BIOPROSPECTING AND CHARACTERISATION OF POTENTIAL BACTERIOCIN PRODUCING *BACILLUS* SPECIES ISOLATED FROM *RASTRINEOBOLA ARGENTEA* (OMENA) WITH ACTIVITY AGAINST BOVINE MASTITIS BACTERIAL PATHOGENS

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Bioprospecting and Characterisation of Potential Bacteriocin Producing *Bacillus* Species Isolated from *Rastrineobola Argentea* (Omena) with Activity Against Bovine Mastitis Bacterial Pathogens

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DECLARATION

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

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The thesis has been submitted for examination with our approval as university supervisors

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DEDICATION

I dedicate this thesis to my family members, my husband Peter, and children Ivy, Ida and Ian for their support and great patience. Special dedication to my mother from whom I get great inspiration.

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ABSTRACT

The inappropriate use of antibiotics has resulted in an increase in drug-resistant pathogenic bacteria. The global problem calls for alternative novel antimicrobial agents with a wide range of clinical significance. Bacteriocins offer an alternative to antimicrobials and have been tried out in food and other industries with limited success. This study sought to explore antimicrobial agents from bacteria inhabiting the commonly consumed *Rastrineobola argentea* from open-air markets in Kisumu Kenya and evaluate their potential for production of crude bacteriocins with antimicrobial activity against bovine mastitis bacterial pathogens. Dried samples were randomly collected from fifteen different fishermen at open-air markets in Kisumu. Using Nutrient agar, 60 bacteria isolates were isolated and characterized using standard microbiological procedures and 54 gram-positive isolates identified as *Bacillus* spp. were further tested for their bacteriocin production capacity. Each isolate was tested for crude bacteriocin production. The extracts were optimized and their physiological and physicochemical parameters determined. On the other hand, the 54 bacteriocin-producing isolates were characterized using molecular techniques by 16S rRNA gene sequencing. All the isolates demonstrated varied utilization of sugars, and 28 isolates showed antimicrobial activity towards the common bacterial standard test microorganisms. Further characterization of the isolates using the analytical profile index system showed that 28 isolates had antimicrobial activity against *E. coli* and *S. aureus* test microorganisms. The analytical profile index system indicated that 20 isolates (71%) were identified as members of the *Bacillus* spp. The crude bacteriocin extracts withstood the effect of the proteolytic protekinase K enzyme, while trypsin and lipase enzymes caused 40% and 15% loss of inhibition, respectively. When tested at different pH levels, the bacteriocin extracts had no inhibition at pH 3-5, but had varied inhibition at pH 6-9 across the test organisms and withstood temperatures of 50 °C–60 °C, showing no loss of activity. The bacteriocin activity decreased by 20% as the temperature increased from 70 °C to 80 °C. Temperatures of 100 °C and 121 °C resulted in a 40% and > 50% reduction in bacteriocin activity, respectively. Metal ions, Cu^{2+}, Fe^{2+} and Zn^{2+} had varying effects on bacteriocin activity against test micro-organisms. The blast analysis of the partial sequences revealed that twenty (20) isolates belonged to *Bacillus subtilis*, with similarities ranging between 96.2% and 99.7%; *Bacillus mycoides* represented 18% (5 isolates) with similarities 99.9% to 100%; and *Bacillus pumilus* constituted 11% (3 isolates) with similarities ranging from 98.4% to 100%. The study has demonstrated *R. argentea* collected from open air markets in Kisumu harbors to a large extent *B. subtilis*, followed by B. *mycoides*, *B. pumilus* and *B. cereus* which have antimicrobial activity against standard laboratory test *E. coli, S. aureus, P. aeruginosa* and *K. pneumoniae*, microorganisms and their corresponding counterparts isolated from mastitis infected animals. Additionally, the *Bacillus* species were able to produce enzymes, ferment carbohydrates, and survive in different temperature and pH conditions besides tolerating presence of metallic ions. *R. argentea* harbours *Bacillus* species from which bacteriocins can be obtained having activity against bovine mastitis bacterial pathogens.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Rampant indiscriminate prescription of antibiotics and inadequate use of drugs has led to increased resistance of pathogenic bacteria in both livestock and humans (Sahoo *et al.*, 2010). This is a major concern to researchers, and it has necessitated the search for novel antimicrobial agents from microorganisms. Such antimicrobial agents are produced by low virulence bacterial strains and having antimicrobial activity on a wide range of clinically significant organisms (Ansari *et al.*, 2012).

The production of an antimicrobial substance by microorganisms is an important factor in microbial ecology (Ansari, 2015). Most of these substances play a key role in bacterial interactions, among them bacteriocins which are highly specific and efficient antagonist against pathogens (Ansari, 2015). Bacteriocin refers to peptides and protein compounds produced by a variety of different microbes and having antimicrobial activity against closely related species (Yang *et al.*, 2014). These antimicrobial agents are gaining more attention as alternative therapeutics in pharmaceutical and preservatives in food industries (Maina *et al.*, 2017). Different kinds of bacteriocins may be produced within same species and which are ribosomally synthesized in the host while the producer strain possesses specific self-protection mechanism (Dobson *et al.*, 2012). The bacteriocins are heterogeneous compounds possessing variability of biochemical properties, molecular weight, activity spectra and mode of action (Salar *et al.*, 2013). According to Ansari *et al.* (2012), BacIB17 bacteriocin produced by *B. subtilis* KIBGE IB-17 possesses inhibitory properties acting as an antimicrobial agent against standard test microorganisms.

Bacteriocins are generally recognized as naturally occurring food preservatives able to influence the quality and safety of foods (Silva *et al.*, 2018). According to Cotter *et al.*

(2013), non-clinical bacteriocin also have applications in controlling animal and foodborne pathogens in livestock.

Members of the genus *Bacillus* are reported to produce a wide arsenal of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocin (Abriouel *et al.,* 2011). Many of the *Bacillus* bacteriocins belong to the lantibiotics, a category of post-translationally modified peptides widely disseminated among different bacterial clades (Abriouel *et al.,* 2011). The presence of *Bacillus* species in food does not always imply spoilage or food poisoning. Considering the high protein, ash and lipid content of *Rastrineobola argentea* (*Omena*), any *Bacillus* bacteria present might have diverse array of antimicrobial peptides with unique chemical structures. To date, no classification scheme has been devised for *R. argentea Bacillus* bacteriocins despite all the classification efforts made with the bacteriocins of LAB. Maina *et al.,* (2015) isolated bacterial strains of *Bacillus spp.* from *R. argentea* and established that the strains produced crude bacteriocins with antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, standard reference strains. However, the studies did not evaluate the extent of bateriocin production and by fully characterised strains of *Bacillus* species. This would only have been achieved using molecular characterisation and testing the *Bacillus* strains on clinical mastitis pathogens rather than reference stock mastitis pathogens which could have lost their virulence. In addition, the presumed mastitis pathogens used by Maina *et al* (2015) had not been characterised using molecular tools which made the interpretation of the results speculative.

Physiochemical factors such as temperature between 30-75°C, a pH of 6.0 and 6.5, and incubation period have been reported as variables affecting the optimal production of bacteriocin (Sure *et al.,* 2016). *B. subtilis* has been found to be capable of producing bacteriocin at a wide range of pH and temperature (Sharma *et al.,* 2011). This phenomenon makes it an ideal strain for the production of bacteriocin on an industrial scale. The identification and production of such bacteriocin and bacteriocin-like compound against a wide spectrum of microbial species are very important for food and pharmaceutical industry (Ansari *et al.,* 2012). In the past two decades, scientists focused

on the bacteriocin produced by the lactic acid bacteria (LAB) (Swetwiwathana & Visessanguan, 2015), due to their potential as natural and safe preservatives (Field *et al.,* 2018). Only a few research teams have reported the use of molecular techniques such as PCR based methods for detection of the structural genes of the bacteriocin precursors (Aleksandrova *et al*. 2010). The extent to which the physiological conditions, chemical structure, and molecular mechanisms of *B. subtilis* secretion of bacteriocins is not well understood.

The need to curb indiscriminate prescription of antibiotics to mastitis infected livestock may call for the need to screen for a large collection of *bacillus* bacterial isolates for potential bacteriocin production.

Handling practices and general processing of *R. argeneta* locally have been found inadequate in maintaining the microbial load of the fish. Such practices as displaying the fish over open drains, exposing them in ambient temperatures and spreading over sacks and mats on the soil makes *R. argeneta* a suitable source for extraction of *bacillus* species.

1.2 Statement of the problem

The most economically costly disease in cattle is mastitis. Bovine mastitis is a disease of major economic importance in the dairy industry globally, affecting the animal health and milk quality particularly in developing countries (Abebe *et al.,* 2016; Ondiek *et al.,* 2018). The economic losses due to bovine mastitis occurring during the first 30 days of lactation for representative US dairy stand at USD 444, with USD 128 in direct cost (diagnostics, therapeutics and veterinary services) while USD 316 indirect costs (milk production loss, premature culling and reproductive costs (Rollin *et al.,* 2015). Furthermore, the loss from decreased milk yield is estimated to be over USD 1 billion yearly (Ondiek & Kemboi, 2018). The milk losses due to mastitis in Embu and Kajiado counties stands at about 43% (Mbindyo *et al.,* 2020). Many antibiotics are licensed for the treatment of mastitis in lactating dairy animals. However, unwanted portions of

antibiotics are released unaltered through the milk of dairy animals and exert serious harmful effects on human health (Pyörälä, 2009). This happens when farmers forget to withhold milk from treated cows for the recommended time. The extra-label drug used which takes the form of increased dose, increased frequency of treatment, use in an unapproved species (Tesfaye, 2019) has been on the increase. Presence of antimicrobial drug residues in dairy milk is one of the major milk contaminant with serious public health implication such as immunogenic reactions in people (Tesfaye, 2019). The indiscriminate use of antibiotics with extra-labelled doses by some veterinarians, quacks, and farmers has increased antibiotic resistance of mastitis-causing pathogens thereby reducing response to antibiotic therapy (Wagner & Erskine, 2013). Brown *et al.,* (2020), reported the presence of β-lactam (7.4%) and tetracycline (3.2%) residues in milk samples marketed for human consumption in Kibera slum in Nairobi. Also, the quantity of antibiotics needed to eliminate mastitis pathogens inhibits the growth of good lactic acid *Streptococci* bacteria essential in cheese and yoghurt making technology; and the residues in milk are a potential threat to human health as may lead to antibiotics resistance (Cao *et al.,* 2007). 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 (Sumi *et al.,* 2015). To the best of the author's knowledge, prior to this study, no classification scheme has been devised for *R. argentea Bacillus* bacteriocins from aquatic environments despite all the classification efforts made with the bacteriocins of LAB from *R. argentea. R. argentea* have high protein, ash and lipid content of any *Bacillus* bacteria present might have diverse array of antimicrobial peptides with unique chemical structures. No studies have evaluated if the *Bacillus* bacteria present in *R. argentea* might have diverse array of antimicrobial peptides with unique chemical structures. Antibiotics, vaccines, bacteriocins, herbal therapy, immunotherapy, and nanoparticle technology have all been tested for efficacy in treating mastitis, but due to the variable response of etiological agents to therapeutic techniques, no single technique has been found to be effective in controlling or treating the disease.

1.3 Objectives

1.3.1 General objective

To bioprospect for bacteriocins from *Bacillus* species isolated from R*astrineobola argentea* (*Omena*) with activity against bovine mastitis bacterial pathogens

1.3.2 Specific objectives

1) To characterise bacteriocin-producing *Bacillus* species from *R. argentea*.
2) To evaluate the *in vitro* antibacterial activity of crude bacteriocins produced by *Bacillus* species isolated from *R. argentea*.
3) To determine the physiochemical properties of the crude bacteriocins produced by *Bacillus* species isolated from *R. argentea*.
4) To determine the phylogenetic relationships among bacteriocin-producing *Bacillus* species isolated from *R. argentea*.

1.4 Hypothesis

1) H_0: *R. argentea* do not harbour bacteriocin-producing *Bacillus* species.
2) H_0: Crude *R. argentea Bacillus* bacteriocins do not have antibacterial activity.
3) H_0: Temperature, pH and metal ions do not have any effect on crude *R. argentea Bacillus* bacteriocines.
4) H_0: *R. argentea* bacteriocin-producing *Bacillus* species are not phylogenetically related.

1.5 Justification

The dairy industry contributes approximately 8% of Kenya's Gross Domestic Product (GDP) with 3.43 billion litres of milk produced annually (Odero-Waitituh, 2017). Kenya is the leading producer of milk in East Africa with approximately 3.2 billion litres of milk produced yearly mostly by about 600,000 small-scale farmers (Mureithi &

Njuguna, 2016). Indiscriminate use of antibiotics in dairy farming for the treatment of various pathogenic bacteria has led to increased antibiotic resistance with immense public health significance (Marshall & Levy, 2011; Sahoo *et al.,* 2010). Among the strategies previously considered is the use of antimicrobial products (bacteriocins) produced by most bacterial strains (Gálvez *et al.,* 2010) in the control of diseases in livestock (Gálvez *et al.,* 2010). Bacteriocins have been reported to offer an advantage over antibiotics because they target very specific organisms (Yang *et al.,* 2014). They are generally considered safe and friendly by consumers besides having a lower impact on food nutrition and organoleptic properties compared with the use of commercial antibiotics and other chemicals. Furthermore, these technologies are cost-effective since they don't require advanced skills or equipment hence can be adopted by developing countries (Gálvez *et al.,* 2010). Bacteriocin are considered safe for infections control since they can be easily degraded by proteolytic enzymes of the mammalian gastrointestinal tract. Nisin and Bovicin HC5 are two major examples of bacteriocins with therapeutic effect against microorganisms responsible for mastitis (Cao *et al*. 2007; Mantovani *et al*. 2001). Studies by Maina *et al.,* (2015) reported that the extent to which the physiological conditions, chemical structure, and molecular mechanisms of *B. subtilis* secretion of bacteriocins is not well understood. Because of their mode of action is targeted, bacteriocins could be effective and safe approach to treat mastitis and prevent the emergence of antibiotic resistant strains of microorganisms. Considering the high protein, ash and lipid content of *R. argentea* any *Bacillus* bacteria present might have diverse array of antimicrobial peptides with unique chemical structures. Additionally, Maina *et al.,* (2015) presumed the two mastitis pathogens obtained from an external source were true mastitis pathogens, but because they had not been characterized using molecular tools it made the interpretation of the results purely speculative. Therefore, novel potent antimicrobial agents derived from bacterial strains of low virulence and poses antibacterial activity against a wide range of significant mastitis-causing strains are needed for improving milk productivity and safety. This is due to the fact that bacteriocins are very specific and they target very specific or closely related species. antibiotics on the other hand will have activity even on the bacteria

necessary for processing of fermented milk products. As a result, the dairy industry could benefit greatly from the development of safe antimicrobial agents from bacteriocins which could improve the livelihood of small holder dairy farmers in Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mastitis

Bovine mastitis (intramammary infection) remains one of the most costly diseases in the dairy industry with economic losses estimated at USD 1.2 to 1.7 billion (Thompson-Crispi *et al.*, 2014; Cao *et al.*, 2007). It is defined as the swelling or inflammation of parenchyma of mammary gland, that is caused by bacteria and its toxins (Sharma *et al.*, 2011). It usually occurs in lactating domestic dairy animals and humans as a result of mechanical, chemical, or thermal injury as an inflammation of the mammary gland during breastfeeding, unusual to the vigorous pull and tug of the infant's suck, the nipples may become sore, cracked, or irritated (Viguier *et al.*, 2009). This phenomenon creates a tiny opening in the breast, through which bacteria can enter (Mass, 2004). The presence of milk in these openings, with high sugar content, provides the bacteria with an excellent source of nutrition. These conditions enable the bacteria to multiply in large numbers causing an infection of the breast. This usually occurs more than two to four weeks after delivery of the baby to approximately 3% to 5% of nursing women (German *et al.*, 2008).

2.1.1 Bacterial causes of mastitis

Mastitis is caused by different bacterial strains also responsible for milk spoilage, they include, *Streptococcus agalactiae, Staphylococcus aureus, Corynebacterium bovis, Staphylococcus epidermidis, Brucella melitensis, Mycoplasma spp* (Vakkamäki *et al.*, 2017; Zastempowska *et al.*, 2016). Also coliforms (*E. coli, Klebsiella* species, and *Enterobacter aerogenes*), *Serratia, Pseudomonas, Proteus* species, environmental *Streptococci,* and *Enterobacter* species are implicated in mastitis (Zeryehun & Abera, 2017). The physical injury to the teat is pre-disposing factor for bacterial invasion, colonization, and infection due to damage of the mucous membranes lining the teat sinus

(Ashraf & Imran, 2020). The pathogenic invasions trigger immunological reactions leading to intramammary infection and inflammation (Abera *et al.,* 2010).

The most common infectious pathogenic bacteria associated with dairy mastitis in cattle in Kenya include *Streptococcus agalactiae*, and *Staphylococcus aureus* (Gitau *et al.,* 2014). These organisms are usually found in the environment of the cow; hence they can easily be contracted by the udder (Zeryehun & Abera, 2017). Mastitis is of tremendous economic importance for the dairy industry, and it is of a concern for public health. These considerations are replicated in sheep and goats and other milk producing females (Edwards & Inya, 2013).

There are two types of mastitis, subclinical, in which no visible symptom are manifested and clinical, where visible symptoms are manifested ranging from mild to severe (Mahlangu *et al.,* 2018). Clinical mastitis is characterized by physical, bacteriological and chemical changes in the milk as well as pathological changes in the glandular tissue of the udder including, heat, pain, swelling of the mammary gland and fibrosis of the udder with visible symptoms ranging from mild to severe (Ashraf & Imran, 2020; Mahlangu *et al.,* 2018; Patil *et al.,* 2015). It is also characterized by abnormal milk secretions with or without local or systemic signs of inflammation (Barlow, 2011), milk discolouration, presences of clots, flakes and increased numbers of leukocytes in milk (Hadef *et al.,* 2018). Other systemic signs associated with clinical mastitis in animals include loss of appetite, fever, dehydration, rapid pulse and depression (Zeryehun & Abera, 2017). The contamination of milk by bacterial from the affected cows render it unsafe for human consumption, it also provides a mechanism of spread of diseases such as tuberculosis, Q-fever, sore-throat, brucellosis and leptospirosis with zoonotic importance (Sharma *et al.,* 2011).

Sub-clinical mastitis on the other hand is usually associated with increased numbers of leucocytic cells in the milk, which are used as indicators of the condition and milk quality (Hadef *et al.,* 2018). It also results in changes in appearance of milk or udder, decreased milk product ion, and presence of bacteria in secretions (Zeryehun & Abera,

2017). The main contributing factor in mastitis is poor hygienic practices that is caused by the bruising of mammary tissue or teats from traumas, nursing, fly bites, or other wounds to the skin which provide an important barrier to infection (Mahlangu *et al.*, 2018). Subclinical mastitis can be determined indirectly using different diagnostic methods such as the California Mastitis Test (CMT), Somatic Cell Count (SCC), the modified white side test, milk electrical conductivity tests, pH and catalase tests (Mahlangu *et al.*, 2018).

2.1.2 Mastitis control and prevention

Mastitis control and prevention should focus mainly on the reduction of pathogens number and type from gaining access to the mammary gland as well as maintaining a physiological balance of the cow for efficient use of broad-spectrum of defence mechanisms (Hamann, 2010). Furthermore, the elimination of existing mastitis infections through therapy, culling is required for reduction of new infection risk.

Proper milking procedures are associated with lower contagious mastitis risk, wearing gloves during milking, using automatic take-offs, and using post-milking teat disinfection (Keefe, 2012).

Milking equipment evaluation schedule should be established with the equipment dealer, and periodic independent system checks should be conducted. The milking machine should be regularly washed and maintained to avoid contamination (Keefe, 2012), prompt treatment of all clinical cases, teat dipping of all teats after every milking, culling of chronically infected cows (Hamann, 2010).

Careful cleaning of the teat end before milking; pre-milking teat disinfection, avoidance of organic bedding material and application of sand; application of internal teat sealants to minimize the new infection rate (Hamann, 2010).

Application of proper dietary supplements of vitamins and trace elements ensures reduction of new infections by pathogenic microorganisms. Long-acting antibiotics such

as azithromycin, clindamycin, cephalexin, dicloxacillin and amoxicillin are recommended for the treatment of mastitis because of the high rate of penicillin resistant staphylococci to reduce cow pain in clinical cases as well as to kill or eliminate the bacterial pathogens (Singh, 2013; Hamann, 2010). Chemotherapy is recommended for fresh subclinical cases caused by contagious pathogens within the first 90 days of lactation (Hamann, 2010).

2.1.3 Management of mastitis and shortcomings

In order to successfully prevent mastitis, good management methods are essential. Because individual cows have significant genetic variety, the resistance can be enhanced by sire selection, which leads to long-term improvements in herd health (Weigel and Shook 2018). The coding area of β-defensin genes can be utilized as a technique for choosing more resistant animals in more ancient breeds that have been least altered for milk yield (Gurao *et al.,* 2017).

There is a stronger link between animal nutrition and infection resistance in mammary tissue (Sharun *et al.,* 2021). Dairy cattle with a negative energy balance are inclined to ketosis, and those animals with clinical ketosis are twice as likely to develop clinical mastitis (O'Rourke 2009). Increased expression of host defense genes can aid recovery from subclinical mastitis when vitamins A, D3, E, and H are supplemented (Sharun *et al.,* 2021).

Lactation therapy involves treating mastitis with antibiotics throughout the lactation phase (Tiwari *et al.,* 2013), however it is not widely used due to its high cost and low efficacy (Pyörälä 2009). Dry cow therapy, on the other hand, is the care of dairy cattle during the dry season. SCC values of 100,000 cells/mL for primiparous cows and 200,000 cells/mL for pluriparous cows are the suggested thresholds for selecting cows for dry cow therapy (Sharun *et al.,* 2021).

Internal teat canal sealant can lower the likelihood of heifers developing subclinical and clinical mastitis during the pre-calving period (Parker *et al*. 2007). When used in conjunction with antibiotic dry-cow therapy, internal teat sealant (ITS) greatly reduced SCC and improved subclinical mastitis prevention (Golder *et al.,* 2016).

The hand-held gadget that creates pulsing pressure waves is used in acoustic pulse therapy (APT), also known as shockwave therapy. These waves can reach deeper tissues and break scar tissue in chronic wounds, allowing for revascularization (Sharun *et al.*, 2021).

2.2 Bacteriocins

2.2.1 Nature and occurrence of bacteriocins

Microorganisms produce an extraordinary array of microbial defence systems. This includes classical antibiotics, lytic agents, metabolic by-products, numerous types of protein exotoxins, and bacteriocins (Rameshkumar *et al.,* 2016; Riley & Wertz, 2002). These antimicrobial substances are important factors in microbial ecology. Most of the substances such as bacteriocin play a key role in bacterial interaction because they are highly specific and efficient antagonist (Garcia-Gutierrez *et al.,* 2018).

Bacteriocins are antibacterial peptides and protein with antibiosis properties produced by several species of Gram-positive and Gram-negative bacteria (Cleveland *et al.,* 2001; Sure *et al.,* 2016). They have antimicrobial properties usually against other closely related species. Within the same species of microbes, different kinds of bacteriocins may be produced (Riley & Wertz, 2002).

Bacteriocins are ribosomal synthesized in the host organism and the producer strain possesses specific self-protection mechanism against this bacteriocin (Maina *et al.,* 2017; Savadogo *et al.,* 2006). They are heterogeneous compounds having variability in biochemical properties, molecular weight, activity spectra and mode of action (Hammami *et al.,* 2013). These antimicrobial peptides are gaining more and more

attention in the scientific community not only as an alternative therapeutic agent for the prevention and treatment of infections but also as preservatives in food industries to avoid deterioration and spoilage of food (Ansari *et al.,* 2012). Any species of the *Bacillus* genus could form part of food contamination microorganisms, but some of the secondary metabolites associated with *B. subtilis* are associated with protective antimicrobial effects.

Originally, bacteriocins were referred to as 'colicins' (Tagg, & Ray, 1995). In 1925, André Gratia described the antagonism action exerted by *E. coli* V towards *E. coli Ø* (Gratia, 2000). This antagonistic effect was later shown to be caused by a bacteriocin which is today known as colicin V or microcin V (Cascales *et al.,* 2007). The term bacteriocin was proposed in 1953 and it described colicin type bacteriocins since these had far been the most widely studied Maina *et al.,* 2017). According to this description, bacteriocin was defined as having a narrow bactericidal spectrum with lethal biosynthesis, intra-specific activity, and attachment to specific cell receptors (Maina *et al.,* 2017).

Bacteriocins are generally recognized as naturally occurring food preservatives able to influence the quality and safety of food (O'Sullivan *et al.,* 2002; Silva *et al.,* 2018). Non-clinically, bacteriocins are also used to control animal and foodborne pathogens in livestock (Bemena *et al.,* 2014). Gram-positive bacteria produced bacteriocins have been largely studied, biochemically and genetically characterized (Ansari *et al.,* 2012). The activity of this bacteriocins is very specific and due to difference in cell wall composition, the activity spectra by Gram-positive bacteria are wider as compared to Gram-negative bacteria (Joseph *et al.,* 2013).

Bacteriocins of Gram-positive and Gram-negative bacteria have evolved differently in terms of size and specificity (Ansari *et al.,* 2012). Currently, studies show that bacteriocins produced by lactic acid bacteria are generally recognized as safe (GRAS) (O'Sullivan *et al.,* 2002). Most members of the genus *Bacillus* also produce bacteriocins with GRAS status and are important microorganisms industrially (Ansari, 2015).

Bacteriocins and other bacteriocin like-inhibitory substances from LAB have been defined, however, there is limited classification system for bacteriocins produced by *Bacillus* species (Abriouel *et al.,* 2011). *B. subtilis* is one of the most important species of genus *Bacillus* commonly occurring in natural environments such as soil and water (Ansari *et al.,* 2012). This bacterium can survive in extreme conditions of heat and desiccation because of the production of the endospore. *B. subtilis* is considered a benign organism as it is non-pathogenic and nontoxigenic to humans, animals and plants, (Ansari *et al.,* 2012). Besides the production of various industrially important enzymes, *B. subtilis* produces a wide variety of antibacterial and antifungal compounds (Ansari *et al.,* 2012; Cowan, 1999).

According to (Riley & Wertz, (2002), almost every bacterial species examined so far produce different kinds of bacteriocins. The genus *Halobacteria*, for example, produce their version of bacteriocins universally i.e. the halocins (Bindiya & Sarita, 2016). According to Saadou &Gharaibeh (2002), *Streptomycetes* generally produce broad-spectrum antibiotics. From the available literature, though limited, it is evident and remarkable that research efforts have been put to isolate bacteriocins of various organisms ranging from Archaea, Gram-positive and Gram-negative bacteria among others (Güllüce *et al.,* 2013).

Both Gram-positive and negative bacteria have the potential to produce bacteriocins (Hammami *et al., 2010).* However, the bacteriocins produced by Gram-positive bacteria have been largely studied and also biochemically and genetically characterized. Bacteriocins of Gram-positive and Gram-negative bacteria have evolved differently in terms of size and specificity. The specificity of bacteriocin activity is due to the difference in cell wall composition (Bindiya & Sarita, 2016). The spectra of activity of Gram-positive bacteria produced bacteriocins are wider as compared to those produced by Gram-negative ones (Lagha *et al.,* 2017). Bacteriocins produced from lactic acid bacteria are currently under extensive study since they are generally recognized as safe (GRAS) (O'Sullivan *et al.,* 2002). Most bacteria species from the genus *Bacillus* are

considered industrially important bacteriocin producers with the history of safe use (Cladera-Olivera *et al.,* 2004).

Most bacteriocins and bacteriocin-like inhibitory substances produced by lactic acid bacteria (LAB) have been classified (Abriouel *et al.,* 2011). However, there is limited classification scheme for *Bacillus* bacteriocins. *B. subtilis,* one of the most important species of genus *Bacillus* that is commonly isolated from water, soil and other environments (Abriouel *et al.,* 2011). They produce endospore that enables them to survive in extreme conditions of heat and desiccation (Abriouel *et al.,* 2011).

2.2.2 Bacteriocins classification

The initial bacteriocins classification scheme was suggested by Klaenhammer (1993). It classified bacteriocins based on factors such as size, mode of action, modification, and activity against Listeria (Table 2.1). Based on these elements the scheme was composed of four major classes (I-IV) of bacteriocins. Klaenhammer, (1993) classified class I as the lantibiotics, class II as small unmodified peptides, class III as larger heat-labile proteins, and class IV as complex bacteriocins with chemical motifs composed of lipids and carbohydrates. Later, class IV was removed as no bacteriocin could fulfil these criteria (Heng & Tagg, 2006). Currently, this classification has been modified in several steps, for example, this classification scheme was redefined by Cotter *et al*., (2005) and proposed a radical change resulting in only two classes, the lantibiotics (class I) and the non-lantibiotics (class II) as well as extensive subdivisions of these two classes. Furthermore, they also suggested that class III should not be classified as bacteriocins but instead be named bacteriolysins.

Table 2.1: Bacteriocin classification overview

Protein-Bacteriocins	Class	Sub-Class	Name	MM (kDa)	Mode of action	Ref.
Gracilicutes						
Escherichia coli	Colicins	Group A		40 to 80	Nuclease/ Pore-forming	
		Group B		40 to 80	Nuclease/ Pore-forming	
Pseudomonas aeruginosa	Pyocins	R-type	Pyocin R2	270 (amino acids)	Pore-forming	
		S-type	Pyocin S1,S2,AP41	75/84/94	Phage-tail like	Riley, & Wertz, J (2002)
		F-type	Pyocin F		Phage-tail like	
Hafnia alvei	Alveicins	Colicin like	Alveicin A, B	408/358 (AA)	Pore forming	Wertz, JE; & Riley, MA. (2004)
Klebsiella pneumonia	Klebicin	Colicin-like	Klebicin C, D	96	Nuclease	James, R. (1988); Jabrane *et al.*, (2002)
Serratia plymithicum	Serracin		Serracin P	66	Phage-tail like	Jabrane *et al.*, (2002)
Xanthomonas campestris	Glynericin		Glynericin A	50	Phage tail like	Heu *et al.*, (2001); Pham *et al.*, (2004)
Yersinia enterocolitica	Enterocoliticin			669	Phage tail like	Strauch *et al* (2001)
Erwinia carotovora	Carotovoricin		Carotovoricin Er	68/76	Phage tail like	Nguyen *et al* (2002)
Firmicutes						
Lactobacillus helveticus	Helveticin J	Class III		37,5	to be defined	Joerger *et al*

						(1990)		
Streptococcus milleri	Millericin	Class III		30	Peptidoglycan hydrolysis	Beukes, *et al* (**2000)**		
Enterococcus faecalis	Enterolysin	Class III		34,5	Peptidoglycan hydrolysis	Nilsen *et al* (2003)		
Staphylococcus aureus	Lysostaphin	Class III		25	Peptidoglycan hydrolysis	Kumar *et al* (2008); Trayer *et al* (1970)		
Peptide-Bacteriocin	**Class**	**Sub-Class**		**Name**	**MM (kDa)**	**Post-translational modification**	**Mode of action**	Ref.
Gracilicutes								
Escherichia coli	Microcin	Class I		Microcin B17	3.1	drastic	intracellular enzymes	
		Class II	IIa	Microcin V	8.8	light	pore-forming	Duquesne *et al* (2007)
			IIb	Microcin E492	7.9	drastic	pore forming	
Firmicutes								
Lactic acid bacteria	Class I	A-type	A1	Nisin	3.5	drastic	pore-forming	Dufour *et al* (2007)
(mainly)	or Lantibiotic		A2	Lacticin 481	3	drastic	pore forming	Dufour *et al* (2007)
		B-type		Mersacidin	2			Jack(2000)
	Class II	class IIa		Pediocin	4.6	light	pore forming	Maqueda *et al* (2008)
		class IIb		Plantaricin E/F	3.5/3.7	light	pore forming	Oppegård (2008)
		Class IIc		carnocyclin A	5.8	cyclic	pore forming	Martinm Visscher, *et al* (2009)

		Class IId	Lactococcin A	5.8	none	pore forming	Diep, *et al* 2007**)**
Cyanobacteria							
Prochloron didemni	microcin –like	-	Patellamides	0.7	drastic		Schmidt *et al* (**2005)**

2.2.3 Chemical and physiological properties of bacteriocins

Bacteriocins are heterogeneous compounds with varying biochemical properties, molecular weight, spectra activity and mode of action (Juturu & Wu, 2018). The study of these antimicrobial peptides is gaining more and more attention not only as an alternative therapeutic agent for the prevention and treatment of infections but also as preservatives in food industries to avoid deterioration and spoilage of food (Ansari *et al.,* 2012). Bacteriocin is relatively thermo- stable up to temperature of 100°C and 121°C although they can withstand temperature up to 121°C. They have demonstrated activity at wide pH range of 2-12, however, the maximum activity has been reported a neutral pH (Sharma *et al.,* 2017), furthermore, most bacteriocins are resistant to acidic pH more than basic pH 12 (Karaoglu *et al.,* 2003). Additionally, the bacteriocins inhibitory activities are not affected by chemical treatment with Tween 80, Urea, and EDTA (Bala *et al.,* 2020). However, studies show that bacteriocins activity is inhibited by proteolytic enzymes trypsin, pepsin and Sodium dodecyl sulfate (Bala *et al.,* 2020).

2.2.4 Mode of action of bacteriocins

In Gram-positive bacteria, the main modes of action are pore-formation and inhibition of cell wall synthesis(Malanovic & Lohner, 2016; Silva *et al.,* 2018). Another mode of action is the enzymatical break down of the cell wall as observed for class IIIa bacteriocins. Other modes of action have also been observed for bacteriocins in Gram-positive bacteria which include quorum sensing and inhibition of spore outgrowth (Brogden, 2005; Liu, 2014). In quorum sensing, nisin and subtilin act as pheromones promoting their expression through a three-component signal-transduction system (Cotter *et al.,* 2005). These two bacteriocins also inhibit spore outgrowth through an

uncommon didehydroalanine residue in position 5, which interacts with the spore-associated factor required for outgrowth (Héchard & Sahl, 2002; Sahl & Bierbaum, 1998).

Many bacteriocins in Gram-negative bacteria have an intracellular function which includes enzymatically modes of action such as DNase and RNase (Cascales *et al.*, 2007). This has not been observed for bacteriocins in Gram-positive bacteria. For some bacteriocins, sensitive cells start lysing soon after exposure to the bacteriocin. This is an effect not caused by the disruption of the membrane potential, but rather the secondary mode of action of these bacteriocins. For the cells to start lysing the cell wall needs to be degraded. This is caused by the autolytic system of the bacteriocin-sensitive cells, not the bacteriocin. The autolytic system causes cell lysis through two diverse mechanisms both of which have been shown to occur with different bacteriocins (David *et al.,* 2016).

The bactericidal spectrum range of bacteriocins depends on their mode of action, presence of the cellular target in a strain, and specificity of the target binding domain (Héchard & Sahl, 2002; Silva *et al.,* 2018). Usually, bacteriocins are regarded as having a narrow bactericidal spectrum, acting on bacteria closely related to the producer. However, should be modified because bacteriocins such as nisins exert their antimicrobial effect on a larger spectrum consequently being effective against strains such as *Listeria* and *Clostridium* (Chikindas *et al.,* 2018). Despite this larger spectrum, the overall bactericidal spectrum remains relatively narrow since bacteriocins produced by Gram-positive strains cannot kill Gram-negative strains under normal growth conditions (Cotter *et al.,* 2005).

2.2.5 Applications of bacteriocins

Settanni & Corsetti (2008) reported that bacteriocins are largely recognized as naturally occurring food preservatives able to influence the quality and safety of foods. Non-clinically, bacteriocins also have applications in control animal and foodborne pathogens in livestock as reported by Diez-Gonzalez, (2007). In the reported cases the bacteriocins

were tested out as intrammaly infusions (Diez-Gonzalez, (2007). *B. subtilis* has been shown to produce a wide variety of antibacterial and antifungal compounds only as an alternative therapeutic agent for the prevention and treatment of infections but also as preservatives in food industries to avoid deterioration and spoilage of food beside the production of various industrially important enzymes (Ansari *et al.,* 2012). The production of antimicrobial substance is an important factor in microbial ecology because they play a key role in bacterial interactions; among them, bacteriocins are highly specific and efficient antagonist (Héchard & Sahl, 2002).

2.2.5.1 Bacteriocins in veterinary medicine

Wipe Out® dairy wipes are a nisin-based udder disinfection that has been authorized by the FDA (Immucell, Portland, ME). Mast Out® (Immucell), a nisin-containing intramammary infusion medication, was also recently submitted for FDA clearance (Ahmad *et al.,* 2017). Lacticin 3147, a highly inhibitory lantibiotic generated by Lactococcus lactis DPC3147, has been studied in teat seal formulations as a dry cow treatment against a variety of mastitis-causing bacteria (Ahmad *et al.,* 2017). Lacticin 3147, a highly inhibitory lantibiotic generated by *Lactococcus lactis* DPC3147, has been investigated in teat seal formulations as a dry cow treatment against a variety of mastitis-causing bacteria (Ahmad *et al.,* 2017).

Klostermann *et al* (2010) found that a 10-minute teat dip treatment with lacticin 3147 reduced levels of Staphylococcus aureus (80%), *Streptococcus dysgalactiae* (97%) and *Streptococcus uberis* (90%) in recent experiments (Ahmad *et al.,* 2017). In mice, lacticin NK34, a partly purified lantibiotic, was tested against infection by *Staphylococcus* spp. isolated from bovine mastitis (Ahmad *et al*., 2017). The minimum lethal dose (MLD) for *Staphylococcus aureus* 69 was 1.53 $X10^9$ CFU/mouse, while the MLD *for Staphylococcus simulans* 55 was 3.59 X 10^9 CFU/mouse (Ahmad *et al.,* 2017).

2.2.5.2 Food preservative applications of bacteriocins

Listeria monocytogenes is a common meat disease that thrives in low moisture environments, even when kept refrigerated (Ahmad *et al.*, 2017). The US government has set a zero-tolerance level for *Listeria monocytogenes* in ready-to-eat foods, which is the most stringent guideline in the world. Gassericin A, a food preservative derived from *Lactobacillus gasseri* LA39, has recently been characterized as stable at 4 °C for 3 months, 37 °C for 2 months, 60 °C for 5 hours, and 100 °C for 30 minutes (Ahmad *et al.*, 2017).

2.2.6 Bacteriocins currently used for treatment of mastitis

Antibiotics are routinely used to prevent and cure mastitis, however bacteriocins are being examined as an alternative (Godoy-Santos *et al.* 2019). Bacteriocins are also the future of treating infections like mastitis in a focused, effective, and safe manner (Ahmad *et al*. 2017). This is one of the strategies for reducing the emergence of antibiotic-resistant bacteria. Nisin and Bovicin HC5 are two important bacteriocins that have been reported to have a well-established therapeutic action against mastitis-causing bacteria (Castelani *et al.*, 2019; Godoy-Santos *et al.*, 2019). *Lactococcus lactis* ssp. lactis produces nisin, a bacteriocin. They show antibacterial activity against a variety of Gram-positive bacteria as well as germs that are found in food. Bovicin HC5 is a bacteriocin produced by *Streptococcus equinus*, a ruminal bacteria (Mantovani *et al*., 2001). Bovicin HC5 inhibits the majority of *streptococcal* and *staphylococcal* strains, however it was ineffective against *E. coli* strains, making it a less effective antibiotic option.

2.3 *Bacillus subtilis*

It is a Gram-positive bacterium found in the soil and the gastrointestinal tract of ruminants and human beings. *B. subtilis*, of the genus *Bacillus*, can form tough and protective endospore making it able to tolerate extreme environmental conditions of temperature and desiccation (Kunst *et al.*, 1997). Typically, its cells are rod-shaped of

about 4-10μm long and 0.25–1.0μm in diameter. *B. subtilis* use their flagella for swarming motility. This motility occurs on surfaces, for example on agar plates, rather than in liquids (Kearns & Losick, 2003). *B. subtilis* are arranged in singles or chains. Cells arranged next to each other can only swarm together, not individually (Kearns & Losick, 2003). For the study of bacterial chromosome replication and cell differentiation, *B. subtilis* is widely studied and researched Gram-positive bacterium (Pavlendová& Barák, 2007).

Historically, it has been classified as an obligate aerobe however, evidence exists that it is a facultative aerobe (Encyclopedia of Life, 2018). *B. subtilis* has been widely used as one of the bacterial champions in secreted enzyme production and used on an industrial scale by biotechnology companies (Yin *et al.,* 2007). Angioi *et al.* (1995) reported that *B. subtilis* containing pharmaceutical preparations has been widely used in the treatment and prophylaxis of intestinal disorders associated with antibiotic therapy or diarrhoea of various origin. According to Horosheva *et al*. (2014), probiotics from *B. subtilis* can be used as antibiotic therapy in the treatment of antibiotic-associated diarrhoea (AAD) and adverse effects related to the use of antibiotics.

Molecular assays of randomly amplified polymorphic DNA PCR assay, denaturing gradient gel electrophoresis analysis, and sequencing of the V3 region of 16S ribosomal DNA have been used to characterize *Bacillus* species and revealed greater variety (Pepe *et al.,* 2003). *B. subtilis* and substances derived from it have been evaluated by different authoritative bodies for their safe and beneficial use in food. In the United States, an opinion letter issued in the early 1960s by the Food and Drug Administration recognized some substances such as carbohydrate and protease enzymes as generally recognized as safe (GRAS). The opinions were predicated on the use of non-pathogenic and nontoxigenic strains of the respective organisms and the use of current good manufacturing practices (Schuster *et al.,* 2002; Sanders & Huis, 1999).

B. subtilis form spores in times of nutrient exhaustion. However, when the nutrients required for the bacteria to grow are abundant, they exhibit metabolic activity (Rose *et al.,* 2007).

These organisms can produce antibiotics during sporulation. Examples of the antibiotics that *B. subtilis* can produce include are polymyxin, difficidin, subtilin, and mycobacillin (Terra Char, 2018). Many *Bacillus* spp can degrade polymers such as protein, starch, and pectin; therefore, they are thought to be an important contributor to the carbon and nitrogen cycles (Alariya *et al.,* 2013). Microorganisms of the genus *Bacillus* are known to produce a wide arsenal of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocins (Abriouel *et al.*, 2011).

Most of the *Bacillus* bacteriocins belong to the lantibiotics, a category of post-translationally modified peptides. L antibiotics are among the best-characterized antimicrobial peptides at the levels of peptide structure, genetic determinants, and biosynthesis mechanisms (Motta *et al.*, 2004). The genus *Bacillus* has also been reported to produce many other non-modified bacteriocins, some of which resemble the pediocin-like bacteriocins of the lactic acid bacteria (LAB), while others show completely novel peptide sequences (Abriouel *et al.*, 2011). *Bacillus* bacteriocins are increasingly becoming more important due to their sometimes-broader spectra of inhibition as compared with most LAB bacteriocins, which may include Gram-negative bacteria, yeasts, or fungi, in addition to Gram-positive species, some of which are known to be pathogenic to humans and/or animals (Field *et al.,* 2015).

B. subtilis as the choice bacteriocin production was because of due to the high protein, ash and lipid content of *R. argentea*. Any *Bacillus* bacteria present might have diverse array of antimicrobial peptides with unique chemical structures. *B. subtilis* as the choice bacteriocin production was because of due to the high protein, ash and lipid content of *R. argentea*. Any *Bacillus* bacteria present might have diverse array of antimicrobial peptides with unique chemical structures. Based on previous studies by Maina *et al.*, 2015; the choice of sampling the *Bacillus* from *R. argentea* and not the water from the

lake or other fishes from lake was informed by the fact that *R. argentea* have high protein, ash and lipid content of *R. argentea,* any *Bacillus* bacteria present might have diverse array of antimicrobial peptides with unique chemical structures however prior to this study, no classification scheme has been devised for *R. argentea* Bacillus bacteriocins despite all the classification efforts made with the bacteriocins of LAB. To date, no classification scheme has been devised for *R. argentea Bacillus* bacteriocins despite all the classification efforts made with the bacteriocins of *Bacillus* LAB.

2.4 *Rastrineobola argentea*

R. argentea is a species of ray-finned fish in the family *cyprinidae* and the only member of the genus *Rastrineobola* (Ahnelt *et al.,* 2006). It is one of the smallest cyprinid fishes found in the Lakes of East Africa especially Lake Victoria basin. *R. argentea* are native zooplanktivores of lakes in East Africa. Currently, *R. argentea* is the most commercially exploited species of Lake Victoria (African-Union, 2014). It is an important commercial fish species of Lake Victoria in Kenya, Uganda, and Tanzania. The local names are Omena in Kenya, Dagaa (Tanzania) and Mukene (Uganda) (African-Union, 2014). *R. argentea* fishing takes place at night using canoes (African-Union, 2014). Drying and preservation are 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 (Sifuna *et al.,* 2008). The dried products are similarly exposed to further contamination during storage, transportation and sale at open-air markets (Sifuna *et al.,* 2008). 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).

R. argentea is harvested from the Lake and sun dried for six to eight hours along the Lake bed (Bille, 2006). It can be eaten directly or transformed into fishmeal and incorporated into livestock and poultry feed thus providing a source of livelihood for the local farmers (Manyala & Ojuok, 2007). Six animal feeds manufacturing companies in Kenya were using 70% of *R. argentea* to produce fish meal, while two other companies

were using 60% Nile perch skeletons for the same purpose (Matsuishi *et al.,* 2006). These commercially produced feeds are not readily available and are expensive to the local farmers (Alacs *et al.,* 2010). *R. argentea* is the most commercially exploited species of Lake Victoria. Overfishing, poor fishing practises such as use of mosquito seine, predation and environmental degradation leading to reduced population size of *R. argentea* (Wagner *et al.*, 2012). Fish in the Lake Victoria basin is imperative as a food resource and a commodity that can earn income from its exploitation and trading thus providing a means of livelihood (Minakawa *et al*., 2008).

Few studies have reported the composition of microorganisms in the locally consumed *R. argentea* (Onyango *et al* 2015; Marijani 2020; Maina *et al* 2021). In most instances the studies have focused on the microbial contamination of *R. argentea*. Incidence of pathogenic bacteria in *R. argentea*, has included contamination by *Staphyloccus* spp, *Shigella* spp, *Salmonella* and *E.coli* bacteria. No previous studies have reported on the availability of *B. subtilis* from *R. argentea* and their presence could be attributed to the drying and processing because majority are aerobic spore formers. To date, no classification scheme has been devised for *R. argentea Bacillus* bacteriocins despite all the classification efforts made with the bacteriocins of *Bacillus* LAB.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

Biochemical analyses were carried out in the Food Microbiology Laboratory at the Department of Food Science and Technology, and molecular work was carried out in the Molecular Biology Laboratory at the Institute of Biotechnology Research (IBR) both at Jomo Kenyatta University of Agriculture and Technology.

3.1.1 Study design

This was a laboratory based cross-sectional study design as seen in the Figure 3.1

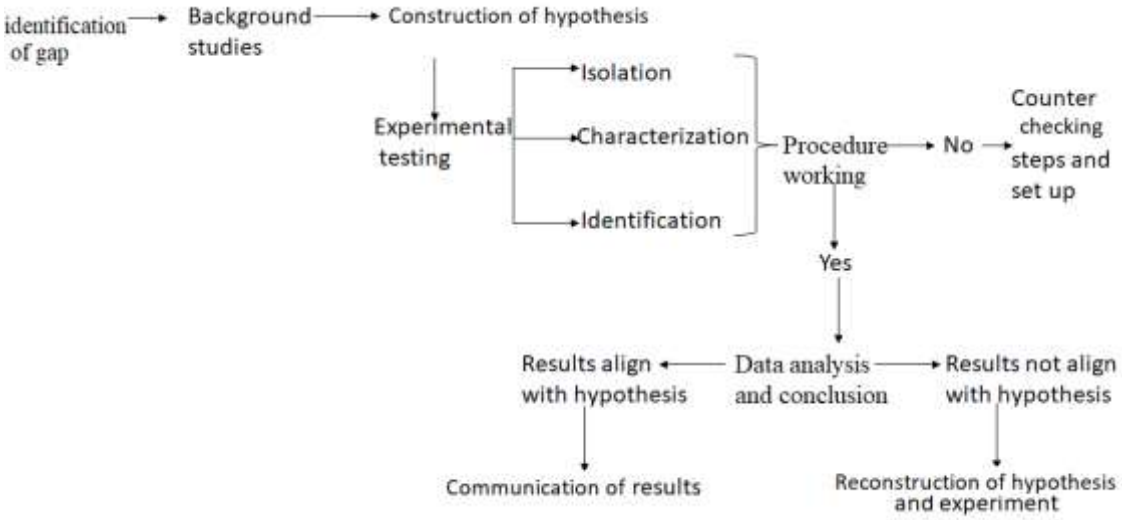

Figure 3.1: Study design

3.2 Sample collection and preservation

Dried *R. argentea* samples weighing 2 kg each were purchased from Dunga fish market, Municipal fish market, Kisumu fish market, Kibuye fish market, and Obunga fish market in Kisumu from fifteen traders. The samples were placed in 9x4 inches zip lock

biohazard polythene bags and immediately transported in sterile sealed cool box at 4^{o}C to the food microbiology laboratory in the Department of Food Science and Technology, Jomo Kenyatta University of Agriculture and Technology for analysis. The samples were stored at -4^{o} C in a refrigerator.

3.3 Isolation of bacteria from *R. argentea*

Nutrient agar (Himedia) was used for cultivation of bacteria from *R. argentea*. It consists of 10 grams peptone, 10 grams meat extract, 5 grams sodium chloride and 12 grams of agar at pH 7.3 ± 0.1 per litre. The medium was prepared by suspending 37 grams in 1 litre of distilled water, brought to boil, mixed well and allowed to stand until completely dissolved and sterilised by autoclaving at 121°C for 15 minutes after which it was then dispensed into 90-mm-diameter polystyrene sterile plastic petri dishes.

Twenty-five grams from each of the fifteen fish samples was placed in a sterile zip-lock bag containing 225 millilitres of sterile peptone water and macerated or homogenized using a stomacher 400 Circulator Homogenizer for two minutes and then heated for 10 minutes at 80^{o}C to destroy vegetative bacteria cell and fungi and facilitate the isolation of *Bacilli* from spores that survive the heat treatment. The liquid supernatant was then serially diluted where 1ml of the liquid supernatant was added to 9ml sterile diluent (distilled water) contained in a test tube. Dilution ratios included: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. These preparations were properly mixed by vortexing, then 100 µl aliquots from different dilutions were transferred to petri dishes containing nutrient agar and spread over the surface with a sterile glass spreading rod. Each dilution series was used to inoculate a series of plates with two plates at each dilution level. This was followed by incubation at 37^{o}C for 24 hours. Sub-culturing was done on nutrient agar to isolate pure cultures.

3.4 Characterization of *Bacillus* producing bacteria species

3.4.1 Long term preservation of the isolates

All bacteria cultures were cryopreserved in the medium containing 15% glycerol (Sigma-Aldrich). The bacteria were stored at –75ºC for long-term storage.

3.4.2 Morphological characterization

Preliminary characterization of the isolates involved the examination of colony morphology and culture features such as colour, pigmentation, elevation, shape, size and growth form (Cappuccino & Sherman, 2014).

3.4.3 Gram staining of the isolated bacteria

Smears of bacteria cultures were prepared and heat-fixed. Slides were placed on the staining rack and flooded with crystal violet. This was allowed to stand for 30 seconds. The slide was then rinsed with water for 5 seconds then covered with Gram's iodine mordant. The slide was allowed to stand for 1 minute and rinsed with water for 5 seconds. Decolourization was done with 95% ethanol for 15 to 30 seconds. This was followed by rinsing with water for 5 seconds. Counterstaining was done using Safranin for about 60 to 80 seconds and the slides were rinsed again with water for 5 seconds. Blot drying was done with bibulous paper and examined using microscope at x100 under oil immersion (Cappuccino & Sherman, 2014). Gram-positive organisms stained blue to purple; Gram-negative organisms stained pink to red. Gram staining results were confirmed by using the 3% KOH test (Halebian *et al.,* 1981). This test was performed by adding a drop of 3% KOH on a slide. A loop full of the bacteria was introduced and mixed thoroughly. Positive results were observed if the culture pulled along with the wire loop when raised up and negative results were recorded if nothing was pulled along the wire loop.

3.4.4 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, steamed for 2-3 minutes as more stain was added cooled and washed under slowly running tap water. This was then counterstained with safranin for 30 seconds. The smears were then washed with tap water, blot dried and observed under immersion oil (Cappuccino & Sherman, 2014). The cell morphology was noted, as well as the spore and colour of spore. Only Gram-positive rods produced spores and this was a good diagnostic test for *Bacillus* species (Brooke *et al.,* 1998).

3.5 Biochemical Tests

Only Gram-positive spore forming rods 54 (90%) isolates presumptive of *Bacillus* species were used in the biochemical test analysis.

3.5.1 Catalase Test

Catalase test was done by scooping a colony of a 24-hour culture, placing it on a glass slide and adding a drop of 3% hydrogen peroxide solution. A positive reaction was indicated by the formation of bubbles, while the absence of air bubbles indicated a negative catalase test (Cappuccino & Sherman, 2014).

3.5.2 Nitrate Reduction Test

Under aseptic conditions, wire loop was used to inoculate each isolate into its appropriately labeled tube by means of a loop inoculation in Trypticase nitrate broth. The last tube served as a control. All cultures were incubated for 24 to 48 hours at 37°C. Five drops of Solution A (sulfanilic acid), was added and then five drops of Solution B (α-naphthylamine), to all nitrate broth cultures. Some of the cultures developed a red colour but to those with no red colour,a small quantity of zinc was added. Observations made and results recorded (Cappuccino & Sherman, 2014b).

3.5.3 Methyl Red-Voges–Proskauer Test (MR-VP)

Methyl red test detects the ability of the isolates to oxidize glucose by detecting the production of sufficient acid as an end product (Harold, 2002). It detects mixed acids, which are the characteristic end products of a particular fermentation pathway that make the medium more acidic (pH≤4.5). This is detected when an indicator is added (Cappuccino & Sherman, 2014b). The Voges Proskauer test is used to identify bacteria that produce non-acidic or neutral end products but not the organic acid products of the glucose fermentation. It specifically detects an intermediate product of the fermentation pathway that yields 2, 3-butandiol known as acetoin, by the addition of Barrit's reagent (4% KOH and 5% alpha naphtol in 95% ethanol). MR-VP broth was inoculated with each of the isolates, in duplicates, shaken and then incubated at 37°C for 72 hours after which, drops of Methyl red indicator for methyl red test or Barrit's reagent for VP test respectively, was added to aliquots of each culture. Positive reactions were those that turned red while negative reactions turned pale yellow for MR test, while for the VP test, positive tests produced a deep red coloration (Cappuccino & Sherman, 2014).

3.5.4 Indole, Motility and Hydrogen sulphide production tests

Indole test identifies isolates with the ability to produce the enzymes tryptophanase that removes the amino group from tryptophan to form Indole, pyruvic acid and ammonia, and cysteine desulfurase, that produces pyruvate, ammonia and hydrogen sulphide from sulphur containing amino acids. Indole reacts with Kovacs reagent (*p*-dimethylamino-benzaldehyde) to form a deep red colour while the Iron in the medium reacts with hydrogen sulphide to produce a black precipitate. The isolates were inoculated in Sulphur-Indole Motility (SIM) agar media by stabbing method in duplicate for replication, and then incubated at 37^{o}C for 48 hours. Two inoculated tubes were used as controls. Kovac's reagent was then added to each of the 48-hour culture. The presence of a cherry red layer in the media indicated positive result for Indole production while negative results showed brown colour. The presence of a black coloration in the media after incubation indicated production of hydrogen sulphide in the media. Lack of

motility was detected by the confinement of the bacteria along the line of inoculation (Cappuccino & Sherman, 2014).

3.5.5 Growth on Simmon's Citrate Agar

This test determines the ability of a microbe to use citrate as the sole source of carbon (Cappuccino & Sherman, 2014). Citrate utilization is indicated by growth accompanied by an alkaline pH (Cappuccino & Sherman, 2014). Simmon's citrate agar slants containing the pH indicator bromothymol blue were inoculated by streaking with the isolates in duplicates and incubated at 37^{o}C for 72 hours. One inoculated tube with the same media served as the control. Positive test was indicated by growth of the bacteria accompanied by colour change in the medium from olive green to Prussian or deep blue. Green colour of medium after incubation was a negative test (Cappuccino & Sherman, 2014).

3.5.6 Triple Sugar Iron Agar Test

Triple Sugar Iron contains three carbohydrates namely, glucose, sucrose and lactose. The media also contains beef extract, yeast extract and peptones which are sources of Nitrogen, vitamins and minerals. Agar and phenol red are also used to solidify the medium and pH indicator respectively. The tubes containing molten agar were angled during preparation and using aseptic technique, the TSI slant was inoculated by first stabbing the butt down to the bottom and then streaking the surface of the slant with appropriate bacterium (Cappuccino & Sherman, 2014). The caps on the tubes were tightly screwed not to permit access of air. Incubation was done aerobically for 18 to 24 hours at 37°C for changes in the butt and on the slant.

A change in the original colour of the medium (reddish-orange) to yellow indicates fermentation of any of the carbohydrates, retention of the red colour in both slant and butt indicates neither glucose, lactose nor sucrose has been fermented. Presence of bubbles in the butt indicates the ability of the bacteria to produce gas. Hydrogen sulfide

production from thiosulfate is indicated by a blackening of the butt as a result of the reaction of hydrogen sulfide with ferrous ammonium sulfate to form a black ferrous sulfide (Cappuccino & Sherman, 2014).

3.5.7 Starch hydrolysis test

Using the wax pencil, starch agar plate was divided into two straight sections. Each of the section was labelled appropriately and aseptically streaked with the respective bacteria. The plates were incubated for 24 to 48 hours at 37^{o}C. Drops of Gram's iodine were then placed on each of the streaking line on the starch agar plate. A clear area around the line of growth showed that starch had been hydrolysed, and the test was positive; while blue colour indicated that starch had not been hydrolysed, and the test was negative (Cappuccino & Sherman, 2014).

3.5.8 Gelatin liquefaction

Gelatine liquefaction detects the breakdown of gelatine to polypeptides and amino acids by enzyme gelatinase. The bacterial isolates were inoculated onto nutrient broth supplemented with 12% gelatine and 1.5% agar, to demonstrate hydrolytic activity of gelatinase. One un-inoculated tube was used as control for each isolate. After 48 hours incubation, the cultures that were totally or partially liquefied when placed in refrigerator at 4^{o}C for 30 minutes were considered positive for gelatine hydrolysis (Cappuccino & Sherman, 2014).

3.6 Screening for antibacterial activity of *Bacillus* species

Antimicrobial activity of the thirty isolated *Bacillus* strains was tested by a well diffusion method (Cappuccino & Sherman, 2014). Wells (10 mm of diameter) in nutrient agar were filled with *Bacillus* species broth cultures and the plates overlaid with a solution of indicator strains; *E. coli ATCC-25922, S. aureus ATCC 25923, K. pneumoniae BAA-1705* and *P. aeruginosa ATCC 15442* by mixing 50µl of strain (24 hours culture on TSB broth at a concentration of 10^{8}cfu/ml) with 200ml 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 activity representing the diameters of the zone of inhibition was expressed in millimeters (Cappuccino & Sherman, 2014).

3.7 Characterization of *Bacillus* species with antibacterial activity using analytical profile index (API)

The bacterial isolate showing the widest zone of inhibition against the target indicator organism was selected for further characterization by the analytical profile index system (API® 50CH B/E, Biomerieux, Inc, France). A 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 at 30^{o}C for 24 hours and then 48 hours. During incubation, carbohydrates fermentation led to acid production resulting in decrease in pH. This was seen by the change in the color of the indicator. 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) (Biomerieux, Inc, France) using manufacturer's instructions.

3.8 Extraction and purification of crude bacteriocin

The potential bacteriocins producing bacterial isolates were sub-cultured in nutrient broth (HiMedia, Laboratories, India) at 30°C for 24 hours. The broth was centrifuged at 15000rpm for 10 minutes after incubation to separate the cells and the supernatant containing crude bacteriocin. The cell-free supernatant was adjusted to pH 6.5 using 1 mol/l NaOH to remove the antimicrobial effects of organic acids and it was used as crude bacteriocin (Elayaraja *et al.,* 2014). Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (C-100 bovineliver, Sigma). Neutralized filtrates were sterilized by filtration and then tested for antimicrobial activity against the indicator organisms using the agar well diffusion method (Kang & Lee,

2005), and their sensitivity tests evaluated using the Kirby-Bauer antibiotic sensitivity assay against *S. aureus, E. coli, P. aeruginosa and K. pneumoniae* according to manufacturer's instructions.

3.9 Physicochemical characterization of bacteriocins from *Bacillus* species

3.9.1 Effect of temperature

Five 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 after heat treatment was evaluated against indicator *S. aureus* and *E. coli* bacteria pathogens by agar–well diffusion assay (Adinarayana *et al.,* 2003).

3.9.2 Effect of pH on crude bacteriocin activity

Five millilitre bacteriocin preparations were tested by adjusting their pH values in the range of pH 3 to 9 with sterile 1N NaOH or 1N HCl (Karaoglu *et al.* (2003). 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.

3.9.3 Effect of proteolytic and lipolytic enzymes on crude bacteriocin activity

Five milliliter aliquots bacteriocin were 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 hours at 37^{o}C and heated for 3 min at 100^{o}C to denature the enzyme and residual activity of bacteriocin (Pilasombut *et al*. 2015).

3.9.4 Effect of metal ions on crude bacteriocin activity

The effect of metal salts on bacteriocin activity was examined by addition 100µl of 2mM, $CuSO_4$, $FeSO_4$, and $ZnSO_4$ (Merck) to 100µl of partially purified bacteriocin

preparation (1mM final concentration). Untreated bacteriocin preparation (positive control). All samples were incubated at room temperature for 2 hours and then tested for residual antimicrobial activity (Adinarayana *et al.,* 2003) by agar-well diffusion assay.

3.10 Determination of phylogenetic relationships among bacteriocin producing *Bacillus* species

3.10.1 DNA extraction

Genomic DNA was extracted from bacterial cells at exponential growth phase grown aerobically in nutrient broth. Prior to extraction, bacterial cells were harvested from broth by centrifuging 1 ml of culture in a 1.5ml Eppendorf tube at 13,000rpm for ten minutes. The pellet was washed by re-suspending the cells in equal volume of TE buffer, centrifuged (Hettich, Micro 200, Germany) for 5 minutes at 13,000rpm and the supernatant discarded. Approximately 50-100 mg of the pellets was used for DNA extraction from each sample. The DNA was extracted in duplicate using Qiagen DNA Isolation Kit (Qiagen, Germany) instructions according to manufacturer's instructions. The DNA quantification was done using spectrophotometer by reading the absorbance at 260 nm and 280 nm used to determine the purity of the DNA.

3.10.2 DNA amplification

Total genomic DNA was extracted according to manufacturer's instructions from overnight cultures grown on nutrient broth at 37^{o}C using QIAamp DNA Mini Kit (Qiagen, Germany). The gene encoding the 16S rRNA was amplified by PCR using bacterial universal primer pair combination of forward primer 27F forward (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R reverse, (5′-GGT TAC CTT GTT ACG ACT T-3′) (Bergmann *et al.,* 2010). Amplification was performed using an advanced Eppendorf 96 AG, model 22331 thermal cycler (Hamburg). Amplification was carried out in a 50µl mixture containing 25µl 3X *Taq* PCR Master Mix (Qiagen, Germany), 2.5µl of each primer, 10µl of DNA template (50ng) and 10µl nuclease free water. The

control contained all the above except the DNA template (Sambrook & Russell, 2001). Reaction mixtures were subjected to the following temperature cycling profiles repeated for 32 cycles: Initial denaturation 94^{o}C for 5 minutes, Denaturation at 94^{o}C for 1 minute, primer annealing at 55^{o}C for 2 minutes, extension at 72^{o}C for 2 minutes and a final extension at 72^{o}C for 10 minutes. Amplification products (7µl) were separated on a 1% agarose gel in 1X TBE buffer and visualized by ethidium bromide (1µg/ml) staining (Sambrook & Russell, 2001).

3.10.3 Agarose gel electrophoresis

Agarose (1.0%) gel (w/v) was prepared by dissolving 1.0 g of agarose powder into 100ml of 1X TBE buffer. The gel solution was stirred, brought to boil in a microwave for 3 minutes to completely dissolve the powder, the cooled gel solution was poured in a casting tray having combs and left for sometimes to gel (polymerise). Ethidium bromide (3µl) was incorporated in the gel to facilitate visualisation of DNA under UV light. The PCR products (7 µl) was mixed with 3µl of loading dye (Bromophenol blue) and loaded into the well and subjected to electrophoresis at 80 V for 45 minutes.

3.10.4 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to the manufacturer's instructions.

3.11 Data Analysis

Data entry management for different colony morphologies, zones of inhibitions and analytical profile index systems were done in Microsoft Excel Spreadsheet. The mean and standard deviation of the triplicate zones of inhibition of test organisms against crude bacteriocins was calculated in excel. The biochemical characterization procedures were done in triplicates. Observations were made on these replicates to define the nature of each of the qualitative tests as either positive or negative. Hierarchical clustering of carbohydrate fermentation profiles of *Bacillus spp* was calculated using dice coefficient

and the tree was constructed using unweighted pair group method with arithmetic mean (UPGMA) with DARwin version 6.0 software. This information was used during the generation of phylogenetic trees to identify bacterial isolates. The 16S rRNA gene sequences of the bacteria isolates were viewed for quality determination and edited using ChromasPro 2.18 software package (http://technelysium.com.au/wp/). The sequences were then compared with available standard sequences of bacteria lineages in the public databases in the National Center for Biotechnology Information (NCBI) gene bank using nucleotide blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find closely related bacterial 16S rRNA gene sequences. The parameters such as percentage similarities, query coverage and E-values were used. This was followed by aligning the sequence using Clustal W software. Phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) with MEGA version 7.0 software package (Kumar *et al.*, 2016). The trees topologies were evaluated using the bootstrap resampling method (Felsenstein, 1985) based on 1000 replicates.

CHAPTER FOUR

RESULTS

4.1 Isolation and characterization of bacteria from *R. argentea*

A total of sixty (60) pure colonies were obtained from *R. argentea* samples from Lake Victoria using nutrient agar medium (Plate 4.1).

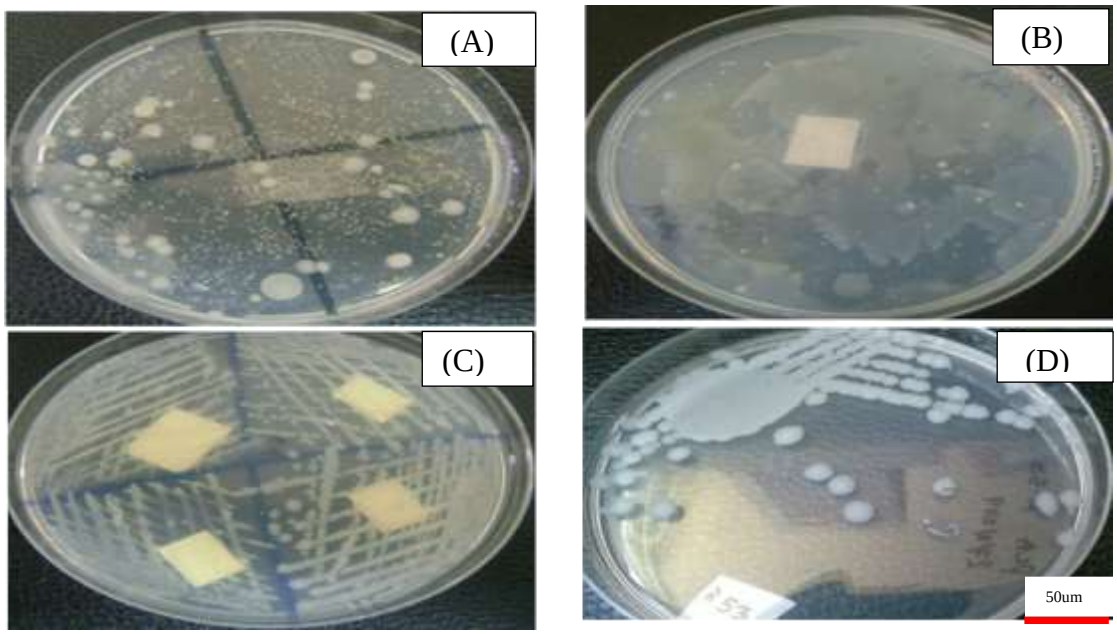

Plate 4.1: Morphological characteristics of bacterial isolates on Nutrient agar

(A) Plate with different colonies before purification of individual colonies (high diversity); (B) Plate with swarming growth cultures; (C) Plate with different isolates; (D) Plate with pure colonies.

Most colonies were smooth or undulated and the colour ranged from white, yellow to cream (Table 4.1). Fifty four (90%) of the isolates were Gram-positive with terminal spore, while 6 (10%) were Gram-negative and with no observable terminal spores. All isolates were rod-shaped. All Gram-negative non-spore formers were eliminated from further investigations.

Table 4.1: Morphological characteristics of bacterial isolates obtained from *R. argentea*

Isolate	Colony characterization				Cell characterization		
	Colour	Form	Elevation	Margin	Gram reaction	Arrangement	Spore formation
R1	White	Irregular	Flat	Undulate	+	Rods	Yes
R2	White	Irregular	Umbonate	undulate	+	Rods	Yes
R3	White	Irregular	Umbonate	Undulate	+	Rods	Yes
R4	White	Irregular	Umbonate	Undulate	+	Rods	Yes
R5	White	Irregular	Flat	undulate	+	Rods	Yes
R6	White	Irregular	Flat	Undulate	+	Rods	Yes
R7	Cream	Irregular	Raised	Undulate	+	Rods	Yes
R8	Cream	Irregular	Raised	Ciliate	+	Rods	Yes
R9	White	Irregular	Flat	Ciliate	+	Rods	Yes
R10	Cream	Irregular	Raised	Undulate	+	Rods	Yes
R11	Cream	Irregular	Raised	Ciliate	+	Rods	Yes
R12	White	Irregular	Raised	Undulate	+	Rods	Yes
R13	White	Irregular	Raised	Undulate	+	Rods	Yes
R14	White	Irregular	Raised	Undulate	+	Rods	Yes
R15	White	Irregular	Raised	Undulate	+	Rods	Yes
R16	White	Irregular	Flat	Undulate	+	Rods	Yes
R17	White	Irregular	Flat	Undulate	+	Rods	Yes
R18	White	Irregular	Flat	Undulate	+	Rods	Yes
R19	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R20	White	Irregular	Flat	Ciliate	+	Rods	Yes
R21	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R22	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R23	White	Irregular	Flat	Undulate	+	Rods	Yes
R24	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R25	Cream	Circular	Flat	Undulate	+	Rods	Yes
R26	Cream	Circular	Flat	Undulate	+	Rods	Yes
R27	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R28	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R29	White	Irregular	Flat	Undulate	+	Rods	Yes
R30	White	Irregular	Flat	Undulate	+	Rods	Yes
R31	White	Irregular	Flat	Undulate	+	Rods	Yes
R32	Cream	Irregular	Flat	Branching	+	Rods	Yes
R33	Brown	Irregular	Flat	Branching	+	Rods	Yes
R34	Brown	Irregular	Raised	Undulate	+	Rods	Yes
R35	Yellow	Irregular	Raised	Entire	+	Rods	Yes
R36	Yellow	Irregular	Raised	Smooth	+	Rods	Yes
R37	Yellow	Circular	Raised	Smooth	+	Rods	Yes
R38	White	Irregular	Raised	Branching	+	Rods	Yes
R39	White	Irregular	Flat	Branching	+	Rods	Yes
R40	White	Irregular	Flat	Branching	+	Rods	Yes
R41	Cream	Irregular	Raised	Smooth	-	Rods	No
R42	Cream	Irregular	Raised	Smooth	-	Rods	No
R43	Cream	Irregular	Flat	Smooth	-	Rods	No
R44	White	Irregular	Umbonate	Ciliate	+	Rods	Yes
R45	White	Irregular	Umbonate	Undulate	+	Rods	Yes
R46	White	Irregular	Umbonate	Undulate	+	Rods	Yes

R47	White	Irregular	Flat	Ciliate	+	Rods	Yes
R48	White	Irregular	Flat	Undulate	+	Rods	Yes
R49	White	Irregular	Flat	Ciliate	+	Rods	Yes
R50	White	Irregular	Umbonate	Ciliate	+	Rods	Yes
R51	White	Irregular	Umbonate	Undulate	+	Rods	Yes
R52	White	Irregular	Flat	Entire	+	Rods	Yes
R53	White	Irregular	Flat	Entire	+	Rods	Yes
R54	Cream	Circular	Raised	Entire	-	Rods	No
R55	Cream	Circular	Raised	Smooth	-	Rods	No
R56	Brown	Circular	Flat	Smooth	-	Rods	No
R57	White	Irregular	Flat	Ciliate	+	Rods	Yes
R58	White	Irregular	Raised	Undulate	+	Rods	Yes
R59	White	Circular	Raised	Ciliate	+	Rods	Yes
R60	White	Irregular	Flat	Ciliate	+	Rods	Yes

4.2. Morphological characterization of bacterial isolates

Colonies grew within 48hrs of incubation at 37°C. The colony morphology of the isolates obtained ranged from circular, undulate, flat, filamentous and branching as indicated in Plate 4.2.

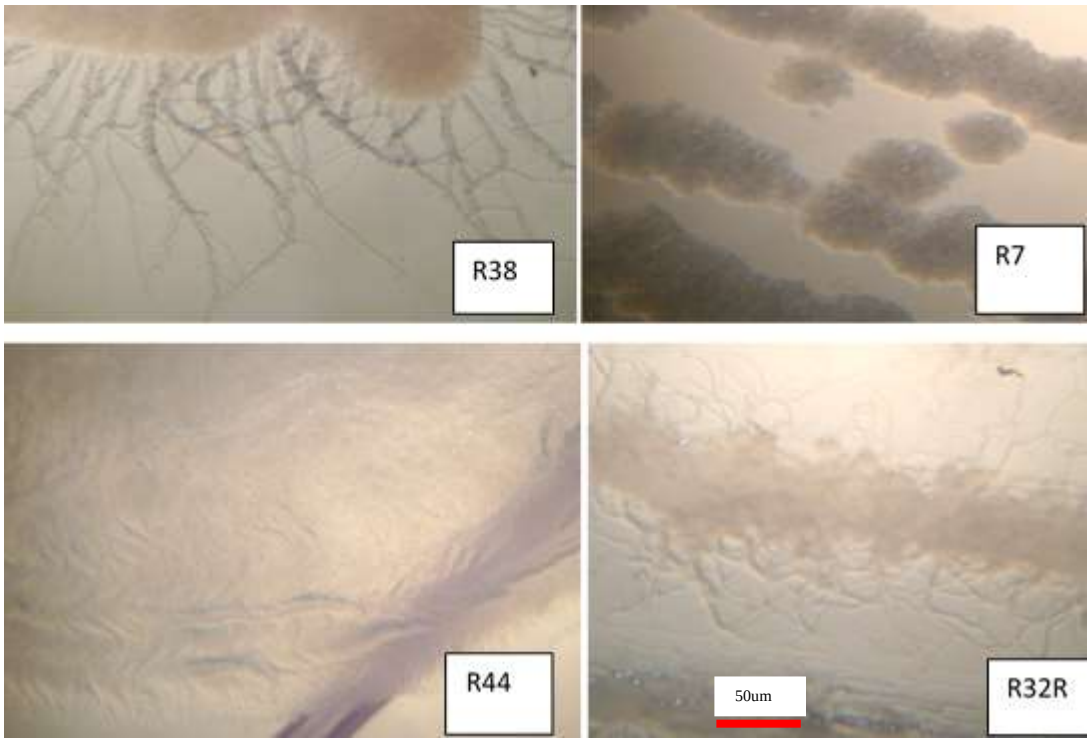

Plate 4.2: Morphological appearances of the bacterial colonies under dissecting microscope

Legend: Irregular, raised and branching (R38), irregular, raised and undulate (R7), irregular, umbonate and ciliate (R44), irregular, flat and branching (R32).

4.2.1 Biochemical characteristics of the presumptive *Bacillus* species

Only 54 (90%) of the Gram-positive spore-forming rods presumptive of *Bacillus* species were used in the biochemical test analysis.

Results for the various biochemical assays namely, catalase test, indole test, starch hydrolysis, nitrate reduction, MR-VP, motility test, triple iron sugar utilization test, gelatine hydrolysis and hydrogen sulphide gas production are shown on Table 4.2.

The results showed that 29 (54%) isolates were motile while 25 (46%) were negative for motility test (Table 4.2). The ability of the isolates to excrete intracellular enzymes was determined through tests on catalase reaction and Voges-Proskauer.

Catalase test revealed that all isolates were positive hence could produce of hydrogen peroxide as an end product of oxidation of sugars. All isolates were positive for the Triple Iron Sugar utilization test and nitrate reduction test except R35, R36 and R37 since the addition of sulphanilic acid and alpha- naphthylamine reacted with nitrite released from nitrate and turned red (Table 4.2). Most isolates were positive for the nitrogen reduction test except isolate R35, R36 and R37 which were negative for extracellular amylase enzyme. Starch hydrolysis test is used to differentiate bacteria based on their ability to hydrolyze starch with the enzyme α-amylase, starch is a polysaccharide consisting of α- D-glucose subunits that exists in two forms amylose (straight chain polymer) and amylopectin (a larger branched polymer with phosphate groups). Since starch is too large to pass through bacterial membrane, these enzymes are needed to hydrolyze it into smaller fragments of glucose molecules making it available for bacteria uptake (Harold, 2002). Therefore, when the bacteria that produce these enzymes are cultivated on starch agar, they hydrolyze the starch around the area of growth. But since both starch and its sugar subunits are invisible in the medium, iodine reagent is used to detect the presence or absence of starch in the around the bacterial growth. Iodine reacts with starch and produces a blue or dark brown color; therefore, any microbial starch hydrolysis was revealed as a clear zone surrounding the growth (Cappuccino and Sherman, 2001; Joanne *et al.*, 2016).

All isolates were negative for indole test. Indole generation by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid was tested for the isolates. Tryptophanase enzyme catalyzes the deamination reaction, during which the amine ($-NH_2$) group of the tryptophan molecule is removed and final products of the reaction are indole, pyruvic acid, ammonia (NH_3), hydrogen sulphide and energy. The test was performed to show the ability of bacteria to split the amino acid tryptophan to indole, pyruvic acid and ammonia with the help of tryptophanase enzyme. The negative

results showed that isolates were unable to produce indole as a result of amino acid tryptophan breakdown attributed to lack of tryptophanase in the cell (Rezwan *et al.*, 2004). The presence of indole can be detected by the addition of Kovacs' reagent which reacts with the indole, producing a bright red compound on the surface of the medium. The test is important in differentiating members of family *Enterobacteriaceae* and genus *Bacillus* (Prescott, 2002).

Gelatin is a collagenous protein a component of animal connective tissues. Gelatin hydrolysis is a test that was used to detect the ability of the isolates to produce proteolytic enzyme (gelatinase) which causes the breakdown of this complex protein derivative to polypeptides. These polypeptides are further converted into single amino acids that bacteria can easily use for their metabolic process. Therefore, bacteria that hydrolyzed gelatin indicated the presence of gelatinase enzymes. All isolates were positive for gelatin liquefaction test (Table 4.2). This test is used to identify and differentiate different species of *Bacillus*, *Clostridium*, *Pseudomonas* and family *Enterobacteriaceae* (Prescott, 2002).

Most isolates were negative for methyl red test showing the inability of the isolates to ferment glucose and produce a lot of mixed acids as end products (Table 4.2) except R34, R35, R36, and R37 were positive.

Table 4.2: Biochemical characteristics of presumptive *Bacillus s*pecies isolated from *R. argentea*

						TSI							
Isolate#	Starch	Catalase	Indole	Motility	Gelatine	Butt	Slant	H_2S	Citrate	MR	VP	Nitrate	Bacterial spp
R_1	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_2	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_3	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_4	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_5	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_6	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_7	+	+	-	-	+	+++	++	-	+	-	+	+	*Bacillus mycoides*
R_8	+	+	-	-	+	++	+++	-	+	-	+	+	*Bacillus mycoides*
R_9	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{10}	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{11}	+	+	-	-	+	+ +	+ +	-	+	-	+	+	*Bacillus mycoides*
R_{12}	+	+	-	-	+	+ +	+ +	-	+	-	+	+	*Bacillus mycoides*
R_{13}	+	+	-	-	+	+++	++	-	+	-	+	+	*Bacillus mycoides*
R_{14}	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{15}	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{16}	+	+	-	-	+	++	+++	-	-	-	+	+	*Bacillus mycoides*
R_{17}	+	+	-	-	+	+++	+++	-	+	-	+	+	*Bacillus mycoides*
R_{18}	+	+	-	-	+	++	+++	-	-	-	+	+	*Bacillus mycoides*
R_{19}	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{20}	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{21}	+	+	-	-	+	+++	+++	-	+	-	+	+	*Bacillus mycoides*
R_{22}	+	+	-	-	+	++	+++	-	+	-	+	+	*Bacillus mycoides*
R_{23}	+	+	-	-	+	++	++	-	-	-	+	+	*Bacillus mycoides*
R_{24}	+	+	-	-	+	+	+++	-	+	-	+	+	*Bacillus mycoides*
R_{25}	+	+	-	-	+	+	+++	-	+	-	+	+	*Bacillus mycoides*
R_{26}	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{27}	+	+	-	-	+	+++	+++	-	+	-	+	+	*Bacillus mycoides*
R_{28}	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{29}	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{30}	+	+	-	-	+	++	++	-	+	-	+	+	*Bacillus mycoides*
R_{31}	+	+	-	-	+	++	+++	-	+	-	+	+	*Bacillus mycoides*
R_{32}	+	+	-	+	+	++	+++	-	+	-	+	+	*B. thuringiensis*

R_{33}	+	+	-	+	+	++	+++	-	+	-	+	+	*B. thuringiensis*
R_{34}	+	+	-	+	+	++	+++	-	+	+	+	+	*B. thuringiensis*
R_{35}	-	+	-	+	+	++	++	-	+	+	+	-	*Bacillus pumilus*
R_{36}	-	+	-	+	+	++	++	-	+	+	+	-	*Bacillus pumilus*
R_{37}	-	+	-	+	+	++	++	-	+	+	+	-	*Bacillus pumilus*
R_{38}	+	+	-	+	+	++	++	-	+	-	-	+	*Bacillus cereus*
R_{39}	+	+	-	+	+	++	++	-	+	-	-	+	*Bacillus cereus*
R_{40}	+	+	-	+	+	++	++	-	+	-	-	+	*Bacillus cereus*
R_{44}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{45}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{46}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{47}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{48}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{49}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{50}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{51}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{52}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{53}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{57}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{58}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{59}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*
R_{60}	+	+	-	+	+	++	++	-	+	-	+	+	*Bacillus subtilis*

Key: (+) Positive/ less colour, (-) Negative, (++) partial colour (+++) intense colour, TSI: Triple Sugar Iron agar, MR: Methyl Red, VP: Voges-Proskauer and H_2S: Hydrogen Sulphide gas. The results were interpreted according to Bergey & Holt (1994) Vos, *et al*. (2011)

4.2.2 Antimicrobial activity screening of the *Bacillus* species isolates against mastitis pathogens

All the 54 isolates were tested for their antimicrobial activity against mastitis causing bacterial pathogens and their ability to inhibit growth of standard microorganisms; *E. coli* ATCC-25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 15442 and *K. pneumoniae* BAA-1705 using agar diffusion method (plate 4.3).

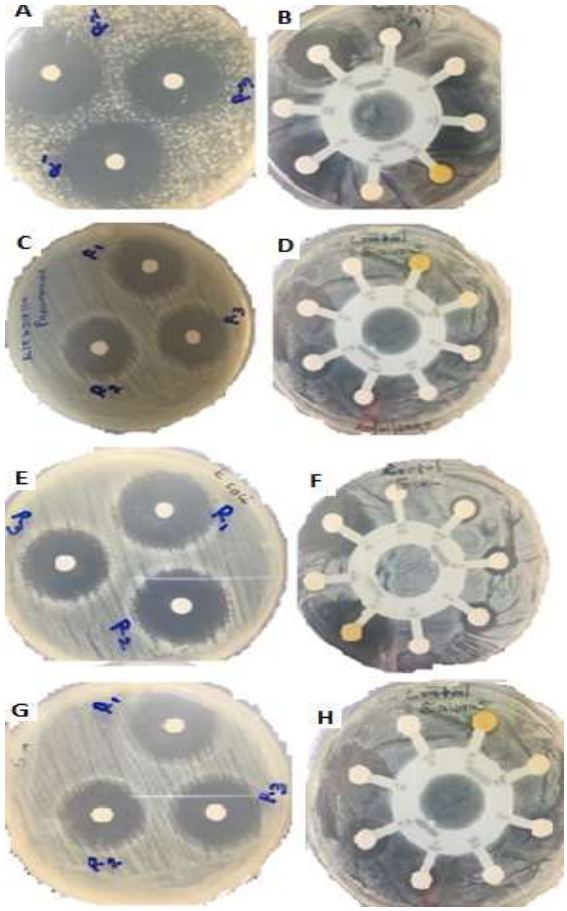

Plate 4.3: Antimicrobial activity against mastitis causing pathogens

(A), zones of inhibition of selected isolates of crude bacteriocin (R1, R2 and R3) on *P. aeruginosa* at pH 7 on nutrient agar; (B), positive control showing zones of inhibition of different antibiotics on *P. aeruginosa;* (C), zones of inhibition of selected isolates of crude

bacteriocin (R1, R2 and R3) on *K. pneumoniae* at pH 7 on nutrient agar; (D), positive control showing zones of inhibition of different antibiotics on *K. pneumoniae;* (E), zones of inhibition of selected isolates of crude bacteriocin (R1, R2 and R3) on *E. coli* at pH 7 on nutrient agar; (F), positive control showing zones of inhibition of different antibiotics on *E. coli;* (G), zones of inhibition of selected isolates of crude bacteriocin (R1, R2 and R3) on *S. aureus* at pH 7 on nutrient agar; (H): positive control showing zones of inhibition of different antibiotics on *S. aureus.*

Out of the 54 *Bacillus* presumptive isolates, twenty-eight (28) isolates showed antimicrobial activity. Twenty-two (79%) and 10 (36%) of the isolates could inhibit the growth of *E. coli* ATCC-25922 and *S. aureus* ATCC 25923 respectively (Table 4.3). Additionally, 25 (89%) and 26 (92%) could inhibit the growth of *P. aeruginosa* ATCC 15442 and *K. pneumoniae* BAA-1705. Isolates with zones of inhibition measuring between 17 to 30mm were considered sensitive, 14 to 16 mm semi-sensitive or intermediate while those below 14mm were considered resistant (based on controls). Thirty-nine percent of the isolates were sensitive against *E. coli* ATCC-25922 (zones of inhibition measuring between 17 to 30mm), while only 7% were able to inhibit *S. aureus* ATCC 25923. The results also showed that 42.8% and 39.2 % of the isolates produced bacteriocins that inhibited *P. aeruginosa* and *K. pneumoniae* BAA-1705 respectively (Table 4.3).

Table 4.3: Antimicrobial activity of supernatant crude bacteriocin products obtained from different *Bacillus* isolates on standard test organisms

Bacillus spp	Isolate	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 15442	*K. pneumoniae* ATCC BAA-1705
B. subtilis	R1	^{s}23.7 ± 2.5	^{s}24.7 ± 1.5	^{s}20.7 ± 1.2	^{s}21.7 ± 1.3
	R2	^{i}16.3 ± 1.5	^{r}2.7 ± 1.2	^{r}1.7 ± 1.1	^{i}12.7 ± 1.1
	R3	^{s}23.3 ± 1.5	^{i}12.7 ± 1.1	^{s}21.7 ± 1.9	^{r}18.7 ± 1.6
	R4	^{s}23.0 ± 1.0	^{s}18.0 ± 1.0	^{i}10.0 ± 1.7	^{s}21.0 ± 1.5
	R5	^{i}15.0 ± 1.0	^{i}14.3 ± 1.5	^{s}18.3 ± 1.4	^{r}1.3 ± 0.9
	R6	^{s}18.0 ± 1.0	^{r}10.0 ± 1.0	^{i}11.0 ± 0.8	^{s}19.0 ± 1.0
	R44	^{s}22.0 ± 1.0	^{r}10.0 ± 1.0	^{r}17.0 ± 1.1	^{s}21.0 ± 1.3
	R45	^{s}23.7 ± 1.5	^{s}20.0 ± 1.0	^{s}20.0 ± 1.0	^{s}20.0 ± 1.0
	R46	^{i}15.3 ± 1.5	^{r}7.0 ± 1.0	^{i}10.0 ± 1.2	^{i}12.0 ± 0.9
	R47	^{r}11.3 ± 0.6	^{r}4.0 ± 1.0	^{r}7.0 ± 0.7	^{i}10.0 ± 1.1
	R48	^{i}14.0 ± 1.0	^{r}7.0 ± 1.0	^{r}9.0 ± 0.6	^{s}17.0 ± 1.4
	R49	^{r}11.0 ± 1.0	^{r}2.7 ± 1.5	^{i}15.7 ± 1.5	^{s}21.7 ± 1.0
	R50	^{s}17.3 ± 1.5	^{r}11.0 ± 1.0	^{r}12.0 ± 1.1	^{i}9.0 ± 0.7
	R51	^{s}25.0 ± 1.0	^{i}15.3 ± 0.6	^{r}19.3 ± 0.9	^{r}11.3 ± 0.4
	R52	^{s}23.7 ± 1.0	^{r}5.3 ± 0.6	^{i}12.3 ± 0.4	^{r}5.3 ± 0.2
	R53	^{i}15.0 ± 1.5	^{r}13.3 ± 1.5	^{s}18.3 ± 1.1	^{r}12.3 ± 1.1
	R57	^{s}24.0 ± 1.0	^{r}12.0 ± 1.0	^{s}18.0 ± 1.2	^{r}11.0 ± 1.0
	R58	^{s}21.3 ± 1.0	^{s}18.7 ± 1.2	^{i}8.7 ± 1.0	^{s}19.7 ± 1.3
	R59	^{s}20.3 ± 1.5	^{s}22.3 ± 1.5	^{s}21.3 ± 1.1	^{i}12.3 ± 1.0
	R60	^{s}23.7 ± 1.5	^{s}25.0 ± 1.0	^{r}15.0 ± 1.3	^{r}5.0 ± 0.2
***B. pumilus* group**	R35	^{r}12.0 ± 1.0	^{s}24.7 ± 1.0	^{r}14.7 ± 1.1	^{s}20.7 ± 1.4
	R36	^{s}17.0 ± 1.0	^{s}21.0 ± 1.0	^{s}19.0 ± 1.2	^{r}11.0 ± 0.8
	R37	^{r}10.3 ± 0.6	^{s}22.0 ± 1.0	^{s}21.0 ± 1.1	^{r}12.0 ± 0.9
***B. mycoides* group**	R7	^{s}17.7 ± 1.5	^{r}1.7 ± 0.6	^{r}11.7 ± 0.4	^{s}18.7 ± 0.7
	R10	^{i}16.7 ± 0.6	NI	NI	NI
	R13	^{i}11.0 ± 1.0	NI	NI	^{r}11.7 ± 0.4
	R14	^{s}17.0 ± 1.0	^{r}12.3 ± 0.6	^{r}11.3 ± 0.2	^{r}12.3 ± 0.3
	R24	^{s}17.3 ± 1.0	NI	NI	NI

Key: NI: no inhibition, r: resistant, s: sensitive, i: semi-sensitive/intermediate. Values are the means ± standard deviations of triplicate measurements; Data represents mean inhibition zones (diameter, mm) against tested bacteria

Isolates that showed antimicrobial activity against *E. coli* ATCC-25922 and *S. aureus* ATCC 25923 were further characterized based on carbohydrates fermentation using API 50 CH B/E kits (Table 4.4).

Table 4.4: Kirby-Bauer Antibiotic Sensitivity assay against *S. aureus, E. coli, P. aeruginosa* and *K. pneumoniae*

Bacteria	Amp	TE25	CoT25	S10	K30	GEN10	SX200	C30
S. aureus	NI	^{i}9.0	NI	^{r}15.0	^{r}14.0	^{i}9.0	NI	^{r}10.0
E. coli	NI	^{s}25.0	NI	^{r}11.0	^{r}11.0	^{i}9.0	NI	^{s}23.0
P. aeruginosa	NI	^{i}8.0	NI	^{r}13.0	^{i}9.0	^{s}19.0	NI	^{s}22.0
K. pneumoniae	NI	^{s}20.1	NI	^{r}14.1	^{i}8.0	^{r}10.1	NI	^{s}23.2

Legend: Amp: Ampicillin; [10ug/ml] TE25 Tetracycline [30ug/ml]; CoT25: Cotrimoxazole [10ug/ml]; S10:Streptomycin; [10ug/ml] K30: Kanamycin[10ug/ml]; GEN10: Gentamicin; SX200 [10ug/ml]: Sparfloxacin [5ug/ml] and C30: Chloramphenicol [10ug/ml]

Key: NI: no inhibition, r: resistant, s: sensitive, i: semi-sensitive/intermediate. Values are the means ± standard deviations of triplicate measurements

4.2.3 Characterization of bacteria using the analytical profile index system

API 50 CHB/E is intended for the identification of *Bacillus* species and related genera as well as Gram-negative rods. It is ready-to-use medium which allows the fermentation of the 49 carbohydrates on the API CH strip to be studied. The 28 isolates that showed antimicrobial activity against *E. coli* ATCC-25922, *S. aureus* ATCC 25923, *K. pneumoniae* BAA-1705 and *P. aeruginosa* ATCC 15442 and were subjected to analytical profile index system identification (Table 4.5).

Table 4.5: Characterization of *Bacillus* sp using the API profile index system

	Carbohydrates fermentation using analytical profile index system (API 50 CH B/E)																													
TEST SUGARS	R1	R2	R3	R4	R5	R6	R7	R10	R13	R14	R24	R35	R36	R37	R44	R45	R46	R47	R48	R49	R50	R51	R52	R53	R57	R58	R59	R60
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	-	+	-	+	+	+
D-ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
D-xylose (DXYL)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-xylose (ADO)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-beta-D-xylopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-alpha-D-mannopyranoside	+	-	-	-	+	-	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	-	-	-	-	+	-	+
Methyl-alpha-D-glucopyranoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-acetylglucosamine	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin ferric citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-cellobiose	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-lactose	+	-	+	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+
D-melibiose	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+
D-saccharose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-melezitose	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-
D-raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amidon	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+
Glycogen	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	-	+	+	+
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentiobiose	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-turanose	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-lyxose (LYX)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-tagatose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium gluconate	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium 2-ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium 5-ketogluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4.2.4 Hierarchical clustering of carbohydrate fermentation profiles of *Bacillus*

The analytical profile index system showed that 20 isolates (71 %) were identified as *B. subtilis*, they include: R1, R2, R3, R4, R5, R6, R44, R45, R46, R47, R48, R49, R50, R51, R52, R53, R57, R58, R59 and R60. On the other hand, 3 isolates were identified as *B. pumilus* representing 11 % (R35, R36 and R37) while 5 (18 %) isolates were identified as *B. mycoides*, they included: R7, R10, R13, R14 and R24 (Figure 4.1).

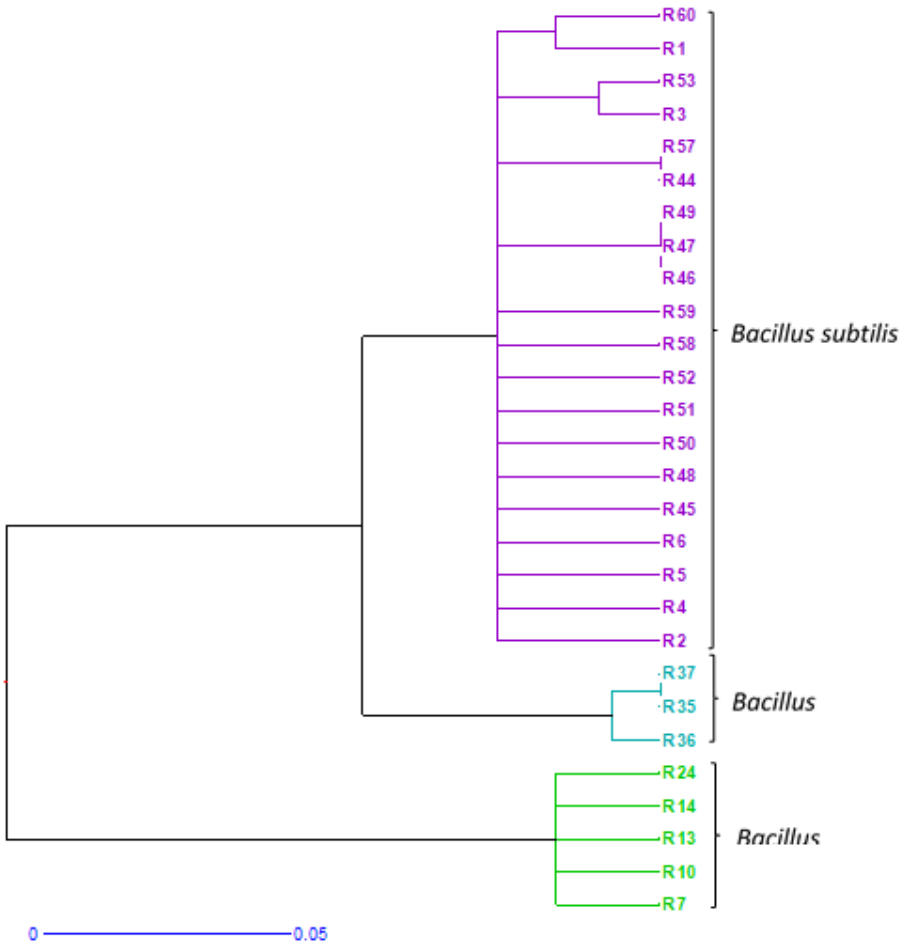

Figure 4.1: Hierarchical clustering of carbohydrate fermentation profiles of *Bacillus* sp.

The clustering was calculated using dice coefficient and the tree was constructed using unweighted pair group method with arithmetic mean (UPGMA).

4.3 Physiochemical characterization of bacteriocins from *Bacillus* species

4.3.1 Effect of pH on crude bacteriocin

The bacteriocin from different isolates were characterized at different pH conditions of pH 3, 4, 5, 6, 7 and 9. The optimum bacteriocin effect on test *E. coli* ATCC-25922; *S. aureus*

ATCC 25923; *P. aeruginosa* ATCC 15442; and *K. pneumoniae* BAA-170 was observed at pH 7 and pH 9 respectively. *S. aureus* ATCC 25923 had the least activity while *P. aeruginosa* ATCC 15442; and *K. pneumoniae* BAA-170 had the most activity. No activity was recorded on pH 3, 4, 5 in all the isolates.

The results were recored based on the presence or absence of zones of inhibition after incubation using well difussion method as shown in table 4.6 below

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Table 4.6: Residual activity of bacteriocin on standard test microorganisms at different pH levels.

	E. coli ATCC-25922						*S. aureus* ATCC 25923						*P. aeruginosa ATCC 15442*						*K. pneumoniae BAA-170*					
	pH 3	pH 4	pH 5	pH 6	pH 7	pH 9	pH 3	pH 4	pH 5	pH 6	pH 7	pH 9	pH 3	pH 4	pH 5	pH 6	pH 7	pH 9	pH 3	pH 4	pH 5	pH 6	pH 7	pH 9
R1	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++
	-	-	-	+	++	++	-	-	-	-	+	+	-	-	-	+	++	++	-	-	-	-	+ +	
R2											+													+
R3	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	-	++	++
R4	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	-	+	++	-	-	-	+	++	++
R5	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	-	++	++
R6	-	-	-	+	++	++	-	-	-	-	+	+	-	-	-	+	++	++	-	-	-	-	+	+
R44	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++
R45	-	-	-	+	++	++	-	-	-	-	+	+	-	-	-	+	++	++	-	-	-	-	+	+
R46	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	-	++	++
R47	-	-	-	+	++	++	-	-	-	-	+	+	-	-	-	+	+	++	-	-	-	+	+	+
R48	-	-	-	+	++	++	-	-	-	-	+	+	-	-	-	+	++	++	-	-	-	-	+	+
R49	-	-	-	+	++	++	-	-	-	-	+	+	-	-	-	+	++	++	-	-	-	-	+	+
R50	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	-	++	++	-	-	-	+	++	++
R51	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	-	++	++
R52	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	+	-	-	-	+	++	++
R53	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++
R57	-	-	-	+	++	+	-	-	-	+	++	+	-	-	-	-	++	+	-	-	-	+	+	+
R58	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	-	++	++	-	-	-	+	++	++
R59	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	-	++	++
R60	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++
R35	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++
R36	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	-	++	++	-	-	-	+	++	+
R37	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++
R7	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+
R10	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
R13	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+	+
R14	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
R24	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	++	-	-	-	-	+	-

Key: E. coli ATCC-25922, *S. aureus* ATCC 25923, *K. pneumoniae* BAA-1705 and *P. aeruginosa* ATCC 15442 at different pH levels. Key: - Shows there was no zone of inhibition, + showed a zone of inhibition (range 11-17mm), ++ (range 18-25m

4.3.2 Effect of different temperatures on crude bacteriocins activity

On exposure of the crude bacteriocin to different temperature levels, the zones of inhibition were measured and residual activity calculated. Table 4.7 below shows the extent to which the bacteriocin lost their activity as temperatures increased. An exposure of the crude bacteriocins to temperatures of 50 ^{o}C and 60 ^{o}C showed no loss of activity based on the initial activity. As the temperature increased to between 70 ^{o}C to 80 ^{o}C it caused a reduction in the bacteriocin activity of about 20%. After exposure to 100 ^{o}C, about 40% of the bacteriocin activity was lost. With an increase of the temperatures to 121 ^{o}C, more than 50% of the activity was lost.

Table 4.7: Effect of different temperature levels on crude bacteriocin activity against test bacterial strains.

	***E. coli* ATCC-25922**						***S. aureus* ATCC 25923**						***P. aeruginosa* ATCC 15442**						***K. pneumoniae* BAA-1705**					
	50 ºC	60 ºC	70 ºC	80 ºC	100 ºC	121 ºC	50 ºC	60 ºC	70 ºC	80 ºC	100 ºC	121 ºC	50 ºC	60 ºC	70 ºC	80 ºC	100 ºC	121 ºC	50 ºC	60 ºC	70 ºC	80 ºC	100 ºC	121 ºC
R1	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R2	-	-	+	+	++	+++	-	-	+	+	+ +	+++	-	-	+	+	++	+++	-	-	+	+	+ +	+++
R3	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R4	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R5	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R6	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R44	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R45	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R46	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R47	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R48	-	-	+	+	++	+++	-	-	+	+	++	+ ++	-	-	+	+	++	+++	-	-	+	+	++	+ ++
R49	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R50	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R51	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R52	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R53	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R57	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R58	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R59	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R60	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R35	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R36	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R37	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++

R7	-	-	+	-	++	+++	-	-	+	+	++	+++	-	-	+	-	++	+++	-	-	+	+	++	+++
R10	-	-	+	-	++	+++	-	-	+	+	++	+++	-	-	+	-	++	+++	-	-	+	+	++	+++
R13	-	-	+	-	++	+++	-	-	+	+	++	+++	-	-	+	-	++	+++	-	-	+	+	++	+++
R14	-	-	+	-	++	+++	-	-	+	+	++	+++	-	-	+	-	++	+++	-	-	+	+	++	+++
R24	-	-	+	-	++	+++	-	-	+	+	++	+++	-	-	+	-	++	+++	-	-	+	+	++	+++

Key: shows - No loss of Residual activity lost, + between 20% Residual activity lost, ++ Between 40% Residual activity lost, +++ More than 50% Residual activity lost. This was based on the percentage of initial activity

4.3.3 Effect of different metal ions on crude bacteriocins activity

Metal ions at the concentration of 1mM were added to a partially purified bacteriocin. Different metal ions were used to find out how they significantly influenced the activity of the crude bacteriocins. All the metal ions did not have a great reduction in the activity of bacteriocins. The residual activity was determined as a percentage ration of the initial bacteriocin activity (Table 4.8).

Table 4.8: Effect of metal ions on crude bacteriocin activity against test bacterial strains

	***E. coli* ATCC-25922**			***S. aureus* ATCC-25923**			***P. aeruginosa ATCC 15442***			***K. pneumoniae BAA-1705***		
Isolate	**Cu^{2+}**	**Fe^{2+}**	**Zn^{2+}**	**Cu^{2+}**	**Zn^{2+}**	**Fe^{2+}**	**Cu^{2+}**	**Fe^{2+}**	**Zn^{2+}**	**Cu^{2+}**	**Fe^{2+}**	**Zn^{2+}**
R1	+++	+++	+	+++	+	++	+++	++	+	+++	++	+
R2	+++	++	++	++	++	++	+++	++	+	+++	++	+
R3	+++	++	+	++	+	++	+++	++	+	+++	++	+
R4	+++	++	+	++	+	++	+++	++	+	+++	++	+
R5	+++	++	+	++	+	++	+++	++	+	+++	++	+
R6	+++	+++	++	+++	++	++	+++	++	+	+++	++	+
R7	++	+	+	+	+	++	++	++	++	++	++	++
R10	++	+	+	+	+	+	++	+	+	++	+	+
R13	++	++	+	++	+	++	++	++	+	++	++	+
R14	++	+	+	+	+	+	++	+	+	++	+	+
R24	++	++	+	++	+	++	++	++	+	++	++	+
R35	+++	++	++	++	++	++	+++	++	++	+++	++	++
R36	+++	++	+	++	+	++	+++	++	++	+++	++	++
R37	+++	++	++	++	++	++	+++	++	+	+++	++	+
R44	+++	++	++	++	++	++	+++	++	+	+++	++	+
R45	+++	+++	++	+++	++	++	+++	++	+	+++	++	+
R46	+++	++	++	++	++	++	+++	++	+	+++	++	+
R47	+++	++	+	++	+	++	+++	++	+	+++	++	+
R48	+++	+++	+	+++	+	++	+++	++	+	+++	++	+
R49	+++	++	+	++	+	++	+++	++	+	+++	++	+
R50	+++	++	++	++	++	++	+++	++	+	+++	++	+
R51	+++	++	++	++	++	++	+++	++	+	+++	++	+
R52	+++	++	++	++	++	++	+++	++	+	+++	++	+
R53	+++	+++	++	+++	++	++	+++	++	+	+++	++	+
R57	+++	++	++	++	++	++	+++	++	+	+++	++	+
R58	+++	++	+	++	+	++	+++	++	+	+++	++	+
R59	+++	++	++	++	++	++	+++	++	+	+++	++	+
R60	+++	++	++	++	++	++	+++	++	+	+++	++	+

Key: +++ Residual activity between (91-100%), ++ Residual activity between (81-90%) and + Residual activity between 71- 80%. This was based on the percentage of initial activity.

4.3.4 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 4.9).

Table 4.9: Effect of enzymes on crude bacteriocin activity against test strains

	E. coli ATCC-25922			*S. aureus* ATCC-25923			*P aeruginosa ATCC 15442*			*K pneumoniae BAA-1705*		
Isolate	**Proteokinase K**	**Trypsin**	**Lipase**	**Proteokinase K**	**Trypsin**	**Lipase**	**Proteokinase K**	**Trypsin**	**Lipase**	**Proteokinase K**	**Trypsin**	**Lipase**
R1	-	+	++	-	+	++	-	+	++	-	+	++
R2	-	+	++	-	+	++	-	+	++	-	+	++
R3	-	+	++	-	+	++	-	+	++	-	+	++
R4	-	+	++	-	+	++	-	+	++	-	+	++
R5	-	+	++	-	+	++	-	+	++	-	+	++
R6	-	+	++	-	+	++	-	+	++	-	+	++
R7	-	+	++	-	+	++	-	+	++	-	+	++
R10	-	+	++	-	+	++	-	+	++	-	+	++
R13	-	+	++	-	+	++	-	+	++	-	+	++
R14	-	+	++	-	+	++	-	+	++	-	+	++
R24	-	+	++	-	+	++	-	+	++	-	+	++
R35	-	+	++	-	+	++	-	+	++	-	+	++
R36	-	+	++	-	+	++	-	+	++	-	+	++
R37	-	+	++	-	+	++	-	+	++	-	+	++
R44	-	+	++	-	+	++	-	+	++	-	+	++
R45	-	+	++	-	+	++	-	+	++	-	+	++
R46	-	+	++	-	+	++	-	+	++	-	+	++
R47	-	+	++	-	+	++	-	+	++	-	+	++
R48	-	+	++	-	+	++	-	+	++	-	+	++
R49	-	+	++	-	+	++	-	+	++	-	+	++
R50	-	+	++	-	+	++	-	+	++	-	+	++
R51	-	+	++	-	+	++	-	+	++	-	+	++
R52	-	+	++	-	+	++	-	+	++	-	+	++
R53	-	+	++	-	+	++	-	+	++	-	+	++
R57	-	+	++	-	+	++	-	+	++	-	+	++
R58	-	+	++	-	+	++	-	+	++	-	+	++
R59	-	+	++	-	+	++	-	+	++	-	+	++
R60	-	+	++	-	+	++	-	+	++	-	+	++

Key: - shows no hallow (no inhibition) after bacteriocin exposure to the enzyme, + shows a reduced hallow (average 60% residual activity) after bacteriocin exposure to the enzyme, ++Shows a slightly reduced hallow (average 85% residual activity) after bacteriocin exposure to the enzyme. This was based on the percentage of initial activity.

4.4 Molecular characterization

4.4.1 PCR amplification of 16S rRNA genes from isolates

Genomic DNA was extracted from 28 selected isolates using QIAamp DNA Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Amplification of 16S rRNA gene with bacterial universal primers bac 27F and bac 1492R (Hogg and Lehane, 1999) yielded an amplification product of approximately 1500bp (plate 4.5). The amplicons were then stained with ethidium bromide and visualised under UV light on 1 % agarose gel (Fig 4.2).

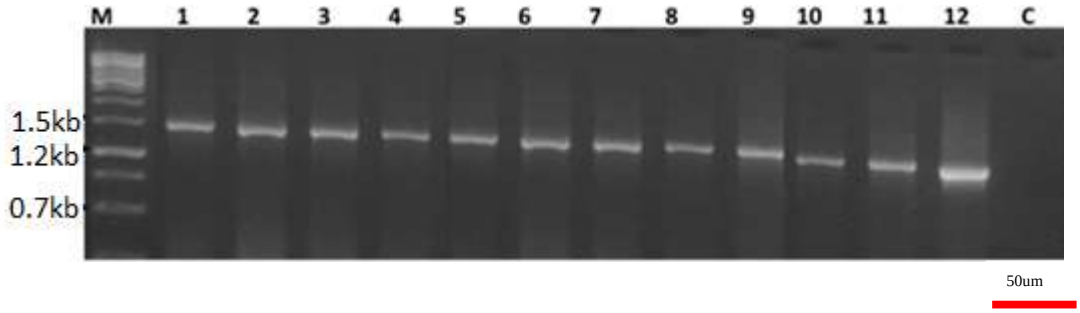

Figure 4.2: Agarose gel photograph of PCR amplification of 16S rDNA of the isolates visualized after ethidium bromide staining.

M (DNA marker), 1 (R1), 2 (R4), 3 (R7), 4 (R14), 5 (R35), 6 (R36), 7 (R37), 8 (R44), 9 (R45), 10 (R51), 11 (R59), 12 (R60) and C (negative control)

4.4.2 Phylogenetic analysis of the sequences

A total of 28 isolates were sequenced and used to construct the phylogenetic tree. The BLAST analysis of the partial sequences showed that 100% of the strains belonged to the genus *Bacillus* within the Firmicutes in the domain bacteria (Table 4.10 with sequence similarities ranging from 96.3% to 100%. Among these were *B. subtilis*, *B. pumilus* and *B. mycoides* (Table 4.10). The result showed twenty (20) isolates or 71% belonged to *B. subtilis* with similarities ranging between 96.2% and 99.7% (Table 4.10). *B. mycoides* represented 18% (five isolates) with similarities between 99.9% and 100% while *B. pumilus* constituted 11% (three isolates) with percentage similarities ranging

from 98.4% to 100% (Table 4.10). However, isolate R1, R2, R3, R37, R53, R57 and R58 had sequence similarity of between 96.2-98.4% and this could represent novel species (Kim *et al.*, 2014) (Table 4.10).

Table 4.10: BLAST analysis results of the isolates nearest neighbours in the data bank and their percentage relatedness

Isolate	Next Neighbour	% Similarity	Query coverage (%)	E-value	Accession number
R1	*Bacillus subtilis* subsp. subtilis strain K-5	99.6	98	0	KJ856901.1
R2	*Bacillus subtilis* strain CC8	96.3	99	0	MK720677.1
R3	*Bacillus subtilis* strain EMB M15	96.7	99	0	KX109607.1
R4	*Bacillus subtilis* strain JSRB 177	99.9	99	0	MN121128.1
R5	*Bacillus subtilis* strain HABIBD2	99.7	99	0	MT102635.1
R6	*Bacillus subtilis* strain FI469	99.7	99	0	MT081484.1
R7	*Bacillus mycoides* strain TH26	100	98	0	CP037992.1
R10	*Bacillus mycoides* strain TH26	100	98	0	CP037992.1
R13	*Bacillus mycoides* strain Gnyt1	99.9	99	0	CP020743.1
R14	*Bacillus mycoides* strain Gnyt1	99.9	99	0	CP020743.1
R24	*Bacillus mycoides* strain TH26	100	98	0	CP037992.1
R35	*Bacillus pumilus* strain 150a	100	100	0	CP027034.1
R36	*Bacillus pumilus* strain SH-B9	98.8	99	0	CP011007.1
R37	*Bacillus pumilus* strain NCTC10337	98.4	98	0	LT906438.1
R44	*Bacillus subtilis* strain IPA 25	99.9	99	0	MK238507.1
R45	*Bacillus subtilis* strain GW 12	99.9	100	0	MK583664.1
R46	*Bacillus subtilis* strain HR10	99.4	99	0	MN893856.1
R47	*Bacillus subtilis* strain EMB M15	99.7	99	0	KX109607.1
R48	*Bacillus subtilis* strain 181203-033_G01	99.8	100	0	MT448935.1
R49	*Bacillus subtilis* strain SY2101	99.8	100	0	MT448726.1
R50	*Bacillus subtilis* strain 181203-033_G01	99.8	100	0	MT448935.1
R51	*Bacillus subtilis* strain 50-1	99.9	99	0	MH475924.1
R52	*Bacillus subtilis* strain MEF4	99.8	100	0	MT415788.1
R53	*Bacillus subtilis* strain BJ-DEBCR-6	96.2	98	0	KU854954.1
R57	*Bacillus subtilis* strain YKF2	96.2	98	0	KU667124.1
R58	*Bacillus subtilis* strain SRSTH1	96.2	99	0	HQ398997.1
R59	*Bacillus subtilis* strain PR10	99.9	99	0	CP040528.1
R60	BRM043908	99.7	99	0	MH305341.1

Phylogenetic analysis of the isolates showed that twenty isolates clustered into species *B. subtilis*. Isolates R1, R2, R3, R4, R5, R6, R44, R45, R46, R47, R48, R49, R50, R51, R52, R53, R57, R58, R59 and R60 were closely related to *B. subtilis* strains (MN134015.1, (MK238507.1, KX109607.1, MK720677.1, MK720677.1, MT081484.1, MH475924.1, MK085082.1 and MK281529.1), isolates R7, R10, R13, R14 and R24 were grouped together with *B. mycoides* strains (CP037992.1) while isolates R35, R36 and R37 were grouped together with *B. pumilus* strain (CP027034.1) (Figure 4.3)

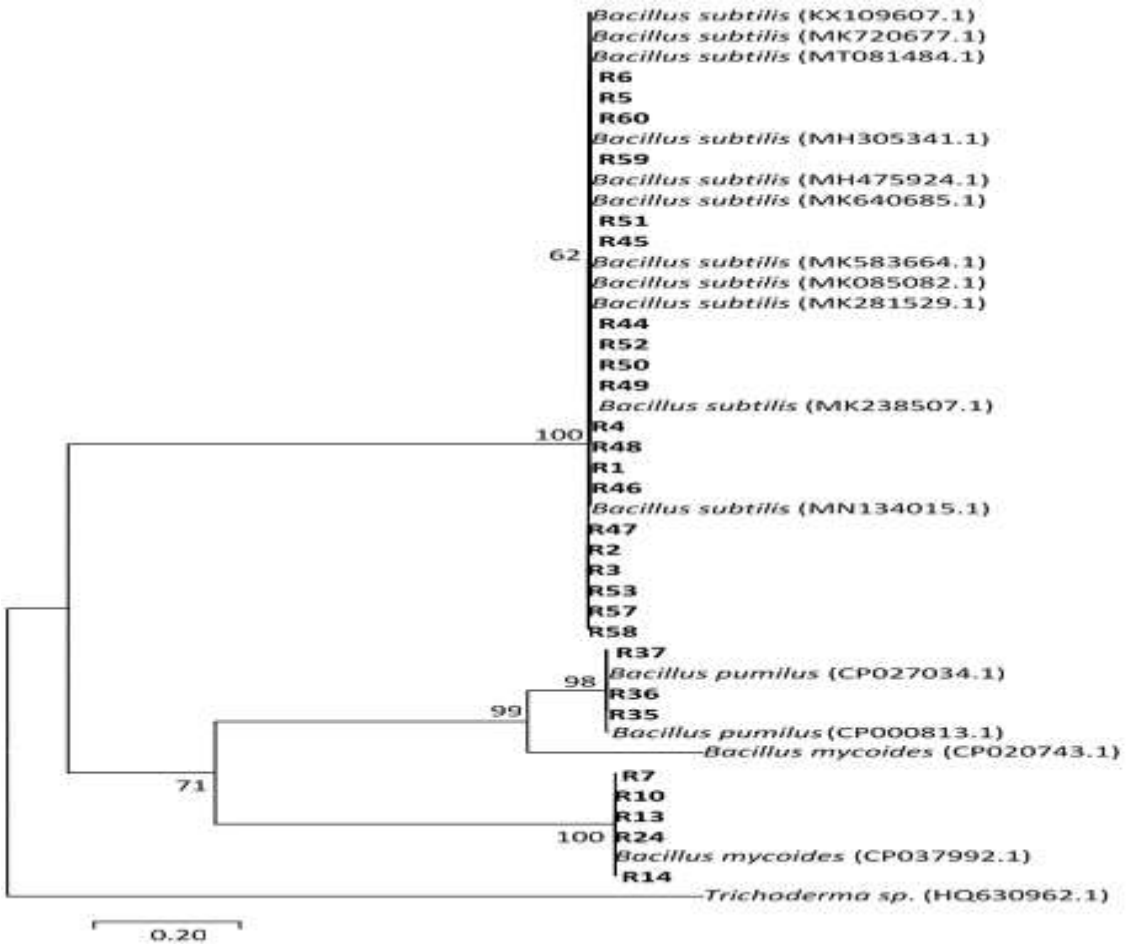

Figure 4.3: Phylogenetic tree based on 16S rRNA gene sequences

Depicts the relationship among the *R. argentea bacillus* isolates and between representatives of other related taxa.

The tree was constructed using MEGA 7.0 software package and the distance matrix inferred by the Maximum Likelihood method based on the Tamura-Nei model. The scale bar indicates 0.2 substitutions per nucleotide position. The number beside the node is the statistical bootstrap value. In brackets are the GenBank accession numbers of closest relatives. The gene sequence of *Trichoderma sp*. (HQ630962.1) was used as an out-group.

CHAPTER FIVE

DISCUSSION

This study aimed to isolate, characterize, and identify bacteriocins producing bacteria isolated from *R. argentea*, a natural source, collected from Lake Victoria with activity against bovine mastitis pathogens. The study contributes to the understanding of the use of bacteriocins in the control of diseases in dairy animal farming and milk production and processing in Kenya. Bacteriocins have been reported to offer an advantage over antibiotics because they target very specific organisms and are considered safe and friendly to the consumers. Previous studies by Maina *et al* (2015) did not identify the extent to which the physiological conditions, chemical structure, and molecular mechanisms of *B. subtilis* secretion of bacteriocins. The studies also failed to identify the microorganisms using molecular characterization which is the gold standard method in organism identification making results obtained purely speculative.

In this study the identification of putative *Bacillus* isolates producing bacteriocins was based on their morphological features, biochemical characteristics, physiochemical and antimicrobial properties, and molecular analysis using 16S rDNA. Nutrient agar was successfully used for the cultivation of bacteriocins producing *Bacillus* species. *Bacillus* are Gram-positive, rod-shaped, motile, catalase-positive, aerobic/ facultative anaerobic bacteria from the division Firmicutes (Wafula *et al.,* 2014).

They are found in varied ecological niche mostly as saprophytic especially in aquatic environments, soil, food, plant surfaces, a few are animal or insect parasites or pathogens (Abriouel *et al.,* 2011; Caulier *et al.,* 2019). They are also known to form heat-resistant endospores when cultured in growth media (Abriouel *et al.,* 2011; Maina *et al.,* 2015). Their ability to form endospores, with diverse physiological properties, as well as their ability to produce various antimicrobial compounds, favor their widespread distribution in nature (Caulier *et al.,* 2019).

The ability of the fifty-four (54) isolates to ferment glucose and produce a lot of mixed acids as end products of fermentation was determined by Triple Iron Sugar utilization test and the results showed that all isolated were positive for this test. This, therefore, indicated the ability of the isolates to carry out glucose fermentation and produce a mixture of acids as end products of fermentation (Table 4.2). The triple sugar iron agar medium can differentiate bacteria based on the fermentation of glucose, lactose, and sucrose (Dhadse *et al.,* 2012; Issazadeh *et al.,* 2013). The preparation of the medium is done as a shallow agar slant with a deep butt, for providing a growth environment for both the aerobic and anaerobic microorganisms. Hence, the microorganisms that can ferment glucose and lactose and/or sucrose were able to turn the medium yellow throughout. Since lactose and sucrose concentrations are more than that of glucose, both the butt and slant remained yellow after 24 hours (Cappuccino & Sherman, 2014). Microorganisms that cannot ferment any of the carbohydrates but uses animal proteins will alkalinize the medium and turn it red. If the organism can use the proteins aerobically and anaerobically, both the slant and butt will appear red. An obligate aerobe will only turn the slant red (Amon *et al.,* 2007; Chen *et al.,* 2008).

The isolates were tested for their ability to produce indole pyruvic acid by deamination of amino acid tryptophan. This is facilitated by an enzyme tryptophanase which catalyzes the deamination reaction, where the amine ($-NH_2$) group of the tryptophan molecule is removed and final products of the reaction are indole, pyruvic acid, ammonia (NH_3), hydrogen sulphide and energy. The positive indole test showed the ability of the isolates to deaminate and hydrolyse amino acids to pyruvic acid and ammonia while the negative results showed that isolates were unable to produce indole as a result of amino acid tryptophan breakdown attributed to lack of tryptophanase in the cell (Rezwan *et al.,* 2004). The presence of indole can be detected by the addition of Kovacs' reagent which reacts with the indole, producing a bright red compound on the surface of the medium. All isolates were negative for this test but positive for hydrogen sulfide gas production. The test is important in differentiating members of the family Enterobacteriaceae and genus *bacillus* (Prescott, 2002). If the organism can use the

proteins aerobically and anaerobically, both the slant and butt will appear red, an obligate aerobe will turn only the slant red (Leboffe., & Pierce, 2019). Hydrogen sulphide gas is produced when microorganisms reduce sulfur compounds. This test thus showed that the isolates were able to reduce sulfur-containing compounds to sulfides during the process of metabolism (Cappuccino & Sherman, 2014).

Starch hydrolysis is a test important for differentiating microorganisms based on their ability to hydrolyze starch with the enzyme α-amylase or oligo-l, 6-glucosidase. Starch is a polysaccharide consisting of a- D-glucose subunits that exist in two forms amylose and amylopectin (a larger branched polymer with phosphate groups). Since starch is too large to pass through the bacterial membrane, these enzymes are needed to hydrolyze it into smaller fragments of glucose molecules making it available for bacteria uptake (Harold, 2002). Therefore, when the bacteria that produce these enzymes are cultivated on starch agar, they hydrolyze the starch around the area of growth. But since both starch and its sugar subunits are invisible in the medium, iodine reagent is used to detect the presence or absence of starch around the bacterial growth. Iodine reacts with starch and produces a blue or dark brown color; therefore, any microbial starch hydrolysis was revealed as a clear zone surrounding the growth (Cappuccino & Sherman, 2014; Joanne *et al.*, 2016). The results showed that all isolates except R35, R36, and R37 were positive for this test (Table 4.2). This test is important in differentiating the species of genera *Bacillus*, *Clostridium*, *Bacteroides*, *Fusobacterium* and members of *Enterococcus* spp (Bergey & Holt, 1994; Vos *et al.*, 2011).

Gelatin is a collagenous protein a component of animal connective tissues produced by hydrolysis of collagen. Gelatin hydrolysis is a test that was used to detect the ability of the isolates to produce proteolytic enzyme (gelatinase) which causes the breakdown of this complex protein derivative to polypeptides (Cappuccino & Sherman, 2014). These polypeptides are further converted into single amino acids that bacteria can easily use for their metabolic process. Therefore, that hydrolyzed gelatin indicated the presence of gelatinase enzymes. This test is used to identify and differentiate different species of *Bacillus, Clostridium, Pseudomonas* and family *Enterobacteriaceae* (Prescott, 2002).

During aerobic respiration, microorganisms produce hydrogen peroxide and, in some cases, an extremely toxic superoxide. Accumulation of these substances will result in death of the organism unless they can be enzymatically degraded. These substances are produced when aerobes, facultative anaerobes, and microaerophiles use the aerobic respiratory pathway, in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production. Organisms capable of producing catalase rapidly degrade hydrogen peroxide. The test showed that all isolates were catalase positive (Table 4.2) and it is important in differentiating aerotolerant strains of *Clostridium*, which are catalase negative, from *Bacillus*, which are catalase positive (Mahon *et al.*, 2015).

The isolates were taxonomically classified based on morphological characteristics, biochemical tests, API kit and 16S ribosomal DNA sequences which placed the isolates to the genera *Bacillus.* Antimicrobial activity of the isolates indicates different variation in terms of inhibiting *E. coli and S. aureus. B. subtilis* were dominant in inhibiting both *E. coli* and *S. aureus* compared to *B. pumilus* group. According to (Gálvez *et al*., 2012) *Bacillus* have been used as a biocontrol in the management of various plant pathogen and diseases. There are reported incidences where *B. subtilis* is used in clinical management of various pathogen compounds (Ansari *et al*., 2012; Cowan, 1999). The effectiveness of inhibition for the most *B. subtilis* varies as shown in the (Table 4.3). The results related to the finding that most of the *B. subtilis* inhibit the growth of other pathogen by either producing enzymes or through competition (Bala *et al.*, 2020; Silva *et al.*, 2018). The results (Table 4.3) correlate with previous findings on the antimicrobial activity of most isolated *B. subtilis* Barlow (2011). Silva *et al.*, (2018 a, 2018b) also established that there is a high chance of the isolated bacteria to prevent food spoilage. The use of bacteria isolated from *Omena* can add a significant impact to the food preservation since the bacteria which are isolated do not have health impact to the consumer and this makes it good alternative in preservation as also found out by Silva *et al*., (2018 a, 2018b).

The analytical profile index or API being a quick identification of clinically relevant bacteria was used for identification as seen in the results (Table 4.4). From the results *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus mycoides* were identified and classified. The result correlated with literature in that most of *Bacillus subtilis* are able to ferment carbohydrates (German *et al.,* 2008).

Temperature has an effect in the bioactivity of bacteriocin. The lower the temperature, the higher the activity and higher the temperature, the lower the bioactivity (Pilasombut *et al.,* 2015). The result agrees to this hypothesis and this was shown after the exposure of the crude bacteriocin to different temperature levels, measuring the zones of inhibition and calculating the residual activity. Table 4.6 shows the extent to which the bacteriocin lost their activity. An exposure of the crude bacteriocins to a temperature of 50 ^{o}C and 60 ^{o}C showed no loss of activity based on the initial activity. As the temperature increased to between 70 ^{o}C to 80 ^{o}C it caused a reduction in the bacteriocin activity of about 20%. After exposure to 100 ^{o}C, about 40 % of the bacteriocin activity was lost. With an increase of the temperatures to 121 ^{o}C, more than 50 % of the activity was lost. The result justifies the required temperature for stability of bacteriocins during food processing are because they act like enzymatic proteins which are sensitive to certain temperature limits. The essence of testing the temperature spectrum was to understand the *in vitro* temperatures where the bacteriocin effect would be optimum for animal treatment application. The preferred mode of bacteriocin delivery to animals is through intramammaly infusions.

The results show the decrease of bioactivity of the bacteriocin as the temperature increases. The optimum temperature recorded was at 60 °C for crude bacteriocin activity (Table 4.6). The activity gradually declines beyond 60 °C. The result relates with Adinarayana *et al.,* (2003) who investigated the optimum temperature and thermal stability of purified enzymes. According to Dhandapani & Vijayaragavan (1994), at temperature of 55 °C is the optimum temperature for alkaline protease from *B, strearothermophilus* and 60 °C for protease derived from *Bacillus spp* B21-2. Therefore, it indicates that *Bacillus spp* work well at maximum temperature of 60 °C. Above the

optimum temperature, there was declined in the bacteriocin activity affecting their action against the target microorganisms.

According to Adinarayana *et al.,* (2003), pH influences the bacteriocin activity. The result (Table 4.6) indicates that there was activity at a pH range of 6.0-9.0. The activity varies across the *Bacillus* spp and the test organism. The optimum inhibition was recorded from pH 9.0. pH had an effect on the activity of the spectrum but the acidity varied across the *Bacillus* spp tested and the test organism. pH 9 had the optimum inhibition across the *Bacillus* spp tested and the test organisms. The findings are in accordance with previous findings showing that *Bacillus* spp does well in alkaline rather than acidic (Adinarayana *et al.,* 2003). According to (Adinarayana *et al.,* 2003) for instance, the activity of *Bacillus* spp *Thermus aquaticus, Xanthomonas maltophila* and *Vibro metscnikovii* is good at pH 10.5.

It has also been established that metal ions have stimulatory and inhibitory effects on bacteriocins (Adinarayana *et al.,* 2003). From the results, (Table 4.7), Cu^{2+} had highest inhibitory effect to the bacteriocin activity while Zn^{2+} had the least inhibitory effect. According to Adinarayana *et al.,* (2003), metal ion such as $Ca^{2+,}$ Mg^{2+} and Mn^{2+} increase protease activity by stabilizing the bacteriocin. This might be due to the activation of enzymes by the metal ions. The cations have also been reported to increase thermal stability of the *Bacillus* sp (Adinarayana *et al.,* 2003). Metal ions protect the bacteriocins against thermal denaturation and play a big role in temperature regulation and maintaining the active conformation of the enzymes especially at a higher temperature (Adinarayana *et al.,* 2003).

Bacillus spp were active on Gram-negative (*E. coli, P. aeruginosa* and *K. pneumoniae*) than Gram-positive (*S. aureus*). This is in agreement with previous studies that reported that various *Bacillus* spp are more active against Gram-positive bacteria than Gram-negative bacteria (Briers and Lavigne, 2015). The susceptibility may be due to structural differences in the cell wall of these classes of bacteria. Cells of Gram-negative bacteria are surrounded by an additional outer membrane, which provides them with a

hydrophilic surface that functions as a permeability barrier for many substances including natural compounds (Hemaiswarya *et al.*, 2008; Briers and Lavigne, 2015). An additional contribution to intrinsic resistance in Gram-negative bacteria is provided by efflux pumps (Eps) which actively pump out a broad spectrum of compounds.

The bacteriocin from *Bacillus* spp gives us reason to investigate why it is more effective against Gram positive bacteria than Gram-negative bacteria compared to other bacteria in the management of mastitis pathogen.

The results also indicate that bacteriocin produced by the *Bacillus* species can be used as an alternative to antibiotics in the management of the mastitis. Bacteriocins produced by bacteria isolates from tiger prawn indicated the highest bacteriocin activity against bacteriocin activity against *P. stutzeri* (Feliatra, *et al*. (2018). Bacteriocins producing bacteria isolated from the freshwater fish possess antagonistic activity against common most fish pathogen *A. hydrophila* (Vijayabaskar & Somasundaram 2008; Giri, Sukumaran, Sen, Vinumonia, Banu & Jena 2011; Banerjee, Dora & Chowdhury 2013), but the mechanism behind the same is still unknown. The isolated LAB species conformed by the methyl red and catalyzed negative test by Jack et al. (1995). The isolated *Bacillus* sp. produces hydrogen peroxidase, other organic acids and bacteriocin (Klaenhammer, 1988; Daeschel, 1989). Enterocin A was the most commonly detected bacteriocin among E. faecium strains from fish viscera (Sarra 2013). *Bacillus* spp. have been successfully used as probiotics in the aquaculture of black tiger shrimp in Thailand (Soltani *et al* 2019).

The BLAST results showed that all of the isolates were from the genus *Bacillus* with majority being *B. subtilis.* Other isolates were *B. pumilus* and *B. mycoides* (Table 4.9). *Bacillus* species are among the most commonly found aerobic eubacteria. The isolates formed three clusters of *Bacillus* on the phylogenetic tree namely; *B. subtilis* (twenty-one isolates), *B. pumilus* (three isolates) and *B. mycoides* (eight isolates). Members of the genus *Bacillus* that have also been previously isolated from Lake Magadi clustered with *Bacilli* members that are alkali tolerant, obligate alkaliphilic while others grow at

pH range of 5-10 (Baumgarte, 2003). Some of these include; *B. alcalophilus, B. silvestris, and B. pseudofirmus.* These were isolated from forest soil, waste water, bauxite waste, garden soil, brine/mud in Wadi Natrun, Lake Bank soil, riverbank soil, horse and elephant manure, rotting wood, solar salterns and saline soils. This is an indication that many alkaliphilic or alkalitolerant members of the *Bacilli* are fairly ubiquitous (Baumgarte, 2003). The genus *Bacillus* has many species that exist; hence the heterogeneity in physiology, ecology and genetics of the genus *Bacilli.* This genus has a vast diversity of physiological types such as degraders of most substrates from plants and animals, antibiotic producers, heterotrophics, nitrifiers, denitrifiers, nitrogen fixers, acidophiles, alkaliphiles, thermophiles and psychrophiles among others.

There are several validly published new species shown to be genetically and phenotypically distinct from other *Bacillus* species that have not been described in the *Bergey's* Manual of Systematic Bacteriology. *B. subtilis* produces the proteolytic enzyme subtilis in. *B. subtilis* spores can survive the extreme heat during cooking. *B. subtilis* is responsible for causing ropiness a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough. Isolate R35 clustered closely with *B. pumilus* strain 150a (CP027034.1) and scored 100 % similarity. *B. pumilus* is a ubiquitous Gram-positive, aerobic, rod-shaped endospore-forming bacterium that can be isolated from a wide variety of soils, plants, and environmental surfaces, and even from the interior of Sonoran desert basalt (Benardini *et al.,* 2003). The tradeoff between spoilage in food and bacteriocin effect on pathogens is that Bacillus use the antimicrobial bacteriocins to antagonize competition from other fastidious growing pathogenic microorganisms. This is an added advantage to allow the survival of the *Bacillus* bacteria.

Bacteriocin-based products have been successfully tested in the past. Nisin has been used as a teat disinfectant in the commercial product as stated by Ross (2005), where Wipe-Out® Dairy Wipes (Immucell Corporation) were incorporated with bacteriocins.

The outcome of the in vitro physiological tests of temperature, pH and metal ions activity suggests the crude bacteriocin products are safe for intramammaly infusions, or they could be packaged as milking jelly's ingredients. The study can be scaled by harvesting the crude bacteriocin and packaging them as intramammaly infusions for treatment of mastitis or as milking jelly ointments for control of causing mastitis bacteria. Before scaling out of the bacteriocins is carried out, in vivo trials are required to understand if the optimum physiological application conditions in dairy animals. Due to the complexity of undertaking animal tests with dairy animals the in vivo testing off the bacteriocin optimum physiological conditions was not carried out and is recommended for further studies.

Considering the extensive costs of a disease such as mastitis to the dairy industry, research directed towards viable and safe alternatives should be considered. Bacteriocins can thus be viewed as a real treatment solution to augment other management strategies and reduce the amount of antibiotics used in the treatment of mastitis.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

1. The study has demonstrated *R. argentea c*ollected from open air markets in Kisumu harbors to a large extent *B. subtilis*, followed by *B. mycoides* and *B. pumilus*.
2. The *Bacillus* isolates showed antimicrobial activity against standard laboratory test *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae*, microorganisms and their corresponding counterparts isolated from mastitis infected animals.
3. The *Bacillus* spp were able to produce enzymes, ferment carbohydrates, and survive in different temperature and pH conditions besides tolerating presence of metallic ions.
4. Molecular characterization of the isolates indicates that all of them belong to domain Bacteria with 90% of the isolates affiliated to microorganisms belonging to the genus *Bacillus spp.*
5. It can be concluded that *R. argentea* harbours *Bacillus* species from which bacteriocins can be obtained having activity against bovine mastitis bacterial pathogens

6.2 Recommendations

1. Further research on isolation and characterization of the bacteriocin compounds produced by these microorganisms is of great importance in reference to their antibacterial abilities and hence their usability in food preservation.
2. Further analysis of bacteria is necessary for complete characterization and identification of more *bacillus* strains by carrying out whole genome sequencing of bacteriocin producing *bacillu*s.

3. Further research on *in vivo* testing experiments are required to understand the optimum physiological application conditions in dairy animals before scaling out the bacteriocin production.
4. Further research on how the crude bacteriocin can be harvested and packaged as intramammaly infusions for treatment of mastitis or as milking jelly ointments for control of causing mastitis bacteria and even used as a means of food preservation and safety of foods.

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APPENDICES

Appendix I: Journal Article I

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Isolation and Identification of Bacteriocin-Producing *Bacillus* spp from *Rastrineobola argentea* (Omena) with Activity against Bovine Mastitis Bacterial Pathogens

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Abstract Rampant use of antibiotics has increased resistance of pathogenic bacteria in both animals and humans. This has triggered the investigation of novel antimicrobial agents produced by a bacterial strain of low virulence with antimicrobial activity with a wide range of clinical significance. This study sought to extract bacteriocins from *Bacillus* spp that were isolated and identified from Omena (*Rastrineobola argentea*) and to evaluate the in vitro antimicrobial effect of the obtained bacteriocin against bovine mastitis pathogens. Samples were collected from Lake Victoria using a completely randomized design method followed by isolation of the bacteriocin producing *Bacillus spp*. Characterization and identification of isolates was done by Gram staining, morphology and Biochemical tests which included catalase, nitrate reduction, methyl Red,-Voges-Proskauer, indole, motility and hydrogen sulphide, growth on Simmons citrate agar, triple sugar iron agar and starch hydrolysis. Antimicrobial activity was done using disc diffusion methods on the nutrient agar and zone of the inhibition measured after 24hours. An analytical profile index system (API 50CH BE, Biomerieux, Inc, France), was also used for identification. Molecular characterization was done by extracting genomic DNA using Qiagen DNA isolation Kit (Qiagen Germany) using the manufacturer's instructions. The gene encoding the 16S rRNA was amplified by PCR using universal bacterial primers pair combination of forwarding primer 27F forward (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R reverse, (5′-GGT TAC CTT GTT ACG ACT T-3′) about *Escherichia coli* gene sequence. A total of 60 pure isolates were obtained from Omena samples from Lake. Victoria. Morphological characteristics showed varied colour, form, shape and elevation of the pure colonies. About 54 isolates (90%) were gram-positive with spores, while 6 (10%) were gram-negative. Biochemical tests showed varied results among the bacterial isolates. 28 isolates showed antimicrobial activity on *E. coli* and *S. aureus* with inhibition measuring between 17-30mm. Analytical profile index system also showed that 20 isolates (71%) were identified as *Bacillus subtilis*, three isolates (11%) identified as *Bacillus pumilus* and five isolates (18%) *Bacillus mycoides*. The Blast analysis of the partial sequences showed100% of the strains of genus *Bacillus* within the Firmicutes in the domain bacteria. Twenty (20) isolates belong to *Bacillus subtilis*, *Bacillus mycoides* represent 18% (5 isolates) while *Bacillus pumilus* constituted 11% (3 isolates). This study contributes to understanding the use of bacteriocin in the control of diseases in dairy animal farming in Kenya.

Keywords: Bacillus Spp, bacteriocin, Rastrineobola argentea, Lake Victoria

Appendix II: Journal Article 2

Antimicrobial Activity and Characteristics of Bacteriocin Producing *Bacillus subtilis* against Mastitis Pathogens

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Abstract The use of drugs and antibiotics has increased the resistance of pathogenic bacteria in both animals and humans. This has been a significant problem and therefore triggers the investigation of novel antimicrobial agents produced by a bacterial strain of low virulence and having antimicrobial activity with a wide range of clinical significance. The use of bacteriocin has been extensively used in food industries, animals, and pharmaceutical industries. This is because it has been linked to antimicrobial activity, which has specific self-protection mechanisms. This study sought to evaluate antimicrobial activity and characteristics of bacteriocin producing *Bacillus subtilis* against Mastitis pathogens. For the screening of the isolates for bacteriocin properties against mastitis pathogens, antimicrobial activity was done using well diffusion methods on the nutrient agar. The results were obtained after 24hours and 48hours. Physiochemical characterization of the bacteriocin from *Bacillus subtilis* was determined at different temperatures of 60°C to 121°C for 15 minutes and monitor the effect of the temperature. The bacteriocin was also prepared at different pH (3-9) and incubated at room temperature; each sample's residual activity was determined against the indicator organisms. Metal ions (Cu^{2+}, Zn^{2+}, and Fe^{2+}) on crude bacteriocin activity were determined to assess the residual antimicrobial activity by agar well diffusion assay. The results showed that bacteriocins from *Bacillus subtilis* were effective against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. Physiochemical characterization showed that bacteriocin from different isolates had no inhibition from pH 3-5 and varied inhibition from pH 6-9 across the test organisms' isolates. On the temperature, crude bacteriocins at a temperature of 50°C to 60°C showed no activity loss based on initial activity. As temperature increases to 70°C to 80°C, there is reduced the bacteriocin activity of about 20%. 100°C had a 40% loss of the bacteriocin activity and 121°C with more than 50% loss of the activity. On metal ions, Cu^{2+}, Fe^{2+}, Zn^{2+} had a varied effect on bacteriocin activity against test organisms.

Keywords: *Bacillus subtilis, Bacteriocin, mastitis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae*

Appendix III: Journal Article 3

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Bacteriocins: Limiting Factors to Optimum Activity

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Abstract:

Bacteriocins are described as ribosomally synthesized antimicrobial peptides lethal to bacteria other than the producing strain. They are the most abundant of antimicrobial compounds produced by bacteria. These antimicrobial peptides offer an advantage over by targeting specific organisms and are generally regarded as safe for humans. The crude bacteriocins have been found to be affected by the presence of proteolytic enzymes like trypsin, temperature, pH, salts, and ions like copper or iron. These antimicrobial agents are gaining attention not only as alternative therapeutics in the pharmaceutical industry but also as a bio-preservative in food industries and in agriculture for control of bovine mastitis pathogens. These applications fundamentally depend on their antimicrobial effects and a vast understanding of their activity and factors inhibiting their mode of action. In this review factors perceived to be consequential to either activating, inactivating or maintaining the optimal activity of bacteriocins were identified and discussed. This comprehensive review delved into these factors with the aim of in-depth understanding of bacteriocins and their application for extensive exploitation. The remits of this detailed review include aspects of re-assuring public faith in bacteriocins and providing adequate information to users on their activity under the various condition in order to make informed choices before use. This review will help in restoring confidence in bacteriocins as a substitute to conventional antibiotics presents a considerable commercial challenge to the pharmaceutical industry. This indulgence will help develop innovative strategies towards the industrial application of bacteriocins.

Keywords: bacteriocins, antimicrobial peptides, antimicrobial activity, bacteriocidal spectrum