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# Microbial Strains and Bioactive Exopolysaccharide Producers from Thai Water Kefir

Vijitra Luang-In[1]*, Worachot Saengha[1], Manatchanok Yotchaisarn[1], Monika Halaslova[2], Piyachat Udomwong[3], and Sirirat Deeseenthum[1]

[1]*Natural Antioxidant Innovation Research Unit, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Khamriang, Kantarawichai, Mahasarakham 44150, Thailand*
[2]*Institute of Biotechnology and Food Science, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Radlinského 9, 81237 Bratislava, Slovakia*
[3]*International College of Digital Innovation, Chiang Mai University, Chiang Mai 50200, Thailand*

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**The aims of this novel work were to determine the microbial strains and exopolysaccharide (EPS) producers in water kefir from Nakhon Ratchasima Province, Thailand. Thirty-three microbial strains were identified using 16S rRNA gene analysis consisting of 18 bacterial strains, as 9 strains of acetic acid bacteria (AAB), 9 strains of lactic acid bacteria (LAB), and 15 yeast strains. All bacteria were able to produce EPS with a diverse appearance on agar media containing different sugars at a concentration of 8%. Culture supernatants from AAB and LAB showed 31–64% 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity with the highest antioxidant activity of 64% from *Acetobacter pasteurianus* WS3 and WS6. Crude EPS from *A. pasteurianus* WS3 displayed the highest ferric reducing antioxidant power at 280 mM $FeSO_4$/g EPS, greatest anti-tyrosinase activity at 20.35%, and highest EPS production of 1,505 mg EPS/L from 8% sucrose. These microbes offer beneficial health implications and their EPSs can be used as food additives and cosmetic ingredients.**

**Keywords: Antioxidant, exopolysaccharide, kefir, lactic acid bacteria, tyrosinase**

## Introduction

Functional foods have become increasingly popular, with homemade fermented products gained attention of health conscious consumers worldwide [1]. In Thailand, the majority of the population still live in rural areas and the villagers ferment foods including Thai indigenous vegetables, fruits or fish using local wisdom passed down from generation to generation. In the past 10 years, kefirs have become more widely recognized as alternative fermented products that Thai house-holds can easily prepare at low cost from drinking water containing brown sugar fermented with kefir grain for only 1–3 days at room temperature and this is called water kefir. In addition, milk kefir can also be prepared by inoculating cow milk with kefir grains [2]. Kefirs are light-sour, alcoholic and aromatic drinks made from kefir grains as starter cultures, consisting mainly of lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeasts [3, 4]. Evidence of the beneficial health properties of kefirs in addition to their nutritive values (rich in amino acids, vitamin K, B12, organic acids) including antioxidation, anti-inflammation, and antimicrobial activity has emerged [5], rendered kefirs as probiotic products. Kefir starter cultures or so-called kefir grains are made of exopolysaccharides (EPSs) produced by some bacteria such as *Lactobacillus hilgardii* TMW 1.828 in water kefir from Germany [6], and *L. kefirano-*

***Corresponding author**
Tel: +66(0)43754085, Fax: +66(0)43754086
E-mail: vijitra.l@msu.ac.th
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*faciens* [7]. Some *Acetobacter* sp. also can produce EPS such as *Acetobacter tropicalis* SKU1100 [8]; however, *Acetobacter* sp. from kefir has not been reported as an EPS producer. Microbial EPSs are polymers, primarily consisting of carbohydrates biosynthesized by bacteria [9], fungi [10] and cyanobacteria [11]. EPSs are grouped into two types as (1) Heteropolysaccharides with high molecular mass composed of numerous sugar monomers, synthesized by the orchestrated action of various glycosyl transferases, and (2) Homopolysaccharides with lower molecular mass consisting of one sugar monomer synthesized by one or few glycosyl transferases [12]. The antioxidant activities of these microbial EPSs are not clearly understood, however they have typically been attributed to the presence of excessive numbers of different monomers, high hydrophobicity and a strong anionic nature [13]. Although kefirs have existed in Thailand for some time, knowledge concerning their properties is scarce. Recently, the first report of Thai milk kefir from Kamphaeng Phet Province was documented [14] but Thai water kefir has yet to be studied. Also, knowledge of EPS producers in Thai water kefir is still limited. Thus, here the microbial strains of Thai water kefir from Nakhon Ratchasima Province, commonly homemade by local villagers, was determined using 16S rRNA gene analysis and EPS producers were identified. The antioxidant and anti-tyrosinase activities of bacterial EPSs were also examined.

## Materials and Methods

### Water kefir preparation

A water kefir grain was purchased on-line from a supplier (JibKefirWater) in Nakhon Ratchasima Province, Thailand. This kefir grain is commonly used to produce water kefir by local villagers in the region. The water kefir grain was prepared in a sucrose solution (100 g/l) in 100 ml sterile distilled water in a 250 ml flask aerobically incubated at 37℃ without shaking for 2 days. Kefir grain was propagated under laboratory conditions twice to eliminate the environmental effects from the supplier and the process was repeated to obtain water kefir.

### Growth conditions of microorganisms

Microbes from water kefir were grown aerobically on three media including De Man, Rogosa and Sharpe (MRS) agar (HIMedia Laboratories, India) supplemented with bromocresol purple, glucose, yeast extract, calcium carbonate (GYC) agar at 37℃ and yeast extract peptone dextrose (YPD) agar (HIMedia Laboratories, India) at 30℃ for selection of LAB, AAB and yeasts, respectively as previously described [14]. Positive colonies were streaked five times on selective media to obtain pure isolates. Pure isolates were identified using PCR-based 16S rRNA and 26S rRNA gene analyses for bacteria and yeasts, respectively. All microbial isolates were deposited in the Natural Antioxidant Innovation Research Unit, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Thailand (WDCM 1160).

### Microbial identification and phylogenetic tree construction

Genomic DNA isolation from the selected microbes was performed as previously reported [14]. Bacteria were identified by partial sequencing of the 16S rRNA amplified with the universal forward primer AmpF (5’-GAGAGTTTGATYCTGGCTCAG-3’) and the reverse primer AmpR (5’-AAGGAGGTGATCCARCCGCA -3’), and a PCR program of 94℃ for 2 min, 32 cycles of 94℃ for 45 s, 54℃ for 45 s, 72℃ for 1 min and a last step at 72℃ for 7 min. Yeasts were identified by partial sequencing of the 26S rRNA using the primers NL1 (5’-GCATATCAATAAGCGGAGGAAAAG-3’) and NL4 (5’-GGTCCGTGTTTCAAGACG G-3’). The PCR reaction mixture (25 μl) consisted of 0.1 mM of each deoxynucleoside 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 in a 0.8% (w/v) agarose gel (Vivantis, Malaysia), cleaned-up using 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. Bootstrapped phylogenetic trees of all isolated bacteria based on 16S rRNA partial sequences and yeasts based on 26S rRNA partial sequences were constructed using MEGA 7.0 software [15] with maximum likelihood method using Kimura 2-parameter [16] at 1,000 Bootstrap replications. The phylogenetic trees were drawn by FigTree [17].

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### Screening for microbial EPS producers

Screening was performed using the disk diffusion method [18]. Each identified isolate was cultured overnight in their corresponding broth till the culture reached $OD_{600nm}$~0.4. Bacterial culture (20 µl) was then pipetted on a sterile disk plated on modified MRS agar without glucose (for LAB and AAB), and modified YPD agar without dextrose (for yeasts) containing 8% specific sugar (glucose, sucrose or lactose) to screen for EPS-producing capacity from identified LAB, AAB and yeasts. Plates were aerobically incubated for 3 days at 37℃ for bacteria and 30℃ for yeasts. EPS producers were identified as having a slimy, ropy or glossy appearance and could be pulled up using an inoculating loop [14]. Negative controls included MRS medium with no bacteria, *Lactobacillus* sp. isolated from Thai Isan sausage and *Escherichia coli* DH5α which do not produce EPS.

### Scavenging of DPPH free radical by bacterial supernatants

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity of bacterial isolates was determined from their supernatants possibly containing EPS released from bacterial cells. LAB cultures (10 ml) in modified MRS broth (without glucose) and AAB cultures (10 ml) in LB broth (pH 7.0) were grown on 8% sucrose till $OD_{600nm}$~0.7 was reached, and then centrifuged at 16,100*g* for 30 min. Briefly, 1.0 ml of bacterial supernatant 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 broth containing 8% sucrose and DPPH solution whereas the blanks contained only ethanol. Absorbance of the resulting solution was measured in triplicate at 517 nm. Scavenging ability of bacterial supernatant was defined as:

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

### Extraction of crude EPS

Each culture from the four selected bacteria with high DPPH scavenging activity from the above assay; *A. pasteurianus* WS3, *A. pasteurianus* WS6, *P. pentosaceus* WS11 and *L. casei* WS13 was collected at 72 h during a stationary phase ($OD_{600nm}$~0.7). The cultures were centrifuged at 16,100*g* at 4℃ for 30 min, and then two volumes of cold absolute ethanol were added to the supernatant and stored at 4℃ for 24 h. After centrifugation, the EPS precipitate was washed twice with absolute ethanol. The fresh cell pellet was weighed before drying to constant weight in an oven at 60℃. Dried EPS (200 mg) was dissolved in 10 ml sterile distilled water, and 20% (v/v) trichloroacetic acid was added to remove protein contamination as a precipitate at 4℃ for 1 h. The mixture was then centrifuged at 16,100*g* for 10 min. The supernatant (EPS fraction) was partially purified using Vivaspin 10 kDa MWCO membrane ultrafiltration (Sigma-Aldrich, Singapore) and freeze dried. The dried crude EPS was weighed and kept at -20℃ until required.

### Ferric reducing antioxidant power (FRAP) assay

The antioxidant activity of each bacterial EPS was evaluated using FRAP assay with some modifications to the previous method [19]. FRAP reagent was prepared in 300 mM acetate buffer pH 3.6 by adding acetic acid, 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM iron (III) chloride solution ($FeCl_3$) at proportions of 1:1:10 (v/v/v), respectively. A total of 100 µl EPS solution (20 mg/ml) was added to 3,000 µl of the FRAP reagent, mixed well and incubated for 15 min at room temperature. Absorbance was measured at 595 nm using a spectrophotometer with iron (II) sulfate ($FeSO_4$) used as a standard. Results were expressed as mM $FeSO_4$/g DW.

### $ABTS^{\bullet+}$ antioxidant assay

The $ABTS^{\bullet+}$ antioxidant assay was carried out with some modifications to the previously reported method [20]. A total of 100 µl EPS sample (20 mg/ml) was added to 1,000 µl of 2,2'-Azino-di(3-ethyl-benzthiazoline sulfonic acid ($ABTS^{\bullet+}$) radical cation solution and mixed well. Absorbance was measured at 734 nm after 15 min incubation at room temperature. The control included only water and $ABTS^{\bullet+}$ solution whereas the blank contained only water. The antioxidant ability of bacterial EPS was defined as:

$$ABTS^{\bullet+}\text{ antioxidant activity (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}}] \times 100$$

### Anti-tyrosinase activity of EPS

This was performed according to a previous report [21]. Ten microliters of EPS extract (20 mg/ml) was mixed with 20 µl of 1000 U/ml mushroom tyrosinase (Sigma-Aldrich, Singapore) in 110 µl of phosphate buffer pH 6.5 in a microplate, and incubated at 37℃ for 10 min at room temperature. Ten microliters of 10 mM levodopa (L-DOPA) was added to the mixture and incubated at 37℃ for 40 min at room temperature. Absorbance at 490 nm was recorded using a spectrophotometer with a buffer mixed with L-DOPA and tyrosinase as a control. All experiments were performed in triplicate. Tyrosinase inhibition was calculated from the equation below:

$$\text{Tyrosinase inhibition (\%)} = [1 - (A_{490nm}\ \text{sample})/(A_{490nm}\ \text{control})] \times 100$$

### Scanning electron microscopic analysis

A scanning electron microscope (SEM) was used to study the surface morphology of Thai water kefir grain and crude EPS extract. Dried water kefir or EPS (2 mg) was coated with a 30 nm layer of gold before observation under SEM (Carl Zeiss, Leo/1450) at accelerating voltage of 5 kV at the Faculty of Engineering, Khon Kaen University, Thailand.

### Statistical analysis of data

Measurements were obtained in triplicate as means ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's honest significant difference (HDS) test by SPSS software (version 19) at $p < 0.05$.

**Table 1. Microbial enumeration on three media.**

| Medium/ Microbes | Microbial enumeration (CFU/ml) | Selected colonies/ total colonies |
|---|---|---|
| MRS/LAB | $6.2 \times 10^8$ | 31/62 |
| GYC/AAB | $6.2 \times 10^6$ | 31/62 |
| YPD/Yeast | $1.0 \times 10^8$ | 50/100 |

## Results

### Microbial strains in Thai water kefir

The first report of microbial consortia in Thai milk kefir has recently been published [14], however microbes in Thai water kefir have yet to be elucidated. Thus the aim of this study was to determine the microbial strains in Thai water kefir. Thai water kefir grain appeared clear, light brown-orange and minute (Fig. 1A) from Nakhon Ratchasima Province (Fig. 1B) [14°58'29.99" N 102°05'60.00" E] and contained Gram-positive rod, coccal, and Gram-negative rod bacteria (Fig. 1C) and yeasts (Fig. 1D). Sixty-two colonies ($6.2 \times 10^5$ CFU/ml from $10^{-4}$ dilution) were grown on MRS agar supplemented with bromocresol purple (Table 1) with yellow zone indication of lactic acid production by putative LAB after 3 days of incubation at 37℃, and 62 colonies ($6.2 \times 10^8$ CFU/ml from $10^{-6}$ dilution) were grown on GYC agar with a clear zone indicating acetic acid production by putative AAB that cleared out calcium carbonate. One hundred yeast colonies ($1.0 \times 10^8$ CFU/ml from $10^{-5}$ dilution) were grown on YPD agar at 30℃. Half the colonies on each plate were then selected, based on different colony morphology and Gram stained, then streaked through 5 pas-

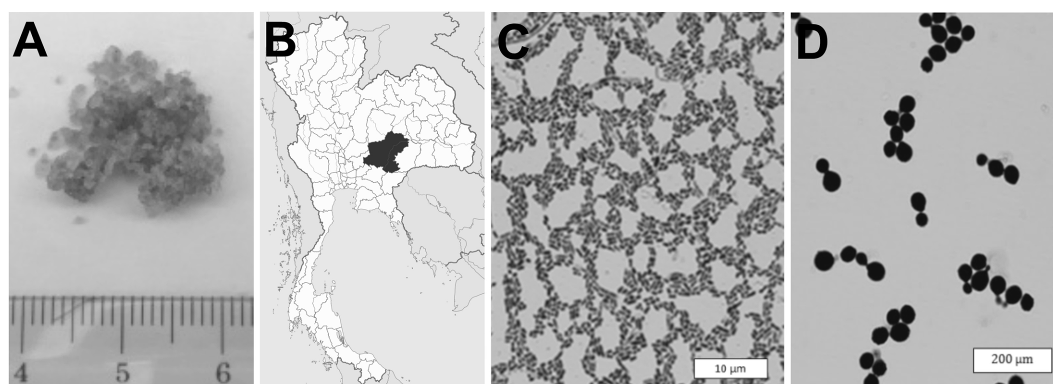


**Fig. 1. Thai water kefir.** (A) Size of water kefir grain. (B) Location of Nakhon Ratchasima Province in Thailand. (C) Magnification at 1000X of LAB in water kefir under microscope. (D) Magnification at 1000X of yeasts in water kefir under microscope.

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**Table 2. Eighteen strains identified out of 62 bacterial isolates and 15 strains identified out of 50 yeast isolates from Thai water kefir.**

| No. | Bacterial strains | Isolates (%)[a] | Accession no.[b] | % identity[c] | Origin of closest relative[d] |
|---|---|---|---|---|---|
| 1 | *Acetobacter pasteurianus* WS1 | 13 (21.31%) | LC336429.1 | 99% | MF179549.1 *Acetobacter pasteurianus* L1, fermented tofu whey, China |
| 2 | *Acetobacter pasteurianus* WS2 | 12 (19.67%) | LC336430.1 | 99% | KX622697.1 *Acetobacter pasteurianus* JBARES-MG-3, makgeolli (Korean fermentation alcohol, Korea |
| 3 | *Acetobacter pasteurianus* WS3 | 7 (11.48%) | LC336431.1 | 97% | KY287771.1 *Acetobacter pasteurianus* DI10SB1, fermentation of Mezcal in Oaxaca, Mexico |
| 4 | *Acetobacter pasteurianus* WS4 | 7 (11.48%) | LC336432.1 | 87% | KT283052.1 *Acetobacter pasteurianus* AS1.41, hinese vinegar Pei, China |
| 5 | *Acetobacter pasteurianus* WS5 | 4 (6.56%) | LC336433.1 | 98% | KX424632.1 *Acetobacter pasteurianus* 1HSc, palm sap, Italy |
| 6 | *Acetobacter pasteurianus* WS6 | 1 (1.64%) | LC336434.1 | 100% | AB906418.1 *Acetobacter pasteurianus* SKU129, santol, Thailand |
| 7 | *Acetobacter pasteurianus* WS7 | 1 (1.64%) | LC336435.1 | 98% | KX424631.1 *Acetobacter pasteurianus* 1RMc, palm sap, Italy |
| 8 | *Acetobacter* sp. WS8 | 1 (1.64%) | LC336436.1 | 96% | KP205437.1 *Acetobacter* sp. SLV-203, Korean traditional vinegar, Korea |
| 9 | *Acetobacter tropicalis* WS9 | 1 (1.64%) | LC336437.1 | 99% | MG225384.1 *Acetobacter tropicalis* LAABBM7A, fermented beans, Brazil |
| 10 | *Pediococcus pentosaceus* WS10 | 4 (6.56%) | LC336438.1 | 98% | KU892402.1 *Pediococcus pentosaceus* PPOF134, traditional cereal-based fermented food, Nigeria |
| 11 | *Pediococcus pentosaceus* WS11 | 1 (1.64%) | LC336439.1 | 98% | KP189225.1 *Pediococcus pentosaceus* V3-100, fermentation of wheat for lemzeiet production, Algeria |
| 12 | *Pediococcus pentosaceus* WS12 | 1 (1.64%) | LC336440.1 | 94% | JQ446485.1 *Pediococcus pentosaceus* QN1D, traditional fermented foods, Ecuador |
| 13 | *Lactobacillus casei* WS13 | 3 (4.92%) | LC336441.1 | 100% | MF429431.1 *Lactobacillus casei* CAU2903, rushan, China |
| 14 | *Lactobacillus casei* WS14 | 1 (1.64%) | LC336442.1 | 99% | MF354567.1 *Lactobacillus casei* CAU:2544, soil, China |
| 15 | *Lactobacillus casei* WS15 | 1 (1.64%) | LC336443.1 | 99% | KM485569.1 *Lactobacillus casei* MJM60422, food, Korea |
| 16 | *Lactococcus hircilactis* WS16 | 1 (1.64%) | LC336444.1 | 100% | KJ201026.1 *Lactococcus hircilactis* DSM 28960, milk, Italy |
| 17 | *Lactococcus lactis* WS17 | 1 (1.64%) | LC336445.1 | 98% | HM218132.1 *Lactococcus lactis* subsp. *lactis* NM26-6, naturally fermented dairy products, China |
| 18 | *Lactococcus lactis* WS18 | 1 (1.64%) | LC336446.1 | 98% | KT260999.1, *Lactococcus lactis* RCB787, bat guano, India |
| | Total bacteria | 62 (100%) | | | |

sages to obtain purified isolates. Some colonies grown on GYC agar were identified as LAB and some on MRS agar were identified as AAB. A BLAST search determined that 9 strains of *Acetobacter* spp. were predominant as well as 9 strains of LAB including *Lactobaciilus* spp., *Lactococcus* spp. and *Pediococcus* spp. with 13 yeast strains as mainly *Saccharomyces* spp. and 2 strains of *Meyerozyma guilliermondii* (Table 2). Most of these bacteria shared high similarity (98–100%) with closest relatives found in Asian countries including China, Thailand, Korea and India. Most *S. cerevisiae* strains are extremely similar (99–100% identity) with closest relatives found in China. *M. guilliermondii* found in our work was similar (98% identity) to yeasts isolated from cultivated kiwi fruit land and fruit skin in China. Phylogenetic analysis of bacteria (Fig. 2A) showed two main clusters of bacteria classified into LAB and AAB. *L. hircilactis* WS16, as an outlier, was evolutionarily different from other LAB and AAB. LAB can be grouped into 3 sub-groups as *L. casei*, *Pc. pentosaceus* and *L. lactis*. The reference strain *L. casei* KLDS 1.0720 (EU626005.1) was evolutionarily similar to our *L. casei* strains. However, another reference strain, *L. lactis* NS32 (EU194346.1) found in Brazilian kefir [4] was evo-

**Table 2. Continued.**

| No. | Yeast strains | Isolates (%)[a] | Accession no.[b] | % identity[c] | Origin of closest relative[d] |
|---|---|---|---|---|---|
| 19 | *Saccharomyces cerevisiae* TC1 | 10 (20%) | LC336447.1 | 100% | EU556339.1 *Saccharomyces cerevisiae*, China |
| 20 | *Saccharomyces cerevisiae* TC2 | 9 (18%) | LC336448.1 | 99% | KJ850219.1 *Saccharomyces cerevisiae* L4, distiller's yeast, China |
| 21 | *Saccharomyces cerevisiae* TC3 | 8 (16%) | LC336449.1 | 99% | KJ850221.1 *Saccharomyces cerevisiae* L6, distller's yeast, China |
| 22 | *Saccharomyces cerevisiae* TC4 | 5 (10%) | LC336450.1 | 100% | KT922993.1 *Saccharomyces cerevisiae* NS-G-24, wine, Spain |
| 23 | *Saccharomyces cerevisiae* TC5 | 3 (6%) | LC336451.1 | 100% | JQ824874.1 *Saccharomyces cerevisiae* LCBG-3Y4, spontaneous fermentation of mescal, Mexico |
| 24 | *Saccharomyces cerevisiae* TC6 | 2 (4%) | LC336452.1 | 99% | HM107783.1 *Saccharomyces cerevisiae* q5, Chinese rice wine, China |
| 25 | *Saccharomyces cerevisiae* TC7 | 2 (4%) | LC336453.1 | 99% | HM107797.1 *Saccharomyces cerevisiae* 4, Chinese rice wine, China |
| 26 | *Saccharomyces cerevisiae* TC8 | 2 (4%) | LC336454.1 | 100% | JQ824873.1 *Saccharomyces cerevisiae* LCBG-3Y8, spontaneous fermentation of mescal, Mexico |
| 27 | *Saccharomyces cerevisiae* TC9 | 2 (4%) | LC336455.1 | 100% | HM191635.1 *Saccharomyces cerevisiae* NL4, Musalais grape wine, China |
| 28 | *Saccharomyces cerevisiae* TC10 | 2 (4%) | LC336456.1 | 100% | HM191636.1 *Saccharomyces cerevisiae* NL5, Musalais grape wine, China |
| 29 | *Saccharomyces cerevisiae* TC11 | 1 (2%) | LC336457.1 | 100% | JQ512832.1 *Saccharomyces cerevisiae* 29, distillery, Spain |
| 30 | *Saccharomyces cerevisiae* TC12 | 1 (2%) | LC336458.1 | 100% | HM191649.1 *Saccharomyces cerevisiae* NL18, Musalais grape wine, China |
| 31 | *Saccharomyces cerevisiae* TC13 | 1 (2%) | LC336808.1 | 100% | HM123751.1 *Saccharomyces cerevisiae* SL7-5-2, fermentation solution, China |
| 32 | *Meyerozyma guilliermondii* TC14 | 1 (2%) | LC336809.1 | 99% | KJ794675.1 *Meyerozyma guilliermondii* B-NC-13-OZ01, kiwi fruit producing area, China |
| 33 | *Meyerozyma guilliermondii* TC15 | 1 (2%) | LC336810.1 | 99% | JX041892.1 *Meyerozyma guilliermondii* DSSL-1, surface of fruits, China |
| | Total yeasts | 50 (100%) | | | |

[a]Number of isolates and abundance percentage (%) = (identified number of isolates/total isolates) x 100
[b]GenBank accession no. of strains on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed)
[c]Based on BLAST search results, identity (%) of strains compared to the closest relatives.
[d]Based on BLAST search results, origin of the closest relatives.

lutionarily diverged from our *Lactobacillus* and *Pediococcus* strains. AAB can be grouped into 2 sub-groups as *A. tropicalis* and *A. pasteurianus*. *A. tropicalis* WS9 isolated from this study appeared to be evolutionarily similar to *A. tropicalis* NRIC 0312 (AB032354.1) in Belgian kefir [22], but different from *A. orientalis* (AB052707) found in Brazilian kefir [4]. The reference strain *A. pasteurianus* subsp. *pasteurianus* (X71863.1) found in Belgian kefir [22] was evolutionarily similar to our *Acetobacter* sp. WS1, *A. pasteurianus* WS5 and WS6. In addition, the phylogenetic analysis of yeasts (Fig. 2B) showed two main clusters of yeasts classified into *S. cerevisiae* and *M. guilliermondii*. All of our *S. cerevisiae* strains from this work were evolutionarily similar to the two reference yeast strains, *S. cerevisiae* VI2 (EU441887.1) found in white wine in Italy and *S. cerevisiae* CHFY0901 (EU649673.1) from bioethanol production in South Korea. Another reference strain, *S. cerevisiae* (EU019225.1) isolated from Koumiss in Xinjiang of China seemed to be evolutionarily different from other *Saccharomyces*; however similar to *Pichia guilliermondii* HK58-2 (EF197951.1). The two isolates of *M. guilliermondii* (TC14 and TC15) are in a separate group from *S. cerevisiae* and *P. guilliermondii* indicating that

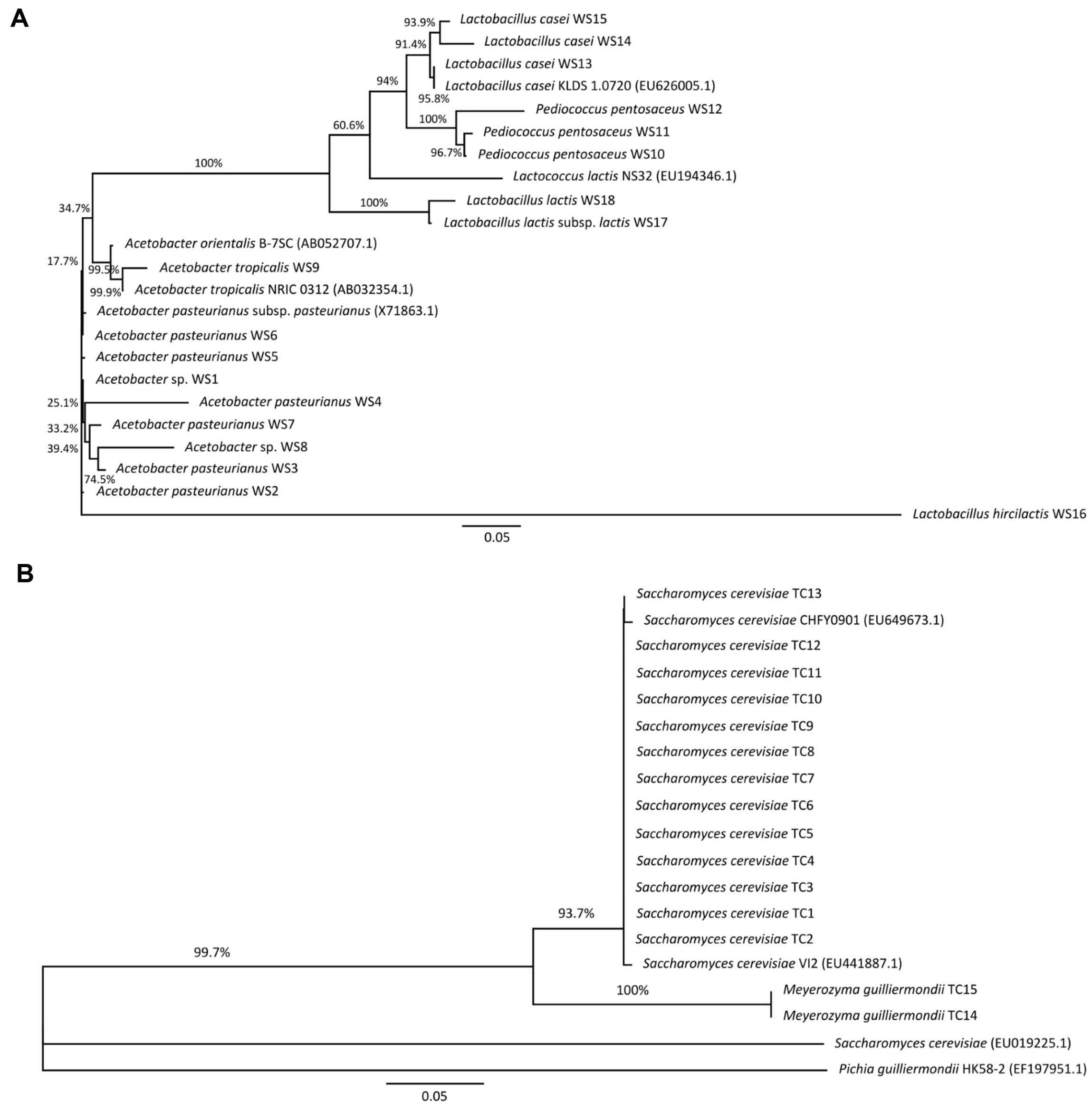



**Fig. 2. Phylogenetic trees of microbes.** (A) It was constructed from bacterial 16S rRNA partial sequences (ca. 1,500 bp). (B) It was constructed from yeast 26S rRNA partial sequences (ca. 600 bp) based on Kimura 2-parameter using Maximum Likelihood method with 1,000 Bootstrap replications MEGA7. The phylogenetic trees were drawn using FigTree. The horizontal bar at the bottom of the figure represents a distance of 0.1 substitutions per site.

they were evolutionarily different.

### Bacterial EPS producers on sugars

Since little was known about microbes responsible for EPS production in kefir grains, our aim was to identify EPS producers in Thai water kefir based on the disk diffusion method using 3 different sugars (80 g/l glucose, sucrose or lactose separately) in modified MRS agar without glucose (for bacteria) and modified YPD agar without dextrose (for yeasts). Many authors have reported on EPS-producing strains isolated from Tibetan kefir, with most identified as LAB such as *Leuconostoc pseudomesenteroides* [23] and *L. plantarum* [24]. Here, all LAB and AAB strains isolated in this study were found to be EPS producers, except when lactose was used. However, some bacterial strains including *A. pasteurianus* WS4, WS6, SW7, *L. casei* WS15 and *L. lactis* WS18 were unable to produce EPS on lactose

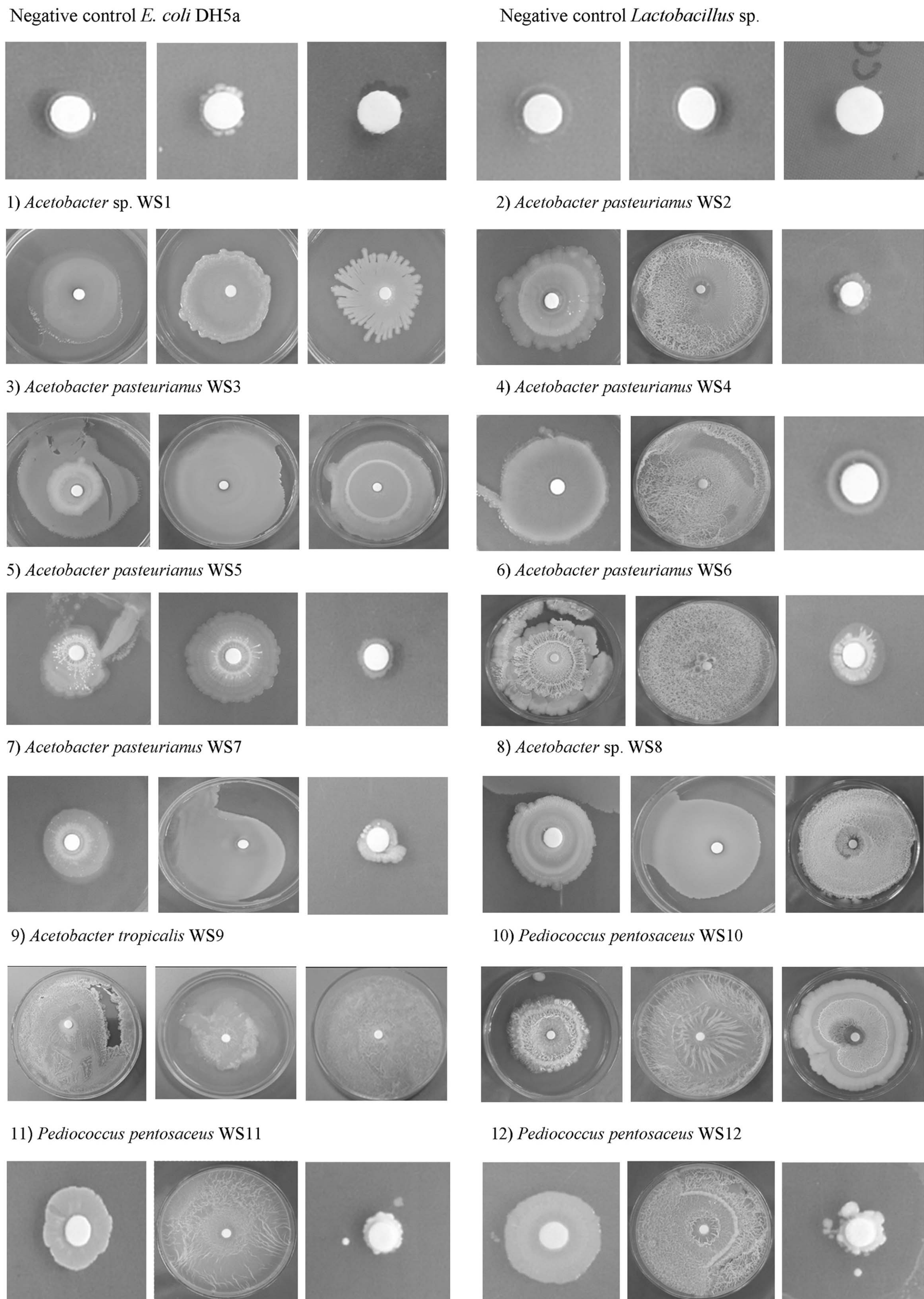
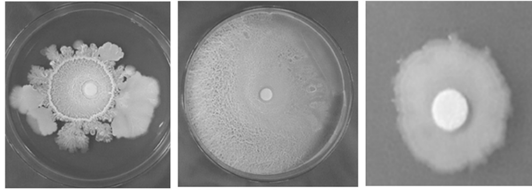
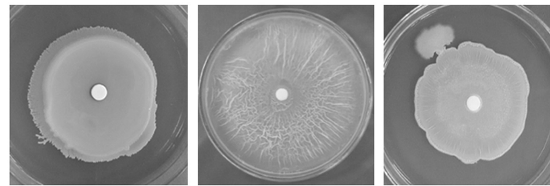



**Fig. 3. EPS production from microbes.** AAB and LAB were grown on 80 g/l glucose (the first column), sucrose (the middle column) and lactose (the third column) containing agar after 3 days of incubation at 37℃.

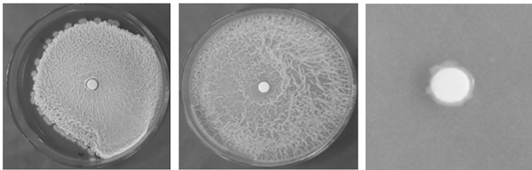
13) *Lactobacillus casei* strain WS13



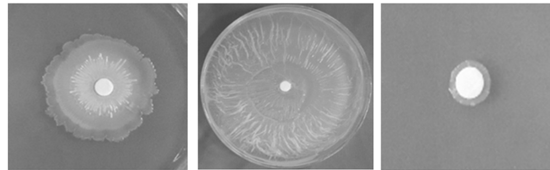
14) *Lactobacillus casei* strain WS14



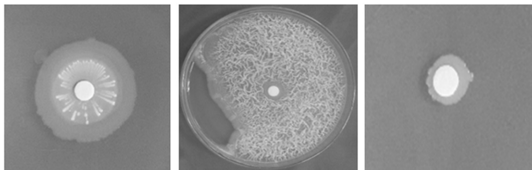
15) *Lactobacillus casei* strain WS15



16) *Lactococcus hircilactis* WS16



17) *Lactococcus lactis* subsp. lactis WS17



18) *Lactococcus lactis* WS18

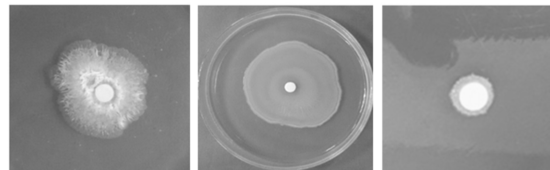


**Fig. 3. Continued.**

(Fig. 3). Different EPS appearances from different sugars were observed in the same bacteria, while diverse EPS appearances from the same sugar were observed in different bacteria (Fig. 3).

### Antioxidant and anti-tyrosinase activities of bacterial EPSs

Antioxidant activities of all bacterial supernatants containing EPS were assessed using the DPPH scavenging assay. The three highest DPPH scavenging activities were observed in *A. pasteurianus* WS3, WS6 and WS1 supernatants at 64, 64 and 62%, respectively, whilst the lowest activity was exhibited by *L. hircilactis* WS16 at 31% (Fig. 4). Most AAB and LAB showed 35–50% scavenging activity. The four representative isolates with highest DPPH scavenging activity from each genus (Fig. 4) including *A. pasteurianus* WS3, *A. pasteurianus* WS6, *Pc. pentosaceus* WS11 and *L. casei* WS13 were selected for EPS production and determination of antioxidant and anti-tyrosinase activities of crude EPSs in further experiments. Crude EPS extracts from 4 bacteria were produced from 8% sucrose in LB media (pH 7.0) for 72 h. *A. pasteurianus* WS3 produced the highest EPS concentration of 1,505 mg DW/L followed by *A. pasteurianus*

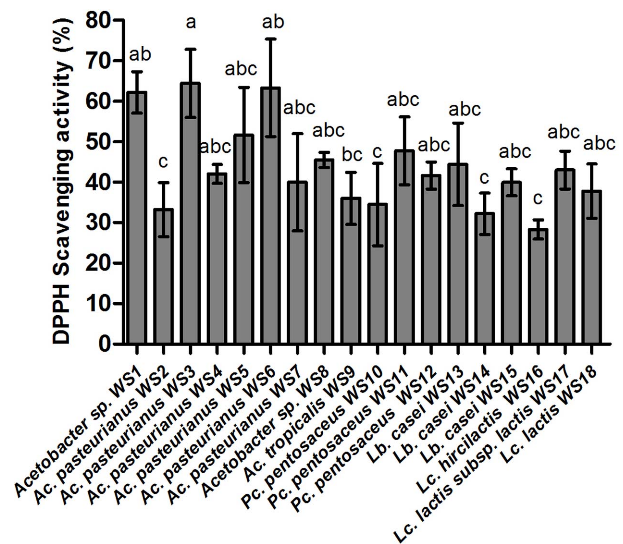



**Fig. 4. DPPH scavenging activity (%) of supernatants of eighteen EPS-producing bacteria isolated from Thai water kefir grown on 8% sucrose for 24 h.** Statistical significance ($p < 0.05$) was indicated by different letters using Tukey's HSD test.

WS6 (336 mg DW/L), *L. casei* WS13 (225 mg DW/L) and *Pc. pentosaceus* WS11 (176 mg DW/L) (Table 3). Reductive ability of EPS as an antioxidant was measured by

**Table 3. Dry weight of EPS, its antioxidant and anti-tyrosinase activity.**

| Bacterial EPS | Crude EPS[a] (mg DW/L) | FRAP ($FeSO_4$ mM/g) | $ABTS^{\cdot+}$ (% antioxidant activity) | Tyrosinase inhibition (%) |
|---|---|---|---|---|
| *Acetobacter pasteurianus* WS3 | 1,505 ± 3 | 280 ± 31 | 87.83 ± 0.71 | 20.35 ± 2.52 |
| *Acetobacter pasteurianus* WS6 | 336 ± 10 | 141 ± 24 | 86.12 ± 0.67 | 8.17 ± 0.31 |
| *Pediococcus pentosaceus* WS11 | 176 ± 8 | 132 ± 8 | 74.73 ± 1.32 | 3.89 ± 1.27 |
| *Lactobacillus casei* WS13 | 225 ± 5 | 150 ± 19 | 92.40 ± 0.36 | 12.56 ± 1.12 |

[a]Crude EPSs were obtained from bacterial cultures in 8% sucrose-containing media (1 L) for 72 h. Letters indicate significant differences between values in the columns ($p < 0.05$) using Tukey's HSD test.

investigating the $Fe^{3+}$-$Fe^{2+}$ transformation in FRAP assay in the presence of TPTZ forming an intense blue $Fe^{2+}$-TPTZ complex with an absorption maximum at 595 nm [19]. *A. pasteurianus* WS3 EPS exhibited the highest FRAP value of 280 mM $FeSO_4$/g which was insignificantly different from those of the other 3 bacterial EPSs ranging from 132–150 mM $FeSO_4$/g (Table 3). However, ABTS assay results showed that *L. casei* WS13 EPS exhibited significantly higher antioxidant capacity of 92.49% than EPSs from *A. pasteurianus* WS3 and WS6 (87.83% and 86.12%, respectively). *A. pasteurianus* WS3 EPS showed the highest anti-tyrosinase inhibition activity at 20.35% with significant difference among 4 bacterial EPSs (Table 3).

**SEM analysis**

The SEM micrographs showed that water kefir grains had ovoid structures of the predominant *Acetobacter* spp. accounting for 77.06% abundance among the bacterial population adhered to each other on the grainy and uneven surface covered with pores (Fig. 5A) presumably offering water-holding capacity [25]. The EPS surface morphology of *A. pasteurianus* WS3 showed a smooth thin film (Fig. 5B).

## Discussion

This is the first report on finding yeast *M. guilliermondii* in microbial strains of kefir. *M. guilliermondii* has been shown to have antifungal properties, producing aromatic flavors during the bread-making process [26] and is found in fermented drinks made by malt, corn and oats called 'Boza' in Eastern Europe [27] indicating it may play a role in producing unique aromas and also alcohol in kefir drinks. In addition, our finding concurred with previous reports that showed LAB including *L. casei, L. higardii, L. hordei, L. nagelii, L. citreum, L. mesenteroides* [28], *L. lactis* subsp. *cremoris* H414 [29], and *L. lactis* subsp. *cremoris* B891 [30] as EPS producers. In addition, *A. fabarum* and *A. syzygii* were found in German kefir and *A. orientalis* in Brazilian milk kefir [4]; however, in this work *A. tropicalis* was identified indicating its presence in kefir of a tropical country. *A. tropicalis* was also found to produce EPS [31]. In the previous report, Tibetan milk kefir that contained 15 culturable microbes including yeasts (*Kazachstania unispora* and *S. cerevisiae*), AAB (*A. okinawensis*) and LAB (*Leu. pseudomesenteroides* and *L. lactis* subsp. *lactis*) [23]. Only certain strains of *A. okinawensis*, *K. unispora* and

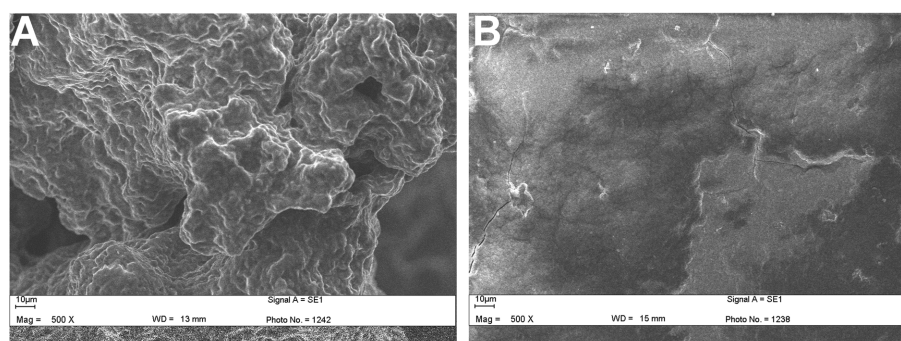



**Fig. 5. Scanning electron micrographs.** (A) Thai water kefir grain. (B) Crude EPS from *A. pasteurianus* WS3. Magnification at 500X.

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*Leu. pseudomesenteroides* from all reported strains were able to produce EPS. This indicated that this EPS-producing capacity seems to differ at the subspecies level. In addition, *L. lactis* subsp. *lactis* did not produce EPS from skim milk containing lactose as a carbon source [23]. Similarly, our *L. lactis* WS17 only produced EPS upon sucrose and glucose, but not lactose (Fig. 3). Similar to the previous study [14], sucrose seemed to be the most efficient sugar substrate, producing EPS with the largest diameter on the agar plates by most bacteria, while lactose was the least efficient, producing the smallest sizes of EPS. These EPSs were thought to be important in the formation and integrity of kefir grains. The type of sugar substrate has a huge impact on EPS productivity and may also influence the composition of EPS and hence its antioxidant activity and anti-tyrosinase activity. Strain characteristics may also be attributed to EPS production. Previously it was shown that EPS production by LAB depended on the sugar substrate present in the culture medium and its regulation of the EPS biosynthetic pathway [32, 33]. Inhibition of the activity of some key enzymes for EPS synthesis caused by certain sugar derived metabolites might also decrease EPS synthesis [34]. This may be why lactose was the least efficient sugar for EPS production in our case.

Molecules responsible for antioxidant activity in the bacterial cell-free supernatants could be extracellular EPSs. Monosaccharides including galactose, glucose, rhamnose and mannose are effective reductive agents as they have a hidden aldehyde moiety [35]. The radical scavenging activity of our EPS, via electron transfer and hydrogen atom transfer mechanisms from FRAP and $ABTS^{\bullet+}$ assays, respectively may be based on their reductive nature. This might be attributed to the hydroxyl group and other functional groups in EPS, such as -COOH, C=O and -O-, which can donate electrons to reduce the radicals to a more stable form, or react with the free radicals to terminate the radical chain reaction [36].

The EPS production yield by *Acetobacter* sp. WS3 and *L. casei* WS13 was higher than those reported recently by other researchers [22, 31, 38–44]. EPS production by *Acetobacter* sp. SKU1100 isolated from fruits in Thailand, under static culture condition reached a maximum of 1,230 mg EPS/L at 37℃ after 3 days of cultivation using 0.5% glucose and 10% potato extract as substrates [31]. *A. okinawensis* RJ-4 from Tibetan kefir produced approximately 380 mg EPS/L from lactose within 24 h [22]. *L. casei* LC2W with probiotic effects showed EPS production at 150.3 mg/l from 32-h fermentation in MRS medium with 2% glucose [38]. A considerable variation in EPS production was observed in *L. casei* depending on sugar substrate used and culture conditions. For example, *L. casei* CG11 produced EPS at 130–250 mg/l with lactose as the poorest carbon source and glucose as the most efficient in basal minimum medium [39], while Mozzi et al. reported EPS production of 488 mg/l for *L. casei* [40]. Bioactivities of the EPS in this study were similar to the previous findings indicating that the partially purified EPS from *L. sakei* Probio 65 (10–200 mg/ml) exhibited potent efficacy on inhibiting tyrosinase enzyme by 13.17–62.85% and displayed a considerable amount of antioxidant efficacy on scavenging DPPH by 49.56% [41]. The hydroxyl groups in EPS structure could form a hydrogen bond to the active site of tyrosinase enzyme, resulting in steric hindrance or changed conformation and thus causing it to have a lower catalytic activity [42]. Our *A. pasteurianus* WS3 EPS showed anti-tyrosinase activity of 20.35% which was much higher than that of EPS from the mushroom *Fomitopsis feei* (2.13, 4.89, 8.84% tyrosinase inhibition from 25, 50 and 100 mg/ml extract) [43]. Similar to our finding, EPSs (1–10 mg/ml) from *Bacillus licheniformis* strains isolated from Kimchi exhibited anti-tyrosinase activities in a range of 22.5–37.0% which was significantly higher than that of beta-glucan (6.0–17.0%) at the same concentrations [44].

The reducing potentials of EPS antioxidants are linked with their electron donating abilities of semi-acetal at C1 of the reducing end of a polysaccharide and thus can reduce $Fe^{3+}$ to $Fe^{2+}$. In comparison with the previous report [45], three EPSs from endophytic bacterium *Paenibacillus polymyxa* EJS-3 exhibited FRAP values of 10.79, 3.37, and 4.38 mM $FeSO_4$/g which were over 10-fold lower those found in our EPSs ranging from 132–280 mM $FeSO_4$/g (Table 3) suggesting stronger reducing power. However, the positive control, ascorbic acid, showed a much higher FRAP value of 11,919 mM $FeSO_4$/g, suggesting that its antioxidant power was much stronger than that of EPSs in their work and our work. The appearance and structure of our *A. pasteurianus* WS3 EPS by SEM analysis is similar to the EPS

produced by previously reported strain *A. okinawensis* RJ-4 [22] exhibited a compact structure with the appearance of a smooth surface with a thin film covering, indicating specific characteristics of materials used to make plasticized films [46]. Other appearances of bacterial EPSs can also be observed. For example, *B. tequilensis* PS21 had a grainy appearance with an uneven surface, covered with pores [47].

To conclude, our findings have shed light on microbial strains, EPS producers of Thai water kefir as distinct from Thai milk kefir and have shown differences in microbial strains with kefirs from other countries. These differences make the kefir products of each country very different in terms of flavor and health benefits. Microbes isolated from our study might provide a fundamental information for developing new kefir grains with desired properties as novel functional foods. Their EPS-producing capacity also offers antioxidant activity and anti-tyrosinase activity for future potential biotechnological applications including food additives, animal feed, cosmetic ingredients, and biodegradable films.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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