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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*

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1. Introduction

Rampant prescription of antibiotics and inefficient use of drugs has led to increased resistance of pathogenic bacteria day by day in both animals and humans [1]. This is a significant concern to researchers, which has triggered the investigation of novel antimicrobial agents produced by bacterial strains of apparent low virulence [2]. The production of an antimicrobial substance by microorganisms is an essential factor in microbial ecology [2]. Most of these substances play a crucial role in bacterial interactions, including bacteriocins, which are highly specific and efficient antagonists [3]. Bacteriocin refers to peptides and protein antibiotics produced by various microbes and have antimicrobial activity against closely related species [4]. These antimicrobial agents are gaining more attention as alternative therapeutics in pharmaceutical and preservatives in food industries [5]. Different bacteriocin may be produced within the same species and are ribosomal synthesized in the host while

the producer strain possesses a specific self-protection mechanism [3]. The bacteriocins are heterogeneous compounds possessing variability of biochemical properties, molecular weight, activity spectra, and action mode [6].

According to Ref [7], BacIB17 bacteriocin produced by *B. subtilis* KIBGE IB-17 possesses inhibitory properties acting as an antimicrobial agent against different pathogenic species. As reported by ref [8], bacteriocins are gaining more attention as an alternative therapeutic agent for the prevention and treatment of infections. Bacteriocins are generally recognized as naturally occurring food preservatives able to influence foods' quality and safety [9]. According to ref [10], non-clinical bacteriocin also have applications to control animal and foodborne pathogens in livestock.

Members of the genus *Bacillus* are known and reported to produce a vast arsenal of antimicrobial substances, including peptide and lipopeptide antibiotics and bacteriocins [11]. Many of the *Bacillus* bacteriocins belong to the lantibiotics, a category of post-translationally modified peptides widely disseminated among different bacterial clades [11]. In their study, ref [12] isolated bacterial strains of *Bacillus* spp. from Omena (*Rastrineobola argentea*) and established that the strains produce crude bacteriocins with antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, the bovine mastitis pathogens.

In the past two decades, the scientist paid a special attention and a significant focus on the bacteriocin produced by the lactic acid bacteria (LAB) [13]. This was due to their potential as natural and safe preservatives [14]. Only a few research teams have reported on the use of molecular techniques such as PCR based methods for detecting the structural genes of the bacteriocin precursors [15]. Therefore, there are still significant gaps that urgently need to be filled, such as physiological, chemical, and molecular characterization coupled with determining the efficacy of bacteriocin isolated from *B. subtilis* against bovine mastitis pathogens. In this regard, it is of paramount importance to use advanced and accurate molecular techniques to screen an extensive collection of bacterial isolates for potential bacteriocin production.

2. Materials and Methods

2.1. Sample Collection and Preservation

Two kilograms samples of ***Rastrineoebola argentea*** were purchased from fishermen in Lake Victoria using a completely randomized design from fifteen different fishermen. Samples were immediately transported in sterile sealed tins in the cooler boxes (at 4°C) to Food Microbiology Laboratory in the Department of Food Science and Technology, Jomo Kenyatta University of Agriculture and Technology for analysis. They were stored under refrigerated conditions. Molecular work was carried out in the Molecular Biology Laboratory at the Institute of Biotechnology Research (IBR), and biochemical analyses were carried out in the Food Microbiology Laboratory at the Department of Food Science.

Isolation of bacteriocin producing *Bacillus* Spp from *Rastrineoebole argentea*

Nutrient agar from Himedia was utilized for the development of bacteriocin creating bacterial species. It comprises 10 grams peptone, 10 grams meat separate, 5 grams sodium chloride and 12 grams of agar at pH 7.3 ± 0.1 per litre. The medium was set up by suspending 37 grams in 1 litre of purified water, brought to bubble, blended well and permitted to remain until broke down and disinfected via autoclaving at 121°C for 15 minutes, after which it was then administered into 90-mm-measurement polystyrene clean plastic Petri dishes. Twenty-five grams from each fish sample was placed in a sterile zip-lock bag containing 225 millilitres of sterile lactose broth 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 and fungi and to make easier the isolation of *Bacilli* from spores that survive the heat treatment. The liquid supernatant was then serially diluted with 1ml of liquid supernatant added to 9ml test tube containing sterile lactose broth. Dilution ratios included: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. These preparations were properly mixed by vertexing, 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 (prepared according to manufactures instruction) to isolate pure cultures.

2.2. Characterization of *Bacillus* Species

Species of genus *bacillus* are rod capsules that are endospores producing Spp. They grow aerobically although some are falcutative in most cases in nutrient agar .They are characterized by their rod-shaped cell morphology and catalase production (Ogura *et al.*, 2010).

2.2.1. Cultural and Morphological Characterization

Preliminary characterization of the isolates involved examining colony morphology and culture features such as colour, pigmentation, elevation, shape, size and growth form [16].

2.2.2. Gram staining of the Isolated Bacteria

Smears of bacteria culture were arranged and heat-fixed. Slides were set on the staining rack and overflowed with gentine violet. This was permitted to represent 30 seconds. The slide was then flushed with water for 5 seconds at that point covered with Gram's iodine stringent. The slide was permitted to represent one moment and washed with water for 5 seconds. Decolonization was finished with 95% ethanol for 15 to 30 seconds. This was trailed by washing with water for 5 seconds. Counterstaining was finished utilizing Safranin for around 60 to 80 seconds, and the slides were flushed again with water for 5 seconds. Smudge drying was finished with bibulous paper and analysed utilizing magnifying instrument at x100 under oil submersion [17]. Gram-positive life forms stained blue to purple; Gram-negative creatures stained pink to red. Gram

staining results were affirmed by utilizing the 3% KOH test (Halebian et al., 1981). This test was performed by adding a drop of 3% KOH on a slide. A circle loaded with the microbes was presented and blended thoroughly. Positive outcomes were noticed if the way of life pulled alongside the wire circle when raised, and adverse outcomes were recorded if nothing was pulled along the wire circle

2.2.3. Spore Staining

Spore staining was finished by making smears of given societies on clean slides, air-dried and heat-fixed. This was then overwhelmed with malachite green. At that point, slides warmed to steaming for 2-3 minutes, adding more stains to the smear occasionally, after which smears were washed under gradually pursuing faucet water cooling. This was then counterstained with Safranin for 30 seconds. The smears were then washed with faucet water; blotch dried and saw under submersion oil [17]. The cell morphology was noted, just as the spore and shade of spore. Gram-positive poles created spores, which was a decent demonstrative test for *Bacillus* species [18].

2.2.4. Biochemical Tests

Gram-positive spore-forming rods 54 (90%) isolates presumptive of *Bacillus* species were used for biochemical test analysis. Cultures used for biochemical test were 24-hours old. Both positive and negative controls were set up alongside the cultures. Catalase, nitrate reduction, Methyl Red-Voges–Proskauer, indole, motility, hydrogen sulphide production, growth on Simmon's citrate agar, Triple Sugar Iron Agar, starch hydrolysis test, and gelatine liquefaction/hydrolysis tests were performed according to ref [19]. All biochemical tests were done in triplicate

2.2.5. Screening of Isolates For Bacteriocins Efficacy against Mastitis Pathogens

All isolate were screened for bacteriocins efficacy against **mastitis pathogens**. A disc diffusion technique tried antimicrobial action of the thirty confined *Bacillus* strains. Wells (10 mm of breadth) in supplement agar were brooded with both cultures of *Bacillus* for 24 hours. The plates were then independently overlaid with an answer of pointer strains; *Sstaphylococcus aureus* (ATCC–25923) and *Escherichia coli* (ATCC-25922) by blending 50µl of strain (24 hours culture on TSB stock at a convergence of 108cfu/ml) with 200ml of Mueller Hinton Agar (Oxford, Hampshire, UK). After the overlays set, the plates were hatched for 24 hours and afterwards analyzed for a zone of restraint around the well. The action addressing the measurements of the hindrance zone was communicated in millimetres.

2.3. Characterization of Bacterial Growth on Different Carbohydrates

The bacterial detach showing the largest hindrance zone against the objective marker living being was chosen for additional portrayal by the logical profile record framework (API® 50CH B/E, Biomerieux, Inc, France) as per the producer's directions. Momentarily, the suspension was made in the mode for the microorganisms to be tried, and every one of the cylinders was immunized with the microorganism to be tried and afterwards hatched at 30°C for 24 hours and afterwards 48 hours. During brooding, starches ageing prompted corrosive creation bringing about diminishing in pH. The adjustment saw this in the shade of the marker. A positive test compares to fermentation delivered by red phenol marker contained in the medium evolving yellow. The biochemical profile was gotten for the strain after the last perusing was recognized utilizing the apiwebTM ID programming with the information base (14.0) (Biomerieux, Inc, France) utilizing producer's directions.

2.4. Production and Purification of Bacteriocin

Potential bacteriocins delivering bacterial separates were sub-cultured in supplement stock (HiMedia, Laboratories, India) at 30°C for 24 hours. Centrifugation was done at 15000rpm for 10 minutes after brooding to isolate the cells and the supernatant containing rough bacteriocin. Sans cell supernatant was changed following pH 6.5 utilizing one mol/l Sodium hydroxide to eliminate the antimicrobial impacts of natural acids. It was utilized as rough bacteriocin [19]. Inhibitory movement from hydrogen peroxide was wiped out by expanding 5 mg/ml catalase (C-100 bovine liver, Sigma). Killed filtrates were disinfected by filtration. Afterwards they were tried for the antimicrobial movement against the pointer creatures utilizing the agar well dispersion strategy as depicted by Kang and Lee, (2005).

2.5. Molecular Characterization

2.5.1. DNA Extraction

Genomic DNA was extracted in duplicate from bacterial cells at the exponential growth phase grown aerobically in nutrient broth using Qiagen DNA Isolation Kit (Qiagen, Germany) according to the manufacturer's instructions. Bioline molecular agarose of 0.8% was run for band detection. A spectrophotometer was used for DNA quantification. Absorbance was measured at 260nm and 280nm to determine DNA purity.

2.5.2. DNA Amplification

All out genomic DNA was separated from unadulterated societies utilizing QIAamp PCR Kit (Qiagen, Germany) was used for amplification as per the manufacturer’s manual. Marker ge 16S rRNA was amplified using forward and reverse primer. Forward primer 27F sequence used was (5’-AGA GTT TGA TCC TGG CTC AG-3’) and reverse primer 1492R sequence was (5’-GGT TAC CTT GTT ACG ACT T-3′) according to Bergmann et al. (2010). The control excluded DNA, according to Sambrook and Russell, (2001). Response combinations were exposed to the accompanying temperature cycling profiles rehashed for 32 cycles: Initial denaturation 94°C for 5 minutes, denaturation at 94°C for one minute, groundwork strengthening at 55°C for 2 minutes, expansion at 72°C for 2 minutes and the last augmentation at 72°C for 10 minutes. Purification of PCR products was done using QIAquick PCR purification Kit protocol

(Qiagen, Germany) according to the manufacturer's instructions. Polymerase chain reaction purified amplicons were run on 1% (w/v) molecular grade agarose gel mixed with 1µg/ml ethidium bromide in 1X TBE cradle according to ref [17].

3. Data Analysis

Data entry and 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 zone of test organisms' inhibition against crude bacteriocins was calculated using Ms. Excel. The Biochemical characterization procedures were done in triplicates. Observations were made on these triplicates defined each of the qualitative tests' nature as either positive or negative. Phylogenetic trees were drawn using Mega version 7. The 16S rRNA gene sequences of the bacteria isolates were viewed for noise determination and edited using ChromasPro 2.18 software package (http://technelysium.com.au/wp/). The sequences were compared with available sequences of bacteria lineages in the National Center for Biotechnology Information (NCBI) database using nucleotide BLASTn (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 sequences alignment using Clustal W software. Phylogenetic trees were constructed using the Maximum Likelihood Parsimony method based on the Tamura-Nei model [20] with MEGA version 7.0 software package [21]. Trees topologies were evaluated using the bootstrap resampling method [22] based on 1000 replicates.

4. Results

4.1. Isolation and Characterization of Bacteriocin Producing *Bacillus* spp. from *Rastrineoebole agentea*

A total of sixty pure isolates were obtained from ***Rastrineoebola argentea*** samples from Lake Victoria. Isolation of pure isolates was done on nutrient agar medium (Plate 1).

4.2. Morphological Characterization of Bacterial Isolates

Morphological characterization was based on classical macroscopic attributes of colour, form, shape, and pure colonies' elevation. The isolates' colony morphology ranged from circular, undulate, and flat (Plate 1). They were smooth or undulate, and the colour ranged from white, yellow to cream (Table 1). A total of 54 (90%) of the isolates were Gram-positive with terminal spore, while 6 (10%) were Gram-negative and did not form spores. All isolates were rod-shaped. All Gram-negative non-spore formers were discarded.

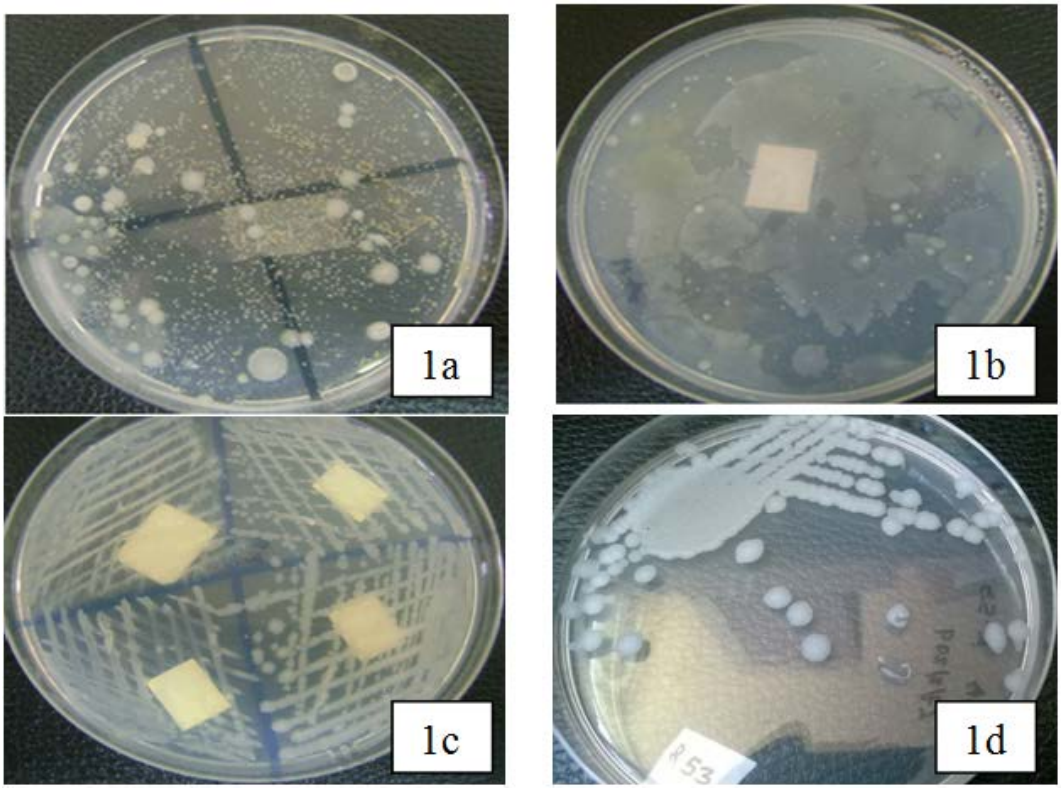

Plate 1. (1a) Nutrient agar medium plate with different colonies before isolation of individual colonies (high diversity). (1b) Nutrient agar medium culture plate with swarming growth. (1c) Culture plate with different isolates. (1d) Culture plate with pure/individual colonies

Table 1. Cultural/Colony and morphological characteristics of isolates obtained from *Rastrineobola agentea*

Isolate	Colony characterization				Cell characterization		
	Colour	**Form**	**Elevation**	**Margin**	**Gram reaction**	**Arrangement**	**Spore formation**
R_1	White	Irregular	Flat	Undulate	+	Rods	Yes
R_2	White	Irregular	Umbonate	undulate	+	Rods	Yes
R_3	White	Irregular	Umbonate	Undulate	+	Rods	Yes
R_4	White	Irregular	Umbonate	Undulate	+	Rods	Yes
R_5	White	Irregular	Flat	undulate	+	Rods	Yes
R_6	White	Irregular	Flat	Undulate	+	Rods	Yes
R_7	Cream	Irregular	Raised	Undulate	+	Rods	Yes
R_8	Cream	Irregular	Raised	Ciliate	+	Rods	Yes
R_9	White	Irregular	Flat	Ciliate	+	Rods	Yes
R_{10}	Cream	Irregular	Raised	Undulate	+	Rods	Yes
R_{11}	Cream	Irregular	Raised	Ciliate	+	Rods	Yes
R_{12}	White	Irregular	Raised	Undulate	+	Rods	Yes
R_{13}	White	Irregular	Raised	Undulate	+	Rods	Yes
R_{14}	White	Irregular	Raised	Undulate	+	Rods	Yes
R_{15}	White	Irregular	Raised	Undulate	+	Rods	Yes
R_{16}	White	Irregular	Flat	Undulate	+	Rods	Yes
R_{17}	White	Irregular	Flat	Undulate	+	Rods	Yes
R_{18}	White	Irregular	Flat	Undulate	+	Rods	Yes
R_{19}	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R_{20}	White	Irregular	Flat	Ciliate	+	Rods	Yes
R_{21}	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R_{22}	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R_{23}	White	Irregular	Flat	Undulate	+	Rods	Yes
R_{24}	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R_{25}	Cream	Circular	Flat	Undulate	+	Rods	Yes
R_{26}	Cream	Circular	Flat	Undulate	+	Rods	Yes
R_{27}	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R_{28}	Cream	Irregular	Flat	Undulate	+	Rods	Yes
R_{29}	White	Irregular	Flat	Undulate	+	Rods	Yes
R_{30}	White	Irregular	Flat	Undulate	+	Rods	Yes
R_{31}	White	Irregular	Flat	Undulate	+	Rods	Yes
R_{32}	Cream	Irregular	Flat	Branching	+	Rods	Yes
R_{33}	Brown	Irregular	Flat	Branching	+	Rods	Yes
R_{34}	Brown	Irregular	Raised	Undulate	+	Rods	Yes
R_{35}	Yellow	Irregular	Raised	Entire	+	Rods	Yes
R_{36}	Yellow	Irregular	Raised	Smooth	+	Rods	Yes
R_{37}	Yellow	Circular	Raised	Smooth	+	Rods	Yes
R_{38}	White	Irregular	Raised	Branching	+	Rods	Yes
R_{39}	White	Irregular	Flat	Branching	+	Rods	Yes
R_{40}	White	Irregular	Flat	Branching	+	Rods	Yes
R_{41}	Cream	Irregular	Raised	Smooth	-	Rods	No
R_{42}	Cream	Irregular	Raised	Smooth	-	Rods	No
R_{43}	Cream	Irregular	Flat	Smooth	-	Rods	No
R_{44}	White	Irregular	Umbonate	Ciliate	+	Rods	Yes
R_{45}	White	Irregular	Umbonate	Undulate	+	Rods	Yes
R_{46}	White	Irregular	Umbonate	Undulate	+	Rods	Yes
R_{47}	White	Irregular	Flat	Ciliate	+	Rods	Yes
R_{48}	White	Irregular	Flat	Undulate	+	Rods	Yes
R_{49}	White	Irregular	Flat	Ciliate	+	Rods	Yes
R_{50}	White	Irregular	Umbonate	Ciliate	+	Rods	Yes
R_{51}	White	Irregular	Umbonate	Undulate	+	Rods	Yes
R_{52}	White	Irregular	Flat	Entire	+	Rods	Yes
R_{53}	White	Irregular	Flat	Entire	+	Rods	Yes
R_{54}	Cream	Circular	Raised	Entire	-	Rods	No
R_{55}	Cream	Circular	Raised	Smooth	-	Rods	No
R_{56}	Brown	Circular	Flat	Smooth	-	Rods	No
R_{57}	White	Irregular	Flat	Ciliate	+	Rods	Yes
R_{58}	White	Irregular	Raised	Undulate	+	Rods	Yes
R_{59}	White	Circular	Raised	Ciliate	+	Rods	Yes
R_{60}	White	Irregular	Flat	Ciliate	+	Rods	Yes

4.3. Biochemical Tests of the Isolates

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 in Table 2.

Table 2. Biochemical characteristics of bacterial isolates obtained from *Rastrineobola argentea*

						TSI						
Isolate#	**Starch**	**Catalase**	**Indole**	**Motility**	**Gelatine**	**Butt**	**Slant**	**H_2S**	**Citrate**	**MR**	**VP**	**Nitrate**
R_1	+	+	-	+	+	++	++	-	+	-	+	+
R_2	+	+	-	+	+	++	++	-	+	-	+	+
R_3	+	+	-	+	+	++	++	-	+	-	+	+
R_4	+	+	-	+	+	++	++	-	+	-	+	+
R_5	+	+	-	+	+	++	++	-	+	-	+	+
R_6	+	+	-	+	+	++	++	-	+	-	+	+
R_7	+	+	-	-	+	+++	++	-	+	-	+	+
R_8	+	+	-	-	+	++	+++	-	+	-	+	+
R_9	+	+	-	-	+	++	++	-	+	-	+	+
R_{10}	+	+	-	-	+	++	++	-	+	-	+	+
R_{11}	+	+	-	-	+	+ +	+ +	-	+	-	+	+
R_{12}	+	+	-	-	+	+ +	+ +	-	+	-	+	+
R_{13}	+	+	-	-	+	+++	++	-	+	-	+	+
R_{14}	+	+	-	-	+	++	++	-	+	-	+	+
R_{15}	+	+	-	-	+	++	++	-	+	-	+	+
R_{16}	+	+	-	-	+	++	+++	-	-	-	+	+
R_{17}	+	+	-	-	+	+++	+++	-	+	-	+	+
R_{18}	+	+	-	-	+	++	+++	-	-	-	+	+
R_{19}	+	+	-	-	+	++	++	-	+	-	+	+
R_{20}	+	+	-	-	+	++	++	-	+	-	+	+
R_{21}	+	+	-	-	+	+++	+++	-	+	-	+	+
R_{22}	+	+	-	-	+	++	+++	-	+	-	+	+
R_{23}	+	+	-	-	+	++	++	-	-	-	+	+
R_{24}	+	+	-	-	+	+	+++	-	+	-	+	+
R_{25}	+	+	-	-	+	+	+++	-	+	-	+	+
R_{26}	+	+	-	-	+	++	++	-	+	-	+	+
R_{27}	+	+	-	-	+	+++	+++	-	+	-	+	+
R_{28}	+	+	-	-	+	++	++	-	+	-	+	+
R_{29}	+	+	-	-	+	++	++	-	+	-	+	+
R_{30}	+	+	-	-	+	++	++	-	+	-	+	+
R_{31}	+	+	-	-	+	++	+++	-	+	-	+	+
R_{32}	+	+	-	+	+	++	+++	-	+	-	+	+
R_{33}	+	+	-	+	+	++	+++	-	+	-	+	+
R_{34}	+	+	-	+	+	++	+++	-	+	+	+	+
R_{35}	-	+	-	+	+	++	++	-	+	+	+	-
R_{36}	-	+	-	+	+	++	++	-	+	+	+	-
R_{37}	-	+	-	+	+	++	++	-	+	+	+	-
R_{38}	+	+	-	+	+	++	++	-	+	-	-	+
R_{39}	+	+	-	+	+	++	++	-	+	-	-	+
R_{40}	+	+	-	+	+	++	++	-	+	-	-	+
R_{44}	+	+	-	+	+	++	++	-	+	-	+	+
R_{45}	+	+	-	+	+	++	++	-	+	-	+	+
R_{46}	+	+	-	+	+	++	++	-	+	-	+	+
R_{47}	+	+	-	+	+	++	++	-	+	-	+	+
R_{48}	+	+	-	+	+	++	++	-	+	-	+	+
R_{49}	+	+	-	+	+	++	++	-	+	-	+	+
R_{50}	+	+	-	+	+	++	++	-	+	-	+	+
R_{51}	+	+	-	+	+	++	++	-	+	-	+	+
R_{52}	+	+	-	+	+	++	++	-	+	-	+	+
R_{53}	+	+	-	+	+	++	++	-	+	-	+	+
R_{57}	+	+	-	+	+	++	++	-	+	-	+	+
R_{58}	+	+	-	+	+	++	++	-	+	-	+	+
R_{59}	+	+	-	+	+	++	++	-	+	-	+	+
R_{60}	+	+	-	+	+	++	++	-	+	-	+	+

Key: (+) Positive/ less colour, (-) Negative, (++) partial colour (+++) intense colour, MR: Methyl Red, VP: Voges-Proskauer and H_2S: Hydrogen Sulphide gas. The results were interpreted according to ref [23].

4.4. Screening of the Isolates for Bacteriocins Properties against Mastitis Pathogens

All 54 isolates were tested for their antimicrobial activity against mastitis-causing pathogens. They were used to check their ability to inhibit common microorganisms' growth; *Escherichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923, using the agar diffusion method (Plate 2). The zone of inhibition on inoculated plates was observed and measured. Out of the 54 *Bacillus* presumptive isolates, twenty-eight (28) isolates showed antimicrobial activity. Out of which 22 (79%) and 10 (36%) of the isolates could inhibit the growth of *Escherichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923, respectively (Table 3). The result showed that 39% of the isolates were sensitive against *Escherichia coli* ATCC-25922 (zone of inhibition measuring between 17 to 30mm), while only 7% were able to inhibit *Staphylococcus aureus* ATCC 25923. Additionally, 25% of the isolates were sensitive against *Escherichia coli* and *Staphylococcus aureus* (Table 3). Hence, those isolates that showed antimicrobial activity against *E. coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923 were further characterized based on carbohydrates fermentation using API 50 CH B/E kits.

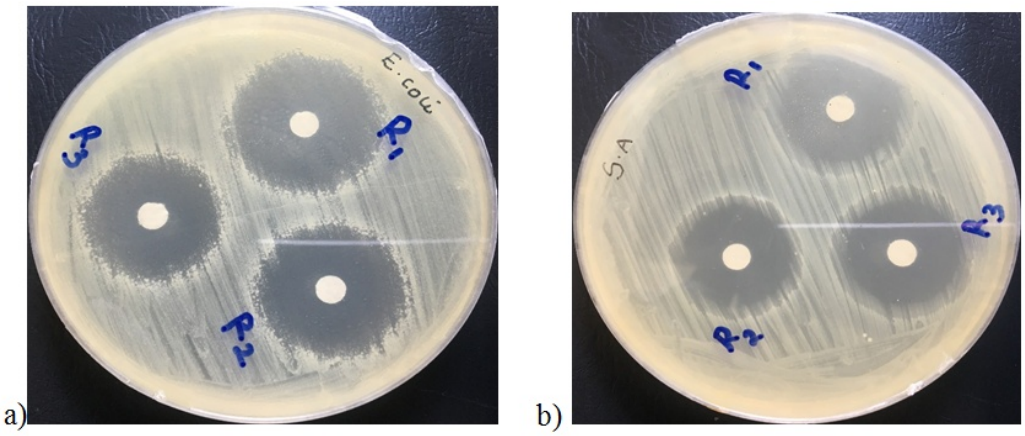

Plate 2. a) Zone of inhibition of selected isolates of crude bacteriocin on *Escherichia coli* ATCC 25922 (R1, R3) and **b)** *Staphylococcus aureus* ATCC 25923 (R2) at pH 7 and 9 on nutrient agar

Table 3. Antimicrobial activity of supernatant (crude bacteriocin) obtained from different *Bacillus* isolates inhibition zone (diameter, mm) against tested bacteria

Bacillus spp	Isolate	Escherichia coli ATCC-25922	Staphylococcus aureusATCC 25923
Bacillus subtilis	R1	^{s}23.7 ± 2.5	^{s}24.7 ± 1.5
	R2	^{i}16.3 ± 1.5	^{r}2.7 ± 1.2
	R3	^{s}23.3 ± 1.5	^{r}12.7 ± 2.1
	R4	^{s}23.0 ± 1.0	^{s}18.0 ± 1.0
	R5	^{i}15.0 ± 1.0	^{i}14.3 ± 1.5
	R6	^{s}18.0 ± 1.0	^{r}7.0 ± 1.0
	R44	^{s}22.0 ± 1.0	^{r}10.0 ± 1.0
	R45	^{s}23.7 ± 1.5	^{s}20.0 ± 1.0
	R46	^{i}15.3 ± 1.5	^{r}7.0 ± 1.0
	R47	^{r}11.3 ± 0.6	^{r}4.0 ± 1.0
	R48	^{i}14.0 ± 1.0	^{r}7.0 ± 1.0
	R49	^{r}11.0 ± 1.0	^{r}2.7 ± 1.5
	R50	^{s}17.3 ± 1.5	^{r}11.0 ± 1.0
	R51	^{s}25.0 ± 1.0	^{i}15.3 ± 0.6
	R52	^{s}23.7 ± 1.0	^{r}5.3 ± 0.6
	R53	^{i}15.0 ± 1.5	^{r}13.3 ± 1.5
	R57	^{s}24.0 ± 1.0	^{r}12.0 ± 1.0
	R58	^{s}21.3 ± 1.0	^{s}18.7 ± 1.2
	R59	^{s}20.3 ± 1.5	^{s}22.3 ± 1.5
	R60	^{s}23.7 ± 1.5	^{s}25.0 ± 1.0
B. pumilus group	R35	^{r}12.0 ± 1.0	^{s}24.7 ± 1.0
	R36	^{s}17.0 ± 1.0	^{s}21.0 ± 1.0
	R37	^{r}10.3 ± 0.6	^{s}22.0 ± 1.0
B. mycoides group	R7	^{s}17.7 ± 1.5	^{r}1.7 ± 0.6
	R10	^{i}16.7 ± 0.6	NI
	R13	^{i}11.0 ± 1.0	NI
	R14	^{s}17.0 ± 1.0	^{r}12.3 ± 0.6
	R24	^{s}17.3 ± 1.0	NI

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

4.5. Characterization of Bacteria Using the Analytical Profile Index System

The 28 isolates that showed antimicrobial activity against *E. coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923 and were subjected to analytical profile index system identification, as shown in Table 4.

The analytical profile index system showed that 20 isolates (71 %) were identified as *Bacillus 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, three isolates were identified as *Bacillus pumilus* representing 11 % (R35, R36 and R37) while 5 (18 %) isolates were identified as *Bacillus mycoides*, they included: R7, R10, R13, R14 and R24 (Table 4).

Table 4. 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	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4.6. Molecular Characterization

4.6.1. PCR Amplification of 16S rRNA Genes From Isolates

Amplification of the 16S rRNA gene yielded amplicons with bands of approximately 1500bp on 1% agarose gel as shown in Plate 3.

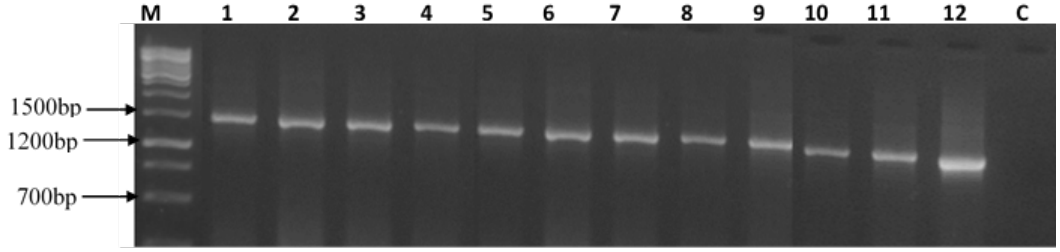

Plate 3. A 1% agarose gel photograph of PCR amplification of 16S rDNA of the isolates visualized after ethidium bromide staining; M (DNA marker), 1 (R1), 2 (R4), 3 (R7), 4 (R14), 5 (R35), 6 (R36), 7 (R37), 8 (R44), 9 (R45), 10 (R51), 11 (R59), 12 (R60) and C (control)

4.7. Phylogenetic Analysis of the Sequences

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 5), with sequence similarities ranging from 96.3% to 100%. Among these were; *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus mycoides* (Table 5). The result showed that twenty (20) isolates, or 71%, belonged to *Bacillus subtilis*, with similarities ranging between 96.2% and 99.7% (Table 5). *Bacillus mycoides* represented 18% (five isolates) with similarities between 99.9% and 100%, while *Bacillus pumilus* constituted 11% (three isolates) with percentage similarities ranging from 98.4% to 100% (Table 5). However, isolate R1, R2, R3, R37, R53, R57, and R58 had sequence similarity of between 96.2-98.4%, representing novel species [24]; (Figure 1). Phylogenetic analysis of the isolates from *Rastrineoebole argentea* showed that twenty isolates clustered into species *Bacillus subtilis*. Isolates R1, R2, R3, R4, R5, R6, R44, R45, R46, R47, R48, R49, R50, R51, R52, R53, R57, R58, R59 and R60 were near related to *Bacillus 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 with *Bacillus mycoides* strains (CP037992.1) while isolates R35, R36 and R37 were grouped with *Bacillus pumilus* strain (CP027034.1).

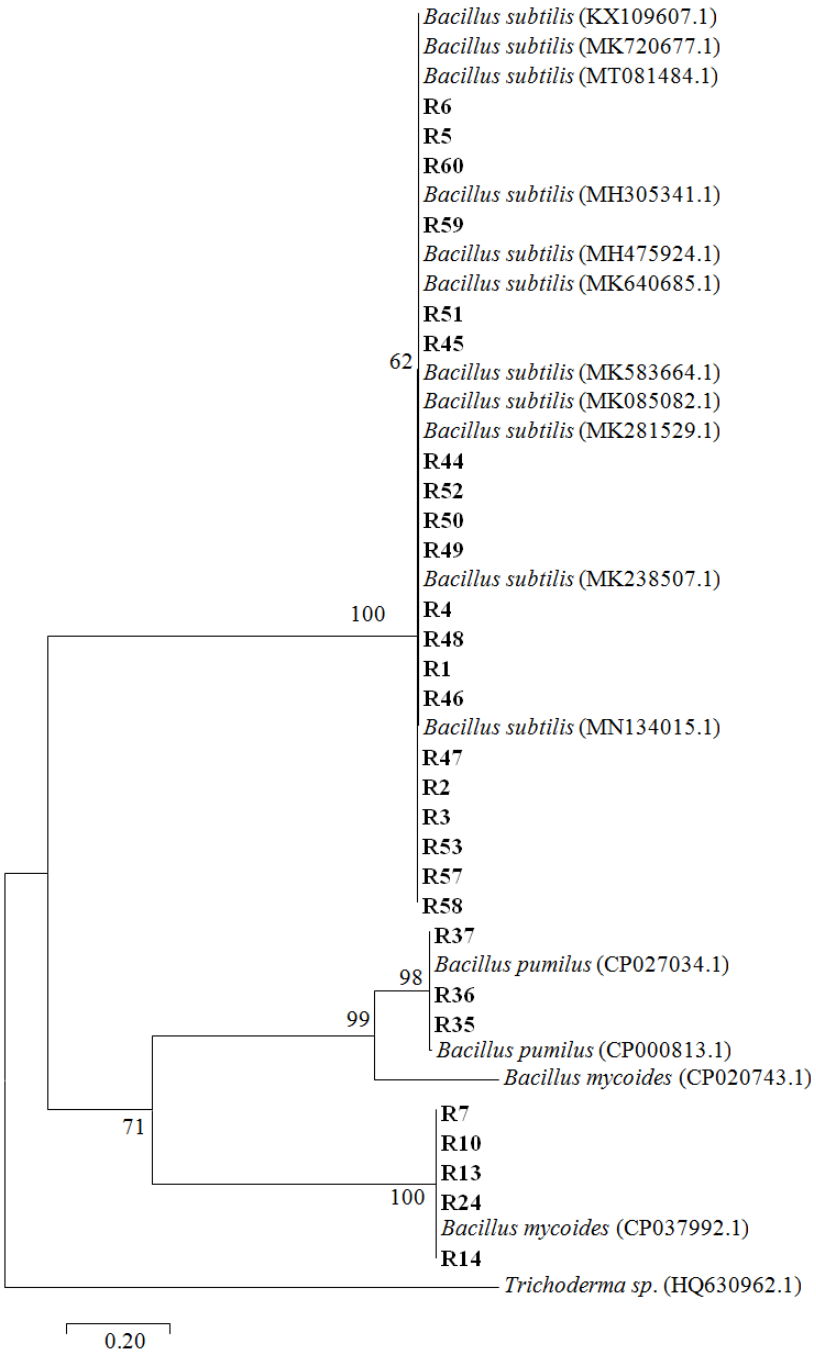

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship among the *Restreneoebole agentea* isolates and between representatives of other related taxa. The scale bar indicates 0.2 substitutions per nucleotide position. The number beside the node is the statistical bootstrap 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

Table 5. 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	0.0	KJ856901.1
R2	*Bacillus subtilis* strain CC8	96.3	99	0.0	MK720677.1
R3	*Bacillus subtilis* strain EMB M15	96.7	99	0.0	KX109607.1
R4	*Bacillus subtilis* strain JSRB 177	99.9	99	0.0	MN121128.1
R5	*Bacillus subtilis* strain HABIBD2	99.7	99	0.0	MT102635.1
R6	*Bacillus subtilis* strain FI469	99.7	99	0.0	MT081484.1
R7	*Bacillus mycoides* strain TH26	100	98	0.0	CP037992.1
R10	*Bacillus mycoides* strain TH26	100	98	0.0	CP037992.1
R13	*Bacillus mycoides* strain Gnyt1	99.9	99	0.0	CP020743.1
R14	*Bacillus mycoides* strain Gnyt1	99.9	99	0.0	CP020743.1
R24	*Bacillus mycoides* strain TH26	100	98	0.0	CP037992.1
R35	*Bacillus pumilus* strain 150a	100	100	0.0	CP027034.1
R36	*Bacillus pumilus* strain SH-B9	98.8	99	0.0	CP011007.1
R37	*Bacillus pumilus* strain NCTC10337	98.4	98	0.0	LT906438.1
R44	*Bacillus subtilis* strain IPA 25	99.9	99	0.0	MK238507.1
R45	*Bacillus subtilis* strain GW 12	99.9	100	0.0	MK583664.1
R46	*Bacillus subtilis* strain HR10	99.4	99	0.0	MN893856.1
R47	*Bacillus subtilis* strain EMB M15	99.7	99	0.0	KX109607.1
R48	*Bacillus subtilis* strain 181203-033_G01	99.8	100	0.0	MT448935.1
R49	*Bacillus subtilis* strain SY2101	99.8	100	0.0	MT448726.1
R50	*Bacillus subtilis* strain 181203-033_G01	99.8	100	0.0	MT448935.1
R51	*Bacillus subtilis* strain 50-1	99.9	99	0.0	MH475924.1
R52	*Bacillus subtilis* strain MEF4	99.8	100	0.0	MT415788.1
R53	*Bacillus subtilis* strain BJ-DEBCR-6	96.2	98	0.0	KU854954.1
R57	*Bacillus subtilis* strain YKF2	96.2	98	0.0	KU667124.1
R58	*Bacillus subtilis* strain SRSTH1	96.2	99	0.0	HQ398997.1
R59	*Bacillus subtilis* strain PR10	99.9	99	0.0	CP040528.1
R60	*Bacillus subtilis* strain BRM043908	99.7	99	0.0	MH305341.1

5. Discussion

Nutrient agar was successfully used for the cultivation of bacteriocins producing *Bacillus* species. *Bacillus* is Gram-positive, rod-shaped, motile, catalase-positive, aerobic/facultative anaerobic bacteria from the division Firmicutes [25].

They are found in a varied ecological niche, mostly as saprophytic, especially in aquatic environments, soil, food, plant surfaces; a few are animal or insect parasites or pathogens [11]. They are also known to form heat-resistant endospores when cultured in growth media [26]. Their ability to form endospores with various physiological properties and produce various antimicrobial compounds favour their widespread distribution in nature [27,28].

The Triple Iron Sugar utilization test determined the isolates' ability to ferment glucose and produce a lot of mixed acids as end products of fermentation, and the result showed that all isolated were positive for this test. Therefore, this indicated the isolates' ability to carry out glucose fermentation and produce a mixture of acids as end products of fermentation. The triple sugar iron agar medium can differentiate bacteria based on the fermentation of glucose, lactose, and sucrose [29,30]. The medium's preparation is done as a shallow agar slant with a deep but for providing a growth environment for both the aerobic and anaerobic microorganisms. Hence, the microorganisms that can ferment glucose and lactose and sucrose could 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 [17]. 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 turn only the slant red [31].

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 the final products of the reaction are indole, pyruvic acid, ammonia (NH_3), hydrogen sulphide and energy. The positive indole test showed the isolates' ability to deaminate and hydrolyze amino acids to pyruvic acid and ammonia. In contrast, the negative results showed that isolates were unable to produce indole due to amino acid tryptophan breakdown attributed to lack of tryptophanase in the cell [32]. 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 medium's surface. All isolates were negative for this test but favourable for hydrogen sulfide gas production. The test is essential in differentiating the family Enterobacteriaceae and genus bacillus [33]. 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 [34]. Hydrogen sulphide gas is produced when microorganisms reduce sulfur compounds. This test thus showed that the isolates could reduce sulfur-containing compounds to sulfides during the process of metabolism [19].

Starch hydrolysis is a test important for differentiating microorganisms based on their ability to hydrolyze starch with the enzyme a-amylase or oligo-l, 6-glucosidase; the 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 [35]. Therefore, when the bacteria that produce these enzymes are cultivated on starch agar, they hydrolyze the starch around the area of growth. However, since both starch and its sugar subunits are invisible in the medium, and 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 colour; therefore, any microbial starch hydrolysis was revealed as a clear zone surrounding the growth [36]. The results showed that all isolates except R35, R36, and R37 were positive for this test. This test is essential in differentiating the species of genera *Bacillus*, *Clostridium*, *Bacteroides*, *Fusobacterium* and members of *Enterococcus* spp.

Gelatin is a collagenous protein, a component of animal connective tissues produced by hydrolysis of collagen. Gelatin hydrolysis is a test used to detect the isolates' ability to produce proteolytic enzyme (gelatinase), which causes the breakdown of this complex protein derivative to polypeptides [17]. These polypeptides are further converted into single amino acids that bacteria can easily use for their metabolic process. Therefore, 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 [33]. During aerobic respiration, microorganisms produce hydrogen peroxide and, in some cases, extremely toxic superoxide. Accumulation of these substances will result in the 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 showed that all isolates were catalase positive and it is important in differentiating aerotolerant strains of Clostridium, which are catalase negative, from Bacillus, which are catalase positive [5].

The isolates were taxonomically classified based on morphological characteristics, biochemical tests, API kit, and 16S ribosomal DNA sequences of their genomic DNA placed the isolates to the genera *Bacillus*. The antimicrobial activity of the isolates indicates different variation in terms of inhibiting E *coli* and *S. aureus*. *Bacillus subtilis* were dominant in inhibiting both *E. coli* and *S. aureus* compared to *B. pumilus* group. Bacillus has been used as both biocontrol in the management of various plant pathogen and diseases. There is reported incidence were *B. subtilis* are used in clinical perspective in the management of various pathogen. The rate of inhibition for most *B. subtilis* varied. The results also related to the finding by the mechanism behind this is that most of the *B subtilis* inhibit further pathogen growth by either producing enzymes or competition [37]. The results also correlate with previous findings on the antimicrobial activity of most isolated *B. subtilis*. Therefore, there is a high chance of isolated bacteria to prevent food spoilage. Because food spoilage, both raw and processed, faces many challenges, the use of bacteria from omena can significantly impact food preservation. Additionally, the isolated bacteria do not have a health impact on the consumer, making them a good alternative in food preservation. Most companies have advocated for the use of alternative methods for food preservation and management.

The logical profile record or API is a grouping of microbes dependent on biochemical tests, permitting quickly recognizable proof. This framework is created for fast recognizable proof of clinically significant microorganisms. Along these lines, I just realized microscopic organisms could be distinguished. One of the API frameworks is explicit for separating individuals from the Gram-negative bacterial family Enterobacteriaceae and is called API-20E. Different API framework is explicit for Gram-positive microbes, including *Staphylococcus* species, *Micrococcus*, *Bacillus* species, and related life forms. Programming interface test strips comprises wells containing dried out substrates to distinguish enzymatic movement, typically identified with the maturation of sugar or catabolism of proteins or amino acids by the vaccinated life forms. A bacterial suspension is utilized to rehydrate every one of the wells, and the strips are hatched. During brooding, digestion produces shading changes that are either unconstrained or uncovered by the expansion of reagents. For instance, when sugars are aged, the pH inside the good reductions and that change are shown by an adjustment in the pH pointer's shade. All sure and adverse test outcomes are arranged to get a profile number, which is then contrasted and profile numbers in a business codebook (or on the web) to decide the bacterial species' ID. From the outcomes, *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus mycoides* were recognized and characterized. The outcome related to writing in that the more significant part of *Bacillus subtilis* can age carbs (German et al., 2008).

The BLAST results showed that all of the isolates were from the genus *Bacillus* within the subtilis species. All of the isolates in this study were affiliated to microorganisms belonging to the genus *Bacillus.* Among these were; *Bacillus subtilis***,** *Bacillus pumilus, Bacillus mycoides*. *Bacillus* species are among the most commonly found aerobic, eubacterial alkaliphiles and selected environments

The isolates formed three clusters of *Bacillus* on the phylogenetic tree, namely; *Bacillus subtilis* (twenty-one isolates), *Bacillus pumilus* (three isolates) and *Bacillus mycoides* (eight isolates). Members of the genus *Bacillus* that have been previously isolated from Lake Magadi, L Naivasha clustered with *Bacilli* members that are alkali tolerant, obligate alkaliphilic while others grow at a pH range of 5-10 [17]. Some of these include; *Bacillus agaradhaerens, Bacillus clarkia, Bacillus alcalophilus, Bacillus silvestris, Bacillus haloalkaliphilus and Bacillus pseudofirmus.* This is an indication that various Lakes contains members of the *Bacilli [19]*. The genus *Bacillus* has many species that exist; hence the heterogeneity in physiology, ecology and genetics of the genus *Bacillus*

This genus has a vast diversity of physiological types such as degraders of most substrates from plants and animals, antibiotic producers, heterotrophic, nitrifiers, denitrifiers, nitrogen fixers, acidophiles, alkaliphiles, thermophiles and psychrophiles, among others [38].

Several validly published new species are genetically and phenotypically distinct from other *Bacillus* species that have not been described in *Bergey's* Manual of Systematic Bacteriology. *B. subtilis* produces the proteolytic enzyme subtilisin. *Bacillus subtilis* spores can survive extreme heat during cooking. *B. subtilis* is responsible for causing ropiness, a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in sour bread dough. Isolate R35 clustered closely with *Bacillus pumilus* strain 150a (CP027034.1) and scored 100% similarity. *Bacillus pumilus* is a ubiquitous Gram-positive, aerobic, rod-shaped endospore-forming bacterium that can be isolated from various soils, plants, and environmental surfaces, even from the interior of Sonoran Desert basalt (Benardini *et al.*, 2003).

Bacteriocin is a peptide that is active against other bacteria and against which the producer has a specific immunity mechanism. They are produced by all significant lineage of bacteria and archaea and contain a heterogeneous group of peptides regarding the size, structure and mode of action. They play the following function; antimicrobial activity by either inhibiting competing strains or through signaling cells of the host immune system [9]. In recent years antimicrobial protein produced by Gram-positive bacteria has a great interest in their potential use as a food preservative [9].

According to ref [39], *Bacillus subtilis* which was isolated from soil inhabited bacterium was found to have the ability to produce antimicrobial compounds, which were also to use found to be safe. *B. subtilis* was found to have bacteriocin with the potential to produce so many different antibiotics. On the analysis of physicochemical characterization, the purified bacteriocin from *Bacillus subtilis* showed varied temperature, pH, enzyme and chemicals. The partially purified bacteriocin from B. subtilis was reported to have antimicrobial activity towards all the bacteria; isolated (*Klebsiella* spp). Therefore, this proved that the bacteriocin from B. subtilis has high antimicrobial activity [39]. Some of the reported studies, such as ref [40], showed that *B. subtilis* have bacteriocin which been isolated, characterized and screened for antimicrobial activity.

Safeguarding food improves the timeframe of realistic usability and food quality to dispense with food-related ailment and item decay, particularly by utilizing food-added substances.

There is a development of buyer interest for safe food without adjusting its wholesome quality . Bio protection of foods by the utilization of regular or controlled microbiota and additionally their antimicrobial is increasingly being preffered. *Bacillus sp*., the Gram-positive microscopic organisms, that create inhibitory substances like cyclic peptides and bacteriocin, with a broad antimicrobial range and a background marked by safe use in food is gaining increased attention *Bacillus* spores are additionally being utilized widely as probiotic food supplements where they are utilized in human as dietary enhancements and feed for domesticated animals and hydroponics as development promoters [41]. An epic idea of multi-target food protection has arisen comparable to jump innovation expressing microbial wellbeing. The healthful characteristics of nourishments depend on joined additive elements (called obstacles including *Bacillus sp*.) that microorganisms present in the food cannot survive. They were subsequently prompting microbial development restraint by upsetting their homeostasis and metabolic depletion and evading braid response by microscopic organisms. *Bacillus pumilus* and *Bacillus mycoides* have isolated and screened for the bacteriocin, which has played a significant role in the food industry [11]. Bacteriocin produced by *Bacillus pumilus* has antibacterial activity against *Staphylococcus aureus*, among other pathogens. *Bacillus pumilus* can produce a noble antibiotic called pumilin, which significantly impacts the food industry [12].

6. Conclusion

Rastrineobolla argentea (Omena) was found to be contaminated with *Bacillus* spp. that produced bacteriocin effective against either the Gram positive. *Bacillus* species isolated and identified from Omena (*Rastrineobola argentea*). included *Bacillus mycoides, subtilis, pumilus* and *lentus* as the most predominant species.

"According" to ref [39], the bacteriocin from B. subtilis has high antimicrobial activity and that members of the genus *Bacillus* are known to produce a wide arsenal of antimicrobial substances, including peptide and antibiotics, and bacteriocins.

The bacteriocin produced from the bacillus species obtained from *Rastrineobola argentea* are heat stable at 100^0C but lose more than 50% of their activity after exposure at autoclaving temperature for 15 minutes. They can therefore withstand pasteurization temperature without significant loss of their activity and they remained stable on boiling at such temperatures.

The crude bacteriocins obtained are proteineous and they were resistant to proteolytic enzymes and susceptible to trypsin and to lipase.

The crude bacteriocins could work in environment contaminated with 1mM copper or iron but their activity is greatly reduced by zinc ions at the same concentration.

The crude bacteriocins are also influenced by variation in pH ranges.

A slightly basic environment is best for the action of the bacteriocin with the optimum activity being achieved at pH 7. Acidic environment of pH 5 and below render the bacteriocin completely inactive.

These properties could be exploited for commercial applications of this bacteriocin.

Stability in neutral pH range (up to pH 9), resistance to proteolytic cleavage but sensitivity to trypsin and heat stability at physiological temperatures allow the crude bacteriocin from *Bacillus* extracted from Omena to be a competitive advantage of antimicrobials preparations. Based on the presented results therefore, it may be concluded that the antimicrobial substance from the culture supernatant of *B. subtillis* and *B. pumillus* is a peptide with a diverse spectrum of antimicrobial activity. Stability in alkaline and neutral pH range (up to pH 9),

resistance to proteolytic cleavage but sensitivity to trypsin and heat stability at physiological temperatures allow bacteriocin from *B. subtillis* and *B. pumillus* to be a competitive advantage of bacteriocin preparations for treatment of mastitis pathogens.

The bacteriocins obtained from bacillus isolated from *Rastrineobola argentea* can therefore be used to treat mastitis caused by *Escherichia coli, Staphylococcus aureus Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, the bovine mastitis pathogens as established by the sensitivity tests.

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