PREVALENCE, ANTIMICROBIAL PATTERNS AND EVALUATION OF DIFFERENT METHODS FOR CONTROLLING PATHOGENIC MICROBES IN WATER AND FISH FROM LAKE VICTORIA AND ITS BASIN IN KENYA

By

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ABSTRACT

Fish is a very important foodstuff in developing countries due to its high protein content and other nutritional value. Fish contamination by microbes, mainly bacteria and fungi, may lead to food poisoning characterized by gastrointestinal diseases, vomiting, diarrhea, acute renal failure and nausea. Developing countries are worst affected by food-borne illnesses due to the presence of pathogenic microbes that may grow in fish. Antimicrobial resistant enteric bacteria have been reported in various aquatic environments including lakes, rivers, drinking water and sewage. Food including fish contaminated with antimicrobial-resistant bacteria is a major threat to public health. Prevalence of fungi and moisture content in sun-dried fish have been a concern due to mycotoxins produced by fungi. This study was designed to determine the prevalence and antimicrobial susceptibility patterns of *Escherichia coli, Salmonella enterica* serovar Typhimurium and *Vibrio cholerae* O1 isolated from water and two fish species *Rastrineobola argentea* (Dagaa) and *Oreochromis niloticus* (Nile tilapia) in the Lake Victoria basin of western Kenya. It also assessed the levels of infestations of dried fish by fungi and moisture contents and finally compared and evaluated the traditional non-selective methods of brine salting and sanitizing with chlorinated solution with new *Moringa oliefera* plant extracts and bacteriophage treatments which can be used for preserving *Rastrineobola argentea* (Dagaa) and *Oreochromis niloticus* (Tilapia) fish and controlling the spread of fish-borne pathogenic microbes. Using a cross-sectional study design, water and fresh or sun-dried fish (*Rastrineobola argentea* and *Oreochromis niloticus*) samples were randomly collected from three fish landing beaches (Dunga, Luanda Rombo and Sirongo) and from three markets (Kisumu municipality, Luanda and Bondo), in the Lake Victoria Basin of western Kenya and taken to the laboratory within 4 hours for processing and microbiological analysis using conventional standard procedures. Statistical difference in the prevalence of enteric bacteria in water or fish samples between the beaches or markets, and effectiveness of the different preservatives was determined by two way Analysis of Variance (ANOVA) with $p \leq 0.05$ value considered statistically significant. *Escherichia coli, S. enterica* serovar Typhimurium and *V. cholerae* O1 were isolated from water and fish. The results showed that water samples from Dunga beach were the most contaminated (282 CFU/ml) and that from Sirongo beach was least contaminated (193 CFU/ml). Fish samples (*Rastrineobola argentea* and *Oreochromis niloticus*) from Kisumu market had the highest total viable bacteria count (218 CFU/g) and those from Sirongo beach had the lowest viable count (130 CFU/g). Out of 162 samples analyzed, 133 (82.1%) were contaminated with various enteric bacterial species. *S. enterica* serovar Typhimurium was the most prevalent with 49.6% among the isolates followed by *E. coli,* 46.6% and the least was *V. cholerae,* 2.8%. Dunga beach had the highest number of bacterial species isolates, 33.8% followed by Kisumu market, 15.85% and the least was Sirongo beach with, 11.3%. The difference in prevalence between the enteric bacteria species, and the beaches or markets was statistically significant, ($p < 0.01$), by two way ANOVA. All the isolates were sensitive to ciprofloxacin. *E. coli* was resistant to ampicillin, tetracycline, cotrimoxazole, chloramphenical and gentamicin while *S. enterica* ser. Typhimurium exhibited resistance to ampicillin, tetracycline, and cotrimoxazole. *V. cholerae* O1 was resistant to tetracycline and ampicillin. Paired two-tail t-test showed that sun-dried *R. argentea* and *O. niloticus* from the markets had significantly higher fungal species isolates compared to fish from the beaches (P = 0.012) sun-dried *R. argentea* from the markets had significantly higher fungal species isolates compared to sun-dried *O. niloticus* from the

markets ($p < 0.00001$) and moisture content of fish (dagaa and tilapia) from the beaches was significantly lower (mean = 13.3%) compared to moisture content of the fish from the markets (mean = 14.3%, $p = 0.03$). A total of 11 fungi species were isolated from the sun-dried *R. argentea* and *O. niloticus* from different sampling beaches and markets. *Mucor* spp and *Penicillium* spp were the most prevalent fungi species isolated from dagaa and tilapia respectively. The results of the preservative experiments showed that with increase in salt concentration and time, there was a high reduction of microbial load in *R. argentae* and *O. niloticus*. The effectiveness of chlorinated solution (sodium hypochlorite) against the bacteria decreased with time but increased with increase in concentration. As *M. oleifera* n-hexane and ethanol extracts concentration and time increased, there was significant decrease in microbial load ($p < 0.001$). Bacteriophage suspension was the most effective, followed by sodium chloride at 12% concentration and chlorine solution even at 200ppm was the least active while the *M. oleifera* plant extracts solutions were moderately effective against the bacteria in fish. The results from this study show that water and fish from some parts of Lake Victoria and its basin are contaminated with antibiotic resistant bacteria, which are of great public health concern. The results also show that other than the traditional sodium chloride, bacteriophage suspension and *M. oleifera* plant extracts can also be used as antimicrobial agents for processing and preservation of fish. The significance of the study was to introduce innovative methods in reducing pathogenic food-borne microbes while handling fish in fish landing beaches and fish markets. It was also significant to show which pathogens were resistant to the antibiotic administered against their infections.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

The challenges of safe food production and delivery in the 21st century are unique in human history while opportunities for food contamination are many. Some of these opportunities arise from human negligence or ignorance of hygiene. As food is being transported to different locations, contamination may occur and spread rapidly if the food is not handled under good hygienic conditions (Ashok, 2008).

According to the World Health Organization (WHO, 2002), food-borne infections are the major causes of illness and death worldwide. Ingestion of foods contaminated with bacteria, viruses and parasites is a major world public health issue, especially in developing countries and a significant proportion of diarrheal diseases are of food-borne origin (Rosek *et al.,* 2003). Those susceptible to food-borne infections are particularly infants, children, the elderly, pregnant women and immunocompromised individuals (Lund and O'Brien, 2011). The World Health Organization estimates that approximately 2–3% of cases of food-borne disease outbreaks leads to long-term ill health (WHO, 1999).

Micro-organisms harboured in foods evolve and new ones emerge. All infectious agents causing diarrhea are efficiently spread by the faecal-oral route (Okoh and Osode, 2008). According to epidemiologic investigations, consumption of faecally contaminated food and drinking water are the most common causes of diarrheal infection (WHO, 2002). Fresh fish is prone to microbial contamination by pathogenic microorganisms (Novotny *et al.,* 2004: Sujatha *et al.,* 2011). Therefore, it is important to treat or preserve fresh fish to inactivate pathogenic microorganisms. Pathogenic and potentially pathogenic bacteria associated with

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fish and shell fish include *Mycobacteria, Streptococcus iniae, Vibrio vulnificus,* and other *Vibrio* spp., *Aeromonads,* and *Salmonella* spp. (Chattopadhyay, 2000).

The US Centers for Disease Control and Prevention (CDC) (2001) reported that in the past fifteen years, several important diseases of previously unknown cause have been determined to be complications of food-borne infections. Examples are Guillain-Barre syndrome, which can be caused by *Camplyobacter* infection, and hemolytic uremic syndrome (acute kidney failure in children), caused by *E. coli* O157:H7 (Wong, *et al*, 2000; Gould, *et al.,* 2009). In addition, *Salmonella* infection may produce reactive arthritis and serious infections, while *Listeria* can cause meningitis and stillbirths. According to CDC, many sick people do not seek medical attention, and since many who do are not tested, numerous cases of food-borne illnesses go undiagnosed (CDC, 2001). The CDC estimates that thirty-eight cases of salmonellosis occur for every case that is diagnosed and reported to public health authorities (CDC, 2006).

In many developing countries, bacteraemia due to invasive non-typhoid salmonellosis has been associated with increased risk in death, particularly in children with severe malaria and among immunocompromised adults, particularly those with HIV/AIDS (Dropulic and Lederman, 2009). The main source of infections has been contaminated food of animal origin (Velge *et al.,* 2004). Cholera has been endemic in the developing countries of Asia and Africa and has caused epidemics in them (WHO, 2011), the same to the Middle East, and in South and Central America (CDC, 1999). In 2004, fewer cases of cholera worldwide were reported to the World Health Organization than in any year in the previous decade 101,383 cases from 56 countries (WHO, 2004). Microbial contamination of water is the largest and immediate health hazard (Arnone and Walling, 2007). Surface water quality is subjected to frequent

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dramatic changes in microbial quality because of the variety of activities on the watershed (Okoh *et al.*, 2005). *Vibrio cholerae*, the etiologic agent of cholera, is autochthonous to various aquatic environments, but despite intensive efforts its ecology remains an enigma. As fish carrying bacteria swim from one location to another they serve as vectors on a small scale (Sujatha *et al.*, 2011). Senderovich *et al.*, (2010) have confirmed the occurrence of *V. cholerae* in some marine species of fish and suggested that fishes are reservoirs of *V. cholerae*. An outbreak of cholera caused by *Vibrio cholerae* was recorded in western Kenya between June 1997-March 1998 (Shapiro *et al.*, 1999).

The use of antibiotics in livestock, fish farming, and poultry has accelerated the development of antibiotic resistant bacteria, complicating the treatment for both animals and human (Van den Bogaard and Stobberingh, 2000). A number of studies have indicated that microorganisms develop resistance to human medicine due to over use of the antimicrobials in veterinary, animal husbandry, as well as agricultural practice (Barton, 2000; Velge *et al.*, 2004). Chemotherapeutic selection may have additional consequences for virulence evolution through the acquisition of virulent genes (Velge *et al.*, 2004). In many developed countries, most antimicrobial resistant *Salmonella* infections are acquired from eating contaminated foods of animal origin (Angulo *et al.*, 2000; Gorman and Catherine 2004). Raw, undercooked or poorly processed fish can be contaminated by pathogenic microbes that cause diseases such as cholera, typhoid, bacillary dysentery, staphyloenterotoxicosis, listeriosis, and aflatoxicosis (Feldhusen, 2000; John *et al.*, 2003; Butt *et al.*, 2004).

Moulds are common contaminants of agricultural commodities, foods, beverages and feeds. Moulds grow under moist conditions better than bacteria and for this reason, moulds are often associated with sun-dried and smoked fish (Santour *et al.*, 2002). Dried fish products

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undergo contamination from varied fungal flora. The most predominant genera that have been reported include the *Aspergillus spp., Penicillium spp., Rhizopus spp., Mucor spp., Fusarium spp., Wallemia spp.,* and *Cladosporium* spp. (Obeyamiji *et al.,* 2008). Under favourable conditions during harvesting, processing and storage of food commodities, moulds produce toxic metabolites known as mycotoxins (Sulyok, 2006; Bhat and Miller, 2010) which are currently of considerable concern to global food safety because of their ubiquity and potential deleterious effect on human and animal health. Considerable importance has been attached to aflotoxins in foods and feeds because of their carcinogenic, mutagenic and teratogenic nature (CDC, 2004; 2005) and various researchers have reported dangerous levels of aflatoxin in dried fish (Mitchell, 2007; Adebayo-Tayo *et al.,* 2008).

Human infections by pathogenic pathogens in fish are usually through contact with infected or infested fish while handling them, water or other constituents of fish life environment (Acha and Szyfres, 2003). Most of human gastroenteritis results from ingestion of contaminated food products such as undercooked fish, shell fish, beef, pork, eggs, and milk (Esaki *et al.,* 2004). The current study was therefore designed to determine the prevalence and antimicrobial patterns of enteric microbes (*E. coli, S. typhimurium,* and *V. cholerae*) in water and fish, investigate fungal infestations and moisture contents of dried fish, and compare different non-selective methods for preserving fish from Lake Victoria and its basin in Kenya.

1.2 Statement of the Problem

The prevous studies done by Onyango *et al.* (2008; 2009) indicates that the water of Lake Victoria is contaminated with various bacteria and this could contaminate fish which are in the same environment. Also storm waters and effluents going into the Lake Victoria,

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(Onyango, 2009) can contaminate the lake waters and fish in the water or fish processed at the beaches using contaminated or untreated water.

Fish can be involved in both passive and active transfer of a range of diseases causing pathogens to humans. The identification of *Streptococcus iniae* in tilapia as the cause of sore throat in North America (FDA, 2006) has raised concern about fish-borne infections. The ban on fish export from Kenya in 1996 to EU countries was due to *Salmonella* spp., detection on fresh fish export from Kenya (Henson *et al.,* 2000). In this study, it was therefore important to determine the prevalence of *E. coli, S. typhi* and *V. cholera* in water and fish (*Rastrineobola argentea* and *Oreochromis niloticus*) from the beaches and markets of Lake Victoria basin, Kenya

Fish can also harbour antimicrobial resistant microbes. In the previous studies, Onyango *et al.,* (2008; 2009) found that the of high antibiotic resistance pattern of *Salmonella* spp isolated from the fish landing beaches were resistant to commonly used antibiotics used for treating salmonellosis and diarrhoeagenic diseases in the local community. The high prevalence of resistance to tetracycline, ampicillin and co-trimoxazole in *E. coli* in the lake basin region has also been reported by Sifuna *et al.* (2008), in which *E. coli* demonstrated resistance mostly to ampicillin and tetracycline. It was therefore important to determine the antimicrobial patterns of *E. coli, S. typhimurium* and *V. cholerae* isolated from water and fish (*Rastrineobola argentea* and *Oreochromis niloticus*) from the beaches and markets of Lake Victoria basin, Kenya

Several studies on the assessment of the risk potential of dried fish have reported the detection of aflatoxins as potential natural contaminants in dried and smoked fish products (Santour *et al.,* 2002; Mitchell, 2007; Adebayo-Tayo *et al.*, 2008; Owaga *et al.*, 2010). It

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should always be an urgent need to screen fish before human consumption as fish are vehicles of pathogen and various researchers have reported dangerous levels of aflatoxin in dried fish (Bennett and Klich, 2003). In this research, it was therefore important to determine the prevalence of fungal infestations in sun-dried *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya.

The moisture content of dried fish products adversely affects the presence of fungal growth on dried fish and fishery products. Fungal species such as *Apergillus* sp., *Penicillium* sp., *Mucor* sp., *Rhizopus* sp., and *Fusarium* are pathogenic to humans and also cause food spoilage. (Felicia and Jamila, 2003). Since fungi grow better in moist conditions it was of significance to determine the moisture content of sun-dried *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya.

Studies have indicated that salt can significantly inhibit the bacterial contamination of fish and also significantly increase its shelf life (Kofi, 1992). In addition, chlorinated water is commonly used for sanitizing fish during processing in factories (Park *et al.,* 1991). Doughari *et al.,* (2007) found that 100 mg/ml of *M. oleifera* plant extracts inhibit the growth of *S. typhi,* and Suarez *et al.,* (2003) demonstrated the antibacterial and antifungicidal activity of *M. oleifera* leave extract and its antibiotic activity identified as pterygospermin, a bacterial and fungicidal compound. Therefore, it was found necessary to evaluate the effective of *Moringa oliefera* plant extracts for preservation of fish in comparison to the traditional methods, i.e., salting and sanitizing by use of chlorinated solution. The use of phages in the treatment of bacterial infections or in prophylaxis is an attractive alternative to existing therapies (example, antibiotics), because unlike broad-spectrum antibiotics, lytic phages target a particular host

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and are unlikely to illicit resistance in untargeted bacterial strains (Mathur *et al.*, 2003; Sulakvelidze and Kutter, 2005). Also unlike chemical therapeutic agents, phages are not susceptible to the onset of bacterial resistance because they have the ability to evolve with their host (Sulakvelidze and Kutter, 2005). Bacteriophage applied directly to food has no harm to human (USFDA/CFSAN, 2006). However, these traditional methods of fish preservation had not been compared with bacteriophage, chlorinated solution and also with *Moringa oleifera* plant extracts in controlling pathogenic microbes associated with *R. argentea* (dagaa) and *O. niloticus* (tilapia). Therefore it was important to compare the traditional non-selective methods of brine salting and sanitizing with chlorinated solution, with new *Moringa oleifera* plant extracts and bacteriophage treatment for preserving fish *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya in this thesis research.

1.3 Justification and Significance of the Study

New food-borne pathogens are being identified from old familiar pathogens, and new pathogens are being discovered (Lone and Hans, 2004) such as reactive arthritis listeriosis. Raw, undercooked or poorly processed or handled fish can be contaminated with pathogenic microbes that cause diseases such as cholera, dysentery, typhoid, diarrhea and aflatoxicosis. Untreated or inadequately treated domestic sewage is usually the primary source of microbial pathogens in the lake waters, and some disease-causing micro-organisms can persist for months in fish or within the digestive tracts or gills of fish (Novotny *et al.*, 2004; Sujatha *et al.*, 2011). Sewage contamination of fish harvesting areas is the major reason for the presence of *E. coli*, however, contamination can occur through the use of non-portable water or ice in the fish landing beaches/points or fish markets (Kumar *et al.*, 2005). Fish landing

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environment has been identified as a major source of fish quality problems in Kenya (Nanyaro and Makene, 1998). Fish has been found to be a source of *Salmonella* infection (Massete, 1999). Salmonellosis was the most frequent laboratory diagnosed food-borne infection by The Food-borne Diseases Active Surveillance Network (Food Net) in 2001 (CDC, 2002). Fish such as *R. argentea* (dagaa) and *O. niloticus* (tilapia) are foods that are consumed by many people living around Lake Victoria since they are nutritious and have high protein content (Abila, 1998; Abdullahi *et al.,* 2001). Fish from L. Victoria represents 85% of Kenya's fish supply and constitutes 25% of total catch from Africa's inland fisheries (Gitonga, 2006).

This study was not only designed to assess the prevalence of fungal infestations in sun-dried *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets but also determine the moisture contents of the sun-dried *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) The study further compared and evaluated the traditional non-selective methods of brine salting and sanitizing with chlorinated solution, with new *Moringa oleifera* plant extracts and bacteriophage treatment for preserving fish *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya.. This is because it is important to show that use of selective antibiotics treatment of patent infections which can lead to drug resistant strains can be avoided by use of non-selective preservatives such as salt, chlorine, *Moringa oleifera* plant extracts and even bacteriophage treatment which can be used to control the spread of the pathogenic microbes.

Dried fish products undergo contamination from varied fungal flora. Under favourable conditions during harvesting, processing and storage of fish, moulds produce toxic

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metabolites known as mycotoxins (Adebayo-Tayo *et al.*, 2008) which are currently of considerable concern to global food safety because of their ubiquity and potential deleterious effect on human health.

By unwinding the intricacy of pathogenic food-borne microbes, prevalence and antimicrobial patterns of *E. coli, Salmonella typhimurium* and *V. cholerae*, prevalence of antimicrobial, prevalence of fungal infestation and moisture content on sun dried fish and food preservation, the results from this study can provide new methods of reducing pathogenic food-borne microbes in Kenya, particularly in various parts of the fish landing beaches and markets. The results from this study can also provide valuable information to agencies and legislators, Ministry of Health, Ministry of Fisheries, FAO and WHO that are involved in making policy decisions on food safety in Kenya and other parts of the world.

1.4 Main Objective

Determine the prevalence and antimicrobial patterns of enteric microbes (*E. coli, S. typhimurium*, and *V. cholerae*) in water and fish, investigate fungal infestations and moisture contents of dried fish, and compare different non-selective methods for preserving fish from Lake Victoria and its basin in Kenya.

1.4.1 Specific Objectives

1. To determine the prevalence of *E. coli, S. enterica* serova Typhimurium and *V. cholerae* in water and fish (*Rastrineobola argentea* and *Oreochromis niloticus*) from the beaches and markets of Lake Victoria basin, Kenya.

2. To determine the antimicrobial patterns of *E. coli*, *S. enterica* serova Typhimurium and *V. cholerae* isolated from water and fish (*Rastrineobola argentea* and *Oreochromis niloticus*) from the beaches and markets of Lake Victoria basin, Kenya

3. To determine the prevalence of fungal infestations in sun-dried *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya.

4. To determine the moisture content of sun-dried *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya.

5. To compare the traditional non-selective methods of brine salting and sanitizing with chlorinated solution, with new *Moringa oleifera* plant extracts and bacteriophage treatment for preserving fish *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya.

1.5 Null Hypothesis

1. *Escherichia coli, S. enterica* serova Typhimurium and *V. cholerae* are not prevalent in water and fish (*Rastrineobola argentea* and *Oreochromis niloticus*) from the beaches and markets of Lake Victoria basin, Kenya.

2. *Escherichia coli, S.enterica* serova Typhimurium and *V. cholerae* isolated from water and fish (*Rastrineobola argentea* and *Oreochromis niloticus*) from the beaches and markets of Lake Victoria basin, Kenya, are not resistant to antibiotics commonly used in the western Kenya region

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3. Fungal infestation of sun-dried *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya is not common.

4. There are no differences in moisture contents of sun-dried *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya.

5. There are no differences in the effectiveness of non-selective methods of brine salting, sanitizing with chlorinated solution, and the new *Moringa oleifera* plant extracts, and bacteriophage treatment for preserving fish *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) from the beaches and markets of Lake Victoria basin, Kenya.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Fish-borne Pathogenic Microbes

Fishing is a very ancient activity of man and dates back to the times of Pharaohs and even earlier. Fish is a very important foodstuff in developing countries, due to its high protein content and other nutritional values. Threats to public health from fishery are diverse. Reportedly, fish had been implicated in the irregular occurrence of influenza pandemics, cholera, typhoid and diarrhea (Lone, 2004). Contamination of fish with pathogenic microbes may result from water pollution, unhygienic processing areas, and lack of medical check-ups for fish processors (King *et la.,* 2000).

Gram *et al.* (1990) reported that fish has a distinctive complex microbiology, which is contributed by various factors. The surface flora on fishery products reflects the environmental flora where the products have passed through (Gram *et al.,* 2002). The bacterial flora found on newly caught fish is dependent on the water in which the fish is caught. Members of the *Vibrionaceae* (*Vibrio* and *Photobacterium*) and the *Aeromonadaceae* (*Aeromonas spp.*) are also common aquatic bacteria and typical of the fish flora (Gram *et al.,* 2002; Novotny *et al.,* 2004). Potential source of bacterial pathogens for human beings are often bacterial species facultatively pathogenic for both fish and man and may be isolated from fish without apparent symptoms of disease. The infection source may be fish kept either for food or as a hobby (Acha and Szyfres, 2003). Coliform bacteria are in general, undesirable in foods. Their presence in some foods (water and fish) is indicative of sewage contamination and hence of the possible presence of enteric pathogens, and their growth in foods results in contamination (Arnon and Walling, 2007).

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Food poisoning is a general term for health problem arising from eating contaminated food. Food may be contaminated by bacteria, viruses, environmental toxins or toxins present within the food itself. Food poisoning agents that are commonly associated with fish and fish products include *Escherichia spp., Salmonella spp., Vibrio spp., Staphylococcus aureus, Bacillus cereus, Listeria monocytogens* and *Clostridium perfringens* (Kvenberg, 1991). Food poisoning can be a long term problem. Symptoms of food poisoning usually involve nausea, vomiting or/and diarrhea (Lund and O'Brien, 2011).

Every year, millions of people suffer from bouts of vomiting and diarrhea associated with eating contaminated fish products (Novotony, 2004). Many cases are mild and pass so rapidly that they are never diagnosed. However, the true incidence of diseases transmitted by fish as human food is not known (Lone, 2004). There are many reasons for this. In a country like Kenya, there is no obligation to report on food-borne diseases to public health authorities. Untreated or inadequately treated domestic sewage is usually the primary source of microbial pathogens in the lake waters, and some disease-causing micro-organisms can persist for months in or on fish or within the digestive tracts or gills of fish (Thampuran, *et al.,* 2005; Rio-Rodriguez, *et al.,* 2008). Raw, undercooked or poorly processed fish can be contaminated by pathogenic microbes that cause diseases such as cholera, typhoid, bacillary dysentery, staphyloenterotoxicosis, listeriosis, and aflatoxicosis (Feldhusen, 2000; John *et al.,* 2003; Butt *et al.,* 2004).

One way to determine food or water quality is to measure the presence of *E. coli*, which is the main member of a large family *Enterobacteriaceae* which is composed of aerobic and facultative anaerobic, Gram-negative, non-spore forming rods that live in the gastrointestinal tract of most warm-blooded animals (Edberg *et al.,* 2000) and ferment lactose

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within 24-48 h at 35 ^{0}C. In general, coliform bacteria can be divided into faecal and non-faecal groups. The faecal coliform can grow at a higher temperature (44 ^{0}C ± 2 ^{0}C) than non-faecal coliform (Figueras and Borrego, 2010). The presence of *E. coli* in normal human intestines and faeces has led to using the bacteria as an indicator of faecal pollution in water (Edberg *et al.,* 2000; Hurst *et al.,* 2002; Tallon *et al.,* 2005). Most *E. coli* strains are harmless, but several cause diarrhea. In severe cases, people, especially children and the elderly, have died from ingesting water or food contaminated with *E. coli* (APHA/AWWA/WEF, 1998; Tharannum *et al.,* 2009).

2.2 Pathogenic Bacteria

2.2.1 Escherichia coli

Escherichia coli, originally known as *Bacterium coli commune*, was identified in 1885 by the German pediatrician, Theodor Escherich (Karmali, 1989; Weagant *et al.,* 1995). *E. coli* is a member of the *Enterobacteriaceae* family (Levine, 1987). The bacterium *Escherichia* species is Gram negative rods, about 0.5 µm in diameter and motile. *E. coli* is a genetically and phenotypically diverse species whose strains are identified on the basis of 'O', 'H' and sometimes 'K' antigens, which together constitute the serotype (Wani *et al.,* 2004).

2.2.1.1 Epidemiology and pathogenicity of E. coli

Based on toxigenicity, *E. coli* is now classified into five distinct groups: enteropathogenic, enterohemorrhagic, enteroaggregative, enteroinvasive and enterotoxigenic (Prescott *et al.,* 1999). Each of these subspecies of *E. coli* carries virulence gene. Enteroinvasive *E. coli* (EIEC) penetrates and grows within epithelial cells of the colon resulting into severe inflammation, bloody, mucus laden diarrhea and fever. Enteroaggregative *E. coli* (EAEC) causes non-bloody diarrhea without inflammation and

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adhering to intestinal mucosa. Enterotoxigenic *E. coli* (ETEC) which causes diarrhea by producing heat-labile and/or heat-stable enterotoxin infection symptoms include watery diarrhea without blood and can be very severe. Enteropathogenic *E. coli* (EPEC) causes profuse watery diarrhea, vomiting and a slow grade fever. For enterohemorgenic *E. coli* (EHEC), most outbreaks are caused by a single serotype O157:H7. It produces a Shiga-like toxin which causes bloody diarrhoea; toxins produced by this microbe escapes into blood stream, kills erythrocyte and also damages endothelial cells lining the blood vessel leading to hemolytic uremic syndrome (Prescott *et al.*, 1999).

Escherichia coli can cause a variety of different types of diseases which include traveler's diarrhoea, dysentery, hemolytic uremic syndrome, bladder and kidney infections (Pulz *et al.,* 2003). Diarrhoea caused by *E. coli* is probably the most important cause of children's diarrhea in developing countries (Schlundt, 2002). Though *E. coli* contamination of tropical seafood is quite common (Kumar *et al.*, 2005), the distribution of different pathogenic types in seafood is poorly studied except for a previous document on the presence of Shiga toxin-producing *E. coli* (Kumar *et al.*, 2001) in Indian seafood. *E. coli* can survive for many weeks in salted fish at 6-8% (ICMSF, 1998). Most strains of *E. coli* are not pathogenic and food quality control laboratory should be able to differentiate such strains from pathogenic ones. The presence of pathogenic *E. coli* in seafood reflects secondary contamination, as *E. coli* are known to be associated with warm blooded animals, and are not known to be present in the environment as a natural flora. Sewage contamination of fish harvesting areas is the major reason for the presence of *E. coli*, but contamination can occur through the use of non-portable water or ice in the fish landing beaches/points or fish markets (Kumar *et al.,* 2005).

2.2.2 *Salmonella*

Salmonella species are Gram-negative, motile, facultative anaerobe, rods shaped bacterium belonging to the *Enterobacteriaceae* family. It was first discovered in 1885 by the American veterinary surgeon, Daniel E. Salmon (Salmon, 1884). *Salmonella* isolates have been generally classified according to the Kauffmann-White scheme based on the presence of specific antigens contained in the envelope, cell wall and flagella referred to as Vi, O and H antigens, respectively (Velge *et al.*, 2005). *Salmonella* also contains extrachromosomal DNA, termed plasmids, which are important due to the presence of genes that confer antibiotic resistance and pathogenecity. More recently, a change of nomenclature was proposed in order to minimize improper medical treatments due to confusion between serotypes (Chen-Hsun and Lin-Hus, 2007). The new nomenclature recognizes all *Salmonella* isolates as a single species. *Salmonella* remains the primary cause of reported food poisoning world wide and recent years have seen massive outbreaks. The genus *Salmonella* consists of more than 2,600 serotypes that cause a wide spectrum of illnesses in humans (Garcia-Del Portillo, 2000).

In the United States, an estimated 1.41 million cases and more than 500 human deaths occur annually due to salmonellosis (Santos *et al.*, 2003). It is estimated that 600 deaths occur annually from *Salmonella* infections primarily among the elderly and very young (Katherine, 2004) and more than one third of all cases occur in children younger than 10 years, while the incidence in children younger than 1 year is ten times higher than in the general population (Katherine, 2004). Approximately 95% of these infections are food-borne. The two predominant agents associated with food-borne nontyphoidal salmonellosis are *S. enterica* serotype Enteritidis and *S. enterica* serotype Typhimurium (Kidgell *et al.*, 2002). In many countries where sanitation is poor, typhoid and paratyphoid fevers, which are transmitted by faecal-oral route, are an important cause of gastroenteritis illness (John *et al.*, 2003).

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2.2.2.1 Epidemiology and pathogenicity of Salmonella

Salmonella is one of the most important food-borne pathogens, being responsible for about half reported cases and outbreaks of food-borne diseases in US (Butt *et al.*, 2004). Approximately 13 million cases of salmonellosis occur worldwide annually with 70% of the reported cases occurring in Africa (Murugkar *et al.*, 2005). *Salmonella* is well established as one of the most important causes of food-borne illness worldwide and transmission is usually by the faecal-oral route (Adams and Moss, 1995). It is generally believed that like *E. coli*, *Salmonella* are not present in the natural aquatic environment but are derived as a result of sewage contamination. However, unlike *E. coli*, *Salmonella* is associated with a number of non-human hosts, for example, reptiles (Winfield and Groisman, 2003). *Salmonella* has been reported to survive and persist in the aquatic environment (Ekperigin and Nagaraj, 1998) and this can contaminate fish. This bacterium has been detected in environmental soil samples collected from both agricultural and recreational areas (Winfield and Groisman, 2003) and it has been reported that *Salmonella* can survive and even multiply in this ecosystem in contrast to *E. coli* that has a half life span of 3 days in soil (Winfield and Groisman, 2003). Compared to other bacteria, *Salmonella* has high survival rates in aquatic environments (Chu-Yu *et al.*, 2010). It outlives both *Staphylococcus aureus* and the waterborne *Vibrio cholerae* in ground water and in heavily eutrophied river water (DiRita, 2001). *Salmonella* has been detected in the gut of tilapia and carp (Ogbondeminu, 1993).

In humans, *Salmonella* are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a food-borne infection/intoxication. Salmonellosis is characterized by diarrhea, abdominal pain, nausea, vomiting and fever which generally appear 12–36 h after ingestion (Nester *et al.*, 2004). The main source of infections has been contaminated food of animal

origin. Following oral ingestion, *Salmonella* colonizes the intestine and invades the intestinal mucosa. Invasion of enterocytes and mast cells results in the extrusion of infected epithelial cells into intestinal lumen followed by villus blunting and loss of absorptive surfaces (Wallis and Eduoard, 2000). *Salmonella* also elicits a polymorphonuclear (PMN) leukocyte influx into infected mucosa and induce watery diarrhae, which may contain blood (Wallis and Eduoard, 2000).

There is very little information on *Salmonella* food poisoning (gastroenteritis) arising from the consumption of fish in Africa despite the unsanitary fish processing practices observed in many countries. The ban of fish export from Kenya in 1996 to other EU countries was due to *Salmonella* spp detection on fresh fish exports from Kenya (Henson *et al.*, 2000).

2.2.3 *Vibrio cholerae*

The bacterium *Vibrio* species belong to the Vibrionaceae family. Vibrios are Gram negative, facultatively anaerobic, non-spore forming, rigid, about 1.4-2.6 μm long, curved rods that are actively motile with a single polar flagellum. *Vibrio cholerae* was first isolated in pure culture by Robert Koch in 1883, although it had been seen by other investigators including Pacini, who is credited with describing it first in Florence, Italy, in 1854 (Alam *et al.*, 2006). *Vibrio cholerae* is traditionally classified by somatic O group (with >150 O types currently recognized), by biotype (classical and El Tor), and by serotype (Ogawa, Inaba, and, rarely, Hikojima) (Sack *et al.*, 2004).

Until the 1990s, *V. cholerae* strains belonging to serogroup O1 (referred to as *V. cholerae* O1) were believed to be the sole etiologic agents of cholera. Strains of the other serogroups are collectively known as non-O1 *V. cholerae*. However, since late 1992, *V. cholerae* serogroup O139 has emerged as an additional etiologic agent of cholera in the Indian

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subcontinent (Ramamuthy *et al.,* 2003). Cholera caused by *V. cholerae* O139 is indistinguishable from that caused by *V. cholerae* O1. These serogroups are known as epidemic *V. cholerae* strains.

Vibrio cholerae is a mesophilic organism that grows in the temperature range of 10 ^{0}C to 43 ^{0}C, with optimum growth at 37 ^{0}C. The pH optimum for growth is 7.6 although it can grow in the pH range of 5.0 to 9.6. *V. cholera* live both in marine and fresh water habitat and in association with aquatic animals (Feldhusen, 2000). The association of *Vibrio cholerae* with planktons notably copepods, provides further proof for the origin of cholera, as well as explanation for the sporadic occurrence of cholera epidemics (Fitnat, 2007).

2.2.3.1 Epidemiology and pathogenicity of Vibrio

Cholera remains a major epidemic disease. There have been seven great pandemics, the latest, which started in 1961, invaded the Western Hemisphere (last century) with a massive outbreak in Peru in 1991. Cholera has been endemic in the developing countries of Asia and Africa and has caused epidemics in Asia, the Middle East, South and Central America (CDC, 1999). In 2004, fewer cases of cholera worldwide were reported to the World Health Organization than in any year in the previous decade (101,383 cases from 56 countries) (WHO, 2004). For 2008 alone, a total of 190,130 cases were notified from 56 countries, including 5,143 deaths. The true burden of the disease is estimated to be 3–5 million cases and 100,000–120,000 deaths annually (WHO, 2011). Other serogroups of *V. cholerae* may cause diarrheal disease and other infections but are not associated with epidemic cholera. *Vibrio parahaemolyticus* is an important cause of enteritis associated with the ingestion of raw or improperly prepared seafood (FDA/CFSAN, 2001).

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Vibrio cholerae is the aetiological agent of cholera, a waterborne and severe diarrheal disease, with high morbidity and mortality if left unchecked (Sack *et al.,* 2004). Among the members of the genus, 12 species have so far been reported to be pathogenic to humans, where eight of these may be associated with food-borne infections of the gastrointestinal tract (Oliver and Japer, 1997). Of more than 200 *V. cholerae* serogroups that exist, only serotype O1 and O139 are associated with the epidemiological features and clinical syndrome of cholera. However, organisms of *V. cholerae* serogroups other than O1 and O139 (non-O1 and non-O139 serogroups) have been associated with sporadic cases of food-borne outbreaks of gastroenteritis, but have not spread in epidemic form (Heyman, 2004). The most important virulent factor associated with *V. cholerae* O1 and O139 serogroups is the cholera toxin or choleragen, which is responsible for the profuse diarrhea and a pilus colonization factor known as toxin co-regulated pilus (Faruque and Mekalanos, 2003). Vibrios are sensitive to acid and colonize the small intestine bowel, where they secrete the potent cholera enterotoxin. This toxin binds to plasma membrane of intestinal epithelial cells and releases an enzymatically active subunit that causes a rise in cyclic adenosine monophosphate (cAMP) production. The resulting high intracellular cAMP level causes massive secretion of electrolytes and water into the intestinal lumen (Kenneth, 2009).

Non-O1 and non-O139 serogroups are generally nontoxigenic. Most of these food-borne cholera infections are caused by *V. cholerae* and *V. parahaemolyticus* and to a lesser extent by *V. vulnificus* (FDA/CFSAN, 2001). Although *V. cholerae* is known to be a human pathogen, the bacterium constitute part of the normal aquatic flora in the estuarine and brackish waters and are able to persist in the absence of human host (Lip *et al.,* 2002; Reidl

and Klose, 2002). All species are typically of marine or estuarine environments and most require NaCl (2-3%) to grow (Morris, 1990).

Cholera remains a public health threat globally causing hundreds of thousand cases every year (Heymann, 2004). However, this threat is much reduced in places with safe water supply and good standards of hygiene and sanitation. Cholera is an acute intestinal infection. Its incubation period ranges from a few hours to five days, usually two to three days. Although asymptomatic infection is more common, clinical illness may be exhibited. Symptoms include a sudden onset of profuse painless watery diarrhea that can quickly lead to rapid dehydration, metabolic acidosis, potassium depletion, ultimately vascular and circulatory collapse, hypoglycaemia in children, renal failure and death if treatment is not promptly given (Nester *et al.,* 2004). Cholera is transmitted through ingestion of food or water contaminated with the bacterium, especially via faeces or vomitus of infected persons, directly or indirectly (Mugoya *et al.,* 2008).

The *Vibrio* bacteria are responsible for cholera with abrupt onset of massive diarrhea, vomiting and muscle cramps due to the potent exotoxin, the cholera toxin. The infective dose is believed to be approximately 10^6 cells (Kaysner, 2000) although some researchers state that ingestion of as much as 10^{11} cells are required to make up for the rapid reduction by gastric acids. Senderovich *et al.,* (2010) have confirmed the occurrence of *V. cholerae* in one marine species of fish and suggested that fishes are reservoirs of *V. cholerae*. As fish carrying bacteria swim from one location to another, they serve as vectors on a small scale.

Cholera affects only humans and main source of the bacteria during epidemics are the faeces of acutely infected people. However, the bacterium persists in the environment and is

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often found attached to planktons and *V. cholerae* have been found to survive for long periods of time in river waters (Violeta, 2007). It was T. Fujino of Japan in 1951 who demonstrated that *Vibrio parahaemolyticus* was an agent of food poisoning (Fujino, 1951). It is a major problem in Japan where many sea foods are consumed raw. *Vibrio* spp., are natural inhabitants of marine aquatic environments both in temperate and tropical regions with most human infections acquired by exposure to such environments or to food derived from them. In 1997, a ban on fish from East African countries and Mozambique was imposed by the EU as a result of cholera outbreaks in these countries (Henson *et al.*, 2000).

2.2.4 Use of antibiotics for controlling microbes and the problem of drug resistance

Several antibiotics exist for controlling microbial infections in man and other animals. Different classes of antibiotics are used for treating different types of bacterial and fungal infections in man. Antibiotics such as norfloxacin, erythromycin, neomycin, tetracycline, ampicillin, gentamycin and chloramphenicol, are effective against both Gram positive and Gram negative bacteria, and drugs such as amphotericin B, nystatin, are effective against fungal infections and can be used for controlling even the fish-borne infection in humans. However, the widespread use and abuse of some of these antibiotics for the treatment of infectious diseases has led to some microbes developing resistance to the drugs (Alanis, 2005). According to the WHO criteria, drug resistance is defined as drug resistance in previously treated cases if the patient has been treated for months or more, whereas it is regarded as primary if there is no history of previous treatment (Van den Bogaard and Strobbertingh, 2000).

Though antibiotics are the mainstay for controlling infectious diseases in humans, whether the infections are fish-borne or from other sources, it would be unwise to let people eat infected fish and then seek chemotherapy. This is why this study aimed to determine the antimicrobial patterns of *E. coli, S. typhimurium* and *V. cholerae* isolated from water and fish (*Rastrineobola argentea* and *Oreochromis niloticus*) from the beaches and markets of Lake Victoria basin, Kenya.

2.3 Mould Infestation in Fish

Moulds are common contaminants of agricultural commodities, foods, beverages and feeds. Fungal contaminations are common problem and it adversely affects the quality of cured fish (Patterson and Ranjith, 2009). They produce enzymes that degrade carbohydrates, fats and proteins, thereby resulting in softening and flavour deterioration of foods (Bennett and Klich, 2003). Insects are also known to cause mould contamination by carrying the spores on their bodies (Tatfeng *et al.*, 2005). Dried fish products undergo contamination from varied fungal flora. The most predominant genera that have been reported include the *Aspergillus spp., Penicillium spp., Rhizopus spp., Mucor spp., Fusarium spp., Wallemia spp.,* and *Cladosporium* spp., (Obeyamiji *et al.,* 2008). Under favourable conditions during harvesting, processing and storage of food commodities, moulds produce toxic metabolites known as mycotoxins (Sulyok, 2006; Bhat and Miller, 2010). Mycotoxins are currently of considerable concern to global food safety because of their ubiquity and potential deleterious effect on human and animal health. Considerable importance has been attached to aflotoxins in foods and feed because of their carcinogenic, mutagenic and teratogenic nature (Bankole and Adebanjo, 2003; CDC, 2004; 2005; Bhat and Miller, 2010).

Production of aflotoxins is primarily associated with growth of *Aspergillus flavus* and *Aspergillus parasiticus* (Bennett and Klich, 2003). However, the growth of the *Aspergillus* spp., and the production of aflatoxins depend on factors such as the fungal strain, competing flora, substrates, temperature and relative humidity conditions (Santour *et al.*, 2002). Favourable conditions for mould growth and aflatoxin production are temperature of 28 ^{0}C-30 ^{0}C and relative humidity of 90% (Santour *et al.*, 2002). Several studies on the assessment of the risk potential of dried fish have reported the detection of aflatoxins as potential natural contaminants in dried and smoked fish products (Owaga *et al.*, 2009).

It should always be an urgent need to screen fish before human consumption as fish are vehicles of pathogen and various researchers have reported dangerous levels of aflatoxin from fungal infestation in dried fish (Bhat and Miller, 2010). *Aspergillus* spp., and *Penicillium* spp., are among the fungi species that produce toxins which are carcinogenic (Mari and Riccioli, 2004). Sun-dried fish (dagaa and tilapia) are popular in the lake basin region because they are protein rich and are some times eaten raw but mostly used in cooking most native soup. Moulds have been observed to grow on them and hence the need for this study to determine the prevalence of fungal infestation in sun-dried *R .argentea* and *O. niloticus* from the beaches and markets.

2.3.1 *Aspergillus spp*

The fungus producing aflatoxin was identified as early as 1961, as *Aspergillus flavus* in a peanut meal for turkeys in England (Kuhn and Ghannoum, 2003). Studies have revealed that aflatoxins are produced primarily by some strains of *A. flavus* and most if not all are strains of *A. parasiticus* and *A. niger* (Bennett and Klich, 2003). There are four major aflatoxins: B1, B2, G1 and G2, plus two additional metabolic products M1 and M2 that are of

significance as direct contaminants of foods and feeds. Post harvest production of aflatoxin in fish is favoured by warm temperature and high humidity (Bennett and Klich, 2003).

Aflatoxicosis, the disease caused by aflatoxin consumption, is primarily a hepatic disease. In fact, aflatoxin causes liver damage and a recurrent infection as a result of immunity suppression. Aflatoxin B, the commonest of the aflatoxin is produced by *Aspergillus flavus* (Bennett and Klich, 2003), and studies have related exposure to humans of aflatoxin B1 to cancer risk. The induction of cancer by aflatoxins has been extensively studied (CDC, 2004; 2005). Aflatoxin B1, aflatoxin M1, and aflatoxin G1 have been shown to cause various types of cancer in different animal species. However, only aflatoxin B1 is considered by the International Agency for Research on Cancer (IARC) as having produced sufficient evidence of carcinogenicity in experimental animals to be identified as a carcinogen (Turner *et al.*, 2000).

In 1988, the IARC placed aflatoxin B1 on the list of human carcinogens. This is supported by a number of epidemiological studies done in Asia and Africa that have demonstrated a positive association between dietary aflatoxins and liver cell cancer. Although aflatoxin B1 synergies hepatitis B and C infections in the causation of liver cancer (Turner *et al.*, 2000), both laboratory and epidemiological data have established the role of aflatoxin in liver carcinogenesis. It has been shown that aflatoxin B1 exposure occurs through the consumption of mold contaminated groundnuts, grains and animal feed (CDC, 2004; 2005), which can be transmitted transplacentally. Aflatoxin contaminations have also been linked to male infertility. Recently, in a study conducted by Uriah *et al.*, (2001) in Nigerian men, the blood and aflatoxin levels ranged from 700 to 1393 ng/ml and 60 to 148 ng/ml in infertile and fertile men, respectively. Some species also cause otomycosis to human (Tang *et al.*, 2006).

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2.3.2 *Penicillium* spp.

Penicillium spp., has been associated with the production of mycotoxins particularly aflatoxin (Bennett and Klich, 2003). Ochratoxin A (OTA), a mycotoxin produced as secondary metabolite by *Penicillium* and *Aspergillus* is nephrotoxic and carcinogenic (Pitt, 2001), though it was first isolated from cultures of *A. ochraceus* (CDC, 2004; 2005). Depending on the dosage, OTA may be carcinogenic, genotoxic, immunotoxic or teratogenic (CDC, 2004; 2005). It has recently been a matter of concern because of the demonstrated carcinogenicity of these compounds (Bennett and Klich, 2003; CDC, 2004; 2005). *Penicillium* spp. are occasional causes of infection in humans and the resulting disease is known generally as penicilliosis.

2.4. Moisture Contents in Dried Fish

The moisture content of dried fish products adversely affects the presence of fungal growth on dried fish and fishery products. Fungal species such as *Apergillus* sp., *Penicillium* sp., *Mucor* sp., *Rhizopus* sp., and *Fusarium* are pathogenic to humans and also cause food spoilage. (Felicia and Jamila, 2003). Since fingi grow better in moist conditions it is always of significance to determine the moisture content of sun-dried fish for long term storage and preservation. This is because the conditions that contribute to fungal growth and production of aflatoxins in fish are hot and humid climate and moisture content of 16% (Adebayo-Tayo *et al*, 2008).

2.5 Methods for Preservation of Fish

Fish preservation is the method of extending the shelf life of fish and other fishery products. Historically, the fish curing methods of drying, salting, smoking, and pickling which were non-selective, were used both as separate processes and in various combinations

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(FAO, 2005; 2008). Fish were usually salted down immediately to prevent spoilage, but today's boats commonly bring in unsalted fish. Fish that are to be cured are usually first cleaned, scaled, and eviscerated. Fish that are to be salted are packed between layers of salt or immersed in brine (Patterson and Ranjitha, 2009). Fish to be smoked are preserved by drying and, when the fish are close enough to the source of heat, by heat penetration. Fish are dried under controlled conditions of temperature, humidity, and air velocity, but drying is the least common method of fish preservation because the dried product is relatively unappetizing and re-hydrating it is slow (FAO, 2008).

2.5.1. Traditional methods of preservation of fish

2.5.1.1. Salting

Salting has been used for many years to preserve fish and fishery products (Bellargha et al., 2007; Patterson and Ranjitha, 2009). The primary objective is to select the halophilic micro-organisms which will affect a controlled degradative process on the organic compounds in the fish muscle to bring out the desired flavours in the product. Salting has no adverse effects on the value of the fish protein and bacterial growth can be significantly retarded by the presence of sufficient quantities of common salt (sodium chloride). When fish is placed in a brine solution, the salt penetrates the fish and water is extracted from the tissues by osmosis. At salt concentrations of 6-10% in the fish, the activity of most bacteria that cause contamination and spoilage is inhibited (Bahri et al., 2006). Since fish contains 70-80% water, the amount of brine used must be adjusted accordingly. The higher the salts content in the fish, the longer the shelf life (Patir et al., 2006).

Traditional methods involve rubbing salt on the flesh of the fish or making alternate layers on fish which causes the problem of un-uniform application of salt. Brining takes care

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of this problem, which involves immersing the fish into pre-prepared solution of salt; the advantage is that salt concentration can be more easily controlled and salt penetration is more uniform (Clement and Saheed, 2004). The principle effect on micro-organisms is due to the lowering of water activity through sodium chloride itself in higher concentrations which may be lethal to some bacteria due to osmotic effects (Clement and Saheed, 2004).

Control of pathogenic microbes by salting can be advantageous because it is non-selective and will not lead to development of microbial resistance to humans. This is because salt is chemically unrelated to the antibiotics which are used for treating microbial infections in humans and can also be effective in the control of microbes which can be mechanically transmitted by insects such as beetles, houseflies and cockroaches. Moreover, salting also leads to repellency of insects such as houseflies and can also reduce infestation of fish by beetles (Osuji, 1975). Therefore, brine salting was tested as one of the non-selective methods for controlling fish-borne pathogens in this study.

2.5.1.2 Chlorinated solution

Chlorinated solutions can be used as a method of sanitizing or elimination of bacteria and moulds from fish. Sanitizing agents such as sodium hypochlorite have generally been proven effective in reducing overall bacterial populations as well as numbers of specific bacterial pathogens on fish and other food products (Wei *et al.,* 1996). Sodium hypochlorite is a chemical compound consisting of sodium, oxygen, and chlorine that has been used for centuries for bleaching and disinfecting. Hypochlorite was first produced in 1789 in Javelle, by a Frenchman Berthollet, by passing chlorine gas through a solution of sodium carbonate. The resulting liquid, known as "Eau de Javelle" or "Javelle water" was a weak solution of sodium hypochlorite (Chalmers, 1978).

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Historically, chlorine is one of mankind's most trusted weapons in the war against infectious waterborne diseases. Drinking water chlorination, first introduced in Great Britain in the early 1900s and shortly afterwards in the US, immediately improved the quality of life wherever it was employed (Park *et al.,* 1991). Chlorine is a highly effective yet inexpensive weapon available to control food-borne diseases all over the world.

Chlorinated solutions have also been used for sanitizing fish products especially during processing and their effectiveness in controlling pathogenic microbes should also be investigated (Park *et al.,* 1991). Chlorine is commonly used at concentrations of 50-200ppm with a contact time of 1-2 min to sanitize produce surface (CFSAN/FDA, 2001).The inhibitory or lethal activity depends on the amount of free available chlorine in the solution (in the form of hypochlorous acid, HOCl, present in bleach and chlorine solutions) that comes in contact with microbial cells. Free chlorine disinfects by chemically disrupting bacterial cell walls and membranes through oxidation of a chemical group known as the thiol group (WHO, 1998). The concentration of the fast-acting, antimicrobial hypochlorous acid, the chemical species providing free available chlorine to disinfectant solutions, is a function of pH. Between pH 6.5 and 7.0, HOCl exists as 95-80% of the free chlorine concentration. At pH greater than 8, the free chlorine concentration is less than 20%. It has also been approved that the paper test strips and colorimetric kits used to monitor chlorine do not distinguish between the presence of HOCl and a far less active chemical species, hypochlorite (OCl-). Therefore, to maintain a given free chlorine concentration, it is necessary to monitor and adjust the pH of disinfectant solutions (Suslow, 2000).

2.5.2. Potential or new methods of preservation of fish

2.5.2.1. Moringa oleifera plant extracts

Moringa oleifera is the best known of the thirteen species of the genus *Moringa* and the family moringaceae (Folker *et al.,* 1999). *Moringa oleifera* is a fast growing, aesthetically pleasing small tree which is adapted to arid and sandy conditions. The species is characterized by its long, drumstick shaped pods that contain its seeds (Folkard *et al.,* 1999). It is currently being examined as a bio-enhancer of drugs and nutrients because of its production of compounds with antibiotic activity. The seed of *Moringa* are considered to exhibit antipyretic and antimicrobial activities, used for water purifying and are also a good source of non-desiccating oil (Anwar *et al.,* 2007). Its antibiotic property is identified as pterygospermin, a bactericidal and fungicidal compound (Anwar and Bhanger 2003; Anwar *et al.,* 2007). Studies have shown that an aqueous extract made from seeds is equally effective against the skin infecting bacteria *Staphylococcus aureus* as the antibiotic neomycin (Silvestre *et al.,* 2000; Suarez *et al.,* 2003). It has detoxifying effects, which may come from moringa's ability to purify water. It acts as a coagulant attaching itself to harmful material and bacteria.

Doughari *et al.,* (2007) found that 100mg/ml of *M. oleifera* plant extracts inhibit the growth of *S. typhi,* and Suarez *et al.,* (2003) demonstrated the antibacterial and antifungicidal activity of *M. oleifera* leave extract and its antibiotic activity identified as pterygospermin, a bacterial and fungicidal compound. The control of these organisms by the extracts in foods would reveal the potentials of these extracts as preservatives. The oil extracted from moringa seed is called Ben oil. Solvents such as hexane, ethanol, methanol, petroleum ether and acetone are used in extraction of oil from vegetables, flowers and oil seeds. Hexane is often used for vegetable oil extraction mainly due to its efficiency and ease of recovery (Akaranta

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and Anusiem, 1996). Other solvents such as acetone, ethanol and iso-propanol are also often used in oil extraction (Dunford and Zhang, 2003).

2.5.2.2 Bacteriophages

Bacteriophages were discovered independently in 1915 by Frederick Twort, an English bacteriologist and physician (Twort, 1915), and in the year 1917 by Felix d'Herelle, a self-taught French-Canadian bacteriologist (d'Herelle, 1917). However, it was d'Herelle who first coined the term "bacteriophage" to describe a "microbe" that attack bacteria and was capable of killing them (d'Herelle, 1917). d'Herelle was also the first to recognize that this agent of bacterial death was actually a virus (d'Herelle, 1917; Summer, 2001).

Bacteriophages or "phages" are viruses that invade bacterial cells and, in the case of lytic phages, disrupt bacterial metabolism and cause the bacterium to lyse. Bacteriophages (phages) are viruses that exclusively target and reproduce within bacterial cells. The host specificity of viruses offer an enticing technology for fighting infections caused by bacteria or for the treatment of environments contaminated with pathogenic bacteria (Hagens and Loessner, 2007). The use of phages in the treatment of bacterial infections or in prophylaxis is an attractive alternative to existing therapies (example, antibiotics), because unlike broad-spectrum antibiotics, phage target a particular host and are unlikely to illicit resistance in untargeted bacterial strains (Mathur *et al.*, 2003; Sulakvelidze and Kutter, 2005). Also unlike chemical therapeutic agents, phages are not susceptible to the onset of bacterial resistance because they have the ability to evolve with their host (Sulakvelidze and Kutter, 2005). Phage lambda or M13 is normally found in stool in 1-4% of stool samples from humans and about

every third stool sample from diarrhea patients yield a coliphage (Chibani-Chennoufi *et al.,* 2004).

Phage is an important alternative to antibiotics in the current era of multidrug resistant pathogens. Phage therapy is the therapeutic use of lytic bacteriophages to treat pathogenic bacterial infections (Chanishvili *et al.,* 2001; Weber-Dabrowska *et al.,* 2003; Jikia *et al.,* 2005). Not long after their discovery, bacteriophages were successfully used to treat certain bacterial diseases, such as dysentery (Michael and Rotem, 2005). Bacteriophage may present a likely strategy for reducing the presence of pathogenic microbes. Raya and colleagues (2006) demonstrated that a single dose of bacteriophage specific to *E. coli* O157:H7 given to sheep led to a two-log reduction (99%) of the pathogen.

Purified high-titer phage lysates have been used for the species-specific control of bacteria during the pre-harvest and post-harvest phases of food production and storage. Studies have demonstrated significant efficacy of phages against *E. coli*, *Acinetobacter* spp., *Pseudomonas* spp. and *S. aureus* (McGrath and van Sinderen, 2007). Phages are harmless to humans, animals and plants and target only bacterial cells (Harald, 2005). FDA and USDA have approved various phages against different bacteria like *Listeria monocytogenes, Campylobacter jejuni* and *E. coli* (FDA, 2006). Phages have been proposed as natural antimicrobial agents to fight bacterial infections in humans, in animals or in crops of agricultural importance (Harald, 2005). Due to bacteriophage specificity on bacteria they have been employed in this study as preservative against pathogenic bacteria. Therefore the use of bacteriophage suspension in the preservation of fish should be investigated.

CHAPTER THREE

3.0 STUDY SITES AND METHODS

3.1 Study Sites

Lake Victoria is the second largest fresh water lake in the world, with an area of 69,000 sq km (Abila and Jansen, 1997). The lake is shallow with a maximum depth of 84 m and a mean depth of 40 m. It has a catchment area of 193,000 Km^2 shared between Kenya, Uganda and Tanzania Figure 1.
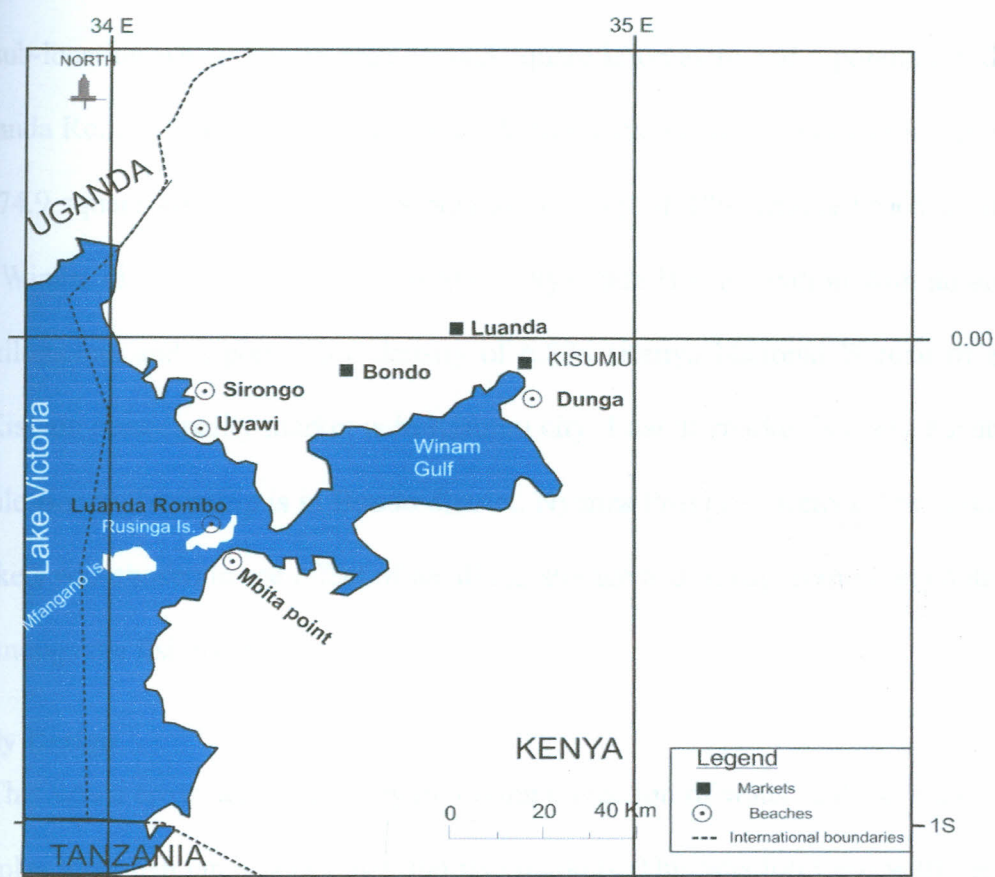

Figure 1. A map of Lake Victoria basin of Kenya showing the sampling sites, courtesy of Dr. John Gichuki of KMFRI.

Kenya owns 6%, Uganda 45% and Tanzania 49% of the total area. The basin supports over 30 million people. The major portion of Kenyan water of L. Victoria is a narrow gulf known to

various authors by several names; the Victoria Nyanza, Kavirondo gulf, Nyanza gulf and Winam gulf. Winam gulf lies south of the Equator between 006 S – 00 32'S and 34013' - 340 52'E at an altitude of 1134 m above sea level and covers an area of 1,920 sq. Km (approximately 6% of whole lake) between 6 Km and 30 Km (Abila and Jansen, 1997). This study was carried out in three fish landing beaches namely Sirongo, Luanda Rombo and Dunga in the Lake Victoria Basin, and three markets: Kisumu Municipality, Luanda, and Bondo (Figure 1).

Sirongo beach is in Bondo district, Nyangoma division, Central Sakwa location, Uyawi sub-location which has an area of 34.5 square kilometers and a population density of 234. Luanda Rombo beach is in Suba district, Mbita division, Wanyama sub-location with an area of 74.9 square kilometers and a population density of 189. Dunga beach is in Kisumu district, Winam division, West Kolwa location, Nyalenda B sub location with an area of 4.7 square kilometers and a population density of 6,886 (Kenya National Bureau of Statistics, 2010). Kisumu municipality market is in Kisumu city; Luanda market is along Kisumu-Busia road while Bondo Township is in Bondo district, Nyanza Province, Kenya. The three beaches and markets was chosen to see effect of handling, storage and transportation on contamination of fish amongs the fish folks.

3.2 Study Design

This was a cross-sectional study involving collection of water and fresh and sun-dried fish samples from fish landing beaches and fish markets. This was followed with the isolation of pathogenic enteric bacteria from fresh fish, determination of antimicrobial profiles, assessment of fungal infestation and moisture content of dried fish and the evaluation and comparison of different methods for preserving fish and controlling the enteric bacteria in fish

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in the laboratory.

3.3 Samples Collection Procedures

3.3.1 Collection of water samples

Water samples (250ml per sample in clean sterile bottles) were randomly collected from the fish landing beaches and from the markets. Within each fish landing beach, or from each market, the water samples were taken from three different locations, and at each location, samples were taken from three different points, meaning that nine (9) samples (water) were taken from each location. A total of 54 samples of water were collected.

The water samples from the beaches were collected from different points, separated by at least 10-15 meters along the lake shorelines, and from 10-20 liter plastic containers (or buckets) used for storing water for washing fish by different fresh fish traders in the markets. All the collected water and fish samples (in clearly labeled containers or plastic bags, respectively) were then transported in cooler boxes with ice packs within four (4) h of collection to Maseno University School of Public Health and Community Development, Department of Biomedical Science and Technology laboratory for microbiological analysis.

3.3.2 Collection of fresh *Rastrineobola argentea* and *Oreochromis niloticus* samples

Using a cross-sectional study design, one kilogram of fresh fish samples (500-600 pieces of *Rastrineobola argentea*) and whole fresh *Oreochromis niloticus* per sample per site respectively. The samples were randomly collected from the fish landing beaches and from the markets and placed in well labeled clean sterile plastic bags. The fresh fish samples from the fish landing beaches were collected (bought) from fishermen from landed fishing boats, and those from the markets collected (bought) from the fish traders. Within each fish landing

beach, or from each market, the fish samples were taken from three different locations, and at each location, samples were taken from three different points, meaning that nine (9) samples were collected from each fish landing beach or market. For fresh dagaa and tilapia 54 samples for each fish was collected. All the collected fish samples were placed in clearly labeled containers or plastic bags and then transported in cooler boxes with ice packs within four (4) h of collection to the Maseno University School of Public Health and Community Development, Department of Biomedical Science and Technology laboratory for microbiological analysis.

3.3.3 Collection of sun-dried *Rastrineobola argentea* and *Oreochromis niloticus* samples

For fungal infestation analysis, also 500-600 pieces of sun-dried *Rastrineobola argentea* (dagaa) and whole *Oreochromis niloticus* (Nile tilapia) per site were also randomly purchased from the three different beaches (Dunga, Luanda Rombo and Sirongo) and markets (Kisumu, Luanda and Bondo) and placed in clearly labeled clean sterile plastic bags. All the collected fish samples (whether fresh or sun-dried in plastic bags) were then transported in cooler boxes with ice packs within four (4) h of collection to the Maseno University School of Public Health and Community Development, Department of Biomedical Science and Technology laboratory for analysis.

3.4 Sample Processing and Microbiological Analysis

The water and fresh fish samples from the field were unpacked and coded for laboratory analysis. 100mls aliquots from each of the 250ml water samples, and weighed ten grams of fish samples either 10-15 whole pieces of *Rastrineobola argentea*, or cut muscles with skin from lateral lines of *Oreochromis niloticus* were used for analysis. The fish samples were macerated for 3 minutes in 90 ml saline (0.85% NaCl) in a blender (Sanyo^{TM}) and

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topped up with saline to make up 100 ml of fish slurry. The 100ml water samples and the resultant 100 ml slurry stocks were respectively transferred into sterile labeled 250ml flasks in readiness for bacteriological analysis.

Total viable non-specific bacterial counts were carried out using the aerobic plate count method which employs plate count agar according to AOAC method 966.23 (1995) with colony forming units determination. All the experiments were performed in triplicate. Briefly, using a micropipette and sterile tips, aliquots of 1 ml of the various sample preparations (i.e. from the 100 ml water samples or fish slurry) were aseptically inoculated into sterile test tubes containing 9mls of plate count agar, vortexed, and then poured or plated into sterile Petri dishes and allowed to set for about 15 minutes. The Petri dishes were then inverted and incubated at 37^0 C (Gallenkamp, Germany) for 24 h. By using Quebec colony counter, the number of colony forming units (CFUs) was counted, and the means from triplicate experiments for each sample determined and recorded. The results were expressed as CFU per millitre of water or fish slury.

Escherichia coli analysis of the various sample preparations (i.e. from the previous 100 ml water samples or fish slurry) was performed using most probable number (MPN) procedure which detects the coliform bacteria as indicators for faecal contamination (Tharannum *et al.,* 2009; Scott, 2010) followed by biochemical assay for species identification. The technique utilizes selective and differential liquid media into which aliquots of serial dilutions of samples are inoculated. The technique involves three successive steps, namely, presumptive test, confirmed test and complete test (SAP, 1999; Tharannum *et al.,* 2009; Scott, 2010).

In the presumptive test in this study, 10 ml of phenol red lactose broth (HiMedia Lab. Pvt. Mumbai, India) was added into each of 3 sets of 25 ml tubes (with inverted Durham's tubes' inserts). Each set contained three tubes (i.e., there were 9 tubes in total). The tubes were sterilized by autoclaving. The tubes were allowed to cool and then inoculated with a ten-fold difference in water and fish samples inoculums volumes, i.e., 0.1 ml, 1 ml, and 10 ml per tube and incubated at 37 ^{0}C (Gallenkemp, Germany). After 48 h, the tubes were examined for acid and gas production. Change of phenol red lactose broth to yellow indicated acid formation from lactose (SAP, 1999; Tharannum et al., 2009; Scott, 2010). Change in lactose broth to yellow and formation of gas in Durham tube is a positive test. No colour change and no gas in the Durham tubes represent a negative test. Each set was scored for the number of positive tubes and the score of all the three sets recorded and used with the standard Most Probable Number (MPN) table (Appendix 1) to determine the number of coliforms in the water or fish slurry samples.

The presumptive test was then followed by the confirmative test, the complete test and IMViC tests. The confirmative test was performed by streaking a positive presumptive tube onto Eosin Methylene Blue agar (EMB) (HiMedia Lab. Pvt. Mumbai, India). This agar contains lactose and the dyes eosin Y and methylene blue. When E. coli grows on EMB, it ferments so much acid that the two dyes precipitate out in the colony producing a metallic green sheen appearance. A positive confirmative test shows the presence of green sheen colonies on EMB streaked from a positive presumptive test.

The complete test was performed by inoculating a tube of phenol red lactose broth with green sheen colonies from positive confirmative tests. Simultaneously, a loop of colony was streaked onto a slant of nutrient agar. Both tubes were incubated at 37 ^{0}C for 48 h. The

culture on the nutrient agar was analyzed by Gram staining.

The presumptive positive samples from the above tests were subjected to further biochemical assays. The biochemical tests performed included indole production, methyl red, Vogues-Proskauer test, citrate (IMViC) test, oxidase production and catalase production. These biochemical tests were performed as per standard microbiological methods (Cappuccino and Sharaman, 2007). Analytical grade chemicals and reagents obtained from HiMedia were used in all the tests. An *E. coli* ATCC 25922 culture obtained from Kenya Medical Research Institute (KEMRI) courtesy of microbiology laboratory technologists was used as a control for the biochemical tests.

The isolation of *Salmonella* spp. from the various samples' (water and fish) was done by resuscitation method of Harrigan (1998). Aseptically, 2ml of the water sample or fish slurry (from the 100ml stocks) was enriched in selenite F media (HiMedia Lab. Pvt. Mumbai, India) and incubated at 37 ^{0}C for 24 h. For identification of *Salmonella*, positive selenite F cultures were touched at the top and streaked on Xylose lysine desoxycholate agar and Salmonella Shigella agar (HiMedia Lab. Pvt. Mumbai, India) and incubated at 37 ^{0}C for 24 h. Suspected colonies were identified based on characteristic colony morphology, Gram staining, and motility tests (Cheesbrough, 2006). The Xylose lysine desoxycholate agar colonies which are pink-red with black centres were streaked onto nutrient agar and incubated at 37 ^{0}C for 24 h and the resultant colonies again streaked on triple sugar iron (TSI) agar and incubated at 37 ^{0}C for 24 h. Slants with H_2S production, i.e. blackening at the butt, were taken as positive for *Salmonella*. Positive isolates for *Salmonella* were streaked on nutrient agar and incubated at 37 ^{0}C for 24 h. Further biochemical screening for *Salmonella* was carried out using the indole, methyl red, Voges-Proskauer and citrate (IMViC) reaction and confirmed by

slide agglutination test following Kauffmann-White scheme serotyping (Kauffmann, 1997) (Appendix II).

The isolates were serotyped by slide agglutination with homologous *Salmonella* O and H group antisera (Bio-Rad, Marne-la-Coquette, France) (Popoff *et al.*, 2000; Popoff and Minor, 2003). Briefly, using a clean microscope slide, a drop of antiserum was placed on the slide at one end and a drop of sterile normal saline (0.85% NaCl, Chemoquip) placed at the opposite end of the same slide, 3 to 4 colonies were suspended in 0.3 ml sterile saline and cell suspension was made. Onto the drops of serum and normal saline, one loopful of the cell suspension was placed, and then mixed well. The slide was shaken gently for one minute. Normal saline served as the control. Agglutination observed within one minute was regarded as positive for *Salmonella* and for the same isolate, monovalent was used employing the same procedure.

Further, H antigen was used for tube agglutination using cultures grown for 8 h at 37 ^{0}C in heart infusion broth which were diluted with an equal volume of saline containing 1% formaline. Antigen suspension of 0.5 ml was added to 0.05 ml of each specific H serum. The tubes were then shaken well for 2 minutes, allowed to stand in a water bath at 50-52 ^{0}C for 1 h, then observed for agglutination. Agglutination of the suspension in tubes signified a positive result and a confirmation for *Salmonella enterica* serovar Typhimurium.

The isolation of *Vibrio cholerae* from the various samples' (water and fish) was performed by enriching 2mls of water sample or fish slurry (from the 100 ml stocks) using 3 tubes with alkaline peptone water (pH 8.6) and incubated at 37 ^{0}C for 24 h. Top surface of alkaline peptone water culture was touched with a loop and inoculated on Thiosulphate Citrate Bile Salt (TCBS) agar (Fluka, Sigma-Aldrich, Switzerland) plates which is a

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differential and selective media for *Vibrio* species and then incubated at 37 ^{0}C for 24 h. From TCBS agar plates, sucrose-fermenting yellow, shiny colonies measuring 2 to 4 mm in diameter suspected for *V. cholerae* were sub-cultured in brain heart infusion agar and incubated at 37 ^{0}C for 24 h and then subjected to biochemical tests i.e. indole, methyl red, Voges Proskauer and citrate, triple sugar iron agar, oxidase, catalase and string tests (Cheesbrough, 2006).

For identification of the serotypes, slide agglutination serology test was done using polyvalent O1 and O139 antisera (Denka Seiken Co. Ltd, Tokyo, Japan) for confirmation of *V. cholerea*. A drop of O1 and O139 antisera was placed on two different slides at one end and a drop of sterile normal saline (0.85% NaCl) placed at the opposite end of each slide. Using an inoculation loop, 2 to 3 discrete colonies were picked from TSI agar plates and emulsified in 0.3 ml sterile saline and mixed thoroughly to make cell suspension. The slide was then tilted back and forth to observe agglutination within 30 seconds to 1 minute. If clumping appeared on cell suspension which had antisera, then this was regarded as positive to Ogawa antiserum and negative to Inaba antiserum as a conformation test of *V. cholerae* O1 (NCID, 1999).

All the confirmed isolates of *E. coli, S. enterica* serovar Typhimurium, and *V. cholerae* O1 were streaked on tryptic soy agar plates and incubated at 37 ^{0}C for 24 h in readiness for antimicrobial susceptibility testing.

3.5 Antibiotic Susceptibility Testing
Kirby-Bauer disc diffusion method (Wikler, 2006) was used to test the sensitivity of the antibiotics against the pure isolated pathogens from fish and water. Isolated bacteria (*E. coli, Vibrio* spp., and *Salmonella* spp.,) from the samples were subjected to disk diffusion test.

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The bacterial isolates 4 to 5 well isolated colonies from overnight tryptic soy agar plates were selected with inoculation loop and transferred to a tube of sterile saline 2 ml and vortexed thoroughly. The bacterial suspension was then compared to 0.5 McFarland standard and viewed against a sheet of white paper on which sharp black lines are drawn. The bacterial turbidity was either reduced by adding sterile saline or increasing by adding more bacterial growth to march the 0.5 McFarland standard. The *E. coli* ATCC 25922 was used as control. The bacterial density suspension was adjusted to equal that of the freshly prepared 0.5 McFarland turbidity standard solutions. The turbidity standard was prepared as follows: a 1% v/v solution of sulphuric acid was prepared by adding 1 ml concentrated sulphuric acid to 99 ml of distilled water and mixed well in a clean 250 ml conical flask and by dissolving 0.5 g of dehydrated barium chloride in 50 ml of distilled water in 100 ml conical flask separately. 0.6 ml of barium chloride solution was added to 99.4 ml of sulphuric acid solution and mixed well. 2 ml of the turbid solution was transferred to screw-cap tube same as the one used for preparing the test and control inocula (Cheesbrough, 2006).

Sterile cotton swab was dipped into the standardized bacterial test suspension and pressed firmly against the inside wall of the test tube just above the suspension level, rotated the the swab to remove excess liquid. Streaked the swab over the entire surface of medium three times, rotating the plate after each application to ensure an even distribution of the inoculums on Mueller-Hinton agar plate. After the agar surfaces had dried for about 5 min, the antibiotic test disks: ampicillin 10 mcg, chloramphenical 30 mcg, erythromycin 15 mcg, gentemicin 10 mcg, norfloxacin 10 mcg, ciprofloxacin 5 mcg, methicilin 5 mcg, tetracycline 30 mcg and cotrimoxazole 23.75 mcg were impregnated individually using heat sterilized forceps and then gently pressed down onto the agar under aseptic condition. The plates were

left to stand for ten minutes then placed in a 37 ^{0}C incubator for 16-24 h. After incubation, antibiotic activity was determined by the width of the zone of no growth around the antibiotic disk using a transparent ruler. The diameter of the zones of complete inhibition including the diameter of the disk was recorded in millimeters. The measurement was done undersurface of the plate without opening the lid. Kirby-Bauer test results were interpreted using the protocol of Clinical and Laboratory Standards Institute (CLSI, 2007). The zone was interpreted according to their diameters as being sensitive, intermediate or resistant (Appendix III). The choice of antibiotic was based on the pathogens and range of localy available antibiotics, in the Kenya region.

3.6 Determination of Fungal Infestation of Sun-dried Fish and Moisture Contents
3.6.1. Determination of fungal infestation of sun-dried fish

The sun-dried fish samples from the field were unpacked and coded for laboratory analysis. Fish samples from the different sampling sites were surface sterilized with 70% ethanol and rinsed three times with autoclaved distilled water (Christianah and Fagade, 2010). The fish samples 10 g dagaa pieces or 10g of tilapia tissue portion from each beach or market was grinded aseptically in a porcelain mortar and mixed in 90 ml of sterile peptone water. From this mixture, 10 fold serial dilutions were prepared to 10^6 and 1 ml aliquots of samples dilutions were spread onto sabouraud dextrose agar (SDA) supplemented with Oxytetracycline to inhibit bacterial growth (FDA, 2001). The plates were incubated at 28 ± 2^{0}C and examined daily for 7 days and counted the colonies. All observed colonies were subcultured in PDA to obtain pure cultures which were subsequently isolated and identified using morphological characteristics, spore formation and production of fruiting bodies according to Yuen-Wan *et al.,* (1999) methods. Each different appearing fungal culture isolate

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was transferred with a sterile needle to a sterile slide, teased apart and stained with lactophenol cotton blue and examined macrobiologically and microscopically (Yuen-Wan *et al.*, 1999). Microscopic examination was done using an iroscope LMOD MX-T. Fungal culture isolates were identified to species level by assessment of the sporulating bodies and mycelial growth (Ochei and Kolhatkar, 2000).

3.6.2. Moisture content determination of sun-dried fish samples

The moisture content was determined by oven-drying of 5g fish tissue according to AOAC method 950.46 (1995). Briefly, 5 g of fish tissue either *Rastrineobola argentea* or lateral line of *Oreochromis niloticus* was oven dried at 105 °C until a constant weight was reached (Nielsen, 1994), and resultant weight measured following the methods outlined in Cockerell *et al.* (1971) which determines the weight difference between dry and wet material and the moisture content expressed as percentage of the wet tissue.

3.7. Comparison of the Traditional Non-selective Methods of Brine Salting and Sanitizing with Chlorinated Solution, with New *Moringa Oleifera* Plant Extracts and Bacteriophage Treatment for Preserving Fish

3.7.1 Preparation of the preservatives

Commercial sodium chloride (common salt) was weighed into 3 g, 6 g, and 12 g and dissolved in 100 ml distilled water in sterile 250 ml conical flasks, respectively, to make different concentrations of the brine solutions and each filtered through 0.45 micron millipore filtration units (Millipore, USA) ready for experiments. Commercial sodium hypochlorite (3.85%) was used to make various parts per million (ppm) solutions according to calculations of Orindah (2002). Briefly, 0.13 ml, 0.26 ml, 0.39 ml, and 0.52 ml of commercial sodium hypochlorite (3.85%) were mixed with 100 ml distilled water in sterile 250 ml conical flasks

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to make 50 ppm, 100 ppm, 150 ppm and 200 ppm of chlorinated solutions, filtered through 0.45 micron filter units and then the filtrates used for the experiments immediately.

Four kilograms of dry seeds of *Moringa oleifera* were collected from Baringo district in Kenya and brought to Maseno University, Biomedical Science and Technology laboratory. The dry seeds were aired under shade at ambient temperature on an open lab tray, this was done until a constant weight was achieved for a period of five as days has been previously done for other plant materials and discoloured seeds discarded. Two kilograms of the seeds was ground into fine powder using Sanyo^{TM} electric blender and placed into closed labeled container ready for extraction with n-hexane and ethanol.

For n-hexane extraction, 1 kg fine powder and 1.5 lt of n-hexane were mixed in a sterile conical flask and shaken at 120 rpm for 72 h using Orbital shaker SOI (Stuart Scientific, UK). The supernatant was filtered using Whatman filter paper No. 91 (Whatman Ltd, China), and the filtrate rotar vaporized under reduced pressure using Eyela rotovapourizer (Tokyo Rikakikai, Japan) to get the n-hexane extract pastes (Dunford and Zhang, 2003). The remaining cake from n-hexane extract was air dried at room temperature for 12 h to remove the n-hexane and then mixed with 1.5 liter of analytical grade ethanol, and shaken at 120 rpm for 72 h, filtered through Whatman filter paper No. 91, and rotar vaporized under reduced pressure using Eyela rotovapourizer (Tokyo Rikakikai, Japan) for ethanol to dry out, hence ethanol extract (Dunford and Zhang, 2003). The n-hexane and ethanol extracts pastes were then dissolved in DMSO (1mg/5ml DMSO for n-hexane extract and 1mg/2ml DMSO for ethanol extract) to yield liquid solutions, which were further diluted in distilled water to make 20mg/100ml, 40mg/100ml, 60mg/100ml and 80mg/100ml solutions (i.e., 20µg/ml, 40µg/ml, 60µg/ml and 80µg/ml final concentrations) for the experiments.

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Two litres of sewage sample for bacteriophage isolation was collected at the Kisat sewage treatment plant, Kisumu city, western Kenya, and taken to Biomedical Science and Technology Laboratory, Maseno University, for bacteriophage isolation. In the laboratory, the sewage sample was passed through Whatman filter paper No. 10 to remove the sludge and 40ml of filtrate added into each of the three flasks, each containing *E. coli*, *Salmonella typhimurium* and *Vibrio cholerae*. The inoculums were incubated at 37 ^{0}C for 24 h. After 24 h, 10 ml of each of the sewage-bacteria-bacteriophage culture was added into each of the four 15 ml centrifuge tubes and centrifuged at 2000 rpm for 10 min. The supernatant was filtered through 0.45 micron filters using Millipore filtration unit (Millipore, USA). The filtrate was transferred to 15 ml centrifuge tubes labeled "enriched phage prep" with the bacterium name (*E. coli*, *Salmonella typhimurium* and *V. cholerae*) and stored at 4 ^{0}C. Ten fold serial dilution of each of "enriched phage prep" was done into a series of 4 tubes using sterile PBS (i.e. 0.1ml phage into 0.9 ml PBS) from the original and subsequent diluted enriched phages giving 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, and 1×10^{-4}, dilutions. To each tube, 0.5 ml log-phase *E. coli*, *S. typhimuriun and V. cholerae* which had separately been cultured in nutrient broth for 8 h, was added to the corresponding tubes and incubated at 37 ^{0}C for 10 min, and then added to 5 ml top agar 'soft agar' at 45 ^{0}C votexed; and poured quickly to bottom agar "hard agar" (Adams, 1959). After the plates had cooled, they were incubated at 37 ^{0}C for 24 h. At the end of incubation, the number of plaque per plate was counted and recorded. Only *E. coli* gave visible plaques on the plates and were subsequently used for the subsequent experiments. Also, only the plates with between 30-300 plaques were used and from the selected plates. Using sterile Pasture pipette, the agar surrounding plaque were pierced and the "plugs" picked out of the agar and from each plate, all placed into labeled (indicating number of plaque per

plate) test tube with 1 ml Phosphate buffered saline and 1 ml chloroform, vortexed and then stored in the refrigerator at 4 ^{0}C as the high titre stock if not diluted immediately for testing. The number of plaques per ml of high titre stock was calculated using the following formula:

No. of phages in 1ml (PFU/ml) = No. of plaques counted x dilution factor
Volume of phage sample (1 ml)

Only 4 plates yielded between 30-300 plaques and were used. The following are the final counts and dilutions from the four plates which were used for the treatment experiment: 142 pfu X 10^1 = 1.42 x10^3, 130 pfu x 10^2 = 1.30 x 10^4, 125 pfu x 10^3 = 1.25 x 10^5 and 110 pfu x 10^4 = 1.10 x 10^6.

3.7.2. Samples processing, preservative treatments and bacterial analysis

The fresh fish samples from the field were unpacked and coded for laboratory analysis. Weighed ten grams of fish samples (either 10-15 whole pieces of *R. argentea*, or cut muscles with skin from lateral lines of *O. niloticus*) were used for analysis. The fish samples were macerated for 3 min in a blender (Sanyo, China) to make slurry. The resultant slurry was respectively transferred into sterile labeled 250 ml flasks in readiness for preservative treatments with the already prepared 100 ml of different sodium chloride solutions: 3%, 6%, 9% and 12%; sodium hypochlorite solutions: 50ppm, 100ppm, 150ppm and 200ppm; and *M. oleifera* n-hexane and ethanol extract solutions: 20µg/ml, 40µg/ml, 60µg/ml and 80µg/ml, respectively, bacteriophage suspension 1.42 x10^3, 1.30 x 10^4, 1.25 x 10^5 and 1.10 x 10^6 PFU/ml was prepared before bacteriological analysis. In all, the five (5) fish slurry for either *Rastrineobola argentea* or *Oreochromis niloticus* in well labeled 250 ml flasks, according to the treatment solution and concentrations including the control flasks, were made in readiness for the treatments and bacterial analysis experiments. Each of the respective prepared

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preservative solution was then added to the respective appropriately labeled 250ml flask containing the respective fish slurry up to 100ml mark, mixed thoroughly, and timed for the 0hr, 2hrs, 4hrs, 6hrs and 8hrs experimental treatment durations. Double distilled water was used as control for all the treatment experiments.

Bacterial analysis from the timed fish samples experiments was done using most probable number (MPN) of coliform determination method (SAP, 1999; Tharannum et al., 2009; Scott, 2010), and the aerobic plate count method which employs plate count agar according to AOAC method 966.23 (AOAC, 1995) with colony forming units (CFU) determination. The MPN method was used to determine the total coliforms while the aerobic plate count was used to determine all viable bacteria. For MPN analysis, 10mls of phenol red lactose broth (HiMedia Lab. Pvt. Mumbai, India) was added into each of 3 sets of 25ml tubes (with inverted Durham's tubes' inserts). Each set contained three tubes. The tubes with media were sterilized by autoclaving. The tubes were allowed to cool and then inoculated with a ten-fold difference in respective fish samples mixture (from the 250ml flasks) i.e., 0.1ml, 1ml, and 10ml per tube and incubated at 37 ^{0}C (Gallenkemp, Germany) to determine the number of coliforms in the respective fish samples, with the end point determined from the most probable number McCardys MPN table (SAP, 1999; Tharannum et al., 2009; Scott, 2010) (Appendix 1).

Total viable bacterial counts from the fish preservative treatment experiments was also done using the aerobic plate count method which employs plate count agar according to AOAC method 966.23 (1995) with colony forming units (CFU) determination. All the tests were done in triplicate. Briefly, using a micropipette and sterile tips, aliquots of 1 ml (or 1000µl) of the various fish/preservative treatments (from the 250 ml flasks), were aseptically

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inoculated into sterile test tubes containing 9 ml of molten plate count agar at approximately 35-40 ^{0}C, vortexed, and then plated into sterile Petri dishes and allowed to set for about 15 min. The loaded Petri dishes were then inverted and incubated at 37 ^{0}C (Gallenkamp, Germany) for 24 h. By using Quebec colony counter, the number of colonies per plate (i.e. colony forming units, CFUs) was counted, and the means from the triplicate experiments for each fish/preservative treatment determined and recorded. The recorded mean values were multiplied by 10 to take care of the dilution factor of 1:10 to express the final results as CFUs per gram of fish sample.

3.8. Data Analysis and Presentation

Data entry and analysis was performed using Excel for windows 2003. Analysis of Variance (ANOVA) was used for determining the differences in prevalence of *E. coli, S. typhimurium* and *V. cholerae* between the various beaches and markets. Descriptive statistics (percentages) was used to show the differences of the antimicrobial resistance patterns between water, *R. argentae* and *O. niloticus.*

Total number of fungi species isolated from each site was not log transformed calculated and compared between sites, between the beaches and the markets, and variations determined by ANOVA, and t-test, respectively. Differences in the total number of fungi species infesting dagaa or tilapia was determined by student's t-test. Significant differences were accepted at $p < 0.05$. Single way ANOVA was used to determine differences in percentage moisture contents between the two fish species or between fish from beaches and markets was determined by paired two tail t-test, with $p < 0.05$ considered statistically significant.

The fish/preservative treatments and microbiological data (MPN and CFU) from the three fish landing beaches (Dunga, Luanda Rombo and Sirongo) and three markets (Kisumu municipality, Luanda and Bondo) were used in the final results presentation and statistical analysis. Only the means of 0 hour and 8 hours experiments for the various preservative treatments and controls were compared for the two fish species and data presented in graphs.

One factor analysis of variance (ANOVA) was used for determining the effectiveness of various preservative concentrations, with $p<0.05$ taken as statistically significant. The results of CFU determination was only used for the tabulation of final comparisons of the antibacterial activities of highest concentrations of the sodium chloride (12%), chlorinated

solution (200ppm), *M. oleifera* plant extracts (80 μg/ml), and bacteriophage suspension 1.10 x

10^6 respectively and only the means of the 8 h treatment duration data was used.

3.9 Study Limitation

In this study, the microbes were three enteric bacteria (*E.coli, Salmonella* entrica

Typhimurium and *Vibrio cholerae* O1) species were isolated from fish (*R. argentea* and *O.*

niloticus) and water in three fish landig beaches (Sirongo, Luanda Rombo and Dunga) and

fish markets (Kisumu, Luanda and Bondo) and fungi isolated from dried fish. The

antimicrobial susceptibility test was done for three bacteria and not for fungi. Comparison

was done on non-selective methods of brine salting and sanitizing with chlorine solution with

new and novel *Moringa oleifera* plant extracts and bacteriophage. Bacteriophage lytic to *E.*

coli was the only isolated phage used in the study as a preservative.

3.10 Research Approval

The approval for the thesis research was given by School of Graduate Studies, on 6 December

2009, (see Appendix IV).

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence and Antimicrobial Susceptibility Pattern of Enteric Bacteria Isolated from Water and Fish in Lake Victoria Basin, Kenya

Water samples from Dunga beach were the most contaminated with total viable counts of 320 CFU/ml, followed by water samples from Kisumu market 280 CFU/ml and then Luanda market 214 CFU/ml, Figure 2. Water samples from Sirongo beach were the least contaminated with 189 CFU/ml. Fish samples from Kisumu market showed the highest total viable count with 216 CFU/g for *R. argentea* and 193 CFU/g for *O. niloticus* followed by Dunga beach 198 CFU/g for *R. argentea* and 179 CFU/g for *O. niloticus*. Sirongo beach amd Luanda Rombo beach recorded the lowest viable colony counts for *R. argentea* 132 CFU/g and *O. niloticus*, 162 CFU/g, respectively (Figure 2).
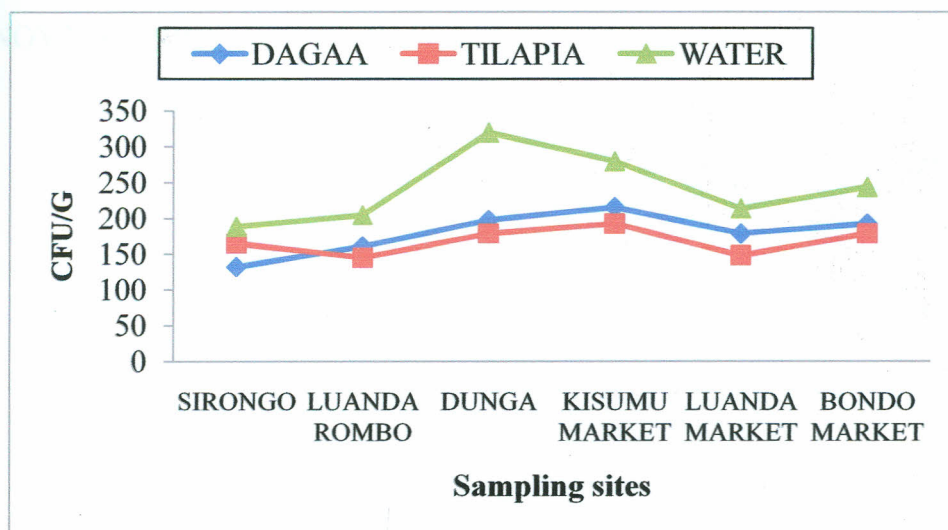

Figure 2. The total coliform count from water, *R. argentea* and *O. niloticus* from various sampling sites
Legend: CFU/g- Colony forming units per gram.
          Sirongo, Luanda Rombo and Dunga are fish landing beaches
          Kisumu market, Luanda market and Bondo are fish markets

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Out of 162 samples analysed, 133 (82.1%) were contaminated with various bacteria species (Table 1). *Salmonella enterica* serovar Typhimurium was the most prevalent with 49.6% among the isolates followed by *E. coli* 46.6% and the least prevalent was *V. cholerae* 3.8%. Dunga had the highest number of bacteria isolated 33.8% followed by Kisumu market with 19.9% and the least was Sirongo beach 11.3%. Out of 45 isolates from Dunga, *Salmonella* Typhimurium isolates were 48% while 44.4% were *E. coli* and *V. cholerae* were 6.7%. From Kisumu market, out of the 21 isolates, 52.4% were *Salmonella* Typhimurium, 42.9% were *E. coli* and 4.8% were *V. cholerae*. On the other hand, Sirongo beach yielded the lowest number of microbes with a total of 11.3% isolates, and out of these 53.3% were *E. coli*, and 46.7% were *Salmonella enterica* serovar Typhimurium. The differences in prevalence between the bacteria species (*S. enterica* serovar Typhimurium, *E. coli* and *V. cholerae*), and between the various beaches and markets combined was significant by two way ANOVA, P < 0.01.

Table 1: **Number of samples contaminated with enteric bacteria (*S. enterica* serovar Typhymurium, *E. coli*, and *V. cholerae* O1) from water and fish samples collected from different beaches and markets in the Lake Victoria Basin of Kenya.**

Sampling location and type (number *)	No. of samples positive for bacteria species			Total
	S. enterica ser. Typhymurium	*E. coli*	*V. cholerae* O1	
Dunga beach				
Water	5	8	2	
R. argentea	8	5	1	
O. niloticus	9	7	0	
Total	22	20	3	45
Sirongo beach				
Water	2	4	0	
R. argentea	3	2	0	
O. niloticus	2	2	0	
Total	7	8	0	15
Luanda Rombo beach				
Water	2	5	1	
R. argentea	3	3	0	
O. niloticus	3	2	0	
Total	8	10	1	19
Kisumu Fish market				
Water	3	3	0	
R. argentea	4	4	0	
O. niloticus	4	2	1	
Total	11	9	1	21
Luanda Fish market				
Water	3	3	0	
R. argentea	2	3	0	
O. niloticus	4	2	0	
Total	9	8	0	17
Bondo Fish market				
Water	4	2	0	
R. argentea	3	2	0	
O. niloticus	2	3	0	
Total	9	7	0	16
Grand Total	66 (49.6%)	62 (46.6%)	5 (3.8%)	133

* The water, *R. argentea* and *O. niloticus* samples were nine (9) from each of the beaches or markets

Antimicrobial susceptibility results for *E. coli*, *S. enterica* serovar Typhimurium and *V. cholerae* O1 are shown in Table 2. In water, over 60% of *S. enterica* serovar Typhimurium isolates were resistant to co-trimoxazole, ampicillin and tetracycline. Over 70% of *E. coli* were resistant to co-trimoxazole, tetracycline and ampicillin and for *V. cholerae*, O1 resistance was over 66% to ampicillin, tetracycline and co-trimoxazole. All the water isolates were sensitive to norfloxacin and ciprofloxacin. However, in *R. argentea* all the isolates were 100% sensitive to ciprofloxacin. Over 68% of *E. coli* isolates were resistant to tetracycline, ampicillin and co-trimoxazole. Of the 23 *S. enterica* serovar Typhimurium isolates, 73% were resistant to co-trimoxazole, tetracycline and ampicillin while in *V. cholerae* O1 isolates, 100% were resistant to ampicillin and tetracycline.

Out of 18 *E. coli* isolated from *O. niloticus*, over 50% were resistant to ampicillin and co-trimoxazole and over 85% of *S. enterica* serovar Typhimurium isolates were resistant to tetracycline and cotrimoxazole, and 100% of *V. cholerae* isolates were resistant to co-trimoxazole and tetracycline. All the isolates from water, *R. argentae* and *O. niloticus* were sensitive to ciprofloxacin.

Out of the 133 bacteria isolates 47 isolates were from water, 43 isolates were from *R. argentea* and another 43 were from *O. niloticus*. I water samples *E. coli* isolates were 25, *Salmonella enterica* serova Typhimurium 19 isolates and *V. cholerae* 3. From *R. argentea* samples *E. coli* isolates were 19, *S. enterica* serova Typhimurium isolates 23 and 1 *V. cholerae* O1 isolate. *O. niloticus* samples had 18 *E. coli* isolates, *S. enterica* serova Typhimurium 24 isolates and 1 for *V. cholerae* O1.

Table 2: Antimicrobial resistance patterns of *E. coli, S. enterica* serovar Typhimurium and *V. cholerae* O1 isolates from water and fish (*R. argentea* and *O. niloticus*) samples

Antibiotic	Number (%) of isolates resistant to antibiotic			Total
	Water			
	E. coli n = 25	*S. enterica* ser. Typhimurium n = 19	*V. cholerae* O1 n = 3	47
Ampicillin	16 (64)	13 (68.4)	2 (66.7)	
Chloramphenicol	7 (28)	5 (26.3)	0	
Erythromycin	4 (16)	2 (10.5)	0	
Gentamicin	8 (32)	3 (15.8)	0	
Norfloxacin	0	0	0	
Ciprofloxacin	0	0	0	
Methicillin	4 (16)	3 (15.8)	0	
Tetracycline	19 (76)	12 (63.2)	2 (66.7)	
Co-trimoxazole	20 (80)	14 (73.7)	2 (66.7)	
	R. argentea			
	E. coli n =19	*S. typhimurium* n =23	*V. cholerae* O1 n =1	43
Ampicillin	14 (73.7)	14 (60.9)	1 (100)	
Chloramphenicol	5 (26.3)	8 (34.8)	0	
Erythromycin	0	3 (13.0)	0	
Gentamicin	4 (21.1)	3 (13.0)	0	
Norfloxacin	1 (5.3)	1 (4.3)	0	
Ciprofloxacin	0	0	0	
Methicillin	2 (10.5)	5 (21.7)	0	
Tetracycline	14 (73.7)	16 (69.6)	1 (100)	
Co-trimoxazole	12 (63.2)	17 (73.9)	0	
	O. niloticus			
	E. coli n =18	*S. typhimurium* n =24	*V. cholerae* O1 n =1	43
Ampicillin	13 (72.2)	10 (41.7)	0	
Chloramphenicol	7 (38.9)	9 (37.5)	0	
Erythromycin	2 (11.1)	1 (4.2)	0	
Gentamicin	3 (16.7)	1 (4.2)	0	
Norfloxacin	2 (11.1)	0	0	
Ciprofloxacin	0	0	0	
Methicillin	2 (11.1)	3 (12.5)	0	
Tetracycline	2 (11.1)	17 (70.8)	1 (100)	
Co-trimoxazole	12 (66.7)	16 (66.7)	1 (100)	

Legend:

n= number of bacteria isolated from water and fish

% = No. of isolates ÷ Total number of isolates X 100

4.2 Prevalence of Fungi Contamination and Moisture Content on Sun-dried Fish

Figure 3 shows fungi species isolated from sun-dried *R. argentea* and *O. niloticus* from different beaches and markets in the Lake Victoria basin of Kenya. Sun-dried *R. argentea* and *O. niloticus* from the markets had significantly higher fungal species isolates (mean = 5.41) compared to fish from the beaches (mean = 4.06, p = 0.012, paired two-tail t-test). Also, sun-dried *R. argentea* from the markets had significantly higher fungal species isolates (mean = 6.0) compared to sun-dried *O. niloticus* from the markets (mean = 4.9, p<0.00001), though there was no significant statistical difference in fungal species isolate counts from *R. argentea* (mean = 4.0) compared to the counts from *O. niloticus* (mean = 4.2 from the beaches, p = 0.56).
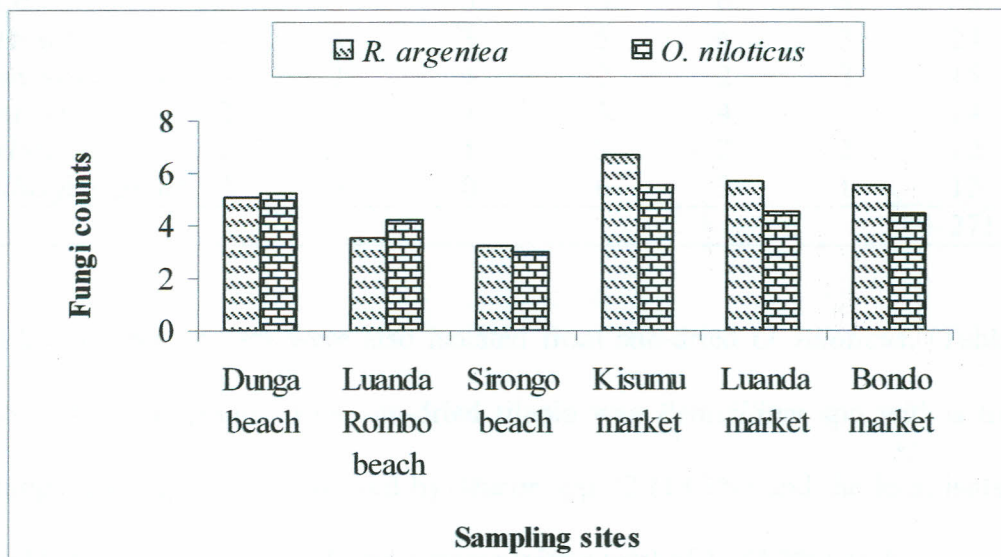

Figure 3: Fungi species isolated from sun dried *R.argentea* and *O. niloticus* from different beaches and markets
Legend: Sirongo, Luanda Rombo and Dunga are fish landing beaches
   Kisumu market, Luanda market and Bondo are fish markets

A total of 11 fungi species were isolated from the sun-dried *R. argentea* from different sampling beaches and markets (Table 3). The most isolated fungi species from sun-dried dagaa was *Mucor* spp with total fungal isolate counts of 44 (16.2%) followed by *Rhizopus oryza*, 35 (12.9%) while the least isolated fungi was *Aspargillus flavus* and *Aspergillus fumigates*, 12 (4.4%) each.

Table 3: Frequency of fungi species isolated from different sampling sites on sun dried *R. argentea*

Fungi isolates	Dunga beach	Luanda Rombo beach	Sirongo beach	Kisumu Market	Luanda Market	Bondo Market	Total	%
Mucor spp.	8	8	5	8	7	8	44	16.2
Rhizopus oryza	7	3	5	4	8	8	35	12.9
R. stolonifera	4	5	4	6	7	6	32	11.8
Penicillium spp	6	4	3	8	4	6	31	11.4
Alternaria spp	5	3	6	7	4	5	30	11.1
Aspergillus niger	2	2	3	5	6	5	25	8.5
Fusarium solani	4	4	3	5	4	3	23	8.5
F. chlamydosporium	5	2	1	2	2	3	15	5.5
R. micosporus	2	1	1	3	4	3	14	5.2
Aspergellus flavus	1	0	1	3	2	2	12	4.4
Aspergillus fumigatus	2	0	0	6	3	1	12	4.4
							271	100

Eleven fungi species were also isolated from sun-dried *O. niloticus*, (Table 4). The most isolated fungi species from sun-dried tilapia was *Penicillium* spp with a total of 35 (15%) fungal isolate counts, followed by *Mucor* spp 32 (13.7%) and the least isolated fungi were *F. chlamydospnonium* and *R. micosporus* with a total of 10 (4.3%) each.

Table 4: Frequency of fungi species isolated from different sampling sites on sun dried

O. niloticus

Fungi isolates	Dunga beach	Luanda Rombo beach	Sirongo beach	Kisumu market	Luanda market	Bondo market	Total	%
Penicillium spp.	7	7	3	8	4	6	35	15
Mucor spp.	9	8	2	0	7	6	32	13.7
R. stolonifera	4	6	2	6	5	3	26	11.2
R. oryza	5	1	5	6	4	3	24	10.3
Aspergillus flavus	4	4	4	7	3	1	23	9.9
Alternaria spp	2	3	3	6	3	4	21	9
Aspergillus niger.	5	2	3	2	5	3	20	8.6
A.fumigatus	4	3	0	6	3	1	17	7.3
Fusarium solani	1	3	2	6	3	0	15	6.4
R. micosporus	5	0	1	0	2	2	10	4.3
F. chlamydosporium	1	1	1	3	2	10	32	4.3
							233	100

The moisture content of fish (dagaa and tilapia) from the beaches was significantly lower (mean = 13.3%) compared to moisture content of the fish from the markets (mean = 14.3%, p = 0.03), paired two-tail t-test. Moisture content of sun-dried dagaa from the beaches was significantly lower (mean = 13.3%) compared to moisture content of sun-dried dagaa from the markets (mean = 14.9%, p = 0.049), and also moisture content of sun-dried tilapia form the beaches (mean = 13.2%) was significantly lower than moisture content of sun-dried tilapia from the markets (mean = 13.6%, p = 0.03). There was no significant difference on moisture content between *R. argentea* and *O. niloticus*, (Table 5).

Table 5: Moisture content of sun-dried *R. argentea* and *O. niloticus* from different sampling beaches and markets

Sampling sites	Moisture content %	
	R. argentea	*O. niloticus*
Dunga beach	14.4	14.1
Luanda Rombo beach	13.4	13.3
Sirongo beach	12.3	12.3
Kisumu market	15.3	14.6
Luanda market	14.6	13.6
Bondo market	14.6	12.6

4.3 Antimicrobial Activities of Brine Salting, Chlorinated Solution, *Moringa oleifera* Plant Extracts, and Bacteriophage Treatments on Microbes in Fish

4.3.1 Brine Salting

With increase in salt concentration and time, there was high reduction of bacterial load on *O. niloticus* and *R. argentae* fish samples, Figure 4. At time 0h and a concentration of 0% salt concentration the bacterial load on tilapia was 205.7 MPN/g and at 0h and salt concentration of 12% bacterial load decreased to 125.2 MPN/g, $P=0.04$, single factor ANOVA. At the end of the experiment at 8h, the bacterial load in the control (0% salt concentration) rose to 362.2 MPN/g and at salt concentration of 12%, the microbial load decreased significantly to 65.7 MPN/g, $P=0.00002$, single factor ANOVA. In the case of dagaa, the control had a microbial load of 241.6 MPN/g and this decreased significantly to 140 MPN/g, $P=0.007$. At the end of 8h, the control recorded 536.6 MPN/g and at a high salt concentration microbial load decreased to 74.5 MPN/g with a significance of $P=0.00001$.
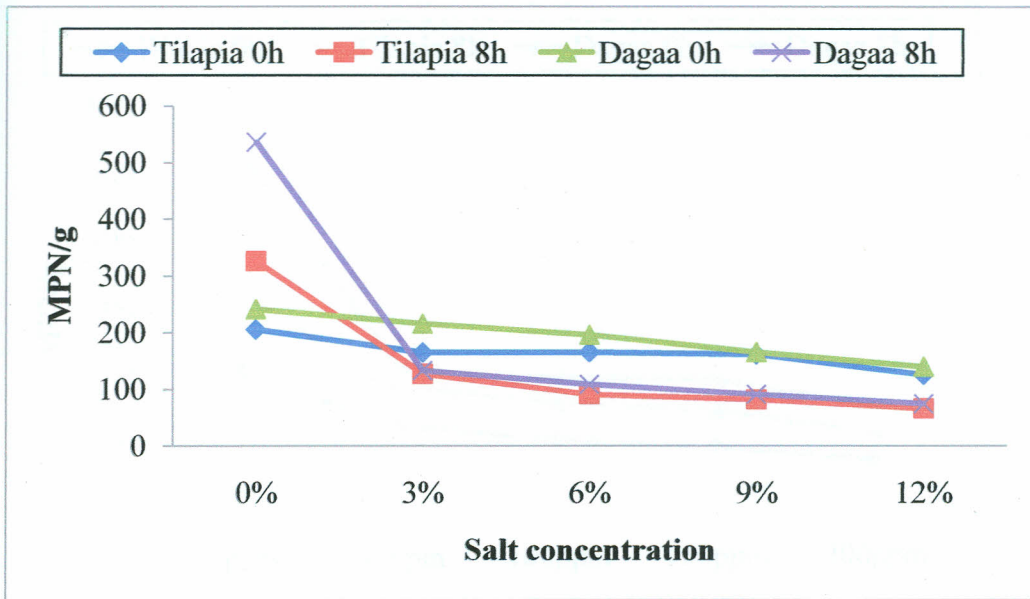
Figure 4: Antibacterial activities of salt solutions on bacteria in *O. niloticus* and *R. argentea* fish samples

Legend: MPN/g- Most probable number per gram.

4.3.2 Chlorinated solution

The effectiveness of chlorinated solution on bacteria in *O. niloticus* and *R. argentea* fish samples decreased with time but increased with increase in concentration, Figure 5. The microbial load in tilapia at 0 h was 205.7 MPN/g and at 200ppm it decreased to 72 MPN/g., P= 0.00001, single factor ANOVA. In dagaa samples, microbial load at 0 h was 241.7 MPN/g and this decreased at high concentration of chlorine 200ppm to 111.5 MPN/g, P=0.0002. At the end of the experiment 8 h, the bacterial load on dagaa at time 0 h was 536.6 MPN/g and at high chlorinated solution concentration it reduced to 151.5 MPN/g, P=0.00001, single factor ANOVA.
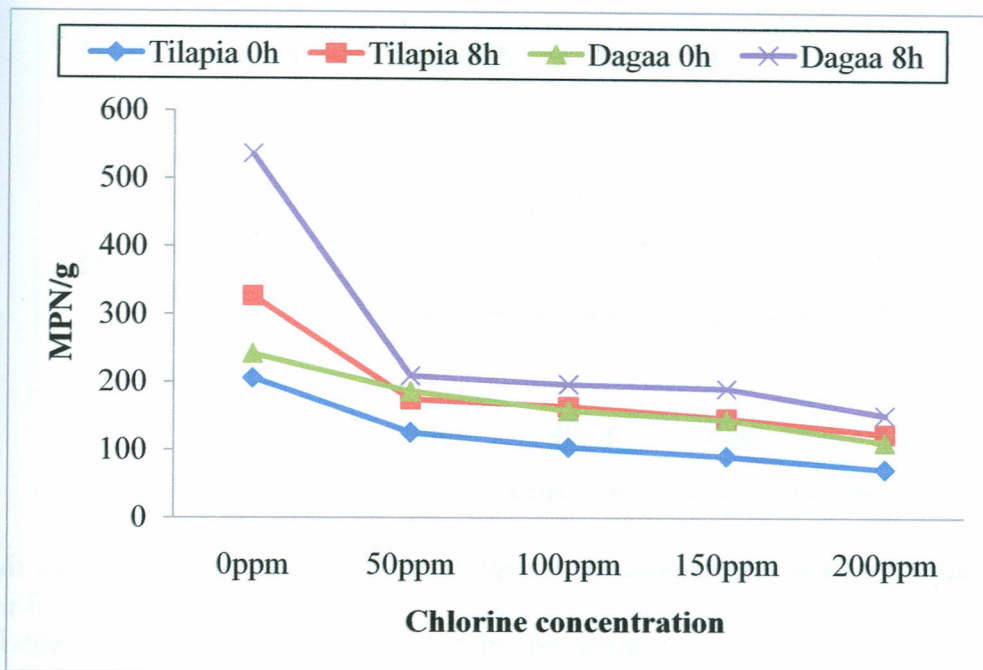
Figure 5: Antibacterial activities of chlorinated solution in *O. niloticus* and *R. argentea* fish samples
Legend: MPN/g- Most probable number per gram

4.3.3 *Moringa oleifera* plant extracts

As *Moringa oleifera* n-hexane extract concentration and time increased, there was decrease in bacterial load in *O. niloticus* and *R. argentea* fish samples, Figure 6. In tilapia control samples, microbial load at 0h was 205.8 MPN/g and this decreased to 148.3 MPN/g at a concentration of 80µg/ml, P = 0.002, single factor ANOVA. At 8h the control tilapia samples had a microbial load of 326.2 MPN/g which decreased to 87 MPN/g at a concentration of 80 µg/ml, P = 0.00001. For dagaa samples, microbial load at 0h was 241.7 MPN/g and this decreased to 189.1 MPN/g at a concentration of 80 µg/ml, P = 0.05 which was less significant. At the end of the experiment 8h, microbial load was 536.6 MPN/g and this decreased to 120.8 MPN/g when the concentration was 80µg/ml was used, P=0.00001 was highly significant.
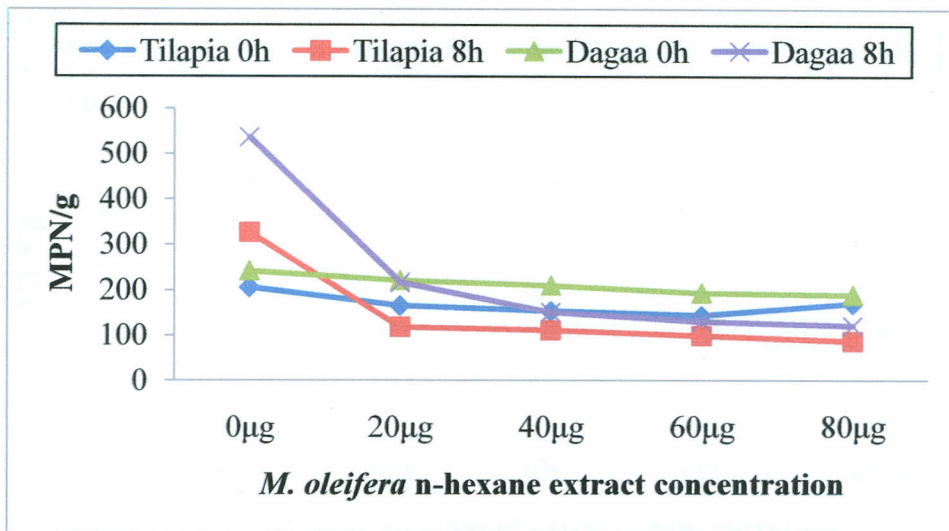
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Figure 6: Antibacterial activities of *M. oleifera* n-hexane extract in *O. niloticus* and *R. argentea* fish sample
Legend: MPN/g- Most probable number per gram

As *Moringa oleifera* ethanol extract concentration and time increased, there was decrease in bacterial load in *O. niloticus* and *R. argentea* fish samples, Figure 7. In tilapia control samples, microbial load at 0 h was 205.8 MPN/g and this decreased to 169.7 MPN/g at a concentration of 80µg/ml, P = 0.04. At 8h the control tilapia had a microbial load of 326.3 MPN/g which decreased to 114.7 MPN/g at a concentration 80 µg/ml, P = 0.00003. For the dagaa microbial load at 0h was 241.7 MPN/g and this decreased to 196.7 MPN/g at a concentration of 80 µg/ml, P=0.06. At the end of the experiment 8h, microbial load was 536.6 MPN/g and this decreased to 144.3 MPN/g concentration of 80µg/ml, P = 0.00001, single factor ANOVA.
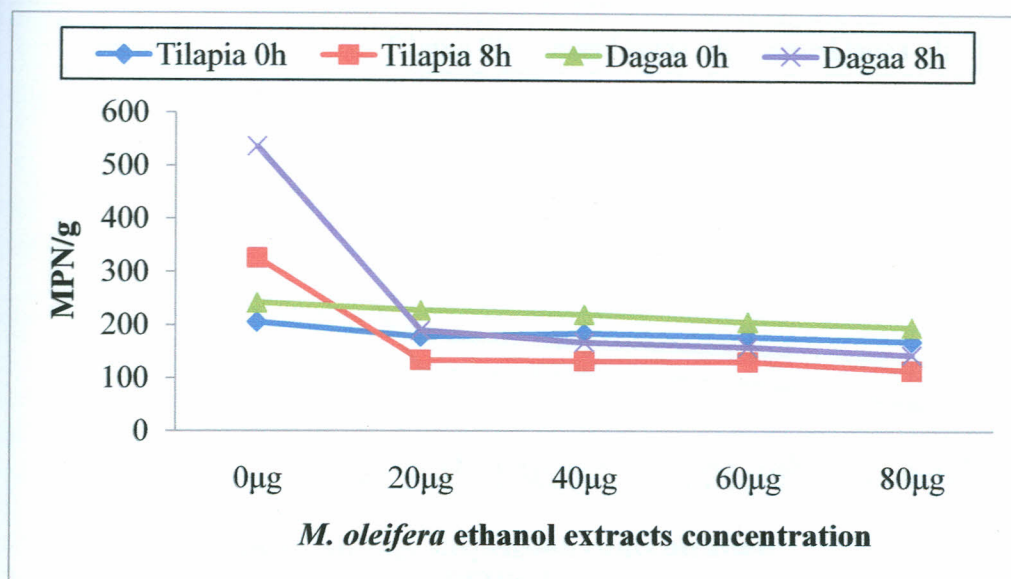
Figure 7: Antibacterial activities of *M. oleifera* ethanol extract on bacteria in *O. niloticus* and *R. argentea* fish samples
Legend: MPN/g- Most probable number per gram

4.3.4 Bacteriophage suspension

With increase in bacteriophage suspension concentration and time, there was decrease in bacterial load in *R. argentea* samples (Figure 8). In tilapia control samples the microbial load at 0h was 205.8 MPN/g and this decreased to 127.1 MPN/g at a concentration of 10^6 PFU/ml, P = 0.00012. At 8h the tilapia control had a microbial load of 326.2 MPN/g which decreased to 35.45 MPN/g at a concentration of 10^6 PFU/ml, P = 0.00001. Incase of dagaa, microbial load in control at 0h was 241.6 MPN/g and this decreased to 110.1 MPN/g at a concentration of 10^6 PFU/ml, P = 0.00014. At the end of the experiment 8h the microbial load in control was 536.6 MPN/g and this decreased to 50.1 MPN/g at a concentration of 10^6 PFU/ml, P = 0.00001.
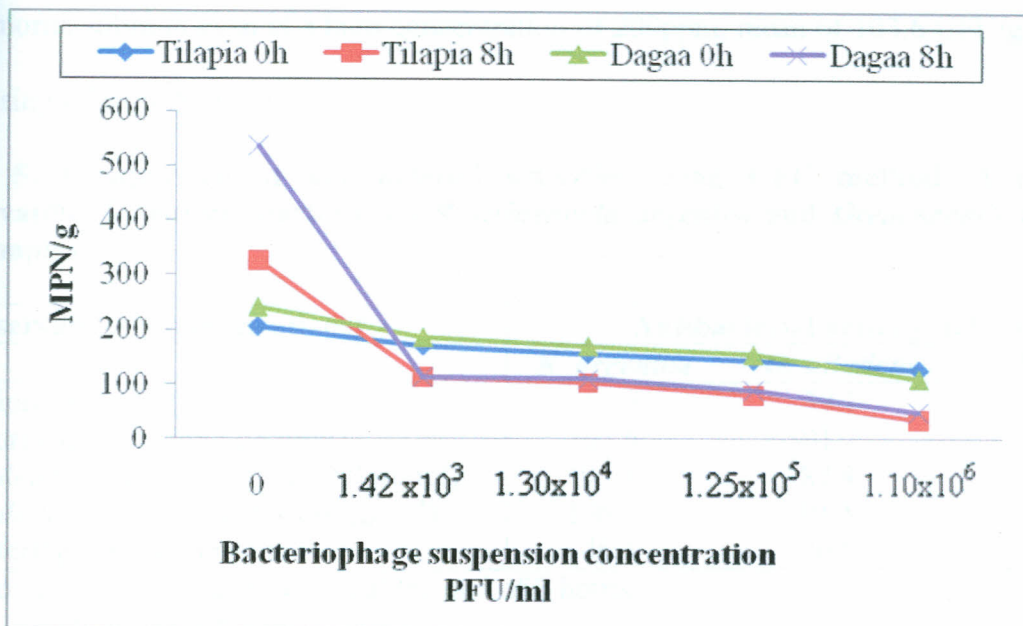
Figure 8: Antibacterial activities of bacteriophage suspensions in *O. niloticus* and *R. argentea* fish samples.

Legend: MPN/g- Most probable number per gram

PFU- Plague forming units

Similar results for the two fish species (Table 5), in the case of tilapia samples after 8 hours, bacteriophage suspension at concentration of 10^6 PFU/ml was the most effective in reducing bacterial load (mean of 36.3 CFU/g) followed by 12% of sodium chloride solution (mean of 39.1 CFU/g), then 80 µg/ml *M. oleifera* n-hexane and ethanol extracts (mean of 87.4 CFU/g and 91.5 CFU/g), respectively, while sodium hypochlorite solution, even at 200ppm, was also the least effective (mean of 101.7 CFU/g), P = 0.00001. For the dagaa fish samples after 8 hours of preservation, bacteriophage suspension concentration of 10^6 was the most effective on bacterial load (mean of46.1 CFU/g) followed by 12% of sodium chloride solution (mean of 59.9 CFU/g), then 80 µg/ml *M. oleifera* n-hexane and ethanol extracts with a means of 95.4 CFU/g and 106.2 CFU/g, respectively. The least effective preservative was the sodium

hypochlorite solution even at a high concentration of 200ppm, mean of 103.6 CFU/g, the P = 0.002, single factor ANOVA.

Table 5: Comparison of antibacterial activities using CFU method of different preservatives in enteric bacteria on *Rastrineobola argentea* and *Oreochromis niloticus* fish samples

Preservatives (concentrations)	Antibacterial activity in CFU/g		
	R. argentea	*O. niloticus*	Mean
Sodium chloride (12%)	59.9	39.1	49.5
Chlorinated solution (200ppm)	103.6	101.7	102.7
M. oleifera n-hexane extract (80 µg/ml)	95.4	87.4	91.4
M. oleifera ethanol extract (80 µg/ml)	106.2	91.5	98.9
Bacteriophage suspension	46.1	26.5	36.3

Legend: The CFU/g was recorded at the end of 8 hours
CFU- Colony forming units

CHAPTER FIVE

5.0 DISCUSSIONS

5.1 The Prevalence of *E. coli, S. enterica* Typhimurium and *V. cholera* O1 in *Rastrineobola argentea* (dagaa) and *Oreochromis niloticus* (tilapia) Fish and in Water from the beaches and markets

Total coliform counts results from this study has shown that water samples from Dunga beach and Kisumu market were more contaminated and the water samples from Sirongo beach were the least contaminated. Similarly, fish samples from Kisumu market and Dunga beach were the most contaminated, and fish samples from Sirongo beach were the least contaminated, suggesting that contaminated water may contribute to the microbial load in the fish. The prevalence data from the most probable number (MPN) method and subsequent bacteria species (*E. coli, S. enterica* serova Typhimurium and *V. cholerae*) identification and isolation from water and fish also showed that Dunga beach and Kisumu market samples had the highest number of bacteria isolated while Sirongo beach had the lowest number. Dunga beach is at the shores of Lake Victoria in Kisumu town within Winam Gulf with significantly higher human population (KNBS, 2010) and the lake water at the beach could be more polluted compared to lake water at Sirongo, which is in Bondo county, a distance from an urban setting and is less populated. The lake generally receives large quantities of treated and untreated waste water discharged from human and industrial sources (Onyango *et al.*, 2009). Furthermore, rivers and rainfall could introduce enteric pathogens from distant sources into shore water (Baudart *et al.*, 2000) hence contributing to lake water pollution. Discharge of untreated municipal effluent into rivers and the lakes, compounded by

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lack of awareness on good hygiene practices, can also directly contribute to the degradation of river and lake water quality for habitats and domestic use (Robin *et al*., 2004).

The reason why fish from Kisumu market were more contaminated was not determined by this study but it could be attributed to the poor sanitary conditions of storage, handling and processing of fresh fish in the market or unhygienic fish transportation methods from the beaches to the market. This suggests that Kisumu municipality authorities therefore need to enforce higher standards of hygiene in the market and Kenya Fisheries Department should also set up high standards for transporting fresh fish from the beaches to the markets.

Results from this study showed that *Salmonella* was the most prevalent pathogen which may contribute to the high prevalence of diarrhoea in Kisumu region (Annual Bulletin, 2007). *V. cholerae* though not very prevalent was also isolated from water and fish. *V. cholerae* O1 is not only able to survive but also able to grow in fresh water habitats (Vital *et al*., 2007). *V. cholerae* O1 found in the waters of cholera endemic areas exists in biofilm-like aggregates in which cells are in conditional viable state (Islam *et al*., 1994). They are metabolically impended cells which can regain its metabolic activity under specific *in vitro* conditions. Such vibrio cells might play a critical role in the transmission of pathogens (Watnick and Kolter, 1999).

The results from this study further showed that fish collected from the beaches were contaminated with enteric bacteria due to use of contaminated water collected directly from the lake for processing fish by local artisanal fish processors as a result of lack of piped water. This could further be enhanced by transportation of fish in dirty fishing boats and dirty packaging baskets by the fisher folks. The fish can also be contaminated through zooplankton and phytoplankton including algae (Worden *et al*., 2006). Results from previous studies have

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shown that zooplanktons, algae and phytoplanktons are normally together in the harvested fish in the nets hence possibility of fish contamination (Reidl and Klose, 2002; Worden *et al.*, 2006).

5.2 The Antimicrobial Patterns of *E. coli, S. entrica* Typhimurium and *V. cholerae* O1 Isolated from Water and Fish from Beaches and Markets

Results from this study have also shown that the enteric bacteria isolated from water and fish were resistant to some antibiotics. All the bacteria isolated from water were sensitive to norfloxacin and ciprofloxacin; and all the bacteria isolates from *R. argentea* were sensitive to ciprofloxacin. In this study therefore, a multidrug resistance pattern was observed for *E. coli,* and *S. enterica* serovar Typhimurium against cotrimoxazole, tetracycline and ampicillin. However, the bacterial species were susceptible to the antibiotics ciprofloxacin and norfloxacin. Resistance to cotrimoxazole, tetracycline and ampicillin might be related to their over use as opposed to norfloxacin and ciprofloxacin which are not used for treating enteric infections (Eduardo *et al.*, 1995). The emergence and dissemination of antimicrobial resistance among *E. coli, Salmonella enterica* serovar Typhimurium and *Vibrio cholerae* O1 strains is an increasing global health problem that is complicating the therapeutic management of severe salmonellosis and diarrhogenic diseases (Sirinavin and Garner, 2000). The findings from this study are consistent with those reported previously by Onyango *et al.*, (2008; 2009) of high resistance pattern of *Salmonella* spp. isolated from the fish landing beaches to antibiotics frequently used for treating salmonellosis and diarrhoeagenic diseases in the local community. The high prevalence of resistance to tetracycline, ampicillin and co-trimoxazole in *E. coli* in the region has also been reported by Sifuna *et al.*, (2008), in which *E. coli* demonstrated resistance mostly to ampicillin, and tetracycline. Similar results were reported

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by Sack (2001) and Shapiro *et al.,* (1999) which attributed resistance to use of tetracycline for mass prophylaxis during cholera outbreaks. The resistant pattern reported in this study can also be linked to use of these drugs in veterinary practise, a factor that can lead to resistance in human as previously reported (Laxminarayan, 2002). Due to widespread acquisition of resistance, it is important that the susceptibility tests are routinely done to guide antibiotic treatment and policy (Hart and Kariuki 1998; Gallarda *et al.,* 1999; Kariuki *et al.,* 2011). Antimicrobial resistance genes can be readily transmitted between commensal *Enterobacteriaceae* and enteropathogens *in vivo* and *in vitro* (Blake *et al.,* 2003), hence these organisms may cause hard to treat persistent diseases in humans, if interventions and proper policies are not instituted in a timely manner.

The finding from this study implies that a high percentage of cases of diarrhea in western Kenya may be caused by antimicrobial-resistant bacteria to the cheaper and commonly used antibiotics, thus illustrating the effect of longstanding, unregulated antimicrobial use. A survey of enteric pathogens isolated from patients attending selected district hospitals in western Kenya revealed high prevalence of resistance to commonly used antibiotics including tetracycline, cotrimoxazole and ampicillin (Kakai, 2009). This suggests that the strains circulating in the human population are also found within the aquatic and fish environment. Most enteric pathogens easily share genes for antimicrobial resistance, and the continuous selective pressure applied by the over-the-counter availability of these antimicrobials, as well as the prescription of these agents at most clinic visits, has potentially lethal consequences for a region plagued by epidemics of cholera. Judicious use of antimicrobial therapy requires the education of health workers and patients, adequate laboratory diagnostic capabilities, and government regulation (Kariuki *et al.,* 2011).

None of the *E. coli, Salmonella enterica* serovar Typhimurium, and *V. cholerae* O1 were resistant to ciprofloxacin. Several studies have shown that ciprofloxacin offers advantages in the treatment of salmonellosis, reaching high concentrations in serum and faeces (Threfall *et al.*, 2001). Widespread use of ciprofloxacin for treating all *E. coli, S. enterica* serovar Typhimurium, and *V. cholerae* O1 infections should be discouraged to avoid selection of resistant strains. This study provide valuable information to the Ministries of Medical Services and Public Health, Fisheries and other agencies in making policy decisions aimed at reducing microbial contamination of fish and water, and the indiscriminate use of antibiotics. There is need for research on antibiotic susceptibility surveillance in the aquatic environments where fresh fish and water are obtained for human consumption.

5.3 Fungi Infestation on Sun-dried *R. argentea* (dagaa) and *O. niloticus* (tilapia)

Sun-dried *R. argentea* and *O. niloticus* from the markets had significantly higher fungal species isolates compared to fish from the beaches. Also, sun-dried *R. argentea* from the markets had significantly higher fungal species isolates compared to sun-dried *O. niloticus* from the markets, however, there was no significant statistical differences in fungal species isolate counts from *R. argentea* compared to the counts from *O. niloticus* from the beaches. The reason why market samples had more fungal counts could be due to storage condition since they are heaped and put in gully bags in poorly ventilated stores (Eyo, 1983). Further infestation by insects may spread the fungal spores to new fish stock in the stores (Mitchell, 2007). Due to long term storage fungi developed and the way fish are displayed on dirty nylone bags in the market centers will further increase fungal contamination on the fish, and also poor handling and hygiene may accelerate the contamination (Onyango *et al.*, 2009; Adebayo-Tayo *et al.*, 2008).

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The beaches recorded lower fungal isolates for the two fish species, especially for *R. argentea,* probably due to the short storage period. Immediately after sun-drying of *R. argentea* within eight (8) hours or less, depending on sun shine, they are often transported to the markets or immediately sold at the beaches for local consumption as per the experience during the study. This duration does not allow fungal spores to sporulate on sun-dried fish. For *O. niloticus,* it could be because the fish mongers at the beaches are better at processing fish leading to less spoilage as compared to fish mongers in the markets.

A total of 11 fungi species were isolated from the sun-dried *R. argentea* and *O. niloticus* from different sampling beaches and markets. The most isolated fungi species from sun-dried dagaa was *Mucor* spp. followed by *Rhizopus oryza* while the least isolated fungi were *Aspergillus flavus* and *A. fumigatus*. The most isolated fungi species from sun-dried tilapia was *Penicillium* spp. followed by *Mucor* spp. and the least isolated fungi were *F. chlamydospnonium* and *R. micosporus*. *A. flavus* is known to produce aflatoxins (CDC, 2004; 2005). Aflatoxins are highly carcinogenic, causing hepatoma and have also been associated with acute hepatitis in man, as demonstrated in the developing countries (CDC, 2004; 2005). It is important that both the artisanal fishermen and the marketers adapt a better method of preservation, sun-drying technology and fish product storage facilities should be well-ventilated. Improper sun-drying of fishes could lead to fungal attack, insect infestation, fragmentation and degradation of the products (Eyo, 1992). Mould growth is encouraged by damp, so cured fish which has not been fully dried is susceptible, particularly after a period of storage (Kumolu-Johnson and Ndimele, 2011).

From the current study, most of the fungi isolated were contaminants rather than originating in the fishes samples. Owaga *et al.,* (2010) also isolated *Aspergillus* spp.

Penicilium spp. from *R. argentea* which had been washed, treated and dried at specific temperature.

5.4 Moisture Contents on Sun-dried Fish *R. argentea* (dagaa) and *O. niloticus* (tilapia)

The moisture content of fish (dagaa and tilapia) from the beaches was significantly lower compared to moisture content of the fish from the markets. Moisture content of sun-dried dagaa from the beaches was significantly lower compared to moisture content of sun-dried dagaa from the markets, and also moisture content of sun-dried tilapia from the beaches was significantly lower than moisture content of sun-dried tilapia from the markets. Fish at the beach can have low moisture content than those in market stores because those in the stores may have accumulated humidity probably due to poor aeration. At the beaches the fish are probably sun-dried since this is their practice while in the market, they are kept in gunny bags and hipped in the store with poor ventilation (Eyo, 1992). During storage of sun-dried fish products, good storage practices are not often adhered to by fish folks, the stores are not well ventilated and pests can easily gain access into the stores (Adebayo-Tayo *et al.*, 2008). The environment in which fish are displayed on dirty nylon sheets in the markets is not always hygienic and this is another avenue for fungi contamination and higher moisture content (Adebayo-Tayo *et al.*, 2008).

The findings of Santour *et al.*, 2002 showed that moulds have the ability to survive harsh conditions and low moisture content. The implication of this report is that most of the fish presently on sale in the markets are infected with moulds and are partially acceptable for human consumption, thus, most consumers might consume fungal metabolites and their prolonged intake may constitute a health hazard (Owaga *et al.*, 2009). Though in Kenya, most fish are properly cooked before eating, once the fish and fishery products are contaminated

with aflatoxin, boiling will have no effect on the potency of the toxic materials. Since aflatoxins have been found to be heat-stable with a melting point of between 268 to 269 ^{0}C (Sulyok, 2006; Bhat and Miller, 2010). It is therefore important that both the fishermen and the fish mongers take necessary precautions in preventing contamination of the fish and fishery products to reduce possible contamination and in the process reduce the risk of mycotoxins that are deleterious to human health.

5.5 Antibacterial Activities of Brine Salting, Chlorinated Solution, *Moringa oleifera* plant Extracts, and Bacteriophage Treatment on Microbes in Fish

The results from this study have shown significant effectiveness of increased sodium chloride concentration (12%) at reducing bacterial load in *R. argentea* and *O. niloticus*. These results are consistent with traditional antibacterial effects of common salt for preserving food. Common salt preserves food in different ways but mainly by inhibiting bacterial growth through dehydration, chloride ion effect, oxygen removal, and carbon dioxide sanitization of growth of preolytic enzyme (Bahri *et al.*, 2006). At a concentration of $\geq$3% (w/v), NaCl generally inhibits the growth of *Salmonellae* (D'Aoust, 2001). The preservative effect of salt is mainly due to the decrease in water activity and thus prevention of growth of many spoilage micro-organisms along with formation of a more membranous surface which further inhibits the growth of micro-organisms (Ahmed *et al.*, 2010).

Results from this study shows that the higher the concentration of sodium hypochlorite solution, the more effective it is in inhibiting bacterial growth in fish. However, with increase in time, the less effective it becomes. This may be due to the amount of organic materials (fats and proteins), in fish tissues which combine with chlorine ions rather than with bacteria (Suslow, 2000). Sodium hypochlorite solution also gets oxidized and looses strength with time (Suslow, 2000). Though chlorine is always reported to be a highly effective and

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inexpensive solution used in the control of food-borne diseases, especially during food processing, this study found that it was not effective in reducing bacterial load in fish sample and therefore, may not be recommended for treating fish for long-term storage.

Higher concentrations of *Moringa oleifera* n-hexane or ethanol extracts were effective in reducing bacterial loads as demonstrated in the current study. These results show that *M. oleifera* has antibacterial activity even on fish and can be used for fish preservation. The compound pterygospermin in *M. oleifera* seeds have antibiotic property which enhance the elimination of bacteria (Anwar and Bhanger, 2003).

For the first time, results from this study show the effectiveness of *Moringa olaifera* plant extracts against bacteria in fish samples. This means that *M. oleifera* plants which easily grows in most arid and sandy conditions as found near the Lake Victoria basin of Kenya, can be exploited to provide extracts for preserving fish for long-term storage and safety for human consumption.

These results additionally show that for the two fish species, *R. argentea* and *O. niloticus* fish species 12% sodium chloride solution was the most effective in reducing bacterial load, compared to chlorinated solution even at the highest concentration of 200ppm. These results are encouraging because common salt (sodium chloride) is cheap and easily available and should therefore be adopted by the fisher community for routine processing and preservation of fish. It is also fortunate that salt has no effect on the value of fish protein and when used for preserving fish even at a higher concentration, recipes for desalting can be adopted during fish preparation and cooking for human consumption.

Bacteriophage represents a quickly diluted preservative incase of absence of the target bacterium and an amplifiable preservative in the presence of the target pathogen. Phage

preservation is novel technique which challenges the current preservative or sanitization techniques. The bacteriophage used here are said to be active due to *in vivo* replication of the phage on the pathogen. Species specificity is the rule for bacteriophage and is one of the major assets of bacteriophage suspension in food preservation. The specificity of bacteriophage would decrease chances of bacterial mutations for resistance as is common with non-specific sanitizers. If bacteria mutate to resist the bacteriophage, the bacteriophage will in turn mutate to continue to infect the bacterial host. Bacteriopahge need their bacterial host to survive, therefore, survival of the bacteria is crucial to the bacteriophage's co-existence. Bacteriophage biocontrol strategy usage in pre-harvest phases of food production and post-harvest storage of food under a variety of environmental conditions should be optimized. Bacteriophage can be applied directly to food and be eaten by the consumer with no harm to the properties of food or to humans (USFDA/CFSAN, 2006).

From the results, the current study shows that the concentration of bacteriophage at the time of application is crucial for better results or efficacy, that is, applying more bacteriophage suspension generally resulted in greater inactivation of microbes. This is in accordance with results of other studies showing that higher bacteriophage numbers yielded better results (Leverentz *et al.*, 2004; Carlton *et al.*, 2005). From the data it suggests that for optimum efficacy, the bacteriophage suspension concentration should not be less than 10^5 PFU/g at the time of application. The concentration of phage must be high enough to ensure the contact of the passively diffusing virus particles with their host cells diffuse almost freely. This situation may vary in different food matrix. Thus, protocols for application of bacteriophage in any food production setting and environment must be individually optimized not only with respect to the phages and target organisms, but also by considering specification

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of food matrix. It should be noted that it is necessary to use a sufficiently high bacteriophage

concentration from the start without relying on self amplification of bacteriophage.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The highest prevalence of bacteria pathogens in water and fish was found in Dunga beach and lowest in Sirongo beach. There was high prevalence of resistance to commonly used antibiotics including tetracycline, cotrimoxazole and ampiciline.

2. The highest fungal prevalence was shown in Kisumu market fish samples *R. argentea* and *O. niloticus* and Sirongo beach fish samples had the least fungal infestation.

3. Fish form the beaches had low moisture content as compared to those from the markets.

4. The bacteriophage suspension was the most effective preservative followed by sodium chloride, then *M. oleifera* plant extracts and the least effective was sodium hypochloride solutions against bacteria in fish.

6.2 Recommendations from Study Results

1. Judicious use of antimicrobial therapy needs the education of health workers and patients, government regulation and proper laboratory diagnosis so that the spread of antimicrobial resistance is reduced.

2. The priorities for microbiological monitoring should be reassessed periodically

3. Mandatory monitoring of antimicrobial usage in humans and animals, preferably according to medical diagnostics and antimicrobial categories, is recommended as this would enable better epidemiological analyses of occurrences and trends of

antimicrobial resistance and would provide a basis for conducting risk assessment as well as for implementing and evaluating interventions.

4. From this research there should be an improvement in food safety by exploring novel methods and technologies, for example by use of bacteriophage for specificity of phages renders them ideal candidates for applications designed to increase food safety during the production process.

5. It is recommended that GMP, GHP and HACCP be applied effectively and monitored closely to decrease the proportion of fish with high prevalences and/or concentrations of *E. coli, Salmonella typhimurium, Vibrio cholerae* O1, *Aspergillus spp* and *Penicillium spp*.

6.3 Recommendations for Policy Formulation

1. The government should establish centers for surveillance of food-borne diseases which may originate from fish so as to allow public health officials to recognize trends, detect outbreak, pinpoint the causes of these outbreaks, and develop effective preventive and control measures. To reduce food-borne transmission of cholera, and salmonellosis, it is suggested that foods should be prepared, served and eaten in a hygienic environment free from faecal contamination.

2. Sanitary conditions of fishing, storage, transport and handling of fish should be monitored by both local government and central government.

3. GMP, GHP, HACCP and official controls should be applied effectively and closely monitored to decrease the risk of contamination by pathogenic bacteria and fungi and other relevant zoonotic agents in fish and fishery products.

6.4 Suggestions for Future Research

1. There is need for research to develop new and more effective antimicrobials for preserving food products such as fish in order to ensure that consumers have access to safe fish and fishery products.

2. The bacteriophages have an advantage over traditional antimicrobials such as salt and chlorine sanitizers as shown from this thesis research and more studies are needed to validate these findings in a real life situation.

3. Although there was high prevalence of microbes in the fish, its association with human diseases in the study area is still unclear. To understand the emergence of fish-borne infection, it is important to investigate the disease agent as well as its interactions with its environmental reservoir and vector such as fish.

4. Further studies, should be done on sensory aspects of preserved fish products using salting, chlorinated solution, *Moringa oleifera* plant extracts and bacteriophage treatment and their shelf life determined.

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