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Diversity of Bacteriocin-encoding Gene Families and the Activity Spectrum among *Bacillus amyloliquefaciens* Isolates

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BACTERIOCINS are considered as ideal candidates for several health care applications due to their limited range of activity and rapid degradability by proteolytic enzymes. Eight bacteriocin-producing *Bacillus amyloliquefaciens* isolates were screened by polymerase chain reaction (PCR) using four sets of primers designed specifically to detect bacteriocin-producing genes on their chromosomes. Gene encoding for *Amylocyclicin* was detected in four isolates. A phylogenetic data analysis of the four *Amylocyclicin*-predicted proteins placed them in a separate node with their closest relatives, *B. amyloliquefaciens* and *Bacillus velezensis* strain FZmhtB, which until recently, was a member of the *B. amyloliquefaciens* species. Surprisingly, *Subtilosin* producing gene was detected in two of the previously mentioned isolates indicating that they contain multiple bacteriocin encoding genes, an unusual phenomenon for *Bacillus amyloliquefaciens* isolates. The remaining four isolates lacked any known bacteriocin gene family and are anticipated to contain novel gene types. The most potent of these four isolates was chosen for further large-scale production and extraction of its bacteriocin. Antibacterial activity of the extracted bacteriocin was detected in the protein fraction under the membrane cut-off value of <10,000kDa against gram-negative and gram-positive indicator bacterial isolates, with a larger average inhibition zone diameter observed for the gram-positive isolate. Furthermore, SDS-PAGE analysis of the partially purified active bacteriocin fraction revealed a protein fragment with a relative molecular weight between 7 and 7.5kDa. The PCR assay in this study provided coverage for all known *B. amyloliquefaciens* bacteriocins allowing the quick and easy screening for the presence of bacteriocin-encoding genes.

Keywords: Antibacterial activity, *Bacillus amyloliquefaciens*, Bacteriocin genes, Phylogenetic analysis, Polymerase chain reaction (PCR) screening.

Introduction

Bacteriocins are low-molecular weight peptides that are produced by various bacterial species during the primary phase of growth. They possess limited antimicrobial activity against closely related species and are easily degraded by proteolytic enzymes in the gastrointestinal tract, making them safe for human consumption with supposedly no adverse effects on the normal microbiota (Drider et al., 2016; Rodrigues et al., 2019). Bacteriocins are naturally synthesized by ribosomes as biologically inactive pre-peptides that are modified post-translationally by the bacteriocin gene cluster prior to export (Rodríguez & Dodd, 1996).

Bacteriocins target the bacterial cell membrane by binding to the hydrophobic regions of the cytoplasmic membrane lipids resulting in the formation of pores and the loss of selective permeability (Moll et al., 1999). Nevertheless, the formation of channels within the cell membrane is not the only

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mode of bacteriocin biological activity. Other mechanisms include attachment of bacteriocins to lipid II (peptidoglycan precursor) and inhibition of cell wall biosynthesis, as well as interaction with the anionic polymers associated with the bacterial cell wall and activation of the release of autolytic enzymes (Bierbaum & Sahl, 1987; Brötz et al., 1995).

Several previous studies have focused on the production of bacteriocins by gram-positive bacteria, with lactic acid bacteria-related bacteriocins receiving special attention (Zommiti et al., 2016; Wyszyńska & Godlewska, 2021). Bacteriocin-associated genes of gram-positive bacteria are organized in a multi-gene operon that encodes the structural protein as well as the additional products required for posttranslational modifications, translocation to the exterior of the cell, and strain self-protection (Klaenhammer, 1993). Similar to lactic acid bacteria, the genus *Bacillus* comprise numerous species with a history of extensive use in the production of numerous bioactive metabolites such as bacteriocins (Arguelles-Arias et al., 2009; Hatata & Fahmy, 2016). Furthermore, the majority of *Bacillus* bacteriocins belong to the previously described classes (class I, class II-a, and class II-b) of gram-positive bacteriocins (Franz et al., 2007), whereas others possess entirely novel peptide sequences (Dischinger et al., 2009; Wang et al., 2014).

Although the majority of described bacteriocins from gram-positive bacteria are still produced by lactic acid bacteria, their increased sensitivity to proteases, low solubility above pH 6, and the emergence of resistant strains, indicate a need for alternative producer bacteria (Garsa et al., 2014; Assoni et al., 2020). *Bacillus* represented an alternative bacterial genus to be investigated for the production of bacteriocins with superior properties compared to the majority of lactic acid bacteria. (Abriouel et al., 2011; Xin et al., 2015).

Whilst few recent studies have reported bacteriocins synthesis by *B. amyloliquefaciens* the majority of these studies have identified novel bacteriocins with a broad antibacterial spectrum, thermal stability over the wide pH ranges and lower sensitivity to proteolytic enzymes compared to other bacteriocins (Sutyak et al., 2008; Halimi et al., 2010; Herzner et al., 2011; He et al., 2012; Arguelles-Arias et al., 2013; Scholz et al., 2014; Lim et al., 2016; Kurata et al., 2019), leaving the door wide open for further investigations.

Given the findings of the previous reports, the present study sought to identify the bacteriocin-producing gene(s) in eight bacteriocin-producing *B. amyloliquefaciens* isolates recovered from crusty dried goat milk (Hanafy et al., 2016). Identify the family type of the produced bacteriocin(s), compare and detect any relevant variation (if found) between their sequences and those of previously published sequences, and determine if any of these isolates contain gene(s) that may encode for a novel type of bacteriocin. Tentative identification and characterized of the corresponding protein(s) will be considered.

Materials and Methods

Bacillus amyloliquefaciens isolates

Eight isolates of *B. amyloliquefaciens* were recovered from crusty dried goat milk during a previous investigational study (Hanafy et al., 2016). Prior to testing, the cultures were maintained on nutrient agar, sub-cultured on Luria-Bertani (LB) broth media and incubated at 37°C.

Detection of bacteriocin genes

Primer design

In order to survey bacteriocin gene families expressed in all eight *Bacillus amyloliquefaciens* isolates, approximately 22 bacteriocin gene(s) sequence(s) were downloaded from the GenBank database. Sequences alignment in the database revealed that genes with identical sequences were frequently represented as distinct bacteriocins with unique accession numbers. After grouping highly homologous sequences (to avoid redundancy), the DNA sequences were condensed into four distinct bacteriocin gene families found in the *Bacillus* genus. Subsequently, specific PCR primers were designed using the complete gene sequence of each family (Supplementary data). The primers were designed using the software program PRIMER3Plus (Untergasser et al., 2007). Primary names for primer pairs with homologous genes were chosen subjectively (Table 1).

TABLE 1. Sequences of the PCR primers used to detect of bacteriocin-encoding gene families present in the studied *Bacillus amyloliquefaciens* isolates

No.	gene name	Forward primer			Reverse primer			Product length
		Sequence (5'->3')	Length	Tm	Sequence (5'->3')	Length	Tm	
B_1	*Uberolysin*	TTACCAAGCAGCTGCG-TATTTT	22	59	TAGTAAAATCTAATA-AAAAGTC	22	45	329
B_2	*Subtilosin*	ATGACTTACGTAAAT-GAAATGA	22	51	TCAAGATGCTCCCCCC-GTCCCT	22	67	1281
B_3	*Mersacidin*	ATGAGTCAAGAAGCTAT-CATTCG	23	56	TTAACAAATA-CATTCAGAAGTTAG	24	51	207
B_4	*Amylolisin*	ATGAATGAGAAAATG-TATCGT	21	50	TTAACACCAGCAAGT-GTGAGT	21	57	183

DNA extraction

The eight isolates of *B. amyloliquefaciens* were cultured overnight in LB broth at 37°C for 18h. After incubation, bacterial DNA was extracted using the QIAamp Mini Kit (QIAGEN Inc, Netherlands) according to the manufacturer's protocol for isolating genomic DNA from gram-positive bacteria. Based on UV absorbance, the spectrophotometer was used to determine the molar concentration and purity of the nucleic acid DNA, yielding a final concentration of 5µg/mL and a purity A260/A280 ratio of 1.8–2.1. The DNA was subsequently stored at –20°C until use.

PCR amplification

Subsequently, using the primers listed in Table 1, PCR amplification was performed to detect the presence of a gene or genes related to the expression of various bacteriocins produced by *Bacillus* spp. The optimal annealing temperatures for the primer pairs used in this study were determined by performing multiple PCR trials in separate reactions. During the preliminary optimization reactions, the concentrations of each primer pair and $MgCl_2$ were also adjusted to achieve satisfactory amplification of each target region. Amplification cycle profiles were conducted under the following conditions: an initial denaturation step at 95°C for 5min, followed by 30 cycles of denaturation at 95°C for 1min., primer annealing temperature was modified for each primer set (1st set annealing at 50°C for 45s; 2nd set annealing at 54°C for 1min; both 3rd and 4th set annealing at 52°C for 40s) and primer extension at 72°C for 1min with a final extension step at 72°C for 7 min. The PCR reaction was conducted in a final volume of 50µL containing template DNA (200ng/µL). A concentration of 10 pmol was used for *Uberolysin* B_1 forward primer, *Subtilosin* B_2 reverse primer, *Mersacidin* B_3 forward and reverse primers, and *Amylolisin* B_4 forward and reverse primers in the PCR reaction mixture. Whereas for *Uberolysin* B_1 reverse primer and *Subtilosin* B_2 forward primer concentrations of 20 and 30pmol were used, dNTP mix (each at 10mM), PCR buffer 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$ (which was increased to 2mM with the first and second set of primers), 1.25 unit/50µL of *Taq* DNA polymerase (New England Biolabs, USA) and ultra-pure PCR water to the final volume of 50µL.

The amplified PCR products were separated on 1.5% (w/v) agarose gel at a constant voltage of 5 V/cm (~100–110V) for 30-45min. The agarose gel was stained with 0.5µg/mL ethidium bromide and the DNA bands were photographed using gel documentation system under UV transillumination (GelDoc 2000, Bio-Rad, UK). The sizes of the resulting DNA bands were determined by comparing them to molecular weight markers of 1kb and 100bp (Invitrogen Life Technologies, USA). All PCR products were purified with the Pure-Link PCR purification kit (Invitrogen, USA) prior to DNA sequencing.

Sequence analysis and phylogeny

The PCR products were sequenced at GenoScreen sequencing facility (Genoscreen, France) using Big-Dye terminator cycle sequencing. Using FinchTV (Geospiza, Inc.), trace sequence data was viewed and edited. Proteins encoded by each open reading frame (ORF) were predicted using the online ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). The predicted proteins were used as the query in a blast search analysis against the non-redundant protein database http://www.ncbi.nlm.nih.gov/BLASTP/ to identify the similarities between the predicted proteins and the bacteriocin genes from various *Bacillus* isolates that were published in the GenBank database. Together with our data

MEGA7 software was used to reconstruct a phylogenetic tree using highly similar protein sequences (Kumar et al., 2016). The evolutionary history was inferred using the maximum likelihood technique and the JTT matrix-based model (Jones et al., 1992). The consensus tree was derived from 1000 bootstrap replicates. Next to each branch the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (Felsenstein, 1985). The Neighbor-Joining method was used to construct the initial tree(s), while the JTT model was employed to estimate pair-wise distances. Discrete Gamma distribution was used to model differences in evolutionary rate between sites. The analysis included 16 sequences of amino acids. In total there were 82 positions in the final dataset.

Evaluation of the antibacterial potency

Bacillus amyloliquefaciens isolates that did not contain any know bacteriocin gene families were chosen to determine the most potent antibacterial producer isolate. This was performed using the modified cross-streak method on Müller-Hinton agar as described by Velho-Pereira & Kamat (2011) against two pathogenic isolates. *Staphylococcus aureus* and *Escherichia coli*. Pathogenic isolates used in this study was kindly provided by the Department of Diagnostic Laboratory and Blood Bank at King Fahd hospital in Al-Madinah city, KSA, at which they were characterized and identified as *Staphylococcus aureus* and *Escherichia coli*.

Bacteriocin production

The most potent isolate from the previous experiment was chosen for large-scale production of bacteriocin in de Man Rogosa and Sharpe (MRS) broth medium with a working volume of 2L, pH adjusted to 6.8, and NaCl concentration of 0.9% (w/v). This was accomplished by inoculating the media with 1% (v/v) of the active *Bacillus amyloliquefaciens* culture (cellular suspensions from 12-18h MRS cultures, incubated at 35ºC, adjusted to an absorbance of 1–1.2 at 600nm, and incubating it for 12–18h at 35ºC under shaking conditions of 150rpm/min (Hanafy et al., 2016).

The cell free supernatant (CFS) was obtained by centrifuging at 10,000rpm for 20min at 4°C followed by filtration through a Millipore syringe filter, with a pore diameter of 0.45µm (Sigma Millex, USA). Total protein in the CFS was then fractionated by Vivaspin 20 ultrafiltration centrifugal concentrators (Sartorius Co., Germany), with the most appropriate membrane cut-off values (10,000 MWCO, 30,000 MWCO and 50,000 MWCO). The concentrator was filled to maximum capasity, inserted into a fixed angle rotor benchtop centrifuge (Beckman, USA), and centrifuged at 6000rpm for 10 to 30min (depending on the MW cut-off value). The concentrated protein fractions were recovered from the bottom of the concentration pocket. The bacteriocin activity in each concentrated fraction was determined using agar well diffusion inhibition assays against the two pathogenic isolates (*Staphylococcus aureus* and *Escherichia coli*).

Partial purification of bacteriocin

The active protein fraction was partially purified using the gel filtration technique. Active crude CFS obtained after fractionation was applied onto Sephadex G-75 dextran gel (Sigma-Aldrich, USA) column (15mL, 20mm Í 200mm). The dextran powder was suspended in 100 mM Tris-HCl (pH 8.0) buffer, and the column was eluted with the same 100mM Tris-HCl (pH 8.0) buffer at a flow rate of 1mL/min. Elutes were collected at a constant volume of 1mL. The absorbance of each collected elute was measured at 280nm using a UV-visible spectrophotometer. Protein concentration of the selected elutes was estimated using Quick Start Bradford protein assay (Bio-Rad, UK). Antimicrobial activity of the selected elutes was examined individually using well diffusion inhibition assays as previously described.

Determination of the inhibitory concentration for the partially purified bacteriocin

The inhibitory concentration for the partially purified bacteriocin was determined using agar well diffusion inhibition assays. For determination of minimum inhibitory concentration, the partially purified bacteriocin(s) were serially diluted (bi-fold dilutions) in the same elution buffer employed in the partial purification step. Then 100 µl of each concentration was used against the two indicator isolates utilized in this study.

Molecular weight estimation of the partially purified bacteriocin

The molecular weight of the bacteriocin was determined using 12% discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a double slab

vertical Mini-PROTEAN electrophoresis cell (Bio-Rad, UK). The gel was run at a constant voltage of 120 – 150 for 90–120 min. The gel was then soaked in an isopropanol fixing solution and shaken at room temperature for 30 to 60 min. Then stained with the Coomasie Blue solution for 2 h. A gel documentation system was utilized to scan and photograph the stained gels. Using the mobility of a low molecular weight protein marker (Invitrogen life technologies, USA), ranging from 3–98kDa, the molecular weight of the bacteriocin polypeptide was determined.

Results

PCR detection of bacteriocin genes

The presence of bacteriocin-producing genes on the chromosomes of all eight *Bacillus amyloliquefaciens* isolates was rapidly determined using PCR. Four isolates (Ba_1, Ba_2, Ba_5 and Ba_6) amplified a single 329 bp PCR band with the first set of primers indicating the presence of the *Amylocyclicin*-type bacteriocin gene family in all four isolates (Fig. 1 panel a). Similarly, PCR fragments (~1281bp) were amplified from the DNA of isolates (Ba_2 and Ba_6) using the second set of primers, flanking the *Subtilosin*-type bacteriocin gene family, indicating its presence in both isolates. In addition to the specific 1281 bp PCR band, another fragment with a size of <500 bp was amplified from the Ba_2 isolate (Fig. 1 panel b). Both bands were separated and extracted using a gel band purification kit for later sequencing, whereas only one nonspecific PCR product was amplified from a single isolate (Ba_4) using the third set of primers. Although the size of the produced band (~400bp) did not match the expected PCR product size of the *Mersacidin*-type bacteriocin gene (~200bp), it was sent for sequencing as a precaution (data not shown). No PCR amplicon were obtained from any of the isolates using the fourth set of primers designed to detect the presence of the *Amylolisin*-type bacteriocin gene family.

DNA sequence analysis and phylogeny

Sequence analysis of amplicons derived from Ba_1, Ba_2, Ba_5, and Ba_6 isolates amplicons using the first set of primers (specific for the *Amylocyclicin* gene) amplified the full-length sequence of their target gene. High similarity percentage (93-95%) with *Uberolysin/Carnocyclin* family circular bacteriocin form the *Bacillus* group was observed when a BLAST search was conducted using the translated nucleotide sequence query of the four isolated genes against the protein databases hosted by GenBank (Table 2).

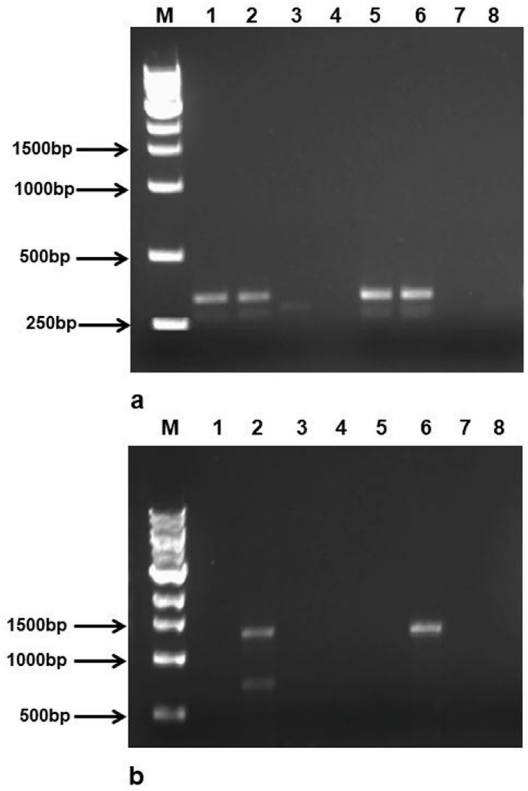

Fig. 1. Agarose gel electrophoresis. Lanes (M), DNA ladder marker; lanes 1-8 represent Ba_1–Ba_8 *Bacillus amyloliquefaciens* isolates [PCR products from the amplification of Amylocyclicin panel a: (lanes 1, 2, 5 and 6) and Subtilosin panel b: (lanes 2 & 6)]

In contrast, sequence analysis, of fragments amplified from isolates Ba_2 and Ba_6 with the second primer set (designed to amplify the *Subtilosin* gene) revealed that their sequences share high similarity with *Subtilosin* biosynthesis protein (ablE) gene which is involved in the initial step of subtilosin maturation.

The four sequences of the *Amylocyclicin/ uberolysin* gene and one representative sequence of the *Subtilosin* biosynthesis gene that were obtained in the study along with their annotations have been deposited at GenBank under the accession numbers (ON820649, ON820650, ON820651, ON820652 and ON820653).

TABLE 2. BLAST search results of the translated protein sequence of the four isolated *Amylocyclicin/Uberolysin* gene with the best matches in the protein GenBank database including their protein names accession numbers and similarities based on 90%-similarity cutoff value

Sample Name	Accession No.	Best match (BLAST search)	Accession No.	Identity (%)
Ba_1	ON820649.1	uberolysin/carnocyclin family circular bacteriocin [*Bacillus* group]	WP_003151973.1	95.5
		uberolysin/carnocyclin family circular bacteriocin [*Bacillus amyloliquefaciens*]	WP_063174629.1	94.1
		uberolysin/carnocyclin family circular bacteriocin [*Bacillus subtilis*]	WP_003222927.1	93.9
Ba_2	ON820650.1	uberolysin/carnocyclin family circular bacteriocin [*Bacillus* group]	WP_003151973.1	95.7
		uberolysin/carnocyclin family circular bacteriocin [*Bacillus velezensis*]	WP_070082305.1	94.2
		uberolysin/carnocyclin family circular bacteriocin [*Bacillus amyloliquefaciens*]	WP_041481666.1	93.1
Ba_5	ON820651.1	uberolysin/carnocyclin family circular bacteriocin [*Bacillus* group]	WP_003151973.1	95.5
		uberolysin/carnocyclin family circular bacteriocin [*Bacillus subtilis*]	WP_014477869.1	94.7
		Bacteriocin class IId cyclical uberolysin [*Bacillus amyloliquefaciens* DSM 7]	CBI44140.1	93.8
Ba_6	ON820652.1	circular bacteriocin, circularin A/uberolysin family [*Bacillus velezensis* FZB42]	ABS75254.1	95.8
		uberolysin/carnocyclin family circular bacteriocin [*Bacillus amyloliquefaciens*]	WP_045509383.1	94.6
		Bacteriocin class IId cyclical uberolysin [*Bacillus amyloliquefaciens*]	AEB64818.1	93.1

The four predicted *Amylocyclicin* proteins and twelve of their closely related amino acid sequences from the database were utilized in a maximum likelihood-based phylogenetic data analysis. The resulting topology grouped our protein sequences with their closest relative the uberolysin/carnocyclin family circular bacteriocin from *Bacillus amyloliquefaciens*, "*B. velezensis* FZmhtB" and other related sequences from the *Bacillus* group into a distinct node (Fig. 2).

Furthermore, the sequences of the nonspecifically amplified PCR products from Ba_2 and Ba_4 isolates revealed a 91% similarity with the "zinc-permease uptake protein" of *B. subtilis* and a 93% similarity with the "Potassium transporting ATPase subunit B" of *B. cereus,* respectively.

Evaluation of the antibacterial potency

Since Hanafy et al. (2016) had previously established the optimal the optimized conditions for large-scale production of bacteriocin from all eight *Bacillus amyloliquefaciens* isolates, the same protocol was used in the present investigation. The four isolates (Ba_3, Ba_4, Ba_7 and Ba_8) that did not contain any of the bacteriocin genes expressed in the genus *Bacillus* were screened to determine the most potent isolate. Ba_3 and Ba_8 isolates exhibited the highest antibacterial activity against the two indicator strains, with minor differences favoring the Ba_3 isolate. Therefore, the Ba_3 isolate was chosen for large-scale bacteriocin production and extraction (Fig. 3a).

Detection of bacteriocin activity in the extracted fractions

Our results indicated that none of the fractionated proteins with nominal membrane cut-off values ranging from ~ <50,000 kDa – >10,000 kDa exhibited antibacterial activity against both indicator isolates, as determined by the results. Antibacterial activity was detected against the two indicator isolates in the protein fraction under membrane cut off value of <10,000 kDa, with a higher zone of inhibition against *S. aureus* than *E. coli* **(**Fig. 3b).

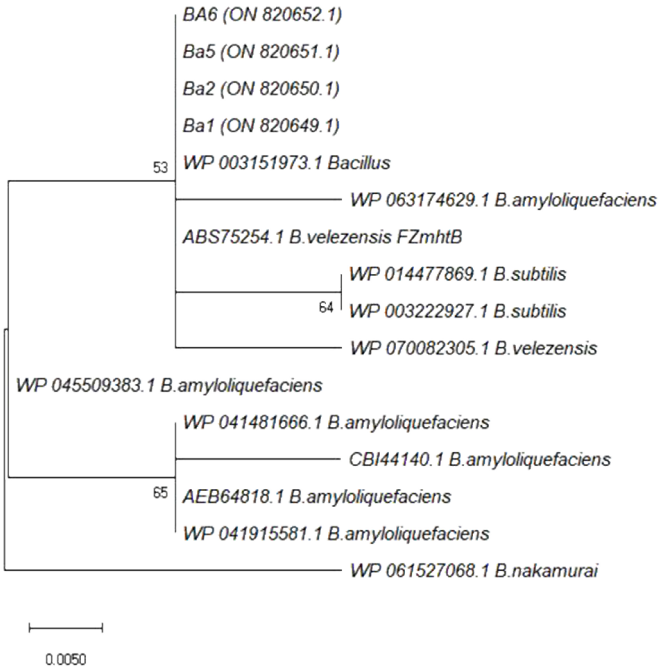

Fig. 2. Likelihood phylogenetic tree based on the amino acid sequences of the *Amylocyclicin/uberolysin* gene, illustrating the phylogenetic relationship between the predicted proteins from isolates Ba_1, Ba_2, Ba_5, and Ba_6 and other representatives of the *Bacillus* group from the GenBank database [All sequences were aligned to the bacteriocin gene of *Bacillus nakamurai* (GenBank accession WP 061527068.1). The tree was depicted to scale, with branch lengths indicated by in the number of substitutions per site with bootstrap values indicated on each node. The bootstrap values were inferred from 1000 replicates]

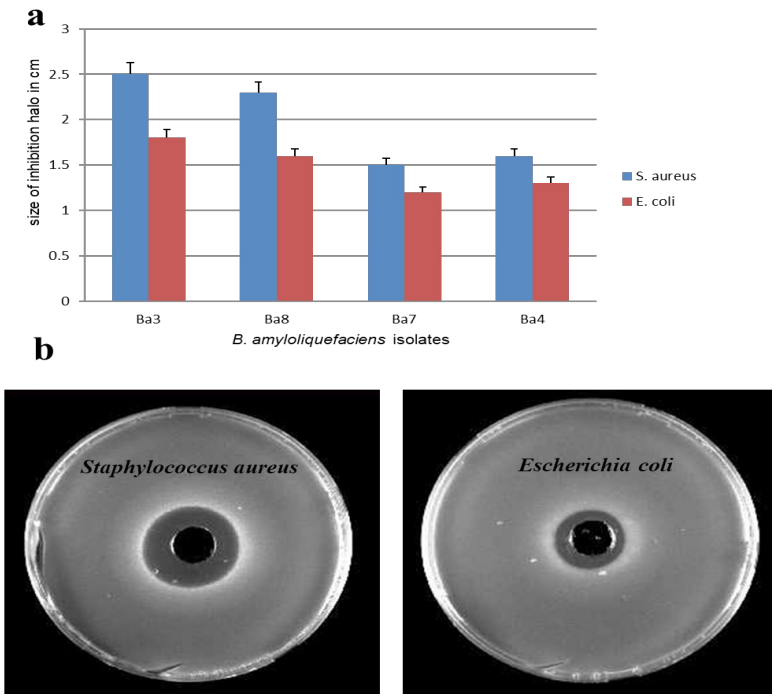

Fig. 3. Panel (a) depicts the antimicrobial activity of the antimicrobial substances produced by the four isolates against the indicator organisms *S. aureus* and *E. coli.* Panel (b) demonstrates the antibacterial activity of the protein fraction <10,000kDa from the CFS of the Ba_3 isolate against indicator strains of S. *aureus* and *E. coli*

Partial purification of bacteriocin

Bacteriocin from the bioactive fraction (<10 kDa) of the Ba_3 isolate was partially purified using the gel filtration chromatographic technique. Twenty-two of the 80 elutes with a sharp peak at 280 nm were collected separately in order to measure their concentration and evaluate their antimicrobial activity using well diffusion inhibition assays. Four of the partially purified elutes (5-8) exhibited antibacterial activity against both indicator isolates (Fig. 4).

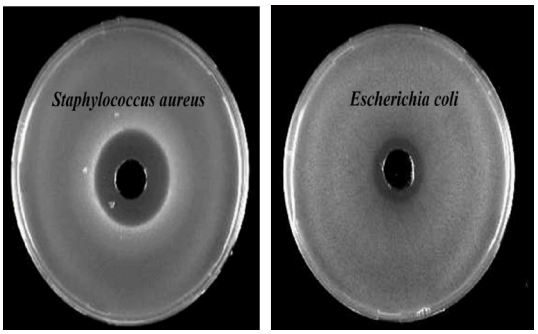

Fig. 4. Demonstrates the antibacterial activity of the four of the partially purified elutes (5-8) from the protein fraction <10,000kDa of the CFS of the Ba_3 isolate against *S. aureus* and *E. coli* indicator isolates

The bioactive elutes (5-8) constituted a negligible portion (16.74%) of the total proteins in the partially purified crude extract (Fig. 5). Table 3 lists the concentrations of all protein elutes.

Inhibitory concentration for the partially purified bacteriocin

The minimum inhibitory concentration of the four (5-8) elutes (containing the partially purified bacteriocin) was further investigated after combining them. Other proteins without antibacterial activity were discarded after their concentrations were measured. The inhibitory concentration value of the partially purified bacteriocin is the lowest concentration capable of inhibiting bacterial growth. The bacteriocin inhibitory concentration for gram-positive *S. aureus* was approximately 7µg/mL with an average inhibition zone of 1cm in diameter. While none of the tested dilutions exhibited antibacterial activity against gram-negative *Escherichia coli*, it is proposed that the inhibitory concentration for *Escherichia coli* is the same as that of the pooled bioactive elutes (~14µg/mL) without further dilution (Fig. 6).

Characterization of bacteriocin via SDS-polyacrylamide gel electrophoresis

The relative molecular weight and purity of the partially purified bacteriocin obtained from the Ba_3 isolate were determined using SDS-PAGE. After each fractionation step, samples were drawn from the CFS (Fig. 7). As depicted in lane D of Fig. 5, the apparent molecular weight of the partially purified bacteriocin was between 7 and 7.5kDa, representing the active elutes obtained directly from the purification procedure using the gel filtration chromatographic technique.

Discussion

The diversity and frequency of bacteriocins-producing genes as well as their activity spectrum vary substantially between bacterial populations. Therefore, the search for new antagonistic bacteriocin-producing isolates is ongoing. Studying the genetic basis of bacteriocins produced by gram-positive bacteria is advancing rapidly. It is now relatively simple to use specific PCR screening techniques to determine whether bacteriocin genes are present in the bacterial isolates especially when multiple species are present in the samples (Więckowicz et al., 2011; Kubašová et al., 2020).

The results of the rapid PCR screening for bacteriocin-producing genes present on the chromosomes of *B. amyloliquefaciens* isolates revealed the presence of the *Amylocyclicin* gene family in four of the eight isolates. Two of the previously mentioned four isolates contained another bacteriocin gene family (*Subtilosin*). These results are considered very interesting since the presence of multiple structural genes encoding for different types of bacteriocins has been reported in numerous lactic acid bacterial genera, with some isolates containing as many as four distinct bacteriocin genes (Henning et al., 2015; Farias et al., 2021). However, to our knowledge, this is the first time that *Bacillus amyloliquefaciens* isolates with multiple bacteriocin genes have been reported. In addition, these results explain why these two isolates exhibited a higher degree of bactericidal activity than the remaining six during Hanafy et al.'s (2016) previous study.

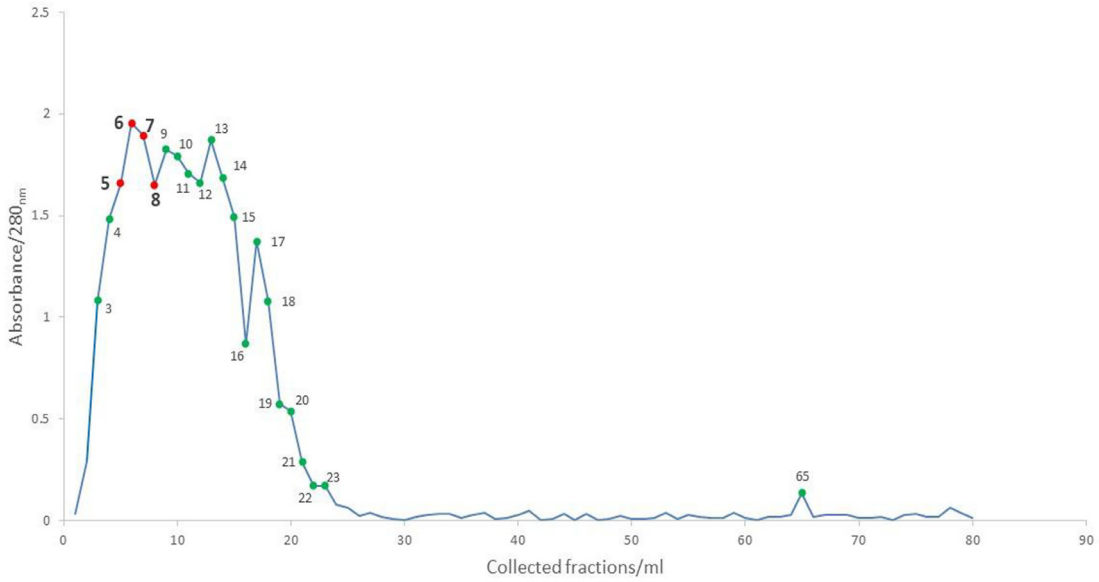

Fig. 5. Elution profile of crude bacteriocin produced by *Bacillus amyloliquefaciens* Ba_3 isolate on Sephadex G-75 dextran gel filtration chromatography; absorbance was set at λ= 280nm for detection of proteins fractions 5–8 (indicated by red dots) that represent bacteriocin activity

TABLE 3. The protein concentration of elutes with a characteristic sharp peak at 280nm as determined by the Bradford protein assay at 595nm

Gel filtration elutes	Concentration (µg/mL)	% Of elutes among the total detected proteins
Fractions 3, 4	13.73	16.34
Fractions 5-8*	14.07	16.74
Fractions 9-11	9.26	11
Fractions 12-14	10.29	12.24
Fractions 15-17	8.91	10.6
Fractions 18-20	9.26	11
Fractions 21-23	8.91	10.6
Fraction 65	9.60	11.42

* Indicates the antibacterial active pooled fractions

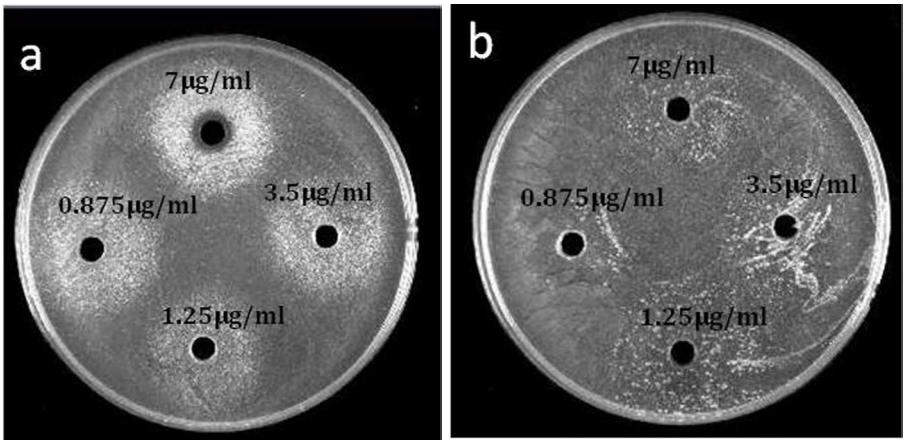

Fig. 6. The minimal inhibitory concentration of the partially purified bacteriocin fraction detected by the agar well diffusion method against the indicator strains *S. aureus* (panel a) and *E. coli* (panel b), where the numbers represent the concentration used in each well

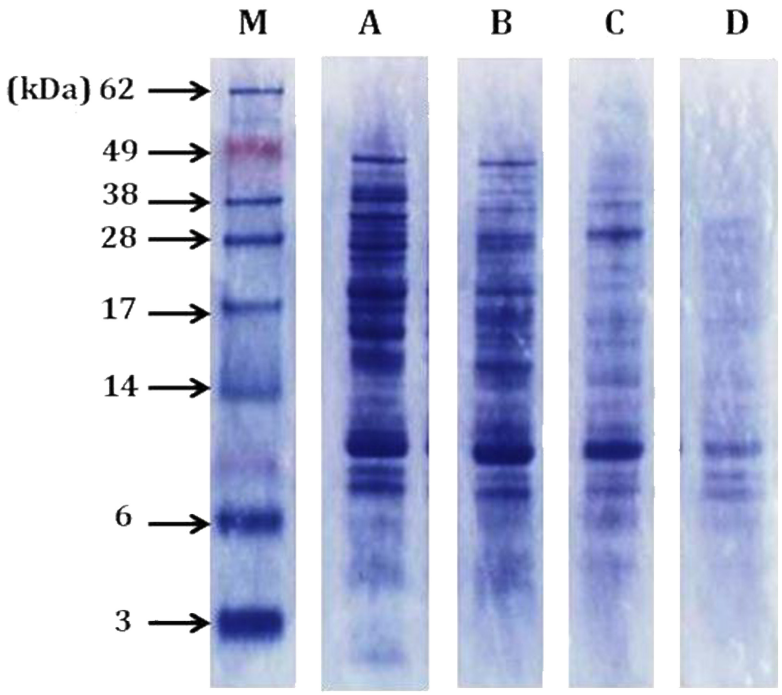

Fig. 7. SDS-PAGE of the purification steps for bacteriocin obtained from *Bacillus amyloliquefaciens* Ba_3 isolate (M) standard low molecular weight protein marker, (A) sample obtained from CSF after nominal membrane cut-off value of <50,000 kDa, (B) sample obtained after nominal membrane cut-off value of <30,000kDa, and (C) sample obtained after nominal membrane cut-off value of <10,000kDa, (D) partially purified bacteriocin obtained directly after gel filtration chromatography

The sequence analysis of the PCR products obtained from the four isolates using the first set of primers revealed similarity to *Amylocyclicin* gene of greater than 95%. Phylogenetic data analysis of the four *Amylocyclicin* predicted amino acid sequences and their most similar sequences in the database grouped them into a distinct node with their closest relatives *B. amyloliquefaciens* and *Bacillus velezensis* strain FZmhtB bacteriocin gene. Until recently, *Bacillus velezensis* was previously classified as a subspecies of *B. amyloliquefaciens* until late 2017, when it was separated into its own species (Fan et al., 2017). This result suggests that the bacteriocin produced by these isolates is more closely related to the bacteriocin produced by the newly evolved species *B. velezensis* than to the bacteriocin produced by the old species *B. amyloliquefaciens*, which is clustered on a distinct branch of the evolved phylogenetic tree. Although all four sequences were clustered together, low bootstrap values were represented at the top of each branch. This may be explained by the considerably high similarity between the short polypeptide sequences of the predicted proteins, which may not provide the optimal environment for good discrimination especially after the removal of 23 residues from the c-terminus of the sequences retrieved from GenBank prior to alignment to ensure that variation in protein length does not influence the phylogenetic results.

Sequence analysis of the amplified PCR products from the second set of primers revealed that, instead of amplifying the designated *subtilosin* gene, the designed PCR primers amplified a fragment that shared more than 94% similarity with *subtilosin* biosynthesis gene "E" which is located in an operon known as "Sbo-alb." This operon encodes the *subtilosin* A protein and is involved in the first step of *subtilosin* maturation, which subsequently indicates the presence of S*ubtilosin* bacteriocin in both isolates. The reason for this misfit in the amplification of the target gene was caused by a mix-up in gene nomenclature. Based on the most recent protein data sequences submitted to GenBank by two different groups, the Vinodkumar group in 2014 under the accession number "AHW82016" and the Tamilselvi group in 2016 with the accession number "AQZ36504.," specific primers were designed for this study. Both groups submitted a

260–270 amino acid residue protein sequence with the assumption that it was the S*ubtilosin* structural gene from *B. amyloliquefaciens*. However, our research's sequence analysis demonstrated that the previously submitted sequences were actually for "*Subtilosin* biosynthesis protein (ablE) gene". Later in 2018, the Kunst group submitted 43 new amino acid residues as the *Subtilosin* A bacteriocin gene from *B. amyloliquefaciens* under the accession number "CAB15763." The number of amino acid residues in this submission coincides with the normal molecular weight range (4–7.5kDa) reported for bacteriocins isolated from gram-positive *Bacillus* spp. (Abriouel et al., 2011; Flühe et al., 2012).

After screening the isolates for the presence of bacteriocin encoding genes normally expressed in *Bacillus*, four isolates did not show any specific amplified PCR product with the four designed sets of primers, indicating that they may contain different structural genes(s) that encode novel type bacteriocin, or structural genes that encode for known bacteriocins that are not typically found in the *Bacillus* species complex. The most potent of these four isolates (considered the most promising) against the indicator isolates was therefore chosen for further production fractionation and partial purification of its bacteriocin, simulating the work of Lim et al. (2016). Although *Mersacidin* and *Amylolisin*-type bacteriocins are frequently detected in *B. amyloliquefaciens* isolates (Halimi et al., 2010; Herzner et al., 2011; He et al., 2012; Arguelles-Arias et al., 2013), none of our eight isolates contained these bacteriocins.

Staphylococcus aureus and *Escherichia coli* were selected in this study are clinical isolates that were classified as drug resistant capable of causing nosocomial infections which by itself represents a challenging point. They were also formerly adopted as indicators to test the antibacterial activity of metabolites produced by microorganisms in previous studies (Hanafy et al., 2016; Hanafy et al., 2022).

Antibacterial activity was detected in the protein fraction under membrane cut-off value of <10,000kDa, which was later confirmed by SDS-PAGE analysis of the partially purified active bacteriocin elutes, which revealed a 7–7.5kDa protein fragment. In addition, after partial purification with Sephadex gel filtration chromatography it was determined that active bacteriocin elutes comprised only a small fraction of the total proteins detected in the partially purified crude <10,000kDa approximately 16.74%. Furthermore, when the partially purified active bacteriocin fraction was examined for its antimicrobial activity against both indicator isolates a larger average inhibition zone diameter was observed for gram-positive indicator isolate. These results indicate that *B. amyloliquefaciens* bacteriocins tend to have small molecular weights and comprise a minor portion of the bacterial cell relative to other proteins, and that their activity is primarily directed against other gram-positive bacteria, three characteristics that have been confirmed by multiple previous studies (Lim et al., 2016; Salazar et al., 2017; Bindiya et al., 2019; Wu et al., 2019).

The bacteriocin produced by the *B. amyloliquefaciens* Ba_3 isolate exhibited broad spectrum antibacterial activity, with the lowest concentration of the partially purified bacteriocin inhibiting the growth of the indicator strains being ~ 7µg/mL for the gram-positive *S. aureus* isolate and ~14µg/mL for the gram-negative *E. coli* isolate. This is a characteristic rarely observed in bacteriocins produced by gram-positive bacteria, as they are predominantly inhibitory towards gram-positive bacteria and less effective against gram-negative bacteria, and only a small number of studies have reported the broad antibacterial spectrum of bacteriocins isolated from *B. amyloliquefaciens* (Sutyak et al., 2008; Scholz et al., 2014; Lim et al., 2016; Salazar et al., 2017; Kurata et al., 2019). Although the reported bacteriocin concentration to inhibit the growth of gram-negative bacteria in this study was twofold higher than that of gram-positive bacteria, it was still unquestionably within the acceptable ranges.

Conclusion

Despite the classification efforts made with bacteriocins to date, no clear classification scheme for bacteriocins produced by *Bacillus* has been established. This is most likely due to the lack of information regarding their amino acid sequences and their extensive diversity. Eight *B. amyloliquefaciens* isolates from previous study were analyzed using PCR primers designed to detect the previously reported bacteriocin structural gene families within the *Bacillus* species complex. Two of the eight *B. amyloliquefaciens* isolates were discovered to

contain *Amylocyclicin* and *Subtilosin* structural genes, an unusual occurrence for *Bacillus* spp. Two others contained only the *Amylocyclicin* gene. While the remaining four isolates did not amplify any of the known bacteriocin genes, they may contain genes encoding for a new bacteriocin(s). In terms of antibacterial activity, Ba_3 was the most potent of these four isolates; thus, it was chosen for further study. In this study, the Ba_3 isolate produced a bacteriocin with a low molecular weight ranging from 7–7.5 kDa and a unique broad-spectrum antimicrobial property at relatively low concentrations. Future work will involve amino acid sequencing of the new active bacteriocin to determine its N-terminal amino acid sequence and using this sequence to design an appropriate probe for isolating, identifying, and elucidating the structural gene involved in its biosynthesis.

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تنوع عائلات الجينات المشفرة للبكتيريوسين والمدى المثبط بين عزلات بكتيريا *Bacillus amyloliquefaciens*

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تعتبر البكتريوسينات مرشحات مثالية للعديد من التطبيقات الخاصة بالعناية بالصحة وذلك بسبب نطاق نشاطها التثبيطي المحدود وقابليتها السريعة للتحلل بواسطة الإنزيمات المحللة للبروتين. في هذه الدراسة تم الاستعانة ثماني عزلات من تحت جنس عصويات *Bacillus amylliquefaciens* قد تم تجميعها من عينات حليب الماعز المجففة في دراسة سابقة و اثبات قدرتها على انتاج البكتيريوسين. وقد تم فحص هذه الثمانية عزلات البكتيرية من تحت النوع *Bacillus amyloliquefaciens* والمنتجة للبكتريوسين بواسطة تقنية تفاعل إنزيم البلمرة المتسلسلة (PCR) وذلك عن طريق استخدام أربع مجموعات من البادئات التي تم تصميمها خصيصًا لاكتشاف الجينات المنتجة للبكتريوسين على الكروموسوم البكتيري للعزلات تحت الدراسة. وقد تم الكشف عن وجود الجين المشفر للبكتريوسين من عائلة الأميلوسيكليسين في أربع عزلات (Ba_1، Ba_2، Ba_5، و Ba_6) من اصل الثمانية وعند اجراء تحليل النشؤ التطوري الوراثي للأربعة بروتينات البكتريوسينية من عائلة الأميلوسيكليسين وجد ان البيانات الوراثية الخاصة بالأربع بكتريوسينات قد انضمت في عقدة منفصلة من عقد شجرة التطورالوراثي مع أقرب أقربائها (أكثر من 95٪ تشابه) من البكتيريوسينات المفروزة بواسطة بكتيريا *B. amyloliquefaciens* strain FZmhtB and *Bacillus velezensis* ، والجدير بالذكر ان بكتيريا *Bacillus velezensis* strain FZmhtB كانت حتى وقت قريب تندرج تحت نوع *B. amyloliquefaciens*. كما تم أيضا في هذا البحث اكتشاف نتائج مثيرة تلخصت في احتواء في عزلتين من العزلات الأربع السابق ذكورها على الجين المنتج للبكتريوسين من عائلة الـ سوبتيلوسين مما يشير إلى احتواء هاتان العزلتان على عدة جينات مشفرة لعائلات مختلفة من البكتريوسين في ذات نفس العزلة، وهي ظاهرة نادرة لا توجد عادة في عزلات هذا النوع من بكتيريا *Bacillus amyloliquefaciens*. وقد افتقرت العزلات الأربع المتبقية إلى وجود أي عائلة من العائلات الجينية المتعارف على وجودها والمشفرة لانتاج البكتريوسين في هذا النوع من البكتيريا ومن ثم فانه من المتوقع أن تحتوي هذه العزلات على عائلات جديدة من الجينات المنتجة لأنواع مختلفة من البكتريوسينات الغير معرفه حاليا. وقد وقع الاختيار على أقوى هذه العزلات الأربعة من حيث القوة التثبيطية (Ba_3) لإنتاج واستخلاص بكتريوسينها على نطاق واسع. وعند الكشف عن النشاط المضاد للبكتيريا مقابل العزلات البكتيرية سالبة الجرام وإيجابية الجرام وجد ان البكتريوسين المستخلص يتواجد في البروتينات تحت حجم أقل من 10 كيلودالتون، وان له نشاط تثبيطي ضد كلا من البكتيريا سالبة وموجبة الجرام مع قوة تثبيطية تميل اكثر نحو موجبة الجرام. علاوة على ذلك، كشف تفريد البكتريوسين المستخلص النشط والمنقى جزئيًا كهربائيا على هلام البولي اكريلاميد SDS-PAGE عن وجوده على شكل حزمة بروتينية بوزن جزيئي نسبي يتراوح بين 7 و7.5 كيلو دالتون. ومما سبق يمكن استنتاج ان مجموعات البادئات التي تم تصميمها واستخدامها في اختبار ال PCR في هذه الدراسة قدمت تغطية لجميع عائلات الجينات المعروفة والمنتجة للبكتيريوسين بواسطة بكتيريا *B. amyloliquefaciens* مما يسمح بكشف السريع والسهل عن وجودها.