ISSN 1330-9862
(FTB-2544)

original scientific paper

Novel Bacteriocinogenic *Lactobacillus plantarum* Strains and Their Differentiation by Sequence Analysis of 16S rDNA, 16S-23S and 23S-5S Intergenic Spacer Regions and Randomly Amplified Polymorphic DNA Analysis

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Received: March 16, 2010
Accepted: May 21, 2010

Summary

Six strains of bacteriocinogenic *Lactobacillus plantarum* (TL1, RG11, RS5, UL4, RG14 and RI11) isolated from Malaysian foods were investigated for their structural bacteriocin genes. A new combination of plantaricin EF and plantaricin W bacteriocin structural genes was successfully amplified from all studied strains, suggesting that they were novel bacteriocin-producing *L. plantarum* strains. A four-base pair variable region was detected in the short 16S-23S intergenic spacer regions of the studied strains by a comparative analysis with 17 *L. plantarum* strains deposited in the GenBank, implying they were new genotypes. The studied *L. plantarum* strains were subsequently differentiated into four groups on the basis of the detected four-base pair variable region of the short 16S-23S intergenic spacer region. Further analysis of the DNA sequence of 23S-5S intergenic spacer region revealed only one type of 23S-5S intergenic spacer region present in the studied strains, indicating it was highly conserved among the studied *L. plantarum* strains. Three randomly amplified polymorphic DNA experiments using three different combinations of arbitrary primers successfully differentiated the studied *L. plantarum* strains from each other, confirming they were different strains. In conclusion, the studied *L. plantarum* strains were shown to be novel bacteriocin producers and high level of strain discrimination could be achieved with a combination of randomly amplified polymorphic DNA analysis and the analysis of the variable region of short 16S-23S intergenic spacer region present in *L. plantarum* strains.

Key words: bacteriocin gene, differentiation, *Lactobacillus plantarum*, ribosomal intergenic spacer region

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Special issue: Probiotics, Prebiotics and Synbiotics

Introduction

Lactobacillus plantarum is a member of industrially important lactic acid bacteria (LAB), which are generally granted the status of Generally Recognized as Safe (GRAS). They are usually used as starter cultures for the production of fermented foods due to their ability to acidify the food environment and to produce bacteriocin to enhance the food safety as well as to standardise the properties of food products (*1*). They are ubiquitous and frequently present in the environments rich in carbohydrates such as food (dairy products, fermented meat, sourdough, vegetables, fruits, beverages), respiratory, gastrointestinal and genital tracts of humans and animals, and in sewage and plant materials (*2*).

LAB, particularly *L. plantarum* species, are able to produce multiple bacteriocins and the combination varies from strain to strain (*3–5*). Generally, beneficial and health promoting characteristics of LAB are strain dependent rather than genus or species dependent (*6*). Therefore, there has been a growing interest among researchers to find new strains with novel antibacterial characteristics to meet specific industrial demands.

For phylogenetic analysis, 16S rDNA sequence is widely used for initial identification of bacterial species including LAB (*7*). However, due to some pitfalls of this technique (*8*), *L. plantarum* has been reported to be misclassified as *L. paraplantarum*, *L. pentosus*, *L. casei*, *L. rhamnosus* or *L. zeae* by 16S rDNA sequence analysis (*9*). Hence, other molecular techniques such as rep-PCR (*10*), amplified ribosomal DNA restriction analysis (ARDRA) (*11*) and species-specific primers based on other parts of ribosomal DNA (*rrn*) operon (*2,9,12*) and *recA* (*13*) have been developed to differentiate bacterial species. However, all of the above-mentioned methods have been shown to be discriminative at the species level and cannot be used to differentiate bacterial strains, particularly the strains that are closely related and isolated from the same ecological niche. In spite of numerous reports on using the 16S-23S intergenic spacer regions (*ITS1*) and 23S-5S intergenic spacer region (*ITS2*) from lactobacilli (*14–16*), only *ITS1* from *L. plantarum* strains has been widely used for identification purpose.

The importance of *ITS1* becomes greater since this spacer region varies in length and DNA sequence polymorphism (*14,15*). As for *ITS2*, it is smaller and could be easily sequenced (*15*). Therefore, the aims of this work are to identify the structural bacteriocin genes responsible for antibacterial activity of six *L. plantarum* strains isolated from Malaysian foods and to differentiate the bacteriocinogenic *L. plantarum* strains by using various molecular tools such as randomly amplified polymorphic DNA (RAPD) analysis, DNA sequence analyses of 16S rDNA and ribosomal intergenic spacer regions.

Materials and Methods

Bacterial strains and growth conditions

Six *L. plantarum* strains (TL1, RG11, RS5, UL4, RG14 and RI11) employed in this study had previously been isolated from Malaysian foods (*17,18*). They were deposited at the BIOTEC Culture Collection (BCC) of BIOTEC Central Research Unit of Thailand (BCC No. 36838–36843). The inhibitory activity and antimicrobial profile of bacteriocin-containing metabolites produced by the studied *L. plantarum* strains were investigated by Thanh *et al.* (*19*), whereas the probiotic characteristics were demonstrated by Loh *et al.* (*20*) and Thanh *et al.* (*21*) on poultry and Loh *et al.* (*22,23*) on rats. Furthermore, Foo *et al.* (*24*) and Lim *et al.* (*18*) reported the probiotic effects of the studied *L. plantarum* strains on rats. The studied strains were grown on de Man-Rogosa-Sharpe (MRS) medium (Merck, Darmstadt, Germany) at 30 °C. Stock cultures were prepared in 15 % (by volume) glycerol and kept at –80 °C for long-term storage.

Identification of L. plantarum strains

The analysis of carbohydrate fermentation pattern for each strain was conducted by using API (bioMérieux, Craponne, France) identification system according to the manufacturer's instructions. The carbohydrate fermentation patterns were analysed by using API 50 CHL v. 5.0 software.

Preparation of total DNA

Genomic DNA was extracted from *L. plantarum* strains according to the method of Leenhouts *et al.* (*25*) with minor modifications. Briefly, bacterial cells were harvested from 1 mL of culture medium. Cell pellet was then resuspended in GTE buffer (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH=8.0) containing 10 mg/mL of lysozyme. The mixture of cell suspension was incubated for 45 min at 37 °C. Lysis solution (20 mg/mL of proteinase K, 20 % (*m/V*) SDS) was then added to the mixture and incubated for 1 h at 60 °C. The proteins were then precipitated by phenol/chloroform/isoamyl alcohol solution. The nucleic acid was precipitated by adding 0.1 mL of 3 M sodium acetate and an equal volume of isopropanol. The DNA pellet was washed with 1 mL of 70 % (by volume) ethanol and air dried. The DNA was then dissolved in 1X TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH=8.0) containing 10 mg/mL of RNase and incubated at 37 °C for 30 min to remove RNA. The purity and concentration of the extracted DNA were then determined spectrophotometry at 260 and 280 nm, respectively. The extracted DNA was stored at –20 °C.

Oligonucleotide primers and PCR

Table 1 (*4,11,14,26–32*) shows the sequence of the primers used in this study, their target genes and annealing temperatures. The PCR reaction mixture was prepared in 25 μL of final volume containing 0.5 μM of each primer (1st BASE Laboratories, Selangor Darul Ehsan, Malaysia), 0.2 mM dNTP, 2 mM $MgCl_2$, 1 U of Taq DNA polymerase, 1X Taq buffer (Fermentas, St. Leon-Rot, Germany) and 0.1–0.5 μg of DNA template. PCR amplification was performed by using a thermal cycler (Mastercycler**,** Eppendorf, Hamburg, Germany) and the thermal profile used was as follows: an initial denaturation step of 94 °C for 3 min, 29 denaturation cycles at 94 °C for 1 min, annealing temperature (as shown in Table 1) for 1 min, 72 °C for 2 min, then followed by a final extension at 72 °C for 7 min. The PCR products were analysed by 1 % (*m/V*) agarose gel electrophoresis and visualised under UV after being stained with ethidium bromide (0.5 μg/mL). GeneRuler™ DNA ladder mix (Fermentas) and 100 bp DNA ladder (Promega, Madison, WI, USA) were used as references for molecular size determination.

Table 1. Primers used for PCR amplification of bacteriocin structural genes, 16S rDNA, 16S-23S and 23S-5S intergenic spacer regions, and randomly amplified polymorphic DNA analyses

Target	PCR primer	Annealing temp./°C	Reference
plnEF	F: 5′ GGC ATA GTT AAA ATT CCC CCC 3′ R: 5′ CAG GTT GCC GCA AAA AAA G 3′	53	*4,26,27*
plnJ	F: 5′ TAA CGA CGG ATT GCT CTG 3′ R: 5′ AAT CAA GGA ATT ATC ACA TTA GTC 3′	51	*4,26,27*
plnK	F: 5′ CTG TAA GCA TTG CTA ACC AAT C 3′ R: 5′ ACT GCT GAC GCT GAA AAG 3′	53	*4,26,27*
plnN	F: 5′ ATT GCC GGG TTA GGT ATC G 3′ R: 5′ CCT AAA CCA TGC CAT GCA C 3′	51.9	*4,26*
plnNC8	F: 5′ GGT CTG CGT ATA AGC ATC GC 3′ R: 5′ AAA TTG AAC ATA TGG GTG CTT TAA ATT CC3′	60	*4,28*
plnS	F: 5′ GCC TTA CCA GCG TAA TGC CC 3′ R: 5′ CTG GTG ATG CAA TCG TTA GTT T 3′	60	*4,29*
plnW	F: 5′ TCA CAC GAA ATA TTC CA 3′ R: 5′ GGC AAG CGT AAG AAA TAA ATG AG 3′	50	*4,30*
16S-23S	F: 5′ GTG GGA ATC GCT AGT AAT CG 3′ R: 5′ GGG TTC CCC CAT TCG GA 3′	55	*31*
23S-5S	F: 5′ CGG TCG AGG ACT TAA CCA AG 3′ R: 5′ GGG AAC AGG TGT ATC CTT C 3′	55	*14*
R1	5′ AGT CAG CCA C 3′	32	*11*
R2	5′ CCG CAG CCA A 3′	32	*11*
R3	5′ ACG AGG CAC 3′	32	*31*
R4	5′ ACG CGC CCT 3′	32	*31*
R5	5′ CCG CCC AAA C 3′	32	*32*
R6	5′ GGC ACG CGT T 3′	32	*32*

Screening for bacteriocin structural genes

The screening for bacteriocin genes present in the studied strains was performed by PCR using total DNA as template. Seven plantaricin (bacteriocins produced by *L. plantarum* species) genes were screened by using the primers listed in Table 1.

Sequence analysis of PCR-amplified ribosomal DNA fragments and bacteriocin genes

After analysing the PCR products with agarose gel electrophoresis, the desired DNA fragments were excised and purified by using gel extraction kit (QIAGEN, Hilden, Germany). The purified fragments were directly sequenced using the same primer sets of PCR (1st BASE Laboratories). The DNA sequence results were processed using BioEdit software (*33*). The homology search of the DNA sequence was performed using BLASTN (*34*), available at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

Randomly amplified polymorphic DNA (RAPD) analysis

Six oligonucleotides of 9- to 10-mer (1st BASE Laboratories), as described in Table 1, were employed either individually or in combinations. Suitable combinations were selected based on their ability to generate discriminative and reproducible DNA banding patterns. The RAPD analyses were repeated three times. The PCR reaction mixtures were prepared as described above. PCR amplification was performed by using a thermal cycler (Eppendorf Mastercycler) with the following thermal profile: denaturation step at 94 °C for 5 min, 34 cycles of 1 min at 95 °C, 1 min at 32 °C, and 2 min at 72 °C; and final elongation step at 72 °C for 5 min. The PCR products were analysed on 1.2 % (*m/V*) agarose gel at a constant voltage of 50 V for 4 h.

Results and Discussion

Identification of L. plantarum strains

The studied isolates were identified as *L. plantarum* I with 99.9 % similarity on the basis of carbohydrate fermentation pattern analysis using API 50 CHL identification system. For further confirmation, the 16S rDNA fragment (1.5 kb) of each strain was amplified (data not shown) and partially sequenced. The BLAST search of partial sequences (563 bp) of 16S rDNA for UL4 and RI11 strains showed 99 % homology to *L. plantarum*, while the percentage of homology was 100 % for strains RG11, RS5, TL1 and RG11. The alignment of the 16S rDNA of the studied *L. plantarum* strains with the 16S rDNA *L. plantarum* sequences deposited in the GenBank (accession numbers: AB11208, D9210, EF536363 and NC004567) revealed three mutation points of insertion, deletion and substitution (Fig. 1). However, the point mutations probably occurred randomly and were not specific to any position. For instance, a point deletion was detected in

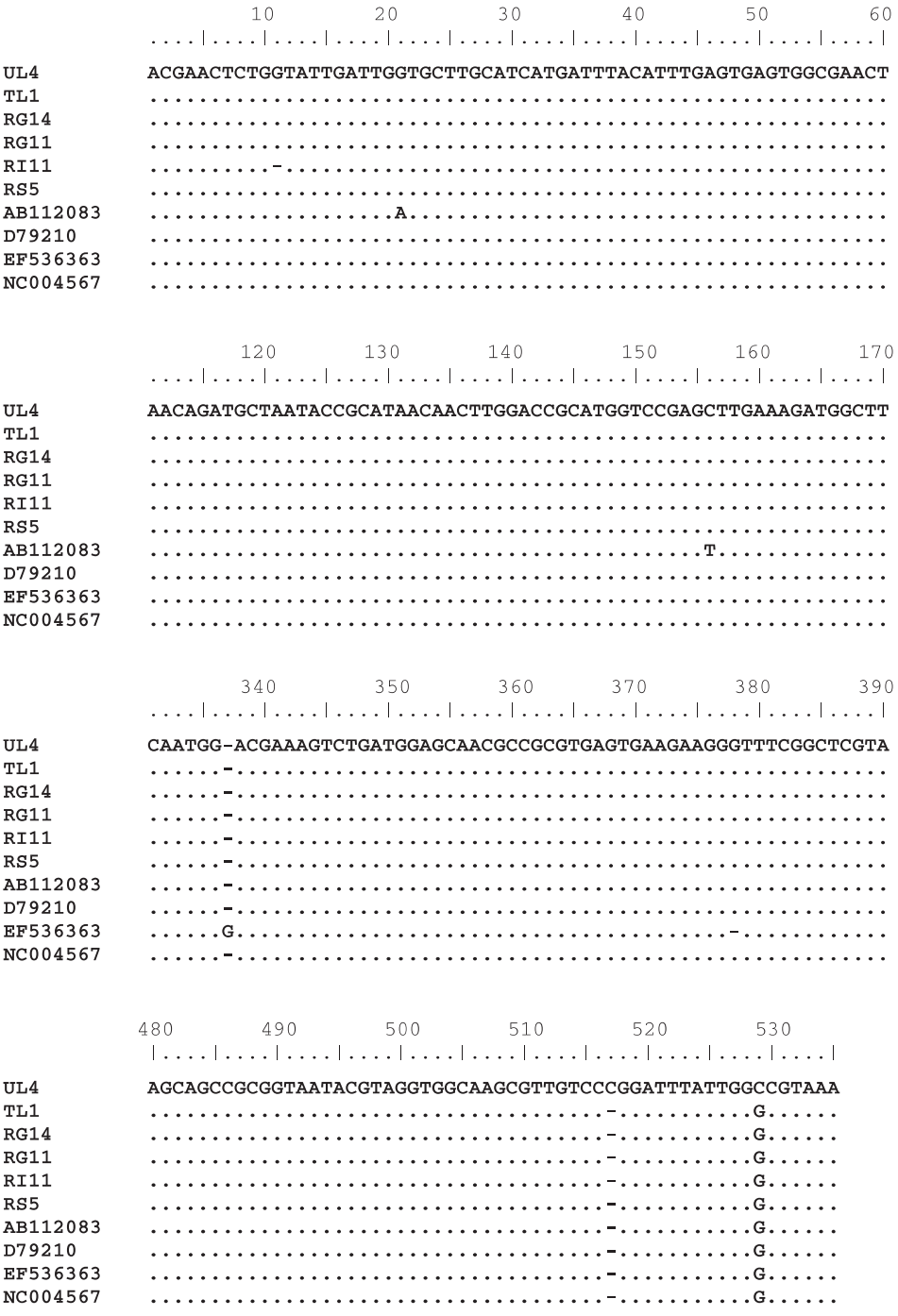

Fig. 1. Alignment of selected 16S rDNA partial sequences amplified from 6 bacteriocinogenic *L. plantarum* strains with 4 strains deposited in the GenBank

strain RI11 at position 11, whereas points of insertion and substitution were observed at positions 517 and 529, respectively, in UL4 strain.

Screening of bacteriocin structural genes

The PCR analyses of bacteriocin structural genes present in the studied *L. plantarum* strains showed consistent results, where *plnEF* and *plnW* were amplified from the genomic DNA extracted from the studied strains (Table 2). The positive results were further confirmed by DNA sequencing analyses, showing 98 and 100 % similarity to the sequences of *plnEF* and *plnW* deposited in the GenBank (data not shown). The DNA sequences of *plnEF* and *plnW* of the studied strains were deposited in the GenBank with the accession numbers from GU138149 to GU138154 and from GU322921 to GU322926, respectively. Nevertheless, the studied *L. plantarum* strains did not harbour *plnJ*, *plnK*, *plnNC8*, *plnN* and *plnS* plantaricin genes (Table 2).

The presence of multiple bacteriocin genes in LAB, particularly *L. plantarum* strains, has been reported (*3*). The surveys of bacteriocin genes present in *L. plantarum*

Table 2. PCR amplification of bacteriocin structural genes present in bacteriocinogenic *L. plantarum* strains

Plantaricin gene	Strain					
	UL4	TL1	RG14	RG11	RI11	RS5
plnEF	+	+	+	+	+	+
plnJ	–	–	–	–	–	–
plnK	–	–	–	–	–	–
plnN	–	–	–	–	–	–
plnNC8	–	–	–	–	–	–
plnW	+	+	+	+	+	+
plnS	–	–	–	–	–	–

strains revealed that the combination of plantaricin genes is strain dependent and varies from strain to strain. For instance, *L. plantarum* strains isolated from an African fermented food harboured four types of plantaricin combinations: 1-*plnEF* and *plnK*; 2-*plnEF*; 3-*plnEF, plnJ*, *plnK* and *plnN*; 4-*plnJ* and *plnK* (*4*). In a similar study, a combination of *plnEF* and *plnJ* was reported for *L. plantarum* J23 (*5*). This is the first report on the concurrent occurrence of both *plnEF* and *plnW* in *L. plantarum* strain (*30*), suggesting that the studied isolates are novel bacteriocin-producing *L. plantarum* strains that possess broad inhibitory spectrum due to the effects of lantibiotic (*plnW*) and heat-stable non-lantibiotic (*plnEF*) bacteriocins (*35*) produced by the studied strains. The results of this study support further the broad inhibitory activity and antimicrobial profile of the studied strains of *L. plantarum*, as reported by Thanh *et al.* (*19*).

Amplification and DNA sequence analysis of ITS1

Two major fragments were amplified (550 and 800 bp, data not shown) from each strain when *ITS1* was used as the target, suggesting that two major forms of *ITS1* were present in the studied strains and designated as *ITS1-S* and *ITS1-L* (*14*). In addition, there was one minor PCR product in all samples. This may be the product of a different *rrn* operon or attributable to the cross-hybridization between multiple amplification products (*31*). According to Nour (*14*), *ITS1-L* in lactobacilli contains $tRNA^{Ile}/tRNA^{Ala}$ encoding genes in tandem arrangement, while *ITS1-S* contains no tRNA gene and is most likely not evolutionary conserved. Thus, further investigations were focused on *ITS1-S* with the aim of finding a variable region that could be used to differentiate the studied strains.

As for GC content, *ITS1-S* from UL4, TL1, RG11 and RS5 contains 30.24 % GC, while RG14 and RI11 contain 29.76 %. Although the length of *ITS1-S* in our study was in agreement with Nour (*14*), who reported the length from 204 to 219 bp with 37 to 41 % GC content for *ITS 1-S*, the GC content of the *ITS1-S* here was found to be relatively low (29.76 and 30.24 %) and the reason is unknown. The complete genome sequence of *L. plantarum* WCFS1 (*36*) contains 44 % of GC content.

The analyses of the DNA sequences of *ITS1-S* fragments revealed that the genes contained 3′ end of 16S rDNA, *ITS1-S* (205 bp) and the 5′ end of 23S rDNA. Both ends were predicted by comparing the 16S and the 23S rDNA sequences of the studied strains with *L. plantarum* WCFS1 (*36*). The DNA sequences were deposited in the GenBank with the accession numbers from GU322927 to GU322932. By aligning the *ITS1-S* sequences of the studied strains with the 17 *L. plantarum* strains available in the GenBank, a four-base pair region at positions 26–29 was identified as a variable region of *ITS1-S* in the studied *L. plantarum* strains (Fig. 2), where *TGCG* was shared among the strains TL1, RG11 and RS5, while UL4, RG14 and RI11 have *CGCT*, *TTCG* and *TGCT*, respectively. These DNA sequences were not observed in the variable regions of the 17 deposited *L. plantarum* strains that were employed for comparative analyses. Therefore, the DNA sequence of the variable regions of *ITS1-S* was used to distinguish the closely related strains of *L. plantarum* into 4 groups, which could not be achieved by using the methods of PCR-RFLP, amplified ribosomal DNA restriction analysis (ARDRA) and $(GTG)_5$-PCR (data not shown). The results obtained in this study also imply that the variable region of *ITS1-S* is a possible powerful tool to discriminate the closely related bacterial strains isolated from the same ecological niche where high degree of similarity is frequently found at the genomic level.

Amplification and DNA sequence analysis of ITS2

Two major fragments (130 and 450 bp) were observed in the PCR products when the *ITS2* regions were amplified (data not shown). Similarly to the amplification of *ITS1*, one to two minor bands were observed, nonetheless, unlike *ITS1*, the minor band patterns were not similar among the studied strains. The presence of minor bands and slight differences in *ITS2* patterns were also reported by Chen *et al.* (*15*), who amplified *ITS2* from *L. plantarum* strains for the comparison of the banding patterns generated by *ITS2* amplification. However, they observed one additional fragment (350 bp), suggesting that the banding pattern generated by *ITS2* amplification from *L. plantarum* strains could be different from strain to strain.

The analyses of DNA sequences for both small and large fragments of *ITS2* showed that they contained the 3′ end of the 23S rDNA, the putative spacer region and the 5′ end of the 5S rDNA (Fig. 3). Both ends were predicted by using the 23S and the 5S rDNA sequences of *L. plantarum* WCFS1 (*36*). The alignments of the DNA sequences revealed that they share 100 % similarity, which was contradictory to the previous reports of the variability of this region due to the absence of functional genes (*14,15*). This has led us to investigate the functional role of the *ITS2* region. Interestingly, the analysis of large fragment DNA sequences using genomic tRNA database (*37*) revealed two tRNA-encoding regions in a tandem arrangement: the region from 205 to 280 encodes for $tRNA^{Val}$ and the region from 282 to 354 encodes for $tRNA^{Lys}$. However, the presence of these tRNA-encoding regions in *ITS2* could not be determined, as the upstream of this region from the positions of 82 to 204 showed 100 % homology to 5S rDNA from *L. plantarum* WCFS1 (*36*). Therefore, the predicted 5′ end of 5S rDNA located at the 3′ end of the large DNA sequence corresponded to additional 5S rDNA sequence at the downstream of *rrnC*

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                                    10        20        30        40        50        60
                                    ....|....|....|....|....|....|....|....|....|....|....|....|
UL4-ITS1-S                          AAGGAATATTACGGAAACCTACACACGCTTCGAAACTTTGTTTAGTTTTGAGAGATTTAA
TL1-ITS1-S                          ........................T..G.............................
RG14-ITS1-S                         ........................TT.G.............................
RG11-ITS1-S                         ........................T..G.............................
RI11-ITS1-S                         ........................T...............................
RS5-ITS1-S                          ........................T..G.............................
ITS1-L.plantarum 83114              ..........................G.............................
ITS1-L.plantarum I18A               ..........................G.............................
ITS1-L.plantarum                    ..........................G.............................
ITS1-L.plantarum                    .........................T...............................
ITS1-L.plantarum ATCC12706          ..........................G.............................
ITS1-L.plantarum ATCC8014           ..........................G.............................
ITS1-L.plantarum ATCC14431          ..........................G.............................
ITS1-L.plantarum ATCC14917          ..........................G.............................
ITS1-L.plantarum NGRI0404           ..........................G.............................
ITS1-L.plantarum NGRI0315           ..........................G.............................
ITS1-L.plantarum NGRI0225           ..........................G.............................
ITS1-L.plantarum NGRI0101           ........................TT..............................
ITS1-L.plantarum 8323               ..........................G.............................
ITS1-L.plantarum JCM1149            ........................T.G.............................
ITS1-L.plantarum WCFS1              ........................A.G.............................
ITS1-L.plantarum EH2                ........................T.G.............................
ITS1-L.plantarum BSO92              ..........................G.............................
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Fig. 2. Alignment of the selected short form of 16S-23S intergenic spacer region of the bacteriocinogenic *L. plantarum* strains with 17 strains deposited in the GenBank. Positions 26 to 29 are the four-base pair variable regions of *ITS1-S* in the studied *L. plantarum* strains

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                    23S rDNA region
                          10        20        30        40        50        60        70        80
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ITS2-L                    ACTTAACCAAGTAATTGGTGTTCCGGTTCATATGAAGATATTTGATATTGTCTAGTTTTGAGAGCACAAGTTTCTCAATA
ITS2-S                    ----------.....................................................................

                                  90        100       110       120       130       140       150       160
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ITS2-L                    GTGTGGTGACGATGGCGAGAAGGATACACCTGTTCCCATGTCGAACACAGAAGTTAAGCTTCTTAGCGCCGAGAGTAGTT
ITS2-S                    .---------------------------------------------------------------------------

                                  170       180       190       200       210       220       230       240
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ITS2-L                    GGGGGATCGCTCCCTGCGAGGGTAGGACGTTGCCATGCGAAATTGGAGGATTAGCTCAGTTGGGAGAGCGTCTGCCTTAC
ITS2-S                    ---------------------------------------------------------------------------

                                  250       260       270       280       290       300       310       320
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ITS2-L                    AAGCAGAGGGTCACAGGTTCGAGCCCTGTATCCTCCATCATGAGCCGTTAGCTCAGTTGGTAGAGCATCTGACTTTTAAT
ITS2-S                    ---------------------------------------------------------------------------

                                  330       340       350       360       370       380       390       400
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ITS2-L                    CAGAGGGTCGACAGTTCGAGACTGTCACGGCTCATTTAACCGCAAGGTTAAATGGATATTATTCAATTTAATATTTAGTT
ITS2-S                    ---------------------------------------------------------------------------

                                  410       420       430
                                  ....|....|....|....|....|....|..
ITS2-L                    TCACATAGTGTGGTGATGATGGCGAGAAGGATACCCC
ITS2-S                    --------------------------------------

                                  5S rDNA region
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Fig. 3. Alignment and analysis of the short and large forms of 23S-5S intergenic spacer regions amplified from 6 bacteriocinogenic *L. plantarum* strains. Positions 82 to 204: 5S rDNA; positions 205 to 280 and 282 to 354 indicate the $tRNA^{Val}$ and $tRNA^{Lys}$ encoding regions, respectively

operon. This finding is in agreement with the previous report of de Vries *et al*. (*38*).

The DNA sequence of a short fragment was subsequently aligned with the DNA sequence of a large fragment (Fig. 3). The full-length sequence of the small fragment was identical to the 3′ end of the large fragment, suggesting that only one type of *ITS2* was present in the genome and the amplification of the large fragment could be attributed to the additional 5S rDNA present in the studied strains. In spite of the high level of similarity among the *ITS2* regions, no encoding gene region was detected in this region. The DNA sequences were deposited in the GenBank with the accession numbers from GU322933 to GU322938.

Randomly amplified polymorphic DNA (RAPD) analysis

The combinations of arbitrary primers were used in the RAPD study since all single arbitrary primers failed to produce discriminative results. The combinations of R1/R2, R1/R3 and R5/R6 resulted in discriminative and reproducible DNA banding patterns (Fig. 4). The combinations were repeatedly analysed for three times in order to study the reproducibility of RAPD analyses. Similar DNA banding patterns were observed, although the intensity of amplified fragments was slightly different when the experiments were repeated. In addition, the reproducibility of the RAPD result was ascertained as similar result was obtained when the analyses were repeated by other researchers (data not shown).

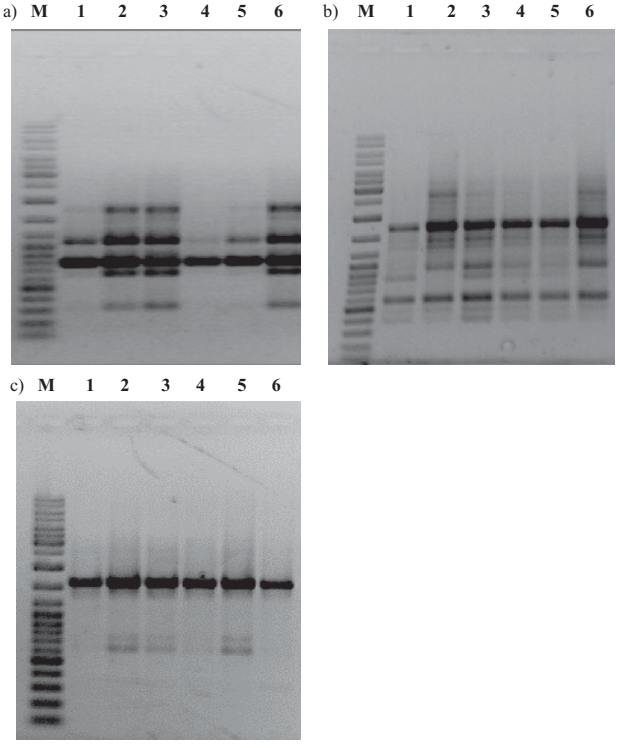

Fig. 4. Randomly amplified polymorphism DNA banding patterns generated by RAPD analyses using combination of primers: a) R1/R2, b) R1/R3, c) R5/R6. Lane 1: UL4, lane 2: TL1, lane 3: RG14, lane 4: RG11, lane 5: RI11, lane 6: RS5, lane M: GeneRuler™ DNA ladder mix (Fermentas)

Fig. 5 summarises the results obtained from RAPD experiments and illustrates the order of primer combinations used in the study. The failure to differentiate the strains by a single arbitrary primer and the need of a series of RAPD approaches with combinations of arbitrary primers indicate that the studied strains are highly similar at the genome level. The literature search showed that the differentiation of strains by RAPD analyses is not common. Nevertheless, the accuracy in differentiation of the bacterial strains is primarily important to achieve, as beneficial and health-promoting characteristics of LAB are strain dependent (*6*).

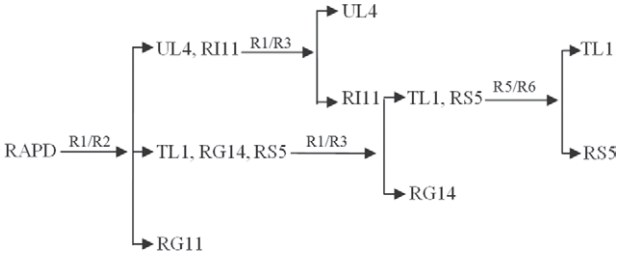

Fig. 5. Summary of RAPD results and the order of primer combinations required for the discrimination of *L. plantarum* strains

Conclusion

In conclusion, the six *L. plantarum* strains harboured the new combination of *plnEF* and *plnW* structural bacteriocin genes, indicating they were novel bacteriocin producers, and the broad inhibitory activity of the studied *L. plantarum* strains (*19*) would warrant their wider application in industry as shown by Lim *et al.* (*18*), Loh *et al.* (*20,22,23*), Thanh *et al.* (*21*) and Foo *et al*. (*24*). The studied *L. plantarum* strains were successfully differentiated by employing the four-base pair variable region of *ITS1-S* and RAPD analyses, although they were highly similar at the genomic level.

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