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Antibacterial activity *Lactobacillus plantarum* isolated from fermented vegetables and investigation of the plantaricin genes

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***Escherichia coli* and *Staphylococcus aureus* are two food pathogens that cause severe food poisonings. Another problem found on a global level is the continuous increase of antimicrobial resistance in bacteria isolated from food. This study aimed to evaluate the antibacterial activity of *Lactobacillus plantarum* against pathogenic bacteria including *E. coli* and *S. aureus* and to study if *L. plantarum* with antibacterial activity contained the most plantaricin genes or not. A total of 50 lactic acid bacteria isolates (LAB) were evaluated for antibacterial activity and identified plantaricin genes by polymerase chain reaction (PCR) methods. Seven LAB isolates with antibacterial activity against *S. aureus* and *E. coli* were identified as *Lactobacillus* based on morphological physiological and biochemical properties. Using species-specific PCR and 16S rRNA gene sequencing, B0039 was identified as *Lactobacillus paracasei*, other isolates were identified as *L. plantarum*. 3 strains tested positive for all the genes in the *pln*ABCD operon. The *pln*EFI operon was detected in four strains. Genes encoding for the two-peptide *pln*J/K were detected only in 2 strains. Finally, the *pln*G/V was also found in 3 strains of *L. plantarm.* The plantarum gene sequences of B0055 were 97 to 100% similarity with the *L. plantarum* WCFS1. The findings suggest that LAB with bacteriocin genes can be used as an alternative mechanism to control drug resistant foodborne pathogens.**

Key worlds: *Lactobacillus*, antibacterial activity, plantaricin gene, *Staphylococcus aureus*, *Escherichia coli*.

INTRODUCTION

Escherichia coli and *Staphylococcus aureus* are two food pathogens that cause severe food poisonings (Bachir and

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Benali, 2012). Many food-poisoning outbreaks of *E. coli* have been associated with contaminated food, such as beef, pork, chicken and water (Wang, 2008). *S. aureus* may produce a number of toxins, the most important ones with respect to foodborne illness belong to the family of heat-stable staphylococcal enterotoxins (SEs). Another public health concern is associated with the increased incidence of antibiotic-resistant strains isolated from poultry meat (Dan et al., 2015). Due to the widespread use of antimicrobials in chicken and pig growth units, the development of resistant strains that can infect humans via the food chain has increased (Mihaiu et al., 2014). As a result, contamination of pathogenic microorganism is recognized as a potential public health concern. As more bacteria become resistant to traditional antibiotics, this leads to emergence and re-emergence of multidrug-resistant pathogens.

Lactic acid bacteria (LAB) have been used in the production of varieties of fermented dairy, vegetables and meat products for many centuries (Man et al., 2014). Recent research revealed that LAB can produce antibacterial substances including organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins (Herna´ndez et al., 2005) to inhibit the growth of a wide range of intestinal pathogens (García-Ruiz et al., 2013). Bacteriocins were defined as antimicrobial peptides or proteins have been observed in many genera of bacteria, including many strains of LAB, which are directed mainly to inhibit the growth of related species (Anyogu et al., 2014). *Lactobacillus rhamnosus* has been reported to interact with intestinal epithelium and prevent the internalization of enterohemorrhagic *E. coli* (Moorthy et al., 2007). *Lactobacillus sake* C2 which produced a bacteriocin strongly inhibited *S. aureus* ATCC 63589 and *E. coli* ATCC 25922 was isolated from traditional Chinese fermented cabbage (Gao et al., 2010). Bacteriocins bacST202Ch and bacST216Ch, produced by *Lactobacillus plantarum* strains isolated from Beloura and Chourico, inhibited the growth of a number of Gram-positive and Gram-negative meat spoilage bacteria (Todorov et al., 2010). *L. plantarum* B0105 isolated from traditional Taiwan fermented mustard, was found to produce bacteriocin inhibiting *Streptococcus mutans* BCRC 10793 (Chen et al., 2013). *L. plantarum* ST71KS was isolated from homemade goat feta cheese and displayed a bactericidal effect against *Listeria monocytogenes* strains 603 and 607 (Martinez et al., 2013).

LAB are very important in ensuring the safety of various foods by production of bacteriocins and other antimicrobial substances. Bacteriocins produced by *L. plantarum* are known as plantaricins (Omar et al., 2008). The genetic determinants for plantaricins were reported as shown for strains *L. plantarum* strain C11 (Diep et al., 2003), WCFS1 (Kleerebezem et al., 2003), NC8 (Maldonado et al., 2003) and J23 (Rojo-Bezares et al., 2008). The *L. plantarum* C11 plantaricin cluster contains five operons (Diep et al., 2003). Two of them (*pln*EFI and *pln*JKLR) code for bacteriocins and immunity proteins, the transport operon (*pln*GHSTUV), which is involved in the secretion of the pheromone and bacteriocins; *pln*ABCD for the signal-transducing pathway, and *pln*MNOP containing genes with unknown functions in bacteriocin synthesis (Diep et al., 2003). In this study, LAB isolated from traditional fermented products, were screened for the antibacterial activity and presence of plantaricin genes in strains were also investigated.

MATERIALS AND METHODS

Bacterial strains

Lactic acid bacteria were isolated from traditional fermented mustard and vegetable samples collected from southern areas of Taiwan. The LAB isolates were characterized based on acid production, Gram stain and catalase test were tested. *Lactococcus lactis* subsp. *lactis* (ATCC 11454) and *L. plantarum* (ATCC 14917) with plantaricin genes as control strain, *S. aureus* (BCRC 12653, BCRC 12654, BCRC12658, BCRC13824 and BCRC 13829) and *E. coli* (BCRC 14825, BCRC 15375 and BCRC 41443), used as test microorganisms in determining antibacterial activity were obtained from Bioresource Collection and Research Center (BCRC), Hsin Chu, Taiwan.

Screening antibacterial activity of LAB

For screening the antibacterial activity of LAB isolates, 1% (v/v) of these cultures were inoculated into 50 ml de Man, Rogosa and Sharpe (MRS) broth individually and incubated at 35°C for 24 h without agitation. Bacterial cells were removed by centrifugation (17,000 g, 10 min, 4°C) and the resulting solution were subjected to filtration with 0.22 µm filter, then, the diameters of inhibition zones were measured using the agar diffusion assay method (Anyogu et al., 2014). Overnight test cultures of *E. coli* and *S. aureus* were diluted in saline solution into 10^8 CFU/ml and 100 µl of dilution were inoculated in nutrient agar medium. Briefly, 100 µl of spent cell-free supernatant (SCS) were placed into wells (10.0 mm in diameter) on nutrient agar plates seeded with the test pathogens. After incubation at 35°C for 14 h, the diameter of inhibition zones was determined. The pH of MRS broth was also adjusted to the same value as blank. The antimicrobial activity was lost after treatment with pepsin, indicating a peptide nature (Rojo-Bezares et al., 2007). The bacterial cell-free supernatants were incubated 37°C overnight with or without pepsin (Sigma, St. Louis, Missouri) at a final concentration of 2 mg/ml. *L. lactis* subsp. *lactis* (nisin producing strain, BCRC 11454) was used as the negative control and *L. plantarum* with plantaricin genes (ATCC 14917) as the positive control. As a blank control, aliquots of MRS broth treated as filtered supernatants were used. All of the tests were repeated 3 times. Diameters of inhibition zones were determined. Reduced inhibition zone (mm) = inhibition zone of SCS - inhibition zone of SCS treated with pepsin.

Strain identification

A polymerase chain reaction (PCR) assay was performed using genomic DNA from strains that showed antibacterial activity. Amplification of 16S rDNA sequences by PCR was performed using the primers 27F- AGAGTTTGATCMTGGCTCAG and 1492R-GGYTACCTTGTTACGACTT described by Tanner et al. (2000). For the PCR identification, genomic DNA was extracted using the

Table 1. Plantaricin gene primers and conditions used in this study.

Target	PCR Primers (5'→3')	Annealing temperature (°C)	Amplicon size (bp)
plnA	F: GTA CAG TAC TAA TGG GAG R: CTT ACG CCA ATC TAT ACG	53	450
plnB	F: TTC AGA GCA AGC CTA AAT GAC R: GCC ACT GTA ACA CCA TGA C	51.5	165
plnC	F: AGC AGA TGA AAT TCG GCA G R: ATA ATC CAA CGG TGC AAT CC	49.5	108
plnD	F: TGA GGA CAA ACA GAC TGG AC R: GCA TCG GAA AAA TTG CGG ATA C	53	414
plnEF	F: GGC ATA GTT AAA ATT CCC CCC R: CAG GTT GCC GCA AAA AAA G	53.2	428
plnI	F: CTC GAC GGT GAA ATT AGG TGT AAG R: CGT TTA TCC TAT CCT CTA AGC ATT GG	52.5	450
plnJ	F: TAA CGA CGG ATT GCT CTG R: AAT CAA GGA ATT ATC ACA TTA GTC	51	475
plnK	F: CTG TAA GCA TTG CTA ACC AAT C R: ACT GCT GAC GCT GAA AAG	52.9	246
plnG	F: TGC GGT TAT CAG TAT GTC AAAG R: CCT CGA AAC AAT TTC CCC C	52.8	453
plnN	F: ATT GCC GGG TTA GGT ATC G R: CCT AAA CCA TGC CAT GCA C	51.9	146
plnV	F: CAG TTT ATT GGC AGC AAT CG R: ATC CAC TCC ATC CAA ACA ATC	54	727
plnNC8	F: GGT CTG CGT ATA AGC ATC GC R: AAA TTG AAC ATA TGG GTG CTT TAA ATT CC	60	207

All the plantaricin primers and annealing temperature are followed from Sáenz et al. (2009).

Genomic isolation kit (GeneMark, Georgin, USA) according to the manufacturer's instructions. Genomic DNA concentration was determined spectrophotometrically (Hitachi, U-2800A, Tokyo, Japan). PCR primers were used to amplify a 1484 bp DNA fragment. The reaction mixture contained 10 µl genomic DNA, 2.5 units of *Taq* polymerase (Promega, Madision, WI), 2 µl each of 10 mM dATP, dTTP, dCTP and dGTP, 5 µl of 10 X reaction buffer (10 mM Tris-HCl (pH 8.3 at 25°C) containing 50 mM KCl, 0.01% Triton X-100, 0.01% gelatin, 6.0 mM $MgCl_2$), and 50 pmol of each primers in a final volume of 50 µl. The DNA was denatured at 94°C for 2 min and amplified for 35 cycles at 94°C for 40 s, 45°C for 50 s and 72°C for 50 s. A final extension incubation of 2 min at 72°C was included. Amplification reactions were performed on a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400, Foster city, CA). The PCR products were purified with Gel/PCR DNA fragments extraction kit (Geneaid, Taipei, Taiwan) and sequenced by automated sequencing core laboratory, National Cheng Kung University (Tainan, Taiwan). Sequence homologies were examined by comparing the obtained sequence with those in the DNA data bases (http//www.ncbi.nim.nih.gov/BLAST) (Todorov et al., 2010).

PCR amplification of plantaricin genes

A PCR assay was performed using total genomic DNA from strains that showed antibacterial activity against test pathogens. PCR amplification of plantaricin genes were carried out, the primers and conditions were specified in Table 1 (Sáenz et al., 2009), with initial

Table 2. Antibacterial activities of lactic acid bacteria spent cell supernatants (SCS) against test pathogens.

Strains LAB	Inhibition zones (mm)							
	E. coli			*S. aureus*				
	BCRC14825	BCRC15375	BCRC41443	BCRC12653	BCRC12654	BCRC12658	BCRC13824	BCRC13829
L. plantarum	21.0 ± 0.00	19.3 ± 0.05	26.6 ± 0.5	18.0 ± 0.0	19.0 ± 0.0	19.3 ± 0.5	12.3 ± 0.5	20.0 ± 0.0
L. lactis	16.3 ± 0.2	18.5 ± 0.0	23.8 ± 0.2	-	-	-	-	-
B0013	22.0 ± 1.0	23.8 ± 0.2	29.0 ± 0.5	18.2 ± 0.02	18.0 ± 0.5	15.2 ± 0.2	14.1 ± 0.7	15.5 ± 0.0
B0039	21.5 ± 0.1	22.7 ± 0.2	29.3 ± 0.2	18.5 ± 0.08	17.8 ± 0.2	16.3 ± 0.2	12.8 ± 0.2	16.0 ± 0.5
B0055	22.2 ± 0.6	24.0 ± 1.0	29.0 ± 0.6	14.0 ± 0.05	14.9 ± 0.1	1.68 ± 0.2	15.5 ± 0.5	15.7 ± 0.2
B0105	20.8 ± 0.2	23.1 ± 0.2	29.3 ± 0.2	14.2 ± 0.02	15.1 ± 0.2	14.2 ± 0.2	13.6 ± 0.7	16.5 ± 0.5
B0125	24.0 ± 0.0	25.6 ± 0.5	29.0 ± 0.0	22.0 ± 0.00	26.6 ± 0.5	22.0 ± 0.0	20.0 ± 0.0	24.0 ± 1.0
B0126	20.9 ± 0.3	22.8 ± 0.7	28.0 ± 0.5	15.2 ± 0.05	15.3 ± 1.0	15.5 ± 1.0	12.9 ± 0.1	18.0 ± 0.4
B0134	20.5 ± 0.5	23.5 ± 0.5	28.3 ± 0.5	14.5 ± 0.03	17.4 ± 0.7	14.2 ± 0.2	12.4 ± 0.3	17.5 ± 0.0

*All of the tests are repeated at least 3 times.

denaturation at 94°C for 3 min and 30 cycles of 94°C for 1 min, annealing at an appropriate temperature (Table 1) for 1 min, 72°C for 30 s, and a final extension at 72°C for 5 min. Amplification reactions were performed on a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400, Foster city, CA). The amplification products were loaded onto a 1.8% agarose gel. After electrophoresis in 1X TBE (Tris-Borate-EDTA) buffer, the gel was stained with ethidium bromide before being photographed by ultraviolet illumination. Sequence homologies were examined by comparing the obtained sequence with those in the DNA data bases (http//www.ncbi.nim.nih.gov/BLAST). The PCR products of plantaricin genes of B0055 were purified with Gel/PCR DNA fragments extraction kit (Geneaid, Taipei, Taiwan) and sequenced by automated sequencing core laboratory, National Cheng Kung University (Tainan, Taiwan).

RESULTS

Screening antibacterial activity of LAB

A total of 50 presumptive lactic acid bacteria isolates obtained from traditional fermented mustard and vegetables were determined with phenotypical and physiological tests. Out of the 50 LAB isolates, only 7 isolates showed antagonistic effect against the test pathogens (*S. aureus* and *E. coli*) (Table 2).

The diameters of inhibition zones against *E. coli* and *S. aureus* ranged between 12 and 29 mm. In some cases, antimicrobial activity reduced after addition of pepsin on the SCS of LAB, the reduction of diameters of inhibition zones against *E. coli* and *S. aureus* ranged between 2 and 7 mm (Table 3).

Strains identification

Amplification of 16S rDNA sequence by PCR was performed using the primers described by Tanner et al. (2000). The identification of LAB isolates with antibacterial activity revealed that strain B0039 was identified as *Lactobacillus paracasei* and other strains were identified as *L. plantarum*. The 16S rDNA nucleotide sequences of the test strains were 99 to 100% similarity with the GenBank access number assigned in Table 4.

Amplification of plantaricin genes

In an attempt to determine whether the strains that showed antibacterial activity against test pathogens carried genes for the production of known plantaricins. A PCR assay was performed using total genomic DNA from strains. Several strains carried at least one or more genes of the plantaricin cluster. *L. plantarum* strains B0013, B0055, B0126 tested positive for the *pln*ABCD operon. The complete *pln*EFI operon was detected in *L. plantarum* B0013, B0055 and B0126, while B0105 was detected only *pln*EF. Genes encoding for the two-peptide *pln*J/K were detected only in *L. plantarum* B0055 and B0126. Finally, the *pln*G/V, which is part of a large operon involved in plantaricin export, was also found in *L. plantarum* B0013, B0055, and B0126. Three strains gave all negative plantaricin genes in PCR analyses (Table 5). Among these positive results, B0055 was

Table 3. Effect of pepsin treatment on the antibacterial activity of LAB SCS against indicators.

Strains	Indicator strains							
	E. coli				*S. aureus*			
	BCRC14825	**BCRC15375**	**BCRC41443**	**BCRC12653**	**BCRC12654**	**BCRC12658**	**BCRC13824**	**BCRC13829**
L. plantarum	-	+	++	-	++	++	-+	++
B0013	++	++	++	+++	+++	++	++	++
B0039	++	++	++	++	++	++	+	++
B0055	++	+++	+++	+	++	++	++	++
B0105	++	+++	+++	-	+	+++	++	+++
B0125	++	++	++	+	++	++	+	++
B0126	++	++	++	+	++	+	+	+++
B0134	++	++	++	++	+++	+	++	+++

*All of the tests are repeated at least 3 times. **Reduced inhibition zone (mm) = inhibition zone of SCS - inhibition zone of SCS treated with pepsin. ***The inhibition zones ≤1 mm, 2–3 mm, 4-5 mm and >6 mm, were classified as strains of no -; little +; mild ++; and strong +++ reduction of inhibition zone, respectively.

Table 4. Identification of LAB strains based on 16S rDNA sequence similarity.

Strains of LAB	Strains	GeneBank acc. no.	Similarity (%)
B0013	*Lactobacillus plantarum*	HM058986	100
B0039	*Lactobacillus paracasei*	HM067019	99
B0055	*Lactobacillus plantarum*	HM058986	100
B0125	*Lactobacillus plantarum*	HM058694	99
B0126	*Lactobacillus plantarum*	HM058986	100
B0134	*Lactobacillus plantarum*	HM058986	100

confirmed by sequencing. The plantarum gene sequences of B0055 were 97 to 100% similarity with the GenBank access number assigned in Table 6.

DISCUSSION

Bacteriocins of LAB are active against Gram-positive bacteria, such as LAB (García-Ruiz et al., 2013) and *S. aureus* (Anyogu et al., 2014; Omar et al., 2008; Sebastià et al., 2011). However, plantaricins produced by *L. plantarum* strains had broad spectra of inhibition activity against Gram-negative bacteria including *E. coli* and *Salmonella* enterica (Anyogu et al., 2014; Omar et al., 2008). Strain *L. sakei* C2 producing a bacteriocin strongly inhibited *S. aureus* and *E. coli* (Gao et al., 2010). In our study, seven strains showed antibacterial activity against test pathogens. Rojo-Bezares et al. (2007) indicated that antimicrobial activity was lost after treatment with trypsin, α-chymotrypsin, papaine, protease, proteinase K, and acid proteases, indicating bacteriocin was peptide nature. Gao et al. (2010) indicated that after treatment by all the three kinds of protease, the antimicrobial activity of cell-free supernatant of strain *L. sake* C2 disappeared and the protein nature of this antimicrobial substance produced by strains C2 was verified. In this study, after treatment by pepsin, the inhibition zones of seven strains selected towards the tested pathogens reduced. It indicated that the substance

Table 5. Plantaricin genes detected by PCR from lactic acid bacteria with antibacterial activity.

Strains	Plantaricin gene											
	***pln*A (450 bp)**	***pln*B (165 bp)**	***pln*C (108 bp)**	***pln*D (414 bp)**	***pln*EF (428 bp)**	***pln*I (450 bp)**	***pln*J (475 bp)**	***pln*K (246 bp)**	***pln*G (453 bp)**	***pln*N (146 bp)**	***pln*V (727 bp)**	***pln*NC8 (207 bp)**
L. plantarum	+	+	+	+	+	+	+	+	+	-	+	-
L. lactis	-	-	-	-	-	-	-	-	-	-	-	-
B0013	+	+	+	+	+	+	-	-	+	-	+	-
B0039	-	-	-	-	-	-	-	-	-	-	-	-
B0055	+	+	+	+	+	+	+	+	+	-	+	-
B0105	-	-	-	-	+	-	-	-	-	-	-	-
B0125	-	-	-	-	-	-	-	-	-	-	-	-
B0126	+	+	+	+	+	+	+	+	+	-	+	-
B0134	-	-	-	-	-	-	-	-	-	-	-	-

Table 6. Identification of B0055 plantaricin gene sequences similarity.

Plantaricin gene	Gene ID	Similarity (%)
*pln*A	1064174	98
*pln*B	1061276	100
*pln*C	1061285	97
*pln*D	1064173	100
*pln*E	1064171	99
*pln*F	1061287	99
*pln*I	1061281	99
*pln*J	1064185	98
*pln*K	1064190	98
*pln*G	1061291	99
*pln*V	1061306	99

with antibacterial activity was sensitive to pepsin, where it might be considered that the bioactive compound produced by some strains might be the protein or peptide nature (Rojo-Bezares et al., 2007; Gao et al., 2010). The variation of the antibacterial activity of our test strains are almost similar to those shown by other works on the antibacterial activity of LAB (García-Ruiz et al., 2013; Anyogu et al., 2014; Gao et al., 2010).Previous studies on the microorganisms of traditional fermented vegetables and fruits had shown that *L. plantarum* was dominant among the isolates from fermented mustards (Chen et al., 2013). All test strains were identified as *L. plantarum* except B0039 as *L. paracasei. L. plantarum* is important in many food fermentations as a component of the natural microflora or as a starter culture (Gao et al., 2010; Omar et al., 2008).

Bacteriocin-producing strains of *L. plantarum* have been reported from many vegetable foods such as fermented cereal doughs, wara (Omar et al., 2008), fermented cassava (Kostinek et al., 2005) and fermented mustard (Chen et al., 2013). Bacteriocins of lactic acid bacteria are active against closely related bacteria. However, activity against Gram-negative bacteria has been described in several cases. Omar et al. (2008)showed that bacteriocins *pln*ABCD encode for the signal-transducing pathway. Variations in this operon have also been reported for the gene clusters described in *L. plantarum* from poto poto with only *pln*C being not conserved (Omar et al., 2008) and *L. plantarum* J23 from grape must, with only *pln*D being conserved (Rojo-Bezares et al., 2008). In this study, *L. plantarum* strains, B0013, B0055 and B0126 tested positive for the *pln*ABCD operon, others are not detected. In other words, variations in *pln*ABCD also exist in our strains. Two of *pln*EFI and *pln*J/K code for bacteriocins to inhibit pathogens. Genes encoding for the *pln*EFI and *pln*J/K were detected in *L. plantarum* B0055 and B0126, while *L. plantantum* B0013 only test positive for *pln*EFI. Omar et al. (2008) indicated that the *pln*EFI operon was detected in thirteen isolates from poto poto, while others only tested positive for *pln*EF or *pln*I. From our results, B0105 detected only positive of *pln*EF. Variations in *pln*J/K operon, including the absence of *pln*K and the presence of *pln*J gene have also been reported in *L. plantarum* J23 (Rojo-Bezares et al., 2008). *Lactobacillus* strains from poto poto also reported the absence of *pln*NC8 plantaricins, while *pln*N gene was absent in some strains (Omar et al., 2008). However, the genes encoding for *pln*N and *pln*NC8 were not detected in any strain. Finally, *pln*G and *pln*V gene, which is part of a large operon involved in plantaricin transport operon (Sáenz et al., 2009) were found in *L. plantarum* strains B0013, B0055, B0126 strains. With antibacterial activity, but showed negative in plantaricin gene, these findings might explain that *L. plantarum* may contain other bacteriocin genes that were not detected in this study. However, *L. plantarum* B0125 and B0134 strains which gave negative results for the PCR amplification should be investigated for possible sequence heterogeneity in the plantaricin operons. Plantaricin gene sequences of B0055 were 97 and 100% similarity with the GenBank access number. The bactericidal effect might be from the production of organic acids and/or in combination with the production of bacteriocin (Lin et al., 2008). In our results, antibacterial activity of selected strains might be from carried genes for the production of plantaricins and production of organic acids.

Conclusion

LAB isolated from fermented vegetalbes seem to have high potentials for production of antimicrobial substances, and also seem to have variations in the plantaricin genes. In this study, fermented vegetables were found to contain LAB and other pathogens. Since *L. plantarum* strains produce bacteriocins that inhibit food-borne pathogens such as *E. coli* and *S. aureus*, the use of these antimicrobial substances as food additives or use of the bacteriocin-producing strains as starters, might contribute to the production of a safer and healthier traditional fermented product (Omar et al., 2008). The findings in this study suggest that LAB producing bacteriocins can be used as alternate mechanism to inhibit the growth of drug-resistant pathogens.

Conflict of Interests

The authors have not declared any conflict of interests.

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