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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 H_2O_2. Removal of EPS showed significantly reduced bioactivity DPPH scavenging activity and resistance to H_2O_2. 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, antimicrobial activity and anti-proliferative activity, anti-mutagenic and anti-carcinogenic properties and it has the potential to become a type of functional food (Guzel-Seydim, Kok-Tas, Greene, & Seydim, 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-producting 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

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of food products. Thus, we aimed to isolate and identify the microbes 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 aerobically 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 +0.05% Bromocresol Purple (BCP) plates to isolate lactic acid bacteria (LAB), containing 2 g/L meat extract, 4 g/L yeast extract, 10 g/L peptone from casein, 1 mL Tween 80, 2.5 g/L K_2HPO_4, 5 g/L sodium acetate, 2 g/L diammonium hydrogen citrate, 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), GYC agar was used (10 g/L yeast extract; 50 g/L D-glucose; 30 g/L calcium carbonate; 15 g/L agar, pH 6.8). For isolation of yeasts the serial dilutions were plated on YPD agar plates (10 g/L peptone from casein, 5 g/L yeast extract, 15 g/L agar, and 20 g/L dextrose, pH at 6.5). After incubation aerobically at 37 °C for bacteria and 30 °C for yeasts for 3 days the viable cell count (CFU/mL) on each agar plate was enumerated on a proper serial dilution. From every suitable serial dilution half of the colonies were picked and streaked five times to obtain pure isolates. Gram staining was conducted to study cell morphology. No yeast cells were observed. Pure bacterial isolates from each type of selective agar plates were grown overnight in the corresponding broths with 15% glycerol added before storage at −80 °C.

2.3. Genomic DNA isolation

For DNA isolation of bacteria overnight cultures (1 mL) were centrifuged at 8000*g* 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' -GAGAGTTTGATYCTGGCTCAG- 3′) and the reverse primer AmpR (5′-AAGGAGGTGATCCARCCGCA -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 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 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* spp. (Larpin, Sauvageot, Pichereau, Laplace, & Auffray, 2002; Song, Jeong, & Baik, 2013). Each identified isolate was cultured overnight in MRS broth and the culture of $OD_{600nm} = 0.4$ (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 aerobically 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, Mutuvulla, Chen, Jiang, & Dong, 2012) with some modifications. Briefly, 1.0 mL of *Bacillus* spp. cells grown overnight in MRS media containing 8% lactose with 10^9 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 8000*g* for 10 min. The scavenging ability was defined as:

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

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* spp. for DPPH free radical. Briefly, cultures of the same 10 strains were centrifuged (6000*g*, 10 min), and the cell pellet was washed twice with distilled water and resuspended in PBS buffer (pH 7.0) to approximately 10^9 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 (6000*g*, 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.

2.7. Resistance to hydrogen peroxide

The method of Li et al. (2012) was used with some modifications. Cultures of 10 selected *Bacillus* spp. grown overnight in MRS