ELSEVIER

Institut Pasteur

Research in Microbiology xx (2015) 1–9

www.elsevier.com/locate/resmic

Original article

Diversity in the antibacterial potential of probiotic cultures *Bacillus licheniformis* MCC2514 and *Bacillus licheniformis* MCC2512

Papanna Shobharani, Radhakrishnan J. Padmaja, Prakash M. Halami*

Food Microbiology Department, CSIR-Central Food Technological Research Institute, Mysore 570 020, Karnataka, India

Received 20 February 2015; accepted 1 June 2015
Available online ■ ■ ■

Abstract

The aim of the present study was to investigate the characteristic diversity and stability of antimicrobial compounds produced by two probiotic strains of *Bacillus licheniformis* (MCC2514 and MCC2512). Antimicrobial compounds from the two strains notably varied, related to stability and potency. The inhibitory spectrum of *B. licheniformis* MCC2512 was higher than MCC2514, but, related to the effect on *Micrococcus luteus* ATCC9341, MCC2514 (LD_{50} = 450 AU ml^{-1}) was more potent than MCC2512 (LD_{50} = 750 AU ml^{-1}). The compounds were thermo-resistant and stable at a wide range of pH and exhibited considerable resistance to digestive enzymes and bile salts (anionic biological detergents), contributing to their appropriate application in various food systems. The isolate *B. licheniformis* MCC2512 gave a positive response to *Bacillus subtilis*-based biosensors BSF2470 and BS168.BS2, confirming the mode of action on the cell wall and subtilin-type, respectively. For *B. licheniformis* MCC2514, the mode of action was characterized by constructing *B. subtilis* reporters that interfered in five major biosynthetic pathways, i.e., biosynthesis of DNA, RNA, protein, the cell wall and fatty acids. *B. licheniformis* MCC2514 responded to the *yvg*S reporter, indicating it as an RNA synthesis inhibitor. Overall, the investigation reveals variability of the antimicrobial compounds from *B. licheniformis* of different origins and for their possible application as biopreservative agents.

Keywords: *Bacillus* sp.; Probiotic; Biosensor; Antibacterial activity; Inhibitory spectrum

1. Introduction

The increasingly fast-paced life style of consumers has increased the need for processed foods and has encouraged their industrialization. Environmental contamination of these processed foods is a serious problem faced by the food industry as it causes enormous economic losses. Chemical preservatives and antibiotics have been extensively used to safeguard processed foods; however, they have a drastic effect upon nutritional properties. Hence researchers have focused on a range of compounds produced by microbial sources that can maintain the nutritional value of products and increase their shelf-life. In addition, the emergence of multidrug-resistant pathogens and imposed restrictions on the use of antibiotics both in clinical settings and food additives have increased the quest for natural antimicrobial compounds from microbial sources. In this regard, probiotic strains with defined health benefits have been extensively studied for their antimicrobial activity. Among them, lactic acid bacteria (LAB) have created a growing interest among food industries because of their biopreservative activities against food-borne pathogens through simple fermentation processes [1,2].

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a number of bacteria that are often effective against closely related species [3,4]. Nisin produced by *Lactococcus lactis* subsp. *lactis* is the only bacteriocin approved by the US-FDA for commercial application in food products, but its use is limited because of weak activity at neutral or alkaline pH. Many non-LAB have also been reported to produce antimicrobial peptides. The genus *Bacillus*

* Corresponding author. Tel.: +91 821 2517539; fax: +821 2517233.
E-mail address: prakashalami@cftri.res.in (P.M. Halami).

http://dx.doi.org/10.1016/j.resmic.2015.06.003
0923-2508/

includes a large number of species known to produce bacteriocins or bacteriocin like substances such as subtilin, sublancin, bacillocin and subtilosin from *Bacillus subtilis* [5–7], coagulin from *Bacillus coagulans* [8], bacitracin and lichenin from *Bacillus licheniformis* [9–12] and megacin from *Bacillus megaterium* [13]. Unlike LAB, the *Bacillus* spp. exhibits broad spectrum inhibitory activity [4,6,11]. Numerous studies have been carried out on *Bacillus* bacteriocins in food products to address food safety aspects and their application in clinical studies [4,10,11,14].

In recent years, whole cell bacterial biosensors containing reporters which can be specifically induced via selected promoters are widely used in identifying specific mode of action (MOA) of antimicrobial compounds [12,15,16]. This is a rapid screening method that is robust, sensitive and very specific. In this context, bacteria have been genetically engineered to respond to the presence of specific chemicals or stress by synthesizing reporter proteins. These bacterial biosensors have been used as a tool in the present study to analyze the MOA of antimicrobial compounds.

Given the wide diversity of *Bacillus* spp., the present work focused on characterizing the antimicrobial compound produced by two strains of *B. licheniformis* (MCC2514 and MCC2512) originating from raw milk from sheep and rhizobial soil of *Hedychium coronarium*, respectively. These two cultures have been previously characterized for their potential probiotic properties [17]. Sheep milk is highly nutritious and represents an ideal growth medium for microorganisms [18]. Similarly, *H. coronarium* or the white ginger lily has high medicinal value. The essential oil from fresh and dried rhizomes is known to have antifungal, antibacterial and cytotoxic activity [19,20], as well as analgestic and anti-inflammatory properties [21].

In the present investigation, we report the differential activity of antimicrobial compounds produced by two strains of *B. licheniformis* in terms of their stability, inhibitory spectrum and mode of action.

2. Materials and methods

2.1. Media chemicals and reagents

All microbial media chemicals, X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside), ONPG (O-Nitrophenyl-β-galactoside) and standard reference antibiotics were purchased from Hi Media Pvt Ltd, Mumbai, India. Sodium chloride, organic solvents such as butanol, toluene, acetone, chloroform and ethanol were purchased from Sisco Research Laboratory, Bangalore, India. Enzymes (trypsin, pepsin, proteinase K, α-amylase), bile salts (sodium glycocholic acid and sodium taurocholic acid) and DEAE cellulose were procured from Sigma–Aldrich Inc, USA. All chemicals used were of analytical grade reagent unless otherwise mentioned.

2.2. Bacterial cultures and growth conditions

Native strains of *B. licheniformis* MCC2514 and MCC2512 previously characterized in the laboratory for their probiotic properties [17] were grown in Luria Bertani (LB) medium at 37 °C under constant shaking (120 rpm). For antimicrobial activity, the indicator organisms such as *Micrococcus luteus* ATCC9341, *Yersinia enterocolitica* MTCC859, *Aeromonas hydrophila* NRRL B445, *Staphylococcus aureus* FRI722, *Salmonella typhimurium* MTCC1251, *Escherichia coli* CFR02 and *Klebsiella* sp. were procured from the American Type Culture Collection (ATCC), USA or the Microbial Type Culture Collection Center (MTCC) Chandigarh, India. *Listeria monocytogenes* ScottA was kindly provided by Dr. A.K. Bhunia, USA. All indicator cultures were grown in brain heart Infusion (BHI) media at 37 °C under constant shaking (120 rpm). Reference standard culture *B. subtilis* 168 and whole cell biosensor *B. subtilis* 168.BS2 (W168 *amy*E::-P_{SpaS}:*lac*Z, P_{SpaRK}-SpaRK), a subtilin-specific reporter, were kindly provided by Prof. K.D. Entian, Germany. The reporter strain *B. subtilis* BSF2470 (CU1065 lial::pMUTIN) [22] was used for confirming cell-wall stress-producing antimicrobial substances. *B. subtilis* ATCC6633 and *Bacillus flexus* MCC2011 were used as positive and negative controls, respectively, for cell-wall stress-inducing antimicrobial compounds.

2.3. Antimicrobial activity of Bacillus isolates

2.3.1. Preparation of the antimicrobial compound (AMC)

Bacillus cultures individually grown in LB broth were centrifuged at 10,000 rpm for 15 min at 4 °C. The cell-free supernatant or crude AMC was filter-sterilized (0.2 μm membrane) and stored at 4 °C until use.

2.3.2. Inhibitory activity of the AMC

Antimicrobial activity of *Bacillus* isolates against various indicator organisms was tested by the agar well diffusion assay as described by Xie et al. [23]. The antibacterial activity was further quantified by the twofold serial dilution method and results were expressed as AU ml^{-1}. A unit is defined as the reciprocal value of the highest dilution at which the zone of inhibition was observed.

2.4. Effect of enzymes on antimicrobial activity

The effect of enzymes including pepsin, trypsin, proteinase K and α-amylase was tested on cell-free supernatant of *Bacillus* spp. An aliquot of cell-free supernatant or crude AMC was treated with the respective enzymes at a final concentration of 2 mg ml^{-1} at 37 °C for 30 min. After incubation, the activity was quantified and expressed as AU ml^{-1} as described elsewhere. An untreated cell-free supernatant and the enzymes alone in the buffer (pH 7.0) served as controls.

2.5. Purification of the AMC

2.5.1. Optimization of the extraction procedure

AMC in the cell-free supernatant (CFS) of test cultures was extracted by various methods to determine a suitable protocol for maximum recovery. Method 1: CFS was precipitated with

Please cite this article in press as: Shobharani P, et al., Diversity in the antibacterial potential of probiotic cultures *Bacillus licheniformis* MCC2514 and *Bacillus licheniformis* MCC2512, Research in Microbiology (2015), http://dx.doi.org/10.1016/j.resmic.2015.06.003

ice-cold ethanol (1:1 v/v) overnight at 4 °C. The precipitate thus obtained was air-dried, resuspended in phosphate buffer (pH 7.0) and checked for inhibitory activity. Method 2: CFS was mixed with 2 volumes of chloroform, stirred thoroughly on a magnetic stirrer for 1 h and left at room temperature for phase separation. The chloroform layer was separated, evaporated under nitrogen and the residue dissolved in phosphate buffer (pH 7.0) was checked for the activity. Method 3: Ammonium sulfate was added to the CFS to a saturation level of 60% under constant stirring. The precipitate obtained was dialyzed against phosphate buffer (pH 7.0) and then monitored for inhibitory activity. Method 4: the CFS was thoroughly mixed with butanol (1:0.5 v/v) and left at room temperature for phase separation. The butanol layer was evaporated and the residue suspended in phosphate buffer (pH 7.0) was tested for activity. Throughout the method, *M. luteus* ATCC9341 was used as an indicator organism to analyze antagonistic activity.

2.5.2. Extraction of AMC by ammonium sulfate precipitation

Percent saturation of ammonium sulfate for initial purification of AMC was determined by subjecting the CFS to various saturation levels of ammonium sulfate (40–80%). The ammonium sulfate saturation point for both cultures was determined with regard to maximum activity and was used for further purification.

2.5.3. Ion exchange chromatography

The dialyzed ammonium-sulfate-precipitated sample (40 mg protein) of each culture was loaded separately onto a DEAE cellulose column (18 × 3 cm) pre-equilibrated with 0.2 mM sodium phosphate buffer (pH 7.0). Protein fractions were eluted with a linear gradient of 0.0–1.0 M NaCl in the same buffer at a flow rate of 1 ml min^{-1}. Fractions of 5 ml were collected and checked for absorbance at 280 nm using a UV spectrophotometer (Multiskan Go, Thermo Fisher Scientific, India). The fraction showing the highest protein content was pooled, lyophilized and monitored for antimicrobial activity.

2.6. Stability of the purified antimicrobial compound

2.6.1. Thermal and pH stability

To determine thermal stability, aliquots of purified AMC were exposed to temperatures ranging from 40 to 100 °C for 30 min. The percent residual activity was calculated by comparing with the untreated sample. Similarly, pH stability of the AMC was analyzed by incubation in various buffers of different pH (2–10) for 30 min, followed by neutralization to pH 7.0, and testing for antimicrobial activity.

2.6.2. Effects of organic solvents

The effects of organic solvents including butanol, chloroform, toluene, acetone and ethanol were tested on the inhibitory activity of the AMC. Briefly, the AMC was incubated with organic solvents at a working concentration of 50% (v/v) for a period of 1 h at 37 °C before being tested for antimicrobial activity.

2.6.3. Effect of bile salts (anionic biological detergents)

The stability of the AMC in the presence of bile salt was determined by incubating the purified AMC in sodium glycocholic acid and sodium taurocholic acid individually at a final concentration of 2 mg ml^{-1} for 1 h at 37 °C and analyzing it for antimicrobial activity.

All experiments were carried out in triplicate and after each treatment samples were tested for antimicrobial activity against *M. luteus* ATCC9341.

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the AMC

Crude AMC, the ammonium sulfate-precipitated sample and fractions purified by ion exchange chromatography of both cultures were subjected to SDS–PAGE [24] along with low range protein markers ranging from 3.5 to 43 kDa. Following electrophoresis, the gel was cut vertically into two halves. The first part was stained with staining solution (0.25% Coomassie brilliant blue R-250, 10% acetic acid, 50% methanol) to determine the molecular weights of separated protein bands. The other part of the gel was assayed for direct detection of inhibitory activity by an overlay assay described by Barboza-Corona et al. [14]. Briefly, the gel was fixed in an isopropanol:acetic acid (25:10) solution for 1 h and washed with double-distilled water in three changes every 30 min. The gel was sterilized by exposing to UV light for 30 min and then aseptically placed in a sterile petri plate and overlaid with BHI medium (1% agar, w/v) pre-inoculated with 1% overnight grown *M. luteus* ATCC9341. The petri plate was incubated at 37 °C for 24 h and observed for the presence of an inhibition zone.

2.8. Effect of the antimicrobial compound on M. luteus ATCC9341 growth

The purified antimicrobial compound at various concentrations (500, 1000, 1500 AU ml^{-1}) was added to BHI media at two growth phases of *M. luteus*. In one set, AMC was added initially at 0 h (6.5 log CFU ml^{-1}) and in the other set, the AMC was added at the beginning of exponential growth phase, i.e., after 6 h (7.18 log CFU ml^{-1}). Culture without AMC served as control. At regular time intervals, an aliquot was drawn, serially diluted and an appropriate dilution was plated on BHI agar. The number of colonies was counted and expressed as colony-forming units per ml (CFU ml^{-1}).

2.9. Specific mode of action (MOA)

2.9.1. Biomarker construction and host strain generation

Whole cell biosensors were constructed similar to the technique of Urban et al. [16] with minor modifications. The upstream regions of *B. subtilis* genes *yhe*I, *yor*B, *ypu*A, *yvg*S and *Fab*HB were amplified (Primers in Table 1) and cloned in front of *lac*Z genes using promoter probe vector pAC6 [25]. The resulting construct carrying the promoter-reporter fusions was subsequently transferred to *B. subtilis* 168 to generate a set of biosensors.

Please cite this article in press as: Shobharani P, et al., Diversity in the antibacterial potential of probiotic cultures *Bacillus licheniformis* MCC2514 and *Bacillus licheniformis* MCC2512, Research in Microbiology (2015), http://dx.doi.org/10.1016/j.resmic.2015.06.003

Table 1
Primers used for amplification of test promoters, target and reference antibiotic tested.

Gene		Sequence (5′–3′)	Promoter region	Target	Reference antibiotic tested
yorB	Forward	GTACGAATTCGGTACCCGGGATATATTGGGATAAAGATTCAGA	532	DNA	Nalidixic acid, Trimethoprin
	Reverse	GTACGGATCCCATACCGTATTTCTCCGATTC			
yvgS	Forward	GTACGAATTCGTTTAATTGGAAGCTGCCAAACC	217	RNA	Rifampicin
	Reverse	GTACGGATCCACAAACATAGATGAAATACTG			
yheI	Forward	GTACGAATTCTTCTTACTATTTTCACTTCCG	529	Protein	Neomycin, Streptomycin
	Reverse	GTACGGATCCCCAGCCAAGCTTTTTCAAAACTG			
ypuA	Forward	GTACGAATTCCCAGTGTCTTTTCCGGCATGTGCC	575	Cell wall	Penicillin, Nisin
	Reverse	GTACGGATCCGCAAAACGGCCGCTGCCAGCATTCC			
FabHB	Forward	GTACGAATTCGCGCCTGTTTGACGTCGCCATTGACCAAAAGC	594	Fatty acids	Triclosan
	Reverse	GTACGGATCCGACATATGAATCACTCCTTATGG			

2.9.2. Chromogenic plate assay

The chromogenic plate assay was carried out using reporter strains by the method described by Burkard and Stein [26]. *B. subtilis* BSF2470 and *B. subtilis* B168.BS2 were streaked on an LB agar plate supplemented with 50 μg ml^{-1} of X-gal. The test cultures were later streaked horizontally to the reporter strain. After 24–36 h of incubation, blue staining was observed at the interjunction of test and reporter culture due to production of β-galactosidase induced by the *lac*Z gene. For specific mode of action studies, pathway-specific reporter cultures including, *B. subtilis yhe*I, *yor*B, *ypu*A, *yvg*S and *fab*HB were inoculated (1% v/v) into LB medium supplemented with X-gal (20 mg ml^{-1}). Subsequently, the standard antibiotics along with test cultures were spotted on the medium plate to analyze β-galactosidase induction. The standard antibiotics included: nalidixic acid and trimethoprin (for *yor*B: inhibition of DNA synthesis), rifampicin (for *yvg*S: inhibition of RNA synthesis), neomycin and streptomycin (for *yhe*I: inhibition of protein synthesis), nisin and penicillin (for *ypu*A: inhibition of cell wall biosynthesis) and triclosan (for *fab*HB: for fatty acid synthesis).

2.9.3. β-Galactosidase induction assay

The CFS of overnight-grown test culture was collected by centrifugation and stored at −20 °C until use. The reporter strain was allowed to grow in LB media until it reached an OD_{600} of ~0.5. Different concentrations (20, 50, 100, 150, 200 μl) of CFS of test culture were added to 2 ml each of reporter strain and incubated for 30 min at 37 °C. After incubation, the cell pellet was collected by centrifugation and resuspended in 1 ml of working Z-buffer (20 mM β-mercaptoethanol, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgSO_4$, pH 7.0) and assayed for β-galactosidase activity as described by Miller [27]. Respective antibiotics were used as positive controls.

2.10. Statistical analysis

All experiments were carried out in triplicate and values were expressed as mean ± standard deviation. Statistica software (6th Version, Statsoft. Inc) was used for data analysis [28]. The significance difference between samples was analyzed by ANOVA performed using Duncan's multiple range test. The value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Inhibitory spectrum of B. licheniformis *MCC2514 and MCC2512*

CFSs of both test cultures (MCC2514 and MCC2512) were analyzed for antimicrobial activity against eight different pathogens. An example of a well-diffusion assay, twofold serial dilution assays and the effect of protease enzymes are shown in Fig. 1. Both the test cultures exhibited inhibitory activity against *M. luteus*, *S. aureus*, *Klebsiella* sp. and *A. hydrophila*, with an inhibition zone ranging from 12 to 24 mm dia. In addition to these pathogenic strains, *B. licheniformis* strain MCC2512 had inhibitory activity against *L. monocytogenes* and *S. typhimurium*.

3.2. Effect of enzymes on the AMC

Cell-free supernatant of test cultures (100 AU ml^{-1} each) was analyzed for sensitivity to several proteases and α-amylase. The activity of the AMC from both cultures was completely lost on exposure to proteinase K, indicating the proteinaceous nature of the compound. On treating the sample with trypsin and pepsin, 100% activity was retained, but with α-amylase, 50% activity was lost.

3.3. Purification of the AMC

The AMC secreted by the test cultures was recovered by various extraction procedures (Table 2). In the case of *B. licheniformis* MCC2514, there was no significant ($p > 0.05$) difference in the activity when extracted with chloroform or butanol. Comparatively, maximum activity (1600 AU ml^{-1}) was obtained with the ammonium sulfate precipitation and ethanol precipitation (1400 AU ml^{-1}), which was 16 and 14 times higher than the crude samples, respectively. Similarly, ammonium sulfate precipitate of *B. licheniformis* MCC2512, showed maximum activity (1600 AU ml^{-1}) that was 16 times

Please cite this article in press as: Shobharani P, et al., Diversity in the antibacterial potential of probiotic cultures *Bacillus licheniformis* MCC2514 and *Bacillus licheniformis* MCC2512, Research in Microbiology (2015), http://dx.doi.org/10.1016/j.resmic.2015.06.003

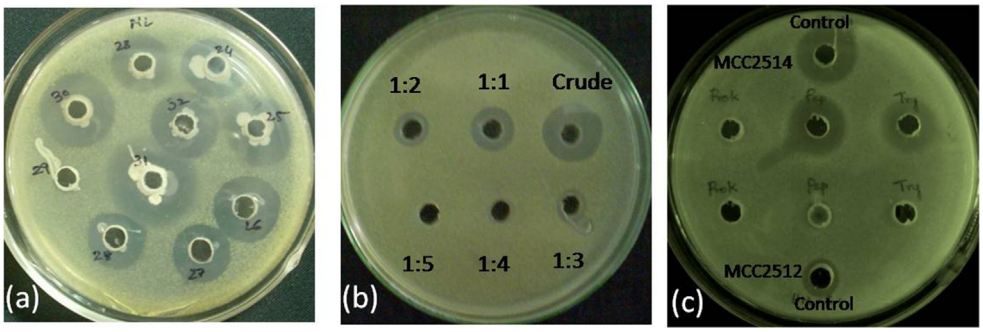

Fig. 1. Antimicrobial activity of *Bacillus* isolates against *M. luteus* ATCC9341. (a) Screening for AMC producing *Bacillus* spp. (b) Broth dilution assay to determine AU units. (c) Effect of protease enzyme on antimicrobial activity.

higher than the crude sample. Hence the ammonium sulfate precipitation method was followed for further purification of the AMC.

Inhibitory activity of the AMC at various saturation levels of ammonium sulfate is presented in Table 2. In the case of *B. licheniformis* MCC2514, maximum inhibitory activity (3200 AU ml^{-1}) was found in the resolved precipitate with 80% saturation, whereas with *B. licheniformis* MCC2512, 70% saturation was found to be optimum (3240 AU ml^{-1}).

Purification of the AMC was further carried out by anion exchange chromatography. The protein fraction of *B. licheniformis* MCC2514 was eluted between 0.18 and 0.3 M NaCl concentration, whereas in the case of MCC2512, the protein was eluted at 0.1–0.25 M NaCl. In each case, fractions showing the highest protein content were pooled, lyophilized and checked for inhibitory activity. According to the data obtained, the maximum AMC concentration (6400 AU ml^{-1}) was recovered from MCC2512 with 128-fold purification and 32% yield, whereas MCC2514 showed 22.5% recovery with a total activity of 45,000 AU ml^{-1}. A comparative result at each step of purification is presented in Table 3.

3.4. SDS–PAGE analysis and activity assay

The molecular weight of AMCs was determined by SDS–PAGE analysis (Fig. 2a). Accordingly, the apparent molecular weight of AMCs from MCC2514 and MCC2512 was found to be 6.5 and 3.5 kDa, respectively. On performing a direct overlay activity assay, the inhibition zones corresponding to their respective protein bands were observed (Fig. 2b and c).

Table 2
Activity of AMC in various extraction methods.

Sample	AU ml^{-1}[a]	
	MCC2514	MCC2512
Control	100 ± 0.02	100 ± 0.02
Ethanol precipitate	1400 ± 0.40	400 ± 0.11
Chloroform extract	200 ± 0.11	1200 ± 0.14
Ammonium sulfate precipitate	1600 ± 0.02	1600 ± 0.20
Butanol extract	200 ± 0.03	800 ± 0.05
Ammonium sulfate saturation (%)		
40	0	0
50	200 ± 0.02	400 ± 0.08
60	1600 ± 0.01	1600 ± 0.11
70	1800 ± 0.11	3240 ± 0.08
80	3200 ± 0.11	1800 ± 0.08

Values are mean ± SD of three individual experiments.
[a] The unit is defined as the reciprocal value of the highest dilution where an inhibition was observed.

3.5. Effect of temperature, pH, organic solvents and bile salts (anionic detergent) on AMC

AMCs from both cultures were found stable at all temperature tested (40–90 °C). At 100 °C, AMCs of MCC2514 and MCC2512 showed 90 and 100% activity, respectively (Table 4). pH sensitivity of AMCs (500 AU ml^{-1}) was evaluated on exposure to various buffers with respective pH (2–10). Data revealed that the AMC secreted by the two strains retained activity under a wide range of pH (Table 4). Nearly 100% residual activity was observed in each case on exposure to pH between 3 and 8. At pH 2.0, MCC2514 and MCC2512 showed 90 and 100% activity, respectively. At high alkaline pH of 9–10, a residual activity of 90% was observed in both cases.

The effect of organic solvents on inhibitory activity of AMCs is presented in Table 4. The substances lost complete activity upon treatment with toluene. In the presence of butanol, 90% reduction in activity was observed for the AMC of MCC2514, whereas the AMC of MCC2412 retained 20% activity. In the presence of bile salts (sodium glycocholate and sodium taurocholate), 70–80% activity was retained in the case of MCC2512, whereas with MCC2514, 60–65% activity was retained. These bile salts, that are biological anionic detergents, are known to unfold the protein structure and affect the three-dimensional conformation of the native protein. The observed results indicate that denaturation or disruption is only partial, and the AMC retained activity even after 1 h exposure to bile salts.

3.6. Effect of the AMC on M. luteus ATCC9341 growth

Fig. 3 represents the effect of AMC supplemented initially at 0 h and after 6 h of bacterial growth. In the first set, the control sample (without AMC) showed growth from 6.4 log to 7.32 log CFU ml^{-1} after 8 h of incubation. On supplementation of AMC at 0 h, a constant decline in cell count was observed with an increase in incubation time. The inhibitory

Please cite this article in press as: Shobharani P, et al., Diversity in the antibacterial potential of probiotic cultures *Bacillus licheniformis* MCC2514 and *Bacillus licheniformis* MCC2512, Research in Microbiology (2015), http://dx.doi.org/10.1016/j.resmic.2015.06.003

Table 3
A comparative data for purification of antimicrobial compound.

Sample		Total protein (mg)	Total activity (AU)	Activity (AU ml^{-1})	Activity (AU mg^{-1})	Purification fold	Recovery (%)
MCC2514	Crude	4369	2,00,000	100	45.8	1	100
	AS	96	64,000	3200	666.7	14.6	32
	IEC	10	45,000	4500	4500	98.3	22.5
MCC2512	Crude	5623	2,00,000	100	35.6	1	100
	AS	148	64,800	3240	437.8	12.3	32.4
	IEC	14	64,000	6400	4571	128.4	32

AS: ammonium sulfate precipitated sample; IEC: fraction purified by ion exchange chromatography.

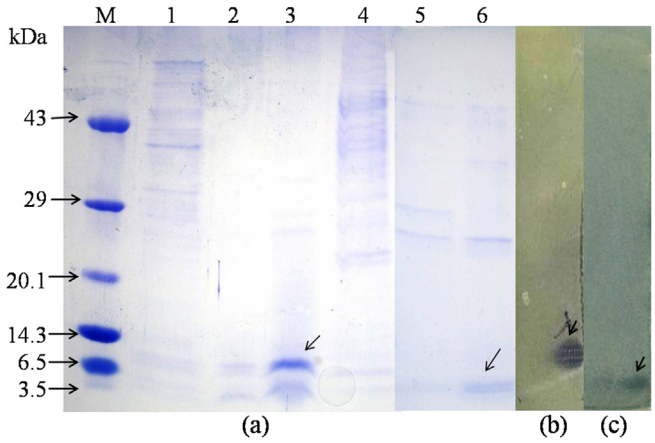

Fig. 2. SDS–PAGE analysis and activity assay of AMCs. (a) Coomassie stained gel, M = protein low range marker; lanes 1–3: *B. licheniformis* MCC2514 – crude, fraction partially purified by ammonium sulfate precipitation and fraction purified by ion exchange chromatography; lanes 4–6: *B. licheniformis* MCC2512 – crude, fraction partially purified by ammonium sulfate precipitation and fraction purified by ion exchange chromatography; (b) and (c) direct overlay assay of SDS–PAGE gel with purified fraction of MCC2514 and MCC2512, respectively, showing inhibition zone against *M. luteus*.

Table 4
Effect of temperature, pH, organic solvent and bile salt on inhibitory activity of AMC.

Treatment	Residual activity (%)	
	MCC2514	MCC2512
Temperature (°C)		
40–90	100 ± 0.11	100 ± 0.01
100	90 ± 0.09	100 ± 0.01
pH		
2	90 ± 0.21	100 ± 0.02
3–8	100 ± 0.02	100 ± 0.03
9–10	90 ± 0.11	90 ± 0.01
Organic solvent		
Butanol	10 ± 0.03	20 ± 0.03
Chloroform	20 ± 0.11	70 ± 0.03
Toluene	–	–
Acetone	50 ± 0.01	50 ± 0.09
Ethanol	80 ± 0.01	30 ± 0.01
Bile salt		
Sodium glycocholate	60 ± 0.23	70 ± 0.02
Sodium taurocholate	65 ± 0.09	80 ± 0.11

Values are mean ± SD of three individual experiments. '–' no activity.

activity increased in a dose-dependent manner. After 8 h of incubation, an almost 5-log reduction was observed in the sample supplemented with AMC (1500 AU ml^{-1}) of MCC2514, whereas a 4-log reduction was observed with MCC2412. From the data, LD_{50} for *B. licheniformis* MCC2514 was determined to be 450 AU ml^{-1}, whereas for *B. licheniformis* MCC2412, LD_{50} was found to be 750 AU ml^{-1}.

In another experiment, AMC added to the growth media of *M. luteus* after 6 h of growth also indicated a decrease of 7–6 log in the sample supplemented with 1500 AU ml^{-1} of AMC.

3.7. Specific MOA of the AMC

The MOA of the AMC produced by *B. licheniformis* strains were evaluated by using pathway-specific whole cell *Bacillus* biosensors. Among the two cultures tested, AMC of *B. licheniformis* MCC2512 exhibited cell-wall stress, inducing a bluish color at the junction of the *B. subtilis* BSF2470 reporter; hence, the AMC was indicative of the presence of a cell wall stress molecule or compound. Subsequently, the compound was tested against the *B. subtilis* B168.BS2 strain, which is a reporter specifically designed for sensing subtilin-type antibiotics. Since the AMC from *B. licheniformis* MCC2512 showed a positive response to the B168.BS2 strain, it was confirmed to be a subtilin-type antibacterial type compound (Fig. 4). The other test culture, *B. licheniformis* MCC2514, which did not induce β-galactosidase in *B. subtilis* BSF2470, was evaluated with other pathway-specific reporters. Among the reporter constructs tested, AMC from *B. licheniformis* MCC2514 responded to *yvgS* promoter-reporter fusion. This reporter is specifically activated in the presence of RNA synthesis inhibitors like rifampicin. Hence, the results indicated that the *B. licheniformis* MCC2514 culture produced an AMC with a specific MOA upon RNA synthesis. In order to investigate the potency of the active compound from *B. licheniformis* MCC2514, quantitative β-galactosidase activity was examined. Maximum β-galactosidase activity (35.2 ± 0.89 U ml^{-1}) was observed with 50 µl of CFS, which was similar to 100 µg of rifampicin (Fig. 4).

4. Discussion

B. licheniformis strains MCC2514 and MCC2512 were previously isolated from raw milk of sheep and rhizobial soil of the medicinal herb *H. coronarium*, respectively [17]. The

Please cite this article in press as: Shobharani P, et al., Diversity in the antibacterial potential of probiotic cultures *Bacillus licheniformis* MCC2514 and *Bacillus licheniformis* MCC2512, Research in Microbiology (2015), http://dx.doi.org/10.1016/j.resmic.2015.06.003

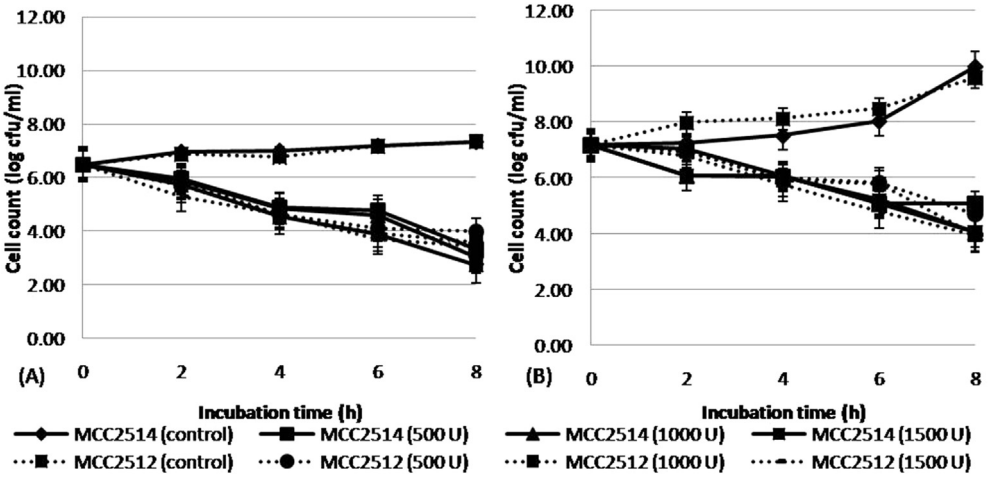

Fig. 3. Effect of antimicrobial compound on *M. luteus* growth: (a) AMC supplemented at 0 h; (b) AMC supplemented after 6 h. In the graph, solid line represents cell count in presence of MCC2514 fractions and dotted lines MCC2512 fractions.

cultures were shown to have potential probiotic properties, including acid bile tolerance, gastrointestinal survival and adhesion ability, along with other functional properties like antioxidant activity, cholesterol-reducing ability and enzyme production [17]. With a view toward their potential probiotic candidacy, the two *Bacillus* strains were analyzed for their possible antibacterial properties.

The result of antimicrobial activity indicates that, although both test cultures were identified as *B. licheniformis*, there was considerable variability in their inhibitory spectrum. Earlier

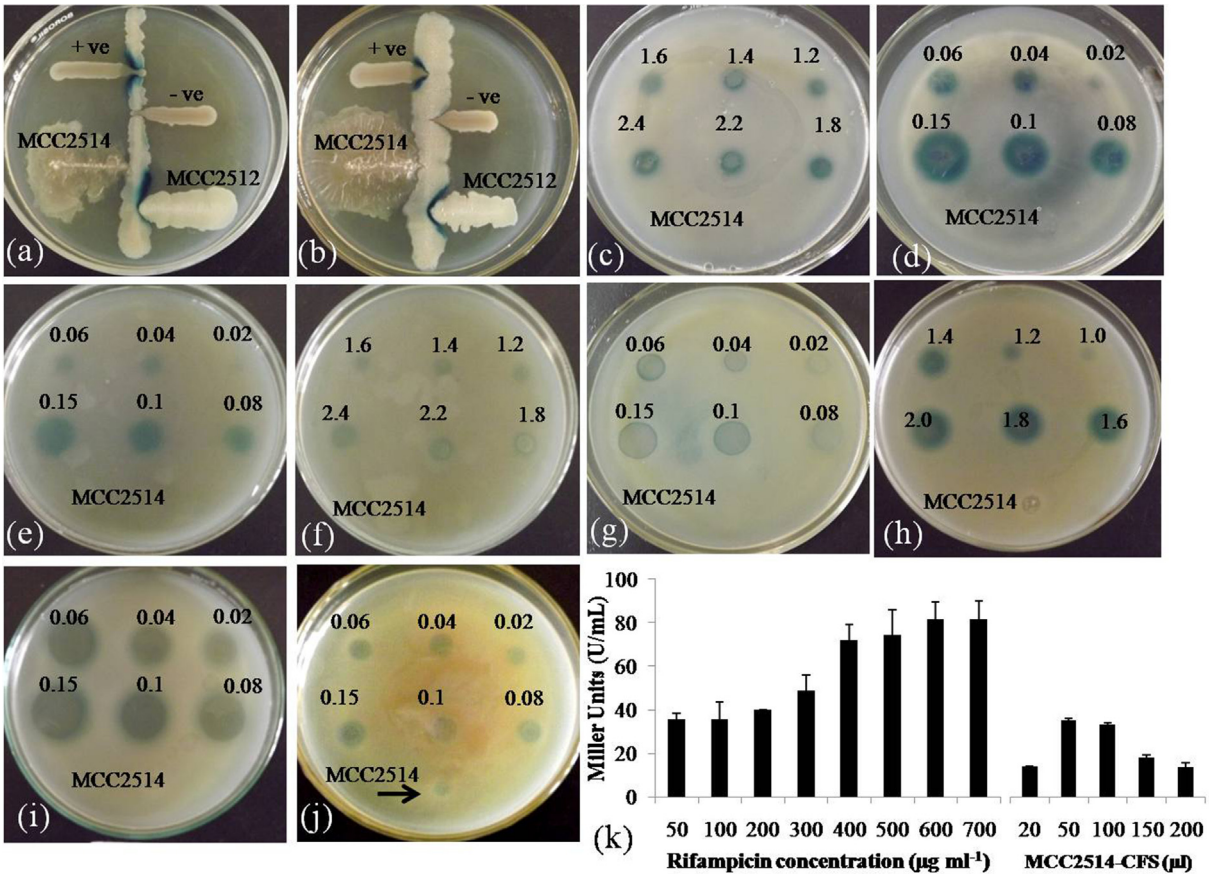

Fig. 4. Mode of action of *Bacillus* spp. by Chromogenic plate assay with cellular biosensor; (a) *B. subtilis* BSF2470; (b) *B. subtilis* B168.BS2 (+ve indicates *B. subtilis* ATCC6633; −ve indicate *B. flexus* MCC2011); (c) *yor*B; with nalidixic acid; (d) *yor*B; with trimethoprin; (e) *yhe*I; with neomycin; (f) *yhe*I; with streptomycin; (g) *ypu*A; with nisin; (h) *yup*A; with penicillin; (i) *fab*HB; with triclosan; (j) *yvg*S; with rifampicin; (k) β-galactosidase induction with rifampicin and CFS of *B. licheniformis* MCC2514. Values indicated on the plates are concentrations of respective antibiotics in micrograms. *B. licheniformis* MCC2514, CFS (5 µl) was spotted on each plate to determine the mode of action. Blue staining (indicated by arrow) in *yvg*S plate by MCC2514 indicates MOA on RNA synthesis.

Please cite this article in press as: Shobharani P, et al., Diversity in the antibacterial potential of probiotic cultures *Bacillus licheniformis* MCC2514 and *Bacillus licheniformis* MCC2512, Research in Microbiology (2015), http://dx.doi.org/10.1016/j.resmic.2015.06.003

reports also point to the broad spectrum inhibitory activity of *Bacillus* spp. Cladera-Olivera et al. [11] identified *B. licheniformis* strain P40 from the Amazon basin with antimicrobial activity against *L. monocytogenes*, *Bacillus cereus* and clinical isolate of *Streptococcus* sp. *B. subtilis* strain LFB112 isolated from Chinese herbs was found to be effective against both Gram-positive and Gram-negative bacteria, including *Escherichia coli*, *Salmonella pullorum*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Clostridium perfringens*, *Micrococcus luteus*, *Streptococcus bovis* and *S. aureus* [23]. A few of the *Bacillus* spp. were also found to be active against phytopathogenic bacteria, like *Xanthomonas oryzae* pv. *oryzae* [29]. He et al. [30] reported on *B. licheniformis* ZJU12 isolated from soil that exhibited a broad spectrum of antagonistic activity against various species of Gram-positive bacteria and fungal pathogens, but not against Gram-negative bacteria.

AMCs produced by the two *B. licheniformis* (MCC2514 and MCC2512) strains were found to be sensitive to proteinase K, but resistant to other protease enzymes tested. As suggested by Bizani and Brandelli [31] as well as von Döhren [32], the cyclic peptide nature of AMC containing unusual amino acids may be the reason for its resistance to proteolytic enzymes. Furthermore, resistance of AMCs to trypsin and pepsin, two vital digestive enzymes, agrees with the possibility that they may prove to be a promising candidate for food formulation destined for oral administration. The result also suggests the possible advantage over sensitive bacteriocin compounds secreted by other *Bacillus* spp. [8,33]. Cladera-Olivera et al. [11] determined a bacteriocin-like compound from *B. licheniformis* strain P40 that is resistant to pronase, but sensitive to pepsin and trypsin. In another study, bacteriocin of *B. subtilis* LFB112 was found resistant to papain, catalase, lysozyme and α-amylase, but sensitive to pepsin, lipase, trypsin, proteinase K and pronase E [23].

The fraction of AMCs resolved on SDS–PAGE and the activity assay indicated molecular weights of 6.4 and 3.5 kDa for MCC2514 and MCC2512, respectively. Several authors have reported a *B. licheniformis*-producing low molecular weight antimicrobial compound [2,9,10,30,33]. However, there exists no report on the 3.5 kDa protein of a subtilin-type antimicrobial compound from *B. licheniformis*. These AMCs were stable at wide ranges in temperature and pH. Similarly to the present study, bacteriocin-like compound from various *B. licheniformis* strains have been shown to be stable at different temperatures (30–100 °C) [23,30,34]. In general, pH sensitivity is considered to be the limiting factor in use of bacteriocins as food preservatives. For example, nisin produced by *L. lactis* is restricted because of its very low activity at neutral or alkaline pH. The present AMCs are resistant to a wide range of pH and are thus advantageous for application in production of diverse food products with varying pH.

Furthermore, the AMCs from both cultures showed considerable stability against various organic solvents tested. Cladera-Olivera et al. [11] showed that organic solvents tested had no significant effect on the antimicrobial activity of bacteriocins from *B. licheniformis*, but in the presence of butanol, the substance lost its inhibitory activity. In addition, AMCs were resistant to bile salts, which are the main inhibitory factors during intestinal passage. Hence, the data point to the stability of AMCs for oral administration.

AMCs from both *B. licheniformis* strains inhibited growth of *M. luteus* ATCC9341 in a dose-dependent manner. A significant variation ($p < 0.05$) was observed in the LD_{50} value for both strains. Overall, results suggest favorable properties of AMCs for application to food preservation and the need to extend the shelf-life of processed foods.

MOA studies indicated that *B. licheniformis* MCC2512 produced a subtilin-type antimicrobial compound that acts on cell wall synthesis. Similarly, large numbers of locally isolated *Bacillus* spp. from food sources have been screened for their specific mode of action using these reporter biosensor strains [12]. Since MCC2514 showed negative results with BS2470 (cell-wall biosensor reporter), it was analyzed for MOA using other pathway-specific biosensors. In preliminary experiments, cellular biosensors constructed with the *lacZ* marker gene were analyzed for their ability to interfere with specific biosynthetic pathways using different concentrations of antibiotics with their respective biosensors (Fig. 4). The results indicated that promoter-reporter fusions represented novel biomarkers specific to their respective biosynthetic pathways. Subsequently, the MOA of *B. licheniformis* MCC2514 was tested against these pathway-specific engineered strains. According to the results obtained, the culture induced β-galactosidase in *yvg*S, which is selectively induced in the presence of RNA synthesis inhibitors such as rifampicin (standard antibiotic tested). In the presence of other biosensors, β-galactosidase was not induced (Fig. 4), indicating specificity towards RNA synthesis.

In conclusion, this study reveals that the AMCs secreted by *B. licheniformis* (MCC2514 and MCC2512) isolated from two different sources (raw milk and rhizobial soil) varied in their activity and stability. The AMC of MCC2514 was active against *M. luteus*, *S. aureus*, *Klebsiella* sp. and *Aeromonas* sp., whereas MCC2512, in addition to these pathogens, showed inhibitory activity toward *L. monocytogenes* and *Salmonella typhimurium*. On comparison of stability towards organic solvents, the AMC from MCC2512 showed 70% retention of inhibitory activity in the presence of chloroform, whereas MCC2514 retained only 20% of its activity. In contrast, the AMC from MCC2514 was more stable than MCC2512 in the presence of ethanol. In the presence of bile salts, antimicrobial activity of MCC2514 was reduced to 60–65%, whereas MCC2512 retained 70–80% activity. The *M. luteus* growth inhibition study shows that the AMC from MCC2514 was more potent than that of MCC2512. With respect to their MOA, the AMC from MCC2512 affected cell-wall biosynthesis, whereas MCC2514 inhibited RNA synthesis.

Conflict of interest

No conflict of interest declared.

Acknowledgments

The authors wish to thank the Director, CSIR-CFTRI for all facilities. SRP thanks the SERB-Department of Science and

Technology, Government of India, New Delhi, for sponsoring the start-up project grant (Project No. SR/FT/LS-203/2009). PMH acknowledges CSIR-DAAD (A/09/08416, WAP-CSIR) for financial assistance for the work carried out in the laboratory of Dr. Thorsten Mascher at Karlsruhe University (KIT), Germany.

References

[1] Delves-Broughton J. Nisin and its uses as a food preservative. Food Technol 1990;44(11):100–11.
[2] Sharma S, Singh RL, Kakkar P. *Bacillus licheniformis* IITRHR2: a novel source of antimicrobial proteinaceous food substance. J Microbiol Antimicrob 2010;2(9):127–33.
[3] Riley MA, Wertz JE. Bacteriocins: evolution, ecology, and application. Annu Rev Microbiol 2002;56:117–37.
[4] Cherif A, Chehimi S, Limem F, Hansen BM, Hendriksen NB, Daffonchio D. Detection and characterization of the novel bacteriocin entomocin 9, and safety evaluation of its producer, *Bacillus thuringiensis* ssp. *entomocidus* HD9. J Appl Microb 2003;95:990–1000.
[5] Paik SH, Chakicherla A, Hansen JN. Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. J Biol Chem 1998;273:23134–42.
[6] Shelburne CE, An FY, Dholpe V, Ramamoorthy A, Lopatin DE, Lantz MS. The spectrum of antimicrobial activity of the bacteriocin subtilosin A. J Antimicrob 2007;59:297–300.
[7] Sutyak KE, Wirawan RE, Aroutcheva AA, Chiindas ML. Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product derived *Bacillus amyliquefaceiens*. J Appl Microbiol 2008;104:1067–74.
[8] Hyronimus B, Le Marrec C, Urdacim MC. Coagulin, a bacteriocin-like inhibitory substance produced by *Bacillus coagulans* I4. J Appl Microbiol 1998;85:42–50.
[9] Pattnaik P, Kaushik JK, Grover S, Batish VK. Purification and characterization of bacteriocin like compound (lichenin) produced anaerobically by *Bacillus licheniformis* isolated from water buffalo. J Appl Microbiol 2001;91:636–45.
[10] Martirani L, Varcamoni M, Naclerio Felice GMD. Purification and partial characterization of bacillocin 490, a novel bacteriocin produced by a thermophilic strain of *B. licheniformis*. Microb Cell Fact 2002;1:678–82.
[11] Cladera-Olivera F, Caron GR, Brandelli A. Bacteriocin-like substance production by *Bacillus licheniformis* strain P40. Lett Appl Microbiol 2004;38:251–6.
[12] Nithya V, Halami PM. Novel whole-cell reporter assay for stress-based classification of antibacterial compounds produced by locally isolated *Bacillus* spp. Ind J Microbiol 2012;52(2):180–4.
[13] Von Tersch MA, Carlton BC. Bacteriocin from *Bacillus megaterium* ATCC 19213: comparative studies with Megacin A-216. J Bacteriol 1998;155(2):866–71.
[14] Barboza-Corona JE, Vazquez-Acosta H, Bideshi DK, Salcedo-Hernandez R. Bacteriocin-like inhibitor substances produced by Mexican strains of *Bacillus thuringiensis*. Arch Microbiol 2007;187:117–26.
[15] Hutter B, Fischer C, Jacobi A, Schaab C, Loferer H. Panel of Bacillus subtilis reporter strains indicative of various modes of action. Antimicrob Agents Chemother 2004;48(7):2588–94.
[16] Urban A, Eckermann S, Fast B, Metzger S, Gehling A, Ziegelbauer K, et al. Novel whole cell antibiotic biosensors for compound discovery. Appl Environ Microbiol 2007;73(20):6436–43.
[17] Shobharani P, Halami PM. Cellular fatty acid profile and H^+-ATPAse activity to assess acid tolerance of *Bacillus* sp. for potential probiotic functional attributes. Appl Microbiol Biotechnol 2014;98:9045–58.
[18] Hardy G. The nutritional value of sheep milk: a natural supplement for clinical nutrition. In: Proceedings, international symposium, development strategy for the sheep and goat dairy sector, Nicosia, Cyprus. Brit. Sheep Dairy News, vol. 17; 2000. p. 23–4.
[19] Joy B, Rajan A, Abraham E. Antimicrobial activity and chemical composition of essential oil from *Hedychium coronarium*. Phytother Res 2007;21:439–43.
[20] Abdul AM, Rowshanul HM, Rezaul KM. Antibacterial and cytotoxic activities of *Hedychium coronarium*. J Koenig. Res J Agric Biol Sci 2009;5(6):969–72.
[21] Lu Y, Zhong CX, Wang L, Lu C, Li XL, Wang PJ. Anti-inflammation activity and chemical composition of flower essential oil from *Hedychium coronarium*. Afr J Biotechnol 2009;8(20):5373–7.
[22] Mascher T, Zimmer SL, Smith TA, Helmann JD. Antibiotic inducible promoter regulated by the cell envelope stress sensing two component system LiaRS of *Bacillus subtilis*. Antimicrob Agents Chemother 2004;48:2888–96.
[23] Xie J, Zhang R, Shang C, Guo Y. Isolation and characterization of a bacteriocin produced by an isolated *Bacillus subtilis* LFB112 that exhibits antimicrobial activity against domestic animal pathogens. Afr J Biotechnol 2009;8(20):5611–9.
[24] Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[25] Jordan S, Junker A, Helmann JD, Mascher T. Regulation of LiaRS-dependent gene expression in *Bacillus subtilis*: identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system. J Bacteriol 2006;188(14):5153–66.
[26] Burkard M, Stein T. Microtiter plate bioassay to monitor the interference of antibiotics with lipid II cycle essential for peptidoglycan synthesis. J Microbiol Methods 2008;75:70–4.
[27] Miller JH. Experiments in molecular genetics. New York: Cold Spring Harbor Laboratory, Cold Spring Harbor; 1972.
[28] Statsoft. Statistics for windows. Tulsa, USA: Statsoft Inc.; 1999.
[29] Beric T, Kojic M, Stankovic S, Topisirovic L, Myers M, Venturi V, et al. Antimicrobial activity of *Bacillus* sp. natural isolates and their potential use in the biocontrol of phytopathogenic bacteria. Food Technol Biotechnol 2012;50(1):25–31.
[30] He L, Chen W, Liu Y. Production and partial characterization of bacteriocin like peptides by *Bacillus licheniformis* ZJU12. Microbiol Res 2006;161:321–6.
[31] Bizani D, Brandelli A. Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A. J Appl Microbiol 2002;93:512–9.
[32] von Döhren H. Peptides. In: Vining LC, Stuttard C, editors. Genetics and biochemistry of antibiotic production. Newton, MA: Butterworth-Heinemann; 1995. p. 129–71.
[33] Paik HD, Bae SS, Park SH, Pan JG. Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thuringiensis* subsp. tochigiensis. J Ind Microbiol Biotechnol 1997;19:294–8.
[34] Kayalvizhi N, Gunasekaran P. Production and characterization of a low-molecular-weight bacteriocin from *Bacillus licheniformis* MKU3. Lett Appl Microbiol 2008;47:600–7.

Please cite this article in press as: Shobharani P, et al., Diversity in the antibacterial potential of probiotic cultures *Bacillus licheniformis* MCC2514 and *Bacillus licheniformis* MCC2512, Research in Microbiology (2015), http://dx.doi.org/10.1016/j.resmic.2015.06.003