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Screening and Characterization of Bacteriocin-Like Inhibitory Substances Produced by Bangladeshi Strains of *Bacillus thuringiensis*

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Abstract

Bacteriocins are ribosomally synthesized antibacterial peptides which have the potential to be used as natural food preservatives as well as alternative to antibiotics. Here, we report the production of bacteriocin-like inhibitory substances (BLIS) from the indigenous strains of *Bacillus thuringiensis*. Deferred antagonism bacteriocin assay and agar well diffusion methods suggested that several of the tested strains have high levels of bacteriocin-like activity against the common food-borne pathogens, *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus*. These bacteriocins are generally produced at the mid-logarithmic phase of growth with optimum temperature of 37 ºC, pH-7.0 and 24 h of incubation. Heat stability assay demonstrated that the bacteriocins produced from the strains are highly heat stable and can retain activity up to 100 ºC. Our study suggests that these bacteriocins may be potential candidates for use as biodegradable natural food preservatives and alternative antimicrobial agents to solve the increasing trends of problems of antibiotic resistance.

Key words: Bacteriocin, *Bacillus thuringiensis*, antimicrobial activity, Bangladeshi strains, food-borne pathogens

Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins which have the potential to inhibit other closely related bacteria, thereby imparting selective advantage to the producer bacteria in the natural environment (Hossain and Biswas 2011). However, the producer strain is immune to its own bacteriocin due to the presence of immunity protein (Hossain and Biswas, 2012). Broadly, bacteriocins can be classified into two major groups such as lantibiotics (which contains post-translationally modified lanthionine groups) and non-lantibiotics (unmodified peptides) (Cotter *et al.,* 2005). While most of the bacteriocins have very narrow spectrum of activity (active against the closely related species), some exhibit broad spectrum of activity (Nissen-Meyer *et al.,* 2009).

Recently, the research on bacteriocins has received intense attention due to the escalating problems of antibiotic resistance among pathogenic bacteria and to control the food spoilage problems caused by undesirable microorganisms present in the food (Anthony *et al*., 2009). Bacteriocins from the Gram positive bacteria hold a great promise as an alternative to antibiotic in food, pharmaceutical and agriculture due to their high level of diversity (Jack *et al*., 1995; Kamoun *et al*., 2005; Rahman *et al*., 2015). Bacteriocins produced from the lactic acid bacteria have been extensively studied due to their GRAS (generally recognized as safe) status and several of them are being used widely in food industries such as nisin, pediocin etc. (Cotter *et al*., 2005). Most of the species of *Bacillus*, which have a history of safe use in food and industry, are also considered as an important source of bacteriocin (Pedersen *et al*., 2002). Several studies have suggested that strains from the genus of *Bacillus* produce a diverse array of bacteriocins which are active against various food-spoilage and pathogenic bacteria such as, subtilin (Jansen and Hirschmann, 1944; Zheng *et al*., 1999; Kindoli *et al*., 2012; Joseph *et al*., 2013) and subtilosin produced by *B. subtilis* (Zheng *et al*., 1999), cerein produced by *B. cereus* (Oscariz and Pisabarro,

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2000), coagulin produced by *B. coagulans* (Hyronimus *et al*., 1998), megacin from *B. megatorium* (Kiss *et al*., 2008), licheniocin from *B. licheniformis* (He *et al*., 2006; Beric *et al*., 2013), and tochicin (Paik *et al*., 1997) and thauricin 7 (Cherif *et al*., 2001), thuricin 439 (Ahern *et al*., 2003), thauricin CD (Rea *et al*. 2010), entomocin (Cherif *et al*,. 2003), bacthuricin F4 (Kamoun *et al*., 2005) from *B. thuringiensis*.

In this study, we attempted to expand the search for novel bacteriocins produced by the renowned bacterium, *B. thuringiensis*, which is widely used in agriculture for the control of many insect pathogens. This bacterium is well-known for its ability to produce crystal protein (Cry, a δ-endotoxin), which causes paralysis of the larval gut and cytolytic toxin (Cyt) (Aronson *et al*., 1986; Crickmore *et al*., 1998; Tounsi *et al*., 2003). As a result, most of the studies on *B. thuringiensis* have focused on its insecticidal activities and very little attention was given for other industrial products (Barboza-Corona *et al*., 2007). The main goal of the present study was to screen and characterize the novel bacteriocins from *B. thuringiensis* (Bt) isolated from different areas of Bangladesh (Asaduzzaman *et al*., 2012; Shishir *et al*., 2014). Here, we report that newly obtained bacteriocins are active against various pathogenic and food spoilage bacteria and are stable in a wide range of temperature and pH.

Materials and Methods

Bacterial strains and culture conditions: 20 indigenous *B. thuringiensis* strains were obtained from the bacterial stock collection center at the Department of Microbiology, University of Dhaka. The strains were isolated from the soil sample of the various region of Bangladesh and identified by 16S rDNA sequence analysis (Asaduzzaman *et al*., 2012; Shishir *et al*., 2014). The bacteria were routinely cultivated and screened for bacteriocin activity in tryptic soy agar or tryptic soy broth (HiMedia, India) at 30 ºC. Indicator bacteria (Table 1) were grown in the same tryptic soy medium at 37 ºC, except *L. monocytogenes* which was routinely cultivated in MRSA medium (Sigma, USA) and *Streptococcus mutans* which was cultivated in Todd-Hewitt medium (Sigma, USA).

Screening for bacteriocin activity: Initially, *Bt* strains were screened for antibacterial activity against the indicator bacteria on tryptic soy agar (TSA) using agar spot antibacterial assay. In brief, bacterial strains were spotted onto TSA plate and incubated overnight at 30 ºC to form colonies. After the growth of producer strains on TSA plate, overnight grown indicator bacteria (10^9 CFU/ml) were spread as soft agar (0.5%) over the TSA plate and the cultures were grown for 24 h at 37 ºC. Antibacterial activity was determined by the presence of growth inhibition around the producer strains.

Bacteriocin production: Bt strains were grown in TSB for 20 h at 150 rpm at 37 ºC. After incubation, bacterial cells were separated by centrifugation at 6000 rpm for 10 min and the supernatant was further sterilized by membrane filtration with 0.22 µm membrane filter and stored at -20 ºC for further studies. Bacteriocin activity was determined out by agar well diffusion method (Jack *et al*., 1995). Briefly, 200 µl of the overnight grown indicator bacteria (10^8cfu/ml) were seeded onto the TSA plate and the well was constructed by a sterile borer. 30 µl of the filter sterilized culture supernatant was loaded into the well and incubated overnight at 37 ºC. Bacteriocin activity was measured as zone of inhibition around the well. Each point of the activity was repeated for at least three times and the average was recorded. The bacteriocin activity was calculated as arbitrary unit (AU) as described by previously (Ahern *et al*., 2003) with some modification and determined as the multiplication of the diameter of inhibitory zones with highest dilution which produced visible growth inhibition.

Effect of temperature on the inhibitory activity of the Bt-BLIS: To investigate the thermostability of the Bt-BLIS, 100 µl aliquots from the filtrated culture supernatants were incubated at a range of temperatures (40, 50, 60, 70, 80, 90 and 100 ºC) for 20 min. After incubation, bacteriocin activity was performed as mentioned above and recorded as the zone of diameter in millimeters.

Mode of action: To determine the mode of action of the observed Bt-BLIS, 100 AU/ml of the bacteriocins were added to mid-logarithmic growth phase (A_{600} of 0.40) of *S. aureus* cells in TSB medium and the culture was incubated at 37 ºC. TSB medium having bacterial cells without any bacteriocin was used as control experiment. Samples were taken at different time intervals

and the viable cells were counted on TSA plates by the standard plate counting method.

Results

Screening of B. thuringiensis strains for antibacterial activity: A total of 20 indigenous *Bt* strains were screened for their antagonist activity against several common food-borne pathogens and food spoilage bacteria by agar spot antibacterial assay and well dilution method (Paik *et al*., 1997). Although most of the strains were able to display some extent of antibacterial activity against one or more indicator bacteria, while four isolates showed remarkable antimicrobial activities and were selected for further analysis (Table 1 and Figure 1a). Successive decrease in the zones of diameter with the increasing dilution of culture supernatant indicated that the observed antibacterial activity was not due to bacteriophage activity (Figure 1b). Culture supernatants from these four strains were sampled at different time intervals and assayed for bacteriocin activity. Based on the relation of growth kinetics and bacteriocin production, selected positive strains can be grouped into two categories (Figure 2). We included JeSa1 and Jsa3 in group I, which were able to produce the considerable level of antibacterial substance at mid logarithmic phase and reached maximum at the early stationary phase. KkSc2 and 34S were included into group B and the antibacterial activity started at the end of logarithmic phase and reached maximum at the middle of stationary phase. Interestingly, we observed a gradual decrease in bacteriocin activity after it reached the peak and lost the activity below detection level after 36 h of incubation.

Table 1. Spectrum of activity of Bt-BLIS. 'U' indicates the instability of the bacteriocin activity when incubated at 80 °C for 20 min.

Indicator/Producer	*Bacillus cereus*	*L. monocytogenes*	*Staphylococcus aureus*	*S. mutans UA159*	*Lactobacillus sp.*	*Bacillus subtilis*
JeSa1	++	+++	+++	+	-	-
KfSa1	-	-	-	-	-	-
KkSc2	-	+++U	++U	-	+U	++U
Ssf1	-	-	-	-	+U	-
JSa1	+U	-	-	-	++U	++U
JSa3	-	++U	++U	-	-	-
Rpsa2	-	-	-	-	-	-
JSb2	-	-	-	-	-	++U
15S	-	-	+U	-	-	-
16S	-	-	+U	-	-	-
20S	-	++U	+U	-	-	-
26S	-	++U	-	-	-	-
34S	-	+++	+++	-	-	
37S	-	-	+U	-	-	-
38S	-	+U	-	-	-	-
40S	-	++	++	-	-	-
41S	-	++	++	-	-	-
42S	-	-	-	-	-	-

Spectrum of inhibitory activity: To investigate the spectrum of inhibitory activity, Bt-BLIS preparations were tested against various indicator bacteria using the well-diffusion method (Table 1). Among the Gram-positive indicator bacteria, *L. monocytogenes* and *S. aureus* were prominently inhibited by the Bt-BLIS produced by most of the strains. JeSa1and Jsa1were able to inhibit the growth of *B. cereus*. JeSa1 was also able to inhibit the prominent dental pathogen, *S. mutans*. KkSc2, Jsa1 and Jsb2 displayed the inhibitory activity against *B. subtilis* and *Lactobacillus* sp. was inhibited by KkSc2, Ssf1 and Jsa1. None of the strains could inhibit the growth of any tested gram-negative bacteria. When Bt-BLIS were tested against other *B. thuringiensis* strains, bactericidal

activities were observed against some of them also (data not shown).

Biochemical properties of the Bt-BLIS: To investigate the biochemical properties of the initially observed bacteriocins, partially purified Bt-BLIS were digested with various enzymes and incubated at various temperatures and pH. The inhibitory activities of the all Bt-BLIS were lost when treated with proteinase K, which indicates the peptide in nature of the observed bacteriocin (data not shown). Treatment with amylase, lipase, RNase didn't cause any inhibition of the bacteriocin activity (data not shown). When bacteriocin samples were incubated at various temperatures ranging from 40 ºC to 100 ºC for 20 min, most of the bacteriocin activities were lost. Only the bacteriocin from JeSa1, 34S, 40S and 41S could retain the activity at high temperature (Table 1). We considered them as heat stable, because they could retain the activity even at 100 ºC (Figure 3).

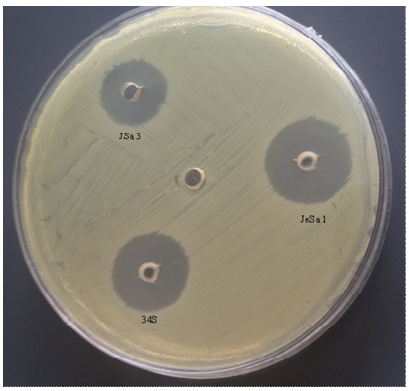

Figure 1(a)

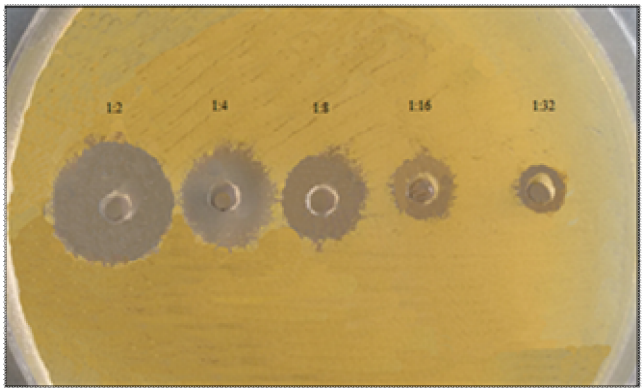

Figure 1(b)

Figure 1. Antagonistic activity of the *Bt* strains against *S. aureus* (1a) and activity at various dilutions of the filtrated culture supernatants against *L. monocytogenes* (1b). Indicator bacteria were grown overnight and adjusted to 0.5 McFarland turbidity standard and swabbed onto the TSA plate. Wells were made on the seeded plate with a sterile borer and 30 µl of the filtrated culture supernatant was placed into the wells and incubated for overnight. After incubation, zones of inhibition around the wells were observed and measured as the millimeter in diameter. This plate is representative of at least three independent experiments.

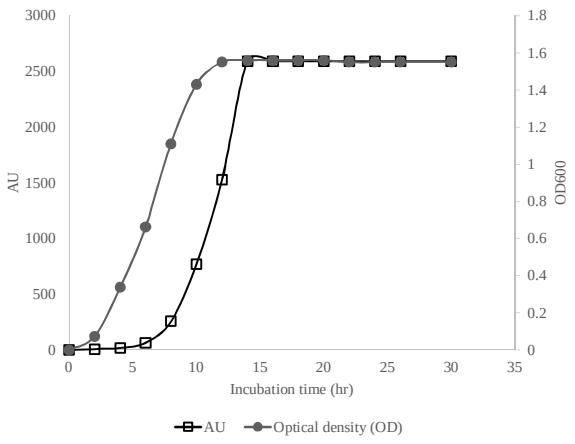

Figure 2(a)

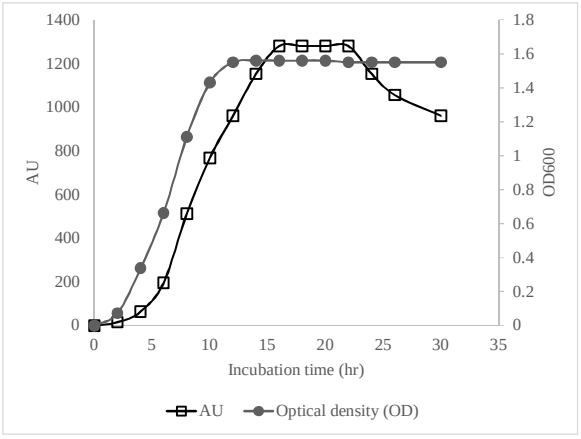

Figure 2(b)

Figure 2. Kinetics of bacteriocin production and the growth of *B. thuringiensis*. Bacteria were grown in tryptic soy broth and samples were collected in duplicate at 2 h intervals. One sample was used for antagonistic activity against *S. aureus* and the other was used for the measurement of optical density at 600 nm. Each point represents the average of three independent experiments. Figure 2(a) represents the growth kinetics of JeSa1 and 2(b) represents the growth kinetics of 34S. Arbitrary unit (AU) is defined as the multiplication of diameter of inhibitory zones with the highest dilution which produced visible growth inhibition by the well-diffusion method.

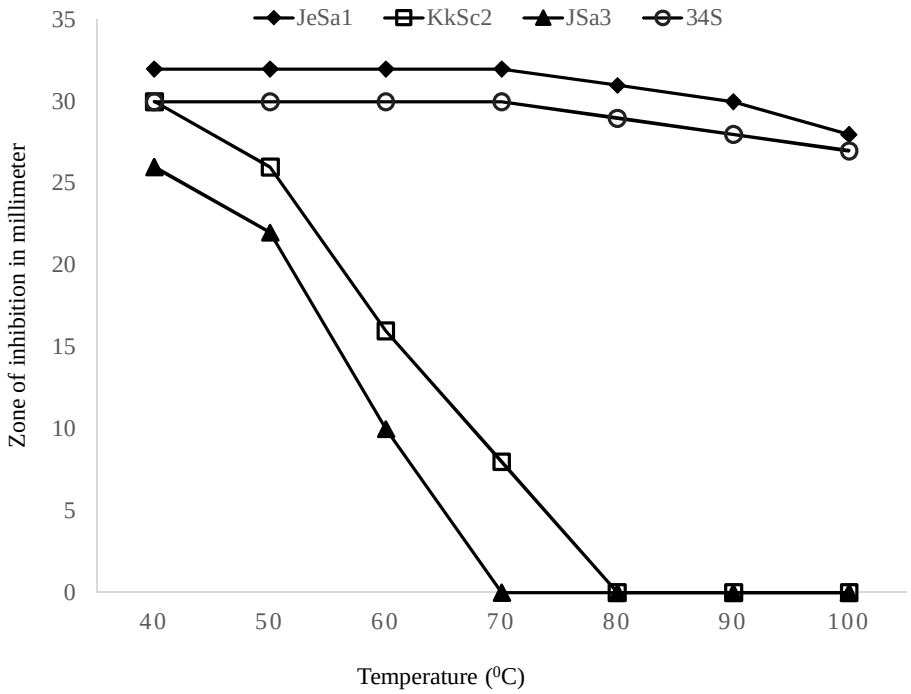

Figure 3. Effect of temperature on the activity of different Bt-BLIS. Filtrated culture supernatants were incubated at different temperatures for 20 min and assayed for growth inhibition against *L. monocytogenes*. Each point represents the average of at least three independent experiments.

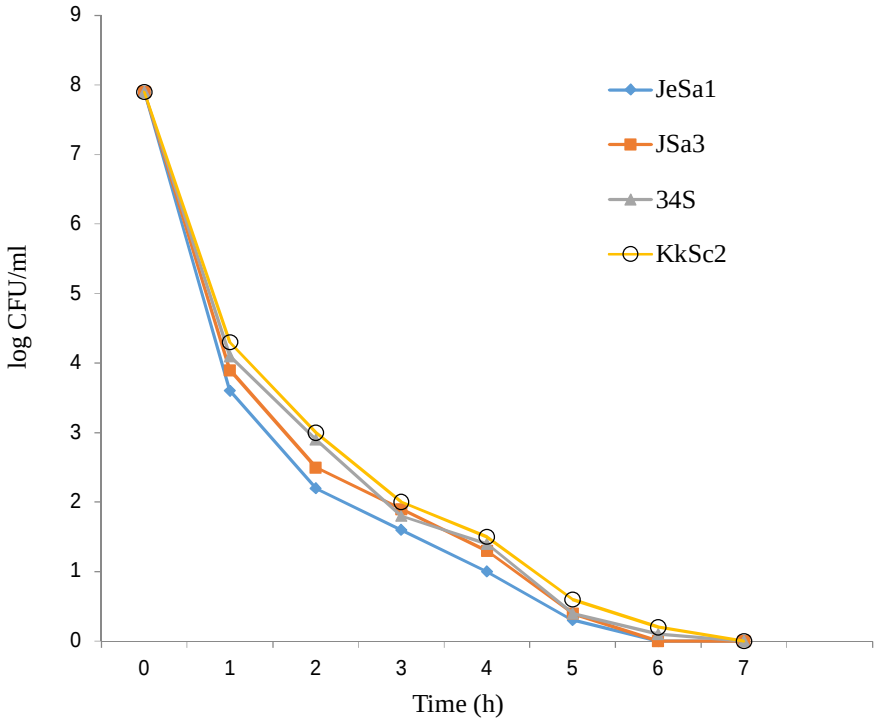

Figure 4. Mode of inhibition of the Bt-BLIS. 100 AU/ml of the bacteriocins were added to mid-logarithmic growth phase (A_{600} of 0.40) of *S. aureus* cells in TSB medium and the culture was incubated at 37 ºC. Samples were taken at different time intervals and the viable cells were counted on TSA plates by standard plate counting method. Each point represents the average of at least three independent experiments.

Mode of inhibition: In order to determine the mode of inhibition, exponentially growing indicator bacteria were incubated with 100 AU/ml of the bacteriocin and assessed for growth and viability. Our study revealed that the addition of bacteriocin caused a drastic decline in growth and viability within 1 h of incubation. A 100-fold reduction of viable cell number was observed within 1 h of growth and demonstrated total growth inhibition after 6 h (Figure 4). Our study indicated that the observed bacteriocin is bactericidal in nature.

Discussion

Increasing trends of health risks posed by the artificial preservatives in food products and the emergence of multi-drug resistant bacteria have raised the demand for the discovery of alternative natural products to alleviate these serious problems. Therefore, the scientific interest has been focused on the development of novel biodegradable preservatives for food products and the new antibacterial agents with pathogen specific activity. The aim of this study was to screen and characterize the novel bacteriocins from *B. thuringiensis* isolated from various areas of Bangladesh.

Among the 20 indigenous *Bt* strains, we found that 17 were able to exhibit bacteriocin-like inhibitory activities directed to the tested indicator bacteria. Based on growth kinetics, we have grouped these bacteriocins into two sub-groups: group 1 includes the strains which were able to produce the considerable level of antibacterial substance at mid logarithmic phase and reached maximum at the early stationary phase and group II which includes the strains that producedthe maximum level of antibacterial activity at the middle of stationary phase. Our results are in consistent with two previous reports (Cherif *et al*., 2001; Barboza-Corona *et al*., 2007), where they reported that strains exhibited maximum bacteriocin-like activity at mid-logarithmic phase to middle of the stationary phase. Our studies also revealed that only four of the observed Bt-BLIS were heat stable, retaining the activity upto 100 ºC. Thuricin 7 and several other bactericions from *Bt* isolates were also reported to be stable at high temperature.

Among the indicator bacteria, Gram positive food-borne pathogens bacteria were prominently inhibited by the tested Bt-BLIS. Food-borne pathogens *L. monocytogenes, S. aureus* and *B. cereus* were inhibited by several Bt-BLIS. Cross activity assay among the *Bt* strains suggested that some of them are active against other *Bt* strains. In contrast with the bacteriocins of lactic acid bacteria, Bt-BLIS displayed relatively narrow spectrum of activity (Jack *et al.,* 1995). Our findings suggested that some of these bacteriocins can be potentially used as food preservative to control food-borne pathogens. In the cross-activity assays performed in this study showed that bacteriocin from one *Bt* strain was able to inhibit the growth other *Bt* strains, which indicates the absence of immunity protein in the susceptible *Bt* strains.

Incubation time, temperature and pH play an important role bacteriocin production. (Kamoun *et al*., 2005; Barboza-Corona *et al*., 2007). Although the highest level of antibacterial activity was obtained when the culture was incubated at 37°C, bacteriocin production was also observed from temperature 25 to 50 ºC and activity diminished at 60ºC (data not shown). Bacteriocin produced at alkaline pH is of particularly important as the pH of most of the food products are neutral to alkaline. Bt-BLIS from our producer strains are capable of producing bacteriocin at neutral to alkaline pH (data not shown). It has been reported that the nisin, widely used commercial food preservative, normally works at acidic pH and very unstable at alkaline condition (Liu and Hansen, 1990). Our result is in accordance with the bacteriocin, bacillocin 490 of *B. licheniformis*, which displayed antibacterial activity between acidic and alkaline pH (Martirani *et al.*, 2002) .

Although they are able to produce entomocidal crystal protein (Aronson *et al*., 1986; Barboza-Corona *et al*., 2007), several studies have reported that *B. thuringiensis* is non-pathogenic and have excellent safety records (Siegel *et al.*, 2001). As our purpose is to develop the protocol for the large scale production and purification of novel antimicrobial agents from *Bt* strains rather than the use of live bacterium, there will be no problem for their industrial use. As these bacteriocins are active against a lot of food-borne pathogens, they have the potential to be used as food preservative which will in turn contribute greatly to the food safety and food security issues thereby. Further studies to clone and identify the structural genes and the proteomics will help to unravel the nature of the

peptides. In addition, the development of recombinant vectors for high-level expression will facilitate the large scale production and purification of these peptides for industrial use.

Conclusions

The current report is based on the screening, isolation and characterization of bacteriocins from local *B. thuringiensis* strains. Our studies revealed that the obtained Bt-BLIS can inhibit several common Gram positive food-borne pathogens. The results suggest that these Bt-BLIS can be used as alternative food preservatives in food industry and as therapeutic agents by the pharmaceutical industries. Further studies on purification, biochemical and genetic characterization are necessary to uncover the exact nature of these antibacterial agents.

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Declaration of interests

The authors declare that they have no conflict of interest.

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