Isolation, Identification and Screening of *Bacillus* species from *Rastrineobola argentea* (Omena) for Production of Bacteriocins active against Bovine Mastitis Pathogens

(Escherichia coli and Staphylococcus aureus)

Juliana Wanjiru Maina

A thesis submitted in partial fulfillment for the degree of Master of Science in Food Science and Nutrition in the Jomo Kenyatta University of Agriculture and Technology

2015

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature: …… Date: ……

Juliana Wanjiru Maina

This thesis has been submitted for examination with our approval as the University Supervisors:

Signature: …… Date: ……

Dr. Julius Maina Mathara

JKUAT, Kenya.

Signature: …… Date: ……

Dr. Gideon M. Kikuvi

JKUAT, Kenya.

DEDICATION

I dedicate this thesis to my family members, my husband Peter, and children Ivy, Ida and Ian for their support and great patience.

ACKNOWLEDGEMENTS

This thesis has been accomplished with encouragement, support and contribution from my supervisors Dr. Maina J. and Dr. Kikuvi to whom I'm deeply indebted. I specifically thank them for having been there throughout the research process to guide me on various issues concerning the research. I owe special thanks to my family members who have been of great support. Special thanks to Jeniffer Wambugu, Senior Technologist in Food Science and Technology Department, who was very helpful and of great encouragement. Above all my thanks go to God for his provision of grace and favors which have been sufficient.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

LAB	Lactic Acid Bacteria
IMIs	Intramammary Infections
SCC	Milk Somatic Cell Count
DHIA	Dairy Herd Improvement Association
MYP	Mannitol-egg Yolk Polymyxin
TC	Total Plate Count
NA	Nutrient Agar
API	Analytical Profile Index
CFS	Cell Free Supernatant
LB	Luria Bertani
BLIS	Bacteriocin-like inhibitory substances
TSB	Tryptic Soy Broth

ABSTRACT

Bacteriocins offer an advantage over antibiotics in that they target very specific organisms and they are generally regarded as safe for humans. The aim of this study was to isolate and identify bacteriocin-producing *Bacillus* species from *Rastrineobola argentea* (Omena) and to screen their bacteriocins for their potential as antimicrobial agents against bovine mastitis pathogens.

A total of 300 *R. argentea* samples were randomly purchased from retailers in Gikomba market-Nairobi. To identify isolated bacteria, colonies were assessed using morphological, biochemical techniques and the API KIT. Further, crude bacteriocins produced by some of the isolates were tested for their antimicrobial activity against the bovine mastitis pathogens *Escherichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC-25923 by well diffusion method. The physicochemical characteristics of the crude bacteriocins produced were also assessed.

The study identified *Bacillus mycoides, subtilis, pumilus* and *lentus* as the most predominant species. Among these, *Bacillus subtilis* and *pumilus* were found to produce crude bacteriocins with antimicrobial activity against *E. coli* and *S. aureus,* the bovine mastitis pathogens.

The crude bacteriocins produced were found to be fully or partially inactivated in the presence of proteolytic enzymes like trypsin and had a lipid moiety since their activity reduced in presence of lipase enzyme. In contrast, their antimicrobial activity was not affected by temperature treatment of up to 100°C or in presence of ions like copper or iron but there was slight reduction in activity with zinc ions. Their activity was optimal at neutral and alkaline pH but reduced significantly at acidic pH.

The bacteriocins produced by these *Bacillus* species have desirable characteristics that make these isolates attractive candidates with potential application for prevention of bovine mastitis pathogens.

Keywords: Bovine mastitis pathogens, antimicrobial activity, *Bacillus*, bacteriocin..

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Mastitis is considered the most economically important disease affecting the dairy industry. Management strategies involve the extensive use of antibiotics to treat and prevent this disease (Diep and Nes, 2002). Prophylactic dosages of antibiotics used in mastitis control programs could select for strains with resistance to antibiotics (Gillor *et al*., 2005). The emergence of multidrug resistant pathogens and imposed restrictions on the use of antibiotic feed additives has intensified the search for novel possible alternatives (Diep & Nes, 2002; Gillor *et al*., 2005). In addition, a strong drive towards reducing antibiotic residues in animal food products has led to research in finding alternative antimicrobial agents. In this regard, much interest has been focused on bacteriocins due to their great potential applications in medicine (Diep & Nes, 2002).

Bacteriocins are ribosomally synthesized antimicrobial peptides lethal to bacterial other than the producing strain (Joerger, 2003) and are the most abundant of antimicrobial compounds produced by bacteria. These antimicrobial peptides are found in all major phylogenetic bacterial lineages (Wertz *et al.*, 2002.) During the last 20 years, bacteriocins of lactic acid bacteria (LAB) have been given much attention because some of them exhibit high activity against pathogenic organisms (Diep & Nes, 2002). In contrast, bacteriocins from *Bacillus* have attracted little attention even though some *Bacillus* species, such as *Bacillus subtilis* and *Bacillus Licheniformis*, are generally recognized as 'safe' bacteria (Teo and Tan, 2005). The primary habitat of most *Bacillus* species is the soil but *B. subtilis , B. cerus, B. firmus* and *B. lentus* have been isolated from both fresh and marine water bodies (Ostensvik *et al*., 2004) and from processed fish (Dube *et al*., 2004). Being capable of producing a large number of antimicrobial peptides, *Bacillus* is an interesting genus to search for inhibitory

substance (Bizaini *et al*., 2005). Unlike lactic acid bacteria (LAB) bacteriocins, which have a narrow antimicrobial spectrum (Jack *et al*., 1995), bacteriocins from *Bacillus* exhibit distinct diversity in their inhibitory activities (Cordovilla *et al*., 1993). For example, several bacteriocins of *Bacillus* show a rather wide antimicrobial spectrum covering Gram-positive and Gram-negative bacteria (Shelburne *et al*., 2007). The production of bacteriocins or bacteriocin-like substances has been described for *B. coagulans, B. brevis, B. lichniformis, B. cerus, B. subtilis, B. amyloliquefaciens* and other *Bacillus* species (Teo and Tan, 2005; Lisboa *et al*., 2006). Although many studies on bacteriocins from *Bacillus* have exhibited their important aspects of food safety (Barboza-Corona *et al*., 2007), few have addressed the potential application of these antimicrobial peptides in animal clinical studies. Some *Bacillus* bacteriocins with strong inhibitory activity against *Staphylococci* have been recommended for further exploration with an aim in use in the control of mastitis in cows (Abriouel *et al.,* 2010). These are mainly from *B. thuringiensis.* Thus, there is a need to further explore these inhibitory substances that have a broad-spectrum antibacterial activity against domestic animals' pathogenic bacteria for use in dairy farming.

1.2 Statement of the Problem

Bovine mastitis remains one of the most important diseases in the dairy industry with economic losses estimated at $1.2 to $1.7 billion (Wells and Ott, 1998) to dairy producer per year in the United States alone. In Kenya, Bovine mastitis is known to be a problem in dairy herds and a major cause of economic loss in the dairy industry (Omore *et al*., 1996). Intramammary infusion of antibiotics is the main approach to treatment of mastitis on many dairy farms (Sears and McCarthy, 2003). Due to increased antibiotic resistance of mastitis pathogens (Wagner *et al*., 2006), reduced responses to antibiotic therapy have become very common in tertiary practice. According to Wagner *et al.* (2006), this situation has caused indiscriminate use of antibiotics with extra-labeled doses by some veterinarians in their practice. Due to the extensive use of antibiotics on dairy farms, antibiotics residues in milk have become a subject of public concern. In addition, the quantity of antibiotics needed to eliminate mastitis pathogens inhibits the growth of lactic *Streptococci*, the bacteria essential in cheese and yogurt making technology; and the residues in milk are a potential threat to human health as may lead to antibiotics resistance (Omore *et al*., 1996). Consequently, there is an increasing need for alternative approaches to mastitis treatment and management. Members of the genus *Bacillus* carry tremendous importance because of their antimicrobial activity since they produce a variety of peptide antibiotics representing several different basic chemical structures (Von, 1983).

1.3 Justification for the Study

Although normally very effective, the use of antibiotics to control mastitis has some disadvantages. Milk is normally withheld for a period of time following antibiotic administration, with concomitant economic losses. In countries where losses incurred by the dairy industry due to mastitis have been quantified they are often enormous. For example in USA herds monitored over a 12 month period in 1993 showed losses ranging from $161 to $322 per lactating cow per year. In 1997 losses were estimated at $185 per cow per year and a total cost of $1.8 billion (Schroeder, 2007). Data from South Africa indicate that in the year 1978 the losses were estimated to be R150 per cow per year and in 1987 – 1988 the figure had risen to approximately R400 per cow per year (Pieterse, 2008).

In Kenya, the losses due to mastitis amount to about $0.3 million annually (Omore, *et al*., 1996). According to Omore *et al*., (1996), severe economic losses due to mastitis occur from reduced milk production, treatment cost, and increased labor, milk withheld following treatment and premature culling. It is recognized that if this disease is diagnosed in early stages, a greater portion of this loss can be avoided (Omore, *et al*., 1996)

Prophylactic dosages of antibiotics used in mastitis control programs could select from strains with resistance to antibiotics (Passantino, 2007) which could enter the food chain through dairy food products making treatment of human pathogens more challenging. Broad-spectrum bacteriocins produced by *Bacillus* species of bacteria may provide valuable alternatives to antibiotics for the treatment of this disease. These natural inhibitors are particularly suitable for such applications given their use and acceptance as preservatives in foods (Delves-Broughton, 1990). Moreover, few studies have addressed the potential application of these antimicrobial peptides in animal clinical studies.

1.4 Hypothesis

Rastrineobola argentea (omena) sold at Gikomba market in Nairobi, Kenya harbors *Bacillus* species which produce bacteriocins that can be used in the control of mastitis pathogens.

1.5 Objectives

1.5.1 General Objective

To isolate and identify the bacteriocinogenic *Bacillus* species associated with *Rastrineobola argentea* (omena) sold at Gikomba Market in Nairobi and investigate the potential of the bacteriocins in the control of mastitis pathogens.

1.5.2 Specific Objectives

1. To isolate and identify the *Bacillus* species associated with *Rastrineobola argentea* (Omena) sold at Gikomba Market in Nairobi.

2. To screen, select and identify bacteriocin producing *Bacillus* species among the isolates with inhibitory activity against indicator bacteria.

3. To determine the physicochemical characteristics of the bacteriocins produced by the selected isolates.

4. To determine the activity of isolated bacteriocins against mastitis causing pathogens.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mastitis

2.1.1 Background Information

The general health and well-being of individuals depends largely on the meeting of basic nutritional needs. Milk and milk products form an important part of the daily nutrition. Increase in global population coupled with increasing demands for milk as an economic food and as an industrial raw food product has necessitated an increase in production of milk with consequent increase in risk of infection. Low milk production has been attributed to disease in dairy cattle of which mastitis accounts for the largest economic losses on dairy farms in most countries (Petrovski *et al*., 2006). Mastitis is the inflammation of the mammary gland in response to bacterial invasion. Improving udder health and decreasing the incidence of udder infection and inflammation of dairy herd's results in increased milk production as huge losses are directly or indirectly incurred through loss of milk during treatment periods, culling of cows and death of clinically infected cattle (Pieterse, 2008).

2.1.2 Development of Mastitis and Invasion of the Udder

A basic knowledge of mammary glands anatomy and physiology is necessary to understand how mastitis develops (Bingo, 2012). The interior of each quarter is composed of a teat cistern, gland cistern, milk ducts and glandular tissue. The glandular tissue or secretion portion contains millions of microscopic sacs called alveoli. Each alveolus is lined with milk-producing epithelial cells and is surrounded by muscle cells that contract and squeeze milk from the alveolus during milking. Blood vessels bring nutrients to each alveolus, where epithelial cells convert them into milk. Between milking, milk accumulates in the alveolar spaces, milk ducts and cisterns. During milking, the accumulated fluid is removed through the teat ducts (Molloy *et al.*, 2008). Mastitis results once bacteria pass through the

teat duct and multiply in milk-producing tissues. Microorganisms breach the teat duct in several ways. Between milking, microorganisms may pass through the teat duct by multiplying inside the duct, or by physical movement resulting from pressure placed on the teat end as the cow moves about. During machine milking, microorganisms may be propelled into or through the teat duct into the teat cistern (Bingo, 2012).

The potential for invasion is greatly increased by bacteria that reside in or colonize the teat duct. Such colorizations occur in lactating and dry cows, and the colonizing bacteria may survive for months, serving as sources of bacteria for infecting the gland. The practice of dipping teats with effective bactericides before and after each milking greatly reduces teat duct colonization (Molloy *et al.*, 2008). Cows contract udder infection at different ages and stages of the lactation cycle. Cows also vary in their ability to overcome an infection once it has been established. The cows' environment influences the numbers and types of bacteria they are exposed to and their ability to resist these microorganisms (Molloy *et al.*, 2008). However, through appropriate management practices, the environment can be controlled to reduce this exposure and enhance resistance to udder disease (Molloy *et al.*, 2008).

Bovine mastitis is mainly caused by bacterial infection. *Staphylococcus aureus*, *Streptococcus agalactiae*, *dysgalactiae, uberis* and various Gram-negative bacteria including *Escherichia coli, Pseudomonas* and *Klebsiella* are the common pathogens in the bacterial infection. However, more than 130 micro-organisms have been reported to cause disease in the mammary gland of cows (Hillerton and Watts, 2005). *Staphylococcus aureus* infections remain the largest mastitis problem in most dairy farms. The cure rate with antibiotic therapy during lactation is very low. Many "staph" cows become chronic and have to be culled (Fox, 2012).

2.1.4 Classification of Types of Mastitis

Clinical and subclinical mastitis are two major forms of mastitis in dairy cows (Christ *et al.,* 2007). Clinical mastitis results in alterations in milk composition and appearance; decreased milk production; elevated body temperature; and swelling, redness, or heat in infected mammary glands (Molloy *et al*., 2008). It is readily apparent and easily detected, however detection of subclinical mastitis is more difficult because signs are not readily apparent. Consequently, subclinical mastitis often goes undetected with a tendency to persist, resulting in an elevated milk somatic cell count (SCC) and decreased milk production, which may lead to development of clinical mastitis and a chance for contagious bacteria to spread from infected to uninfected mammary glands (Junqiang *et al*., 2007).

2.1.5 Mastitis control strategies

The 'give point plan' for mastitis control has been used for many years (Giesecke *et al*., 1994) and has been successful in reducing the incidence of mastitis (Christ *et al.,* 2007). The strategy addresses areas where the risk of infection is greatest and promotes the use of treatment at specific times. It includes; teat disinfection after milking, good hygiene and milking procedures with adequate milking equipment, culling chronically mastitis ill cows as well as antibiotic dry cow therapy and prompt treatment of clinical mastitis during dry period and during lactation. The first three can be described as farm management related areas while the last two are specific treatment actions involving use of antimicrobial agents (Pieterse, 2008).

The following strategies have so far been suggested in literature as very crucial in ensuring effective control of mastitis, including proper farm management, improving the milking procedures and the environment, teat dipping, dry-cow therapy, and use of back-flushers. Farm management strategies employed aim maintaining good hygienic practices in the environment. The holding yards and stalls should be kept clean. Another strategy is the culling of the chronic mastitis cows. One should also prevent cows from having access to manure, mud or pools of stagnant water. The stalls should also be properly designed and maintained (Molloy *et al*., 2008).

Observing good practices during milking is very important, as this is most often the route of infection (Molloy *et al*., 2008). The udder should be prepared by washing the teats followed by disinfection and drying with clean towels. Milking personnel hands should be disinfected to prevent transfer of pathogens. All cows should be treated with a teat dip disinfectant post milking to reduce the risk of infection (Giesecke *et al*., 1994). Teat dips are effective against most mastitis organisms. They have been shown to effectively reduce mastitis caused by *Staphylococcus aureus* and *Strep. agalactiae,* the most common types of mastitis (Fox, 2012). However, considerable controversy surrounds the effectiveness of teat dipping on environmental pathogens *E. coli* and *Strep. uberis.* Some research has shown that teat dipping does not control these organisms but can control the spread of contagious mastitis caused by *Staphylococcus aureus* and *Strep. Agalactiae*, *E. coli* and *S. uberis* pathogens found in the cow's surroundings and if mud is udder deep, the teat dip will be removed and a new infection may occur (Pinedo *et al.,* 2012).

Many effective teat dips, including iodine at 0.1 percent, 0.5 percent and 1 percent, and chlorohexidine at 0.5 percent, are available. Hypochlorite at 4 percent with sodium hydroxide content less than 0.05 percent, although not labeled for teat dipping, has been found effective in field trials (Lam *et al.,* 2013). Effective coverage of the teats was also found to be more important than the type of dip used during the same trials.

The whole teat to the base of the udder must be dipped to stop the spread of contagious bacteria (*S. agalactiae, S. dysgalactiae, S. aureus* or *Mycoplasma*) if found in a herd. Hand sprayers are acceptable for herds that have environmental mastitis because teat colonization is not a factor. Hand-held spray bottles are the most ineffective method of getting proper coverage of dip on the cow's teats, so they should not be used for this purpose. Dip cups, on the other hand, give the best coverage (Ogura *et al.,* 2010).

In addition, the environment can be improved to prevent occurrence of mastitis. The milking system should be kept as clean and dry as possible for the cows. The dry-cow environment is as important as the lactating-cow environment. The calving area should be kept clean (Molloy *et al*., 2008). Barrier dips are reported to reduce new coliform infections; however, they do not appear to be as effective against environmental *Streptococci* and the contagious pathogens. Attempts to control environmental mastitis during the dry period, using either germicidal or barrier dips, have also been unsuccessful (Pinedo *et al.,* 2012). Similarly, dry-cow therapy has been recommended for all quarters of all cows at drying-off. This helps control environmental *Streptococci* during the early dry period but has little or no value in controlling coliforms. It is also not effective during the period prior to calving.

The use of backflushers has been adopted to sanitize the liners and claws between milking periods. Most units on the market have four or five cycles. The first cycle is a water rinse, followed by iodine or similar sanitizer rinse, a clear-water rinse and positive air-dry cycle. Research has demonstrated that back-flushers do reduce the number of bacteria on the liners between cows but do not reduce the number of bacteria on teats (Molloy *et al*., 2008). Backflushers also may stop the spread of contagious organisms, but this also can be accomplished at a much lower cost by teat dipping. Backflushing has no effect on environmental pathogens that are encountered between milking sessions.

2.1.5.1 Treatment of Mastitis by use of Antibiotics

Antibiotic therapy is the most widely used approach to intra-mammary infections (IMIs). A product is considered excellent if it has a high cure rate and a minimum withdrawal period (Gruet *et al*., 2001). Antimicrobial agents may be administered either during lactation or during the dry period. The use of antimicrobials during lactation must be carefully considered due to the likely presence of antibiotic residues in milk.

According to Christ *et al.* (2007), loss of milk due to discarding milk contaminated with antibiotics has been cited as a reason why treatments of subclinical mastitis are not suggested during lactation. Treatment is instead postponed until a clinical flare-up occurs or until dry-off. This is a time when antibiotics are often routinely used to treat all quarters in all cows. It is also used to treat existing infections or prevent new infections from developing during this period (Christ *et al.,* 2007).

2.1.5.2 Alternatives to Antibiotic Treatment of Mastitis

The risk involved in the treatment of mastitis has been discussed in terms of the development of antimicrobial resistance but from the commercial standpoint, milk products containing specific levels of antibiotic residues cannot be sold for human consumption (Pieterse, 2008). Elimination of the use of antibiotics for the treatment of mastitis is unlikely and therefore the goal would to reduce their use. Antimicrobials are still necessary to combat bacteria pathogens and the solution could be to focus on research into development of antimicrobial agents that offer some advantage over the antibiotics currently in use.

The future use of antibiotics in treating animals, especially food animals is shifting towards other alternatives because of food safety concerns and business factors such as loss of milk products due to antibiotic contamination (Shryock, 2004). Another driving force in exploring alternatives to antibiotics is the move toward organic dairy which prohibits the use of antibiotics.

Some of the new technologies include antimicrobial peptides produced by plants, animals and insects, bacteriocins produced by bacteria and bacteriophages to treat bacterial diseases in animals and humans (Pieterse, 2008).

2.1.5.3 Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides lethal to bacteria other than the producing strain (Joeger, 2003) and are the most abundant antimicrobial compounds produced by bacteria. They are found in all major phylogenic bacterial lineages (Wertz *et al.,* 2003). Bacteriocins are categorized in several ways, including according to the producing strain and the mechanism of killing. There are several large categories of bacteriocin from Gram-positive bacteria, the colicins (Cascales *et al*., 2007), the microcins, and the bacteriocins from Archaea. The bacteriocins from *E. coli* are called colicins (formally called colicines, meaning coli killers). According to Cascales *et al*. (2007), the bacteriocins of lactic acid-fermenting bacteria are called lantibiotics. This naming system is problematic for a number of reasons including the fact that naming bacteriocins by what they putatively kill would be more accurate if their killing spectrum were continuous with genus or species designations (Cascales *et al*., 2007).

Bizani *et al*., (2005), indicates that alternative methods of classification that include: method of killing (pore forming, DNAse, nuclease, murein production, etc.), genetics (large plasmids, small plasmids, chromosomal), molecular weight and chemistry (large protein, polypeptide with/without sugar moiety, containing atypical amino acids like lanthionine) and method of production (ribosomal, post ribosomal modifications, non-ribosoms). One method of classification groups the bacteriocins into class I, class II a, II b and II c, and class III (Cotter *et al*., 2006). The class I bacteriocins are small heat-stable proteins. The class IIa bacteriocins (pediocin-like bacteriocins) are the largest subgroup and contain an N-terminal consensus sequence Tyr-Gly-Asn-Val-Xaa-Cys across this group. The c-terminal is responsible for species-specific activity, causing cell leakage by permeabilizing the target cell wall. Class II a bacteriocins have a large potential for use in food preservation as well as medical applications due their strong antilisterial activity, the broad range of activity. The

class IIb bacteriocins (two-peptide bacteriocins) require two different peptides for activity. Other bacteriocins can be grouped together as class II c (circular bacteriocins). This has a wide range of effects on membrane permeability, cell wall formation and pheromone actions of the target cells. Class III bacteriocins are large, heat-labile protein bacteriocins (Bizani *et al*., 2005).

During the last 20 years, bacteriocins of lactic acid bacteria (LAB) have been given much attention because some of them exhibit high activity against pathogenic organisms (Diep & Nes, 2002). In contrast, bacteriocins from *Bacillus* have attracted little attention even though some *Bacillus* species, such as *Bacillus subtitis* and *Bacillus licheniformis*, are generally recognized as safe bacteria (Teo & Tan, 2005). Teo and Tan (2005) note that the members of the genus *Bacillus* are rod-shaped, endospore forming aerobic or facultative anaerobic, Gram-positive bacteria. Many species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment (Claus & Berkeley, 1986). The primary habitat of most *Bacillus* species is in the soil but *B. subtitis. B. cereus B. firmus* and *B. lentus* have been isolated from both fresh and marine water bodies (Ostensvik *et al*., 2004) and from processed fish (Dube *et al*., 2004).

Unlike LAB bacteriocins, which have a narrow antimicrobial spectrum (Jack *et al*., 1995), bacteriocins from *Bacillus* exhibit distinct diversity in their inhibitory activities (Cordovilla *et al*., 1993). Several bacteriocins of *Bacillus* show a rather wide antimicrobial spectrum covering Gram-positive and Gram-negative bacteria (Shelburne *et al*., 2007). The production of bacteriocins or bacteriocin-like substance have been described for *B. coagulans, B.brevis, B. lichniforms, B. cereus, B.subtilis, B. amyloliquefaciens* and other *Bacillus* species (Teo & Tan, 2005; Lisboa *et al*., 2006). Bacteriocins offer an advantage over antibiotics in that they target very specific organisms. They are also generally regarded as safe for humans. Bacteriocin residues in milk and milk products would therefore not carry the same level of risk in terms of milk quality; processing of cheese, yogurt, and food safety issues as antibiotics. To date only the *lactococcal* bacteriocin, nisin has been

developed for commercial application while the lantibiotic, lacticin 3147 has been extensively used for dry cow therapy (Pieterse, 2008).

2.1.6 Effects of Mastitis on Milk Production, Composition and Quality

Mastitis reduces milk yield and alters milk composition. The magnitude of these changes in individual cows varies with the severity and duration of the infection and the causative microorganisms (Pinedo *et al*., 2012). Mastitis almost always is caused by bacteria. These microorganisms produce toxins that can directly damage milk-producing tissue of the mammary gland. The presence of bacteria initiates inflammation within the mammary tissue in an attempt of the latter to eliminate the invading microorganisms (Fox, 2012). Consequently, the inflammation contributes to decreased milk production and is primarily responsible for the compositional changes observed in milk from infected quarters and cows. In general, compositional changes involve an increase in blood components in milk and a decrease in normal milk constituents (Pinedo *et al*., 2012).

The Dairy Herd Improvement Association (DHIA) has adopted an SCC scoring system that divides the SCC of composite milk into 10 categories from 0 to 9 known as linear scores. The DHIA programs determine the SCC on each milking cow each month and report the SCC or the linear score. Linear scores can be used to estimate production losses, but the average linear score for the lactation most accurately reflects reduced milk yield. Cows with higher lactation average SCC scores produce less milk (Lam *et al.,* 2013). Production losses in older cows are about double those of first-lactation cows. Determining the exact amount of milk lost at a specific SCC or linear score or for any one cow is not possible. However, the fact remains that elevated SCCs result in major losses to dairy producers, and an elevated SCC is usually due to the presence of intramammary infection (Lam *et al.,* 2013).

Mastitis not only negatively affects milk yield production, as discussed above, but has a negative impact also on milk composition and its physico-chemical characteristics. These alterations are attributed to changes in vascular permeability due to the inflammatory

process and the damage of epithelial cells that are responsible for the synthesis of milk components, as well as changes in the enzymatic action of somatic cells or micro-organisms in the infected mammary gland (Kitchen, 1981).

For lactose, Bansal *et al.,* (2005) determined that lactose content was higher in healthy quarters than in quarters with high SCC. This result is in agreement with other studies (Pyorala, 2003; Jones, 2006).

Casein, the major milk protein of high nutritional quality, declines and lower quality whey proteins (which derive from the blood mammary barrier disruption) increase, which together adversely affects dairy product quality, such as cheese yield, flavour and quality.

For minerals, because of the increasing vascular permeability and the damage caused by the inflammatory process, blood-borne electrolyte concentrations in milk change. Na+ and Cl- increase in mastitic milk, while K+, normally the predominant mineral in milk, declines. Because most calcium in milk is associated with casein, the disruption of casein synthesis contributes to lowered calcium in milk (Jones, 2006).

These alterations affect milk quality directly through changes in technical and hygienic milk quality, resulting in less efficient processing of milk, and might result in products with less favourable properties. Examples are unstable and rancid taste of milk, a lower cheese yield and a decreased shelf life, which means economic damage to the dairy industry (Hogeveen, 2005).

2.2 *Bacillus* Species

Species of genus *Bacillus* are rods capsules of producing endospores and they grow aerobically although some are facultative in most cases in nutrient agar and are catalase positive (Lutz *et al.,* 2006). Bacteria belonging to genus *Bacillus* belong to class 1 of phylum *firmicuses*, i.e. *Bacilli* members of the genus *Bacillus* are Gram-positive, aerobic

and endospore–forming bacteria that are characterized by their rod-shaped cell morphology and catalase production (Ogura *et al.,* 2010). They are found in diverse environments such as the soil, clays, dust, aquatic environments, vegetation, food and gastro intestinal tracts of various insects and animals (Nicholson, 2002). This ability to survive and grow in such different ecosystems is based on the production of their robust endospores their diversity in physiological properties and their growth requirements.

Bacillus species exhibit quite diverse physiological properties such as the ability to degrade many different substrates derived from plant and animal sources including cellulose, starch, proteins etc. (Lutz *et al.,* 2006). Some *Bacillus* species are heterotrophic, thermophiles, acidophiles or alkalophiles (Priest, 1993; Slepecky & Hemphill, 2006). This diversity is reflected by considerable diversity of *Bacillus* strains, which allow those bacteria, to colonize a wide variety of ecological habitats. Members of *Bacillus* Group are considered good producers of antimicrobial substances including peptides, lipopetide, antibiotics and bacteriocins (Stein, 2005).

The prevalence of *Bacillus* species in food does not always imply spoilage or food poisoning; some species or strains are used in human and animal food production. *Bacillus subtilis* stains are used in Natto in East Asian fermented food (Hosoi and Kiuchi, 2003) and as a starter culture for fermenting soybeans in West Africa (Terlabie *et al.*, 2006). A Non-toxigenic *Bacillus cereus* species with probiotic properties is also used as an animal feed additive (Lodemann *et al.*, 2008). On the other hand *Bacillus* species or strains have been implicated in food poisoning and food spoilage. These include *B. cereus, Bacillus licheniformis*, and *Bacillus sphaericus* (Jay *et al.*, 2005; Granum, 2007). Therefore a rigorous selection process is required for the selection and development of *Bacillus* probiotic candidates (Barbosa *et al.*, 2007) or starter cultures, considering the intra species divergent virulence characteristics.

2.3 *Rastrineobola argentea*

Rastrineobola argentea is a species of ray-finned fish in the family *cyprinidae* and the only member of the genus *Rastrineobola*. *Rastrineobola argentea* are native zooplanktivores of lakes in East Africa (Manyala *et al*., 1992). It is an important commercial fish species of Lake Victoria in Kenya, Uganda and Tanzania. The local names are omena (Kenya) dagaa (Tanzania) and Mukene (Uganda). *Rastrineobola argentea* fishing takes place at night using canoes. For preservation, drying is usually done on the ground at the landing beaches leading to contamination with sand, flies and possibly microorganisms especially *Bacillus* species whose primary habitat is the soil. The dried products are similarly exposed to further contamination during storage, transportation and sale at open-air markets (Abila & Jansen, 1997). It is a relatively cheap source of animal protein for humans and livestock and it is marketed at retail markets as whole sun dried fish (Sifuna *et al*., 2008). Omena has widely been incorporated as part of ingredients in animal feeds.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of *Rastrineobola argentea* samples

A total of 300 *Rastrineobola argentea* (omena) samples were purchased in regular consumer packages (250g tins) from Gikomba retail market in Nairobi. The sampling was randomly done where samples of 250g tins each, were collected from 12 random Omena traders. Completely random design was used because the experimental units were essentially homogeneous and very experimental unit had the same probability of receiving any treatment. Randomization was performed using a random number table that consisted of 12 units that were given 25 treatments.

The samples were immediately transported in tins that were sealed to a microbiology laboratory at Department of Food Science and Technology, Jomo Kenyatta University of Agriculture and Technology for analysis. Samples were stored under refrigerated conditions.

3.2 Isolation and Identification of *Bacillus* Species

Twenty five grams from each fish sample was placed in a bag containing 225 millilitres of lactose and macerated using a stomacher 400 Circulator Homogenizer for two minutes and heated for 10 minutes at 80°C to destroy vegetative bacteria and fungi and to make easier the isolation of *Bacilli* from spores that survive the heat treatment. Ten-fold serial dilutions of the liquid supernatant were prepared with the same diluents up to 10^{-5}. Isolations of *Bacilli* from Selected strains were examined for the presence of spores under immersion oil after straining in safranin after growth on nutrient agar at 37°C for 48hrs. Microscopic examination included Gram stain to detect the presence of spores, after which the purified colonies were stored at 4°C. The colonies are purified by streaking a distinct colony on a nutrient agar plate and incubated at 37ºC for 48Hrs.

Biochemical tests were then carried out according to Harrigan (1976) in *laboratory methods in Food and Dairy Microbiology* procedures to identify the *Bacillus* species (Deak & Timar, 1988). The biochemical tests included: catalase test, nitrate reduction test, Voges Proskauer reaction, indole production test, growth on Simmons citrate agar, sugar fermentation test, motility hydrolysis of starch and casein. The strains to be studied further were identified by API 50 CH kits at 30°C according to the manufacturer's instructions (API system, BioMeriux S.A., Marcy Petoile). The isolates were preserved at 4°C on Nutrient agar (NA) slants and also in glycerol then stored in liquid nitrogen.

3.2.1 Gram Staining

Clean glass slides were obtained and using the sterile technique a smear of each of the microorganisms from an 18-28 hours culture (while micro-organisms were still young) was prepared, and heat- fixed. The smear was gently stained with basic dye crystal violet and left for 1–2 minutes. This was then rinsed rapidly with water, followed by treatment with gram's iodine solution and left for 1 minute which increased interaction between the bacteria cell and the dye so that the dye was more tightly bound or the cell was more strongly stained.

The iodine was then poured off, blotted and the slide or smear decolorized by washing with 95% ethanol until no more stain ran from the side. The slide was then washed well with water and stained with safranin for 30 seconds, which was then washed well and dried. Preliminary characterization by Gram staining was done (using safranin) on each of the isolates using the method of Dussault (1955) and observed under a light microscope at ×100 (Keast *et al*., 1984) and this was observed under oil immersion. The Gram staining technique was used to categorize the isolates into Gram negative and Gram positive (Cappuccino & Sherman, 2010).

3.2.2 Spore Staining

Spore staining was done by making smears of given cultures on clean slides which were air dried and heat fixed. This was then flooded with malachite green, then slides heated to steaming for 2-3 minutes adding more stains to the smear from time to time after which smears were washed under slowly running tap water after cooling. This was then counterstained with safraine for 30 seconds. The smears were then washed with tap water, blot dried and observed under immersion oil (Cappuccino & Sherman, 2010).

3.2.3 Biochemical Tests

Bacteria accomplish their biochemical activities (growth and multiplication) using raw materials obtained from the environment. The biochemical transformations that occur inside and outside the bacteria are governed by enzymes. The tests in this section are of general usefulness in the characterization and identification of bacteria.

3.2.3.1 Catalase Test

The catalase test was very useful in differentiating between groups of bacteria. The purpose of catalase test was to find out if the microbe had catalase protective enzymes. Microorganisms growing on aerobically incubated plates possess the enzyme catalase as reported by Whitten bury (1964).

To test for catalase, a slant was set in an inclined position and several drops of 3% solution of H_2O_2 on the growth on the slant were pipetted. The appearance of bubbles indicated a positive test while the absence of the bubbles indicated a negative test. The effervescences caused by liberation of free oxygen as gas bubbles were an indication of presence of catalase enzyme in the culture under test. Catalase breaks down H_2O_2 into water and oxygen and oxygen gas was detected as bubbles (Cappuccino & Sherman, 2010).

3.2.3.2 Nitrate Reduction Test

Nitrate peptone water was the medium used in this test, it consisted of peptone water with the addition of 0.002 – 0.2 % potassium nitrate analytical reagent grade (Payne, 1973) .The medium was distributed in tubes each with an inverted Durham tube and sterilized by autoclaving of 15 min at 121^{O}C. This was inoculated for broth culture and incubated together with a sterile control tube at the optimum growth temperature for 2-7 days.

One millimeter of each reagent was added to culture and to the control tube. The presence of nitrite was indicated by the development of a red color within few minutes. The control tube showed little or no coloration. A negative result was confirmed by the addition of a small quantity of zinc dust to the tube. This reduced to nitrite and nitrate was still present. The development of a red color indicated that some nitrate remained. If the addition of zinc did not result in the development of color, therefore no nitrate remained, since the nitrate had been reduced by the culture beyond the nitrite stage. The presence of gas in Durham tube indicated the formation of gaseous nitrogen. This microbe can reduce nitrate to nitrite.

3.2.3.3 Voges – Proskauer Test

This is a test for the production of acetylemethylcabinol from glucose. An alkali was added to the inoculated medium after incubation .Any acetylmethylcarbinol present became oxidized to dicecetyl. The diacetyl combined with arginine, creatine to give a rose colouration and the medium used was glucose phosphate broth. After inoculation, incubation was done at the optimum growth temperature for 2-7 days. The development of a red colouration constituted a positive reaction. Test reagents used included O'Meara's modification (1931) and Barritt's modification (1936), which was added to the inoculated medium after incubation. Barritt's reagent consisted of a mixture of alcoholic α-naphthol and 40% potassium hydroxide solution.

The test was performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth which had been inoculated with bacteria. A cherry red color would indicate a positive result, while a yellow-brown color indicated a negative result (Barry & Feeney, 1967).

3.2.3.4 Indole-Production Test

Peptone water was dispensed in 5 millilitre amounts in test tubes and sterilized by auto clarity for 15 min at 121°C. Inoculation was then done as for broth culture, young agar slope cultured and this was incubated at optimum growth for 2-7 days. Kavocs indole reagent was then added, the tube shaken gently and allowed to stand. A deep red colour developed in the presence of indole which separated out the alcohol layer. Cultures that produced a red colouration with Kovacs reagent were Indole positive (MacFaddin & Jean, 2013).

3.2.3.5 Growth on Simmon's Citrate Agar

This medium contained bromthymol blue as a pH indicator, and agar. It was leveled as slopes with a 1-inch butt. The slope culture was inoculated by streaking over the surface with a loop full of peptone water culture. This was incubated at the optimum temperature for a period of up to 7 days. There was a colour change from green to bright blue. When no growth occurred and citrate was not utilized, the colour of the medium remained unchanged (Cappuccino & Sherman, 2010).

3.2.3.6 Sugar Fermentation

Some organisms are capable of fermenting sugars such as glucose and manitol. During fermentation substrates such as carbohydrates were meant to undergo anaerobic dissimilation and produce organic acids in sugar that changes the culture to yellow at slightly acidic pH after incubation. Carbohydrates that have been fermented with the production of acid waste will cause phenol red to turn yellow indicating a positive reaction. The culture that was not capable of fermenting carbohydrate substrate did not turn indicator and the tubes appeared red indicating negative reaction.

3.2.3.7 Motility Hydrolysis with Starch and Casein

a) **Starch Hydrolysis Test**

Motility was determined using the semi-solid motility test medium. For the non- motile organisms, growth occurred along the line of inoculation and was very sharp and defined. Motile organisms swim away from the slant line and growth occurred throughout the tube rather than being concentrated along the line of inoculation. Dyes which change color as the

bacteria grow could make the rest easier to read non-motile organisms especially on addition of casein. When casein was added, clear zones which were visible after incubation of the plates were presumptive evidence of motile organisms (Wilson & Miles, 1975).

b) Casein Hydrolysis Test

Milk agar which consisted agar with addition of skimmed milk to 10% was sterilized by autoclaving for 20 minutes at 115°C. This was cooled before pouring to the plates. Inoculation was done to the poured dried plate of the medium by streaking once across the surface and incubated for growth at 37°C for 48 hrs. Clear zones which were visible after incubation of the plates were evidence of casein hydrolysis (Smith *et al.,* 1991).

3.3 Isolation of Bacteriocin-producing *Bacillus* Species

Antimicrobial activity of the twenty seven (27) isolated *Bacillus* strains was tested by a well diffusion method. Wells (10 mm of diameter) in nutrient agar was incubated with *Bacillus* strains for 24 hours. The plates were then separately overlaid with a solution of indicator strains; *staphylococcus aureus* (ATCC – 25923) and *Escherichia coli* (ATCC- 25922) by mixing 50µl of strain (24 hours culture on TSB broth at a concentration of 108 cfu/millilitre) with 200 mililitres of Mueller Hinton Agar (Oxford, Hampshire, UK).

After the overlays solidified, the plates were incubated for twenty four hours and then examined for a zone of inhibition around the well. The zone of inhibition was expressed in millimeters. The bacterial isolate showing the widest zone of inhibition against the target indicator organism was selected for further identification by the Analytical profile index system (API KIT).

3.4 Bacterial Identification using the Analytical Profile Index System (API KIT)

The elongated flap of the incubation tray containing micro tubes was labeled. The capsules were filled with the prepared bacterial suspension. API 50CH B/E medium was used in the suspension. The suspension was made in the medium for the microorganisms to be tested and each of the tubes was inoculated with the microorganism to be tested and then incubated. During incubation the carbohydrates fermented in acids which produced a decrease in pH. This was seen by the change in the color of the indicator. The suspension of the API 50 CH B/E medium was prepared by transferring twice the number of drops of the suspension into the ampule. This was then homogenized and incubated for 30^{o} C for 24 hrs. and then 48 hrs.

A positive test corresponds to acidification released by phenol red indicator contained in the medium changing yellow. The biochemical profile was obtained for the strain after the final reading was identified using the apiweb^{TM} identification software with the database (14.0) using manufacturer's instructions. The unknown microorganisms were identified by profile recognition system (Cappuccino & Sherman, 2010).

3.5 Preparation of Crude Bacteriocins

The strain(s) which were selected as potential bacteriocins producers were grown in nutrient broth at 30°C for 24 hours. After incubation, the broth was centrifuged at 5000 rpm for 10 minutes to separate the cells and extract the bacteriocin. The culture was adjusted to pH 7.0 by means of 1M NaOH to exclude the antimicrobial effect of organic acid and the supernatant was dialyzed for 24 hours at 4°C.

Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/millilitre catalase (C-100 bovineliver, Sigma). Neutralized filtrates were sterilized by filtration and then tested for antimicrobial activity against the indicator organism using the agar well

diffusion method as previously described. In order to test the sensitivity of CFS inhibitory substances to proteinase k and lysozyme (Eurobio, France), CFS was incubated for 24 hours with enzymes (1 mg/millilitre final concentrations) in a 10 µm potassium phosphate buffer pH 7 at 37°C. Supernatant was used as a crude bacteriocin. The cell free supernatant (CFS) was maintained at pH 7.0. Bacteriocin activity in the supernatant was tested by agar well diffusion assay (Geis *et al*., 1983) and then further subjected to ammonium sulphate precipitation.

3.6 Ammonium Sulphate Precipitation

The cell-free supernatant was used as starting material for protein precipitation. Ammonium sulphate was gradually added to a final concentration (w/v) for 40%, 60%, 70% and 80% respectively. After stirring on a magnetic stirrer, it was kept undisturbed at 4°C overnight. Precipitates formed were collected by centrifugation at 10,000 xg for 10 min and redissolved in 20 milliliter potassium phosphate buffer with pH 6.0. The precipitates were collected at every step by centrifugation at 6,000 rpm for 45 min and redissolved in 20 milliliters of nutrient broth (pH 7.0) and assayed for maximal bacteriocin activity (Harris, 1989) and aliquots stored for physiochemical characteristic tests. Inhibition zone of different fractions was recorded in comparison with the crude bacteriocin.

3.7 Detection of Inhibitory Activity of Crude Bacteriocins against Mastitis Pathogens

Cell free culture supernatants obtained from the bacteriocin producers by centrifugation of cultures at 5,000 rpm at 4°C for 10 min were adjusted to pH 6 with IN NaOH and stored at -20°C until use. The antimicrobial activity of the supernatant was determined by the well diffusion method. 100 µl of the supernatant were placed in wells (8 millimeters in diameter) cut in Luria Bertani (LB) agar plates (20 milliliter) seeded (1% v/v) with the following bovine mastitis pathogens; Gram positive (*Staphylococcus aureu*) and Gram-negative (*Escherichia coli)* organisms. These verified strains were obtained from the Central

veterinary laboratories in Kabete, Kenya and had been isolated from mastitis cases. The plates were incubated at 37°C for 24 hours after which the diameters (in mm) of the zones of growth inhibition were then measured.

3.8 Physiochemical Characterization of Bacteriocins from *Bacillus* Species

3.8.1 Effect of Temperature

A volume of 5 milliliters of bacteriocin in different test tubes were overlaid with paraffin oil to prevent evaporation and then heated for 15 minutes at 60°C, 70°C, 80°C, 100°C, and at 121°C for 15 minutes under pressure. Residual bacteriocin activity was evaluated against indicator bacteria and pathogens at each of these temperatures by agar – well diffusion assay as described earlier (Adinarayana *et al.,* 2003). The growth inhibition zone in diameter was measured by using clear meter rule and the percent inhibition calculated based on the initial zone of activity before exposing to different temperatures.

3.8.2 Effect of pH on Crude Bacteriocin Activity

According to the method described by Karaoglu *et al.,* (2003), sensitivity of partially purified bacteriocin 5 milliliter preparation to different pH values was tested by adjusting the pH of the bacteriocin in the range of pH 3 to 9 with sterile IN NaOH and IN HCl. After 2 hours of incubation at room temperature, residual activity of each of the samples was determined against the indicator organism by agar-well diffusion assay. The growth inhibition zone in diameter was measured by using clear meter rule and the percent inhibition calculated based on the initial zone of activity of the same ilsolates that had inhibition before adjusting the pH.

3.8.3 Effect of Proteolytic and Lipolytic Enzymes on Crude Bacteriocin Activity

A 5millilitre aliquot of bacteriocin preparation was taken in test tubes and treated with lipase (Bacterial source), proteinase K (Fungal source) and trypsin (Animal source) (Sigma) each at a final concentration of 1mg/millilitre. The test tubes with and without the enzyme (control) were incubated for 2 hrs. at 37^{o}C and heated for 3 min at 100^{o}C to denature the enzyme and residual activity of bacteriocin for both the control and the samples were

assayed as described by Nakamura *et al*. (1983). The growth inhibition zone in diameter was measured by using clear meter rule and the percent inhibition calculated based on the initial zone of activity before exposing the crude bacteriocins to different enzymes of equal concentrations.

3.8.4 Effect of Metal Ions on Crude Bacteriocin Activity

The effect of metal salts on bacteriocin activity was examined by addition 100 µl of 2mm, $CuSO_4$, $FeSO_4$, and $ZnSO_4$ (Merck) to 100 µl of partially purified bacteriocin preparation (1 mM final concentration). Untreated bacteriocin preparation (positive control). All samples were incubated at room temperature for 2hrs and then tested for residual antimicrobial activity (Adinarayana *et al.,* 2003) by agar-well diffusion assay. The growth inhibition zone diameter was measured by using clear meter rule and the percent inhibition calculated based on the initial zone of bacteriocin activity before addition of the metal ions of equal concentrations.

3.9 Data Analysis

Means and standard deviations were calculated from independent replicate tests and subjected to analysis of variance using SPSS statistical package (Statsoft, 1995).

Statistical analysis of data was represented by the means ofstandard deviation.

CHAPTER FOUR

RESULTS

4.1 Morphological characterization of bacteria isolated from *R. argentea*

A total of 300 *Rastrineobola argentea* samples were obtained from the field, cultured in mannitol-egg yolk polymyxin agar and incubated. Colonies that were able to grow within 2-5 days of incubation at 37ºC were considered. All plates had growth and colonies with distinct morphology from a single plate were transferred to a nutrient agar plate for purification. Morphological characterization was based on classical macroscopic techniques of color, margin, and elevation of pure colonies. The colonies were found to be either pink or yellow in colour (Appendix 1). A total of 90 distinct isolates were obtained. The general appearance of colonies was as shown in the table in Appendix 2.

MYP Agar Base contained meat extract and peptones that supply nitrogen, vitamins and minerals. Mannitol was the carbohydrate source and phenol red the pH indicator. Microorganisms that were able to ferment Mannitol which produced acid that lowered the pH of the media resulted in yellow colonies. This is characteristic of some *Bacillus* species such as *Bacillus subtilis*. Production of pink colonies meant that the bacteria did not ferment Mannitol and so were Mannitol-negative (pink colonies).

The form, elevation, and margin of the colonies as indicated in Appendix 2, showed that some colonies were smooth, rough, raised, flat, undulate or entire. Microscopy after Gram staining revealed that 90 (91%) of the isolates were rods while 8 (9%) were cocci arranged as a chains or cluster. All cocci isolates were Gram negative and were discarded. The rods were Gram positive. Terminal spores were present in 82 (91%) of the rods while the rest had no spores. The cells ranged from shot rods to long rods.

4.2 Biochemical Tests

The ninety (90) Gram positive rod isolates presumptive of *Bacillus* species were used in the biochemical tests. The biochemical tests included catalase test, Nitrate reduction, Starch hydrolysis, Casein hydrolysis, Voges Proskauer, Growth on Simmon Citrate, indole production and fermentation of glucose sugar and mannitol (Table 1). None of the isolates was positive for all the biochemical tests. Ninety (90) pure isolates that could utilize most of the substrates used were selected for characterization. Among these, 27 isolates exhibited antimicrobial activity against the test organisms used and these were investigated further by the API Kit.

Table 1: Percentage positive and negative isolates on different biochemical tests

TEST	REACTION (n= 90)	
	Positive (%)	Negative (%)
Catalase	98	2
Nitrate Reduction	43	57
Voges Proskauer	5	95
Indole Production	52	48
Growth on Simmon's Citrate	80	20
Glucose Fermentation	58	42
Mannitol Fermentation	74	26
Starch Hydrolysis	87	13
Casein Hydrolysis	72	28

4.3 Screening of isolates for antimicrobial activity

The 90 isolates were then tested for their antagonistic activity against test bacteria. They were used to check on their ability to inhibit growth of standard microorganisms; *Escherichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923 using Agar diffusion. The zone of inhibition on inoculated plates was observed and measured. Out of the 300 samples, 90 isolates that were presumptive to be Bacillus, twenty seven (27) isolates had antimicrobial activity with an extraction rate of 27%. Only 25(28%) and 18 (20%) of the isolates could inhibit the growth of *Escherichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923 respectively.

Table 2: Antimicrobial activity of *Bacillus* isolates on *E.coli* ATCC-25922 and Staphylococcus *aureus* ATCC 25923 as measured by means ±sd of zones of inhibition

Isolate serial No.	Zone of Inhibition on Microorganism	
	Zone on *E. coli*	Zone on *Staphylococcus aureus*
$S10_3$	24mm	22mm
$S40_1$	24mm	25mm
$S34_1$	24mm	26mm
$S60_1$	23mm	26mm
	22mm	26mm
$S52_2$	22mm	None
$S62_2$	20mm	None
$S62_3$	22mm	20mm
$S58_1$	20mm	24mm
$S54_1$	19mm	None
S13	19mm	None
$S53_1$	18mm	24mm
S02	18mm	18mm
$S35_1$	18mm	29mm
$S24_1$	17mm	26mm
$S06_3$	16mm	24mm
$S23_1$	15mm	24mm
$S44_2$	14mm	11mm
$S04_1$	13mm	17mm
$S09_2$	12mm	None
$S22_2$	12mm	None
$SO8_1$	12mm	None
$S03_1$	10mm	None
$S46_1$	None	25mm
$S37_1$	None	15mm
$S16_1$	11mm	None
$S60_2$	24mm	24mm

The standard deviation = 0.5

Most of the isolates (59.26%) that showed antimicrobial effect could inhibit both *E. coli* and *Staphylococcus aureus.* A small percentage (7.41%) was only effective against *Staphylococcus aureus* but not against *E. coli* while 33.33% could inhibit growth of *E. coli* but not *Staphylococcus aureus*. Antimicrobial activity against *E.coli* ATCC-25922 and

Staphylococcus aureus ATCC 25923 were further identified using API 50 CH kits to give *Bacillus mycoides, subtilis, pumilus* and *lentus* as the most predominant species (Figure 1).

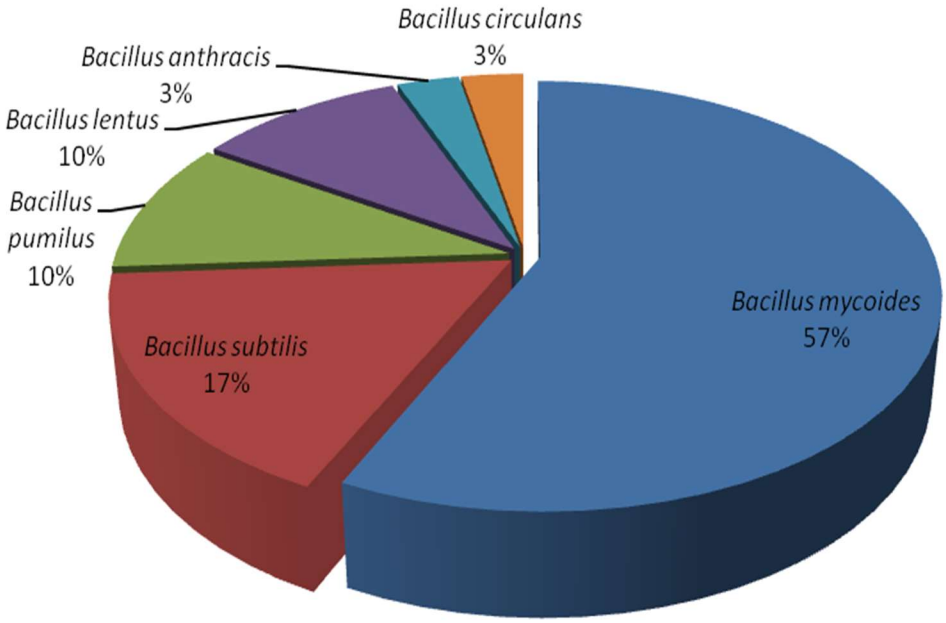


Figure 1: Isolates with antimicrobial activity after identification with API Kit

Fourteen (14, 57%) Isolates were identified as *Bacillus mycoides;* five (5, 17%) as *Bacillus subtilis*; three (3, 10%) as *Bacillus pumilus*; three (3, 10%) as *Bacillus lentus;* one (1, 3%) *Bacillus anthracis* and one (1, 3%) $S54_1$ was identified as *Bacillus circulans.*

Bacillus anthracis was causiouly handed in a hood a mmeditedy autoclaved at 121^{O} C for 15minutes and discarded, since it is dangerous to man and even animals

The supernatants from the twenty seven (27 isolates that showed antimicrobial effect were also tested against the same standard microorganisms. It was noted that not all supernatants had antimicrobial effect. Only 8 (37.04%) of the supernatant obtained that showed antimicrobial effect against the standard micro-organism. They were effective against both *Eshirichia coli* and *Staphylococcus aureus.* The zones of inhibition were taken as the antimicrobial effect of the crude bacteriocin from the *Bacillus* species (Table 2).

Table 3: Zone of inhibition of supernatant (crude bacteriocin) obtained from different *Bacillus* Isolates on *E. coli* ATCC-25922 and *S. aureus* ATCC 25923

***Bacillus* species**	**Isolate**	***Eschorihia. coli***	***Staphylococcus. aureus***
Bacillus subtilis	1. $S52_2$	24mm	None
	2. $S62_2$	22mm	None
	3. $S16_1$	13mm	None
	4. $S10_3$	25mm	24mm
	5. $S58_1$	22mm	25mm
Bacillus pumilus	1. $S23_1$	16mm	26mm
	2. $S44_2$	15mm	13mm
	3. $S46_1$	None	26mm

The bacteriocins were therefore from *Bacillus subtilis* (five, 62.5%) and *Bacillus pumilus* (three, 37.5%) as identified by the API 50 CH KIT (Table 3). The average increase of the zone of inhibition was 1.8mm and 1.5mm for *Bacillus subtilis* against *E. coli* and *Staphylococcus aureus* respectively after extraction of the crude bacteriocin. Inhibition from *Bacillus pumilus* crude bacteriocin increased by an average of 1mm and 1.67mm against *E. coli* and *Staphylococcus aureus* respectively when compared with inhibition by the isolates.

4.4 Characterization of the Crude Bacteriocin

The bacteriocin was characterized in respect to its activity at different environments. These include their ability to inhibit growth of *Eschirichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923 at a range of pH (Table 4 and 5), after exposure to different temperatures (Plate 2) or enzymes (Table 8 and 9) and in presence of different metal ions (Table 6 and 7).

Table 4: Microorganism identified using the API Kit that showed antimicrobial activity

Isolate	**Microorganism**	**Percentage of Microorganism in Isolate as identified**

		using API Kit (%)
S522	*Bacillus subtilis*	99.3
S461	*Bacillus pumulus*	99.6
S231	*Bacillus pumilus*	58.4
S442	*Bacillus pumilus*	99.9
S161	*Bacillus subtilis*	99.6
S541	*Bacillus subtilis*	42
S103	*Bacillus subtilis*	57.5
S581	*Bacillus subtilis*	99.8

The percentages indicated were in terms of identification by the API kit.

4.4.1 Effect of pH to crude bacteriocins activity

The residual activity of the crude bacteriocin at different pH is shown in Table 4 and 5. The zone of inhibition obtained is as shown in figure 2.

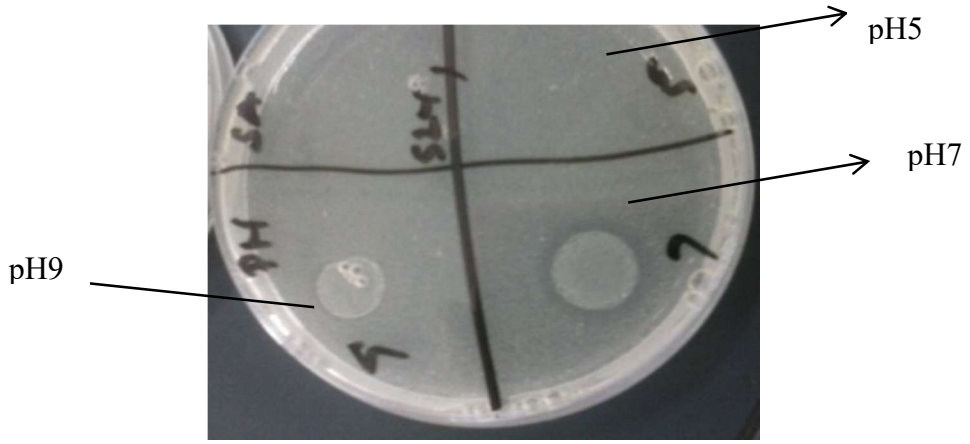


Figure 2: Inhibition of crude bacteriocin on *Staphylococcus aureus* ATCC 25923 after exposure to pH 5, 7 and 9.

Table 5: Residual activity of bacteriocin on *E. coli* ATCC-25922 at different pH

Source of Bacteriocin		**pH of Media**				
***Bacillus* species**	**Isolate**	**pH 3**	**pH 4**	**pH 5**	**pH 7**	**pH 9**
Bacillus subtilis	$S52_2$	-	-	-	+	+
	$S62_2$	-	-	-	+	+
	$S16_1$	-	-	-	+	+
	$S10_3$	-	-	-	+	+
	$S58_1$	-	-	-	+	+
Bacillus pumilus	$S23_1$	-	-	-	+	+
	$S44_2$	-	-	-	+	+

Key: - No zone of inhibition; + Zone of inhibition present (Mean=19.57mm, range=11-25mm the standard deviation from the means =0.5.)

Table 6: Residual antimicrobial activity of crude bacteriocins on *Staphylococcus aureus* ATCC 25923 at different pH

Source of crude bacteriocin		**pH of Media**				
***Bacillus* species**	**Isolate**	**pH 3**	**pH 4**	**pH 5**	**pH 7**	**pH 9**
Bacillus subtilis	$S10_3$	-	-	-	+	+
	$S58_1$	-	-	-	+	+
Bacillus pumilus	$S23_1$	-	-	-	+	+

$S44_2$	-	-	-	+	+
$S46_1$	-	-	-	+	+

Key: - No zone of inhibition; + Zone of inhibition present (Mean=19.57mm, range=11-25mm, standard deviation from the means =0.5).

There was a slight decrease in the zone of inhibition by an average of 2mm at pH 9 as compared to what was exhibited by crude bacteriocin before pH adjustments from pH 7. The activity of the bacteriocins against both *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 was completely lost at acidic environment of pH 5 and below.

4.4.2 Effect of Different Temperatures on Crude Bacteriocin Activity

A bar graph showing the extent to which the bacteriocin lost their activity is shown in Figure 3. Shows the zone of inhibition by the crude bacteriocin on the indicator organisms after heat treatment at different temperatures. Temperature of up to 80ºC did not cause a reduction in the bacteriocin activity. After exposure to 100ºC, about 40% of the bacteriocin activity was lost. However, at 121ºC, more than 50% of the activity was lost.

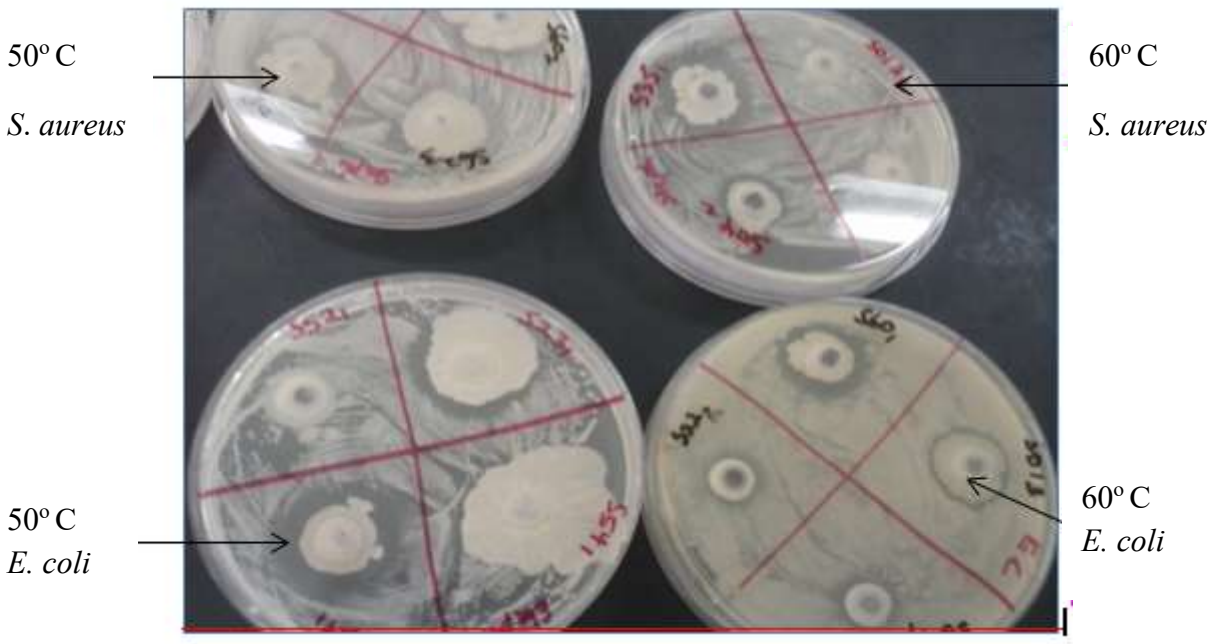


Figure 3: Zone of inhibition by crude bacteriocins on E. coli ATCC 25922 and S. *aureus* ATCC 25923 after heat treatment at different temperatures

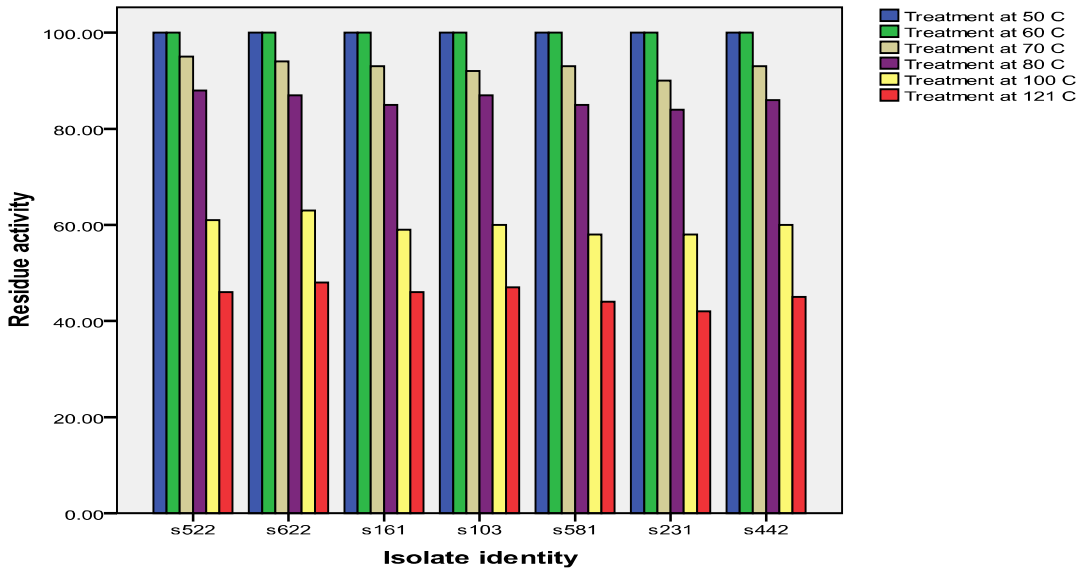


Figure 2: Effects of crude bacteriocins activity after exposure to different temperatures.

4.4.3 Effect of Different Metal Ions on Bacteriocins Activity.

Metal ions at the concentration of 1mM were added to a partially purified bacteriocin. All the metal ions did not have a great reduction in the activity of bacteriocins (Table 6 and 7). The residual activity was determined as a percentage ratio of the initial bacteriocin activity.

Source of bacteriocin		**Metal ion**		
***Bacillus* species**	**Isolate**	**Cu^{2+}**	**Fe^{2+}**	**Zn^{2+}**
Bacillus subtilis	$S52_2$	+++	++	+
	$S62_2$	+++	++	++
	$S16_1$	+++	++	+
	$S10_3$	+++	++	+
	$S58_1$	+++	++	+
Bacillus pumilus	$S23_1$	+++	++	++
	$S44_2$	+++	++	+

Key: +++ Residual activity between 91-100%; ++ Residual activity between 81- 90%; + Residual activity between 71- 80%

The residual activity was a measure of percentage of the zone diameter in millimeters based on the initial zone of activity.

Table 7: Effect of metal ions on bacteriocin activity against *Staphylococcus aureus* ATCC-25923

Source of bacteriocin		Metal ion		
Bacillus species	Isolate	Cu^{2+}	Fe^{2+}	Zn^{2+}
Bacillus subtilis	$S10_3$	+++	++	+
	$S58_1$	+++	++	+
Bacillus pumilus	$S23_1$	+++	++	++
	$S44_2$	+++	++	+
	$S46_1$	+++	++	+

Key: Scoring of level of activity with +++ Residual activity between 91-100%; ++ Residual activity between 81- 90%; + Residual activity between 71- 80%

The residual activity was a measure of percentage of the diameter of the zone in millimeters based on the initial zone of activity.

4.4.4 Effect of Enzymes on Crude Bacteriocins Activity

The bacteriocins were exposed to two proteolytic and one lipolytic enzyme. The bacteriocin portions exposed to Proteokinase K did not show any zone of inhibition while those exposed to trypsin lost 40% of their activity. Lipase caused 15% loss of the bacteriocin activity (Table 8 and 9)

Table 8: Effect of enzymes on bacteriocin activity against *E. coli* ATCC-25922

Source of bacteriocin		Enzyme		
Bacillus species	Isolate	Proteokinase K	Trypsin	Lipase
Bacillus subtilis	$S52_2$	-	+	++
	$S62_2$	-	+	++
	$S16_1$	-	+	++
	$S10_3$	-	+	++
	$S58_1$	-	+	++
Bacillus pumilus	$S23_1$	-	+	++
	$S44_2$	-	+	++

Key: - No inhibition; + residual activity (Mean=up to 60%); ++ residual activity (Mean ≥ 80%) based on the percentage of initial activity measured as the diameter of the zone in millimeters.

Table 9: Effect of enzymes on bacteriocin activity against *Staphylococcus aureus* ATCC 25923

Source of bacteriocin		Enzyme		
Bacillus species	Isolate	Proteokinase K	Trypsin	Lipase
Bacillus subtilis	$S10_3$	-	+	++
	$S58_1$	-	+	++
Bacillus pumilus	$S23_1$	-	+	++
	$S44_2$	-	+	++
	$S46_1$	-	+	++

Key: - No inhibition; + residual activity (Mean=up to 60%); ++ residual activity (Mean ≥ 80%) based on the percentage of initial activity measured as the diameter of the zone in millimeters.

CHAPTER FIVE

DISCUSSION

5.1 Omena as a Source of *Bacilllus*

There was growth in all after heat treatment to eliminate vegetative cells. *Bacillus* are known to form endospores that are resistant to heat (Abriuoel *et al*., 2011) and therefore can germinate when cultured in growth media even after heat treatment. The growth was therefore suspected to be from *Bacillus* spores that germinated after incubation at 37^0C.

Bacillus are found in such diverse environments because of their ability to produce endospores, their wide physiological properties and growth requirements (Abriouel *et al*., 2011).They are known to be heterotrophic, thermophiles, acidophiles or alkalophiles (Priest, 1993). They have been isolated from Chinese herbs (Xie *et al.,* 2009), sea water (Jayaraman *et al.*, 2012), milk (Mohankumar & Murugalatha, 2011) and other environments (Nicholson, 2002). The *omena* once harvested are preserved by sun drying on the ground, sometimes under direct contact with the soil. They are transported in gunny bags or boxes which could be of various hygiene conditions. At the open air market, they are placed on bags and sold in tins whose bacteriological quality is unknown. The initial source of contamination of the fish with *Bacillus* is therefore not established.

5.2 Isolation and Identification of Bacteria from *Rastrineobola argentea*

Bacillus species are mainly described as Gram positive aerobic or facultative, spore forming rods according to Bergey's Manual of Determinative Bacteriology. Some are Gram negative but they appear positive due to presence of the spore at the capsule (Claus & Berkeley, 1986). Smith *et al.* (1952) has shown that *Bacillus* exhibit varied morphological characteristics and thus these observations alone are not confirmatory and so other tests

needed to be carried out. Endospores presented as swollen capsule may or may not be present (Abriouel *et al.*, 2010).

Microorganisms that were able to ferment Mannitol which produced acid and lowered the pH of the media resulted in yellow colonies. This is characteristic of some *Bacillus* species such as *Basillus subtilis*. Production of pink colonies meant that the bacteria did not ferment Mannito and so were Mannitol-negative (pink colonies).

The *Bacilli* are known to be catalase positive. They can also hydrolyze several sugars (Priest, 1993). The spore morphology and biochemical tests can help classify the Bacillus genus into several groups. These are, *Bacillus polymyxa* group (I), *Bacillus subtilis* group (II), *Bacillus brevis* group (III), *Bacillus sphaericus* group (IV), and thermophiles (V and VI) (Deak and Timar, 1988). All these bacteria produce acids from a range of sugars. Some in this group are facultative anaerobes but *Bacillus subtilis* is generally regarded as an aerobe. However, it can grow and sporulate slowly under strict anaerobic conditions in presence of glucose and nitrite as a terminal electron acceptor (Priest, 1993). Three characteristics distinguish *Bacillus pumilus* from *Bacillus subtilis*: starch hydrolysis, nitrate reduction, and a biotin requirement. *Bacillus subtilis* can hydrolysis starch, reduce nitrate to nitrite and does not require biotin during growth unlike *B. pumilus* (Lovett & Young, 1969).

The identification of the isolates with antimicrobial effect with the API KIT and online apiweb^{TM} identification software gave six *Bacillus* species.

The profile recognition system has been used successfully for identification of unknown microorganisms (Cappuccino & Sherman, 2010). Various species have been found to be most prevalent in different environments. According to Parvathi *et al.* (2009), *Bacillus pumilus*, *cereus* and *sphaericus* were most numerous species in that order in the marine environment they researched in. *Bacillus cereus, licheniformis, pumilus* and *subtilis* are prevalent in soils, particularly low-nutrient soils (Priest, 1993). Deak and Timar (1988)

noted that the most aerobic spore-formers from foods were *B. subtilis, pumilus* and *licheniformis*.

5.3 Screening for Crude Bacteriocins

Supernatant with antimicrobial activity against *E. coli* and *Staphylococcus aureus* was obtained from only 33% of the *Bacillus* species identified, that is, *Bacillus subtilis* and *pumilus*. Supernatant from *Bacillus mycoides*, *lentus*, *anthrancis* and *circulans* did not show any inhibitory activity against any of the test microorganisms in this research. Bacteriocins from the latter four *Bacillus* species have been identified in work from other researchers. A *Bacillus mycoides* strain isolated from whey was shown to produce a BLIS active against food-borne pathogens such as *L. monocytogenes* and *L. mesenteroides* (Sharma & Gautam, 2008). Sharma *et al.* (2006) reported a BLIS active against *L. monocytogens* and *Staphylococcus aureus* produced by a food-grade *B. lentus* NG121 strain isolated from *Khameera*, a traditional fermented food from Himachal Pradesh (India). *B. circulans* strain NRRL B-30644 produced different bacteriocins with anti-*Campylobacter* activity (Svetoch *et al.*, 2005). However, heterocycloanthracins which are putative peptides mainly from *Bacillus anthracis* and *cereus* were not shown to have any antimicrobial (Haft, 2009).

5.4 Crude Bacteriocin Activity on Bovine Mastitis Pathogens

Most of the isolates found with antimicrobial effect were against both *E. coli* and *Staphylococcus aureus* and were 16 (59.26%) while 2 (7.41%) could not inhibit growth of *E. coli. Staphylococcus aureus* could not be inhibited by 9(33.33%) isolates. This indicates that *Staphylococcus aureus* was *more* resistant to the inhibition by the isolates as compared to *E. coli.* The former is Gram positive and the latter is Gram negative. Gram negative bacteria are known to be highly resistant to existing treatments (Drider & Rebuffat, 2011). The ability of some isolates to inhibit both microorganisms showed the possibility of their metabolites to be used for mastitis. Xie *et al.* (2009) found *Bacillus subtilis* strain LFB112 to have inhibitory activity against most pathogenic strains, including some important animal

pathogens and two multidrug resistant clinical isolates of *Escherichia coli* and *Salmonella pullorum*, as well as against a yeast strain.

Bacteriocins from some *Bacillus* species have shown wide inhibitory spectrum against both Gram negative and Gram positive bacteria (Sharma *et al.*, 2011; Bizani & Brandelli, 2002; Cherif *et al.,* 2003). However, some bacteriocins have shown a narrow inhibitory spectrum against either some Gram positive or Gram negative bacteria (Abriouel *et al.*, 2011; Lee *et al*., 2001). The bacteriocin in this research shows its application to both Gram positive and negative bacteria. This is useful in treating mastitis which is caused by both Gram positive and negative bacteria.

Bacteriocins from *Bacillus subtilis* that have been identified include rhizocticin, surfactin, mycosubtilin, betacin and other uncharacterized antimicrobial agents. They have been found to be effective against bacteria and fungi (Abriouel *et al.,* 2011).

Bacillus subtilis LFB112 from Chinese herbs produces a BLIS active against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases, including *E. coli, Salmonella Pullorum*, *P. aeruginosa, Pasteurella multocida, C. perfringens, M. luteus, Streptococcus bovis* and *Staphylococcus. aureus* (Xie *et al.*, 2009). Several BLIS from *Bacillus thuringiensis* showed antimicrobial activity against a collection of *Staphylococcus aureus* isolates from dairy sources. The best results were reported for morricin 269, followed by kurstacin 287 *Bacillus pumilus* (Barboza-Corona *et al.*, 2007). These results which are similar to what was obtained in this research show a great potential in bacteriocins from *Bacillus* in use for treating mastitis.

This antimicrobial activity was against sensitive indicator bacteria *L. monocytogenes, L. mesenteroides* and *L. plantarum* where it increased by 200%, 333% and 175% respectively (Sharma *et al.,* 2011). The bacteriocins from the *Bacillus* species in this research show a similar trend. This is because Bacteriocin from the isolates identified as *Bacillus subtilis*

could inhibit the growth of *E. coli* and *Staphylococcus aureus*. Bacteriocin from all *Bacillus pumilus* isolates was effective against *Staphylococcus aureus* and showed a wide clearance zone.

5.5 Physiochemical Characterization of Crude Bacteriocin Produced by Isolates

The activity of the bacteriocin was highest at pH 7 and it decreased slightly at a slightly alkaline environment. Bacteriocins differ greatly with respect to their sensitivity to different pH range. A BLIS from *Bacillus mycoides* was stable over a wide pH range of 4–11 (Sharma & Gautam, 2008). Bacteriocin from Chinese herbs work by Xie *et al.* (2009) was stable at pH (3–10). The bacteriocins in this research work best at a range of pH 7 and pH 9.

Entomocin 110 obtained from *B. thuringiensis subsp. entomocidus* HD110 and entomocin 9 which retained 53% and 72% of their activity respectively even after autoclaving (Cherif *et al.,* 2008; Cherif *et al.*, 2003). The BLIS from *Bacillus mycoides* isolated by Sharma and Gautam (2008) was stable at 100 °C. Xie *et al*. (2009) reported the bacteriocin from Chinese herbs as heat stable.

The addition of copper ions at the concentration of 1mM to the bacteriocins did not reduce the latter inhibitory activity against the indicator microorganisms. Addition of iron ions at the same concentration caused a slight decrease of between 10-19% while Zinc ions caused a decrease by between 15-30%. Presence of trace elements in food would not significantly reduce the activity of the bacteriocin (Cappucino & Sherman, 2010). The presence of metal ions however was important to note since they could interact with the bacteriocin when used as part of ingredients or could interfere with absorption of some oral drugs used by the animals.

The antimicrobial activity of the crude bacteriocins in this work were affected by proteinase K. This was similar to *L. fermentum* RMM701, *S. bovis* RMM703 and *S. bovis* RMM902 (Joerger, 2003) which were isolated from raw milk samples from dairy farms in Chiang Mai province. They all produced bacteriocins against *Streptococcus dysgalactiae* DMST10953,

a bovine mastitis pathogen. These bacteriocins were also found to be inactivated by proteolytic enzymes such as proteinase K and subtilisin A, suggesting that the substances could be antimicrobial peptides.

5.6 Conclusions and Recommendations

5.6.1 Conclusions

Bacillus species were isolated and identified from Omena (*Rastrineobola argentea*). These included *Bacillus mycoides, subtilis, pumilus* and *lentus* as the most predominant species.

Among the species identified, *Bacillus subtilis* and *pumilus* were found to have antimicrobial activity against *E. coli* and *Staphylococcus aureus*.

The crude bacteriocins produced by the selected isolates were heat stable at 100^0C and can therefore withstand pasteurization temperatures without significant loss of activity. However, more than 50% of the crude bacteriocin activity was lost after exposure to 121^0C for 15 minutes.

The crude bacteriocins' activity was optimum at pH 7 and pH 9. They were sensitive to proteokinase K but resistant to trypsin and lipase. They worked in environment contaminated with 1mM copper or iron but their activity can be greatly reduced by zinc ions at the same concentration.

Stability in neutral pH range (up to pH 9), sensitivity to proteokinase K cleavage but resistance to trypsin and lipase, heat stability at physiological temperatures allows the crude bacteriocins from *Bacillus* extracted from Omena to be a competitive advantage of antimicrobials preparations.

5.6.2 Recommendations

This study recommends that:

1. The identified organisms should be investigated further for purification and molecular characterization of their bacteriocins.
2. Further studies be done on the ability of the bacteriocins to inhibit bovine mastitis pathogens in *vivo.*

REFERENCES

Abila, R.O. and Jansen, G.K. (1997). From the local to global markets: The fish export and fish meal industries of Lake Victoria-structure, strategies and socioeconomic impacts in Kenya. IUCN Eastern Africa Programme –socio-economic of Lake Victoria Fishers.

Abriouel, H., Franz, C.M.A.P., Omar, N.B. and Gálvez, A. (2011). Diversity and application of *Bacillus* bacteriocins. *FEMS Microbial Rev* 35, 201-232.

Adinarayana, K., Ellaiah, P., and Prasad, D.S. (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. AAPS *Pharm. Sci. Tech*., 4(4), 56.

Bansal, B. K., Hamann, J., Grabowski, T. N. and Singh, K. B. (2005). Variation in the composition of selected milk fraction samples from healthy and mastitic quarters, and its significance for mastitis diagnosis. *Journal of Dairy Research,* 72(2), 144–152.

Barboza-Corona, J. E., Vazquez-Acosta, H., Bideshi, D. K. and Salcedo-Hernandez, R. (2007). Bacteriocin-like inhibitor substances produced by Mexican strains of *Bacillus thuringiensis. Arch. Microbiol*. 187, 117-126.

Barrit, M. (1936). The intensification of the Voges-Proskauer reaction by the addition of a-naphthol. – *J. Path. Bact*. 42, 441-454.

Barry, A.L. and Feeney, K.L. (1967). Two Quick Methods for the Voges Proskauer Test. *Appl. Microbiol*. 15, 1138-1141.

Bingo, (2012). Relations between electrical conductivity, somatic cell count, California mastitis test and some quality parameters in the diagnosis of subclinical mastitis in dairy cows. *Turkish Journal of Veterinary & Animal Sciences*, 36(1), 49-55.

Bizaini, D., Dorminguez, A.P.M. and Brandelli, A. (2005). Purification and partial chemical characterization of the antimicrobial peptide cerein 8A. Lett. *Appl. Microbiol.* 41, 269-273.

Cappuccino, J. G. and Sherman, N. (2010). Microbiology: a Laboratory Manual, 6th edn. Menlo Park, CA: Benjamin/Cummings.

Cascales, E., Buchanan, S.K., Duche, D., Kleanthous, C., Lloubes, R., Postle, K., Riley, M., Slatin, S., and Cavard, D. (2007). Colicin biology. *Microbiology and molecular biology reviews: MMBR*, Vol.71, No.1, pp. 158-229, ISSN 1092-2172

Cherif, A.*et al*,. (2003). Detection and characterization of the novel bacteriocin entomocin 9, and safety evaluation of its producer, *Bacillus thuringiensis ssp. entomocidus* HD9. J. *Appl. Microb.* 95, 990-1000.

Cherif. A et al., (2008). Characterization and partial purification of entomocin 110, a newly identified bacteriocin from *Bacillus thuringiensis* subsp. *entomocidus* HD110. *Microbiol Res* 163, 684–692.

Christ, W. L., (2007). Mastitis and its control. Kentucky:University of Kentucky.

Claus, D. and Berkeley, R.C.W. (1986). Genus *Bacillus* in Bergeys manual of systematic bacteriology. Sneath, PH ed. The Williams and Wikins company Baltmore.

Cordovilla, P., Valdivia, E., Gonzalez-Segura, A., Galvez, A., Matinez-Bueno, M. and Maqueda, M. (1993). Antagonistic action of the bacterium *Bacillus licheniformis* M-4 toward the *amoeba Naegleria fowleri. J. Eukaryot Microbiol.* 40, 323-328.

Cotter, P. D., Hill, C. and Ross, R.P. (2006). "What's in a name? Class distinction for bacteriocins". *Nature Reviews Microbiology* 4 (2).

Craven, N. (1987). Efficacy and Financial value of antibiotic treatment of bovine clinical mastitis during lactation – a review. *Br. Vet. J.* 143, 412-422.

Deak. T. and Timar, E. (1988). Simplified identification of aerobic spore formers in the investigation of foods. *Int. J. Fd. Microbiol.,* 6,115-125.

Delves –Broughton, J. (1990). Nisin and its uses as food preservative. *Food Technol*.44, 100-112.

Diep, D.B. and Nes, I.F. (2002). Ribosomally synthesized antibacterial peptides in Gram positive bacteria. *Curr. Drug T*. 3, 107-122.

Drider, D. and S. Rebuffat. (2011). Bacteriocins from Gram-Negative Bacteria: A Classification? p. 55-72. Prokaryotic Antimicrobial Peptides. Springer New York.

Dube, S., Dlamini, N.R. and Muzungu, W. (2004). Level and types of aerobe spore forming bacteria associated with Matemba, (*Limnothrissa miodo*). *AJFAND 4* (2).

Dussault, H. P. (1955). An improved technique for staining red halophilic bacteria. *J Bacteriol* 70, 484–485.

Fox, L. (2012). Mycoplasma mastitis: causes, transmission, and control. The Veterinary Clinics of North America. *Food Animal Practice*, 28(2), 225-237.

Geis, A *et al*., (1983). Potential of lactic *streptococci* to produce bacteriocin. Appl. Environ. Microbiol., 45, 205-211

Giesecke, W. H., Du Preez, J.H. and Petzer, I.M. (1994). Practical mastitis control in dairy herds. Durban South Africa: Butterworth publisher..

Gillor, O., Nigro, L.M. and Riley, M.A. (2005). Genetically engineered bacteriocins and their potential as the next generation of antimicrobials. *Curr. Pharm. D*. 11,1067-1075.

Gordon. D.M., and O'Brien, C.L. (2006). Bacteriocin diversity and the frequency of multiple bacteriocin production in *Escherichia coli. Microbiology* 152, 3239–3244

Granum, P.E. (2007). *Bacillus cereus*. Food Microbiology, Fundamentals and Frontiers. 3rd Ed (Doyle MP & Beuchat LR, eds), pp. 445–455. Washington, DC: ASM Press.

Gruet, P., Maincent, P., Berthelot, X. and Kaltsatos, V. (2001). Bovine mastitis and intramammary drug delivery: review and perspectives. *Advanced Drug Delivery Reviews,* 50, 245–259.

Haft, D.H. (2009). A strain-variable bacteriocin in *Bacillus anthracis* and *Bacillus cereus* with repeated Cys-Xaa-Xaa motifs. *Biol Direct*, 4, 15.

Harris, E.L.V. (1989). Concentration of the extract: Protein Purification Methods. In: A Practical Approach. (Eds.): New York.E.L.V. Harris and S. Angal. IRL Press .

Heip J. *et al*., (1975). Purification of the *Agrobacterium radiobaeter* 84 Agrocin. *Arch. Int. Physiol. Biochem.*, 83, 974-976.

Hillerton, J.E. and Kliem, K.E. (2002). Effective treatment of clinical mastitis to minimize the use of antibiotics. *J Dairy Sci,* 85*,* 1009 – 1014.

Hogeveen, H. (2005). Mastitis is an economic problem. pp. 1–13, *in:* Proceedings of British Mastitis Conference (2005) Stoneleigh. Institute for Animal health/ The Dairy Group.

Hosoi,T.and Kiuchi,K. (2003). Natto – A food made by fermenting cooked soybeans with *Bacillus subtilis* (*natto*). In: Handbook of Fermented Functional Foods. Farnworth ER, editor. Boca Raton, Fla: CRC Press.

Iqbal, A., Ali, S.A., Ahmad S. and Rasool, S.A. (1999). Isolation and partial purification of Bac201: a plasmid associated bacteriocin like inhibitory substance from *Staphylococcus aureus* AB201. *J. Basic Microbiol*., 39, 325-336.

Jack, R.W., Tagg, J.R. and Ray, B. (1995). Bacteriocins of Gram-positive bacteria .*Microbiol .Rev*,59, 171-200.

Jay, J.M., Loessne,r M.J. and Golden, D.A. (2005). Modern Food Microbiology. 7th Ed. New York: Springer.

Jayaraman, G., Rajesh, D. and Karthikeyan, S. (2012). Isolation and partial characterization of a new bacteriocin from *Bacillus pumilus* DR2 isolated from sea water. CIB Tech. *Journal of Microbiology,* 133-41

Joerger, R. D. (2003). Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poult. Sci*. 82, 640-647.

Jones, G. M. (2006). *Understanding the basics of mastitis*. Virginia Cooperative Extension, Publication 404-233. 7 p. Virginia State University, USA.

Juanqiang, W., Songhua, H. and liting, C. (2007). Therapeutic Effects of Nisin Z on Subclinical Mastitis in Lactating Cows. Antimicrob. *Agents and Cheothe*r. 51, 3131-3135.

Karaoglu, A., F. Aydin., S. Kilic and A. Kilic. (2003). Antimicorbial activity and characteristics of bacteriocins produced by vaginal *lactobacilli*. *Turk J. Med Sci.,* 33, 7-13.

Keast, J.R., Furness, J.B., and Costa, M. (1984). Origins of peptide and norepinephrine nerves in the mucosa of the guinea pig small intestine. *Gastroenterology*, 86,637-644.

Kerr, A. and K. Htay. (1974). Biological control of crown gall through bacteriocin production. *Physio. Plant Pathol.,* 4, 37-43.

Kitchen, B.J. (1981). Review of the progress of dairy science - bovine mastitis - milk compositional changes and related diagnostic-tests. *Journal of Dairy Research,* 48(1),167–188.

Lam, T. M., Van Den Borne, B. P., Jansen, J. J., Huijps, K. K., Van Veersen, J. L., Van Schaik, G. G., and Hogeveen, H. H. (2013). Improving bovine udder health: A national mastitis control program in the Netherlands. *Journal of Dairy Science*, 96(2), 1301-1311.

Lee, K.H., Jun, K.D., Kim, W.S. and Paik, H.D. (2001). Partial characterization of polyfermenticin SCD, a newly identified bacteriocin of *Bacillus polyfermenticus*. *Letters in Applied Microbiology* 32, 146–151.

Lisboa, M.P., Bonatto, D., Bizani, D., Henriques, J. A. P. & Brandelli, A. (2006). Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazillian Antlatic Forest. *J. Int. Microbiol*., 9,111-118.

Lodemann, U., and Martens, H. (2008): Effects of *Bacillus cereus* var. *toyoi* as probiotic feed supplement on intestinal transport and barrier function in piglets. *Archives of Animal Nutrition* 62, 87–106.

Lovett, P.S. and Young, F.E. (1969). Identification of *Bacillus pumilus subtilis* NRRL B-3275 as a strain of *Bacillus pumils. J. Bacteriol*., 100(2), 658.

Lutz, B., Wiedemann, S. and Albrecht, C. (2006). Degradation of transgenic Cry1Ab DNA and protein in Bt-176 maize during the ensiling process. *J. Anim. Physiol. Anim. Nutr.* 90, 116-123.

MacFaddin and Jean, F. (2013). Biochemical Tests for Identification of Medical Bacteria. Williams & Wilkins, p 441.

Mohankumar, A. and Murugalatha, N. (2011). Characterization and Antibacterial Activity of Bacteriocin Producing *Lactobacillus* Isolated from Raw Cattle Milk Sample. *International Journal of Biology* 3,128-143.

Molloy, E.M., Hill, C. H., Cotter, P.D.C., and Ross, P. (2008). Bacteriocins. Encyclopedia Of Dairy Sciences 2Nd Edition, Four-Volume Set, 420-429.

Mossel, D.A., Koopman, M.J. and Jongerius, E. (1967). Enumeration of *Bacillus cereus* in foods. Appl Microbiol 15, 650–653.

Nakamura, T., N. Yamazaki., H. Taniguchi and S. Fujimura. (1983). Production, purification and properties of bacteriocin from *Staphylococcus aureus* isolated from saliva. *Inf. Imm.,* 39, 609-614.

Nicholson, W.L. (2002). Roles of *Bacillus* endospores in the environment. *Cell Mol Life Sci* 59,410-416

Ogola, H., Shitandi, A. and Nanua, J. (2007). Effect of mastitis on raw milk compositional quality. *Journal of Veterinary Science,* 8(3), 237–242.

Ogura, K., Matsumoto, T., Aoki, Y., Kitabatake, T., Fujisawa, M., and Kojima, K. (2010). IgG4-related tumour-forming mastitis with histological appearances of granulomatous lobular mastitis: comparison with other types of tumour-forming mastitis. *Histopathology*, 57(1), 39-45.

O'meara, R.A.Q. (1931). A simple, Delicated and Rapid Method of Detecting the Formation of Acetylmethyl-carbinol by Bacteria Fermenting Carbohydrate. *J. Pathol. Bact.* 34,401-406.

Omore, A., McDermott, J., Arimi, S., Kyule, M. and Ouma, D. (1996). A longitudinal study of milk somatic cell counts and bacterial culture from cows on smallholder dairy farms in Kiambu District, Kenya, Prev. *Vet. Med*. 29, 77-89.

Osmanagaoglu,O., Y. Beyati, and U. Gunduz. (2001). "Isolation and characterization of pediocin producing *Pediococcus pentosaceus* Pep1 from Turkish vacuum packaged sausages", *Tr. J. Biol.* , 25, 133-143.

Ostensvik, O. C., Heindenreich, B., Sullivan, O. and Granum, P.E. (2004). Cytotoxic *Bacillus* Species Belonging to the *B. Cereus* and *B. Subtilis* Groups in Norweigan Surface Waters. *J appl. Microbiol*. 96, 987.

Parvathi A., Krishna K, Jose J., Joseph N., and Nair S., (2009). Biochemical and molecular characterization of *Bacillus pumilus* isolated from coastal environment in Cochin, India. *Brazilian J. Microbiol*. 40,269-275

Passantino, A. (2007). Ethical aspects for veterinarians regarding antimicrobial drug use in Italy. *Int J Antimicrob Agents* 29, 240-244.

Payne, W. J. (1973). Reduction of nitrogenous oxides by micro-organisms. *Bacteriological Reviews* 37,409-452.

Petrovski, K. R., Trajcv, M. and Buneski, C. (2006). A review of the factors affecting the costs of mastitis. *J. S. Afri. Vet. Asso.* 77, 52-60.

Pieterse, R. (2008). Control of Bacterial pathogens associated with mastitis in dairy cows with natural antimicrobial peptides produced by lactic acid bacteria. Msc. Thesis University of Stellenbosch, South Africa.

Pinedo, P. J., Fleming, C. C. and Risco, C. A. (2012). Events occurring during the previous lactation, the dry period, and peripartum as risk factors for early lactation mastitis in cows receiving 2 different intramammary dry cow therapies. *Journal of Dairy Science,* 95(12), 7015-7026.

Priest, F.G. (1993). Systematics and ecology of *Bacillus*. *Bacillus Subtilis* and Other Gram-Positive Bacteria (Sonenshein AL, Hoch JA & Losick R, eds), pp. 3–16. Washington, DC: American Society for Microbiology,

Pyorala, S. (2003). Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research,* 34, 565–578.

Schroeder, J.W., and Eide, W.D. (2007). Market Analysis for Post-harvest Demand of North Dakota Dairy Products: Objective 1 - Document North Dakota dairy production and processing logistics, area of service and procurement of milk. Funded by the North Dakota Dairy Promotion Commission.

Sears, P. M., and McCarthy, K. K.. (2003). Management and treatment of Staphylococcal mastitis. *Vet. Clin. North Am. Food Anim. Pract.* 19,171–185.

Sharma, N, Kapoor, G. and Neopaney, B. (2006) Characterization of a new bacteriocin produced from a novel isolated strain of *Bacillus lentus* NG121. *Antonie Van Leeuwenhoek* 89, 337–43.

Sharma, N. and Gautam, N. (2008). Antibacterial activity and characterization of bacteriocin of *Bacillus mycoides* isolated from whey. *Ind J Biotechnol* 8,117–121.

Sharma, N. and Kumari, R. (2011). Purification and characterization of bacteriocin produced by B*acillus subtilis* R75 isolated from fermented chunks of mung bean (*Phaseolus radiatus*). *Food Technol. Biotechnol.* 49 (2) 169–176.

Shelburne, Ch.E., An F.Y., Dholpe, V., Ramamoorthy, A., Lopatin, D.E. and Lantz, M.S. (2007). The spectrum of antimicrobial activity of the bacteriocin *subtilosin* A. *J Antimicrob Chemother* 59,297–300.

Shryock, T.R. (2004). The future of anti-infective products in animal health. *Nature Reviews Microbiology* 2, 425–430.

Sifuna, A.W., Njagi, E.N.M., Okemo, P., Munyalo, A., Orinda, G.O. and Kariuki, S. (2008). Microbiological quality and safety of *Rastrineobola argentea* related in Kisumu town markets, Kenya. *East African Medical J*., 85 (10), 509-513.

Slepecky, R. and Hemphill, E. (2006). The genus *Bacillus*. Nonmedical. The Prokaryotes, Vol. 4 (Dworkin M, Falkow S, Rosenberg E, Schleifer K-H & Stackebrandt E, eds), pp. 530–562. Springer, New York.

Smith K. L *et al*., (1991). Costs associated with selected preventive practices and with episodes of clinical mastitis in nine herds with low somatic cell count. *J Am Vet Med Assoc* 199, 190-196.

Smith, N. R., Gordon, R. E. and Clark, F. E. (1952). Aerobic spore forming bacteria. *U.S. Dept. Agr. Monograph* No. 16.

Statsoft (1995). Statistica for Windows. USA: SPSS Inc.

Stein, T. (2005). *Bacillus subtilis* antibiotics structure, syntheses and specific functions. Mol. Microbiol., 56,860 -867.

Svetoch E.A. and Eruslanov B.V. (2005). Isolation of *Bacillus circulans* and *Paenibacillus polymyxa* strains inhibitory to *Campylobacter jejuni* and characterization of associated bacteriocins. *J Food Protect* 68, 11–17.

Teo, A.Y.L. and Tan, H.M. (2005). Inhibition of *Clostridium perfringens* by a Novel Strain of *Bacillus subtilis* Isolated from the Gastrointestinal Tracts of Healthy Chickens. *Appl. Environ. Microbiol*., 71(8): 4185- 4190.

Terlabie N.N., Sakyi-Dawson, E. and Amoa-Awua, W.K. (2006). The comparative ability of four isolates of *Bacillus subtilis* to ferment soybeans into dawadawa. *Int J Food Microbiol* 106,145–152.

The Dagaa (*R. argentea* Pellergrin Fishery in the Kenyan waters of Lake Victoria: In: The report of the first meeting of the working group on Lake Victoria *R. argentea* Kisumu: Kenya Marine and Fisheries Research Institute.

Von Heijne, G. (1983). Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem.* 133 (1), 17–21.

Wagner, S. and Erskine, R. (2006). Antimicrobial drug use in bovine mastitis. In: Antimicrobial Therapy in Veterinary Medicine 4th edn). Giguere S, Prescott JD, Baggot RD et al. (eds). Oxford: Blackwell.

Wells, S.J. and Ott, S.L. (1998). What is the current milk quality in the U.S.? Pages 10–18 in 1998 Natl. Mastitis Counc. Annu. Meet. Proc. Natl. Madison, W: Mastitis Counc.,

Wertz, J.E., Goldstone, C., Gordon, D.M., and Riley, M.A. (2003). A molecular phylogeny of enteric bacteria and implications for a bacterial species concept. *J Evol Biol* 16, 1236–1248

Whittenbury, R. (1964). Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. *J. Gen. Microbiol*. 35, 13-26.

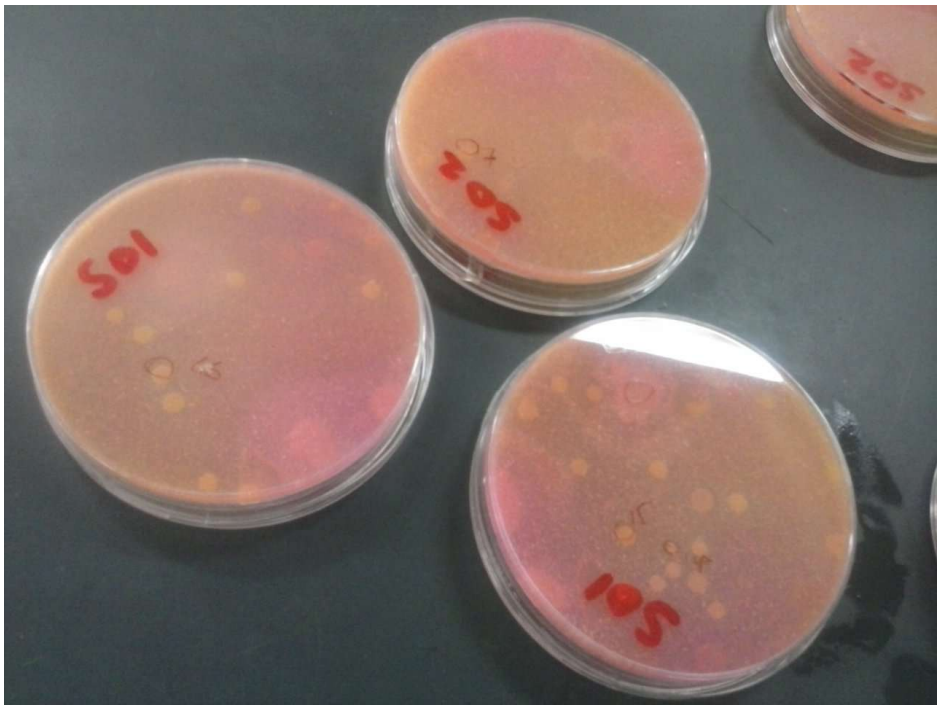
Wilson, G. S. and Miles, A. (1975). Tapley and Wilson's principles of bacteriology, virology and immunity, 6th ed., chap. 14. London: Arnold.

Xie, J., Zhang, R., Shang, C. and Guo, Y. (2009). Isolation and characterization of a bacteriocin produced by an isolated *Bacillus subtilis* LFB112 that exhibits antimicrobial activity against domestic animal pathogens. *African Journal of Biotechnology* 8, 5611-5619

Zheng, G. and Slavik, M. F. (1999). Isolation, partial purification and characterization of bacteriocins produced by newly isolated *Bacillus subtilis* strain. *Lett. Appl. Microbiol.*, 28,363-367.

APPENDICES

Appendix 1: Bacterial colony morphology after incubation at 37ºC for 24hrs



Cultures showing pink and yellow colonies grown in nutrient agar at 37ºC for 24hrs.

Appendix 2: Morphological characterization of *R. argentea* bacterial isolates on nutrient agar

Colony Morphological Characteristics			
Colour	Margin	Elevation	Number (%) (n=98)
Pink	Entire	Raised	0 (0)
Pink	Entire	Flat	2 (0.02)
Pink	Undulate	Raised	0 (0)
Pink	Undulate	Flat	46 (46.94)
Yellow	Entire	Raised	48 (48.97)
Yellow	Entire	Flat	0 (0)
Yellow	Undulate	Raised	2 (0.02)
Yellow	Undulate	Flat	0 (0)

Appendix 3: Preparation of Phosphate Buffer, 0.1M

Solution A Dissolve 13.6g of potassium dihydrogen phosphate (KH_2PO_4) in distilled water, and make up to 1 litre of solution.

Solution B Dissolve 26.8g of disodium hydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$) in distilled water, and make up to 1 litre of solution.

Volume (ml) of solution		
pH	A	B
6.70	52	48
6.81	48	52
7.00	34	66
7.10	28	72
7.30	20	80
7.42	16	81

Distribute and sterilize as required.

Appendix 4: Preparation of Peptone water

(Suitable for the indole test)

Tryptone or tryptose	10.0g
Sodium chloride	5.0g
Distilled water	1 litre

pH 7.2

Dissolve the peptone and sodium chloride in the water by steaming. Adjust to pH 7.2, and dispense in 5ml amounts in 150 X 16mm test-tubes and sterilize by autoclaving at 121°C for 15 min.

Appendix 5: Preparation of Malachite Green

(For staining membrane tilters)

Malachite green	0.1g
Distilled water	1 litre

Appendix 6: Kavoc's Indole Reagent

(To test for indole production in tryptone water)

Amyl or isoamyl alcohol	150ml
p-Dimethylaminobenzaldehyde	10g
Concentrated hydrochloric acid	50ml

Dissolve the aldehyde in the alcohol then slowly add the acid. Store in the refrigerator.

Appendix 7: Nutrient Agar

(A general purpose culture medium for bacteria)

Nutrient broth	1 litre
Agar	15.0g

pH 7.2

Dissolve the agar in the nutrient broth by autoclaving at 121ºC for 20 min. Adjust the pH to 7.2. Filter through the paper pulp (*q.v.*). Distribute as required and sterilize at 121ºC for 20 min.

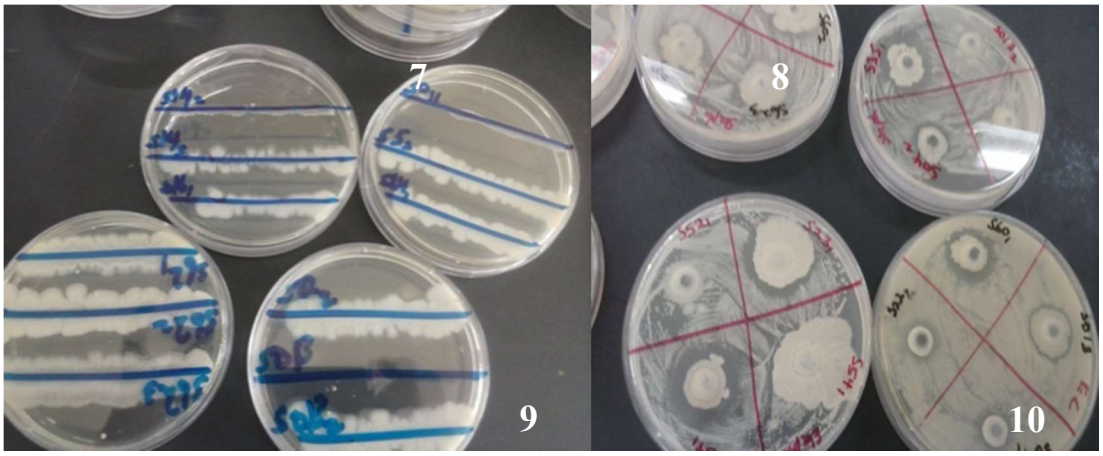
Appendix 8: Simmon's Citrate Agar

(For the determination of citrate-utilization)

Sodium chloride	5.0g
Magnesium sulphate, hydrated ($MgSO_4.7H_2O$)	0.2g
Ammonium dihydrogen phosphate	1.0g

Appendix 9: The plates of the biochemical tests carried out on the selected samples

1: Voges – Proskauer Test; 2: Gram staining and spore staining 3: Catalase Test; 4: Sugar Fermentation; 5: Nitrate Reduction Test; 6: Indole Production Test; 7: Growth on Simmon's Citrate Agar 8: Hydrolysis of starch and casein; 9: Microorganisms showing bacteriocin activity.


7
8
9
10

Appendix 10: **API Kit results after incubation of micro-organisms inoculated in API 50CH B/E at 30°C for 24 Hrs.**

Appendix 11: Microorganism identified using the API Kit

API KIT RESULTS

++	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
$S52_3$	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S46_1$	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S37_1$	-	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S44_1$	-	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-		-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S62_3$	-	+	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S23_1$	-	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
$S04_1$	-	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S60_2$	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S06_3$	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-
$S02_1$	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S08_2$	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S01_3$	-	+	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S62_2$	-	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	-	-	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S22_2$	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
S44	-	+	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
$S40_1$	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S22_1$	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S16_1$	-	+	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	-	+	-	+	+	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S03_1$	-	-	-	-		+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S0_2$	-	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S54_1$	-	+	-	+	-	+	-	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-	+	-	+	-	-	+	+	+
$S53_1$	-	+	-	-	+	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S34_1$	-	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-
$S24_1$	-	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S04_1$	-	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S60_1$	-	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S35_1$	-	+	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S10_3$	-	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S52_3$	+	+	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
$S58_1$		+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

Appendix 12: Summary of Strains of Bacillus as identified by the API Kit.

Microorganism	Number (%) (n=27)
Bacillus subtilis	5 (17)
Bacillus Pumilus	3 (10)
Bacillus mycoides	14 (57)
Bacillus lentus	3 (10)
Bacillus circulans	1 (3)
Bacillus anthracis	1 (3)