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Research Article

Partial purification and characterization of bacteriocin from novel *Brevibacillus borstelensis sp.* isolated from Donkey's lactation

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Abstract
The heightened awareness of ensuring enhanced safety standards is growing in order pertaining to the avoidance of chemical food preservation. Concurrently, there is a discernible increase in the prevalence of antimicrobial resistance among foodborne pathogens, which engenders infections such as food poisoning, gastro-intestinal infections *etc.*. In this regard, bacteriocins present themselves as a compelling alternative for achieving both preservation and safety objectives in the realm of food science. Bacteriocins are ribosomally synthesized proteins that demonstrate inhibitory activities against a diverse spectrum of undesirable microorganisms. In the present study, a bacterium (*Brevibacillus borstelensis* sp.) derived from Donkey milk was scrutinized for its ability to generate a bacteriocin-like inhibitory substance (BLIS). The effectiveness of this substance was assessed against pathogens linked to foodborne/spoilage, specifically *Bacillus subtilis (KK01), Staphylococcus aureus (MRSA) ,Pseudomonas aeruginosa (HCS36),* and *Escherichia coli (O22)* .The evaluation comprised the bit/disc method followed by the Well-diffusion method. Employing a combination of phenotypic, biochemical, and molecular characteristics, including the 16S rRNA gene technique, the bacterial isolate was identified as *Brevibacillus borstelensis strain 3.1* [OR272522.1]. The Bacteriocin-like inhibitory substance produced by *B. borstelensis*was purified for the first time by using the chloroform extraction method, unveiling a molecular mass of 56 kDa. Further analysis examined the BLIS reactions to temperature, pH, proteolytic enzymes, and stability. The Bacteriocin-like inhibitory substance exhibited thermo-stability up to 90 °C, activity at neutral pH, sensitivity to trypsin, and partial stability up to 15 days of storage, indicating its potential as a bio-preservative for food.

Keywords: Antimicrobial activity, Bacteriocin like inhibitory substances (BLIS), Biopreservation, Donkey milk, Microorganisms,16S rRNA

INTRODUCTION

Consumer preferences underscore a demand for natural and minimally processed foods characterized by a fresh taste, ease of consumption, and elevated safety standards. Consequently, ongoing research and development endeavours are steering towards innovations that reduce or replace conventional heat treatments and preservatives. The primary objective is to employ treatments capable of preserving the sensory and nutritional attributes of the product while upholding stringent food safety measures.Alvarez□Sieiro*et al.,* 2016 and Heredia-Castro *et al.,* 2021). Thus, effectively managing these foodborne pathogens in food products necessitates exploring alternatives to chemical preservatives, and bacteriocins or bacteriocin-like substances emerge as promising options. Bacteriocins offer protection against both spoilage-causing agents and foodborne pathogens without posing any adverse effects on health. This has generated heightened attention within academic and industrial sectors, designating the bacteriocin investigation as a central domain that encompasses all the aspects,i.e. purification, large scale production and applications in food industries (Pingitora*et al.,* 2007 and Dicks *et al.,* 2018).

The use of bacteriocins, or antimicrobial peptides derived from bacteria, to combat pathogenic organisms and food spoilage is gaining international attention.

Bacteriocins, which are antimicrobial proteins or protein complexes with bactericidal activity synthesized ribosomally, usually target species intimately associated with the microbes that produce them (Sharma *et al.,* 2008 ; Angelopoulou *et al.,* 2020). According to Klaenhammer (1993) and Noroozi et al. (2019), these chemicals differ in their molecular weight, biochemical characteristics, activity spectrum, and modes of action. Bacteriocins are frequently recognised as secondary metabolites generated by a variety of microorganisms, such as gram-positive Lactobacillus and Enterococcus bacteria. (De Giani *et al.,* 2019).

The Lactobacillus and Enterococcus genera encompass a range of species known for their safe use in diverse beneficial industrial products, such as enzymes, antibiotics, and amino acids (Bizani and Brandelli, 2002; Darbandi *et al.,* 2022). Bacillus and lactic acid bacteria are gaining attention for exploring their bacteriocin-producing potential as preservative and antimicrobial agents. Sharma *et al.*(2014)reported that isolate*BrevibacillusborstelensisAG1*, nested within the Bacillus genus from 'Marcha' (an herbal cake used as a starter culture for fermenting local wine) and reported its potential to produce bacteriocins and efficient food preservative. Many bacteriocinogenic substances are isolated from unconventional sources or from the non-bovine milk sources (Ahern*et al.,* 2003; Chen *et al.,*2003; Chehimi*et al.,* 2007), isolated bacteriocins munditin from Slovak raw goat milk and analyzed their potential against pathogenic bacteria.In the last two decades, there has been a significant development in scientific research on donkey milk composition (Bhardwaj *et al.,* 2020) and microbiology, processing techniques, and health benefits(Kumari *et al.,* 2022).Therefore,the present study evaluated the bacteriocinogenic effect of *Brevibacillus borstelensis* isolated from the donkey milk of Northern-Southern Haryana, India.

MATERIALS AND METHODS

Collection and preservation of donkey milk Samples

Fresh 10 donkey milk samples were collected from the southern-western Haryana (districts, i.e. Bhiwani, Hisar, Sirsa, Fatehabad and Dadri) region and immediately subjected to bacterial culture analysis. 400 microliter raw milk samples were evenly spread on de Man Rogosa Sharpe (MRS) agar plates. These plates were then placed in an incubated at 37 °C for 48 hours. After incubation, colonies with differing morphologies were carefully selected and streaked onto fresh agar plates to obtain pure isolates.The pure cultures of confirmed bacteria isolates were stored in MRS broth with 40% glycerol (v/v) at -80°C.

Initially, six different bacterial colonies were observed on the plates, leading to the screening of bacteriocin-producing isolates through the bit/disc method and the well-diffusion method (Barefoot and Klaenhammer, 1983; Kimura *et al.,* 1998). The screening involved testing against foodborne pathogenic indicators *i.e. Bacillus subtilis (strain KK01), Brevibacillus borstelensis (strain 3.1)*

Staphylococcus aureus ((strain MRSA) , Pseudomonas aeruginosa ((strain HCS36), and *Escherichia coli ((strain O22)* Among all six selected bacterial isolates of donkey milk, only one bacterium was selected for molecular identification and screening based on its strong antagonistic activity against pathogenic strains.

Identification and screening of *Brevibacillus borstelensis* strain

Various biochemical and physiological tests were employed to identify the chosen strain. These tests included morphology, Gram staining, catalase activity, oxidase test, MR-VP test, growth at varying salt concentrations,*etc.* 16S ribosomal RNA (rRNA) sequence analysis was performed using the universal primers 27F and 1492R to obtain additional validation. The manufacturer's instructions were followed for extracting genomic DNA using a DNA extraction kit (HiMedia, India). Each primer (0.3 pmol/µl) and 1 µl of Taq DNA polymerase), 400 µM of each deoxynucleotide triphosphate, 5 µl of 10x reaction buffer, and water were included in the 25 µl Polymerase Chain Reaction (PCR) amplification mixture. The PCR amplification process included 5 minutes of initial denaturation at 94°C, thirty-five cycles of denaturation at 95°C for sixty seconds, two minutes of annealing at 45°C, two minutes of extension at 72°C, and a final ten minutes of extension at 72°C. The resulting PCR products were separated by electrophoresis in 0.8% (w/v) agarose gels in 0.5x TAE buffer at 80 V for 1 h.The PCR amplicons were purified and subjected to sequencing. The bi-directional DNA sequencing reaction of the PCR amplicon was carried out with primers. The PCR products obtained were sequenced on ABI 3730xl with universal 16S rRNA primers.The obtained sequences were aligned and compared to known sequences in GenBank using the online BLAST tool (http,//blast.ncbi.nlm.nih.gov/Blast.cgi). The identified sequences were submitted to the National Center for Biotechnology Information (NCBI) Gen Bank database using BankIt submission tool and strains were conclusively identified as *Brevibacillus borstelensis AG1*.

Purification of bacteriocins isolated from *Brevibacillus borstelensis*

The purification process of the bacteriocin-like inhibitory substance produced by *Brevibacillus brevis*AG1 involved several steps. Initially, a 10% culture of *B.borstelensis AG1*[OR272522.1](1.0 OD) from a pre-prepared starter culture was added to a flask containing

500 mL of MRS broth (pH 7.0) and incubated at 37°C for 48 hours with intermediate mixing. After incubation, the culture was centrifuged at (10,000rpm for 10min. at 4 °C) as per Angelopoulou *et al.* (2020). The resultant supernatant was used for partial purification of BLIS by the ammonium sulfate precipitation method with slight modifications of the protocol of Sharma *et al.*(2014). The solution was centrifuged at 15,000 rpm for 30 minutes at 4 °C after reaching precipitation at 50% salt saturation and then maintained at 4 °C for 12 hours. After precipitation, the final pellet was diluted in 1 mL of 0.1 M sodium phosphate buffer (pH 7.0) and kept 4 °C. A 24 hour period passed before the dialyzed cell-free extract was examined for antimicrobial activity against indicator pathogens. The cell-free culture extract was dialyzed against phosphate buffer (pH 7.0, 0.1 M).The resulting pellet was re-suspended in 3 mL of sodium phosphate buffer (0.1 M, pH 7.0).14µL of protein and 6 µL of sample buffer were applied to a 15% polyacrylamide gel for electrophoresis at 30V for 40 min. and then 60V for 2 hours. A molecular weight marker (10-300kDa) was also loaded. The gel was fixed overnight, washed, and subjected to staining with commassive brilliant blue stain treatment to visualize the bands.

Characterization of Bacteriocin-Like Inhibitory Substance (**BLIS**)

The purified bacteriocin-like inhibitory substance was characterised by slight modification in the protocol (Karthikeyan *et al.,* 2009), including studying its effects under different conditions. The impact of temperature was assessed by exposing the substance to various temperatures (30–90 °C) for 15 minutes, and the pH effect was evaluated by adjusting the pH of the substance (1.5-3) in nutrient broth. Enzyme controls were prepared using phosphate buffer to purify bacteriocin-like inhibitory substances, as mentioned (Choeisoongnern et al., 2019). The potency of the substance was also monitored over several weeks against sensitive indicators using the Well-diffusion method.

RESULTS AND DISCUSSION

Biochemical and physiological identification of each bacterial isolate was done with the help of protocols mentioned in Bergey's Manual and six bacterial isolates were isolated from the donkey milk. The *B.borstelensis* isolate was identified as gram-positive, rod-shaped, with circular and creamish colonies on MRS agar medium, and tested positive for catalase. Through morphological, biochemical examination, and molecular 16S rRNA gene techniques (as the results of sequencing showed in chromatogram (Fig.1), the strain was conclusively identified as *B.borstelensis.* This bacteriumwas chosen for bacteriocin production due to its exceptional antagonistic activity against a majority of the potent spoilage-causing and foodborne pathogens utilized as test indicators. It exhibited significant zones of inhibition against pathogenic bacteria. The sequence analysis yielded the following sequences for *B.borstelensis strain 3.1*

>OR272522.1*Brevibacillusborstelensis strain 3.1*

GAGCGAGTCCCTTCGGGGGCTAGCGGCGGAC-
GGGTGAGTAACACGTAGGCAACCTGCCCGTAAGC
TCGGGATAACATGGGGAAACTCATGCTAATACCGG
ATAGGGTCTTCTCTCGCATGAGAGGAGACGGAAAG

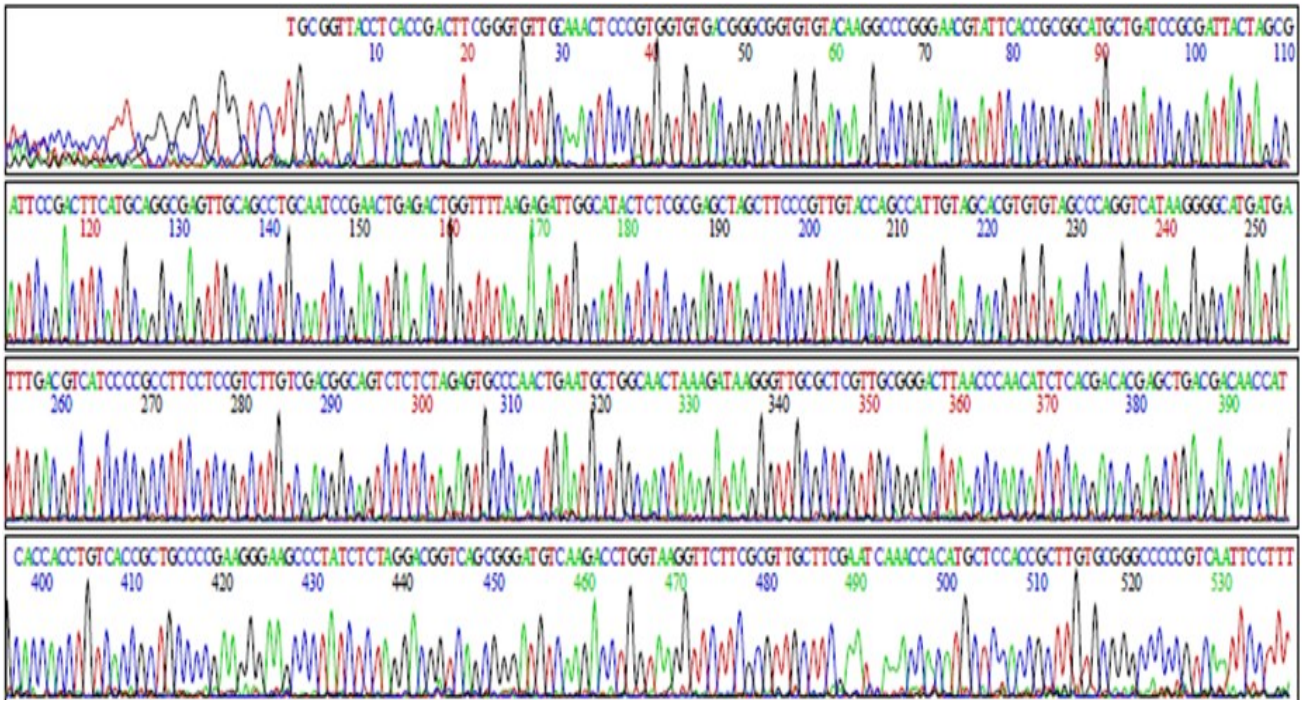

Fig.1. Chromatogram of *Brevibacillusborstelensis strain 3.1*

GTGGCGCAAGCTACCACTTACGGATGGGCCTGCG
GCGCATTAGCTAGTTGGTGGGGTAACGGCCTACCA
AGGCGACGATGCGTAGCCGACCTGAGAGGGTGAC
CGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATTTTCCACAATG
GACGAAAGTCTGATGGAGCAACGCCGCGTGAACG
ATGAAGGTCTTCGGATTGTAAAGTTCTGTTGTCAGA
GACGAACAAGTACCGTTCGAACAGGGCGGTACCTT
GACGGTACCTGACGAGAAAGCCACGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGC
GGCGGCTATGTAAGTCTGGTGTTAAAGCCCGGGG
CTCAACCCCGGTTCGCATCGGAAACTGTGTAGCTT
GAGTGCAGAAGAGGAAAGCGGTATTCCACGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAACACCA
GTGGCGAAGGCGGCTTTCTGGTCTGTAACTGACG
CTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGAT

The sequence of the obtained isolate was submitted to the NCBI database and has been officially registered in the gene bank databases. A phylogenetic tree was constructed using 16S rRNA gene sequence analysis for *Brevibac illusborstelens is* with *Accession number OR272522*, aligning it with reference probiotic bacteria sequences retrieved from NCBI through BLAST analysis, as illustrated in Fig. 2 clearly indicated the close similarity to probiotic strains *Biofidobacterium breve DSM20213 etc.*

The crude bacteriocin-like inhibitory substance demonstrated a maximum activity of 1.5 × 10^6 AU/mL at 35 °C after 48 hours of incubation, as shown in Table 1. Following precipitation with ammonium sulfate, the bacteriocin-like inhibitory substance from *Brevibacillusborstelensis* was concentrated, producing 4.6 × 10^6 AU/mL. This partially purified cell-free bacteriocin-like inhibitory substance from *B.borstelensis* maintained its original antagonistic activity pattern against *Bacillus subtilis (KK01), Staphylococcus aureus (MRSA), Pseudomonas aeruginosa (HCS36*), and *Escherichia coli (O22).*

The molecular mass of *B.borstelensis* was validated through SDS-PAGE, and its estimation was carried out by estimating Rf values, revealing a value of 45kDa (Fig. 5). In comparison, the molecular mass of the bacteriocin laterosporulin produced by *B. revibacillus* sp. strain GI-9, determined through MALDI-TOF experiments, was reported to be 5.6 kDa (Singh *et al.,* 2013). Sharma *et al*.(2014) isolated 12 kDa bacteriocin from *B. borstelensis* AG1. Additionally, the bacteriocin Bac-

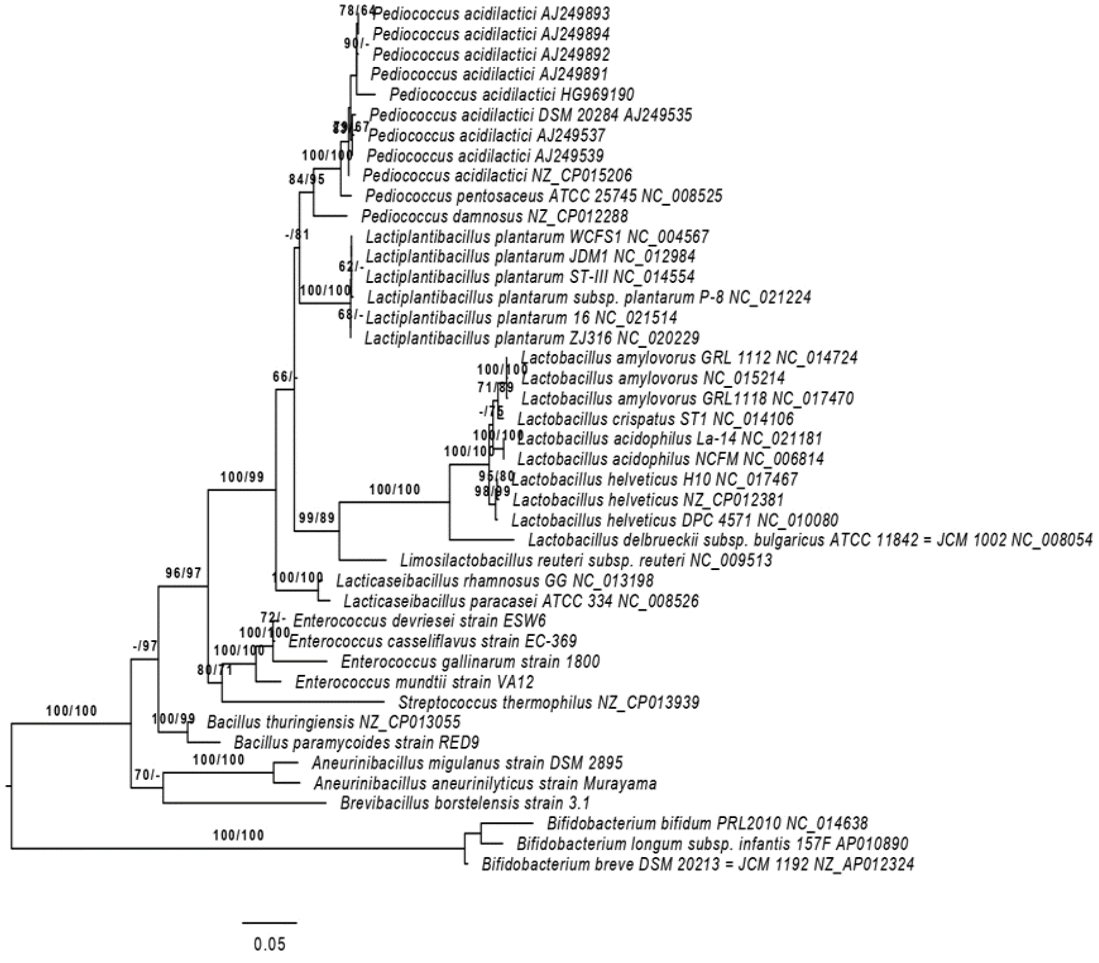

Fig.2. *Evolutionary tree of Donkey milk bacterial isolate Brevibacillusborstelensis 3.1 by the GTR+GAMMA model and the numbers above the branches are support values when larger than 60% from ML (left) and MP (right) bootstrapping*

Table1. Purification of bacteriocin-like inhibitory substances (BLIS) produced by *Brevibacillus borstelensis strain 3.1*

Purification procedure	Amount (ml)	Activity units (AU/ml)	Protein (mg/ml)
Crude culture supernatant	500	1.5×10^5	14.8
BLIS	70	4.5×10^6	10.5

BLIS: Bacteriocin-Like Inhibitory Substance

GM100, purified to homogeneity, was identified as a monomer protein with a molecular mass of 4.375 kDa, as indicated by MALDI-TOF/MS analysis (Ghadbane*et al.,* 2013). Bacteriocins exhibit broad-spectrum antimicrobial activity against pathogenic bacteria as studied by Heredia-Castro *et al.* (2021) isolated the ≤3 kDa bacteriocin from *Lactobacillus fermentum* strains of goat milk. The literature reports a wide range of bacteriocin molecular weights, spanning from very low, such as 2.0 kDa (Martrani*et al.,* 2001), to very high, like 94 kDa (Rajaram *et al.,* 2010).In the present investigation, the molecular weight (56kDa) of the purified bacteriocin from *B. borstelensis* was slightly on the higher side, as shown in Fig.3, suggesting the potential existence of a novel bacteriocin produced by this strain.

The isolated bacteriocin-like inhibitory compound demonstrated thermo-stability and remained active for 10 minutes at 100 °C;however, a progressive activity reduction was noted as the temperature rose. As shown in Fig.4, the zones with diameters of 18 (0), 18 (0.47), and 16 (0.47) mm, respectively, were produced when the bacteriocin-like inhibitory material was treated at 20°C, 30°C, 40 °C, 50 °C, and 60 °C against *Bacillus subtilis (KK01), Staphylococcus aureus (MRSA) ,Pseudomonas aeruginosa (HCS36*), and *Escherichia coli (O22).*

Furthermore, a novel *Bacillus sp.* maintained 70% of its activity after 60 minutes at 100 °C (Kayalvizhi and Gunasekaran, 2010), and the bacteriocin generated by *B. licheniformis* was stable at 100 °C for 10 minutes but lost its activity at 121 °C in 15 minutes (Khalil *et al.,* 2009). If the bacteriocin is used as an antibacterial agent in foods that have undergone heat processing, then this heat stability may be beneficial.

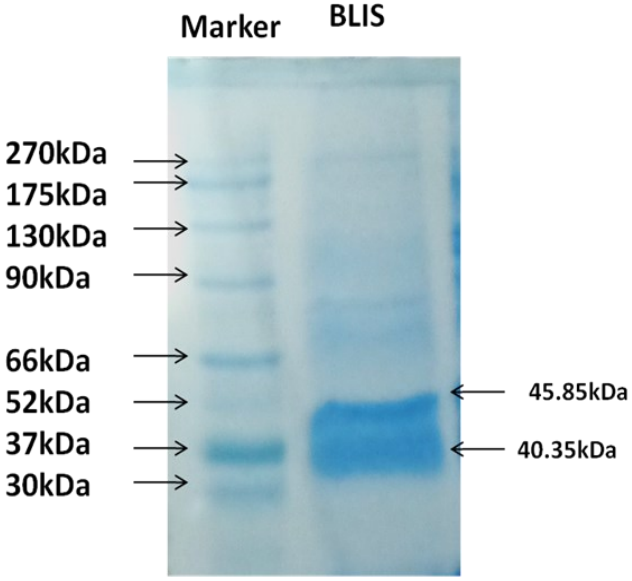

Fig. 3. *SDS-PAGE of purified Bacteriocin produced from Brevibacillus borstelensis*

In our study, bacteriocin-like inhibitory compound of *Brevibacillusborstelensis* strain exhibited its highest effectiveness against the corresponding indicators at pH 6. When the pH was increased from 4.0 to 8, bacteriocin continued to be active; however, when the pH dropped from 3.0 or 2.0 or increased above 10.0, it stopped being active. Ghadbane et al. (2013) performed similar findings in which B. brevis strain GM100 bacteriocin was stable within a pH range of 3–10. The pH range of 3.0-8.0 was found to be active for the purified bacteriocin of *B. amyloliquifaciens*, while a range of pH levels, ranging from 3-10.5, was found to be stable for the thuricin S generated by *Bacillus thuringiensis* (Lisboa *et al.,* 2006; Motta *et al.,* 2007).

Hence, it seems that bacteriocin peptides characterized by pronounced hydrophobicity, wide pH range activity and a net negative charge may be associated with potent antimicrobial activity against both gram-positive and gram-negative bacteria. Indeed, prior research has indicated that increased hydrophobicity in antimicrobial peptides results in heightened disruption of membranes, while greater charge density contributes to enhanced electrostatic interactions between peptides and membranes (Heredia-Castro *et al.,* 2021). Thus, it was found that the bacteriocin was highly active throughout the pH range, which implies that it might be helpful as a good food preservative for a variety of foods with either acidic, neutral or alkaline flavors.

Conclusion

The *B.borstelensis strain 3.1[*OR272522.1*]* produced bacteriocin-like inhibitory compounds that exhibited antimicrobial action against pathogens,*i.e.B. subtilis, S. aureus, P. aeruginosa,* and *E.coli*. The ammonium sulphate (salt saturation) method was used to purify the inhibitory compound that resembled bacteriocin. The purified bacteriocin-like inhibitory substance, isolated first time from donkey milk, the molecular weight (56kDa) demonstrated as a very active substance, 4.5 × 106 AU/mL, and showed high specific antimicrobial activity. The phase of the purification process showed an increase in its titre, and the zone of inhibition's size grew by 80%, 40%, and 62.5% compared to the corresponding indications. The analysis of the bacteriocin-

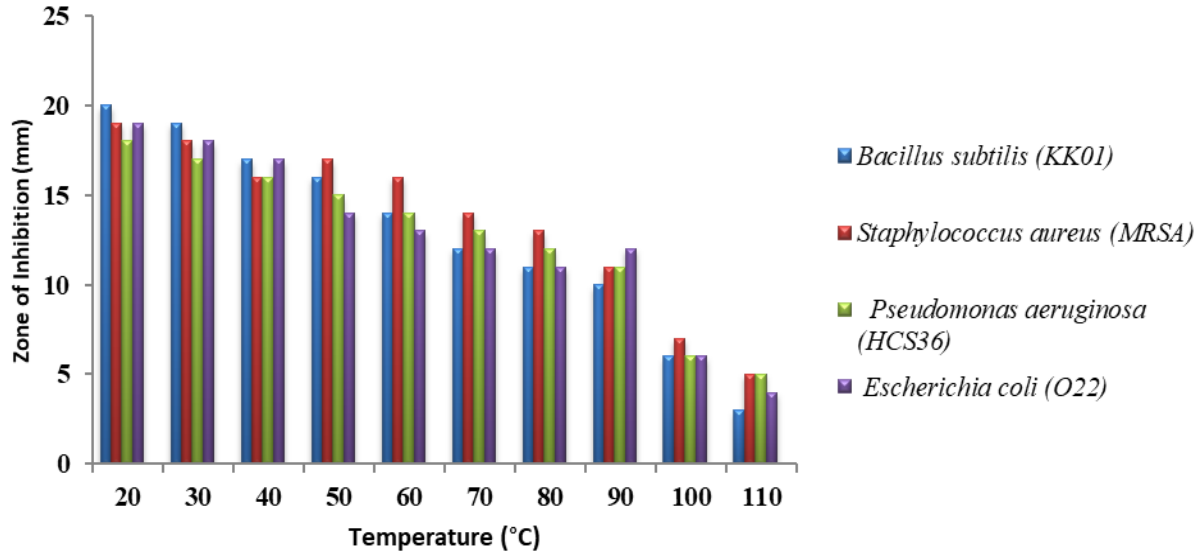

Fig. 4. *Effect of temperature on the activity of BLIS purified from the culture of Brevibacillus borstelensis*

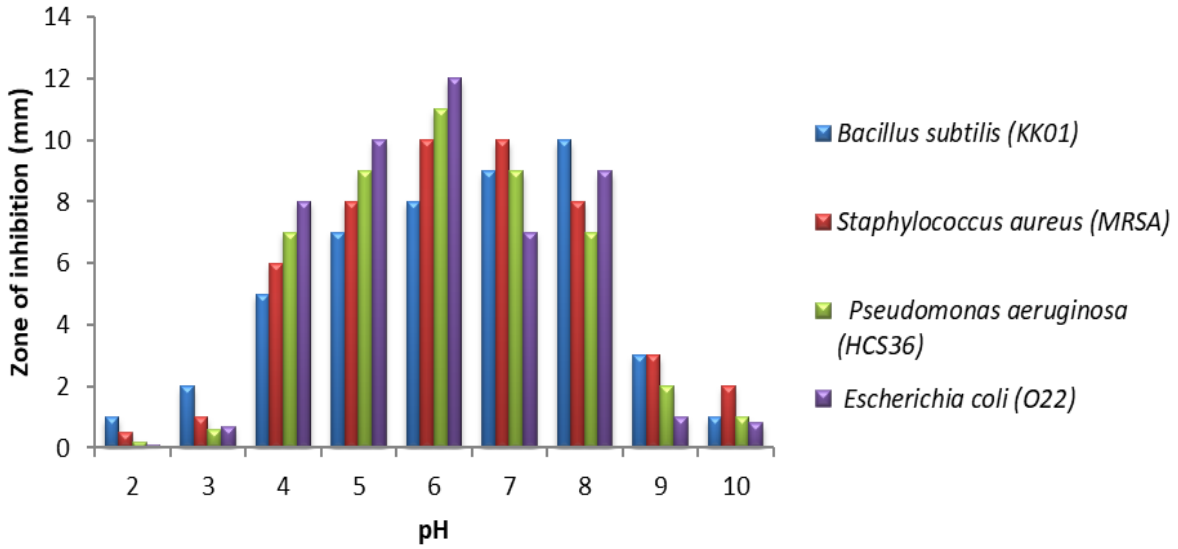

Fig. 5. *Effect of pH on the activity of BLIS purified from culture of Brevibacillusborstelensis*

like inhibitory compound revealed a wide pH tolerance range (4.0–8) and high thermostability up to 100 °C for 10 minutes. The isolated strain of *B.borstelensis strain 3.1* exhibited a bacteriocin-like inhibitory substance due to the unique combination of all the previously described features, making it a much-desired food biopreservative for food preservation in the food and food processing industries.

Conflict of Interest

The authors declare that they have no conflict of interest.

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