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Inhibition Of Bovine Mastitis Pathogens By Bacteriocins Produced By *Bacillus Spp* Isolated From *Rastrineobola Argentea* (Omena)

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Abstract: **Bacteriocins offer an advantage over antibiotics in that they target very specific organisms and they are generally regarded as safe for humans. The aim of this study was to isolate and identify bacteriocin-producing *Bacillus* species from *Rastrineobola argentea* (Omena) and to screen their bacteriocins for their potential as antimicrobial agents against bovine mastitis pathogens. A total of 300 *R. argentea* samples were randomly purchased from retailers in Gikomba market-Nairobi. To identify isolated bacteria, colonies were assessed using morphological, biochemical techniques and the API KIT. Further, crude bacteriocins produced by some of the isolates were tested for their antimicrobial activity against the bovine mastitis pathogens *Escherichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC-25923 by well diffusion method. The physicochemical characteristics of the crude bacteriocins produced were also assessed. The study identified *Bacillus mycoides, subtilis, pumilus* and *lentus* as the most predominant species. Among these, *Bacillus subtilis* and *pumilus* were found to produce crude bacteriocins with antimicrobial activity against *E. coli* and *S. aureus,* the bovine mastitis pathogens. The crude bacteriocins produced were found to be fully or partially inactivated in the presence of proteolytic enzymes like trypsin and had a lipid moiety since their activity reduced in presence of lipase enzyme. In contrast, their antimicrobial activity was not affected by temperature treatment of up to 100°C or in presence of ions like copper or iron but there was slight reduction in activity with zinc ions. Their activity was optimal at neutral and alkaline pH but reduced significantly at acidic pH. The bacteriocins produced by these *Bacillus* species have desirable characteristics that make these isolates attractive candidates with potential application for prevention of bovine mastitis pathogens.**

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

1. INTRODUCTION

Mastitis is considered the most economically important disease affecting the dairy industry. Its management strategies involve the extensive use of antibiotics for its treatment and prevention. It's thought that prophylactic dosages of antibiotics used in mastitis control programs could select for strains with resistance to antibiotics. The emergence of multidrug resistant pathogens and imposed restrictions on the use of antibiotic feed additives has intensified the search for novel alternatives [1]. In addition, a strong drive towards reducing antibiotic residues in animal food products has led to research in finding alternative antimicrobial agents. In this regard, much interest has been focused on bacteriocins due to their great potential applications in medicine [1].

Bacteriocins are ribosomally synthesized antimicrobial peptides lethal to bacterial other than the producing strain [2] and are the most abundant of antimicrobial compounds produced by bacteria. These antimicrobial peptides are found in all

major phylogenetic bacterial lineages [3]. During the last 20 years, bacteriocins of lactic acid bacteria (LAB) have been given much attention because some of them exhibit high activity against pathogenic organisms [1]. In contrast, bacteriocins from *Bacillus* have attracted little attention even though some *Bacillus* spp., such as *Bacillus subtilis* and *Bacillus licheniformis*, are generally recognized as 'safe' bacteria [4]. The primary habitat of most *Bacillus* species is the soil but *B. subtilis, B. cereus, B. firmus* and *B. lentus* have been isolated from both fresh and marine water bodies [5] and from processed fish [6]. Being capable of producing a large number of antimicrobial peptides, *Bacillus* is an interesting genus to search for inhibitory substance [7]. The production of bacteriocins or bacteriocin-like substances has been described for *B. coagulans, B. brevis, B. licheniformis, B. cereus, B. subtilis, B. amyloliquefaciens* and other *Bacillus* species [4][8]. Some *Bacillus* bacteriocins with strong inhibitory activity against *Staphylococci* like *B. thuringiensis* have been recommended for further exploration with an aim in use in the control of mastitis in cows [9]. In the current study, we sought to isolate and characterize *Bacillus* species from Omena (*Rastrineobola argentea*) and further test their bacteriocins for antimicrobial activity against bovine mastitis pathogens.

II. MATERIALS AND METHODS

2.1 Isolation and identification of beneficial microorganisms:

A total of 300 *Rastrineobola argentea* (omena) samples in regular consumer packages (500g) were randomly purchased from retailers in Gikomba market, Nairobi-Kenya. Twenty five grams from each sample was placed in a bag containing 225ml of lactose and macerated using a stomacher 400 Circulator Homogenizer for 2mins and heated for 10mins at 80°C to destroy vegetative bacteria and fungi and to make easier the isolation of *Bacilli* from spores that survive the heat treatment. Ten-fold serial dilutions of the liquid supernatant were prepared with the same diluents up to 10^{-5}. One milliliter of dilutions was mixed with molten (45°C) mannitol-egg yolk polymyxin (MYP) agar and poured into plates. After incubation at 37°C for 24 to 48h, the colonies that appeared on the plates were counted as colony forming units (CFU) per gram fresh weight sample. One milliliter volume from 10^{-5} dilutions was placed on duplicate nutrient agar plates for a total plate count (TC). Morphologically distinct colonies on the spore count plates were purified by streaking them on further nutrient agar (NA) plates. Gram and spore staining were done by conventional methods [10]. Selected strains were examined for the presence of spores by microscopy after growth on nutrient agar at 37°C for 2 to 5 days.

Biochemical tests were then carried out according to a method by Harrigan [11] to identify the *Bacillus* species [12]. The biochemical tests included: catalase test, nitrate reduction test, Voges Proskauer reaction, indole production test, growth on Simmons citrate agar, sugar fermentation test, motility hydrolysis of starch and casein. The strains to be studied further were identified by API 50 CH kits at 30°C according to the manufacturer's instructions (API system, BioMeriux S.A., Marcy Petoile). The isolates were preserved at 4°C on Nutrient agar (NA) slants and also in glycerol then stored in liquid nitrogen till use.

Antimicrobial activity of isolated *Bacillus* strains was tested by well diffusion method. Wells (10mm in diameter) in nutrient agar was incubated with both culture of *Bacillus* for 24 h. The plates were then separately overlaid with a solution of indicator strains; *staphylococcus aureus* (ATCC-5923) and *Escherichia coli* (ATCC-25922) by mixing 50µl of strain (24 h culture on TSB broth at a concentration of 108cfu/ml) with 200ml of Mueller Hinton Agar (Oxford, Hampshire, UK). After the overlays solidified, the plates were incubated for 24 h and then examined for a zone of inhibition around the well. The activity representing the diameters of the inhibition zone was expressed in millimeters.

2.2 Preparation of crude bacteriocins:

Isolates selected as potential bacteriocin producers were grown in nutrient broth at 30°C for 24 h. After incubation, the broth was centrifuged at 5000 rpm for 10 mins to separate the cells and supernatant containing the bacteriocin [13]. The supernatant was adjusted to pH 7.0 by 1M NaOH to exclude the antimicrobial effect of organic acid and dialyzed for 24 h at 4°C. Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5mg/ml catalase (C-100 bovineliver, Sigma). Neutralized supernatants were sterilized by filtration and tested for antimicrobial activity against indicator organisms using the agar well diffusion method [14].

2.3 Detection of inhibitory activity of crude bactericins against bovine mastitis pathogens:

Cell free culture supernatants obtained from the bacteriocin producers by centrifugation of cultures at 5000 rpm at 4°C for 10 mins were adjusted to pH 6 with 1N NaOH and stored at -20°C until use. The antimicrobial activity of the

supernatants was determined by the well diffusion method. Briefly, 100µl of supernatants were placed in wells (8mm in diameter) cut in Luria Bertani (LB) agar plates (20ml) seeded with the following bovine mastitis pathogens; Gram positive (*Staphylococcus aureus* ATCC-25923) and Gram-negative (*Escherichia coli* ATCC-25922) organisms. These verified strains were obtained from the Central Veterinary Laboratories in Kabete, Kenya. The plates were incubated at 37°C for 24 h after which the diameters (in mm) of the zones of growth inhibition were then measured and recorded [13].

2.4 Sensitivity of crude bacteriocins from Bacillus species to temperature, pH, enzymes and metal ions:

A volume of 5ml of bacteriocin in different test tubes were overlaid with paraffin oil to prevent evaporation and then heated for 15 mins at 60°C, 70°C, 80°C, 100°C, and at 121°C under pressure. Residual bacteriocin activity was evaluated against indicator bacterial pathogens at each of these temperatures by agar–well diffusion assay as described [15]. Sensitivity of crude bacteriocin preparation to different pH values was tested by adjusting the pH in the range of pH 3 to 9 with sterile 1N NaOH and 1N HCl according to a method by Karaoglu *et al* [16]. After 2 h of incubation at room temperature, residual activity of each of the samples was determined against the indicator organisms by agar-well diffusion assay.

To test the effect of enzymes, a 5ml aliquot of bacteriocin preparation was taken in test tubes and treated with lipase, proteinase K and trypsin enzymes (Sigma) each at a final concentration of 1mg/ml. The test tubes with and without the enzyme (control) were incubated for 2 h at 37^{o}C and heated for 3 min at 100^{o}C to denature the enzyme and residual activity of bacteriocin for both the control and the samples were assayed as described Nakamura *et al* [17].

The effect of metal salts on bacteriocin 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 was used as a positive control. All samples were incubated at room temperature for 2 h and then tested for residual antimicrobial activity [17] by agar-well diffusion assay.

2.5 Data analysis:

The data obtained were expressed as means of duplicate experiments. All statistical analysis was performed using SPSS ver. 17.0 for Windows software (SPSS, Inc).

III. RESULTS AND DISCUSSION

Identification of isolated bacteria:

A total of ninety eight (98) *Rastrineobola argentea* samples were assayed using morphological, biochemical techniques and the API KIT. The predominant colonies were either pink or yellow in colour, with entire or undulated margins and either flat or raised in elevation 94 (95%) (Table 1). Gram staining revealed that 90 (91%) of the isolates were rods while 8 (9%) were cocci arranged as chains or clusters. All cocci isolates were Gram negative and were discarded. The rods were Gram positive and were used in further investigations. Terminal spores were present in 82 (91%) of the rods while the rest had no spores (Data not shown). The Gram positive rod isolates presumptive of *Bacillus spp* were assessed using biochemical tests. Most isolates were catalase positive, able to grow on Simmon's citrate agar, fermented mannitol and could hydrolyse both starch and casein. Some isolates were negative on Voges-Proskauer test, could not reduce nitrate or ferment glucose (Table 2). The isolates were further matched to known *Bacillus* species using the API KIT profiles and showed that most had high identities (99%) while some had below 50% identity (Table 3). In summary, *Bacillus mycoides, subtilis, pumilus* and *lentus* were the most predominant species. Isolates S371, S041, S602, S063, S021, S082, S13, S222, S401, S221, S031, S601, S351 and S241 were identified as *Bacillus mycoides;* S522, S623, S161,S103, and S581 as *Bacillus subtilis*; S461, S231 and S442 as *Bacillus pumilus*; S622, S092 and S341 as *Bacillus lentus;* S531 as *Bacillus anthracis* while S541 was identified as *Bacillus circulans.*

Bacillus are known to form endospores that are resistant to heat and therefore can germinate when cultured in growth media even after heat treatment [9]. They are found in diverse environments because of their ability to produce endospores, their wide physiological properties and growth requirements [9]. They are known to be heterotrophic, thermophiles, acidophilic or alkalophilic [18]. They have previously been isolated from Chinese herbs [19], sea water [20], milk [21] and other environments [22]. According to the Bergey's Manual of Determinative Bacteriology, *Bacillus* species are mainly described as gram positive aerobic or facultative, spore forming rods. Some are gram negative but they appear positive due to presence of the spore at the capsule [23]. Biochemical tests performed were inconclusive

necessitating the further use of API KIT and online apiweb™ identification software gave six *Bacillus* species. More than half (57%) of the isolates were identified as *Bacillus mycoides* and the rest were *Bacillus subtilis* (17%), *pumilus* (10%), *lentus* (10%), *anthracis* (3%) and *circulans* (3%).

Antagonistic screening with bovine mastitis pathogens:

Supernatants from selected isolates were tested for their ability to inhibit growth of the bovine mastitis pathogens *E. coli* ATCC-25922 and *S. aureus* ATCC 25923. After incubation for 24 h at 37°C most of the supernatants were effective against both *E. coli* and *S. aureus* as shown by clear zones of inhibition whose diameter was measured and recorded (Table 4). However, supernatants from nine (9) isolates (S522, S622, S541, S13, S092, S222, S081, S031 and S161) did not show any activity against *S. aureus* ATCC 25923 while only supernatants from two (2) isolates (S461 and S371) did not show any activity against *E. coli* ATCC-25922.

This indicates that *S. aureus* was more resistant to the inhibition by the isolates as compared to *E. coli* and can be explained by the fact that Gram negative bacteria are known to be highly resistant to existing treatments .The ability of some isolates to inhibit both microorganisms showed the possibility of their metabolites to be used against mastitis pathogens. A study by Xie *et al.*, [19] found *Bacillus subtilis* strain LFB112 to have inhibitory activity against most pathogenic strains, including some important animal pathogens and two multidrug resistant clinical isolates of *E. coli* and *Salmonella pullorum*, as well as against a yeast strain [20]. Several other studies have reported the production of bacteriocins by *Bacillus spp*. previously, and the best-characterized bacteriocins are subtilin of *B. subtilis* [24], megacin of *Bacillus megaterium* [25], lichenin of *B. licheniformis* [26], tochicin of *Bacillus thuringiensis* [27], and the bacteriocins of *Bacillus cereus* [28]. Bacteriocins from some *Bacillus* species have shown wide inhibitory spectrum against both gram negative and gram positive bacteria [29], [7], [30]. However, some bacteriocins have shown a narrow inhibitory spectrum against either some gram positive or gram negative bacteria [9], [31]. The crude bacteriocins in this research show they can find application to both gram positive and negative bacteria especially against treating bovine mastitis pathogens.

Effect of pH, enzymes, temperature and metal ions on activity of crude bacteriocin produced by Bacillus isolates:

The crude bacteriocins from *Bacillus* isolates were assayed for their ability to inhibit growth of *E. coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923 at a range of pH, after exposure to different temperatures or enzymes and in the presence of different metal ions. When isolates from *Bacillus subtilis* and *pumilus* were treated with pH ranging from 3-9 and their supernantants tested against bovine mastitis pathogens there was no zone of inhibition observed with pH below 5 (Table 7). However, there was inhibition observed for pH 7 to 9 with a mean zone of inhibition of 19.57mm and a range of 11-25mm (Data not shown). This shows that the bacteriocins isolated from the *Bacillus* isolates are affected by acidic pH environments but work well in neutral and alkaline environments. Bacteriocins differ greatly with respect to their sensitivity to different pH ranges. A study of BLIS bacteriocin from *Bacillus mycoides* showed it was stable over a wide pH range of 4–11 [32]. In contrast, a bacteriocin isolated from Chinese herbs by Xie *et al.*, was stable at pH (3–10) [19].

When crude bacteriocins were heated at different temperatures and tested against bovine mastitis pathogens, temperatures of up to 80°C did not cause any significant reduction in the bacteriocin activity (Figure 1). However, after exposure to 100°C about 40% of the bacteriocin activity was lost and further increase in temperature to 121°C led to more than 50% loss of activity. This shows that the crude bacteriocins are effective up to 80°C above which their activity is significantly reduced. *Bacillus* bacteriocins are usually thermal stable. A study on Entomocin 110 obtained from *B. thuringiensis subsp. entomocidus* HD110 and entomocin 9 showed that they retained 53% and 72% of their activity respectively even after autoclaving [33], [30]. Also, the BLIS bacteriocin from *B. mycoides* isolated by Sharma & Gautam was stable at 100°C [32]. When the crude bacteriocins from selected *Bacillus* isolates (*B. subtilis* and *pumilis*) were treated with metal ions, there was a similar trend observed in the residual activity of bacteriocins against bovine mastitis pathogens (Table 5). In particular, Cu^{2+} ions had the highest residual activity (91-100%) for all the *Bacillus* isolates tested (S103, S581, S231 and S442) with Zn^{2+} having the least residual activity (71-80%). This shows that the activity of the bacteriocins produced are affected by the presence of Zn^{2+} ions, they would therefore not work well if used with drugs that constitute these ions and the presence of trace elements in food would not significantly reduce the activity of the bacteriocins.

When the crude bacteriocins from *Bacillus* isolates were exposed to proteinase K, trypsin and lipase enzymes, the zones of inhibition reduced significantly. The crude bacteriocins exposed to proteinase K did not show any zone of inhibition while those exposed to trypsin lost 40% of their activity. However, lipase caused only a 15% loss of the bacteriocin

activity (Table 6). This shows that the crude bacteriocins are proteinaceous in nature and it's the reason why they were affected by the presence of proteolytic enzymes. They may have a lipid moiety whose hydrolysis by the lipase enzyme did not lead to significant loss of the crude bacteriocins' activity. A study of two strains of *B. subtilis*, PB3 and PB6 by Alex and Hai-Meng [4] showed they had antimicrobial activity against *C. perfringens* ATCC 13124. They showed that filtrates of these *Bacillus* species contained a proteinaceous antimicrobial factor that was stable in the presence of high heat and in the presence of bile salt and solvents [34].

IV. CONCLUSION AND RECOMMENDATION

This current study identified *Bacillus* species from Omena (*Rastrineobola argentea*), among which, *Bacillus subtilis* and *pumilus* were found to produce crude bacteriocins with antimicrobial activity against *E. coli* and *S. aureus*, the bovine mastitis pathogens. The crude bacteriocins produced by the selected isolates were heat stable at 100°C and could work optimally at neutral and alkaline pH. Their proteinaceous nature, ability to work in environments with ions like copper or iron and the retention of their inhibitory activity after heat treatment are desirable characteristics that make these isolates attractive candidates with potential application for prevention of bovine mastitis pathogens.

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APPENDIX.A

TABLES AND FIGURES:

Table 1: Summary of morphological characterization of isolates obtained from *Rastrineobola argentea*

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

Table 2: Summary of biochemical characterization of isolates obtained from *Rastrineobola argentea*

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

Table 3: Summary of microorganisms identified using the API KIT in isolates obtained from *Rastrineobola argentea*

Isolate	**Microorganism**	**(%)**
S522	*Bacillus subtilis*	99.3
S461	*Bacillus Pumilus*	99.6
S231	*Bacillus pumilus*	58.4
S442	*Bacillus Pumilus*	99.9
S161	*Bacillus subtilis*	99.6
S541	*Bacillus subtilis*	42
S103	*Bacillus subtilis*	57.5
S581	*Bacillus subtilis*	99.8

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Table 4: Antibacterial activity of *Bacillus* isolates against *E. coli* ATCC-25922 and *S. aureus* ATCC 25923.

Isolate No.	Zone of Inhibition	
	E. coli	*S. aureus*
S103	24mm	22mm
S401	24mm	25mm
S341	24mm	26mm
S601	23mm	26mm
S221	22mm	26mm
S522	22mm	-
S622	20mm	-
S623	22mm	20mm
S581	20mm	24mm
S541	19mm	-
S13	19mm	-
S531	18mm	24mm
S02	18mm	18mm
351	18mm	29mm
S241	17mm	26mm
S063	16mm	24mm
S231	15mm	24mm
S442	14mm	11mm
S041	13mm	17mm
S092	12mm	-
S222	12mm	-
SO81	12mm	-
S031	10mm	-
S461	-	25mm
S371	-	15mm
S161	11mm	-
S602	24mm	24mm

Table 5: Effect of metal ions on crude bacteriocin activity against selected bovine mastitis pathogens

Isolate	*Bacillus* species	*E. coli* ATCC-25922			*S. aureus* ATCC-25923		
		Cu^{2+}	Fe^{2+}	Zn^{2+}	Cu^{2+}	Fe^{2+}	Zn^{2+}
S103	*B. subtilis*	+++	++	+	+++	++	+
S581	*B. subtilis*	+++	++	+	+++	++	+
S231	*B. pumilus*	+++	++	+	+++	++	+
S442	*B. pumilus*	+++	++	+	+++	++	+

***Key**: Percentage residual activity +++ (91-100%); ++ (81-90%); + (71-80%)*

Table 6: Effect of enzymes on crude bacteriocin activity against selected bovine mastitis pathogens

Isolate	*Bacillus* species	*E. coli* ATCC-25922			*S. aureus* ATCC-25923		
		Proteinase K	Trypsin	Lipase	Proteinase K	Trypsin	Lipase
S103	*B. subtilis*	-	+	++	-	+	++
S581	*B. subtilis*	-	+	++	-	+	++
S231	*B. pumilus*	-	+	++	-	+	++
S442	*B. pumilus*	-	+	++	-	+	++

***Key**: Percentage residual activity ++ (≥85%); + (≤65%) ; - (No inhibition)*

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Table 7: Effect of pH on crude bacteriocin activity against selected bovine mastitis pathogens

Source of crude bacteriocin		Indicator organism	pH of Media				
Bacillus spp.	**Isolate**		**pH 3**	**pH 4**	**pH 5**	**pH 7**	**pH 9**
Bacillus subtilis	S103	*S. aureus* ATCC-25923	-	-	-	+	+
	S581	*S. aureus* ATCC-25923	-	-	-	+	+
Bacillus pumilus	S231	*S. aureus* ATCC-25923	-	-	-	+	+
	S442	*S. aureus* ATCC-25923	-	-	-	+	+
	S461	*S. aureus* ATCC-25923	-	-	-	+	+
Bacillus subtilis	S522	*E. coli* ATCC-25922	-	-	-	+	+
	S622	*E. coli* ATCC-25922	-	-	-	+	+
	S161	*E. coli* ATCC-25922	-	-	-	+	+
	S103	*E. coli* ATCC-25922	-	-	-	+	+
	S581	*E. coli* ATCC-25922	-	-	-	+	+
Bacillus pumilus	S231	*E. coli* ATCC-25922	-	-	-	+	+
	S442	*E. coli* ATCC-25922	-	-	-	+	+

Key: *- No zone of inhibition; + Zone of inhibition present (Mean=19.57mm, range=11-25mm)*

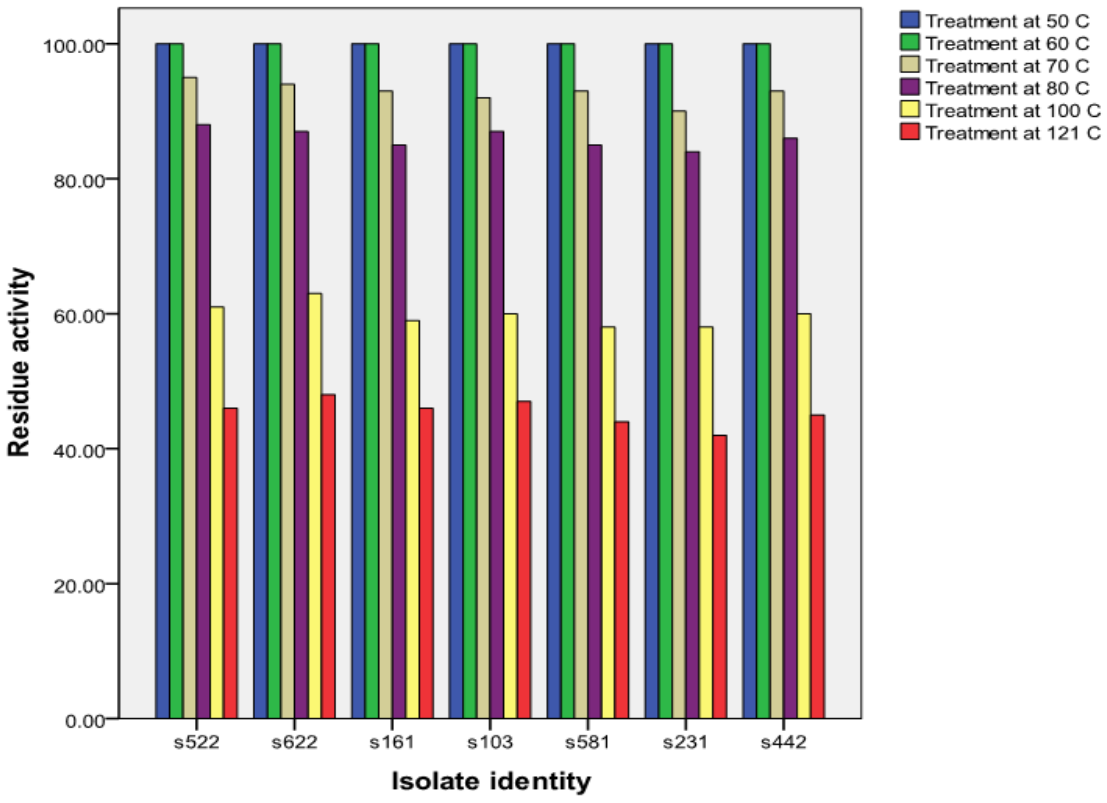

Figure 1: Effect of temperature on activity of crude bacteriocins from selected *Bacillus* isolates obtained from *Rastrineobola argentea*