.548	1.195	19	.247
Pair 3	Pangani - Westlands	26479.211	60777.503	13943.315	-2814.608	55773.029	1.899	18	.074

#### 4.2.3 Total aerobic bacterial count from non-roasted African sausages.

Three of the thirty eight (7.9%) non-roasted African sausage samples had a bacterial load of between  $1.0- 9.9 \times 10^1$  cfu/g , 6/38 (15.79%) samples had a bacterial load of between  $1.0- 9.9 \times 10^2$  cfu/g, 8/38 (21%) samples had a bacterial load of between  $1.0- 9.9 \times 10^3$  cfu/g, 8/38 (21%) samples had a bacterial load of between  $1.0- 9.9 \times 10^4$  cfu/g, 8/38 (21%) samples had a bacterial load of between  $1.0- 9.9 \times 10^5$  cfu/g, 4/38 (10.5%) samples had a bacterial load of between  $1.0- 9.9 \times 10^6$  cfu/g and 1/38 (2.6%) sample had a bacterial load of between  $1.0- 9.9 \times 10^7$  cfu/g.

With respect to individual study sites, 2/11 (18.8%) of samples from Kangemi, 0% from Pangani and 1/13 (7.7%) from Westlands area had a bacterial load of between  $1.0- 9.9 \times 10^1$  cfu/g. 1/11 (9%) of samples from Kangemi, 0% from Pangani and 5/13 (38.5%) from Westlands area had a bacterial load of between  $1.0- 9.9 \times 10^2$  cfu/g. 2/11 (18%) of samples from Kangemi, 2/14 (14.3%) from Pangani and 4/13 (30.8%) from Westlands area had a bacterial load of between  $1.0- 9.9 \times 10^3$  cfu/g.

Three of the eleven (27.3%) of samples from Kangemi, 5/14 (35.7%) from Pangani and 0% from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>4</sup> cfu/g. 1/11 (9%) of samples from Kangemi, 4/14 (28.6%) from Pangani and 3/13 (23%) from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>5</sup> cfu/g. One of the eleven (9%) of samples from Kangemi, 3/14 (21.4%) from Pangani and 0% from Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>6</sup> cfu/g. 1/11 (9%) of samples from Kangemi, 0% from Pangani and Westlands area had a bacterial load of between 1.0- 9.9 x10<sup>7</sup> cfu/g. The results are as given in Figure 9. Table 3 gives the mean total aerobic bacterial count from non-roasted African sausages across the three geographical areas. There was no significant difference (p≥0.05) in mean total aerobic bacterial count across the areas under study.



**Figure 9:** Percent bacterial loads of the sampled non-roasted African sausages sold in the three study sites

**Table 3:** Evaluation of the mean total aerobic bacterial count from non-roasted African sausages across the three geographical areas using paired sample t-test (*P* values evaluated at 95% confidence limits).

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Kangemi - Pangani	-32490.00	1839501.21	554630.48	-1268283.73	1203303.73	-.059	10	.954
Pair 2	Kangemi - Westlands	504456.36	1407287.95	424313.28	-440972.54	1449885.27	1.189	10	.262
Pair 3	Pangani - Westlands	457091.54	851423.04	236142.26	-57418.26	971601.33	1.936	12	.077

### 4.3 Susceptibility of the three most prevalent isolates to lemon juice extract

#### 4.3.1 Inhibitory effect of lemon juice-extract at various concentrations

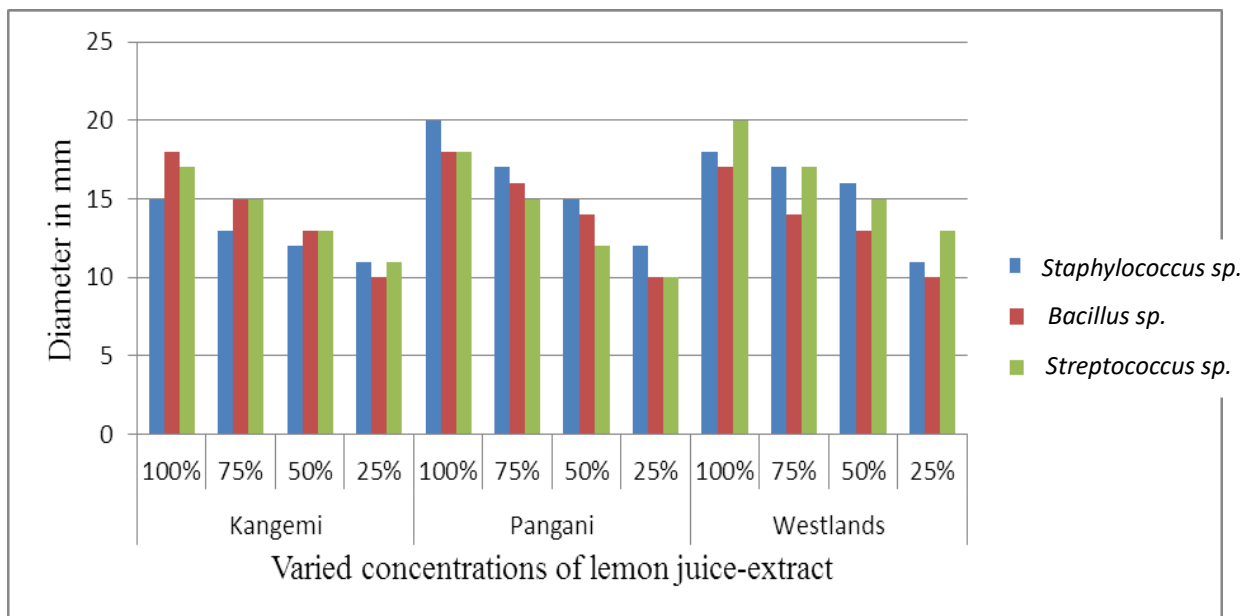
The concentrated extract (100%) showed most effect on all the three bacteria studied and in all the three study areas for both Broth inhibitory method and agar diffusion method. In Kangemi, Pangani and Westlands, the MIC was 50% for the *Staphylococcus* and *Streptococcus* isolates under inhibitory testing. The MIC for bacillus in Kangemi was 75% using broth inhibitory method. Minimum effect being shown by the lowest concentration of 25% using agar diffusion method which, for the three study bacteria gave an inhibition zone of 10 mm. There were also varied responses even for the same bacterial type and same concentration, with respect to the three study areas. The 100% extract had highest effect on *Staphylococcus* isolate from Pangani (inhibition zone of 20 mm), followed by the one from Westlands (inhibition zone of 18 mm), lastly the one from Kangemi (inhibition zone of 15mm). For *Bacillus*, the 100% extract had highest effect on isolate from Kangemi (inhibition zone of 18mm), followed by the one from Pangani (inhibition zone of 18 mm), lastly the one from Westlands (inhibition zone of 17 mm).

For *Streptococcus*, the 100% extract had highest effect on isolate from Westlands (inhibition zone of 20 mm), followed by the one from Pangani (inhibition zone of 18 mm), lastly the one from Kangemi (inhibition zone of 17 mm). The 75% extract had highest effect on *Staphylococcus* isolate from Westlands (inhibition zone of 17mm), followed by the one from Pangani (inhibition zone of 17 mm), lastly the one from Kangemi (inhibition zone of 13 mm). For *Bacillus*, the 75% extract had highest effect on isolate from Pangani (inhibition zone of 16 mm), followed by the one from Kangemi (inhibition zone of 15 mm), lastly the one from Westlands (inhibition zone of 14 mm).

For *Streptococcus*, the 75% extract had highest effect on isolate from Westlands (inhibition zone of 17 mm), while it had same effect on isolates from Pangani and Westlands (inhibition zone of 15 mm). The 50% extract had highest effect on *Staphylococcus* isolate from Westlands (inhibition zone of 16 mm), followed by the one from Pangani (inhibition zone of 15 mm), lastly the one from Kangemi (inhibition zone of 12 mm). For *Bacillus*, the 50% extract had highest effect on isolate from Pangani (inhibition zone of 16 mm), followed by the one from Westlands and Kangemi (inhibition zone of 13 mm). For *Streptococcus*, the 50% extract had highest effect on isolate from Westlands (inhibition zone of 15 mm), followed by the one from Pangani (inhibition zone of 13 mm), lastly the one from Kangemi (inhibition zone of 12 mm). Interestingly, the 25% extract had the same effect on all the three study bacteria and the three study areas (inhibition zone of 10 mm). Overall, the bacterium that showed the most susceptibility to lemon extract was *Staphylococcus*. Inhibitory profiles for the three most prevalent isolates to varied concentrations of lemon juice-extract are as given in Table 4, Figures 10 and 11

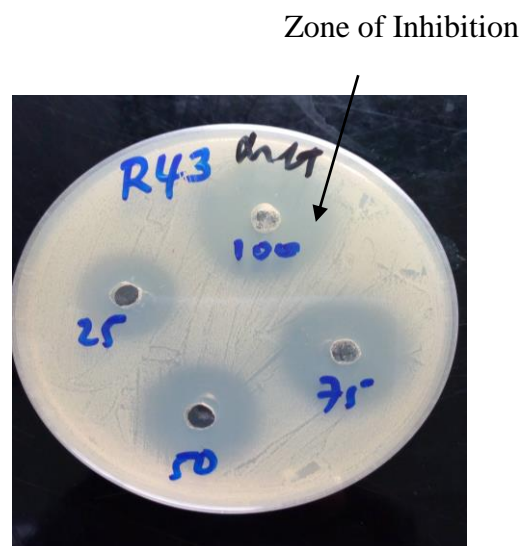
**Table 4:** Minimum Inhibitory Concentration profile of the three most prevalent isolates to varied concentrations of lemon extract using broth inhibitory method

Study site	Minimum Inhibitory concentration		
	Staphylococcus	Bacillus	Streptococcus
Kangemi	50%	75%	50%
Pangani	50%	50%	50%
Westlands	50%	50%	50%



**Figure 10:** Inhibitory profile of the three most prevalent isolates to varied concentrations of lemon extract using agar gel diffusion method

Figure 11 shows inhibitory pattern of one of the tested isolates to the various dilutions of the lemon extract; 50% dilution being the highest dilution/lowest concentration that had inhibitory effect on the test organism, while the susceptibility results show different zones of inhibition with 25%, 50%, 75% and 100% concentrations of lemon extract.



**Figure 11:** Inhibitory pattern of *Bacillus* sp. to the various dilutions of the lemon juice-extract

#### 4.3.2 Mean diameters of the inhibition zones

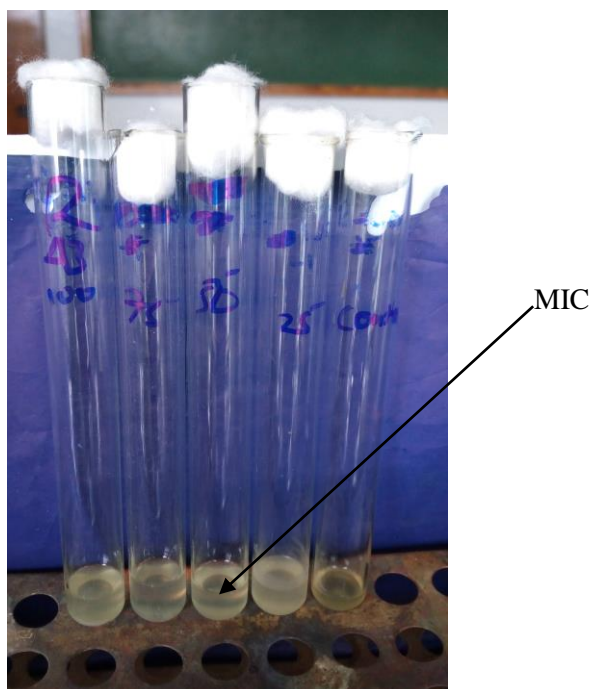
The three tested isolates gave a mean inhibitory zone diameter of 14 mm, with a minimum inhibitory zone of 10mm and a maximum of 20 mm for *Streptococcus*, minimum inhibitory zone of 11 mm and a maximum of 20 mm for *Staphylococcus* and minimum inhibitory zone of 10mm and a maximum of 18 mm for *Bacillus* (appendix V).

#### 4.3.3 Statistical comparison of the mean diameters

When the mean values for inhibitory zones were compared statistically using unpaired student t-test, at 95% confidence level, the *p* values were above 0.05 ( $>0.05$ ), indicating that the mean values were not significantly different (appendix IV).

#### 4.3.4 Minimum Inhibitory Concentration using broth inhibitory testing

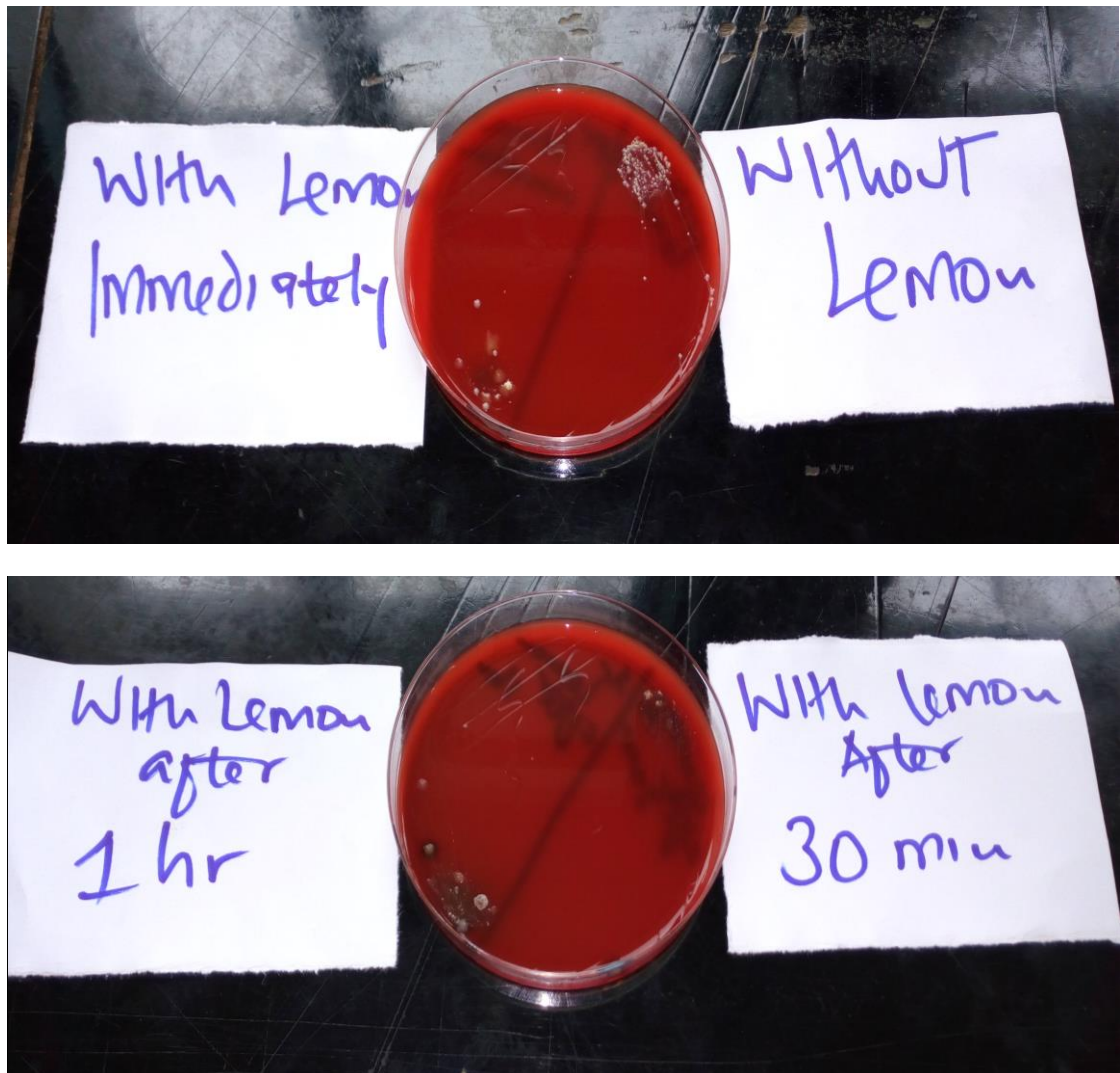
In Kangemi area, six isolates were tested namely; four *Staphylococcus* Spp. and two *Bacillus* sp. In Pangani area, eight isolates were tested namely; one *Staphylococcus* Spp., five *Bacillus* Spp. and two *E.coli*. In Westlands area, five isolates were tested namely; three *Staphylococcus* Spp. and two *Bacillus* Spp. Figure 12 shows the minimum inhibitory concentration for *E.coli* isolated from an African sausage. From left, the dilutions are 100%, 75%, 50% and 25%; the one in extreme right is the control with no lemon juice-extract added



**Figure 12:** Lemon antibacterial sensitivity pattern for *E.coli* – Minimal inhibitory concentration.

#### 4.3.5 Potency of Lemon Juice extract

Figure 13 shows relative amounts of harvested bacteria after the homogenates were treated with lemon juice-extract (100% concentrated). There was almost immediate action on the bacteria, as indicated by growth reduction, compared to the one without lemon treatment. Maximum inhibition was shown after 30 minutes' treatment, after which the lemon effect faded as shown by the growth one hour after the treatment.



**Figure 13:** Bacterial loads before and after lemon juice-extract treatment of roasted and unroasted African sausages homogenates, after specified time periods.

#### **4.4 Pathogenicity of one isolate - *E. coli*, using a mouse model**

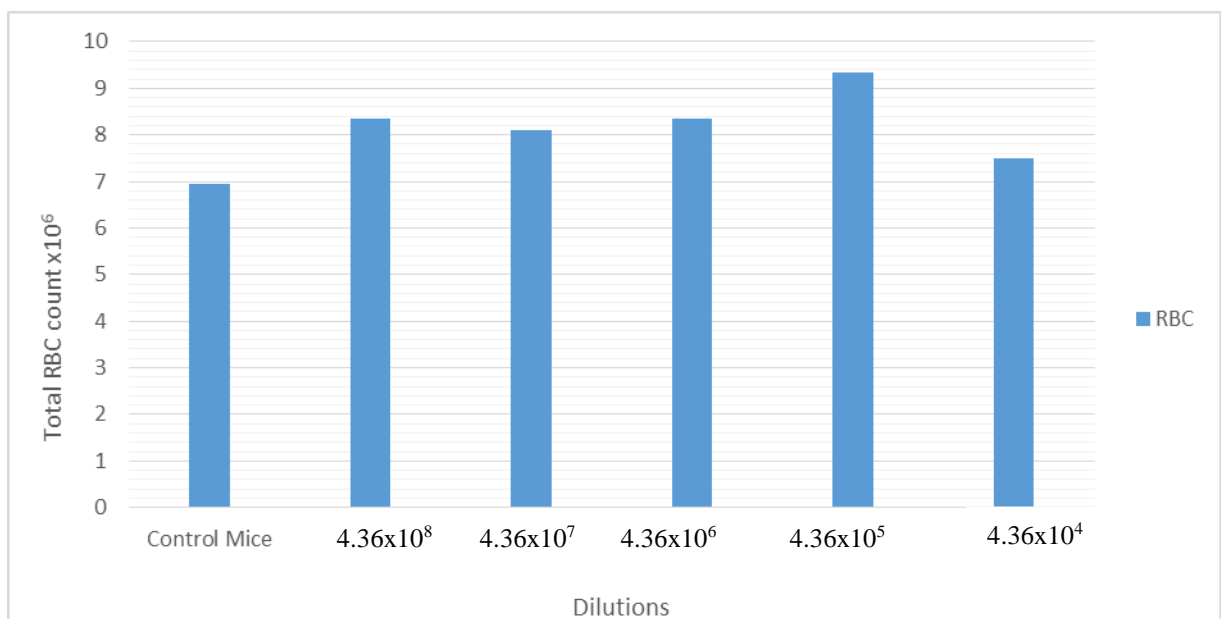
##### **4.4.1 Clinical signs**

When the mice were inoculated with various concentrations ( $4.36 \times 10^8$ ,  $4.36 \times 10^7$ ,  $4.36 \times 10^6$ ,  $4.36 \times 10^5$  and  $4.36 \times 10^4$ ) of *E. coli*, No mortality was observed in all the mice under experiment as all survived and did not show any clinical sign at the end of the 7 day experimental period.



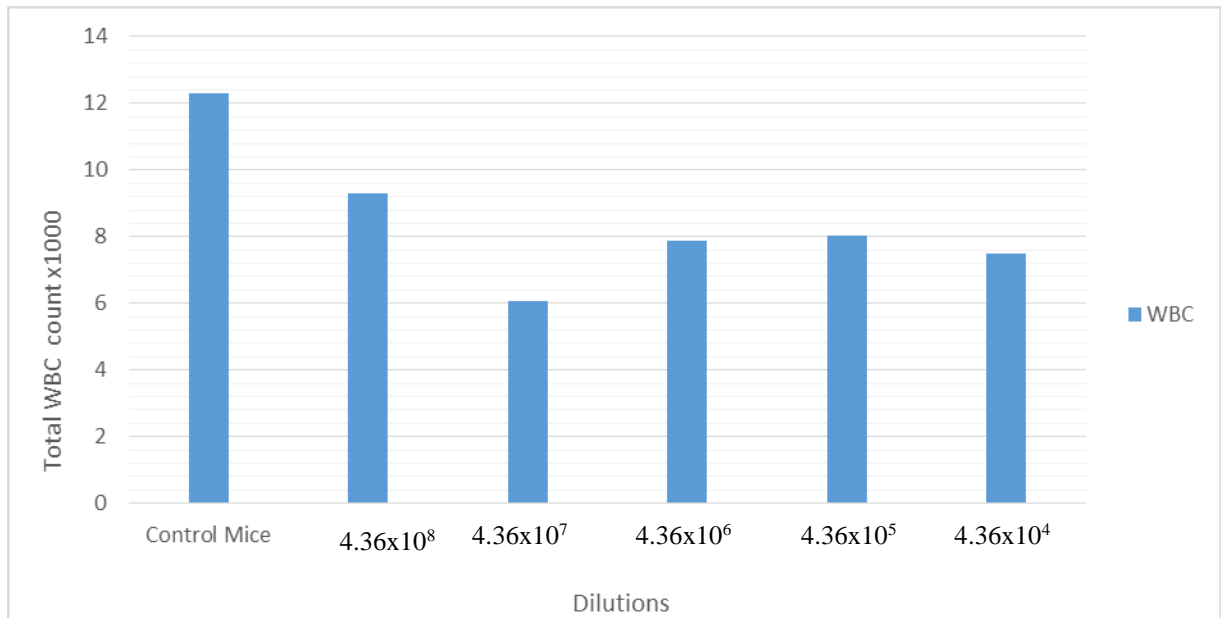
#### 4.4.2 Clinical pathology

Two sets of blood samples were collected at once from each mouse on the 7<sup>th</sup> day of the experiment in vacutainer tubes: one with anticoagulant [Ethylenediaminetetra acetic acid (EDTA)] for haematological investigations and one without anticoagulant for clinical chemistry tests. The blood samples were submitted to Pathologist Lancet Kenya for analysis. The figure 11 shows the investigator assisted by a Veterinary pathologist to collect blood samples for haematological and clinical examination examinations. Figure 14 show the comparison of total erythrocyte count in test and control group of mice subjected to varied concentrations of *E.coli*.



**Figure 14:** Total erythrocyte count variations in control and experimental mice inoculated intraperitoneally with the various concentrations (4.36x10<sup>8</sup>-4.36x10<sup>4</sup>) of test *E. coli*.

Figure 15 shows the comparison of total leucocyte count in test and control group. The values fell within the reference value control and experimental mice.



**Figure 15:** Total Leucocyte Count (TLC) variations in control and experimental mice inoculated intraperitoneally with the various concentrations ( $4.36 \times 10^8$ - $4.36 \times 10^4$ ) of test *E. coli*.

The haematological and biochemical mean values from the mice inoculated with the test *E. coli* were statistical compared using unpaired student t-test at 95% confidence level and the analysis of variance for the test groups. The results/mean values for the control and experimental mice showed no significance variations ( $p > 0.05$ ), while some for serum proteins and BUN values (Chemistry parameters) we slightly different from the control group ( $p > 0.05$ ) (appendices VI, VII, VIII and IX, X and XI).

#### **4.4.3 Necropsy findings**

The peritoneum was white, smooth and shiny. All other organs retained normal colour, size and shape. Organs examined were normal except for accumulation of blood in the venous side of circulation and prominence of lymphoid follicles in the spleen, Peyer's patches and peri-bronchial lymphoid areas.

#### **4.5 Quality control check**

The peritoneum was white, smooth and shiny euthanized control mice. All other organs retained normal colour, size and shape while the organs examined were normal.

## CHAPTER 5: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

### 5.1 Discussion

Five genera of bacteria were isolated, namely: *Staphylococcus* spp., *Bacillus* spp., *Streptococcus* spp., *Proteus* spp. and *Escherichia coli*. *Staphylococcus* organisms were the most predominant bacteria in all the sausage samples collected with a prevalence of 60.2%; various species of *Bacilli* were the second most prevalent at 23.5%, followed by *Streptococci* spp. at 11.65%, *Proteus* spp. at 2.9% and *E.coli* was isolated at 1.9%. The data obtained on isolation of bacteria in the present study where *Staphylococcus* spp. and *Bacillus* spp. were the predominant isolates, agrees with a study by Oluwafemi and Simisaye (2006) and Okonko *et al.*, (2009) who isolated similar organisms from beef sausages and seafood in Nigeria, respectively. However, differences are noted whereby in the present study *Streptococci* spp., *Proteus* spp. and *E. coli* were isolated while in the latter, *Enterobacter* spp., *Pseudomonas* spp. and *Klebsiella* species were isolated from beef sausages and seafood respectively.

The current study showed *Staphylococcus* species prevalence of 60.2%. This was higher than the *Staphylococcus* species recovery at 58.6% from hotels, from restaurants and cafes; report by Berynestad and Granums (2002). According to the reports, the predominant organisms included *Staphylococcus* Spp, *E. coli*, *Bacillus* Sp., *Enterobacter* Spp., *Pseudomonas* Spp. and *Klebsiella* Spp. Carrying-out a similar study on minced meat, sausage rolls and pies, Waites and Arbuthnott (1999) reported a 60.9% prevalence of *Staphylococcus aureus* and 50% for *E. coli*. A study by Yusuf *et al.* (2012), percentage of occurrence of bacterial isolated from the “balangu” meat product showed highest for *Bacillus cereus* with 19.6% which was attributed to aerial spores carried in the air and lower prevalence of *Staphylococcus aureus* at 12.5% occurrence.

A study by Aycicek *et al.* (2005) reported that processed foods were found to be more prone to *Staphylococcus* species contamination. Presence of *Staphylococcus* contamination in some of the samples examined in this study might have resulted from handler's skin or the environment. Among the roasted African sausages, *Staphylococcus* was highest in Kangemi. However, the results of the current study contrast the findings by Orogu and Oshilim (2017) who reported a 30% *Bacillus* occurrence from suya meat. Similar prevalence was obtained by a study by Matos *et al.* (2006) working with dry smoked sausages. Presence of *Bacillus* contamination in some of the samples examined in this study might have resulted from contamination from vendor's skin or the environment. This may have been attributed to contamination from aerial spores carried in the air, throat, hands and nail of food handling persons (Hatakka *et al.*, 2000).

Similar finding on the prevalence of *Streptococcus* spp. was reported by Onuora *et al.* (2015) working on grilled beef. The prevalence of *E. coli* reported in the present study was slightly lower than that reported by Syne *et al.* (2013) and Onuora *et al.* (2015). *E. coli* presence in African sausages have the potential to cause diarrhoea. The incidence of *E. coli* obtained in this study is a cause for public health concern as this bacterium has been implicated in cases of gastroenteritis (1982). The presence of *Proteus* isolates was remarkably higher in a study by Gwinda *et al.* (2014) from beef meat, compared to what was found in the current study. The presence of *Proteus* organisms in the meat samples can obviously be attributed to unhygienic food processing. *Staphylococcus* spp., *Bacillus* spp., *Streptococcus* spp. and *E. coli* are known to produce potent enterotoxins and the ingestion of food containing these toxins can cause a sudden onset of illness within three to four hours, with nausea, vomiting and diarrhea as the major symptoms (2006). There was no significant difference ( $p>0.05$ ) in distribution of these organisms across the three geographical sites studied.

Cases of the food handlers being sources of contamination have been reported: Bebora *et al* (2005) isolated toxin-producing *E. coli* from food handlers in some tourist-class hotels in Nairobi; this implies that the handlers were carriers of the organism. Kuria *et al* (2009) isolated *Salmonella*, *E. coli* and other bacteria from commonly handled coins (currency) in Kenya; an important point to note since African sausages sellers normally simultaneously handle money and the African sausages.

In the present study, it was observed that there was no significant difference in the bacterial load across the three geographical sites studied ( $p>0.05$ ). Bacterial load of between  $1.0 - 9.9 \times 10^2$  and  $1.0 \times 9.9 \times 10^4$  cfu/g was reported in most (54%) of African sausage samples. Similar findings by Clarence *et al.* (2009) reported a bacterial load level of between  $3 \times 10^3 - 2.8 \times 10^4$  cfu/g in beef sausages. In present study there was no significant difference ( $p>0.05$ ) in mean total aerobic bacterial count across the areas under study and between roasted and non-roasted African sausages samples. The level of the viable bacterial load in present study could be attributed to unhygienic processing technique, unhygienic environment and practices such as dirty cutting boards and knives or utensils. Cheesbrough (2006) noted that insects contribute to contamination by mechanical transfer of microorganisms to food products since they are left uncovered and exposed to dust.

The *Citrus Limon* fruit juice-extract showed antibacterial activity against the *Staphylococcus*, *Bacillus* and *Streptococcus* in current study. The susceptibility increasing with increased concentration of the lemon juice-extract. There was no significant difference ( $p>0.05$ ) in the sensitivity patterns across the three genera tested. In the current study, the MIC was 50% for the *Staphylococcus* and *Streptococcus* isolates under inhibitory testing. The MIC for bacillus in Kangemi was 75% using broth inhibitory method.

Report by Adham (2015) revealed that *Citrus limon* juice and combination extract showed activity against all gram positive and negative bacterial strain 100%, followed by *Citrus reticulata* combination extract effect on 80% gram positive, while juice alone and in combination with peel effect on 50% gram negative bacterial strain. Combination of peel and juice extract of *Citrus paradisi* showed effect on 60% gram positive and 50% gram negative bacterial strain. *Citrus sinensis* only effect on 20% gram positive bacteria. The results supported by previously recorded data in which Citrus fruit have high antimicrobial activity against gram positive than gram negative bacterial strain (Al-Ani *et al.*, 2010). The difference in sensitivity of the Gram-positive bacteria compared to that of Gram-negative bacteria to antibiotics and antimicrobial natural products may due to differences in their cell wall composition (Samarakoon *et al.*, 2012).

The highest zone of inhibition of 20 mm was demonstrated by *Staphylococcus* and *Streptococcus* organisms at a 100% lemon juice-extract concentration ; inhibition zones of 17mm were exhibited by *Staphylococcus* and *Streptococcus* organisms at 75% lemon juice-extract concentration , 16mm zone of inhibition was exhibited by *Staphylococcus* organisms at lemon juice-extract concentration of 50% and 13mm zone of inhibition was exhibited by *Streptococcus* organisms at 25% lemon juice-extract concentration. The study confirms that the bacterial susceptibility to varied concentration of lemon juice-extract is directly proportional to the size of the inhibition zone (Garg *et al.*, 2010).

A study by Hindi and Chabuck (2013) reported a susceptibility of between 20-26mm diameter for *Staphylococcus* except *S. epidermidis* and a susceptibility of between 20-28 mm diameters for *Streptococcus*. Similar findings were recorded by Al-Ani *et al.* (2010); they found out that *C. limon* produced a bactericidal activity on *Staphylococcus aureus*, with a

zone of inhibition of 10 mm to 15 mm by 5% and 10% concentrations. The lowest zone of inhibition (10 mm) in this study was observed among *Bacillus* organisms at 25% lemon juice-extract concentration; other inhibition zones demonstrated by these organisms were: 13 mm at 50% lemon juice-extract concentration, 14 mm at 75% lemon juice-extract concentration and 17 mm at 100% lemon juice-extract concentration.

A sensitivity test study by Marzoog *et al.* (2015) using conventional drugs, amoxicillin was the most effective antibiotic on *E.coli* and *Proteus mirabilis* with a 20mm and 22 mm diameter inhibition zone respectively followed by tetracycline at 15mm and 8mm respectively. For, *Staphylococcus*, erythromycin was the most effective antibiotic at 31mm diameter inhibition zone followed by tetracycline at 26mm, gentamycin at 25mm and amoxicillin at 15mm. Differences in susceptibility between *Staphylococcus*, *Bacillus* and *Streptococcus* organisms can be explained by differences in the nature and extent of cell membrane damage or disruption of different cellular targets (Winniczuk and Parish, 1997). To demonstrate potency of lemon juice extract on a whole piece of African sausage, this study compared the amount of bacterial growth on a culture medium. The African sausage treated with lemon juice extract and streaked immediately and after 30 minutes showed significantly reduced amount of growth compared to the untreated “sausage” and one which was lemon juice-extract treated but streaked after 1 hour.

This therefore has shown that lemon juice-extract can be used to reduce bacterial load on African sausages, best results being within half an hour of application. Sprinkling of the juice-extract on the African sausages is, therefore encouraged, more so since it also adds flavor to the meat product and improves on appetite (personal experience). Organic acids in lemon have been identified as a possible antibacterial component (Kawaii *et al.*, 2000).



Giuseppe *et al.* (2007) noted presence of limonoids in *C. limon*, a component that is antibacterial. Other antimicrobial components identified are terpenoid, alkaloid and phenolic compounds which interact with enzymes and proteins of the microbial cell membrane disrupting their structures and changing their permeability (Friedman *et al.*, 2004; Tiwari *et al.*, 2009; Burt, 2004; Gill and Holley, 2006). The results in the present study suggests that lemon fruit juice-extract could be a valuable natural antimicrobial compound as natural preservatives for the food industry to preserve food because it exerts its activity even at very low concentrations/high dilutions. Using it will avoid application of health hazards of chemical preservatives/industrial additives.

*Escherichia coli* isolate was chosen for a pathogenicity study using mouse model. Some strains of this organism are pathogenic and can cause serious food poisoning in humans (Cheesbrough, 2006). The aim was to determine if it was pathogenic to the experimental mice. After intraperitoneal injection of isolated *E.coli* strain, no mortality was observed in all the experimental mice studied at the end of experimental period. None of the mice presented with any clinical signs. The results for the leukocyte count and erythrocyte count fell within the reference value for mice (Rosenthal, 2002) and the results for the control and experimental mice showed no significant variations ( $p>0.05$ ).

In differential leucocyte count, the study found no significant decrease or increase in mean percentage values of neutrophils, basophils, lymphocytes, eosinophil and monocytes in the controls and in test mice inoculated with various dilutions of test organism. The test isolate did not have any statistically significant effect on haematological and biochemical means ( $p \geq 0.05$ ). The serum protein and BUN parameters were within the reference range (Rosenthal, 2002).

These slight variations of serum proteins and BUN in the present study may be possibly attributed to normal body regulatory physiology or accelerated protein catabolism because of the stress of the infection and fever and/or may be secondary to the increase in globulin concentration since colloid osmotic pressure has to be maintained within normal limits by a regulatory mechanism as seen in increase in serum protein levels (Benjamin, 1978). In the present study, the results for BUN and serum protein were within the reference ranges for the mice in test and control experimental mice groups.

The congestion observed could be associated with euthanasia method used i.e. diethyl ether. Proliferation of lymphoid tissues attributed to non-specific immune response to an agent in the animals' environment as the findings were obtained in all animals including non-infected controls. Invasive *E. coli* causes peritonitis and urinary tract infection characterized by suppurative lesion comprising dead cells surrounded by a haemorrhagic zone. Systemic reactions include neutrophilia, but when it becomes overwhelming, a neutropenia with toxic granulation expected. The local inflammation may turn into bacteraemia, septicaemia with subsequent localization in internal organs especially those with end-artery circulatory system such as kidney, heart and brain.

Non-invasive *E. coli* and even commensals are capable of establishing an infection once mechanical barrier of the skin or mucus membrane is breached as occurs with perforations, surgical wound or bedsores (Kumar *et al.*, 2009). Lack of systemic reactions in mice following intraperitoneal inoculation with *E. coli* isolated from sausages may be attributed possibly due to the ability of peritoneal phagocytes to inhibit and/or kill *E. coli* (Al-Mariri, 2008). The control mice euthanized before and after experiments showed no apparent sign of infection. Their peritoneum was white, smooth and shiny.

All other organs retained normal colour, size and shape while the organs examined were normal. This showed that the mice were free from organism(s) of interest.

## 5.2 Conclusions

- The present study reveals the fact that roasted and non-roasted African sausages sold at retail outlets in Nairobi County are contaminated with bacteria pathogens. The presence of bacteria which are potentially zoonotic to humans in the roasted and non-roasted African sausages is of public health significance.
- The susceptibility pattern of the bacterial isolates from African sausages with lemon juice extract shows the high incidence of sensitive bacterial contaminants in meat.
- Diluted lemon extract juice has antibacterial activity at 25% which is the minimum inhibitory concentration in present study.
- *E.coli* bacterial load of between  $4.36 \times 10^4$  –  $4.36 \times 10^8$  obtained from African sausages proved non-pathogenic to mice.
- The *E.coli* used in pathogenicity studies was not pathogenic to experimental mice.

## 5.3 Recommendations

- Comprehensive study is needed to sample more eatery meat points in Nairobi and other areas in order to isolate, characterize and establish the bacterial load and the pathogenicity of isolated organisms to humans.
- There is also need to establish the sources of bacteria due to high bacterial load determined in the current study.
- Relevant regulatory body in conjunction with African sausage vendors should lay down food safety measures for the African sausages

- Further studies are needed to establish the mode of action of lemon juice extract whether bacteriostatic or bactericidal and how heat stable it is to determine if it can be incorporated as one of the ingredient in preparation of African sausages.

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## CHAPTER 7: APPENDICES

### Appendix I: Ethical clearance of proposal by Biosafety, Animal use and Ethics committee



## UNIVERSITY OF NAIROBI

### FACULTY OF VETERINARY MEDICINE

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Mr Henry Karoki Wambui  
c/o Dept. of Vet. Pathol. Microbiol. & Parasitol.

REF:FVM BAUEC/2016/114

20/09/2016

Dear Mr Karoki,

**RE: Approval of Proposal by Biosafety, Animal use and Ethics committee**

**Antibacterial effect of lemon extract on bacteria isolated from African sausages (mutura) sold in Nairobi County and pathogenicity of the most prevalent bacterium  
By Henry Karoki Wambui (J56/73504/2014)**

We refer to the above revised proposal that you submitted to our committee for review. We have now reviewed the proposal and are satisfied that you have satisfactorily addressed most of the issues regarding animal transport and husbandry, methods of euthanasia and biosafety measures to be observed.

We hereby approve your study as per your revised proposal

A handwritten signature in blue ink, appearing to read 'Rodi O. Ojoo'.

Rodi O. Ojoo BVM/M.Sc Ph.D  
Chairman, Biosafety, Animal Use and Ethics Committee,  
Faculty of Veterinary Medicine



**Appendix II** – Prevalence of the five genera of bacteria isolated from African sausages  
sampled from Pangani, Kangemi and Westlands, Nairobi County

Type of Organisms	Pangani		Kangemi		Westlands		Total
	Roasted	Non-roasted	Roasted	Non-roasted	Roasted	Non-roasted	
<i>Bacillus</i>	6	7	2	5	1	3	<b>24</b>
<i>Staphylococci</i>	12	10	15	6	8	11	<b>62</b>
<i>Streptococci</i>	3	2	3	0	1	3	<b>12</b>
<i>E. Coli</i>	1	1	0	0	0	0	<b>2</b>
<i>Proteus</i>	0	2	1	0	0	0	<b>3</b>
No Growth	1	0	7	1	11	0	<b>20</b>
<b>Total</b>	<b>23</b>	<b>22</b>	<b>28</b>	<b>12</b>	<b>21</b>	<b>17</b>	<b>123</b>

**Appendix III - Quantification of bacterial load of the African sausages sampled from**

**Pangani, Kangemi and Westlands, Nairobi County**

Bacterial Load	Pangani		Kangemi		Westlands	
	Roasted	Non-roasted	Roasted	Non-roasted	Roasted	Non-roasted
$1.0 - 9.9 \times 10^1$	3	0	10	2	9	1
$1.0 - 9.9 \times 10^2$	3	0	1	1	7	5
$1.0 - 9.9 \times 10^3$	7	2	4	2	1	4
$1.0 - 9.9 \times 10^4$	4	5	3	3	2	0
$1.0 - 9.9 \times 10^5$	2	4	3	1	0	3
$1.0 - 9.9 \times 10^6$	0	3	2	1	0	0
$1.0 - 9.9 \times 10^7$	0	0	1	1	0	0

**Appendix IV** – Evaluation of mean bacterial load across the three geographical areas among the roasted and non-roasted African Sausages using paired student t-test (p values evaluated at 95% confidence limits)

**Paired Samples Test**

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Pair 1 PR - KR	-0.71	3.30	1.25	-3.77	2.34	-0.572	6	0.588
Pair 2 KR - WR	0.71	3.09	1.17	-2.15	3.58	0.611	6	0.564
Pair 3 PR - WR	0.00	4.00	1.51	-3.70	3.70	.000	6	1.000
Pair 4 PNONR - KNONR	0.43	1.90	0.72	-1.33	2.19	0.596	6	0.573
Pair 5 KNONR - WNONR	-0.29	2.43	0.92	-2.53	1.96	-0.311	6	0.766
Pair 6 PNONR - WNONR	0.14	3.29	1.24	-2.90	3.18	0.115	6	0.912
Pair 7 PR - PNONR	0.71	2.98	1.13	-2.05	3.47	.633	6	0.550
Pair 8 KR - KNONR	1.86	2.85	1.08	-0.78	4.50	1.722	6	0.136
Pair 9 WR - WNONR	0.86	3.76	1.42	-2.62	4.34	.603	6	0.569

Abbreviations/Acronyms

PR- Pangani roasted,

PNOR- Pangani non-roasted,

KR- Kangemi roasted,

KNOR – Kangemi non-roasted,

WR – Westlands roasted,

WNOR – Westlands non-roasted

**Appendix V** – Determination of Mean diameters of the inhibitory zones to lemon juice extract, measured in mm of three isolates from the three main areas of study.

<b>Isolates</b>	<b>N</b>	<b>Minimum</b>	<b>Maximum</b>	<b>Mean</b>	<b>Std. Deviation</b>
Staphylococcus	12	11	20	14.75	2.958
Bacillus	12	10	18	14.00	2.954
Streptococcus	12	10	20	14.67	2.995

**Appendix VI - Comparison of mean inhibitory zones to lemon juice extract of the three isolates studied using unpaired student t-test (p values evaluated at 95% confidence limits)**

Isolates		Levene's Test for Equality of Variances		t-test for Equality of Means			
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference
<i>Staph sp.</i>	Equal variances assumed	1.630	0.248	-1.722	6	0.136	-3.250
	Equal variances not assumed			-1.722	4.448	0.153	-3.250
<i>Bacillus sp.</i>	Equal variances assumed	0.000	1.000	-0.209	6	0.842	-0.500
	Equal variances not assumed			-0.209	5.999	0.842	-0.500
<i>Streptococcus sp.</i>	Equal variances assumed	0.643	0.453	0.115	6	0.912	-0.250
	Equal variances not assumed			0.155	5.519	0.913	-0.250

**Appendix VII** – Average haematological and biochemical parameters of the test mice and control group after inoculation of various concentration of test organism (*E.coli*)

Parameters	Control mice	Treatments – Varied concentrations of test organism				
		4.36x10 <sup>8</sup>	4.36x10 <sup>7</sup>	4.36x10 <sup>6</sup>	4.36x10 <sup>5</sup>	4.36x10 <sup>4</sup>
RBC	6.95	8.34	8.1	8.345	9.35	7.505
WBC x1000	12.3	9.315	6.085	7.86	8.04	7.505
HB g/dl	12.7	15.3	14.75	15.6	17.75	14.9
HCT %	31.3	36.85	35.8	35.85	42.5	32.5
MCV (fl)	44.45	43.95	44.15	43	45.5	43.3
MCH (pg)	18.55	18.35	18.2	18.7	19	19.95
MCHC g/dl	42.15	42	41.3	43.5	41.75	45.95
RDW %	30.5	32.85	32.5	32.2	32.25	33.85
Platelets x1000	229	205	425.5	205	550.5	387
Neutrophil %	38.8	22.75	52.35	47.35	39.5	29.45
Lymphocytes %	55.85	72.55	40.4	45.45	53	63.7
Monocytes %	0.95	0.95	1.35	1.25	0.95	1.9
Eosinophils %	4.1	3.4	4.9	5.85	5.9	4.7
Basophils%	0.3	0.35	1	0.2	0.65	0.25
Serum Protein	10.95	6.65	10.7	5.8	14.6	12.35
BUN	15.26	13.45	7.1	11.2	24.75	6.1

Abbreviations/Acronyms

- RBC- Red blood cells
- MCV – Mean cell Volume
- HB – Hemoglobin
- HCT – Hematocrit
- WBC – White blood cells
- MCHC – Mean Cell Haemoglobin Concentration
- RDW – Red cell distribution Width
- BUN – Blood Urea Nitrogen

**Appendix VIII** – Haematological and biochemical parameters of individual test mice and control group after inoculation with test organism (*E.coli*)

Treatmnt	Parameters									
	RBC	WBC	MCV	MCH	Neu %	Lym %	Mon %	Eos %	serum Prot	BUN
Control 1	5.68	14.4	41.2	19.9	47.1	49.2	0.4	3.1	11.7	15.96
Control 2	8.22	10.2	47.7	17.2	30.5	62.5	1.5	5.1	10.2	14.56
10 <sup>1a</sup>	8.92	8.53	47.3	18.3	18.6	75.3	0.9	4.7	7.3	12.89
10 <sup>1b</sup>	7.76	10.1	40.6	18.4	26.9	69.8	1	2.1	6	14
10 <sup>2a</sup>	8.38	7.19	45.5	18.1	56.7	34.4	1.5	7	10.1	6.1
10 <sup>2b</sup>	7.82	4.98	42.8	18.3	48	46.4	1.2	2.8	11.3	8.1
10 <sup>3a</sup>	9.26	12.13	42.8	18.6	46	49.4	0.8	3.6	6.3	10.6
10 <sup>3b</sup>	7.43	3.59	43.2	18.8	48.7	41.5	1.7	8.1	5.3	11.8
10 <sup>4a</sup>	9.52	10.44	44.9	18	28.3	66.3	1.4	3.6	14.6	23.2
10 <sup>4b</sup>	9.18	5.64	46.1	20	50.7	39.7	0.5	8.2	14.6	26.3
10 <sup>5a</sup>	7.12	6.83	43.8	21.1	33.2	61.3	1	4.4	11.8	6
10 <sup>5b</sup>	7.89	8.18	42.8	18.8	25.7	66.1	2.8	5	12.9	6.2

Abbreviations/Acronyms

- RBC- Red blood cells
- MCV – Mean cell Volume
- HB – Hemoglobin
- HCT – Hematocrit
- WBC – White blood cells
- MCHC – Mean Cell Haemoglobin Concentration
- BUN – Blood Urea Nitrogen

**Appendix IX:** Means for haematological and biochemical parameter for test mice inoculated with the test organism - *E. coli*

<b>Parameters</b>	Minimum x10 <sup>6</sup>	Maximum x10 <sup>6</sup>	Mean	Std. Deviation
RBC	5.68	9.52	8.0983	1.07861
WBC	3.6	14.4	8.518	3.1113
HB	11.3	18.4	15.167	1.8763
HCT	23.4	42.7	35.800	5.8659
MCV	40.6	47.7	44.058	2.2645
MCH	17.2	21.1	18.792	1.0587
MCHC	36.0	48.3	42.775	3.6646
Platetes x1000	73	738	333.67	186.711
Neutrophil %	18.6	56.7	38.367	12.4130
Lymphocytes %	34.4	75.3	55.158	13.3394
Monocytes %	0.4	2.8	1.225	0.6384
Eosinophils %	2.1	8.2	4.808	2.0147
Basophils%	0.1	1.6	.458	0.4166
Serum Protein	5.3	14.6	10.175	3.2656
BUN	6.00	26.30	12.9758	6.51704

Abbreviations/Acronyms

- RBC- Red blood cells
- MCV – Mean cell Volume
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- WBC – White blood cells
- MCHC – Mean Cell Haemoglobin Concentration
- BUN – Blood Urea Nitrogen



**Appendix X:** Descriptive statistics for haematological and biochemical parameters for control and test mice inoculated with various concentrations of test organism – *E.coli*

Parameters	N	Minimum x10 <sup>6</sup>	Maximum x10 <sup>6</sup>	Mean	Std. Deviation
RBC	12	5.68	9.52	8.0983	1.07861
WBC	12	3.6	14.4	8.518	3.1113
HB	12	11.3	18.4	15.167	1.8763
HCT	12	23.4	42.7	35.800	5.8659
MCV	12	40.6	47.7	44.058	2.2645
MCH	12	17.2	21.1	18.792	1.0587
MCHC	12	36.0	48.3	42.775	3.6646
RDW	12	29.9	37.8	32.358	2.3122
Platetes x1000	12	73	738	333.67	186.711
Neutrophil %	12	18.6	56.7	38.367	12.4130
Lymphocytes %	12	34.4	75.3	55.158	13.3394
Monocytes %	12	.4	2.8	1.225	.6384
Eosinophils %	12	2.1	8.2	4.808	2.0147
Basophils%	12	.1	1.6	.458	.4166

Abbreviations/Acronyms

- RBC- Red blood cells
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**Appendix XI:** Comparing mean values of the hematological and biochemical parameters after inoculating test and control mice with various concentration of test organism using unpaired student t-test (*p* values evaluated at 95% confidence limits).

Parameter	Treatments – Varied concentrations of test organism				
	4.36x10 <sup>8</sup> cfu/g	4.36x10 <sup>7</sup> cfu/g	4.36x10 <sup>6</sup> cfu/g	4.36x10 <sup>5</sup> cfu/g	4.36x10 <sup>4</sup> cfu/g
RBC	0.424	0.470	0.467	0.202	0.716
WBC x1000	0.315	0.120	0.449	0.313	0.162
HB g/dl	0.270	0.298	0.306	0.082	0.258
HCT %	0.620	0.639	0.655	0.292	0.895
MCV (fl)	0.924	0.940	0.700	0.781	0.760
MCH (pg)	0.896	0.820	0.922	0.814	0.513
MCHC g/dl	0.985	0.905	0.847	0.956	0.619
Platelets x1000	0.814	0.391	0.885	0.246	0.132
Neutrophils %	0.226	0.285	0.416	0.965	0.413
Lymphocytes %	0.146	0.227	0.311	0.866	0.382
Monocytes %	1.00	0.556	0.714	1.000	0.436
Eosinophils %	0.711	0.764	0.551	0.547	0.463
Basophils%	0.811	0.369	0.423	0.323	0.624
Serum Protein	*0.049*	0.819	*0.029*	*0.040*	0.808
BUN	0.179	*0.022*	*0.048*	*0.031*	0.271

\* mean values that were significantly different from the control group ( $p \leq 0.05$ )

#### Abbreviations/Acronyms

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**Appendix XII:** Analysis of the differences among group means (ANOVA) for hematological and biochemical parameters in test and control mice inoculated with the test *E. coli*.

Parameters		F	Sig.
RBC	Between Groups	1.324	0.367
WBC	Between Groups	0.881	0.545
HB	Between Groups	2.575	0.140
HCT	Between Groups	0.826	0.574
MCV	Between Groups	0.195	0.953
MCH	Between Groups	0.575	0.720
MCHC	Between Groups	0.301	0.895
RDW	Between Groups	0.306	0.893
Platelets x1000	Between Groups	1.374	0.351
Neutrophils %	Between Groups	2.928	0.112
Lymphocytes %	Between Groups	2.906	0.113
Monocytes %	Between Groups	0.544	0.740
Eosinophils %	Between Groups	0.327	0.880
Basophils%	Between Groups	1.199	0.409
Serum Protein	Between Groups	35.892	0.000**
BUN	Between Groups	60.131	0.000**

\* - values which show a statistically significant effect ( $\leq 0.05$ )

Abbreviations/Acronyms

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