MICROBIOLOGICAL QUALITY AND SAFETY OF *RASTRINEOBOLA ARGENTEA* (OMENA) SOLD IN KISUMU TOWN MARKETS

BY

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DECLARATION

This thesisis my original work and has not been presented for a degree in any other University or any other award.

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To wife Rose, and children Eugene and Faith

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CDC	Centre for Disease Control and Prevention
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
EEC	European Economic Community
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. co*li
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EU	European Union
FAO	Food and Agriculture Organization
FAO/WHO	Food and Agriculture Organization/ World Health Organization
FBDs	Food Borne diseases
FC	Faecal coliform
FDA	Food and Drug Administration
FDA/CFSAN	Food and Drug Administration / Centre for Food Safety and Applied Nutrition
FPC	Fish Protein Concentrate
GHP	Good Hygiene Practices
GMP	Good Manufacturing Practices
GOK	Government of Kenya
HACCP	Hazard Analysis and Critical Control Point
HIV/AIDS	Human Immunodeficiency Virus/ Acquired Immunodeficiency Disease Syndrome
HUS	Haemolytic Uremic Syndrome
KEBs	Kenya Bureau of Standards
L-EMB	Levines' Eosin-Methylene Blue Agar
L T	Lauryl Tryptose
LVB	Lake Victoria Basin
MAR	Multiple Antibiotic Resistance
MID	Minimum Infection Dose

NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
NRC	National Research Council
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
RA	Risk Analysis
RASFF	Rapid Alert System for Food and Feeds
RSHM	Refik Saydam Hygiene Centre
SEA	Staphylococcal Enterotoxin A
SPS	Sanitary and Phytosanitary
TBT	Technical Barriers to Trade
TPC	Total Plate Count
T.SHS.	Tanzanian Shilling
UK	United Kingdom
UNICEF	United Nations International Children Emergency Fund
USDA	United States Department of Agriculture
VP	Vogas-proskauer
VTEC	Verocytotoxic *E. coli*
VRBA	Violet Red Bile Agar
WTO	World Trade Organization
XLD	Xylose Lysine Deoxycholate

ABSTRACT

The Lake Victoria *Rastrineobola argentea* (omena) is today among the most important commercial fish species, ranking second to Nile perch, *R. argentea* is a relatively cheap source of animal protein. Sundried omena has also recently found use in the animal feed industries. The sun drying of omena is carried out at the fish landing sites along Lake Victoria; the fish is spread over fishing nets and left to dry for 6 to 8 hrs. During the drying process the fish is exposed to contamination from soil, animals and personnel processing the fish. This study was to investigate microbiological quality and safety of omena sold in retail markets in Kisumu town. The study design was based on random and repeated cross sectional sampling. The study was carried out in Kisumu town, targeting 6 markets; Oile, Jubilee, Kibuye, Kondele, Nyalenda and Manyatta. Total plate count (TPC), faecal coliform (FC) and presence or absence of *Salmonella*, *Shigella* and *E. coli* were used to assess the microbiological quality of the fish. Bacterial isolates were identified by biochemical and serological techniques. Antimicrobial susceptibility to ampicillin 10µg, tetracycline 30µg, cotrimoxazole 25µg, augumentin 30µg, gentamicin 10µg, kanamycin 30µg, cefuroxime 30µg, chloramphenicol 30µg, nalidixic acid 30µg and norfloxacin 10µg was performed using the disk diffusion method. *In-vitro* conjugation and plasmid finger printing experiments were carried out to determine transferable antimicrobial resistance and the type of plasmids involved in the enteric pathogens. A total of 60 fish samples were analyzed. The findings show that the products are of low microbial quality, TPC 7.34 ± 0.30 cfu/g and FC 4.13 ± 0.32 cfu/g at 95% confidence interval (CI). All the fish were found to be contaminated with *E. coli*, and in addition 6.67% of the products sampled tested positive for *Salmonella*. *Shigella* was absent in all samples analyzed. 26.53% of *E. coli* isolates tested were resistant to two or more antimicrobial agents tested, with the highest level of resistance detected against cotrimoxazole at 38.76%. The *E. coli* multiple antibiotic resistance (MAR) index was 0.084 indicating that the contamination was not originating from a high - risk source. A plasmid of approximately 5.6kb was commonly isolated from *E. coli* isolates that showed resistance to ampicillin. Plasmids isolated were not transferable by conjugation. The presence of *Salmonella* species and occurrence of MDR *E. coli* were identified as some of the possible health risks that may be associated with omena displayed for sale in Kisumu town markets. It is important that public health workers create awareness for the need to institute good hygiene practices (GHP) and hazard analysis critical control point (HACCP) as for the purpose of ensuring fish products are produced and sold under hygienic conditions.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The fisheries sub-sector is of socio-economic importance, especially in the Lake Victoria Basin (LVB). The sub-sector provides employment, income and export earnings to the riparian communities (GOK 2000). In the year 2000, the country's fish production amounted to 202,639 metric tons valued at over KSh 7.9 billion. In the same year the country earned about Ksh 4 billion through exports. The sub-sector is also of critical importance in meeting the food security and nutritional requirements of riparian populations (Abila & Jansen 1997).

Kenya produces fish from two major sources namely capture and aquaculture fisheries. Capture fisheries account for the bulk of the production and covers fresh water (lakes, rivers, dams) and marine waters (GOK 2000). Lake Victoria is the second largest lake in the world with an approximate area of 69,000 square kilometres. The lake accounts for over 90% of fish production in Kenya (GOK 2000). The lake is shared between three East African countries: Tanzania (49%), Uganda (45%) and Kenya (6%) and has over 300 species of fish (Abila & Jansen 1997). Fish production in Kenya is exploited mainly by small-scale fishermen who generally account for 90% of the total fish catch, while commercial fisheries accounts for only 10% (GOK 2002).

Rastrineobola argentea (omena) is among the most important commercial species of Lake Victoria and ranks the second after the *Lates niloticus* (Nile Perch). In the year 2002 a total of 35,455 metric tons of omena valued at KSh 661 million was landed (GOK 2002). It is a relatively cheap source of animal protein as nourishment for man and livestock. Its demand is substantially increasing, as other protein sources like meat are getting expensive. In Tanzania, meat retails at TSh 1200/kg, but omena retails at TSh 400/kg (Nsinda 2003). In Kenya, meat retails at KSh 160/kg but omena retails at Ksh 40/kg (GOK 2002).

At the artisanal fishery along Lake Victoria, omena is basically processed by sun drying on top of fishing nets at the beaches for the purpose of preserving it and giving it a longer shelf life. The Kenya Bureau of Standards (KEBs) specifications for dried omena recommend that only fish of acceptable quality and fit for human consumption should be processed. It further specifies that the fish should be protected against contamination from dirt, sand and insects and the dried product should have a moisture content of between 5 - 7 % (Kenya Bureau of Standards 1998). However, studies have found that the final product may be heavily impregnated with sand particles and faecal matter; therefore pose a potential health risk (Abila & Jansen 1997).

The unilateral changes in the Codex Alimentarius, and demands by importers have pointed to the increasing importance of food safety and quality. As a result, effective and

adequate sanitary practices and regulation to control food borne diseases (FBDs) and ensure quality and safety of fish and fish products has been developed to enable market access for exports of fish and fish products from Kenya. However, most food safety hazards on fish and fishery products sold locally has not been characterised and understood. Food safety has also not received considerable public attention locally for several reasons; such as lack of appropriate technological expertise to trace many FBDs to specific pathogens in the various foods. Secondly, because of low incomes, consumers have not demanded higher levels of quality and safety. Thirdly, consumers often cannot detect food hazards at the time of purchase and thus cannot always indicate their demand for safe food through purchase decision. Therefore the government needs to set public policies that address these issues, possibly by setting standards and institutions for testing safety and enforcement.

Identification and characterization of possible food hazards of public health interest form an important aspect of hazard analysis. Hazard analysis is an important element of risk assessment, a component of risk analysis (RA), and provides a framework to finding appropriate solutions to public health and food safety. RA has been recognized by the joint FAO/WHO food standards programme as a necessary step to ensure that standards for food safety and public health actually reduce risk and have a scientific basis.

Antimicrobial agent resistance among pathogenic bacteria has also been recognised as an

emerging worldwide problem in both human and veterinary medicine (Ogan & Nwiika 1993, DePaola, Peeler & Rodrick 1995). Children, the elderly, and those with weakened immune systems (including cancer, HIV/AIDS, and transplant patients) are particularly vulnerable because their immune systems are not as vigorous as those of healthy adults.

In Kenya the data on health risks associated with consumption of fish and fish products particularly *Rastrineobola argentea* (omena) is lacking. This study therefore seeks to assess the microbial quality of sundried omena and the associated public health risks (safety). The study sought to determine the level of bacterial contamination of the products, the response of isolates to antimicrobial agents and presence of resistance genes by plasmid DNA fingerprinting and conjugation experiments. The information generated will assist in the understanding of health risks associated with omena products sold in Kisumu town. It will further help in the evaluation of the existing sanitary practices and guidelines and suggest possible measures that can improve omena quality and safety for human consumption and livestock feeds. The findings will also help in the reduction of post-harvest losses due to microbial contamination and help fish traders to increase their incomes.

1.2 Industrial utilization of fish products

1.2.1 Fishmeal

Fishmeal is the end product obtained by drying and grinding fish or fish waste to which no other matter has been added. The bulk of the world's production of fishmeal is used for incorporation into compound feeds for livestock such as poultry, pigs and fish. In Kenya the animal feeds industry started using omena as the main source of crude protein in feeds in the early 1990's (Abila & Jansen 1997)

Contaminated animal feeds have been identified as important sources of pathogens among food animals (Ranken 1984). Jones et al. (1991) found that in the United States of America, animal feeds might be the ultimate source of S*almonellae* to breeder/multiplier houses. Colibacillosis is the common bacterial infection in poultry (Barnes & Gross 1997) and is caused by *E. coli* serotypes O_1 and O_{55} (Allan, Hurk, Van & Potter 1993). An important measure of fishmeal quality therefore should be the absence of food-poisoning microorganisms that may contaminate animals meant for human consumption (Ranken 1984). The microbiological concerns of fishmeal industry have mainly been directed to preservation of raw materials and contamination of the finished product (Hobbs & Roberts 1993).

1.2.2 Fish protein concentrate

Fish protein concentrate (FPC) is any stable fish preparation intended for human consumption, in which the protein is more concentrated than in the original fish (FAO corporate document repository http://www.fao.org/wairdocs/tan/x5917e01.htm). FAO has classified FPC in to three types; Type A, is a virtually odourless and tasteless powder having a maximum total fat content of 0.75%; type B, is a powder having no specific limit as odour or flavour, but may have fishy flavour and a maximum fat content of 3% and type C, is normal fish meal produced under satisfactorily hygienic conditions (FAO corporate document repository http://www.fao.org/wairdocs/tan/x5917e01.htm).

The manufacture of FPC involves removal of the water and some or all the fat. The methods currently in use involve use of chemical solvents to remove the water, fat and fishy-tasting components; ethanol, propanol or ethylene dichloride may be used (FAO corporate document repository http://www.fao.org/wairdocs/tan/x5917e01.htm).

FPC contains about 80% proteins and therefore, the production of FPC represents one of the ways by which underutilised fish may be upgraded in value, in that it provides acceptable high protein adjuncts that can be added to a variety of foods.

1.3 Microbial health risks associated with food

1.3.1 Food borne diseases

Food borne diseases (FBDs) have a major public health impact (Käfterstein 2003). The World Health Organisation (WHO) defines FBDs as "diseases of infectious or toxic nature caused by, or thought to be caused by the consumption of food or water" (Le Loir, Baron & Gautier 2003). More than 250 FBDs have been described. FBDs contribute to high health care cost and reduced access to export market (Bryan 1978, Turnbull 1979, WHO 2002). In the United States of America each year, seven food borne pathogens (*Campylobacter jejuni*, *Clostridium perfringens*, *E. coli* O157: H7, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus*, and *Toxoplasma gondii*) cause an estimated 3.3 – 12.3 million infection cases and up to 3900 deaths. Their cost in human illness has been estimated at US$ 6.5 – 34.9 billion annually (WHO, 1997). Similarly FBDs have also been identified as some of the most important underlying factors for malnutrition and, indirectly, for respiratory tract infections in developing countries (Käfterstein 2003). Repeated episodes of FBDs over a period of time could lead to malnutrition, with a serious impact on the growth and immune systems of infants and children and therefore vulnerable to other diseases, including respiratory tract infections (Käfterstein 2003). As a consequence, FBDs have been viewed as potential threats to quality of life, food security and economic growth of developing countries (Pinstrup-Anderson 1999, WHO 2002, FAO/WHO 2005).

The epidemiology of FBDs is rapidly changing as newly recognized pathogens emerge and well-recognised pathogens increase in prevalence or become associated with new food vehicles (Käfterstein 2003). The factors contributing to the emergence of FBDs are: changes in human demographics, behaviour, technology, industry, international travel and commerce; microbial adaptation; economic development and land use; and the breakdown of public health measures (Institute of Medicine 1992).

FBDs vary in their health consequences depending on the disease agent, the stage of treatment, and the duration of the illness, as well as the age and susceptibility of the individual. Acute symptoms include: diarrhoea, vomiting, abdominal pain, crumps, fever, and jaundice (Käfterstein 2003). Some FBDs can however cause serious and chronic sequelae on the cardiovascular, renal, articular, respiratory, or immune system. For example, *E. coli* O157:H7 infection may develop haemolytic uremic syndrome (HUS) the most common cause of acute kidney failure in children in the United States. Salmonellosis may cause invasive disease or reactive arthritis (Swerdlow et al. 1990, Altekruse, Hyman, Klontz, & Tollefson 1994, Boyce et al. 1995).

The true incidence of diseases transmitted by food is generally not known, as data are only available for confirmed cases (Huss 1994). According to WHO, over 4000 million episodes of diarrhoea occur worldwide annually, the great majority in the developing world (Loaharanu 2001). Statistics from industrialized countries shows that up to 10% of

the population may suffer annually from a foodborne illness (Loaharanu 2001).

In the United States, between 1973 and 1987 a total of 7,458 FBD outbreaks involving 237,545 cases were reported to Centre for Disease Control and Prevention (CDC) (Bean & Griffin 1990). A specific food vehicle was identified in only 3,699 (50%) of the outbreaks. Of these items seafood was most frequently associated with disease and accounted for approximately 10% (Bean & Griffin 1990). The pathogenic bacteria responsible for fish-associated outbreaks were *C. botulinum, V. parahaemolyticus, Shigella, Salmonella, V. cholerae, C. perfringens* and *S. aureus* (Bryan 1980, 1987). Available data from the EU member states indicate that disease outbreaks from fish and shellfish between 1983 and 1992 ranged from 1.9% of the total food-borne outbreaks in United Kingdom to 12.4% in Denmark (FAO/WHO 1990, 1992, 1995). When the known food source was identified the range of fish and shellfish outbreaks was from 4.45% in the United Kingdom to 16.1% in Finland. From 1987 to 1996, a total of 1475 seafood-borne disease outbreaks resulting in 33,253 cases were recorded in Japan (Japan Ministry of health and Welfare 1997). Fish and shellfish accounted for 93 cases, *V. parahaemolyticus* caused 75% of bacterial disease cases while *Salmonella*, *S. aureus*, *E. coli* and *C. perfringens* were the other major causes.

In Africa little in the way of food borne surveillance is done (WHO 1997). As a result, the data are extremely scarce. However an acute illness directly linked to fish products

was reported in Tanzania in the year 1991, where a botulism outbreak claimed at least 18 lives following consumption of locally made fish meal. In Egypt in 1994 an out break of *E. coli* O157 was traced to hamburgers and dairy products (WHO 1997).

In Kenya food borne illness are a problem although no studies have been carried out to estimate the health impact associated with them (FAO/WHO 2005). Generally food borne outbreaks in Kenya have affected productivity, income generation, expenditure on health and loss of markets due to bans relating to food safety concerns (FAO/WHO 2005). Table 1 below show some incidences related to food borne illnesses that were observed in the year 2004 in Kenya.

Table 1: Incidences of some food borne illnesses reported in Kenya in the year 2004 source (FAO/WHO 2005)

DISEASES	INCIDENCES
Gastroenteritis	722,275
Typhoid	643,151
Dysentery	600,660
Aflatoxin poisoning	323
Brucellosis	198
Cholera	56

1.3.2 Antimicrobial resistance

Resistance to antimicrobial agents among pathogenic bacteria has emerged as an important problem in both human and veterinary medicine (Ogan and Nwiika 1993,

DePaola, Peeler & Rodrick 1995). Infections resistant to antibiotic treatments have serious consequences for public health. Antibiotic-resistant bacteria may keep people sick longer, and sometimes people are unable to recover at all. Children, the elderly, and those with weakened immune systems (including cancer, HIV/AIDS, and transplant patients) are particularly vulnerable because their immune systems are not as competent as those of healthy adults.

The incorporation of antimicrobial agents into commercial livestock and poultry feed at sub-therapeutic doses is believed to be one of the practices that have enhanced selection of resistant bacteria (Van den Bogaard, Lodon, Driessen & Stobberingh 2001). While the use of antibiotics in medicine and agriculture clearly stimulates the proliferation of antibiotic resistance, exposure to environmental pollutants and changes in nutrient composition may also lead to selective pressures favouring certain organisms or genotypes. Recent studies have found that toxic metal exposure may effectively select for antibiotic resistant bacteria (Davidson 1999, McArthur & Tuckfield 2000). Other studies have also indicated that resistance to clinically relevant antibiotics are widespread in aquatic bacteria, including potential human pathogens (Heuer et al. 2002, Furushita et al. 2003, Kelsey et al. 2003). *E. coli*, *Salmonella*, *Shigella, Vibrio*, and other potential human pathogens common in coastal waters and sediments (NRC 1999), have exhibited multi-drug resistance (Denton et al. 1998, Hanberger et al. 2001), suggesting that fish and fishery products that come in contact with such drug resistant bacteria may thus act as possible carriers.

1.4 Safety of fish and fish products

Many people are turning to fish as a healthy alternative to red meat. However, consumption of fish and fish products may also cause disease due to infection or intoxication and therefore the need to protect the public. Standards and regulations have been developed to ensure that food purchased by the consumers is healthy, safe and wholesome. A variety of agencies cooperate to define these standards and regulations. Some of the agencies operate at an international level for example Food and Agriculture Organization (FAO), World Health Organization (WHO) and United Nations International Children Emergency Fund (UNICEF) (Pelczar, Chen & Krieg 1993). In Kenya the Kenya Bureau of Standards (KEBs), Ministry of Health, the Food and Drugs Licensing Board, among others, have developed specific microbiological criteria for food quality assurance.

The way in which public agencies approach food safety has changed considerably during the past decade. While traditional quality control approaches are unable to eliminate quality problems, preventive strategies based on thorough analysis of prevailing conditions have been found to provide assurance that objectives of a quality assurance programme are being met (Huss 1994). In 1995 the FDA mandated the use of hazard analysis critical control point (HACCP) in fish processing plants, thus reflecting the growing importance of preventing and controlling hazards before they reach the consumer (Buzby & Crutchfield 1997). In Kenya the fish processing plants adapted the HACCP system for safety management in 1998 following export bans on fish products to

the EU market (Ogwan'g, Muchiri & Thakor 2005). However, the system is not applied at the artisanal fish processing level and local markets. Therefore fish products produced at this level may be contaminated with pathogens.

Rules that govern international trade are those agreed upon during the Uruguay Round of Trade Negotiations and apply to members of the World Trade Organisation (WTO) (FAO 1998). The Uruguay Round of Trade Negotiations was the first to deal with the liberalization of trade in agricultural products, an area excluded from previous rounds of negotiations. Significant implications for the Codex Alimentarius Commission arise from the Final Act of the Uruguary Round: the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS agreement) and the Agreement on Technical Barriers to Trade (TBT Agreement) (FAO 1998).

With respect to food safety matters, the rules are set out in the Agreement on the Application of Sanitary and Phytosanitary Measures (the SPS Agreement). The overall objective of the SPS Agreement is to permit countries to take legitimate measures to protect the life and health of their consumers (in relation to food safety matters), while prohibiting them from using those measures in a way that unjustifiably restricts trade. It requires that such measures be based on science and implemented in an equitable and transparent manner (FAO 1998). It is against this background that developed countries have instituted measures to protect the public.

In Europe, numerous food laws have been developed by the EU parliament that lay down the health conditions for the production and placing on the market food of animal origin. The Regulation (EU) No 854/2004 of 29 April 2004 lays down specific rules for the organisation of official controls on products of animal origin intended for human consumption, whereas Regulation (EU) No 882/2004 of 29 April 2004 prescribes official controls performed to ensure the verification of compliance with feed and food laws, animal health and animal welfare rules.

The regulation (EU) No 178/2002 of 28 January 2002 lays down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. The general objectives of this food law is to achieve high levels of protection of human life and health and protect consumer interests, but also achieve the free movement in the community of food and feed manufactured or marketed according to the general principles and requirements of the food laws (Regulation (EU) No 178/2002).

The European Food Safety Authority (EFSA) provides scientific advice and technical support for the community's legislation and policies in all fields, which have a direct or indirect impact on food and feed safety (Regulation (EU) No 178/2002). To enable EFSA to perform its task of monitoring the health and nutritional risks of foods effectively, a rapid alert system for notification of a direct or indirect risk to human health

was established (RASFF Annual Report 2005).

The Rapid Alert System for Food and Feeds (RASFF) is used to notify all member countries of the EU when food a product is detected as source of danger to health (RASFF Annual Report 2005). The source of danger and the country of origin are reported. According to the RASFF Annual Report (2005) the numbers of notifications transmitted through the RASFF have risen from 698 in 1999 to 6,897 in 2005, similarly, 195 alerts were related to fish and fish products and represented 20% of all food alerts reported.

In Kenya, the regulations governing the production and placing of fish and fish products on the market is entrenched in the Fisheries (Fish Quality Assurance) Regulations, 2000. The conditions are adapted from the obsolete European Unions Council Directive 91/493/EEC. The regulations provide for various conditions governing landing of fish, processing (filleting and smoking), storage, transport, health and monitoring of production conditions. Other important regulations governing the hygiene and marketing fish products include: KS 05-423: Kenya Code of Practice for Handling, Processing and Distribution of Fish; The Public Health Act, Cap 242 and The Food, Drugs and Chemical Substances Act, Cap 254 of the Laws of Kenya. However, these standards are hardly observed and fish and fishery products are frequently exposed to potential health hazards by the use of untreated water, unhygienic handling, processing and offer for sale.

1.5 Bacteria associated with fresh and dried fish

The surface flora on fishery products reflects the environmental flora where the products have passed through (Shewan 1977). The bacterial flora found on newly caught fish is dependent on the water in which the fish is caught. Psychrotrophic Gram-negative rod-shaped bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella* and *Flavobacterium* dominate the bacterial flora on temperate water fish (Hobbs & Hodgkiss 1982). Members of the *Vibrionaceae (Vibrio* and *Photobacterium)* and the *Aeromonadaceae (Aeromonas spp)* are also common aquatic bacteria and typical of the fish flora (Shewan 1977). Mesophilic *Enterobacteriaceae* have been found to dominate fish caught in polluted waters (Gram 1992).

Bacteria have been found to be involved in the spoilage of fish and fish products (Liston 1980, Gram 1989). In fresh fish the initial loss of quality is brought about by autolytic deterioration due to the action of enzymes present in the gut and in the flesh of the fish while spoilage is mainly due to the action of bacteria (Huss 1994). In salted fish spoilage is by salt-tolerant or halophilic bacteria of the genera *Serratia*, *Micrococcus*, *Bacillus*, *Alcaligenes*, *Psuedomonas* and others, which often cause discolouration, a red/pink colour being common. *Halococcus* and *Hallobacterium*, are strongly proteolytic and produce off-odours and off-flavours in the product (Gram & Huss 1996). Molds are the chief spoilage organisms in smoked and dried fish (Frazier & Westhoff 1988). It has been demonstrated that spoilage rates of fish and hence shelf life depend on the temperature and conditions of storage. Lima dos Santos (1981) noted that deterioration

processes were quicker at higher temperatures. However, Huss (1994) suggested that rapid catch handling on board and storage of products under anoxic conditions (vacuum packed or modified atmosphere packed) or refrigeration and use of antioxidants can prevent quality losses. Similarly Jørgensen, Gibson & Huss (1988) found hygiene measures aimed at controlling contamination of fish and fish products to greatly influence spoilage rates and shelf life.

The importance of fish as vehicle for human infection and intoxication, and the ability of pathogens to survive long periods of storage in frozen fishery products has been documented (Bryan 1980, Huss 1994). Microbial evaluation of on-line and finished products indicate that bacterial contamination occurs primarily through exposure to polluted environments or cross contamination during landing, transport, processing, storage, or during preparation for consumption (Huss 1994, Dillon, Patel & Martin 1994, Ogwan'g, Muchiri & Thakor 2005, Mwambazi 2005). Huss (1994) classified seafood-borne pathogenic bacteria into two groups based on how they contaminate the fish products, as indigenous bacteria or non-indigenous bacteria.

1.5.1 Indigenous bacteria

Indigenous bacteria are common and widely distributed in the aquatic environments in various parts of the world with the water temperature having a selective effect (Huss 1994). Thus the more psychrotrophic organisms (*Clostridium botulinum* types E, B and

F and *Listeria*) are common in arctic and colder climates, while the more mesophilic types (*Vibrio cholerae* and *V. parahaemolyticus*) represent the natural flora on fish from coastal and estuarine environments of temperate or warm tropical zones. Other members of this group are *Aeromonus hydrophila* and *Plesiomonas shigelloides*.

1.5.1.1 *Clostridum botulinum*

Clostrdium botulinum is a Gram – positive, obligatory anaerobic, endospore-forming bacillus. The organism is widely distributed in soil, aquatic sediments and may be found in the intestines of man and other animals including fish (Huss 1980). *C. botulinum* produces toxins, which cause a neuroparalytic illness commonly referred to as botulism. Seven distinct types of botulinum toxins (type A-G) are recognised however, botulism in man is usually caused by types A, B and E and more rarely F and G (Cheesbrough 2000).

In food-borne botulism, the food becomes contaminated with spores from the environment, which are not destroyed by the initial cooking or processing. If the food is then kept in conditions appropriate for growth, the spores may germinate, leading to production of toxin (Brown 2000). Increased speed of toxin production is noted at temperatures above 3^0C until an optimum temperature of 25 – 30^0C is reached. However, the rate of toxin production is also greatly dependent on the actual spore load. Huss (1981) observed that out of the 165 outbreaks of botulism caused by fish products, lightly preserved products (smoked, fermented) represented by far the most dangerous group.

1.5.1.2 *Vibrio* species

The three *Vibrio* species of primary significance in food borne illnesses are *V. cholerae* (serogroup O1, non – O1, and recently, O139), *V. parahaemolyticus*, and *V. vulnificus*. Another species, *V. mimicus* formerly included in the species *V. cholerae* has also been recognized as pathogenic (Davis et al. 1981). The pathogenic species are mostly mesophilic, generally occurring in tropical waters and in highest numbers in temperate waters during summer months (Huss 1994, Eley 1994).

V. *parahaemolyticus* usually causes gastroenteritis or diarrhoea of sudden onset and varying severity (Farmer, Hickman – Brenner & Kelly 1985, FDA/CFSAN 2001). Food poisoning associated with this organism is almost always associated with consumption of raw or lightly cooked contaminated marine fish (FDA/CFSAN 2001). Proper cooking of seafood is the only method currently available to inactivate *V. parahaemolyticus*, though it will not affect any preformed thermostable Kanagawa haemolysin, which would remain stable in the cooked food (Eley 1994). Cooking is obviously not effective, however, if cross-contamination with raw products is allowed to take place following the cooking process (Bryan 1980). Strict hygiene measures are also essential.

V. cholerae occurs in two serotypes, the O1 and the non-O1 (Huss, 1994). The serovars O group 1 contains two serological groups based on variations in the O or somatic antigen factors, namely Ogawa (factor A and B) and Inaba (factor A and C). These

serotypes may exist in either the classical or E1 Tor biotype. *V. cholerae* serogroup O1 is the causative agent of, epidemic cholera (Farmer, Hickman – Brenner & Kelly 1985; Peterson 2002). Other serovars, non-O1, do not present the same health threat as does *V. cholerae* O1 but can be responsible for intestinal infection or gastroenteritis (Morris et al. 1981). Cholera is usually a water-borne disease characterised by gastroenteritis. However, food-borne and nosocomial outbreaks are also important and person – to – person transmission may occur under conditions of extreme crowding and poor hygiene (Farmer, Hickman – Brenner & Kelly 1985). Following ingestion of *V. cholerae* and passage through the stomach, the bacteria multiply and produce an enterotoxin that either stimulates the mucosal cells to secrete large quantities of isotonic fluid, or increases the permeability of vascular endothelium (Varman & Evans 1991).

V. cholerae has been found to occur as a natural resident of aquatic environments in cholera-free areas and its presence is not necessarily associated with fecal contamination or sporadic human infections (Huss 1994). It is therefore to be expected that freshly harvested seafood might be harbouring this pathogenic *Vibrio* spp. In cholera endemic areas, seafood could be contaminated with *V. cholerae* reaching the marine environment through sewage discharge (Bryan 1980). Post harvest contamination could occur due to poor sanitary conditions in processing units and when carriers of *V. cholerae* handle seafoods. Epidemiological investigations have incriminated shellfish for out breaks of cholera infection (Morris & Black 1985). Fish and shellfish form a suitable substrate for survival of *V. cholerae* and other studies have indicated that the organism persists in fish

and shellfish at room temperature for 2 to 5 days and under refrigeration for 1 to 2 weeks (Ganowiak 1990). This allows relatively low initial numbers to increase drastically under improper conditions of harvesting, processing, distribution and storage.

1.5.1.3 *Aeromonas* species

These are Gram-negative, facultatively anaerobic, non-spore forming bacilli, which are oxidase positive. The genus contains species (*A. hydrophila, A. sobria and A. caviae*) pathogenic for frogs, fish and mammals including humans (Huss 1994). The motile *Aeromonas* spp, particularly *A. hydrophila* has received increasing attention as a possible agent of food-borne diarrhoea (Eley 1994). However, the role of *Aeromonas* as an enteric pathogen is not fully clarified. Species of *Aeromonas* produce a wide range of toxins such as cytotoxins, enterotoxins, haemolysins and a tetrodotoxin-like sodium channel inhibitor (Varnam and Evans 1991, Eley 1994). The role of these toxins in producing disease in man is unresolved and currently no method is available for differentiating between apathogenic environmental strains and pathogenic strains.

Aeromonas is ubiquitous in fresh water environments, but may also be isolated from saline and estuarine waters (Hobbs & Roberts 1993). These organisms can also be found in untreated sewage excreted from both symptomatic and asymptomatic persons and from farm animals (Knøchel 1989). There are two major problems associated with the control of *Aeromonas* species, their frequent presence in food and the fact that many strains are

psychrotrophic, meaning that they are capable of reaching high numbers even in refrigerated foods (Eley 1994). Fortunately, these are relatively heat-sensitive organisms and heat processing is usually effective in killing them (Condon, Gracia, Otero & Sala 1992).

1.5.1.4 *Plesiomonas shigelloides*

These are Gram-negative, facultatively anaerobic, non-spore forming bacilli, which are oxidase positive, are widespread in nature but are mostly associated with both fresh and sea water (Arai, Jkejima & Itoh 1980). Due to its mesophilic nature, there is a marked seasonal variation in the numbers isolated from waters, being much higher during warmer periods. Transmission by animals and intestines of fish is common and it is likely that fish are the primary reservoirs of *Plesiomonas shigelloides* (Koburger 1989).

Plesiomonas shigelloides may cause gastroenteritis with symptoms varying from mild illnesss of short duration to severe diarrhoea. However, it is possible that only a few strains carry virulent characteristics as observed by Herrington et al. (1987); volunteers ingesting the organism do not always become ill. Very few foods have been implicated with any degree of certainty in *P. shigelloides* food poisoning (Eley 1994). Those that have been implicated include fish, crabs and oysters.

1.5.1.5 *Listeria* species

Six species of *Listeria* are currently recognised, but only 3 species *L. monocytogenes, L. ivanovii and L. seeligeri* are associated with disease in humans and or animals (Huss 1994). However, human cases involving *L. invanovii* and *L. seeligeri* are extremely rare with only four reported cases. *L. monocytogenes* causes listeriosis, a severe and often fatal illness, to which certain populations (e.g. pregnant mothers, new-borns, immunocompromised individuals and transplant recipients) may be increasingly susceptible (FDA/CFSAN 2003). The organism is ubiquitous and is widespread in the environment, where it may survive for long periods. Its presence has been reported in animal and human faeces, and consequently in sewage. The organism has also been isolated from soil, surface water, vegetation, foods including fish and fishery products and domestic kitchens (Ryser & Marth 1991; Fuchs & Reilly 1992). Most of these environmental strains are probably non-pathogenic.

Listeria. monocytogenes is primarily transmitted to humans through foodstuffs contaminated during production and processing (Ryser & Marth 1991). Frequent isolations from seafood (Rørvik & Yndestad 1991) and the demonstration of growth potential in chilled (4 ^{0}C) smoked salmon (BenEmbarek & Huss 1992, Fuchs and Reilly 1992) are evidence that seafood may be important in the transmission of *L. monocytogenes*. However, so far there have only been a few documented cases of seafood involvement and two cases where seafood involvement was suspect (Lennon et al. 1984, Facinelli et al. 1989, Frederiksen 1991).

Listeria. monocytogenes is particularly difficult to control, since it is widespread in the environment, and because it possesses physiological characteristics (e.g. multiplication at refrigeration temperatures) that allow growth under conditions that are usually adverse for most other pathogenic bacteria. However, the qualitative level of *L. monocytogenes* contamination on fish and fishery products can be maintained at very low levels (<1 - 10/g) by proper GMP and factory hygiene (Huss 1994).

1.5.2 Non-indigenous bacteria

Non-indigenous bacteria consist of *Salmonella* spp, *Shigella* spp, *Esherichia coli*, *Staphylococcus aureus* and *Clostridium perfringens*. Fish caught from unpolluted waters are generally free of these pathogenic bacteria but may become contaminated during subsequent handling and processing (Bryan 1980, Huss 1994).

1.5.2.1 *Salmonella* species

These are Gram -negative, facultatively anaerobic, non-spore forming bacilli that can be split into more than 2000 serotypes according to a system based on somatic (O) capsular (Vi) and flagella (H) antigens, known as the Kauffmann-White scheme (Popoff, Bockemuhl & Brenner 2000). These mesophilic organisms are widely distributed in nature and are commonly found in the intestinal tracts of animals and human beings and in environments polluted with animal or human excreta (Jay 1992, FDA 2003). Survival

in water depends on many parameters such as ecological (interaction with other bacteria) and physical factors (temperature). Rhodes & Kater (1988) demonstrated that *Salmonella* spp can multiply and survive in the estuarine and tropical fresh water environments for weeks. Reilly, Twiddy & Fuchs (1992) suggested that there is a great risk of *Salmonella* infection from fish and shellfish gathered in polluted water. Contamination of fish and fishery products by *Salmonella* may also occur through improper plant sanitation or more probably through improper personal sanitation of workers, who having had salmonellosis often become carriers of the organism for a period of time after symptoms have gone (Ganowiak 1990, Huss 1994).

The principal symptoms of salmonellosis are non-bloody diarrhoea, abdominal pain, fever, nausea and vomiting which generally appear may be 24hrs after ingestion. However, symptoms may vary considerably from grave typhoid-like-illness to more serious complications. The infective doses in healthy individuals vary according to serovars, foods involved and susceptibility of the individuals. There is evidence for a minimum infective dose (MID) of as little as 20 cells (Varnam & Evans 1991) while other studies have consistently indicated > 10^6 cells.

Seafood is however a much less common vehicle for *Salmonella* than other foods, and fish and shellfish are responsible for only a small proportion of *Salmonella* cases (Ahmed 1991). Most seafood is cooked prior to consumption and these products pose minimal

health risks to the consumer except by cross-contamination in kitchens. This is borne out by epidemiological evidence presented by Ahmed (1991), reporting on 7 outbreaks of seafood-borne salmonellosis in USA in the period 1978 – 1987. Three of these outbreaks were due to contaminated shellfish including 2 outbreaks after consumption of raw oysters harvested from sewage-polluted waters.

1.5.2.2 *Shigella* species

The genus *Shigella* is composed of Gram-negative, facultatively anaerobic, non-spore-forming organisms that do not ferment lactose and are non-motile. This genus consists 4 distinct species i.e. *Sh. dysenteriae, Sh. flexneri, Sh. Boydii* and *Sh. Sonnei* (Cheesbrough 2000). The organism is host-adopted to humans and higher primates, and its presence in the environment is associated with faecal contamination (FDA 2001). *Shigella* strains have been reported to survive in water for up to six months (Wachsmuth & Morris 1989).

Shigella food poisoning (shigellosis), which is an infection of the gut, may vary in severity from asymptomatic infection to fulminating dysentery (Guerrant 1985). The severity of symptoms depends on the species of *Shigella* implicated. When the causative organism is *Sh. dysenteriae*, there is often abdominal pain, fever, frequent passage of bloody/fluid stools, and the patient may also have headache, nausea and undergo prostration. The incubation period may vary from 1 – 7 days and symptoms may persist for 10 – 14 days or longer. *Shigella* spp is highly infectious as low infective dose: 100

bacteria or less can produce disease. Death in adults is rare, but the disease in children can be severe (Huss 1994). Recent estimates show that annually about 164.7 million episodes of *Shigelosis* are reported worldwide, the developing countries account for 163.2 million cases with 1.1 million deaths, 61% involving children less than 5 years (Cheesbrough 2000).

The majority of outbreaks of shigellosis worldwide are associated with drinking contaminated water and person-to-person transmission may also spread the disease by the faecal-oral route (Eley 1994, FDA 2003). However, fish and shellfish salads are sometimes implicated as vehicles of outbreaks of shigellosis (Bryan 1980, Huss 1994). This happens only when a human carrier of *Shigella*, who does not practise adequate personal hygiene and whose hands consequently become contaminated with faeces, prepares fish or shellfish products that are not subsequently heated to temperatures that kill shigellae. Eley (1994) proposed that preventive procedures should emphasise on personal hygiene of workers, through cooking or reheating (where applicable) and rapid cooking and proper refrigeration.

1.5.2.3 *Escherichia coli*

Escherichia coli is part of the natural flora of the intestinal tract of man and warm-blooded animals (Huss 1994). Generally the *E. coli* strains are intestinal commensal organisms, which on occasions can become opportunistic pathogens when transferred to

other sites in the body. However, within the species, there are at least 4 types of pathogenic strains, Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. co*li (EIEC), Enterotoxigenic *E. coli* (ETEC) and Enterohaemorrhagic *E. coli* (EHEC)/ Verocytotoxic *E. coli* (VTEC or *E. coli* O157: H7) (Eley 1994). Pathogenic strains of *E. coli* produce disease of the gut, which vary in severity depending on a number of factors such as type of strain, susceptibility of victim and degree of exposure.

E. coli may be isolated in environments polluted by faecal material or sewage, and the organism can multiply and survive for a long time in this environment (Rhodes & Kater 1988, Jimenez, Munir, Toranzos & Hazen 1989). However, it was demonstrated that *E. coli* can also be found in unpolluted warm tropical waters, where it can survive independently (Jimenez, Munir, Toranzos & Hazen 1989). There is no indication that seafood is an important source of *E. coli* infection (Ahmed 1991). Most infections appear to be related to contamination of water or products during processing and subsequent handling.

1.5.2.4 *Staphylococcus aureus*

These are Gram-positive cocci, non-spore forming which grow in clusters, aerobically and anaerobically at optimum temperatures of 37^0C (Hobbs and Roberts 1993). They are coagulase and deoxyribonuclase (DNAse) positive. The organism can be found in air, water, dust, milk and sewage (Le Loir, Baron & Gautier 2003). However, the main

reservoir is the human nose, throat and skin. The human carrier rate may be up to 60% of healthy individuals with an average of 25-30% of the population being positive for enterotoxin – producing strains (Ahmed 1991). Staphylococcal food poisoning is caused by the ingestion of a preformed enterotoxin produced by growth of the bacteria in food (Frazier and Westhoff 1988). The enterotoxin is very stable being resistant to proteolytic enzymes and heat. Eight serologically distinct types (A, B, C_1, C_2, C_3, D, E and F) have so far been recognised (Eley 1994). However, staphylococcal enterotoxin A (SEA) is frequently involved and is found in the food associated with approximately 75% of out breaks due to this organism.

Fish may be contaminated with *S. aureus* via infected food handler or from another source previously contaminated by humans (Ganowiak 1990). To avoid contamination and growth, good sanitary conditions and temperature control are necessary. *Staphylococcus aureas* is mesophilic with a minimum growth temperature of 10^0C, but higher temperatures (>15^0C) are required for toxin production (Huss 1994).

Staphylococcus aureus competes poorly with other food-spoilage microorganisms and raw, naturally contaminated food is unlikely to be toxic unless severely spoiled (White & Hall 1985). Exceptions to this are foods containing fairly high levels of salt, such as seafood, which may select growth of salt tolerant *Staphylococcus*, thus enabling toxic levels to be reached before obvious spoilage has occurred (Bryan 1980). *Staphylococcus*

aureus is particularly resistant to freezing (White & Hall 1985). Therefore, freezing cannot be relied upon to give a significant reduction in the level of contamination from *S. aureus*, and prolonged frozen storage will not reduce the potential for growth during subsequent thawing (White & Hall 1985). Canned seafood has been implicated in several outbreaks of *Staphylococcus* enterotoxin poisoning (Ababouch 1992). This has prompted research to assess the validity of thermal processes used in the fish canning industry. It has been demonstrated that *S. aureus* can survive in thermally processed fish packed in oils.

1.5.2.5 *Clostridium perfringens*

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacillus. The organism is widely distributed, occurring in soil, dust and among the intestinal microflora of warm-blooded animals (Brynestad & Granum 2002). Consequently, the organism is a common contaminant of raw foods and food ingredients; because of its ability to produce resistant spores, which may persist in food products. Based on surface antigens and major toxins produced, five types of *C. perfringens* (A - E) are recognised (Cheesbrough 2000). Human disease is caused by types A and C; other types cause diseases in animals. In food poisoning out-breaks caused by *C. perfringens*, large numbers of the organism (10^6 or more/g) are normally found in the suspected food (Bryan 1980, Eley 1994). The organisms multiply when they reach the gastrointestinal tract followed by sporulation with subsequent release of enterotoxin (Cheesbrough 2000). The toxin damages epithelial cells and inhibits the absorption of glucose, which causes an influx of sodium

and chloride ions and water (Eley 1994). This results in excess fluid movement into the lumen of the gut, leading to diarrhoea. *Clostridium perfringens* can contaminate seafood from water, soil, mud or through contact surfaces (Ganowiak 1990). The organism has received more attention in recent years as a food-borne poisoning in which cooked fish have been implicated.

1.6 Indicators of food safety

In general microbial indicators have been used to assess food safety and sanitation (Jay 1992). Indicator organisms have more often been employed to reflect the microbiological quality of foods relative to product shelf life or their safety from food-borne pathogens. Indicator microorganisms are used to predict the presence of and or minimise the potential risk associated with pathogenic microbes. For instance total and faecal coliforms have been used extensively for many years as indicators for determining the sanitary quality of foods (Caplenas & Kanarek 1984). The faecal coliform group consists mostly of the *E. coli* but some other enterics such as *Klebsiella* spp may be considered as a member of this group (FDA 2003). As a result *E. coli* has emerged as an indicator of faecal contamination. However, recent studies have suggested that *E. coli* may not be a reliable indicator in tropical and subtropical environments due to its ability to replicate in contaminated soils (Solo-Gabriele, Wolfert, Desmarais & Palmer 2000, Desmarais, Solo – Gaberiele & Palmer 2002).

Krumperman (1983) however, developed a procedure which distinguishes between *E. coli* originating from high–risk environments and *E. coli* originating from other sources, thus providing a quantum of definition not possible with conventionally used laboratory procedures. According to Krumperman (1983) the greatest risks of faecal contamination to humans originate from humans, poultry or swine. Human faeces potentially carry all enteric diseases to which humans are susceptible. Poultry is the primary and permanent reservoir for *Salmonella* spp, whereas swine harbour *Salmonella* spp, *Shigella* spp, enteropathogenic *E. coli*, and other disease-causing microorganisms.

Indexing *E. coli* isolates obtained from food according to the frequency with which multiple-antibiotic-resistance (MAR) occur may provide a relatively easy method for making distinction of their origin. Sub-therapeutic use of antibiotics in the mass production of poultry, eggs, and pork has promoted the emergence of and maintains the prevalence of multi drug resistant (MDR) *E. coli* in the faecal environment of these animals (DePaola, Peeler & Rodrick 1995). These practices have resulted in the coexistence of MDR *E. coli* within these major reservoirs of enteric disease for humans. The consequence of this practice has provided a fortuitous opportunity to identify *E. coli* contamination of food originating from these high-risk environments by MAR indexing of *E. coli* isolates obtained from food.

1.7 Problem statement and justification

Although *Rastrineobola argentea* (omena) is of critical importance in meeting the food security and nutritional requirements of riparian communities, there has been relatively little information regarding the bacteriology of sun dried omena and their potential public health risk. Omena fishing takes place at night using canoes without ice for preserving the catch. Drying is usually done on the ground at the landing beaches leading to contamination with sand and microorganisms. As a result, there have been many concerns because the handling, drying, storage and transportation conditions of omena are usually inadequate to ensure food safety (Abila & Jansen, 1997). On the other hand, pathogenic bacteria such as *Staphylococcus* spp, *Shigella* spp, *Salmonella* spp and *Vibrio* spp have been isolated from fish and fishery products (Bryan 1980, Bryan 1987, Mungai Mwatha & Okemo 2002). In the resent past the EU imposed export bans on Kenyan fish in 1996 and 2000 citing poor sanitary conditions at landing beaches and processing facilities (Anonymous 1997), emphasizes the growing concerns of fish products as important vehicles of food borne pathogens.

There is thus, potential health risks associated with the consumption of omena contaminated with pathogenic bacteria. This risk is further compounded by the emergence of bacteria that have become resistant to antibiotics, which are important in the treatment and management of FBDs and the evolution and spread of antibiotic resistance plasmids within the bacterial populations.

The aim of the study was to document the level of bacterial contamination of sundried *R. argentea* sold in Kisumu town markets based on total plate counts, faecal coliform counts, and presence of enteric pathogens including *E. coli, Salmonella and Shigella* species. The study also investigated presence of plasmids as mediators of resistance to antimicrobials among bacterial isolates.

The information gathered will assist in the evaluation of existing sanitary practices and standards and propose appropriate measures that can improve the quality and safety of omena produced for human consumption and livestock feeds. The findings will further help in reducing post-harvest losses due to microbial contamination and allowing fish traders to increase their incomes.

1.8 Hypothesis

There is no potential bacterial health risk associated with omena marketed in Kisumu Town.

1.9 Objectives

1.9.1 General objective

To investigate the bacteriological quality of sun-dried omena supplied and sold in KisumuTown markets.

1.9.2 Specific objectives

i) To determine the hygienic practices of the omena traders at the studied markets.

ii) To determine the total bacterial and faecal coliform loads on sun dried omena.

iii) To isolate and identify some enteric pathogenic bacteria from sun-dried omena.

iv) To determine antimicrobial response of the isolated strains.

v) To determine the role of plasmids as mediators of resistance to antimicrobials among the bacterial isolates.

CHAPHER TWO

MATERIALS AND METHODS

2.1 Study site and design

The area of study was Kisumu town (plate 1). Kisumu town is located in Winam Division in Kisumu District of Nyanza province, which also holds the provincial and District headquarters. Kisumu town is the largest in Western Kenya. The town has a population of over 300,000 (Republic of Kenya, 2002). The main attraction to the town is the availability of jobs and business opportunities, colleges and training institutions. The markets studied included Oile, Jubilee that are in the town centre, Kondele, Manyatta and Nyalenda that are in a slum set up.

The study design was based on an analytical research design. A cross-sectional study approach was adopted based on random sampling. Ten samples were collected from each of the six markets on various dates between June and November 2005.

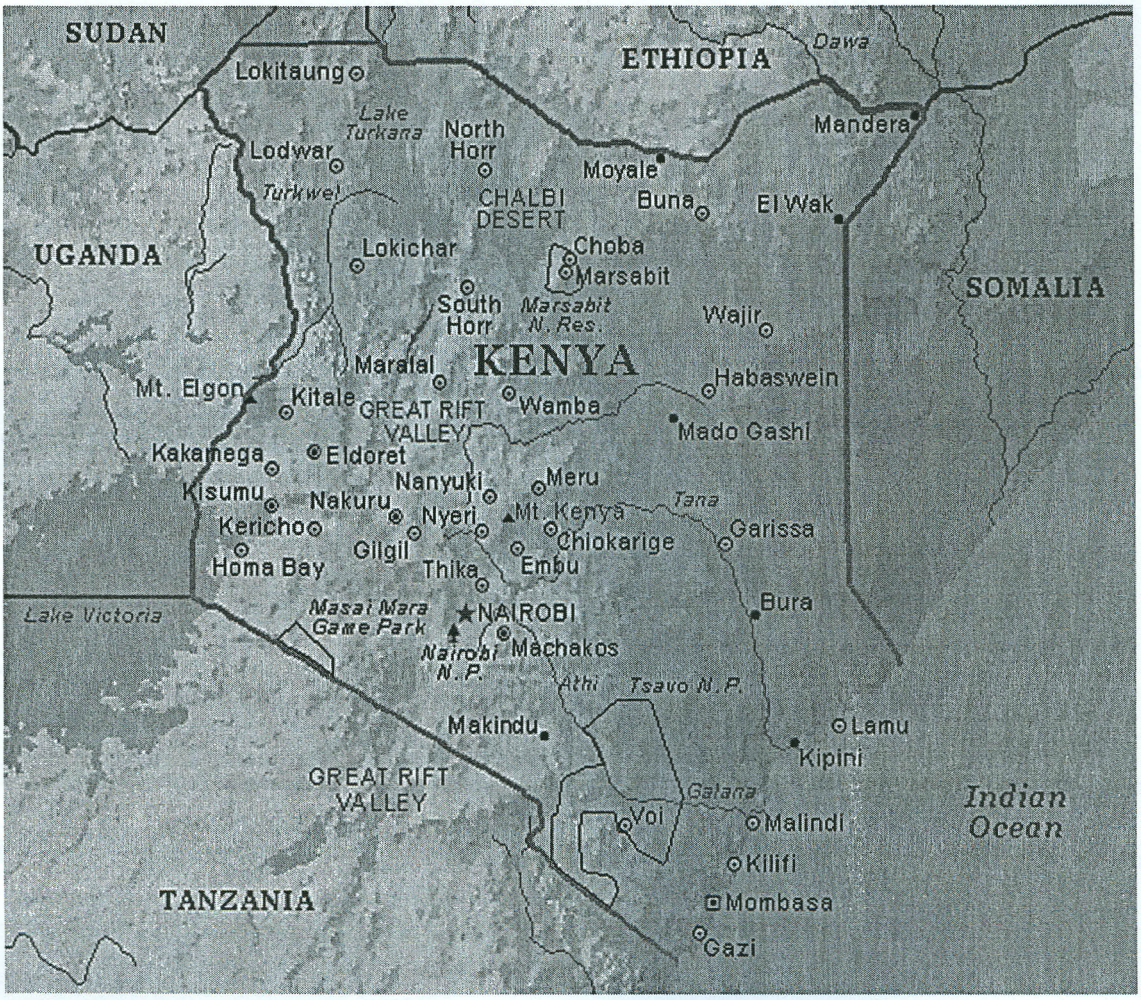

Plate 1: Map of Kenya showing the location of Kisumu town, source: http://www.village-sanctuary.com/Africa/images/map%20of20kenya.gif

2.2 Sampling of fish

The hygienic practices, such as handling, storage and the surrounding environment of the omena selling markets were noted and photographs taken. The fish were then purchased

from the omena traders in regular consumer packages (500g tins - approximately 100 - 150g of omena). The fish products were put in sterile bag, iced and immediately transported to the Fish Quality Control Laboratory in Kisumu for analysis.

2.3 Sample analysis

The samples were analysed for total plate counts (TPC), faecal coliforms (FC), presence of *Salmonella*, *Shigella* and *E. coli*.

2.3.1 Enumeration and identification of bacterial contamination

25g of sample were weighed and aseptically added to 225ml of buffered peptone water (Lab M) and homogenised in a stomacher. The homogenate was then utilised to enumerate for TPC, FC and determine presence of *Salmonella*.

2.3.1.1 Total plate count

TPC was determined as described by FAO (1992). Ten-fold serial dilutions were prepared from the homogenate prepared as described in 2.3.1 above. The diluents were cultured onto plate count agar (PCA) (Oxoid) and incubated at 35°C for 48h. After 48h plates were enumerated and results reported as colony forming units per gram (cfu/g).

2.3.1.2 Determination of faecal coliforms

FCs were determined as described by the FDA (2003). Ten-fold serial dilutions were prepared from the homogenate prepared as described in 2.3.1 above. The diluents were cultured onto Violet Red Bile agar (VRBA) (Oxoid) and incubated at 44.5°C for 48h. After 48h plates were enumerated and results reported as colony forming units per gram (cfu/g). Characteristic purple – red colonies that were 0.5mm or larger in diameter and surrounded by a zone of bile acid precipitate were counted.

2.3.1.3 Isolation and identification of *E. coli*

Suspect colonies from VRBA were transferred to Lauryl tryptose (L T) broth (Oxoid) and incubated at 44.5°C for 48h and examined for gas at intervals of 24h. The gassing L T tube were gently agitated and loopfuls of each suspension transferred to tubes of EC medium and incubated at 44.5°C for 48h and examined for gas production at 24h interval. Confirmation of *E coli* was done by streaking a loopful of the suspension from the gassing EC medium (Oxoid) tube on to Levines' Eosin-Methylene Blue Agar (L-EMB) (Oxoid) and incubated at 35°C for 18 - 24h. Typical *E. coli* colonies on L-EMB agar appear greenish with metallic sheen. Two typical colonies from each L-EMB plates were picked and transferred to PCA slants for morphological, biochemical characterization.

2.3.1.4 Isolation of *Salmonella* species

Isolation and identification of *Salmonella* species was carried out as described by FAO (1992). The remaining homogenate in section 2.3.1 above was then transferred to sterile screw - cap jars and let to stand for 60 minutes at room temperature. The jar caps were loosened and incubated at 35°C for 24h. 1ml of the mixtures were transferred to 10ml of selenite cystine broth (Oxoid) and another to 10ml of tetrathionate broth (Oxoid) and incubated at 35°C for 24h. Both enriched broths were streaked onto Xylose Lysine Desoxycholate (XLD) agar (Lab M) and Bismuth sulfite (BS) agar (Oxoid) and incubated at 35°C for 24h. Typical *Salmonella* colonies on XLD agar appear as pink colonies with or without black centres, whereas on Bismuth sulfite (BS) agar they appear as brown, grey, or black colonies and may have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation. Two typical *Salmonella* colonies from each plate were sub-cultured into Triple Sugar Iron (TSI) agar (Oxoid) slants and incubated at 35°C for 24 and 48h respectively. *Salmonella* in TSI culture typically produce alkaline (red) slant and acid (yellow) butt, gas, with or without production of H_2S (blackening of agar). All presumptive positive cultures were later identified by biochemical characterization and serological reactions.

2.3.1.5 Isolation of *Shigella* spp

Isolation of *Shigella* spp was carried out as described by FAO (1992). Briefly 25g samples were weighed aseptically in 225ml selenite cystine broth (Oxoid) contained in

wide - mouth, screw - cap pint jars. The jar caps were loosened and incubated for 16 h at 35°C. The suspensions were agitated and streaked on MacConkey agar (Oxoid) and XLD agar (Lab M) plates and incubated for 24h at 35°C and examined for typical *Shigella* colonies at appropriate intervals. The jars were incubated for an additional 24h and streaked again on selective agar plate media. The selective plates were incubated for an additional 24h, if no growth was visible. *Shigella* on XLD agar appears as pink colonies without black centres. On Mac Conkey agar they appear as slightly pink and translucent, with or without rough edges. Two or more suspect colonies per plate were incubated in TSI agar slant for 24h at 35°C. Typical *Shigella* colonies were identified by morphological and biochemical characterization.

2.3.1.6 Biochemical characterization of isolates

Gram stain: A loopful of typical colony was spread onto a slide and the film fixed on the slide by dry passing it over the Bunsen burner flame. After the slide had cooled it was flooded with crystal violet solution and allowed to react for 2 minutes. The stain was then flooded with iodine solution. The iodine solution was allowed to react for 2 minutes. The excess iodine was drained off by decolourising using acetone for not more than 5 seconds, and washed off immediately with water. Basic fuchsin counter stain was applied and allowed to react for 2 minutes. The slide was then washed and allowed to dry in the air. *E. coli*, *Salmonella* and *Shigella* are Gram negative (stain red / pink), and short rods (Cheesbrough 2000).

Catalase test: A drop of hydrogen peroxide was placed onto a clean glass slide. A typical colony was picked from the culture and mixed with the hydrogen peroxide drop. Appearance of air bubbles (effervescence) meant a catalase positive test. *E. coli, Salmonella and Shigella* are catalase positive (Cheesbrough 2000).

Oxidase: Using a sterile wire loop, suspect colonies were thoroughly smeared on a 6cm square piece of filter paper impregnated with 1% aqueous tetramethylparaphenylene diamine dihydrochloride solution. Colour changes were then observed (Cheesbrough 2000). A positive test was recorded if transferred cells turn dark purple in 5 to 10 seconds. *E. coli*, *Salmonella* and *Shigella* are oxidase negative; the test was used for the purposes of identification in relation with other test carried out.

Indole production: Tryptone broth (Oxoid) tubes were inoculated and incubated for 24h at 35°C. 0.2 -0.3ml of Kovacs' reagent was added. Appearance of distinct red colour in upper layer was considered positive (FDA 2003).

Voges-proskauer (VP) - reactive compounds: MR-VP broth (Oxoid) tubes were inoculated and incubated for 48h at 35°C. 1ml was transferred to 13 x100mm tubes, 0.6ml of α-naphthol solution and 0.2ml of 40% KOH, was added. The tubes were shaken and let to stand for 2h. Tubes that developed an eosin pink colour were considered

positive (FDA 2003).

Methyl red-reactive compounds: after VP test, MR-VP tubes were incubated for an additional 48h at 35°C. 5 drops of methyl red solution was added to each tube. Those tubes that developed distinct red colour were considered positive whereas those that developed a yellow colour were considered negative (FDA 2003).

Citrate: Koser's citrate broth (Oxoid) was lightly inoculated; avoiding detectable turbidity and incubated for 96h at 35°C. Development of distinct turbidity was considered positive (FDA 2003).

Gas from lactose: LST (Oxoid) tubes were inoculated and incubated for 48h at 35°C. Gas production was considered positive reaction (FDA 2003).

Lysine decarboxylase broth: LD broth (Oxoid) tubes were inoculated with small amount of growth. The tubes were incubated for 48h at 35°C but examined at 24h intervals. Positive test is indicated by purple colour throughout the medium. Negative test is indicated by yellow colour throughout medium (FDA 2003).

Urease test: Urea broth (Oxoid) tubes were inoculated with growth from each presumed-positive TSI slant culture and incubated at 35^0C for 24h. Un-inoculated tubes of the broth were included as controls (Cheesbrough 2000). Inoculated tubes of urea broth that turned purple/red were considered positive, whereas no colour change is considered negative.

2.3.1.7 Serological confirmation: Serological confirmation of *Salmonella* was performed based on the Kauffmann- White scheme (Cheesbrough 2000). The isolates were initially confirmed at the genera level by polyvalent O group A – G (Munex Biotech Ltd. Dartford Kent UK), and then to species level using *Salmonella* O antisera – monovalent group A, factor, 2; group B, factor 4; group D, factor 9, (RSHM antisera, Turkey) and *Salmonella* H antisera monovalent phase 1 factors, a, b, c, d i, g, phase 2, 1 and 2 (Munex Biotech Ltd. Dartford Kent UK). Using a sterilised wire loop, a portion of growth from TSI surface was picked and emulsified in three small drops of physiological saline on the clean sterilised glass microscope slides and mixed thoroughly. A small drop of O antiserum was added to one of the suspensions and a small drop of H antiserum to the second. The third suspension was used as a control for auto-agglutination (roughness). The suspensions and antiserum were mixed thoroughly and the slide was then tilted back and forth to observe the agglutination, which was further confirmed by observing under a light microscope at X 40.

2.3.2 Antimicrobial susceptibility testing

The standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial agent sensitivity profiles of the isolates (NCCLS manual, 1999) for ten antimicrobial agents (ampicillin 10µg, tetracycline 30µg, cotrimoxazole 25µg, augumentin 30µg, gentamicin 10µg, kanamycin 30µg, cefuroxime 30µg, chloramphenicol 30µg, nalidix acid 30µg and norfloxacin 10µg). Initially LB broth was inoculated with the isolates and incubated over night at 37°C to a turbidity of 0.5 Mc Farland standard (10^8 cfu). 150mm Mueller – Hinton medium (Lab M) plates were swabbed with the inoculums and ten commercially prepared antimicrobial agent disks placed on each of the inoculated plates. The plates were incubated at 37°C for 18 to 20h. The diameters (in millimetres) of clear zones of growth inhibition around the antimicrobial agent disks, including the 6mm disk diameter, were measured by using precision callipers (NCCLS manual, 1999). A standard reference strain of *E. coli* (ATCC 25922) was used as a control. The breakpoints used to categorize isolates as resistant to each antimicrobial agent were those recommended by NCCLS (1999) shown in Table 2.

Table 2: Antimicrobial agents used and their ratings in mm

Antimicrobial agent	Resistant	Intermediate	Susceptible
Tetracycline (30μg)	0 – 14	15 – 18	>19
Ampicillin (10μg)	0 – 13	14 – 16	>17
Co-trimoxazole (25μg)	0 – 10	11 – 15	>16
Augumentin (30μg)	0 – 13	14 – 17	>18
Gentamicin (10μg)	0 – 12	13 – 14	>15
Kanamycin (30μg)	0 – 13	14 – 17	>18
Cefuroxime (30μg)	0 – 15	16 – 20	>21
Chloramphenicol (30μg)	0 – 12	13 – 17	>18
Nalidix acid (30μg)	0 – 13	14 – 18	>19
Norfloxacin (10μg)	0 – 12	13 – 16	>17

2.3.3 Plasmid isolation from drug resistant strains

The organisms were grown in 1ml of LB broth and incubated at 37°C in a shaking water bath for 18 – 24h, 500μl were pipetted and put into Eppendorf tubes and spin at 12000xg for 30 seconds at 4^0C in an Eppendorf centrifuge. Plasmids were then isolated using the method of Kado and Liu, (1981). The supernatants were removed by aspiration. The cells were resuspended by vigorously vortexing and 100μl of the alkaline sodium dodecyl sulphate (SDS) mixture added then vortexed again. The tubes were incubated for 30 minutes at 65°C, vortexing them at intervals of 15 minutes and at the end of the

incubation period. 100µl of 1: 1 (v/v) mixture of phenol and chloroform-isoamylalcohol, were added and vortexed at full speed to shear the chromosomal DNA and spinned for 2 minutes in an Eppendorf centrifuge. The tubes were allowed to stand for 5 minutes and 25µl of the supernatant removed and put into a second small Eppendorf tube for plasmid separation. Plasmids were separated by mixing 10µl of plasmid suspension with 3µl of electrophoretic dye (0.07% bromophenol blue, 0.7% sodium dodecyl sulphate and 33% of glycerol in distilled water) and subjected to electrophoresis using 1 % agar for 1-3h at 125 volts, in a standard electrophoretic system. The plasmid bands were stained using aqueous ethidium bromide (1µg/ml) for 10 minutes and then washed continuously in tap water for 10 minutes. The bands were visualised on an ultraviolet transilluminator and photographed with Polaroid film through the UV and red filters. The sizes of the plasmids were estimated by co-electrophoresis with plasmids of known sizes from *E. coli* strains V 517 (NCTC 50193) [53.7, 7.2, 5.6, 3.9, 3.0, 2.7, 2.1 kb] and 39R861 (NCTC 50192) [147, 63, 43.5, 6.9 kb].

2.3.4 Conjugation experiments

Only *E. coli* isolates that were resistant to ampicillin and sensitive to nalidixic acid were recruited for conjugation studies. Bacterial conjugal mating was carried out as described by Yamamoto and Yokota, (1983). Donor bacteria (*E. coli* isolates from omena) and recipient bacteria (*E. coli* K12 nalidixic acid resistant and F-) that had been grown on nutrient agar overnight were sub-cultured in 3ml of tryptic soy broth in bijou bottles and incubated at 37^0C for 3h in a rotating incubator to get into log phase. The Logarithmic

phase cultures of donor and recipient bacteria were then mixed in proportions of 1: 10, respectively by putting 0.5ml of culture into 4.5ml fresh broth. The donor and recipient were then mixed in equal proportions of 2ml in duplicate mixtures. One group of the mixture was incubated at 37^0C and the other at room temperature overnight. The cells were then pelleted by centrifugation (13000xg) for 1 minute in a refrigerated micro-centrifuge using 1.5ml tubes. The pellets were then washed with sterile phosphate buffered saline. The mixture was centrifuged at 13000xg for 1minute and pellet washed again in sterile phosphate buffered saline before centrifuging at 13000xg for 1 minute. The supernatant was removed by aspiration. Using a sterile wire loop the cells were each sub-cultured on MacConkey agar containing 30µg/ml ampicillin and 30µg/ml nalidixic acid to select transconjugants., MacConkey agar containing 30µg/ml ampicillin as control (only *E. coli* isolates from omena were to grow) and MacConkey containing 30µg/ml nalidixic acid as control (only *E. coli* K12 were to grow)

2.3.5 Data analysis

The total bacterial count and faecal coliform counts represented as colony forming units per gram (cfu/g) were converted to their respective Logarithmic_{10} representations. Presence of pathogenic organisms was reported as present or absent. Response to antimicrobial agents was recorded as resistant, intermediate or susceptible as specified in the NCCLS, 1999. Plasmid fingerprinting was presented by use of bands and their molecular weights determined based on known standards. Conjugation studies were reported as plasmids transferred or not transferred.

The MAR index was calculated based on the formula described by Krumperman (1983); a/(b x c), where a; is the aggregate antibiotic resistance score of all isolates from the sample, b; is the number of antibiotics, and c is the number of isolates from the sample.

The data collected was entered in Microsoft Excel (windows XP Professional) spreadsheet and analysed by MINTAB software (version 13) and confidence levels of 5% were considered significant. ANOVA and Kruskal-Wallis Test were used to determine differences between and within markets and antimicrobial agents tested, respectively.

CHAPTER THREE

RESULTS

3.1 Field observations

Fish was displayed for sale on raised wooden racks covered with mats and either manila sucks or polythene paper as shown in Plate 2. Although this practice was common among the markets, it however does not offer protection to the fish products against dust and/or flies. The traders did not observe personal hygiene.

Plate 2: Omena displayed for sale on wooden racks covered with papyrus reed mat and manilla sack at jubilee market: the fish is not protected from flies and dust

Fish stores as shown in Plate 3, lacked pest control measures and the products were stored directly on the floor in open containers. The facilities were also not refrigerated.

Plate 3: A fish store at jubilee market. *Rastrineobola argentea* is packed in manila gunny bag, and an open basket, placed directly on to the store's floor

3.2 Total bacterial and faecal coliform loads on sun dried *R. argentea*

The levels of bacterial contamination are shown in Tables 3 and 4 below. All the markets surveyed showed TPC and FC means above those recommended by the Kenya Bureau of Standards 100,000cfu/g ($\log_{10}$ 5cfu/g) and zero faecal coliforms, respectively for TPC and FC. The market with the highest TPC mean value was Jubilee market with TPC mean $\log_{10}$ 8.55cfu/g; Kibuye market recorded the lowest mean value with TPC mean

log_{10} 6.94cfu/g. Oile market however demonstrated the highest levels FC, log_{10} 4.33cfu/g whereas Kibuye market registered the lowest of log_{10} 3.62cfu/g.

Table 3: Bacterial loads of *R. argentea* at various markets sampled in Kisumu town (n = 10 for each market)

	TPC		
MARKETS	**Range**	**Mean**	**Mean Log10 cfu/g.**
OILE	6.3×10^5- 2.2×10^9	2.4×10^8	7.21 ± 1.11
JUBLILEE	1.0×10^6- 2.4×10^9	7.6×10^8	8.05 ± 1.26
KIBUYE	2.6×10^5- 2.9×10^8	2.8×10^7	6.94 ± 0.87
KONDELE	1.1×10^5- 1.7×10^9	2.4×10^9	7.23 ± 1.24
NYALENDA	3.0×10^5- 2.0×10^9	4.7×10^8	7.36 ± 1.47
MANYATTA	1.4×10^6- 1.2×10^9	1.5×10^8	7.22 ± 0.94

Table 4: Levels of faecal contamination of *R. argentea* at various markets sampled in Kisumu town (n = 10 for each market)

	FEACAL COLIFORMS		
MARKETS	**Range**	**Mean**	**Mean Log10 cfu/g**
OILE	$2.5 \times 10^2 - 6.4 \times 10^5$	1.34×10^5	4.33 ± 1.07
JUBILEE	$1.0 \times 10^1 - 5.0 \times 10^5$	1.87×10^5	4.27 ± 1.54
KIBUYE	$2.3 \times 10^2 - 8.8 \times 10^5$	1.03×10^5	3.62 ± 1.23
KONDELE	$2.0 \times 10^1 - 7.2 \times 10^5$	1.80×10^5	4.33 ± 1.48
NYALENDA	$2.8 \times 10^2 - 8.4 \times 10^5$	1.60×10^5	4.32 ± 1.13
MANYATTA	$1.8 \times 10^2 - 4.0 \times 10^5$	7.92×10^4	3.91 ± 1.08

The study did not find any significant difference between the means $\log_{10}$ TPC and FC for the sampled markets ($p > 0.05$) as shown in Table 5. No statistically significant difference was noted based on the Hsu's Multiple Comparison with the Best (MCB) in this case the smallest mean among the markets.

Table 5: ANOVA one – way test comparing between means of markets for TPC and FC at the level of significance $\alpha = 0.05$

Source of variation	SS	Df	MS	F	P-Value	F. crit.
TPC						
Between groups	6.99	5	1.4	1.03	0.412	2.39
Within groups	73.52	54	1.36			
Total	80.51	59				
FC						
Between groups	4.44	5	0.89	0.55	0.74	2.39
Within groups	86.75	54	1.61			
Total	91.19	59				

Legends: SS = Sum of Squares, MS = Mean Square

Using correlation coefficient to determine association between TPC and FC as variables, positive correlation between TPC and FC was noted among Oile market; 0.347 [p = 0.325], Jubilee; 0.497 [p = 0.144], Kibuye; 0.506 [p = 0.136] and Kondele; 0.771 [p = 0.009]. Negative correlation was noted among Nyalenda market; -0.016 [p = 0.965] and Manyatta; -0.027 [p = 0.942]. Kondele and Kibuye markets recorded a moderately positive correlation between the two parameters tested, whereas Jubilee and Oile markets had weak positive correlation. Nyalenda and Manyatta markets' recorded weak negative correlations. Only Kondele market showed strong statistical evidence ($p < 0.05$) that TPC and FC were correlated. Oile, Jubilee and Kibuye markets showed no significant evidence ($p > 0.05$) that TPC and FC correlated, although there was a weak linear relationship between TPC and FC. Nyalenda and Manyatta markets on the other hand

showed a negative weak linear relationship between the two parameters. All cases therefore showed that it is possible that these two indicators of microbial contamination may influence each other.

3.3 Identification of enteric bacteria isolated from *R. argentea*

The study observed that *E. coli* occurred in all the sampled and analysed representing 100% occurrence whereas; *Salmonella* was only present in four (4) samples representing only 6.67%, *Shigella* spp was found to be absent in all the fish samples tested as shown in Table 6. The study therefore demonstrates that *E. coli* is an important microbial contaminate of *R. argentea* sold in Kisumu town markets, whereas *Salmonella* may be less frequent and *Shigella* probably absent.

Table 6: Percentage occurrence of *E coli*, *Shigella* spp, and *Salmonella* spp, in *R. argentea* sampled (n=60)

Bacteria	Number of samples	% Occurrence
E. coli	60	100
Salmonella spp	4	6.67
Shigella spp	0	0

The four *Salmonella* isolates reported in the study were isolated from samples collected from three markets. Markets that showed positive correlation between TPC and FC had at least one sample among the ten samples tested, test positive for *Salmonella* (Oile and Kibuye), whereas Kondele market that showed a moderate positive relationship between

TPC and FC had two samples test positive for *Salmonella*. The *Salmonalla* serotypes isolated and incidence of occurrence as observed in this study is shown in Table 7.

Table 7: Incidences and serotypes of *Salmonella* as recorded among the markets in Kisumu town (n=10 samples per market)

Markets	Incidences	Serotype
Oile	1	*S. enteritidis*
Kibuye	1	*S. paratyphi B*
Kondele	2	*S. typhymurium, S. enteritidis*

3.4 Antimicrobial response of isolates from *R. argentea*

3.4.1 Antimicrobial response for *E. coli* isolates

All *E. coli* isolates were fully susceptible to chloromphenicol, nalidixic acid and norfloxacin (Table 8). The most frequently encountered resistance was observed against co-trimoxazole (38.8%), tetracycline (20.4%) and ampicillin (12.2%). 47% isolates showed resistance to at least one antimicrobial agent, whereas 26.5% of the isolates tested showed resistance to two or more antimicrobial agents tested.

Table 8: Antimicrobial response of *E. coli* isolates to 10 antimicrobial agents

Antimicrobial agent	Number and percentage of susceptibility of strains (%)		
	Resistant	**Intermediate**	**Susceptible**
Ampicillin	6 (12.24)	0 (0)	43 (87.76)
Tetracycline	10 (20.41)	11 (22.45)	28 (57.14)
Cotrimoxazole	19 (38.76)	2 (4.08)	28 (57.14)
Augmentin	2 (4.08)	5 (10.2)	42 (85.71)
Kanamycin	2 (4.08)	25 (51.02)	22 (44.90)
Gentamicin	1 (2.04)	3 (6.12)	45 (91.84)
Cefuroxime	3 (6.12)	43 (87.76)	3 (6.12)
Chloromphenicol	0 (0)	0 (0)	49 (100)
Nalidixic acid	0 (0)	0 (0)	49 (100)
Norfloxacin	0 (0)	0 (0)	49 (100)

Mean disk diffusion zones sizes for *E. coli* isolates examined showed that Kondele market isolates had the smallest mean diffusion zones for all the agents except for cefuroxime, for which Manyatta market had the smallest mean zone. Overall, the largest diffusion zones (indicating greater susceptibility) were found with chloromphenicol (Table 9).

Table 9: Mean disk diffusion zones for *E. coli* isolates by markets

MARKET	Amp	Tet	Cot	Aug	Kan	Gen	Crx	Chl	Nal	Nor
OILE	20.7	18.8	18.5	20.2	18	18.2	19.9	25.5	20.6	22.3
NYALENDA	18.1	19.9	17.1	19	18.4	17.4	19.6	24.9	19.4	21.1
MANYATTA	17.3	13.3	14.9	19.4	17.4	16.9	17.9	24.1	19.6	22.1
KONDELE	14.9	10.9	14.9	16.3	16.4	15.9	18	23.3	18.4	19.3
KIBUYE	19.9	19.5	17	19.7	16.9	16.8	19.1	24.7	19.8	21.7
JUBILEE	17.4	19.4	16	19.3	16.1	17	18.4	24.7	19.6	21.6

Legends: Cot = Co-trimoxazole, Crx = Cefuroxime, Tet = Tetracycline, Kan = Kanamycin, Aug = Augmentin, Gen = Gentamicin, Amp= Ampicillin, Chl = Chloromphenicol, Nal = Nalidixic Acid, Nor = Norfloxacin

One – way ANOVA test for the difference between markets from which the samples were collected showed significant difference among diffusion zone sizes for tetracycline ($p < 0.0005$) as shown in Table 10.

Table 10: ANOVA one – way test for tetracycline among the *E. coli* isolates by market at the level of significance $\alpha = 0.05$

Source of variation	SS	Df	MS	F	P-Value
Between groups	0.6514	5	0.1303	6.13	0.000
Within groups	0.9135	43	0.0212		
Total	1.5650	48			

Based on Fishers pair wise comparisons for least significant difference (LSD), at family error rate of 0.350, an individual error rate of 0.0500 and critical value of 2.017, statistical differences between means among markets was noted for tetracycline among Manyatta market with respect to Jubilee, Kibuye, Oile and Nyalenda markets whereas Kondele markets with respect Jubilee, Kibuye, Nyalenda and Oile markets. Kondele market also showed statistically significant differences among norfloxacin, ampicillin, augumentin, gentamicin and nalidixic acid drugs. No statistically significant difference was noted between the corresponding means based on the Hsu's multiple comparison with the best (in this case the largest means) for co-trimoxazole, kanamycin, cefuroxime and chloromphenicol Family error rate of 0.0500 and a critical value of 2.33.

The study established that the *E. coli* isolates were resistant to seven (7) of the ten (10) antimicrobial agents tested. The study also demonstrated cross-resistance among some *E. coli* strains with cotrimoxazole, tetracycline and ampicillin being the most frequent. A total of 12 antibiogram patterns as shown in Table 11, the most frequent one was resistance to cotrimoxazole. All cases of ampicillin resistance observed were cross – resistance cases.

Table 11: Resistance antibiogram patterns and frequency of occurrence within the *E. coli* isolates

Resistance pattern	No of strains	Resistance pattern	No of strains
Cot	9	Amp + Cot	2
Crx	1	Tet + Cot	3
Tet	1	Amp + Tet + Cot	2
Cot+Kan	1	Amp + Tet + Aug	1
Tet+Aug	1	Tet + Crx	1
Amp+Tet+Cot+Gen	1	Cot +Kan + Crx	1

Legends: Cot = Co-trimoxazole, Crx = Cefuroxime, Tet = Tetracycline, Kan = Kanamycin, Aug = Augmentin, Gen = Gentamicin, Amp= Ampicillin

The Multiple antibiotic resistance (MAR) index was calculated based on the formula described by Krumperman (1983); a / (b x c), where a; is the aggregate antibiotic resistance score of all isolates from the sample, b; is the number of antibiotics, and c is the number of isolates from the sample.

MAR = 41 / (10 x 49)

MAR = 0.084

Based on the calculated *E coli* MAR index, the *E coli* contamination does not originate from a high-risk source. Based on the Krumperman, (1983) classification, *E coli* from high-risk sources give MAR index of ≥ 0.200.

3.4.2 Antimicrobial response for *Salmonella* Isolates

The *Salmonella* strains tested showed resistance to only one antibiotic tested cefuroxime (25%) as shown in Table 12. However, the isolates were all susceptible to Gentamicin, Cotrimoxazole, Chloromphenicol, Nalidixic acid and Norfloxacin. Intermediate response was observed amongst ampicillin, tetracycline, augmentin, kanamycin and cefuroxime.

Table 12: Antimicrobial response of *Salmonella* isolates to 10 antimicrobial agents

Antimicrobial agent	Number and percentage of susceptibility of strains (%)		
	Resistant	**Intermediate**	**Susceptible**
Ampicillin	0(0)	1(25)	3(75)
Tetracycline	0(0)	2(50)	2(50)
Cotrimoxazole	0(0)	0(0)	4 (100)
Augmentin	0(0)	2(50)	2(50)
Kanamycin	0(0)	2(50)	2(50)
Gentamicin	0(0)	0(0)	4 (100)
Cefuroxime	1(25)	3(75)	0(0)
Chloromphenicol	0 (0)	0 (0)	4 (100)
Nalidixic acid	0 (0)	0 (0)	4 (100)
Norfloxacin	0 (0)	0 (0)	4 (100)

3.5 Plasmid profiling of *E. coli* isolates

Ten *E. coli* strains were selected based on their antibiogram patterns. However six showed resistance to ampicillin. A total of six different plasmid profiles were detected among the *E. coli* isolates as shown in plate 4. All the isolates that showed resistance to ampicillin had a common plasmid of approximately 5.6kb. The most frequent plasmids observed among the isolates were those of approximately 5.6kbp or less. Plasmids of molecular weights greater than 5.6 kb were observed in only 2 isolates.

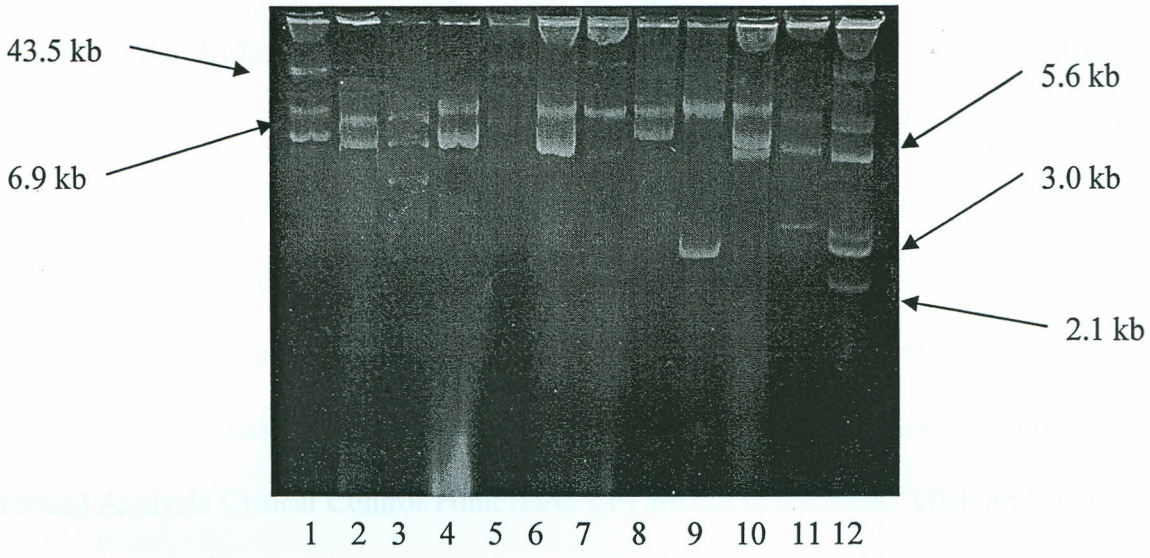

Plate 4: Gel picture showing plasmid DNA profiles for *E. coli* isolates, Lane 1, R39; lane 2 E8(5.6kbp), lane 3 E15 (3; 3.9; 5.6kbp); lane 4 E24 (5.6kbp); lane5 E37 (43.5kbp); lane 6 E12 (5.6kbp); lane 7 E13 (5.6; 43.5kbp); lane 8 E38 (5.6kbp); lane 9 E27 (2.7kbp); lane 10 E45 (5.6kbp); lane 11 E19 (3; 5.6 kbp)and lane 12 V517

Six *E. coli* isolates that were resistant to ampicillin and susceptible to nalidixic acid were recruited for the conjugative studies. However, ampicillin resistance was not transferred to the recepient *E. coli* K12 strain thus suggesting that the isolates may not have been carrying any conjugative plasmids responsible for the transfer of ampicillin resistance.

CHAPTER FOUR

DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 Discussion

The study observed high levels of total plate counts (TPC) and faecal coliform contamination in all the 60 *R. argentea* samples tested including infestation with *E. coli* and *Salmonella*; *Shigella* was absent. This therefore implies that the fish being sold in Kisumu town markets is of low microbial quality and does not comply with the locally laid down standards for *R. argentea* (Kenya Bureau of Standards 1998). The Kenya Bureau of Standards, 1998 specifications recommend that sun dried *R. argentea* should have total count (TPC) of not more than 100,000cfu/g ($\log_{10}$ 5cfu/g), zero coliform counts and no food-poisoning organisms. The findings in this study could be attributed to the operations the fish products go through, which include harvesting, processing, distribution and marketing. In all these operations Good Hygiene Practices (GHP) and Hazard Analysis Critical Control Point (HACCP) are not operational. GHP and HACCP are important tools for managing and ensuring that food products are produced under hygienic conditions and that food safety measures are in place to address all possible risks that may be associated with the food product.

Ogwan'g, Muchiri & Thakor (2005) and Huss (1994) reported that bacterial contamination in fish products occurs primarily through exposure to polluted environments or cross contamination during landing, transportation, processing, and

storage or during consumption. Since drying of omena is usually done on the ground at the landing beaches as reported by Abila & Jensen (1997); such a practice may act as a major source of contamination of omena with sand and microorganisms. Mwambazi (2005) reported that drying of fish on the ground, wooden boards, reeds, grass mats or fishing nets gives no protection from rodents, insects, birds, chickens and other domesticated animals. Since the fish is laid on the ground, water accumulates around it instead of draining away. The inadequacy of drying the fish makes it susceptible to mould and beetle attack including blowflies thus increasing possibilities of faecal contamination. The drying processes are not lethal *per se* to microorganisms although these processes may destroy some (Jay 1992). This is more so if poor quality foods are dried and if proper practices are not followed in the drying steps.

The practice of displaying fish products on raised wooden racks covered with either manila sacks or polythene paper at markets (Plate 2), does not offer any protection as demonstrated with the recovery of bacteria and in high counts. Local public health and fish inspection authorities have for a long time encouraged fish traders to adopt the practice by displaying fish products for sale on racks. The study demonstrates that this practice does not guarantee that the fish products offered to consumers are safe. Fish stores are also not provided with pest control facilities and refrigeration (Plate 3), thus the stored products may be prone to pest attack, and these conditions may further promote microbial infestation and growth.

These findings therefore demonstrate that there are potential health hazards present in omena retailed in Kisumu town markets; improving handling practices along the production and distribution line may go a long way in eliminating the possible hazards.

The occurrence of *Salmonella* in omena products retailed on the markets poses a public health risk not only to humans but also to other food animals such as pigs and poultry that may be fed on contaminated animal feeds. Since most local animal feed industries have mainly been concerned with the physical appearance of the fish products supplied to them and not the microbiological quality as reported by Abila & Jansen (1997), it is possible that fishmeal used by these local industries may be an important source of these pathogens in animal feeds. It is therefore important that the omena products to be used in the animal feed industries be free of pathogenic *E. coli* and *Salmonella* among other pathogens.

Since the study did not find any significant difference in bacterial loads and faecal contamination (TPC and FC) among sampled markets ($p > 0.05$), the study demonstrates that the sources of contamination of the fish products may be common among the markets. However, the relatively weak relationship between TPC and FC among the sampled markets within the study area demonstrates that other than prevailing sanitary conditions at markets, other factors may also have influenced contamination of the products with microbes. TPC and FC are measures of bacterial contamination. TPC is

indicative of the general bacterial load whereas FC indicates possible contamination from external sources such as human or animal feaces. The study however, demonstrates that markets with positive correlation between TPC and FC were likely to have at least one sample test positive for *Salmonella* (Oile, Kibuye and Kondele). *Salmonella* primarily inhabit the intestinal tract of animals; the organisms are excreted in faeces from which they may be transmitted by insects and other living creatures to food.

The study observed that the contamination based on the MAR indexing of *E. coli* was lower than 0.200, suggesting that the contamination may have arisen from a low risk source (Krumperman 1983). The occurrence of *Salmonella* spp in some samples collected from the market and absence of *Shigella* spp, however strongly supports the existence of other high – risk sources within the geographical limits of the study that are not exposed directly to antimicrobials. *Salmonella* have a wide range of warm-blooded animals including, human beings as hosts whereas *Shigella* spp are restricted to higher primates, including humans where they are usually spread by food handlers with poor personal hygiene (FDA 2001). The findings therefore suggest that MAR indexing of *E. coli* alone may not be a good indicator of risk assessment of food products and especially those naturally sourced within the Lake Victoria basin, as there are other risk sources that MAR indexing of *E. coli* may not be able to detect since the intensive rearing of livestock is least practiced in the region.

In this study bacterial isolates showed relatively low level of resistance to antimicrobial agents tested compared to clinical isolates in other studies conducted locally (Kariuki et al. 1999, Kariuki et al. 2005). The study agrees with Österblad et al (1999) who found very low frequency of antimicrobial resistance in *Enterobacteriaceae* isolated from vegetables although very few *E. coli* were isolated suggesting that faecal contamination was rare. It also agrees with Sherley, Gordon & Collignon (2000) who found that *E. coli* was the most common isolate and also showed lowest prevalence of resistance among *Enterobacteriaceae* isolated from wild Australian mammals. The study also agrees with Kariuki et al (2002) who found that all the Non Typhi Salmonella (NTS) isolates from cows, pigs and sewers tested for drug sensitivity to 11 antibiotics were fully susceptible. The low frequencies of antimicrobial resistance observed among the isolates isolated from *R. argentea* in this study could be attributed to low levels of factors such as heavy metals and antimicrobial agents that are known to promote the development of drug resistant microorganisms (Davidson 1999, McArthur & Tuckfield 2000, Van den Bogaard, Lodon, Driessen & Stobberingh 2001,).

The prominence of co-trimoxazole, tetracycline and ampicillin resistance among *E. coli* isolates in the study demonstrates the similarities in the development of resistance among the isolates and the *E. coli* of clinical importance. The study therefore postulates that this exposure to antibiotics could be of a human origin. This is supported by the presence of resistance to co-trimoxazole, a synthetic antimicrobial agent generally used in humans only. The differences in levels of resistance and resistance patterns among isolates could

be due to levels of exposure to the agents or other factors that may have increased or decreased the likelihood of the development and conservation of resistant genes among the bacterial isolates. The findings therefore demonstrate that multi-drug resistant (MDR) bacteria are important potential health hazards that can be associated with omena retailed in Kisumu town markets.

This study found that isolates recovered from Kondele market showed the smallest mean diffusion zones for all the antimicrobial agents except for cefuroxime demonstrating higher levels of resistance compared to isolates from other markets. This may indicate that there were other sources of resistance factors or practices that could have contributed to contamination of fish products or exposure to resistant bacteria within the markets, which were not sampled in the study, such as personnel, and the market surroundings. General observations indicated that the Kondele market is not designated and is situated on a road reserve; no sanitary facilities or solid waste management measures were available and such conditions could predispose the fish products to contamination. Pimentel & Bashore (1998) noted that soil is an important source of contamination of foods by chemical pollutants and pathogens, whereas Rysz & Alvarez (2004) demonstrated that bacteria in the soil could acquire resistance to tetracycline from environmental exposure, possibly creating a reservoir of resistance factors generated outside host animals. Torres, Fernandez-Roblas, Mendez & Soriano 1986, Baquero & Balazquez (1997) and Sherley, Gordon & Collignon (2000), reported that geographical location and physiological differences between species and genera could influence

antibiotic resistance profiles including number of resistances among bacterial isolates. This study concurs with these arguments as possible factors that may have contributed to the relatively high levels of resistance among isolates from Kondele market based on the mean diffusion zones observed and similarly influencing the difference in rates of *E. coli* resistance to tetracycline within the markets.

However, the differences in rates of resistances to tetracycline could also have arisen as a result of species-specific differences in the distribution of resistance genes on the bacterial chromosome or associated plasmid and transposon populations as was reported by Sherley Gordon & Collignon (2000) and Prescott, Baggot & Walker (2000). These findings therefore, suggest that although generally microbial contamination of *R. argentea* sold in Kisumu town markets may occur through a common source other factors may be important in selection of resistance among bacterial isolates within the markets.

Several studies carried out locally have demonstrated occurrence of multi – drug resistant *E. coli* and *Salmonella* among food animals and human in Kenya (Bebora, Oundo & Yamamoto 1994, Kariuki et al. 1999, Kariuki et al. 2002, Kariuki et al. 2005). In all these studies high degrees of resistance to co-trimoxazole, ampicillin and tetracycline, were observed and conjugative plasmids of about 100kb to 110 kb were reported to be responsible for the transfer of resistance among the *E. coli* and *Salmonella* isolates studied. However, in this study a non-conjugative plasmid of approximately 5.6 kb was

more frequent and common among isolates that showed resistance to ampicillin. Other studies have also reported occurrence of small molecular weight plasmids among *Enterobacteriaceae* family (Kariuki et al. 2002, Sherley, Gordon & Collignon 2003, Kariuki et al. 2005).

Sherley, Gordon & Collignon (2003) observed that the majority of *E. coli* wild-type strains carried one or more plasmids, which varied in size, but 45% of the isolates from the plasmid bearing strains, had plasmids smaller than 16 kb in size. Kariuki et al (2002) and Kariuki et al (2005) on the other hand observed that such small molecular weights plasmids (of <15 kb) were non-transferable by conjugation among *E. coli* or *Salmonella* isolates in their studies. Bacteria acquire plasmid mediated antibiotic resistance genes through conjugation or by direct uptake – transformation. However, studies have shown that although plasmids can be acquired through such means, they similarly can easily be lost depending on the antibiotic pressure or alternatively, they can integrate into the chromosomal DNA and become undetectable by the usual plasmid extraction techniques (Weischer & Kolmos 1993).

According to Sherley, Gordon & Collignon (2003) host cells determine plasmid-size limitations and that plasmid evolution differ depending on the species of the host cells, and the size of the plasmids involved, therefore while conjugative transfer may transfer genetic material between species, plasmids are likely to evolve independently within each

species according to size and other limitations. Plasmids also place a metabolic burden on the cell that maintains them and therefore the persistence of plasmids within a population depends on the cost/benefit ratio of plasmid carriage and/or the role in mediating adaptation to local environmental niches (Stewart & Levin 1977, Eberhard 1990). These facts explain the characteristic occurrence of antimicrobial resistance and small molecular weight non conjugative plasmids among the isolates obtained in this study.

Studies investigating the roles antimicrobial resistance transfer by both conjugative and non-conjugative plasmids in *E. coli* causing urinary tract infections, reported that small non-conjugative plasmids of < 10kb could mediate single and cross – resistance to ampicillin, tetracycline, sulphonamide and streptomycin (Vorland, Carlson & Aalen 1985). It is therefore possible to suggest that the 5.6 kb plasmid observed among isolates in this study could be carrying ampicillin resistance genes, although additional research is required as conjugative experiments conducted were not able to demonstrate this. It has however been reported by Miles, McLaughlin & Brown (2006) that small molecular weight plasmids of approximately 12kb could transfer tetracycline resistance among bacteria by transformation processes. Findings in this study therefore, should not be used to demonstrate the likely absence of transferable antibiotic resistance plasmids among *R. argentea* bacterial isolates.

4.2 Conclusions

The study findings have demonstrated that omena displayed for sale on markets in Kisumu town does not comply with the KEBs KS05-1470: 1998 specifications for sundried *R. argentea* and thus possess a health risk to the public.

R. argentea sold on markets in Kisumu town could play a role (to some extent) in transmitting causative agents for some foodborne illnesses, such as *Salmonella* and also be a source of multi-drug resistant (MDR) *E. coli*. Personnel involved in the processing, transportation and marketing of the products including the consumers may be at risk of contracting these infections.

Utilization of omena as fishmeal makes it an important source of these pathogens in animal feeds. Incorporation of an irradiation or a killing step could improve on the safety of omena before use in the animal feeds. The Codex Alimentarius Commission has suggested irradiation at 1.5 or 2.2 kGy for teleost fish and fish products. Alternatively, hygienic handling and preservation of omena, during harvesting, processing and marketing may also eliminate the danger of microbial pathogens from the fishery products.

Applying Good Hygiene Practices (GHP) such that the fishery products are protected from possible contamination; and employing risk management tools such as the Hazard Analysis and Critical Control Point (HACCP), to minimise public health risks are important management systems that can guarantee elimination or control of food hazards.

This study confirms the possibility of a common source of exposure to antimicrobials among the isolates of clinical importance and those contaminating omena displayed for sale on local markets in Kisumu town. The study also showed that other factors may be important in influencing resistance profile and rates of resistance within the markets and tetracycline respectively. The findings further suggest that MAR indexing of *E. coli* alone may not be a good tool for risk assessment among some food products especially those produced and processed in rural areas and environments where intensive rearing of animals is not practiced.

The study also suggests that the *E. coli* isolates contaminating omena in Kisumu town consist largely of plasmids of low molecular weights, which are non-transferable by conjugation and a plasmid of approximately 5.6kb is common among isolates that were resistant to ampicillin and could be responsible for encoding for ampicillin related resistance.

4.3 Recommendations

The study therefore recommends the following: -

- Public health workers should encourage fishermen, processors and traders to adopt HACCP as a risk management tool, among Good Hygiene practices (GHP) at their respective levels for ensuring that fish products are produced under hygienic conditions and that food safety measures are in place.
- Government should allocate funding to go towards the development and adaptation of better methodologies of processing, transporting and displaying omena for sale. Such technologies should be able to address contamination by dust, insects, birds and domestic animals.
- Since omena has become an important protein supplement for animal feeds there is need for government and other stakeholders to fund surveillance studies to detect pathogenic bacteria that may be a source of infestation and spread of antimicrobial resistance among food animals.
- Public health workers should sensitize the public on the health risks associated with omena. Consumers of omena should be advised to cook appropriately to ensure the process kills all microbes and similarly they should avoid cross-contamination from raw omena to other foods.
- The government regulatory bodies should be strengthened to enable them monitor and enforce the relevant regulations. Such an effort would ensure that only products that comply with laid down specifications and standards are allowed on to the market.

- Long term prospective studies to examine isolates from omena, including microbial source tracking, are required to give more accurate, temporal and spatial information on the levels and characteristics of microbial contamination including resistance to antimicrobials among markets.
- Characterization of the small plasmids may be necessary to understand if they play any roles in encoding for drug resistance and possibly plasmid development among the *Enterobacteriaceae* family contaminating omena products.

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APPENDICES

Appendix I: Biochemical tests for characterization of *E.coli*, *Salmonella* and *Shigella*

Biochemical test	**Species**		
	E.coli	***Salmonella***	***Shigella***
VP	+	-	-
Citrate	+	-/+	-
Lysine Decarboxylase	+	+	-
Indole	+	-	-
Oxidase	-	-	-
Methyl red	-	-	+
Catalase	+	+	+
Urea	-	-	-

Appendix II: Results of antimicrobial susceptibility test for *E. coli* and *Salmonella* isolates obtained from *omena* isolates from markets in Kisumu town

Isolate	Amp	Tet	Cot	Aug	Kan	Gen	Crx	Chl	Nal	Nor
E23	22	21	24	20	20	18	19	26	19	21
E12	7	23	6	23	17	20	24	29	20	21
E22	22	20	6	20	18	19	21	28	17	21
E6	23	22	6	21	20	20	22	25	21	20
E27	20	22	29	20	18	18	18	23	20	23
E41	18	20	6	19	18	18	20	19	19	20
E10	21	23	25	21	20	18	25	25	21	22
E40	20	22	6	20	19	20	21	25	19	21
E42	20	19	19	19	14	17	19	21	18	19
E13	6	6	15	13	15	15	16	23	19	20
E4	20	20	26	20	20	18	20	25	23	20
E38	20	20	6	22	19	19	20	25	20	21
E15	20	6	6	19	16	16	18	22	18	19
E6	23	22	27	21	17	17	22	27	19	20
E28	23	21	27	22	20	18	21	30	23	25
E37	19	7	6	19	19	16	19	25	18	20
E14	20	20	23	20	19	17	20	24	20	23
E29	19	19	20	20	15	15	20	25	20	21
E2	18	18	22	17	16	17	20	22	20	22
E1	20	19	25	20	15	17	20	27	22	20

E5	20	20	26	19	21	18	20	25	19	20
E44	19	18	24	19	16	16	18	22	19	21
E25	19	19	23	19	17	14	18	23	21	25
E31	17	18	6	20	14	15	16	23	20	21
E51	17	19	6	19	14	16	17	24	18	20
E30	18	18	23	20	17	15	18	24	22	24
E32	18	20	20	19	17	17	18	25	18	19
E18	21	18	25	19	18	17	18	24	17	20
E27	19	18	6	18	13	14	14	22	19	19
E17	18	18	6	18	12	15	15	23	20	20
E46	20	20	24	18	15	17	18	23	19	21
E8	20	20	24	19	17	17	19	25	20	21
E21	23	22	6	22	19	18	23	25	17	21
E47	21	6	6	20	18	17	19	25	22	24
E20	21	6	20	6	16	16	20	20	14	16
E24	6	7	6	22	17	17	21	29	22	25
E35	20	18	22	19	17	17	20	24	21	26
E43	19	21	6	20	18	17	18	26	18	22
E39	21	20	25	22	22	22	22	26	23	28
E45	6	6	6	16	18	13	15	25	15	15
E36	20	19	24	19	16	17	19	24	21	20
E48	22	20	25	22	20	22	22	27	22	25
E50	20	22	27	20	20	18	20	27	21	23

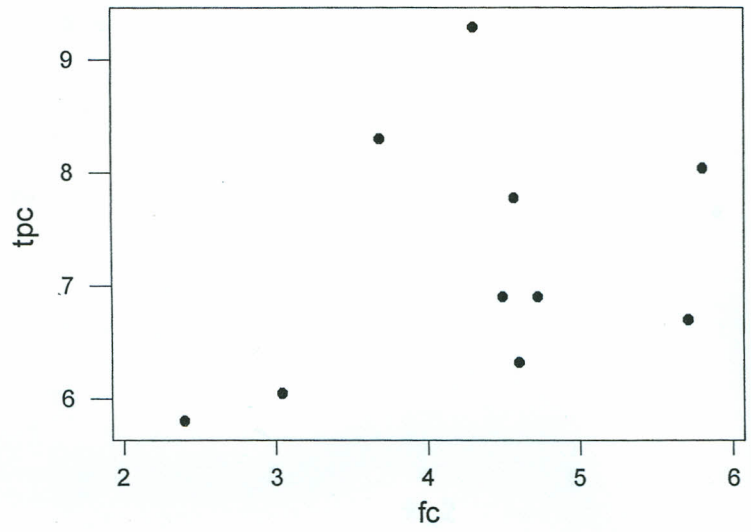
E49	18	17	22	15	14	16	17	22	17	21
E34	18	8	15	18	16	16	7	25	19	23
E3	18	17	22	16	15	16	10	24	20	22
E33	6	18	7	15	16	15	17	25	19	22
E16	22	9	22	19	17	16	22	23	21	25
E19	7	6	6	19	19	19	19	28	21	20
S6	14	20	24	14	17	17	20	22	19	20
S1	20	18	20	21	18	17	14	27	21	24
S2	18	20	18	20	19	19	20	21	20	23
S4	17	17	22	15	16	18	19	23	21	24
EC25922	22	25	29	24	24	21	26	32	25	31

Legend: Amp =ampicillin, tet = tetracycline, cot = cotrimoxozale, aug = augumentin, kan = kanamycin, gen = gentamycin, crx = cefuroxime, chl = chloromphenicol, nal = nalidixic acid and nor = norfloxacin.

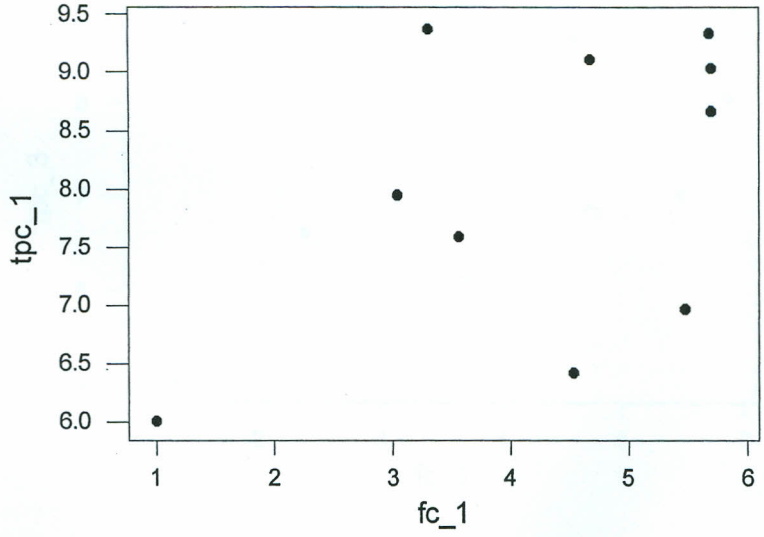
Appendix III: *Salmonella* serotypes isolated from omena samples

	O Antisera		H antisera		
Isolate	**Group**	**factor**	**phase 1**	**phase 2**	**serotype**
S1	B	4	i	1	*S. enteritica* T
S2	D	9	g	-	*S. enteritidis*
S4	D	9	g	-	*S. enteridídis*
S6	B	4	b	2	*S. paratyphi* B

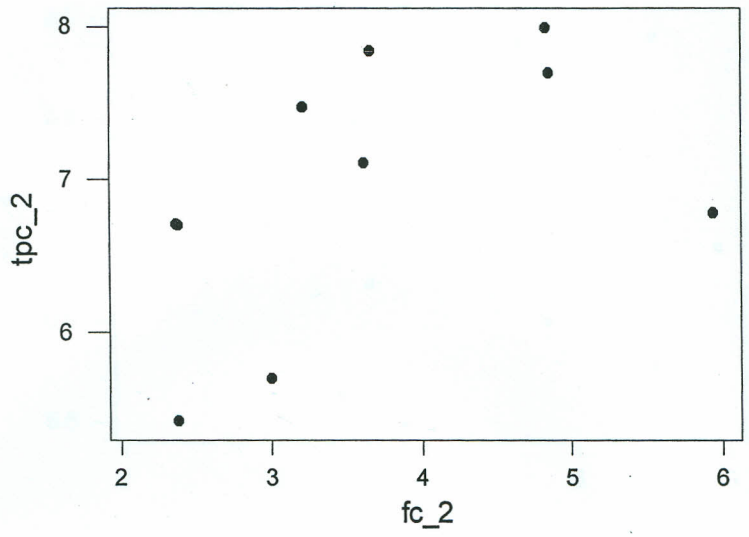
Key: T = Typhymurium.



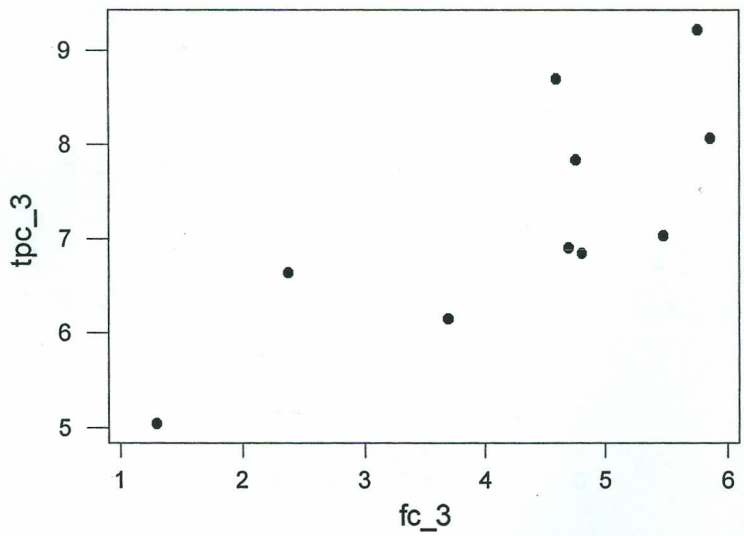
Appendix **IV**: Scattered plot showing relationship between TPC and FC points for Oile market



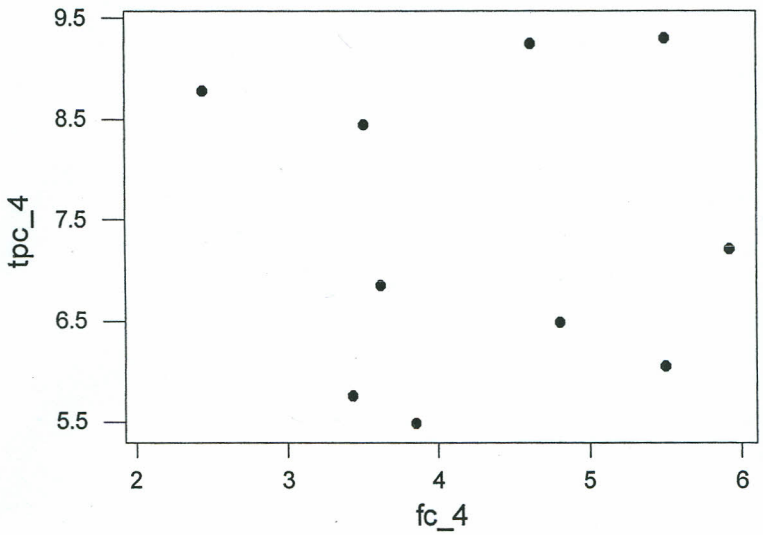
Appendix **V**: Scattered plot showing relationship between TPC and FC points for Jubilee market



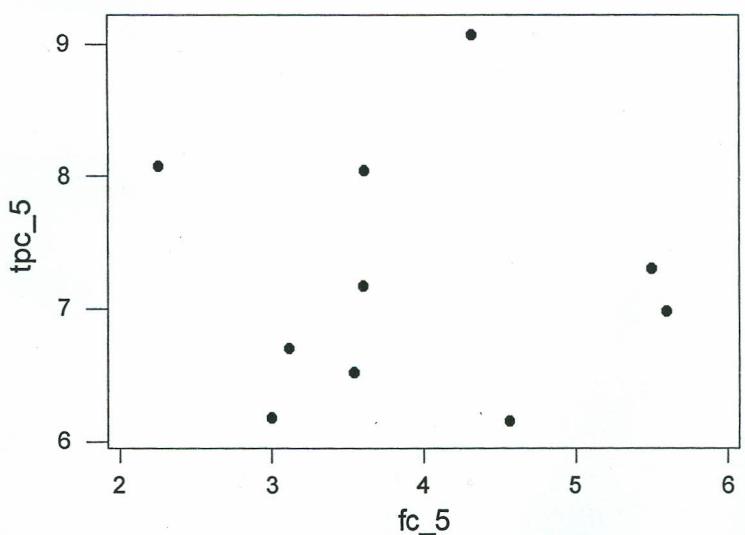
Appendix **VI**: Scattered plot showing relationship between TPC and FC points for Kibuye market



Appendix **VII**: Scattered plot showing relationship between TPC and FC points for Kondele market



Appendix **VIII**: Scattered plot showing relationship between TPC and FC points for Nyalenda market



Appendix **IX**: Scattered plot showing relationship between TPC and FC points for Manyatta market