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

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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. It has triggered the investigation of novel antimicrobial agents produced by various bacterial strains of apparent low virulence having antimicrobial activity wide range of clinically significant organisms [2]. The production of an antimicrobial substance by microorganisms is an essential factor in microbial ecology [3]. Most of these substances play a crucial role in bacterial interactions, including bacteriocins, 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 pharmaceuticals and preservatives in food industries [5]. Different bacteriocin may be produced within the same species and are ribosomally synthesized in the host while the producer strain possesses a specific self-protection mechanism [6]. The bacteriocins are heterogeneous compounds possessing variability of biochemical properties, molecular weight, activity spectra, and action mode [7].

According to ref [2], 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 [5], bacteriocins are gaining more attention as an alternative therapeutic agent for preventing and treating infections and as preservatives in food industries to avoid deterioration and spoilage of food. Bacteriocins are generally recognized as naturally occurring food preservatives able to influence foods' quality and safety [8]. According to ref [9], non-clinical bacteriocin also has 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 bacteriocin [10]. Many of the Bacillus bacteriocins belong to the lantibiotics, a category of post-translationally modified peptides widely disseminated among different bacterial clades [10]. In their study, ref [11,16,17] isolated bacterial strains of Bacillus subtilis. Omena (*Rastrineobola argentea)* established that the strains produce crude bacteriocins antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* bovine mastitis pathogens.

2. Materials and Methods

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

The antimicrobial activity of the twenty isolated Bacillus strains was tested by a well diffusion method. Wells (10 mm of diameter) in nutrient agar were incubated with both cultures of Bacillus for 24 hours. The plates were then separately overlaid with a solution of indicator strains; *Staphylococcus aureus* (ATCC-25923*), Escherichia coli* (ATCC-25922), *Pseudomonas aeruginosa* (ATCC 15442), and *Klebsiella pneumoniae* (BAA-1705) by mixing 50μl of strain (24 hours culture on TSB broth at a concentration of 10^8cfu/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 inhibition zone was expressed in millimetres.

2.2. Production and Purification of 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 1mol/l NaOH to remove the antimicrobial effects of organic acids, and it was used as crude bacteriocin [12]. Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase (C-100 bovine liver, Sigma). Neutralized filtrates were sterilized by filtration and then

tested for antimicrobial activity against the indicator organisms using the agar well diffusion method as described by [18].

2.3. Physiochemical Characterization of Bacteriocins from Bacillus Subtilis

2.3.1. Effect of Temperature

Five millilitres of bacteriocin in different test tubes were overlaid with paraffin oil to prevent evaporation and then heated for 15 minutes at 60°C, 70°C, 80°C, 100°C, and at 121°C for 15 minutes under pressure. Residual bacteriocin activity was evaluated against indicator *S. aureus* and *E. coli, P. aeruginosa and K. pneumoniae* at each of these temperatures by agar – well diffusion assay [13].

2.3.2. 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 [14]. After 2 hours of incubation at room temperature, each of the samples' residual activity was determined against the indicator organism by agar-well diffusion assay.

2.3.3. Effect of Proteolytic and Lipolytic Enzymes on Crude Bacteriocin Activity

Five-millilitre 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°C and heated for 3 min at 100°C to denature the bacteriocin's enzyme and residual activity [1].

2.3.4. Effect of metal Ions on Crude Bacteriocin Activity

The effect of metal salts on bacteriocin activity was examined by addition 100μl of $CuSO_4$, $FeSO_4$, 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 [13] by agar well diffusion assay.

3. Results

## 3.1. Screening of the Isolates for Bacteriocins Properties against Mastitis Pathogens

All the 20 Bacillus 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, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 15442, and *Klebsiella pneumoniae* BAA-1705 using the sensitivity tests method (Figure 1). The Zone of inhibition on inoculated plates was observed and measured. Out of the 20 Bacillus subtilis presumptive

isolates, all isolates showed antimicrobial activity. All twenty (20) isolates could inhibit the growth of *Escherichia coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923, respectively (Table 1). Additionally, all of the twenty isolates could inhibit the growth of *Pseudomonas aeruginosa* ATCC 15442 and *Klebsiella pneumoniae* BAA-1705. Isolates with Zone of inhibition measuring between 17 to 30mm were considered sensitive, 14 to 16mm semi-sensitive or intermediate, while those below 14mm were considered resistant (Based on control). The result showed that all *Bacillus subtilis* were sensitive against *Escherichia coli* ATCC-25922 (Zone of inhibition measuring between 17 to 30mm), inhibit *Staphylococcus aureus* ATCC 25923. For the *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* BAA-1705, the results showed 100% were inhibited. Hence, those isolates that showed antimicrobial activity against *Escherichia* coli ATCC-25922 and *Staphylococcus aureus* ATCC 25923 were further characterized based on carbohydrates fermentation using API 50 CH B/E kits. Inhibition by *Klebsiella pneumoniae* BAA-1705 and *Pseudomonas aeruginosa* ATCC 15442 was observed (Table 1).
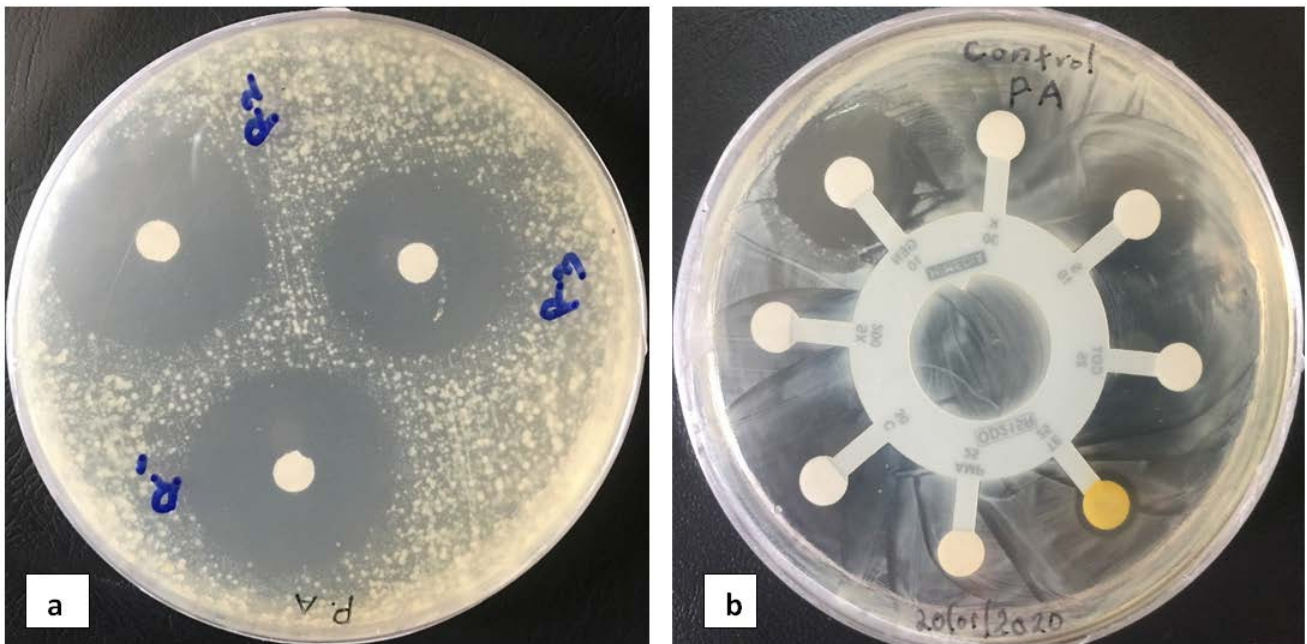
Figure 1. a Zone of inhibition of selected isolates of crude bacteriocin (R1, R2, and R3) on *Pseudomonas aeruginosa* at pH 7 on nutrient agar; **b:** positive control showing Zone of inhibition of different antibiotics on *Pseudomonas aeruginosa*
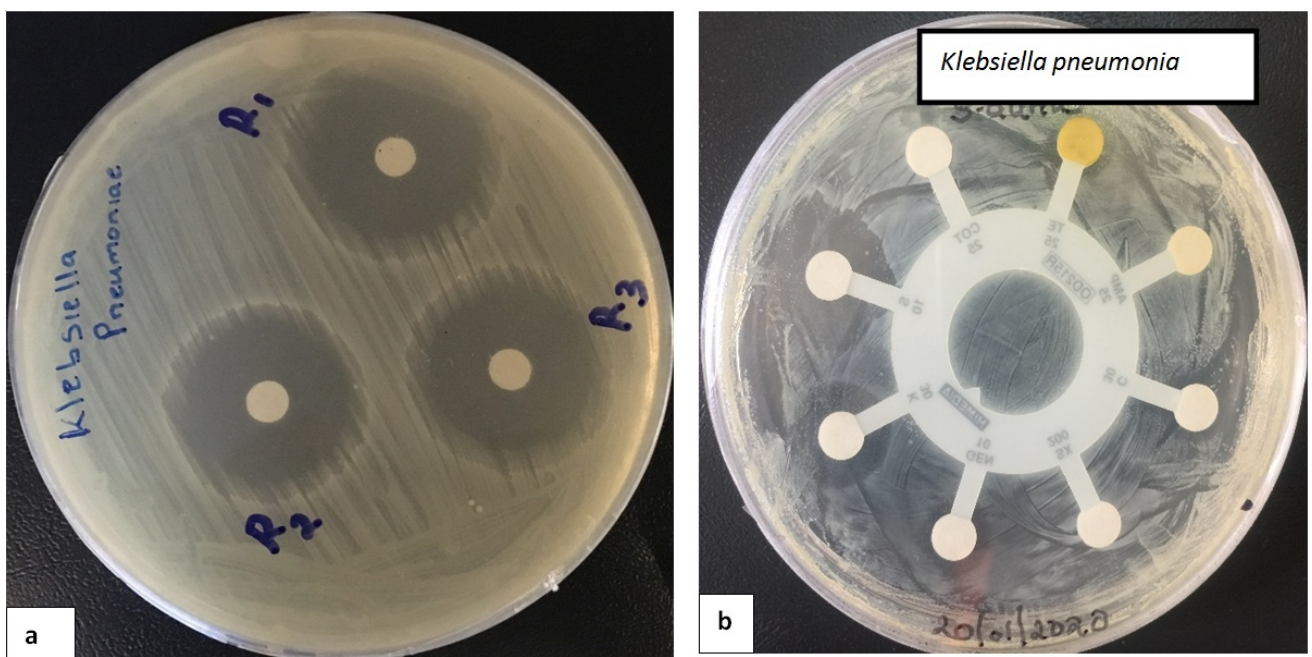
Figure 2. Zone of inhibition of selected isolates of crude bacteriocin (R1, R2, and R3) on *Klebsiella pneumoniae* at pH 7 on nutrient agar
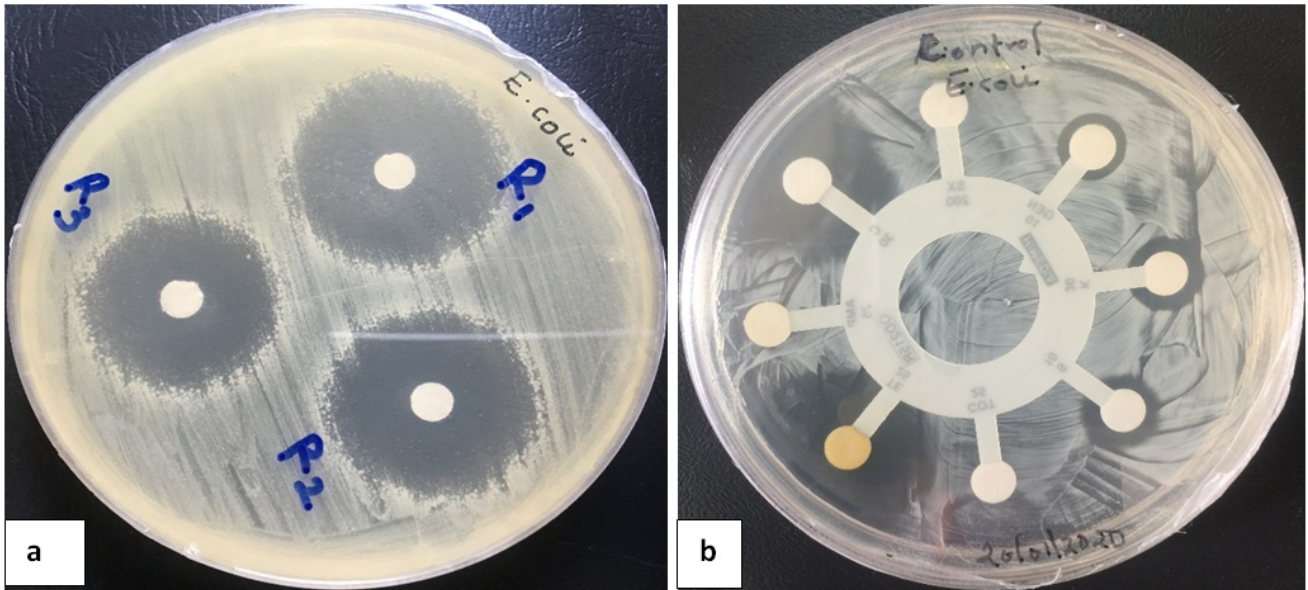
Figure 3. a: Zone of inhibition of selected isolates of crude bacteriocin (R1, R2, and R3) (a) on *E coli* at pH 7 on nutrient agar; positive control showing Zone of inhibition of different antibiotics on *E coli* (**b**)
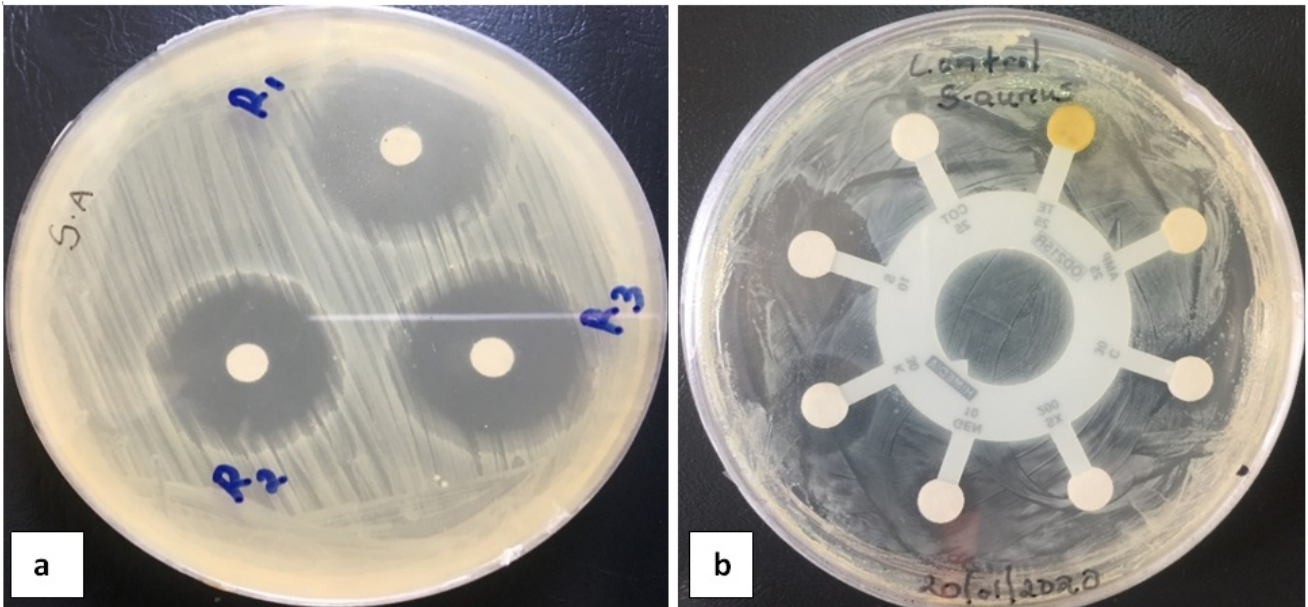
Figure 4. a Zone of inhibition of selected isolates of crude bacteriocin (R1, R2, and R3) on *Staphylococcus aureus* at pH 7 on nutrient agar; **b:** positive control showing Zone of inhibition of different antibiotics on E coli

Table 1a. Antimicrobial activity of supernatant (crude bacteriocin) obtained from different *Bacillus subtilis* isolates inhibition zone (diameter, mm) against tested bacteria

Isolate	*Escherichia coli* ATCC-25922	*Staphylococcus aureus* ATCC 25923	*Pseudomonas aeruginosa* ATCC 15442	*Klebsiella pneumoniae* BAA-1705
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

Isolate	*Escherichia coli* ATCC-25922	*Staphylococcus aureus* ATCC 25923	*Pseudomonas aeruginosa* ATCC 15442	*Klebsiella pneumoniae* BAA-1705
R51	s25.0 ± 1.0	i15.3 ± 0.6	r19.3 ± 0.9	r11.3 ± 0.4
R52	s23.7 ± 1.0	r5.3 ± 0.6	i12.3 ± 0.4	r5.3 ± 0.2
R53	i15.0 ± 1.5	r13.3 ± 1.5	s18.3 ± 1.1	r12.3 ± 1.1
R57	s24.0 ± 1.0	r12.0 ± 1.0	s18.0 ± 1.2	r11.0 ± 1.0
R58	s21.3 ± 1.0	s18.7 ± 1.2	i8.7 ± 1.0	s19.7 ± 1.3
R59	s20.3 ± 1.5	s22.3 ± 1.5	s21.3 ± 1.1	i12.3 ± 1.0
R60	s23.7 ± 1.5	s25.0 ± 1.0	r15.0 ± 1.3	r5.0 ± 0.2

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

Table 1b. Kirby-Bauer Antibiotic Sensitivity assay against *S. aureus*, *E. coli*, *P. aeruginosa*, and *K. pneumonia*

Bacteria	Amp	TE25	CoT25	S10	K30	GEN10	SX200	C30
S. aureus	NI	i9.0	NI	r15.0	r14.0	i9.0	NI	r10.0
E. coli	NI	s25.0	NI	r11.0	r11.0	i9.0	NI	s23.0
P. aeruginosa	NI	i8.0	NI	r13.0	i9.0	s19.0	NI	s22.0
K. pneumoniae	NI	s20.1	NI	r14.1	i8.0	r10.1	NI	s23.2

Legend: Amp: Ampicillin; TE: Tetracycline; CoT: Cotrimoxazole; S: Streptomycin; K: Kanamycin; GEN: Gentamicin; SX: Sparfloxacin and C: Chloramphenicol

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

3.2. Physiochemical Characterization of Bacteriocins from *Bacillus* Subtilis

3.2.1. Effect of pH on Crude Bacteriocin

The bacteriocin from different isolates was characterized at different pH conditions of pH 3, 4, 5, 7, and 9. The results were recorded based on the presence or absence of Zone of inhibition after incubation using the well diffusion method, as shown in Table 2 below.

Table 2. Residual activity of bacteriocin on *E. coli* ATCC-25922, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* BAA-1705, and *Pseudomonas aeruginosa* ATCC 15442 at different pH levels

Source of bacteriocin/Isolate	*Escherichia coli* ATCC-25922						*Staphylococcus aureus* ATCC 25923						*Pseudomonas aeruginosa* ATCC 15442						*Klebsiella 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	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++	-	-	-	+	++	++

Key: - Shows there was no zone of inhibition**,** + showed a zone of inhibition (range 11-17mm), ++ (range 18-25mm).

Table 3. Effect of different temperature levels on crude bacteriocin activity against *E. coli* ATCC-25922, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* BAA-1705, and *Pseudomonas aeruginosa* ATCC 15442

Source of bacteriocin/Isolate	The temperature level of the media																								
	Escherichia coli ATCC-25922						*Staphylococcus aureus* ATCC 25923						*Pseudomonas aeruginosa* ATCC 15442						*Klebsiella pneumoniae* BAA-1705					
	50°C	60°C	70°C	80°C	100°C	121°C	50°C	60°C	70°C	80°C	100°C	121°C	50°C	60°C	70°C	80°C	100°C	121°C	50°C	60°C	70°C	80°C	100°C	121°C
R1	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R2	-	-	+	+	++	+++	-	-	+	+	+ +	+++	-	-	+	+	++	+++	-	-	+	+	+ +	+++
R3	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R4	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R5	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R6	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R44	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R45	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R46	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R47	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R48	-	-	+	+	++	+++	-	-	+	+	++	+ + +	-	-	+	+	++	+++	-	-	+	+	++	+ ++
R49	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R50	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R51	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R52	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R53	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R57	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R58	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R59	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++
R60	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++	-	-	+	+	++	+++

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.

3.2.2. Effect of Different Temperatures on Crude Bacteriocin Activity

The inhibition zones were measured, and residual activity calculated on exposure of the crude bacteriocin to different temperature levels. Table 3 shows the extent to which the bacteriocin lost its activity. Exposure of the crude bacteriocins to a temperature of 50°C and 60°C showed no activity loss based on the initial activity. As the temperature increased to between 70°C to 80°C, it caused a reduction in the bacteriocin activity of about 20%. After exposure to 100°C, about 40% of the bacteriocin activity was lost. With an increase in the temperatures to 121°C, more than 50% of the activity was lost.

3.2.3. Effect of Different Metal Ions on Bacteriocins Activity

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

Table 4. Effect of metal ions on crude bacteriocin activity against *E. coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* BAA-1705 and *Pseudomonas aeruginosa* ATCC 15442

	Metal ions											
	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	++	+	+	+	+	++	++	++	++	++	++	++

	Metal ions											
	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+}
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.

3.2.4. 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 inhibition zone, while those exposed to trypsin lost 40% of their activity. Lipase caused a 15% loss of bacteriocin activity.

Table 5. Effect of enzymes on crude bacteriocin activity against *E. coli* ATCC-25922 and *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* BAA-1705 and *Pseudomonas aeruginosa* ATCC 15442

	Enzyme activity											
	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	-	+	++	-	+	++	-	+	++	-	+	++

Isolate	Enzyme activity											
	E. coli ATCC-25922			*S. aureus* ATCC-25923			*P aeruginosa* ATCC 15442			*K pneumoniae* BAA-1705		
	Proteokinase K	Trypsin	Lipase	Proteokinase K	Trypsin	Lipase	Proteokinase K	Trypsin	Lipase	Proteokinase K	Trypsin	Lipase
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. Discussion

A bacterial suspension is used to rehydrate each of the wells, and the strips are incubated. During incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. For example, when carbohydrates are fermented, the pH within the well decreases, and that change is indicated by a change in the colour of the pH indicator. All positive and negative test results are compiled to obtain a profile number, which is then compared with profile numbers in a commercial codebook (or online) to determine the identification of the bacterial species. The *Bacillus subtilis* were identified and classified. The result correlated with literature in that most *Bacillus subtilis* can ferment carbohydrates [15].

The temperature effects in terms of bioactivity. The lower the temperature, the higher the activity and the higher the temperature, the lower the bioactivity [1]. The result show that the crude bacteriocin can be exposed to different temperature levels, the zones of inhibition were measured, and residual activity was calculated as seen in Table 3. It shows the extent to which the bacteriocin lost its activity. Exposure of the crude bacteriocins to a temperature of 50°C and 60°C showed no loss of activity based on the initial activity. As the temperature increased to between 70°C to 80°C, it caused a reduction in the bacteriocin activity of about 20%. After exposure to 100°C, about 40% of the bacteriocin activity was lost. With an increase in the temperatures to 121°C, more than 50% of the activity was lost. The result justifies the required temperature for the autoclaving media. Bellow 50°C, the media's effectiveness is lower since most of the bacteria have not been fully sterilized. It is also viewed that when temperature increases, the effectiveness of media increases, at 121°C is the optimum temperature that not only kills the microbes in the media but also does not interfere with media composition. That is why it was most effective in closing the bacteriocin activity (Table 3).

The results show the decrease of bioactivity of the enzyme as the temperature increases. The optimum temperature recorded was at 60°C for crude bacteriocin activity (Table 3). The activity gradually declines beyond 60°C. The result relates with that of ref [13], who investigated the optimum temperature and thermal stability of crude purified enzymes. According to Dhandapani & Vijayaragavan (1994), at a temperature of 55°C, it was an optimum temperature for alkaline protease from *B strearothermophilus* and 60°C for protease derived from *Bacillus sp* B21-2. Therefore, it indicates that *Bacillus sp* works well at 60°C and below. Above the optimum temperature, there was declined in the bacteriocin activity and therefore affecting the functioning of the enzymes

According to ref [13], pH plays a big role in bacteriocin activity effectiveness. The result (Table 2) indicates that there was activity from pH 6.0-9.0. The activity varies across the *Bacillus subtilis* and the test organism. The highest inhibition was recorded from pH 9.0. The findings are by previous findings showing that *Bacillus* spp does well in alkaline rather than acidic. For instance, the activity of *Bacillus sp*, *Thermus aquaticus, Xanthomonas maltophila,* and *Vibro metscnikovii* does well in pH 10.5 ref [13].

Metal ions have stimulatory effects, and most of them inhibit the effect of the enzymes. From the results (Table 4), Cu^{2+} had the highest inhibitory to both the pathogen and Zn^{2+} had the least inhibitory effect. According to ref [13], metal ions such as Ca^{2+}, Mg^{2+}, and Mn^{2+} have an increased effect on stabilizing the enzyme's protease activity. This might be due to the activation of the metal ions. The cations also have the effects reported to increase the Bacillus spp thermal stability [13]. Despite that, it is observed that metal ions protect the enzymes

against thermal denaturation and also played a big role when comes to temperature regulation and maintaining the active conformation of the enzymes especially at higher temperature [13].

Based on the results shown in Tables 1, *Bacillus subtilis* demonstrated to be more active on Gram-negative (*E. coli*, *P. aeruginosa* and *Klebsiella pneumoniae*) more than Gram-positive (S. aureus). Studies have reported that various Bacillus spp are usually more active against Gram-positive bacteria than Gram-negative bacteria. 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 [1,3]. 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 ineffectiveness of bacteria as antimicrobial toward Gram-negative pathogens has been proposed to be strongly related to EPs as the combination of bacteria antimicrobials with EPs inhibitors leads to a striking increase in antimicrobial activity [7]. This Bacillus subtilis gives us a discovery to investigate why it is more effective than Gram-negative bacteria than other bacteria in managing mastitis pathogen. The results also indicate that bacteriocin produced by the *Bacillus subtilis* against the test organism show is the best alternative in the management of the mastitis.

Table 1a shows the most commonly used treatments for dry cow and lactation therapy, as well as the prescribed withdrawal time and the potential activity continuum of mastitis pathogens. Formalized the antibiotic groups and antimicrobials used in these remedies work in various ways, and many new semi-synthetic compounds have been created to combat the challenge of antimicrobial resistance. The bulk of antibiotics used are various antibiotics that are effective against both Gram-positive and Gram-negative bacteria. Tetracyclines, such as oxytetracycline, block protein synthesis by binding to the 30s subunit and disrupt aminoacyl-tRNA binding. Tetracycline is bacteriostatic and is most selective against Gram-positive bacteria. Oxytetracycline is an irritant that can be discouraged.

Aminoglycosides (streptomycin, neomycin) block oxidative stress and peptide chain elongation by binding to the 50S ribosomal subunit. Aminoglycosides are often active against Gram-negative bacteria and are often combined with -lactam penicillins in formulations. Polymixin B is an antimicrobial agent that attaches to the cell membrane, causing it to lose its structure and permeability. It is the antibacterial of choice for P. aeruginosa infections.

Macrolide antibiotics (tylosin, lincomycin, and erythromycin) are involved in treating Gram-positive udder infections when administered parenterally or intramammary. They are bacteriostatic and, therefore, battle infection in collaboration with the host immune system. The action mechanism avoids peptide elongation by binding to the 50S ribosomal subunit and inhibiting protein synthesis.

5. Conclusion

Mastitis's economic impact as a recurring disease in dairy farming necessitates further studies into creating novel antimicrobial treatment technologies. Bacteriocins may be used as a substitute and have certain benefits over standard antibiotic treatment. Concerns about human wellbeing are, owing primarily to the rise of antibiotic resistance in pathogenic bacteria.

Bacteriocins are normally active against particular bacterial strains based on target receptors located on susceptible strains' surfaces. When assessing the cause of mastitis, the causative bacteria must be identified, and selective treatment for particular pathogens should be considered. Bacteriocins can easily destroy susceptible species by inducing cell lysis. This prompt intervention will reduce the likelihood of pathogen resistance developing. Antibiotics are usually broad-spectrum, eliminating all Gram-positive or Gram-negative bacteria they are exposed to, not just those that cause infection. Bacteriocins have the advantage of being able to operate on a single target. If a wider range of action is needed, a mixture of two or three bacteriocins can be used to ensure that more than only one pathogen is prevented during treatment.

The bacteriocins defined as the lowest concentration (MIC) should be calculated to minimize bacteriocin used in the treatment substance.

The drug distribution method in a mastitis management technique is crucial, and a teal seal can provide several advantages. For instance, it serves as a physical shield and serves as a preventative measure. By mixing an antibacterial agent with a teat seal, the enzyme is localized in the teat canal, targeting microbes that may be present near the teat opening and preventing bacteria from colonizing the thymus tissue. Bacteriocins have been found to be very specific to certain microorganisms and they have narrow spectra. Identification of mastitis bacterial pathogens and application of a specific bacteriocin to the very microbe is of advantage and found to be a very effective way of mastitis control in bovines.

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