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*Full Length Research Paper*

# Identification of bacterial strains in viili by molecular taxonomy and their synergistic effects on milk curd and exopolysaccharides production

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**Viili, also known as viilia, is a traditional fermented dairy product, which is popular in Finland. However, little research has been conducted on organisms' isolation and their interaction during milk fermentation. In this study, culture-dependent and independent methods had been used to identify isolates. Also, their synergistic effects on milk curd and exopolysaccharides (EPSs) yield was evaluated. When *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Lactobacillus paracasei*, *Bacillus cereus* and *Lactobacillus delbrueckii* were successfully separated and screened from viili by using the combination of conventional microbiological cultivation with polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), they were individually inoculated into sterilized skimmed milk or combined as co-cultures to determine the curd time and the yield of EPSs. Results show that *L. delbrueckii* presented the strongest curding capability at 5 h and combination of *L. paracasei*, *B. cereus* and *L. plantarum* brought the highest yields of EPSs (186.71 mg/L). Moreover, it suggested that the synergistic effects among the isolates had shortened the curding time and increased the production of EPSs.**

**Key words:** Viili, PCR-DGGE, exopolysaccharides, lactic acid bacteria.

## INTRODUCTION

Viili is a healthy dairy product made with a traditional mesophilic mixed-strain starter, mainly composed of exopolysaccharides (EPSs)-producing bacterial communities that confer its strong ropy character (Saxelin et al., 1986) and probiotic effect. Viili originated from Scandinavia and was claimed to have various functional benefits and health-improving potential, for example, reducing the blood cholesterol level of rats (Nakajima et al., 1992) and preventing carcinogens (Kitazawa et al., 1991) mediated by the stimulation of the mitogenic activity of B lymphocytes (Kitazawa et al., 1992). It is generally believed that these EPSs produced during the acidification of milk had contributed to the stability (Van Marle and Zoon, 1995; Yang et al., 2010).

During the last decade, many investigators have identified the various bacteria in dairy products in selective growth medium by morphological and biochemical characteristics (Lin et al., 1999; Simova et al., 2002; Gallagher and Horwill, 2009). However, the results showed that some bacteria were not able to grow in media because of different unfavorable factors such as low pH value and presence of inhibitors produced by the starters. Microorganisms isolated and selected empirically by taxonomists is somewhat restricted because of repetitive encounters with possible duplicate

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**Abbreviations: EPSs,** Exopolysaccharides; **PCR-DGGE,** polymerase chain reaction-denaturing gradient gel electrophoresis.

strains (Fujimorl and Okuda, 1994). In addition, when similar strains are passed through a certain assay system based on their activity, it is difficult to determine whether one of them should be eliminated for it takes time to examine these precisely. Recently, molecular biological methods have been developed to study the microbial biodiversity of dairy food. The polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) are based on direct analysis of the extract of DNA from the microbial environment and do not require cell cultivation (Ercolini, 2004; Ariefdjohan et al., 2010; Liu et al., 2010). Now, this method has been used successfully to evaluate bacterial composition of probiotic products (Lee et al., 2009; Connor et al., 2010), identify or profile bacteria in dairy products (Walter et al., 2001; Nielsen et al., 2005; Theunissen et al., 2005).

As the important metabolites produced by a wide variety of bacteria, EPSs are secreted extracellular microbial polysaccharides associated with the cell surface (Sutherland, 1972) and they play an important role in the rheology, texture and mouthfeel of fermented milk. Now, both rheological and microscopic studies showed that the polysaccharides produced by lactic acid bacteria (LAB) (Schellhaass and Morris, 1985; Gutierrez et al., 2009; Saija et al., 2010) or other bacteria (Van Marle and Zoon, 1995) have an indirect effect on the protein gel electrophoresis formed during acidification of milk, which had influenced the EPSs yield and curding time.

As the key bacteria, LAB play an important role in food fermentation processes (Wood, 1998) for their ability to acidify and preserve foods from spoilage, as well as their involvement in the texture, flavor, and aroma development of food products. Currently, there is a renewed interest in using LAB as probiotic food additives to enhance immune function and prevent gastrointestinal infections (de Roock et al., 2010; Higgins et al., 2010). Unfortunately, many commonly used LAB preparations have been found to be lacking efficacy (Hull et al., 1992; Aza s-Braesco et al., 2010). To gain the valuable probiotics possessing potential commercial application, culture -dependent and –independent methods were applied in the present study, and 5 probiotics had been successfully isolated from viili. And our results indicated that *L. delbrueckii*, the dominant *Lactobacillus* in viili, possessed the highest capability of EPSs yield and milk curding among isolates. However, when grouped with the dominant bacteria (*S. thermophilus*) in viili, they presented the shortest curding time but the lowest EPSs procuction among the combinations. In contrast, the combination of minor bacteria possessed the highest EPSs production and takes the longest time in milk curding, which showed that synergistic effects occurred during the fermentation progress among isolates. And the synergistic effects among isolates in viili or other commercial probiotics may possess a good application prospect for commercial products.

## MATERIALS AND METHODS

### Viili starter and isolates

The viili starter was a generous gift from Dr. Yinghua Lu (Xiamen University). Viili starter was incubated (5%, w/v) and propagated in sterilized milk at 25°C for 20 h and then the starter was transferred into fresh milk and incubated at 25ºC for another 20 h. After this procedure was repeated 3 times, the starter was considered active and used in this study. Then, 10 g of viili starter was homogenized in 90 mL of sterile saline solution (0.85% sodium chloride solution, pH 5.5) in a flask and concentrations of the viable bacteria in suspensions were obtained by serial dilutions. The total bacteria in viili were examined on BHI agar (Jacobsen, 1999) and LB+ skimmed milk agar; the LAB were examined on MRS agar (Todorov and Engell, 2008).

### Identification of the clone and bacterium morphology

The microbes were divided according to their clone and bacterium morphology, as well as the requirements of medium or oxygen.

### DNA extraction and PCR amplification

DNA was isolated according to a bead-beating method (Zoetendal et al., 1998). Samples were suspended in 1 mL TN150 buffer containing 10 mM Tris-HCl (pH 8.0) and 150 mM NaCl in a screw-capped tube, containing 0.3 g of sterile zirconium beads (diameter, 0.1 mm) and 150 µL of phenol. The tubes were bead-beaten at 5000 rpm for 3 min in a mini-bead beater, following phenol-chloroform extraction. The solution was precipitated with ethanol and pellets were suspended in 500 µL of TE. This solution was added to 5 U (1 µL) of DNase-free RNase and incubated at 37°C for 15 min. After phenol-chloroform extraction, DNA was precipitated with ethanol and suspended in 50 µL of TE. Primers 357f 5′-TACGGGAGGCAGCAG-3′and 519r 5′-ATTACCGCGGCTGCTGG-3′were used to amplify the 16S rDNA from the total bacterial DNA; Lac1 5′-GATTYCACCGCTACACATG-3′ and Lac2 5′- AGCAGTAGGGAATCTTCCA-3′ were used for *Lactobacillus*; a GC clamp in primer was used to create PCR products suitable for separation by DGGE (Zoetendal et al., 1998). PCR was performed with the Taq DNA polymerase kit from Life Technologies. Based on the instruction of manufacturer, the PCR reaction (25 µL) used 0.125 µL Tap polymerase (1.25 U), 0.5 µL primers, 1 µL ten-fold diluted DNA template (approximately 1 ng), 2.5 µL ten–fold PCR buffer, 1.5 µL $MgCl_2$ (50 mM ) and lastly UV-sterile water. The samples were amplified in a Biosci PCR system, with 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. Aliquots of 5 µL were analyzed by electrophoresis on an agarose gel (1%) to check the size and amounts of the amplicons.

### DGGE gel

Amplicons of V3 of 16S rDNA were used for sequence-separation by DGGE (Simpson et al., 2004). DGGE was performed using 40 mM Tris-HCl (pH 8.0) as the electrophoresis buffer in a BioRad DGGE system. The electrophoresis was initiated by pre-running for 5 min at a voltage of 220 V, and subsequently run at a fixed voltage of 85 V for 16 h at 60°C. The gel was stained with $AgNO_3$ and developed after completion of electrophoresis. The gel was then covered by cellophane membrane and dried overnight at 60°C.

### Plasmid construction and sequencing

PCR products were subcloned with the pMD18-T vector system I

**Table 1** The cell and colony morphologies of bacteria isolated from viili by using BHI, YPD, MRS and skimmed milk plates both in aerobic and anaerobic condition

| Stain number | Clone morphology | Bacteria morphology | Growth conditions |
|---|---|---|---|
| 1 | Milky white big circular, protuberance, moist | $G^+$, Slender or long rod | Aerobic, MRS medium |
| 2 | Milky white big circular, protuberance, moist | $G^+$, Short rod | Aerobic, MRS medium |
| 3 | Milky white big circular, protuberance, moist | $G^+$, Short rod | Aerobic, MRS medium |
| 4 | Milky white big circular, protuberance, moist | $G^+$, Short rod | Aerobic, skimmed milk medium |
| 5 | Milky white big circular, protuberance, moist | $G^+$, Short rod | Aerobic, skimmed milk medium |
| 6 | Milky white big circular, protuberance, moist | $G^+$, Long rod or short rod | Anaerobic, MRS medium |
| 7 | Milky white big circular, protuberance, moist | $G^+$, Slender or long bacilliform | Anaerobic, BHI medium |
| 8 | Milky white small circular, protuberance, moist | $G^+$, Spherical, single or catenate | Anaerobic, MRS medium |
| 9 | Milky white big circular, protuberance, moist | $G^+$, Long rod | Anaerobic, MRS medium |
| 10 | Milky white small circular, protuberance, moist | $G^+$, Spherical, single or catenate | Anaerobic, YPD medium |
| 11 | Milky white big circular, protuberance, moist | $G^+$, Long rod | Anaerobic, YPD medium |
| 12 | Milky white small circular, protuberance, moist | $G^+$, Spherical, single or catenate | Anaerobic, skimmed milk medium |
| 13 | Milky white big circular, protuberance, moist | $G^+$, Long rod | Anaerobic, skimmed milk medium |
| 14 | Milky white big circular, protuberance, moist | $G^+$, Long rod | Anaerobic, skimmed milk medium |
| 15 | Milky white big circular, protuberance, moist | $G^+$, Long rod | Anaerobic, skimmed milk medium |

(Takara) according to the manufacturer's instructions. Cells of *Escherichia coli* DH5a were electrotransformed with recombinant plasmids by a standard method (Sambrook and Russell, 2001). Selection of transformants was done on LB agar containing 100 mg/mL of ampicillin. Transformants were randomly picked and sequenced in Invitrogen.

#### Curding time

Viili starter, single strain and strain-combinations were incubated in skimmed milk with a 1% inoculum at 40°C, and then the curding condition was observed every 30 min.

#### Separation and purification of the EPSs

After bacterial growth, trichloroacetic acid (Miller and Neuzil 1982) was added to the cultures to a final concentration of 4% (w/v), and the mixture was stirred for 2 h. Cells and precipitated proteins were removed by centrifugation (2200 g, 15 min). The EPSs were precipitated by gradually adding an equal volume of cold ethanol. The precipitate was collected by centrifugation, washed, and then dissolved in water. Finally, the aqueous solutions of the EPSs were again filtered through an Acrocap filter (0.45 µm), dialysed against water overnight at 4°C. The uniformity of the EPSs material was examined by gel-filtration using a column (75×1.5 cm) of Bio-Gel P-30 polyacrylamide gel (exclusion limit 35,000 Da). The sample (1 mg) was loaded onto the column and eluted with 50 mM ammonium acetate with UV monitoring at 280 nm.

#### Statistical analysis

Data of curding time and EPSs yield were submitted to a variance (ANOVA) analysis, and then significant differences between means were determined by the nonparametric Student-Newman-Keuls separation test with significance level ($P < 0.05$) or very significance level ($P < 0.01$).

## RESULTS

### Morphology of colony and bacterium

Viili microbes were evaluated after 48 h incubation in 4 kinds of plates (BHI, YPD, MRS and skimmed milk) in both aerobic and anaerobic condition. The results (Table 1) show that 15 strains were isolated from viili, all of which belonged to Gram-positive bacteria in long rod, short rod or spherical shape. By comparison of their $O_2$ requirements, mediums, colonies as well as cell morphologies, we can determine that at least 4 different strains had been isolated from viili.

### PCR amplification

The bacteria showed various morphological characters when growth conditions (example pH, temperature, nutrition and growth period) had changed, which posed difficulties on clone selection. Therefore, the total bacteria primers and *Lactobacillus* primers were applied to avoid the repetitions in strain isolation. In this study, PCR primers Lac1 and Lac2 were derived for the amplification of 380 bp of the 16S rDNA of *Lactobacilli*. Previous analysis (Walter et al., 2001) showed that the primers would permit most of the *Lactobacillus* species to be different, so both $V_3$ universal primers and *Lactobacillus* specific primers were used to amplify strain DNAs, which lowered the failure chances of amplification.

Results of the PCR amplification (Figure 1A) showed that all the strains could be amplified (about 200 bp) by $V_3$ universal primers which had ensured the quality of bacterial DNAs; when *Lactobacillus* primers were used to amplify the same DNAs (Figure 1B), no bands were found in lanes 3, 4, 9, 11, 13, which indicated that strains

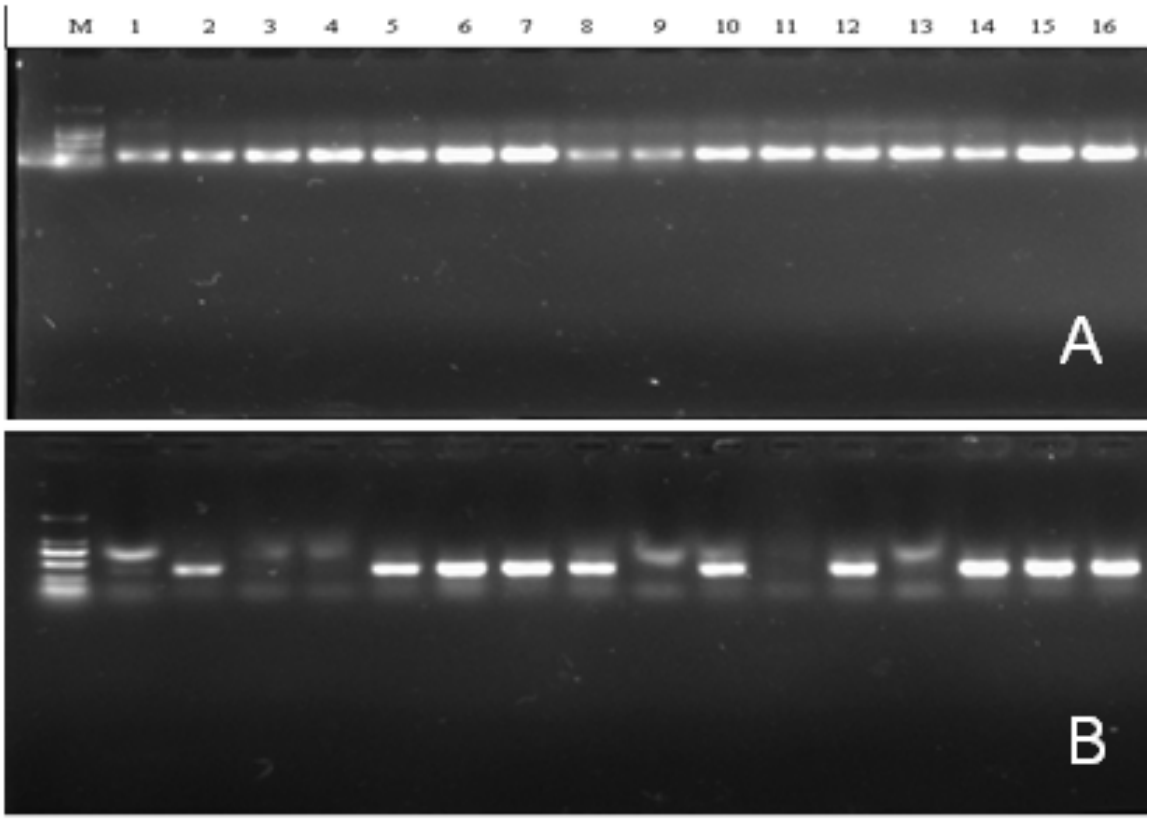


**Figure 1.** The electrophoritic profile of PCR amplification. A, The PCR amplification of total bacteria (about 193 bp); B, The PCR amplification of Lactobacilli (about 380 bp); MW, DL2000 DNA Marker (from top to bottom: 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp and 100 bp); lane 1, viili; lanes 2-16, strains 1-15.

2, 3, 8, 10 and 12 belonged to bacteria but *Lactobacilli*. For viili, the light band amplified by universal primers and the weak band amplified by *Lactobacillus* specific primers showed that the LAB species were subordinate in this fermented food.

## PCR-DGGE analysis

To distinguish the same bacteria, the DGGE of total bacteria and *lactobacillus* was applied (Figures 2 and 3). For DGGE analysis, a GC-clamp of 40 bases was linked to $V_3$ universal primers and *Lactobacillus* specific primers which exhibited a melting behavior suitable for DGGE.

In Figure 2, the dominate bands in lanes 1 and 7 shared the same location in DGGE gel, which indicated that they belonged to the same bacterium; likewise, the strains 4, 5, 9, 11, 13, 14 and 15; 3, 8, 10 and 12 belonged to a same strain, respectively. As the dominant bands of strains 2 and 6 were different from the other bacteria, so they were distinctive. Moreover, the dominant band in viili (band 8) shared the same locations with strains 3, 8, 10 and 12, which indicated that their corresponding strain was the dominant strain in viili starter.

In Figure 3, the strains 1 and 7 shared the same dominant band locations in DGGE gel, they belonged to a same *Lactobacillus*; As above, strains 4, 5, 9, 11, 13, 14, 15 were a same *Lactobacillus*; strain 6 was a different *Lactobacillus*. In the gel, the dominant band of viili located the same position with strain 6, so the strain 6 is the dominant *Lactobacillus* in viili.

Here, these results also indicated that minor species may not be detected by DGGE when presented at <1% of the total population (Felske et al., 1998; Fasoli et al., 2003; Prakitchaiwattana et al., 2004; Theunissen et al., 2005; Renouf et al., 2007).

## 16S rDNA sequencing

From the DGGE results above (Figures 1, 2 and 3), it could be concluded that strains 2, 6, 7, 10 and 14 belonged to different strains. And then these single strains were pure cultured and identified using 16S rDNA sequencing.

Sequences were compared in the Genbank database and 5 different isolates had been obtained named *L. plantarum*, *S. thermophilus*, *L. paracasei*, *B. cereus* and *L. delbrueckii*.

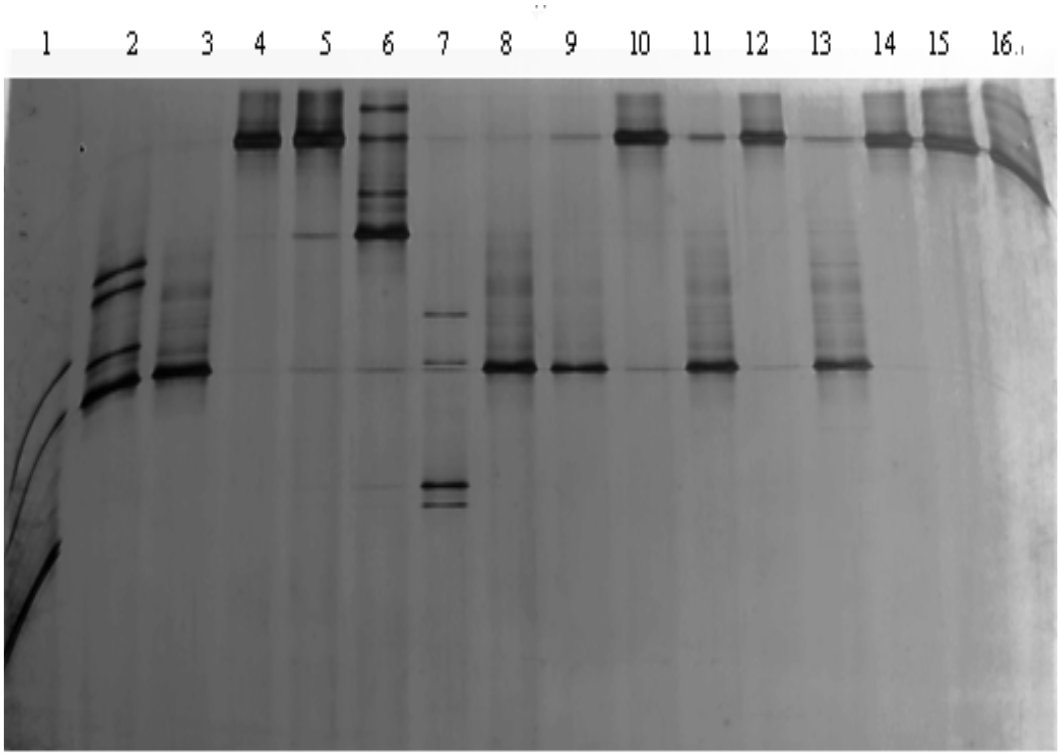


**Figure 2.** Denaturing gradient gelelectrophoresis analysis of bacteria 16S V3 region by using universal bacteria primers. Lanes 1-7, strains 1-7; lane 8, all strains of viili; lanes 9-16, strains 8-15.

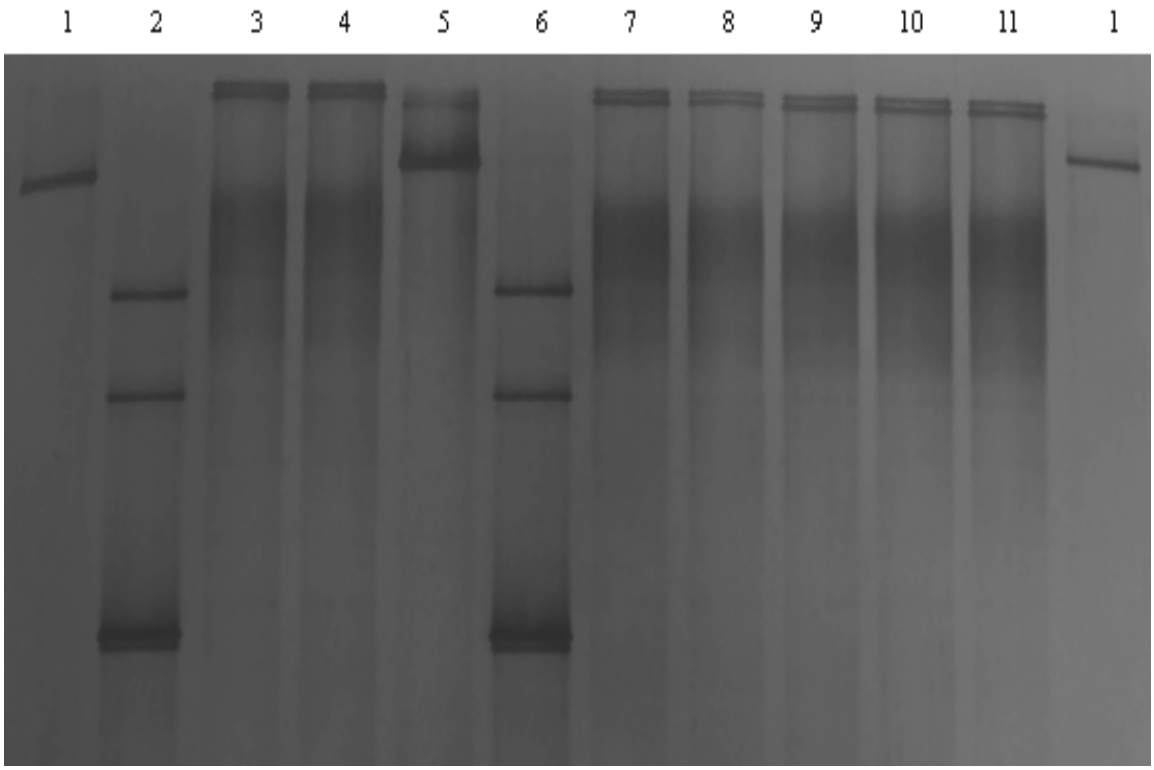


**Figure 3.** Denaturing gradient gelelectrophoresis analysis of bacteria 16S V3 region by using Lactobacillus primers. Lane 1, Lactobacilli of viili; lanes 2-11, strains 1, 4, 5, 6, 7, 9, 11, 13, 14 and 15.

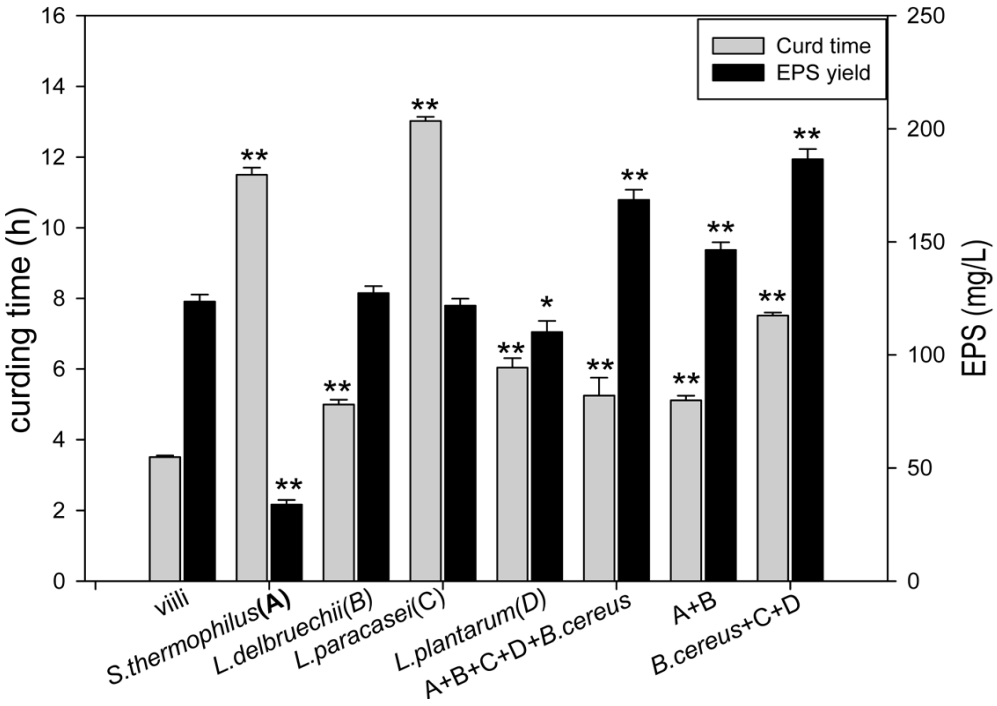


**Figure 4.** The curd time and EPS yield comparison among isolates and combinations. **: p<0.01, *: p<0.05 (n=3).

## Curding time and EPS yield

Several studies indicated that interactions (positive and negative) between LAB and yeasts may modify ripening time and/or the production of essential odours, depending on the combination (Gadaga et al., 2001). In this study, we studied the synergistic effect on milk curd and EPSs yield among organisms in viili.

When curding time was tested, viili possessed the shortest curding time 3.5 h and the dominant LAB (*L. delbrueckii*) could curd at 5 h while *Lactobacillus paracasei* took 13 h to reach the same condition; for EPSs yield, there were no significant difference between viili and isolates except *S. thermophilus*. In this research, 3 co-culture groups were established: The dominat bacteria and dominant LAB (A+B), the subordinate bacteria combination (*B. cereus*+C+D) and the combination of all the isolates (A+B+C+D+*B. cereus*). From Figure 4, we can see that the curding time of these combinations was longer than viili, but they produced much more EPSs. Interesting, though *S. thermophilus* (the dominant bacteria in viili) possessed the overwhelming superiority in viili, its effects on curding time and EPSs yield were inferior to the dominant LAB. Moreover, the minor bacterial combination of *B. cereus*+C+D produced the highest amounts of EPSs.

The EPSs yields of *S. thermophilus* and *L. delbrueckii* were 33.6 and 128 mg/L, respectively. While the EPSs production of their combination was as high as 146.5 mg/L; also, the groups of *B. cereus*+C+D and A+B+C+D+*B. cereus* yielded 186.7 and 146.5 mg/L EPSs, which were much higher than their corresponding single isolate. This indicated that the bacteria producing low EPSs (*S. thermophilus*, *B. cereus* and other bacteria without detection) had enhanced the combinations' capability to yield EPSs. Furthermore, the significantly higher EPSs yield of these 3 combinations than viili indicated that the interactions of yeasts as well as some unknown bacteria in viili had reduced the EPSs production rather than shorted its curd time.

## DISCUSSION

The present study shows that 5 different strains was determined from viili. As a powerful molecular biological tool in bacterial research, PCR-DGGE could quickly classify the same bacteria in DGGE gel, which has simplified the complex steps on bacterial identification. The bands in DGGE gel had also pointed out the dominant bacteria and dominant LAB in viili. The compared among viili, single isolate and isolates combination on curding time and EPSs yield showed that interactions between isolates had enhanced their EPSs production while the yeasts and other bacteria existed in viili (except our isolates) decreased the EPSs yield but shorted the curding time. A further research should be carried out to study the interactions among organisms in viili and other commercial probiotics, which would be of great practical use since desirable effects could be

promoted while those undesirable could be minimized.

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