Exopolysaccharide-producing isolates from Thai milk kefir and their antioxidant activities

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ABSTRACT

This is the first report of microbial diversity in milk kefir from Thailand and isolates with exopolysaccharide (EPS)-producing capacity. Microbes in milk kefir were isolated on selective agars and the purified bacteria were identified using PCR-based 16S rDNA gene analysis. Twenty four strains of Bacillus spp. were identified out of 85 isolates without any lactic acid bacteria, acetic acid bacteria or yeasts. Closest relative isolates of Bacillus amyloliquefaciens SD-32 (100% identity) were predominant (67% abundance) followed by Bacillus sp. DH25 (95% identity, 7% abundance), uncultured Bacillus sp. clone L4 (95% identity, 5% abundance) and the other 21 Bacillus spp. each accounts for only 1% abundance. All Bacillus spp. identified were able to produce EPS on MRS agar with 8% sugar (sucrose, lactose or glucose) using the disk diffusion method suggesting their capacity to utilise sucrose, lactose and glucose as precursors to EPS production however EPS appearances varied depending upon bacterial strain and type of sugar. Selected bacteria grown in 8% sucrose exhibited 18–27% DPPH scavenging activity and resistance to H2O2. Removal of EPS showed significantly reduced bioactivity DPPH scavenging activity and resistance to H2O2. Microbial diversity of Thai milk kefir was unique from other milk kefirs of different origins.

1. Introduction

In recent years, fermented drinks such as water or milk kefir and local fermented beverages made from fruits and vegetables have gained popularity in Thailand due to a variety of health claims in addition to their nutritional value (Urdaneta et al., 2007). Studies regarding kefir's biological activities have established that kefir has anti-inflammatory activity, immune-modulating activity, anti-microbial activity and anti-proliferative activity, anti-mutagenic and anti-carcinogenic properties and it has the potential to become a type of functional food (Gузел-Сейдим, Кок-Тас, Грин, & Сейдим, 2011; Diniz, Garla, Schneedorf, & Carvalho, 2003; Vinderola et al., 2005; Liu et al., 2006; Silva, Rodrigues, Filho, & Lima, 2009). The origin of kefir remains unclear. However, the microflora of kefir has been found to be a consortium of different lactic acid bacteria, acetic acid bacteria and yeasts (Franzetti, Galli, Pagani, & de Noni, 1998; Galli, Fiori, Franzetti, Pagani, & Ottogalli, 1995; Horisberger, 1969; Lutz, 1899; Neve & Heller, 2002; Pidoux, 1989; Ward, 1892). In Thailand, most kefir products available in the market or prepared in local households are of unknown origin and the microbial composition is believed to change over time as the kefir grains are passed on from one generation to the next and the fermentation recipes may vary. To date, a number of publications on different sets of bacteria and yeasts isolated from kefirs from around the world have been produced (Franzetti et al., 1998; Galli et al., 1995; Horisberger, 1969; Lutz, 1899; Neve & Heller, 2002; Pidoux, 1989; Ward, 1892), however there are no data where household kefir consortia originating from Thailand are analysed and identified at subspecies level. In addition, the molecular background of the formation of a stable consortium in these fermented drinks of Thai origin is unknown and microbes capable of producing exopolysaccharide (EPS) and forming the starter culture or the so-called kefir grains used for producing kefir beverage are not yet identified. The knowledge of the composition of the microbiota and identification of EPS-producing microbes are the prerequisites for better understanding the formation and the interactions of a stable consortium of these microbes and for future use as starter cultures. Such EPS may be of interest for use in food and biotechnology applications, information of defined microbial strains for food fermentation is essential for a better control and enhanced quality
of food products. Thus, we aimed to isolate and identify the microorganisms from Thai milk kefir capable of producing EPS and their bioactivities.

2. Materials and methods

2.1. Milk kefir preparation

A milk kefir grain was purchased from Kamphaeng Phet Province, Thailand where homemade milk kefir production was exercised. A grain was propagated under standardised conditions for at least two times to eliminate influences resulting from different cultivation procedures of the suppliers. Milk kefir grain was aseptically incubated in 100 mL pasteurized cow milk at 37 °C for one day and this process was repeated again to obtain kefir milk.

2.2. Media and growth of microorganisms

For preparation of the serial dilutions, 1 g of the strained milk kefir grains mixed with liquid part of milk kefir were diluted with 9 mL of 0.85% NaCl and then homogenised using a sterile mortar and pestle. The mixture was vortexed for 2 min. The different serial dilutions were plated on MRS agar at +0.05% Bromocresol Purple (BCP) plates to isolate lactic acid bacteria (LAB), containing 2 g/L of YPD broth at pH 6.8. For isolation of yeasts the serial dilutions were plated on YPD agar plates (10 g/L peptone from casein, 1 mL Tween 80, 2.5 g/L K2HPO4, 5 g/L sodium acetate, 2 g/L ammonium hydrogen carbonate, 0.2 g/L magnesium sulfate hepta hydrate, 0.038 g/L manganese sulfate monohydrate, 20 g/L glucose, 15 g/L agar, pH at 6.5. To isolate acetic acid bacteria (AAB). YC agar was used (10 g/L yeast extract; 50 g/L d-glucose; 30 g/L calcium carbonate; 15 g/L NaCl (5 M) for removing S-layer proteins, all in each suspension. The pellets were centrifuged at 8000 rpm for 10 min. The scavenging ability was determined according to the method previously described (Li, Mutuvuyla, Chen, Jiang, & Dong, 2012) with some modifications. Firstly, 1.0 mL of Bacillus sp, cells grown overnight in MRS media containing 8% lactose with 109 CFU/mL in phosphate buffered saline (PBS) buffer (pH 7.0), was added to 2.0 mL ethanolic DPPH radical solution (0.05 mM). The mixture was mixed vigorously and incubated at room temperature in the dark for 30 min. The controls included only PBS buffer and DPPH solution whereas the blanks contained only ethanol. The absorbance of the resulting solution was measured in triplicates at 517 nm after centrifugation at 8000g for 10 min. The scavenging ability was defined as:

\[
\text{Scavenging activity(%) } = \left[ 1 - \left( \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \right) \right] \times 100
\]

In parallel, another set of the same bacterial cultures were treated to remove/de-structure EPS to evaluate the presence of EPS on bacterial cellular surface for the scavenging ability of Bacillus sp. for DPPH free radical. Briefly, cultures of the same 10 strains were centrifuged (6000g, 10 min), and the cell pellet was washed twice with distilled water and resuspended in PBS buffer (pH 7.0) to approximately 106 CFU/mL. The cell suspensions were added with pepsin (0.5 mg/mL) for hydrolysing cell surface proteins, sodium-metaperiodate (10 g/L) for oxidising surface polysaccharides, and LiCl (5 M) for removing S-layer proteins, all in each suspension. The mixture was incubated at 37 °C for 30 min and centrifuged (6000g, 10 min) to remove supernatant. The bacterial cells were then washed twice and resuspended in PBS buffer (pH 7.0) for the assay of the DPPH free radical scavenging ability of the strains as described above.

2.3. Genomic DNA isolation

For DNA isolation of bacteria overnight cultures (1 mL) were centrifuged at 8000g for 5 min, the pellet was washed with 1 mL TE-buffer containing 1 mM EDTA, 10 mM Tris, pH 8 and centrifuged again. The pellets were stored at −20 °C. The total DNA isolation was performed from bacterial pellet using the Bacterial Genomic DNA isolation kit (Vivantis, Malaysia) according to the kit’s instructions.

2.4. Bacterial 16S rDNA sequence analysis and phylogenetic tree construction

Bacterial 16S rDNA was amplified with the universal forward primer AmpF (5′-GAGAGTTGTATCTGCTCAG-3′) and the reverse primer AmpR (5′-AAAGGAGGTGATCCARCCGC-3′) and a PCR program of 94 °C for 2 min, 32 cycles of 94 °C for 45 s, 54 °C for 45 s, 72 °C for 1 min and a last step at 72 °C for 7 min. The reaction mixture (25 μL) consisted of 0.1 mM of each deoxyribonucleoside triphosphate, 0.75 U Taq polymerase (Vivantis, Malaysia), 5 pmol of each primer and 1 μg of genomic DNA. The PCR products were resolved by electrophoresis on a 0.8% (w/v) agarose gel (Vivantis, Malaysia) and were cleaned-up using the GF-1 PCR Clean-up kit (Vivantis, Malaysia) according to the manufacturer’s instructions and sent to 1st BASE Co. Ltd (Malaysia) for sequencing. The identities of the isolates were determined on the basis of the highest matching score on BLAST search (Altschul, Gish, Miller, Myers, & Lipman, 1990). A bootstrapped phylogenetic tree of all isolated bacteria based on 16S rDNA sequences was constructed using MEGA 6.0 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) with neighbor joining method using Tamura and Nei model (Tamura & Nei, 1993) at 1000 Bootstrap replications.

2.5. Screening for EPS-producing microbes

This was performed using the disk diffusion method according to Paulo et al. (2012). MRS agar was used for high EPS production by Bacillus sp. (Larpin, Sauvageot, Pichereau, Laplace, & Auffray, 2002; Song, Jeong, & Baik, 2013). Each identified isolate was cultured overnight in MRS broth and the culture of OD600nm = 0.4 (20 μL) was pipetted on a sterile disk plated on MRS agar containing 8% specific sugar (glucose, sucrose or lactose) to screen for EPS-producing isolates and aseptically incubated for 3 days at 37 °C. Isolates with slimy or ropy appearance as potential EPS producers were identified using an inoculating loop to pull the sticky EPS. Negative controls included MRS media without any bacteria, Lactobacillus sp. isolated from Thai Isan sausage and E. coli DH5α that do not produce EPS.

2.6. Scavenging of DPPH free radical

The DPPH radical-scavenging capacity of 10 selected Bacillus spp. was determined according to the method previously described (Li, Mutuvuyla, Chen, Jiang, & Dong, 2012) with some modifications. Firstly, 1.0 mL of Bacillus spp. cells grown overnight in MRS media containing 8% lactose with 109 CFU/mL in phosphate buffered saline (PBS) buffer (pH 7.0), was added to 2.0 mL ethanolic DPPH radical solution (0.05 mM). The mixture was mixed vigorously and incubated at room temperature in the dark for 30 min. The controls included only PBS buffer and DPPH solution whereas the blanks contained only ethanol. The absorbance of the resulting solution was measured in triplicates at 517 nm after centrifugation at 8000g for 10 min. The scavenging ability was defined as:

\[
\text{Scavenging activity(%) } = \left[ 1 - \left( \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}} \right) \right] \times 100
\]
media containing 8% lactose (10⁵ CFU/mL) were inoculated at 1% (v/v) into MRS media containing 1.0 mM hydrogen peroxide (30 wt% solution in water), and incubated at 37 °C for 8 h. The cell growth at OD₆₀₀ₙₙ was measured spectrophotometrically. In parallel, another set of the same bacterial cultures were treated to remove/dest-structure EPS as described above prior to being tested so as to determine whether EPS is responsible for resistance to hydrogen peroxide.

3. Results

3.1. Microbial diversity in Thai milk kefir

The aim of this work was to determine microbial diversity in Thai milk kefir from Kamphaeng Phet province, Thailand that has not been studied before. This Thai milk kefir has been made and consumed in local households and also sold to people who are interested in health-promoting products. The physical characteristics of Thai milk kefir grain were yellow-white and minute, and contained only Gram-positive rod bacteria (Fig. 1). In general, kefir consists of LAB, AAB and yeasts (Farnworth & Mainville, 2003) however we found no colony on MRS agar supplemented with BCP suggesting no LAB was found in this milk kefir (Table 1). Eighty-five colonies (8.5 × 10⁵ CFU/mL) were grown on GYC agar (Table 1) with clear zone indication of acetic acid production that cleared out calcium carbonate and 95 colonies (9.5 × 10⁷ CFU/ml) were grown on YPD agar after 3-day incubation. After that half of colonies on GYC or YPD plates were randomly selected for pure isolation using streak plate method through 5 passages and then Gram staining for morphological study under microscope. All colonies appeared white, circular, convex, smooth, mucoid, and slimy with diameter of 1–2 mm on agar plates. As a result, only Gram-positive rod-shaped bacterial cells of 5 μm × 0.2 μm were observed from all 85 selected pure isolates during Gram staining suggesting there were only bacteria in this kefir. No yeast cells were found suggesting bacteria were grown on YPD agar, not yeasts. These 85 pure bacterial isolates were then identified using PCR-based 16S rDNA gene analysis and subsequently BLAST search. Interestingly, the results showed that out of 85 isolates there were 24 species of Bacillus. Table 2 shows microbial diversity in Thai milk kefir and possible origin of each species. KW1 isolates identified as Bacillus amyloliquefaciens SD-32 (100% identity) were most dominant (67% abundance) followed by KW2 isolates with 95% identity to Bacillus sp. DH25 (7% abundance), KW3 isolates with 95% identity to uncultured Bacillus sp. clone L4 (5% abundance) and the rest of 21 species of Bacillus each accounted for only 1% abundance. Most of the closest relative species of Bacillus were found in Asia i.e. China, Japan, India, Pakistan, Thailand, and Taiwan and a minority was found in South Africa, Argentina and USA (Table 2). Fig. 2 shows a phylogenetic tree of all 24 Bacillus spp. in Thai milk kefir. Bacillus methylotrophicus 3Β_1_1 is closely related to Bacillus sp. C87 and Bacillus sp. LB15 however their EPSs on glucose, sucrose or lactose appeared different (Fig. 3). All B. amyloliquefaciens seemed to be evolved similarly except for B. amyloliquefaciens KW8 yet their EPSs had

Fig. 1. (A) Milk kefir grain from Kamphaeng Phet Province, Thailand. (B) Gram staining of microbes in milk kefir after 1-day incubation in pasteurized cow milk at 37 °C. (C) The location of Kamphaeng Phet Province, Thailand.
These EPSs have sticky characteristics when pulled by an inoculameter of EPSs from lactose and the largest EPSs from sucrose. The results showed that all 24 Bacillus spp. were able to utilise all 3 sugars and produce EPS. The same bacteria produced different EPS appearances from different sugars and different bacteria produced EPSs differently from the same sugar (Fig. 3) despite they are evolutionarily similar. Most bacteria produced the smallest diameters of EPSs from lactose and the largest EPSs from sucrose. These EPSs have sticky characteristics when pulled by an inoculating loop and appeared as biofilms on the surfaces of bacterial cultures in MRS broths with 8% sugar (Fig. 4).

### Table 1
Microbial enumeration on three different media agars.

<table>
<thead>
<tr>
<th>Medium agar</th>
<th>Microbial enumeration (CFU/mL)</th>
<th>Selected colonies/total colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS + BCP</td>
<td>n.d.</td>
<td>0</td>
</tr>
<tr>
<td>GYC</td>
<td>$8.5 \times 10^5$</td>
<td>40/85 (all bacteria)$^a$</td>
</tr>
<tr>
<td>YPD</td>
<td>$9.5 \times 10^7$</td>
<td>45/95 (all bacteria)$^a$</td>
</tr>
</tbody>
</table>

n.d. = not detected.

$^a$ Gram staining images show bacterial morphology of Gram-positive rod cells for all selected colonies without yeasts on YPD agar.

### Table 2
Twenty-four species out of 85 bacterial isolates from Thai milk kefir.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Closest relative</th>
<th>Accession no.$^a$</th>
<th>% identity$^b$</th>
<th>% abundance$^c$</th>
<th>Origin$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>KW1</td>
<td>Bacillus amyloliquifaciens SD-32</td>
<td>AB853319.1</td>
<td>100</td>
<td>67</td>
<td>Antifungal activities against plant pathogens, Japan</td>
</tr>
<tr>
<td>KW2</td>
<td>Bacillus sp. DH25</td>
<td>AB918712.1</td>
<td>95</td>
<td>7</td>
<td>Soil bacteria for controlling tomato wilt, Taiwan</td>
</tr>
<tr>
<td>KW3</td>
<td>Uncultured Bacillus sp. clone L4</td>
<td>FJ863112.1</td>
<td>95</td>
<td>5</td>
<td>Isolated from heavy oil sewage, China</td>
</tr>
<tr>
<td>KW4</td>
<td>Bacillus subtilis MML2483</td>
<td>KF655461.1</td>
<td>91</td>
<td>1</td>
<td>Center for Advanced Studies in Botany, India</td>
</tr>
<tr>
<td>KW5</td>
<td>Bacillus subtilis SY</td>
<td>JQ410791.1</td>
<td>97</td>
<td>1</td>
<td>A Cameroon traditional fermented condiment</td>
</tr>
<tr>
<td>KW6</td>
<td>Bacillus subtilis F-14</td>
<td>KT027665.1</td>
<td>97</td>
<td>1</td>
<td>Salt tolerant rhizobacteria from Salt Range, Pakistan</td>
</tr>
<tr>
<td>KW7</td>
<td>Bacillus subtilis 2C-62</td>
<td>LN885092.1</td>
<td>99</td>
<td>1</td>
<td>Rhizosphere bacteria Island, Indonesia</td>
</tr>
<tr>
<td>KW8</td>
<td>Bacillus amyloliquifaciens BVC15</td>
<td>JQ660598.1</td>
<td>97</td>
<td>1</td>
<td>Straw and goat manure based vermicompost, India</td>
</tr>
<tr>
<td>KW9</td>
<td>Bacillus amyloliquifaciens AD2</td>
<td>KT388663.1</td>
<td>97</td>
<td>1</td>
<td>Soil, South Africa</td>
</tr>
<tr>
<td>KW10</td>
<td>Bacillus amyloliquifaciens MP-18</td>
<td>KJ934385.1</td>
<td>98</td>
<td>1</td>
<td>Different tea growing regions of South India</td>
</tr>
<tr>
<td>KW11</td>
<td>Bacillus sp. LB15</td>
<td>KT216027.1</td>
<td>100</td>
<td>1</td>
<td>Eupatorium odoratum (Siam weed), Thailand</td>
</tr>
<tr>
<td>KW12</td>
<td>Bacillus sp. L. D12_A_P</td>
<td>KT820799.1</td>
<td>100</td>
<td>1</td>
<td>Rice-based fermented beverage (haria), India</td>
</tr>
<tr>
<td>PS13</td>
<td>Bacillus sp. S141</td>
<td>AB909436.1</td>
<td>97</td>
<td>1</td>
<td>PGPR coinoculant on soybean, Thailand</td>
</tr>
<tr>
<td>PS14</td>
<td>Bacillus sp. AR491</td>
<td>LN829575.1</td>
<td>98</td>
<td>1</td>
<td>Cellulose and xylan degrading bacteria, Argentina</td>
</tr>
<tr>
<td>PS15</td>
<td>Bacillus sp. CMU2</td>
<td>EU590016.1</td>
<td>98</td>
<td>1</td>
<td>Antagonists of Lasiodiplodia theobromae, Thailand</td>
</tr>
<tr>
<td>PS16</td>
<td>Bacillus sp. AR493</td>
<td>LN829576.1</td>
<td>99</td>
<td>1</td>
<td>Cellulose and xylan degrading bacteria, Argentina</td>
</tr>
<tr>
<td>PS17</td>
<td>Bacillus sp. O5_001</td>
<td>KJ716480.1</td>
<td>99</td>
<td>1</td>
<td>Epiphytic leaf surface of Oicium sanctum, India</td>
</tr>
<tr>
<td>PS18</td>
<td>Bacillus sp. J-26</td>
<td>KP966464.1</td>
<td>98</td>
<td>1</td>
<td>Diversity of endophytic bacteria, China</td>
</tr>
<tr>
<td>PS19</td>
<td>Bacillus sp. NCTP-1119</td>
<td>LN051461.1</td>
<td>96</td>
<td>1</td>
<td>Epiphytic and desert soil of Pakistan</td>
</tr>
<tr>
<td>PS20</td>
<td>Bacillus sp. BC17</td>
<td>KT361089.1</td>
<td>100</td>
<td>1</td>
<td>Aerobic and anaerobic bacteria from Au-mine, China</td>
</tr>
<tr>
<td>PS21</td>
<td>Bacillus tequilensis xuru17</td>
<td>GQ480486.1</td>
<td>97</td>
<td>1</td>
<td>Technical Center, Xufu Liquor Industry Co. Ltd, China</td>
</tr>
<tr>
<td>PS22</td>
<td>Bacillus tequilensis BVC39</td>
<td>JQ660622.1</td>
<td>97</td>
<td>1</td>
<td>Straw and goat manure based vermicompost, India</td>
</tr>
<tr>
<td>PS23</td>
<td>Bacillus siamensis HQ8720</td>
<td>KT758684.1</td>
<td>98</td>
<td>1</td>
<td>The Coast of Weihai, China</td>
</tr>
<tr>
<td>PS24</td>
<td>Bacillus methylotrophicus 3B_1.1</td>
<td>KT720125.1</td>
<td>100</td>
<td>1</td>
<td>Phoenix facing spacecraft surfaces, USA</td>
</tr>
</tbody>
</table>


$^b$ Identity (%) from BLAST search.

$^c$ Abundance (%) = (identified number of isolates/total isolates)$^*100$.

$^d$ Origins of closest relative species i.e. where each bacterium was isolated from.

3.2. **Bacterial EPS production from different sugars**

All Bacillus spp. isolated from Thai milk kefir were tested for their capacity to produce EPS upon 3 different sugar substrates namely glucose, sucrose, and lactose (8%) in MRS media. The disk diffusion method was used to determine the production of bacterial EPS. The results showed that all 24 Bacillus spp. were able to utilise all 3 sugars and produce EPS. The same bacteria produced different EPS appearance from different sugars and different bacteria produced EPSs differently from the same sugar (Fig. 3) despite they are evolutionarily similar. Most bacteria produced the smallest diameters of EPSs from lactose and the largest EPSs from sucrose. These EPSs have sticky characteristics when pulled by an inoculating loop and appeared as biofilms on the surfaces of bacterial cultures in MRS broths with 8% sugar (Fig. 4).

3.3. **Antioxidant activities of EPS-producing isolates**

Nine EPS-producing Bacillus spp. were chosen to be studied for antioxidant activity of intact cells versus EPS-removed cells using DPPH scavenging assay and resistance to hydrogen peroxide assay. DPPH scavenging assay revealed that bacterial cells with EPS removed or destroyed exhibited significantly lower scavenging activity (8–12%) than those untreated cells (18–27%) (Fig. 5A) indicating ~10–20% reduction in antioxidant capacity of these strains by EPS removal or destruction. Three highest antioxidant isolates are Bacillus sp. KW2, Bacillus sp. KW3 and Bacillus subtilis KW4. Similarly, resistance to H$_2$O$_2$ assay showed that bacterial cells with EPS removed or destroyed had significantly lower growth (OD$_{600\text{nm}} < 0.1$) than those untreated cells (Fig. 5B). Untreated cells with intact EPS seemed to be resistant to 1 mM H$_2$O$_2$ since their growths (OD$_{600\text{nm}}=0.4–0.5$) were very similar to those without 1 mM H$_2$O$_2$ addition (Fig. 5B).

4. **Discussion**

To date there are few reports on microbial diversity in kefirs around the world however not many findings on kefirs in Asia, especially Thailand, have been documented. Our objective was to isolate and identify microbial diversity in Thai indigenous milk kefir. In addition, microbes capable of producing EPS in milk kefir were also identified. These EPS-producing microbes are essential since they are responsible for the formation of kefiran, polysaccharides that make up kefir grains. Without EPS, microbial consortia would not have been formed or existed as kefir grains. There is no confirmed finding yet about the main EPS-producing microbes found in kefirs. It is hoped that the findings of this work will shed the light on these unexplored areas. Bacillus spp. were unable to grow on MRS agar supplemented with BCP (Table 1), however were able to grow on MRS + 8% sugar, GYC and
Korean kimchi was found to produce EPS with antioxidant potential.

Only *Bacillus* spp. were identified in Thai milk kefir from Kamphaeng Phet Province, Thailand. In the literature, Tibet milk kefir also had unusual bacteria *Pseudomonas* sp. as a minority (Jianzhong, Xiaoli, Hanhu, & Mingsheng, 2009). However, Lactobacillus species and yeasts, that have always been reported as prevalent in kefirs (Chen, Wang, & Chen, 2008; Jianzhong et al., 2009; Magalhaes et al., 2010), were not found in our Thai milk kefir. AAB, that have occasionally been detected in kefirs (Chen et al., 2008; Jianzhong et al., 2009; Miguel, Cardoso, Lago, & Schwan, 2010; Dobson, O’Sullivan, Cotter, Ross, & Hill, 2011), were not observed. Interestingly, *Bacillus* sp. was also found along with Lactobacilli, AAB or yeasts in milk kefs from Spain (Angulo, Lopez, & Lema, 1993; Ottogalli, Galli, Resmini, & Volontiero, 1973) whereas our Thai milk kefir contained only *Bacillus* sp. This distinct microbial community of Thai milk kefir from kefirs of Brazil, Tibet, Taiwan, and Ireland (Chen et al., 2008; Dobson et al., 2011; Leite et al., 2012) that mainly consist of LAB and yeasts may lie in culturing environments in Thailand over the years and also *Bacillus* sp., spore-forming species, can be found in soil, water, pond sediments as well as from the intestinal tracts of animals (Hong et al., 2009) and thus they can be easily obtained in kefirs upon passage over many generations. Alternatively, the starter culture of this Thai milk kefir may be composed of distinct components in the first place since the closest relative species of *Bacillus* found in Thai milk kefir were mainly found in Asia i.e. China, Japan, India, Pakistan, Thailand, and Taiwan (Table 2) where *Bacillus* spp. are prevalent in Asian local foods and food ingredients. For example, *Bacillus licheniformis* KS-17 isolated from Korean kimchi was found to produce EPS with antioxidant potential (Song et al., 2013). The novel *B. licheniformis* strain B3-15 has been demonstrated to possess EPS with antiviral and immunostimulant effects (Arena et al., 2006). To date very few reports on metabolisms of *Bacillus* spp. were available and therefore we used metabolic model of *B. subtilis* (Cruz et al., 2000) for discussion. It was found that *B. subtilis* can produce lactate, acetate, acetoin, ethanol, succinate and 2,3-butanediol from the substrates i.e. glucose and pyruvate during anaerobic metabolism (Cruz et al., 2000). This led us to propose that *Bacillus* sp. in our Thai milk kefir were able to produce acetic acid and alcohol from lactose substrate in milk. This was in accordance with our preliminary result with the detection of acetic acid and alcohol by HPLC and GC-MS analyses, respectively without lactic acid in Thai milk kefir (data not shown). *B. amyloliquifaciens* was most dominant in this Thai milk kefir. According to proteome data regarding *B. amyloliquifaciens* on Uniprot (http://www.uniprot.org/), it has beta-galactosidase or 6-phospho-beta-galactosidase for metabolising lactose in milk kefir to glucose and galactose that are further metabolisable. In addition, *B. amyloliquifaciens* also has lactate dehydrogenase to produce lactic acid, acetate kinase to produce acetic acid and alcohol dehydrogenase to produce alcohol. It is not clear yet why no lactic acid was detected in this Thai milk kefir, but we proposed that (1) the lactic acid amount produced may be negligible by HPLC analysis, (2) metabolites generated from several *Bacillus* sp. in Thai milk kefir may inhibit lactate dehydrogenase, responsible for lactic acid production or (3) some *Bacillus* sp. may exhibit Clostridial-type fermentation where pyruvate from glycolysis is oxidised to acetyl-CoA by pyruvate:ferredoxin oxidoreductase in the presence of ferredoxin. The pathway leads to production of acetic acid, ethanol and acetoacetyl-CoA with the latter can be further metabolised to butyric acid (Sikora, Błaszczyk, Jurkowski, & Zielenkiewicz, 2013). In a similar report, large amounts of acetic, propionic and succinic acids were produced by *B. licheniformis* in traditional fermented Chinese liquor called *Daqu* while lactic acid was found in smaller amounts (Yan, Zheng, Chan, Han, & Han, 2013). In addition, 3 out of 18 samples of Doenjangs, traditional Korean fermented soybean paste products with predominant *B. subtilis*, showed no lactic acid production while the other 15 samples showed only 1.53–51.73 mg of lactic acid/100 g in comparison with greater amounts of acetic acid (31.74–178.28 mg/100 g) produced in all samples (Shukla et al., 2010). The authors did not explain the reason for the lack of lactic acid production, but it was possibly due to different metabolic pathways of different *Bacillus* species.

Few *Bacillus* strains have also been considered as promising probiotics in recent years. Compared to the well-established LAB and bifidobacteria (Patel, Ahire, Pawar, Chaudhari, & Chincholkar, 2009), they have also been found to be excellent EPS producers and they exhibit beneficial effects. EPSs can exhibit immunomodulatory, antiviral, antiulcer and antioxidant effects and used as drug carriers (Maugeri et al., 2013; Parikh & Madamwar, 2006) in pharmaceutical industry. EPSs are also used as stabilisers, emulsifiers, gelling agents (Yukseldag & Aslim, 2008) in food industry. Compared to synthetic polymers, bacterial EPS are more prone to natural biodegradation and therefore constitute less to environmental pollution (Patel, Michaud, Singhania, Soccol, & Pandey, 2010) and this has been recognised as its big advantage. *B. subtilis* has already been provided with Generally Recognised as Safe (GRAS) status (Cheon, Kim, Park, Han, & Kim, 2009) and many have been reported for its antioxidant properties. *B. subtilis* NRClAza and and *B. subtilis* MTCC 121 produced EPS with antioxidant or/and antitumor properties (Abdel-Fattah, Gamal-Eldin, Helmy, & Esway, 2012; Vijayabaskar, Babinastarin, Shankar, Savukumar & Anandapandian, 2011). *B. amyloliquifaciens* known for their catalytic properties and degradation of complex macromolecules can form a strong endospore when conditions are not favourable (Rao,
Fig. 3. EPS production by 24 Bacillus spp. using disk diffusion method on MRS agar plate +8% glucose (first column), sucrose (middle column) and lactose (third column).
13) *Bacillus* sp. PS13

14) *Bacillus* sp. PS14

15) *Bacillus* sp. PS15

16) *Bacillus* sp. PS16

17) *Bacillus* sp. PS17

18) *Bacillus* sp. PS18

19) *Bacillus* sp. PS19

20) *Bacillus* sp. C87

21) *Bacillus tequilensis* PS21

22) *Bacillus tequilensis* PS22

23) *Bacillus siamensis* PS23

24) *Bacillus methylotrophicus* 3B_1.1

Fig. 3. (continued)
Sudharsan, Sekaran, & Mandal, 2013). The predominant \textit{B. amyloliquefaciens} in the present work can be considered as an alternative promising probiotic species. Recent study showed that \textit{B. amyloliquefaciens} strain C-1, isolated from ready-to-eat sliced apple samples, had significant antioxidant activities and its EPS had considerable potential to be used as medical compounds or functional additives (Yang et al., 2015). In addition, antitumor activity of bacterial EPS from the endophyte \textit{B. amyloliquefaciens} sp. isolated from \textit{Ophiopogon japonicas} (Chen et al., 2013) was reported. Another group demonstrated that EPS from \textit{B. amyloliquefaciens} LPL061 had potential for industrial use (Han et al., 2015). Our results show that \textit{Bacillus tequilensis} was able to produce EPS for the first time and also support the previous study that a soil isolate, \textit{B. methylotrophicus}, was able to produce EPS (Ezhil, Belur, & Saidutta, 2015). \textit{B. subtilis}, also found in this work, has been used in several commercial probiotics products in Europe, China, USA e.g. Biosporin, Bio-Kult Medilac-Vita, Nature’s First, and Food Primal Defense (Cutting, 2011). Bacilli, often considered soil organisms, should be considered as gut commensals and are being used as probiotic supplements for use in animal feeds, for human dietary supplements as well as in registered medicines in recent years.

This is the first report of \textit{Bacillus} spp. being identified as EPS producers in milk kefir. This is very different from previous reports on EPS producers in kefirs of other countries that usually found LAB such as \textit{Lactobacillus casei}, \textit{Lb. higardii}, \textit{Lb. hordei}, \textit{Lb. nagelii}, \textit{Lactococcus citreum}, \textit{Lc. mesenteroides} capable of producing EPS in water kefir in Germany (Gulitz, Stadie, Wenning, Ehrmann, & Vogel, 2011). These \textit{Bacillus} spp. are thought to play a key role in forming kefir grains in this study as shown by their stickiness and biofilm formation in MRS broth (Fig. 4). In this work, sucrose was a better carbohydrate source for EPS (Fig. 3) as compared to glucose and lactose since EPS was produced on sucrose by most bacteria and had the largest diameter. This finding is in accordance with the recent work showing that \textit{Bacillus} sp. produced highest EPS on sucrose (Shukla, Patel, & Duggirala, 2015). Appearances of the EPSs of each \textit{Bacillus} on different sugars were different possibly due to different kinds of polymers produced from each species on each sugar. Removal or destruction of EPS on these \textit{Bacillus} spp. has lowered their antioxidant activities suggesting bacterial EPS plays a role in antioxidation of kefirs. Their EPS from our work is thought to be secreted by the cells or are just slimes loosely associated to cell surface. This finding is in agreement with a previous study confirming that the cell-surface proteins or polysaccharides of \textit{Lactobacillus plantarum} CS8 were involved in its antioxidant activity, since removing these cell surface compounds led to significant decrease of the DPPH free radical scavenging capacity of the strain (Li et al., 2012). Our results showed higher antioxidant activity (18–27%) in \textit{CFU/mL Bacillus} spp. based on DPPH scavenging.
antioxidant properties can be used as wound healers in medicine, as cosmetic ingredients or food additives. This area is definitely worth exploring in the future.

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References


Cheon, J., Kim, S. B., Park, S. W., Han, J. K., & Kim, P. (2009). Characterization of L-arabinose isomerase in Bacillus subtilis, a GRAS host, for the production of edible tagatose. Food Biotechnology, 23, 8–16.


