Evaluation of Yeast Diversity in Dadih and Dangke Using PCR-RFLP of Internal Transcribed Spacer Region

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Abstract. The dominant indigenous microbes, such as lactic acid bacteria group, mainly determine the quality of naturally fermented milk products. Yeasts have also contributed to the fermentation development, especially in determining the organoleptic or physicochemical characteristics of the products. This study was aimed to evaluate the diversity of yeasts in the naturally fermented milk products from Indonesia, which were dadih and dangke by using PCR-RFLP of ITS region. Two dadih samples used with one sample were collected in three consecutive days. Dangke samples consisted of three made from buffalo milk (sample A-C), and sample D was from cow’s milk. The isolated yeasts were further characterized genotypically using RFLP analysis of the ITS region. The representative isolates of each cluster as a result of the restriction pattern obtained with HinfI and HaeIII enzymes were further identified. A total of 37 yeast isolates (17 isolates from dadih and 22 isolates from dangke) were grouped into three clusters based on the band pattern of RFLP analysis. Based on DNA sequencing analysis, the three species were identified as *Saccharomyces cerevisiae* (group I), *Candida metapsilosis* (group II) and *Kluyveromyces marxianus* (group III). *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* were found in both dadih and dangke samples. Unfortunately, yeasts were not detected in dangke samples A and C. *Candida metapsilosis* was found frequently in dadih, while *Saccharomyces cerevisiae* was the dominant species in dangke. Further investigations are needed to shed light on microbial dynamics since the changes in the abundance and type of microbiota during the fermentation process play a pivotal role in the quality of the final products.

1. Introduction

Besides lactic acid bacteria (LAB), yeasts also play an essential role in naturally fermented milk products, and their diversity and important role have been the subject of intensive studies [1-9]. Yeasts that have been recovered from naturally fermented milk (NFM) products vary in numbers and originally come from a wider range of sources, such as the milk, containers, equipment used and the processing environment (eg airborne contamination) [10]. Common yeast genera used in the food industry are *Candida*, *Saccharomyces*, and *Kluyveromyces* [11]. There are fewer yeasts in fermented milk products compared with bacterial (LAB) numbers; whereas yeasts outnumber bacteria in...
alcoholic fermentation products and play a primary role in the fermentation [12]. However, as secondary microbiota, the presence of yeasts in fermented milk products is inevitable and can be both undesirable and desirable [13].

The occurrence of yeasts in NFM products is common and they play an essential role in determining product quality and safety. The important contribution of indigenous yeast to NFM products as noted by Narvhus & Gadaga [14] is in the development of texture and flavor. In airag, koumiss, and kefir, CO2 produced by yeasts positively affects the aroma and flavor of final products [14, 15]. Besides stimulating the growth of starter microorganisms through the production of amino acids and vitamins, enzymatic activities (lipolytic and proteolytic) of yeasts play a substantial role in the development of desired taste, texture and flavor [14, 16]. In terms of safety, some yeasts also produce natural killer factors that can inhibit the growth of undesired microorganisms [10, 16]. Additionally, Saccharomyces cerevisiae was able to reduce the concentration of aflatoxin M1 in fermented milk [17]. A complex interaction between yeasts and other microbiota especially LAB bring advantages to the quality and safety of final products.

Nowadays, the study of the ecology of yeasts in NFM products is of increasing interest. Particular yeast species are used not only as starter cultures for controlled fermentation processes but also because of their benefits to human health, such as probiotic properties [11, 18]. However to date Saccharomyces cerevisiae var. boulardii is the only yeast probiotic that has proven clinical efficacy [19]. Therefore, interest in the identification and characterization of yeasts from many NFM products is high. In order to obtain novel potential non-LAB organisms, the current work was conducted to isolate and identify the dominant yeast species contributing to fermentation of the dadih and dangke products. Yeast isolates were fingerprinted using the PCR-RFLP method and their identity confirmed using gene sequencing of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers (ITS) regions.

2. Materials and Methods

2.1. Collection of samples
Two dadih samples were collected from two rural areas of Solok Regency and Gadut (Agam Regency), respectively, in West Sumatra Province, Indonesia. To evaluate the microbial succession, samples from three consecutive days of fermentation were used for the dadih samples collected from Solok Regency. Meanwhile, dangke samples were also collected from rural areas in Enrekang Regency, Makassar Province, Indonesia. Four different household producers were sampled (identified as samples A, B, C, and D). Buffalo milk was used in dangke A, B, and C; while dangke D used cow's milk. All the samples were placed in sterile 100 mL propylene tubes. The samples were kept at 4-6°C and transported in a cool box within two days to Microbiology laboratory, Department of Biology, University of Brawijaya, Indonesia. The pH of each sample was measured at the laboratory using a calibrated pH-meter (model 3205, Jenway, UK).

2.2. Isolation and enumeration of yeasts
Ten grams of fermented milk samples (dadih and dangke) were transferred aseptically into 90 mL saline water (0.75% w/v NaCl) and mixed thoroughly. Further tenfold serial dilution (10-1-10-6) was made for each sample and 0.1 mL of appropriate dilution was spread on YMA (yeast mannitol agar) acidified to pH 3.5 with 1 N HCl. All the plates then were incubated aerobically at 37°C for 48 hours. A similar technique was applied to assess the presence of yeasts in bamboo. A fresh bamboo tube was filled with 10 mL of a sterile saline solution (0.75% w/v NaCl) and the solution mixed thoroughly using a sterile spoon and cultured on media as described above. Representative colonies were picked randomly from the plates where the number of colonies fell between 30 and 300. Duplicate plates of each dilution were prepared and the numbers of yeasts detected was calculated as an average value. The yeast colonies were checked for purity by streaking twice on YEPD agar (1% yeast extract, 1% peptone, and 2% of D-glucose). Stock cultures of the isolates were preserved in their preferred
medium containing 15% glycerol (Merck, Darmstadt, Germany) and stored at -80°C until further analysis.

2.3. Phenotypic characterization
The phenotypic characterization of yeast was conducted based on cell morphology and colony characteristics, such as shape, size, margin, elevation, surface, texture and colour. The catalase and oxidase tests (Sigma-Aldrich, St. Louis, US) were also assessed as part of biochemical characterization.

2.4. Genotypic characterization
A total of 39 yeast isolates were grouped into clusters based on the band pattern of RFLP analysis of the internal transcribed spacer (ITS) region. The oligonucleotide primers used in this study were ITS 1 (5’ TCCGTAGGTGAACCTGCG G 3’) and ITS 4 (5’ TCCTCCGCT TATTGATATGC 3’) [20]. A single colony of yeast isolate was used as a DNA template by using a sterile toothpick to sample the colony and place the material into a PCR tube. To denature the yeast cell wall, the colony in the PCR tubes was heated in a Microwave oven (Panasonic) for one minute. After this, 25 µl of reaction mixture for PCR was dispensed into the tubes. The DNA amplification was carried out using a Mycycler thermal cycle (Bio-Rad Laboratories, Hercules, CA, USA) with the following components: 5 µl of 5X PCR buffer, 1.25 µl of 50 mM MgCl2, 2.5 µl of 2.5 mM dNTPs (2 mM each dATP, dCTP, dGTP and dTTP), 0.25 µl of Taq polymerase (5 U/µl) (Promega, Madison, Wis. USA) and 1.5 µl of a 5 µM concentration of each primer. The PCR conditions consisted of 35 cycles (denaturation step at 94°C for 1 min, annealing step at 53°C for 1 min, and elongation step at 72°C for 1 min), initial denaturation step at 94°C for 5 min and additional cycle at 72°C for 5 min as a final extension.

A total volume of 10.2 µl was used for RFLP analysis which comprised 9 µl of amplicons, 1 µl of buffer (composition dependent on the enzymes), and 0.2 µl of restriction enzymes. HinfI and HaeIII enzymes were used for digesting yeasts (New England BioLabs Inc., MA, US). The mixture was incubated at the optimum temperature for the enzyme activity for 24 h. Following this incubation, 5 µl of the RFLP product added with 2 µl of loading dye were dispensed into the wells of the 1.5% (w/v) agarose gels containing 5% (v/v) of ethidium bromide (EtBr), and then electrophoresed in 1 × TAE buffer for 1 h. Gel image was visualized with the Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA). The sizes of the DNA fragments were estimated by comparing their relative mobility with 100 bp of molecular weight ladder (size range: 100-1,000 bp, New England BioLabs Inc.).

2.5. Molecular identification and DNA sequencing analysis
The PCR products from the targeted ITS region were purified for sequencing by using UltraClean PCR clean-up kit (Mo Bio Laboratories Inc, Solena Beach, CA, USA,) according to the protocol described by the manufacturer. The DNA sequence was determined using the Big Dye Terminator version 3.1 cycle sequencing ready reactions (Applied Biosystem, Foster City, CA, USA) at the DNA Sequencing Facility, Flinders Medical Centre, Bedford Park, South Australia. The nucleotide sequences were aligned and used for the analysis of sequence similarity through the Basic Local Alignment Search Tool–BLAST (http://www.ncbi.nlm.nih.gov/blast) performed using the Gen-Bank database (The National Centre for Biotechnology Information-NCBI). A phylogenetic tree based on neighbor-joining with the likelihood model (Tamura-Nei) was constructed using Geneious® 9.1.7 (Biomatters Ltd., New Zealand) and then visualized using FigTree v1.4.2 (Institute of Evolutionary Biology, University of Edinburgh).
3. Results and Discussion

3.1. Predominant yeasts in dadih and dangke

Naturally fermented milk is a unique ecological niche in which only well-adapted species can grow and interact synergistically with each other. Mesophilic LAB and non-LAB in dadih and dangke have been evaluated. In this present study, the structure and dynamics of yeasts involving in these products was also investigated using a culture-dependent approach.

| Samples               | Milk type   | Location | pH value | Colony number (log cfu/mL or g) |
|-----------------------|-------------|----------|----------|---------------------------------|
| Raw milk              | Buffalo milk| Solok    | 6.92     | 7.35±0.24                       |
| Dadih Sianok day 1    | Buffalo milk| Solok    | 5.02     | 8.08±0.00                       |
| Dadih Sianok day 2    | Buffalo milk| Solok    | 4.65     | 6.85±0.05                       |
| Dadih Sianok day 3    | Buffalo milk| Solok    | 4.65     | 6.99±0.42                       |
| Bamboo                | -           | Solok    | ND*      | 4.19±0.42                       |
| Dadih Gadut day 3     | Buffalo milk| Gadut    | 4.85     | 8.05±0.00                       |
| Dangke A              | Buffalo milk| Enrekang | 4.09     | 7.29±0.23                       |
| Dangke B              | Buffalo milk| Enrekang | 4.08     | 7.14±0.47                       |
| Dangke C              | Buffalo milk| Enrekang | 4.26     | 3.75±0.05                       |
| Dangke D              | Cow’s milk  | Enrekang | 5.14     | 7.18±0.29                       |

The viable counts of the yeast from raw buffalo milk, dadih and dangke products, and their pH values are shown in Table 1. The initial count of yeast for the raw buffalo milk sample was 7.35±0.24 log cfu/mL, and then the yeast count gradually increased after 24 h of fermentation (8.08±0.00 24 log cfu/g). A decreased yeast count was observed at day 2 (6.85±0.05 log cfu/g) and slightly increased again at day 3 (6.99±0.42 log cfu/g). Decreasing pH value was also observed from 6.92 (raw buffalo milk) to 4.65 (dadih Sianok days 2 and 3). The viable yeast count of dadih from Gadut (day 3 of fermentation) was higher (8.05±0.00 log cfu/g) than dadih from Sianok (6.99±0.42 log cfu/g), while the pH value was relatively similar (4.65 and 4.85, respectively).

The viable yeast counts of dangke A, B, and D were relatively similar, which were 7.29±0.23 log cfu/g, 7.14±0.47 log cfu/g and 7.18±0.29 log cfu/g, respectively, with pH values ranged from 4.08-5.14. The viable count of dangke C was the lowest number (3.75±0.05 log cfu/g) and a pH value of 4.26. In the first stage of yeast identification, phenotypic characterization (morphological observation, cell-shape, and biochemical tests) was similar for all yeast isolates. All the isolates appeared to be ovoid-shaped, catalase-positive and oxidase-positive.

Concerning yeast enumeration, some literature reports that the number of yeasts in some NFM products usually ranges from 2 to 7 log cfu/mL or g [4, 21-23], substantially in line with the data obtained from this present study. However, viable count for yeasts in dadih from Gadut and dadih Sianok day 1 reached 8 log cfu/g. The data obtained at the present study was in agreement with a previous study [24], in which microbiota in dadih from Gadut was dominated by yeasts instead of LAB. This decrease was affected by the increase in LAB counts due to lactic acid production [25].
3.2. Molecular characterization and identification

Table 2. Grouping of yeasts based on ITS-PCR RFLP and identification by the 5.8S rRNA gene and ITS region gene sequencing

| Groups | Number of isolates | ITS-PCR (bp) | RFLP (bp) | Similarity (%) | Identification (gene sequencing) | Accession Number |
|--------|--------------------|--------------|-----------|---------------|----------------------------------|-----------------|
| I      | 5                  | 13           | 800       | 50, 110, 310  | Closely similar to Saccharomyces cerevisiae isolate KDLYH4-1 | KF710035         |
| II     | 10                 | 0            | 500       | 80, 260, 300, 500 | Candida metapsilosis CBS 2916 | KY102207         |
| III    | 2                  | 9            | 750       | 80, 120, 185, 240 | Kluyveromyces marxianus CBS 1557 | KY103803         |
| Total  | 17                 | 22           |           |               |                                  |                 |

A total of 39 yeast strains (17 strains from dadih and 22 strains from dangke) were characterized and identified using PCR amplification of the 5.8S rRNA gene and ITS region (ITS1 and ITS2) combined with RFLP and gene sequencing. The PCR products of all yeast strains showed a high length variation in this region for the different groups: 800 bp for group I, 500 bp for group II, and 750 bp for group III (Table 2). The restriction analysis results were consistent with cellular and colony appearance, which differentiated the yeast isolates into three groups as well (Table 2, Fig. 1A). The restriction enzymes HinfI (Fig. 2) and HaeIII (Fig. 3) successfully digested the amplicons and confirmed the presence of three different groups (species) indicates that the use of HaeIII and HinfI to digest the ITS-PCR products could provide a reproducible result. Based on DNA sequencing analysis, the three species were identified as *Saccharomyces cerevisiae*, *Candida metapsilosis*, and *Kluyveromyces marxianus*. However, the nucleotide homology of group I in the GenBank database was only 90% of the similarity level, which was close to *Saccharomyces cerevisiae* KDLYH4-1 (accession number: KF710035). In this case, yeast isolated from baker’s yeast was used as a reference strain. Both amplicon and RFLP fragments (digested using HaeIII) showed a similar size and profiles (Fig. 13B) confirming that they correspond to each other. A phylogenetic tree was also constructed to establish the genetic relationship between the representative yeasts and the type strains or submitted strains on the GenBank database (Fig. 4).
**Figure 1.** PCR amplification of the 5.8S rRNA gene and ITS region of selected yeasts (A) & Confirmation of ITS-PCR RFLP analysis between *Saccharomyces cerevisiae* SL1.1 and *Saccharomyces cerevisiae* isolated from baker’s yeast (B).

A) Lane M: 100 bp DNA marker; lane 1: H₂O; lane 2: *Kluyveromyces marxianus* SL2.13; lane 3: *Candida metapsilosis* B.1; lane 4: *Saccharomyces cerevisiae* SL1.1; B) lane 1 and 3: *Saccharomyces cerevisiae* SL1.1; lane 2 and 4: *Saccharomyces cerevisiae* (baker’s yeast).

**Figure 2.** Gel electrophoresis of PCR-amplified 5.8S rRNA gene and ITS region digested with *Hinf1*

Lane M: 100 bp DNA marker; lane 1: *Kluyveromyces marxianus* D2.26; lane 2: *Kluyveromyces marxianus* D2.24; lane 3: *Kluyveromyces marxianus* D2.23; lane 4: *Kluyveromyces marxianus* D2.22; lane 5: *Kluyveromyces marxianus* D2.21; lane 6: *Kluyveromyces marxianus* DC.16; lane 7: *Kluyveromyces marxianus* DC.15; lane 8: *Saccharomyces cerevisiae* DC.14; lane 9: *Kluyveromyces marxianus* DC.12; lane 10: *Kluyveromyces marxianus* DC.11; lane 11: *Saccharomyces cerevisiae* DC.10; lane 12: *Candida metapsilosis* GD.21; lane 13: *Candida metapsilosis* GD.20; lane 14: *Candida metapsilosis* GD.19; lane 15: *Candida metapsilosis* GD.18; lane 16: *Candida metapsilosis* GD.16; lane 17: *Kluyveromyces marxianus* SL2.13; lane 18: *Candida metapsilosis* SL0.14; lane 19: *Candida metapsilosis* SL0.12; lane 20: *Saccharomyces cerevisiae* DC.13; lane 21: *Saccharomyces cerevisiae* DC.8; lane 22: *Saccharomyces cerevisiae* DC.7; lane 23: *Saccharomyces cerevisiae* D2.2.
Figure 3. Gel electrophoresis of PCR-amplified 5.8S rRNA gene and ITS region digested with HaeIII
Lane M: 100 bp DNA marker; lane 1: *Kluyveromyces marxianus* B.8; lane 2: *Kluyveromyces marxianus* D2.24; lane 3: *Kluyveromyces marxianus* D2.23; lane 4: *Kluyveromyces marxianus* D2.22; lane 5: *Kluyveromyces marxianus* D2.21; lane 6: *Kluyveromyces marxianus* DC.16; lane 7: *Kluyveromyces marxianus* DC.15; lane 8: *Saccharomyces cerevisiae* DC.14; lane 9: *Candida metapsilosis* GD.15; lane 10: *Kluyveromyces marxianus* SL2.13; lane 11: *Kluyveromyces marxianus* DC.12; lane 12: *Kluyveromyces marxianus* DC.11; lane 13: *Saccharomyces cerevisiae* DC.10; lane 14: *Candida metapsilosis* GD.21; lane 15: *Candida metapsilosis* GD.20; lane 16: *Candida metapsilosis* GD.19; lane 17: *Candida metapsilosis* GD.18; lane 18: *Candida metapsilosis* SL0.14; lane 19: *Candida metapsilosis* SL0.12; lane 20: *Saccharomyces cerevisiae* DC.13; lane 21: *Saccharomyces cerevisiae* DC.8; lane 22: *Saccharomyces cerevisiae* DC.7; lane 23: *Saccharomyces cerevisiae* D2.

Figure 4. Neighbor-joining tree showing the phylogenetic relationships among yeast isolates from dadih and dangke samples and the type isolates based on ITS1, 5.8S rRNA gene, and ITS2 sequences. *Pichia (P.) jadinii* was used as an outgroup.
Saccharomyces cerevisiae and Kluyveromyces marxianus were found in both dadih and dangke samples. Unfortunately, yeasts were not detected in dangke samples A and C. Saccharomyces cerevisiae was the dominant strain in dangke, while Candida metapsilosis was found frequently in dadih. Interestingly, the identity of yeasts (two isolates) in the inner part of the bamboo was found to be C. metapsilosis B.1 and K. marxianus B.8.

The most predominant yeast in dadih was C. metapsilosis, while S. cerevisiae predominated in dangke followed by K. marxianus. Meanwhile, C. metapsilosis was only present in dadih. The occurrence of yeasts in dadih has been reported before with different species, namely Candida stelimalicola and Pichia jadinii [26]. Yeasts in dangke have also been reported, namely Candida sp., Saccharomyces sp., Geotrichum sp. and Rhodotorula sp., with Candida sp. as the dominant species [27]. Saccharomyces cerevisiae is the most frequently reported yeast in a range of NFM products, such as fermented goat milk from Tajikistan [28], fermented milk matsoni from Georgia and Armenia [29], Tarak from Korea [30], Tibetan kefir grains [31], Sameel milk from Saudi Arabia [32], NFM from Tibetan Plateau of China [9], Nunu from Ghana [22], Amabere amaruranu from Kenya [33], yak milk dreg from Tibet [34] and Shubat from Kazakhstan [35]. Kluyveromyces marxianus was also identified in some of those NFM products, apart from Tarak, Nunu, Sameel milk, amabere amaruranu, and fermented milk products from Tibetan Plateau of China. Moreover, this yeast species was also reported in Koumiss [15]. Interestingly, C. metapsilosis has not been reported in any NFM products before, thus this is the first record from NFM products.

Concerning RFLP analysis of the 5.8S-ITS region, the RFLP profile of K. marxianus was consistent with previous research [28, 36]. The RFLP profile of S. cerevisiae in this present study compared with those reported in previous studies exhibited a slightly different pattern [28, 37]. Digestion of HaeIII produced an identical pattern, but HinfI gave a completely different pattern. As a result, the strain of S. cerevisiae in this study was considered to be different from the strains covered in the database. However, this strain displayed a similar RFLP pattern with Baker's yeast as shown in Fig. 1B, although only one restriction enzyme used (HaeIII). In contrast, the database for RFLP profiles of the 5.8S-ITS region for C. metapsilosis has been not documented so far. The available RFLP profile targeted a different region, the D1/D2 domain [38]. These authors also reported that C. metapsilosis grouped as endophytic yeast. This report was in agreement with the result of the present study, in which C. metapsilosis and K. marxianus also found in bamboo tubes.

4. Conclusion

Three yeast species were obtained from dadih and dangke samples, namely S. cerevisiae, C. metapsilosis, and K. marxianus, with C. metapsilosis as the predominant yeast in dadih and S. cerevisiae in dangke. Although the viable count of yeasts can be determined from the isolation medium, the yeast isolates derived from this study may not be fully representative of the full yeast flora of dadih and dangke. The total picture of the microbes involved in the fermentation processes of these products is still unclear. Further investigations are needed to shed light on microbial dynamics, and diversity analysis of more dadih and dangke samples. This would contribute to a better understanding of the fermentation process since the changes in the abundance and type of microflora during the fermentation process play a pivotal role in the quality of the final products.

Acknowledgements

We would like to thank Mr. Dasril Abalga and Ms. Andi Fauziah Yahya for assisting in collecting the dadih and dangke samples.

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