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Microbiological characterisation of whey-based kefir beverages after Bod ljong cheese-making at different fermentation temperature

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Abstract. Whey is generally disposed into sewage which creates major problem of pollution besides the loss of valuable nutrients. Therefore, the aim of the present work is to develop whey-based beverage after Bod ljong cheese-making. Microbiological characterisation was characterized at different fermentation temperatures using culture-dependent on selective media and culture-independent PCR-DGGE and sequencing of dominant bands. The dominant microbiota, as revealed by PCR-DGGE, was composed by yeast affiliated to Kluyveromyces marxianus, Kluyveromyces lactis, Saccharomyces cerevisiae, and bacteria affiliated to Lactococcus lactis and Acetobacter lovaniensis. Results indicated that conventional culture method and PCR-DGGE could be combined to describe in maximal detail the microbiological composition for future implementation of whey-based kefir beverages.

Abbreviations

- PCR-DGGE: Polymerase chain reaction-denaturing gradient gel electrophoresis
- LAB: Lactic acid bacteria
- MAB: Mesophilic aerobic bacteria
- MRS: Man, Rogosa and Sharp
- PDA: Potato dextrose agar
- CFU: Colony forming units
- ANOVA: Analysis of variance
- SPSS: Statistical package for the social sciences
- DNA: Deoxyribonucleic acid
- rRNA: Ribosomal ribonucleic acid

1. Introduction

Bod ljong cheese is a semi-hard cheese exclusively manufactured in the Qinghai-Tibet Plateau regions of China. The acidic cheese curd is formed by addition of Tibetan kefir grains and small quantities of rennet and stored in a cool well-ventilated place for approximately a month, after which the cheese is characterized by a pleasant acid taste and a mild aroma[1]. Tibetan kefir grains are small irregularly shaped, yellowish-white and resemble miniature cauliflower blossoms and clusters of microorganisms.
held together by a matrix of lactic acid bacteria, acetic acid bacteria, and yeasts coupled with casein and polysaccharides [2-4]. Kefir grains have traditionally been used as starter cultures for the production of the kefir that has a slightly acidic taste, yeasty flavor and creamy consistency [5, 6]. Since certain yeasts along with the LABs from kefir grains are able to metabolize lactose, the natural mixed culture kefir was chosen for whey exploitation [7, 8].

Cheese whey results from the precipitation and removal of milk casein in cheese making processes [9]. On average, for the production of 1 kg of cheese 10 kg of milk are used, which produces 9 kg of cheese whey as a byproduct. This is equivalent to 5 million tons a year of whey worldwide [10-12]. In the whey, water is the major composition accounting to 90-92% of the total composition and the proximate composition of whey on a dry weight basis is lactose (65-75%), nitrogenous compounds (proteins, peptides and amino acids, 8-11%), fat (2-8%), and minerals (9-13%) [13, 14]. Recent research has focused on the development of technologies that employ whey as a raw material for producing foods or food additives of added value [15, 16]. The aims of this study were to evaluate the microbiological characterisation of whey-based kefir beverages at different temperature after Bodljong cheese-making.

2. Materials and methods

2.1. Production of whey-based kefir beverages
Cheese whey was obtained from Bodljong cheese-making with Tibetan kefir grains as the starter and lactose (Bright Dairy & Food co., ltd, Shanghai, China) was dissolved in the whey to a lactose concentration of 46 g/L. Deproteinised cheese whey was made by autoclaving at 115 °C for 10 min the cheese whey solution, followed by aseptic centrifugation (2000g for 15 min) to remove fines and cream. Kefir grains isolated from a commercial Tibetan kefir drink were used in the present study. The inoculum was prepared by cultivating kefir grains in pasteurized skimmed milk, renewed every 24 h for 7 days. After that, the grains were washed with sterile distilled water and the grains (50 g) were inoculated in 250 mL of prepared cheese whey solution. Subsequently, the aseptic filtration whey was incubated at three temperatures (20 °C, 30 °C, and 37 °C) for 12 h. The pH values were measured every 0.5 h. Then all the inoculated whey reached the target pH, after which the obtained whey-based kefir beverages were stored at 4 °C for 24 h.

2.2. Isolation of microbial strains by culture-dependent approach
Bacteria and yeasts were enumerated by method of Hu et al. [17]. 10g of each sample was homogenised in 90 mL of cold sterile 0.1 % peptone solution. Serial decimal dilutions were prepared in the same diluent and inoculated in triplicate by surface spreading on specific solid media. The total MAB, LAB, lactobacillus, lactococcus, yeasts, acetobacter in selective mediums were counted and all media for bacterial enumeration were supplemented with 0.4 mg/mL nystatin (Sigma-Aldrich, USA). Gram staining and catalase tests were performed for confirmation of lactic acid bacteria. The results of the viable counts were expressed as means of log CFU/g sample ± standard deviations. The data collected were subjected to one-way ANOVA using SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA), and differences were considered non-significant at p > 0.05.

2.3. DGGE analysis of the whey beverage
The DGGE analysis of final products was determined according to the method described by some researchers with modifications [18-20]. The microbial DNA was extracted from whey-based kefir beverages samples using SK8233 gDNA Miniprep kit (Songon, Shanghai, China). The bacterial community DNA was amplified with primers 338f (5'-ACT CCT ACG GGA GGC AGC AGC AG-3') and 518r (5'-ATT ACC GCG GCT GCT GG-3') spanning the V3 region of the 16S rDNA gene [11]. The D1 domain of the 26S rRNA gene of yeasts was amplified using the primers NL1-GC (5'-GCG GGC CGC GGC ACC GCC GGG ACG CGC AG-3') and a reverse primer LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3'), as reported
by Lievore et al. [9]. The predominant DGGE bands were excised and re-amplified using the primers without GC clamp. The PCR amplicons were then sequenced (Applied Biosystems, Foster City, CA, USA). Sequences were used as a query sequence to search for similar sequences from GenBank by means of the blast program (http://www.ncbi.nlm.nih.gov/BLAST/) [21]. Sequences showing 97% similarity or higher were deemed to belong to the same species [22].

3. Results And Discussion

3.1. DGGE fingerprinting of bacterial and yeast communities

The V3 region of the 16S rDNA gene of the bacteria and D1 region of the 26S rRNA gene of yeasts were amplified, and representative DGGE fingerprints are shown in Figure 1a and 1b. After Blast analysis, sequence results showed 98-100% identity with sequences retrieved from GenBank accession numbers. Figure 1a DGGE band A was clearly identified as Lactococcus lactis, band B as Lactobacillus kefir, band C as Lactobacillus parakefir, band D as Lactobacillus kefiranofaciens, band E as Acetobacter lovaniensis. Figure 1b DGGE band A was clearly identified as Kazachstania exigua, band B as Kazachstania turicensis, band C as Kluyveromyces lactis, band D as Kluyveromyces marxianus, band E as Saccharomyces cerevisiae. PCR-DGGE analysis showed that Lc. lactis (Figure 1a band A) was the dominant bacteria in the whey-based kefir beverages at different temperatures, as also indicated by the plating results (Table 2). The representative of Lc. lactis could be differentiated according to the migration distances of its respective 16S rDNA fragments. Figure 1a band E in the DGGE analysis corresponded to A. lovaniensis. Interestingly, this was the only specie of non-lactic acid bacteria found by culture-independent methods. In fungal analysis, PCR-DGGE showed a good correlation with the culture-dependent methods. Figure 1b band E represented the Saccharomyces sensu stricto group. Among them, S. cerevisiae was the most probable strain identified according to culture-based isolations; this species was the most commonly recovered yeast in the whey-based kefir beverages.

![Figure 1 DGGE profiles of bacterial 16S rDNA gene V3 fragments (a) and yeast 26S rRNA gene D1 region (b) amplified from whey-based kefir beverages at different temperatures](image)

3.2. Enumeration and identification of isolates by a culture-dependent method

In order to establish the different species of bacteria and yeasts presented in the whey-based kefir beverages samples, a representative number of isolates from each culture medium were identified (Table 1). All the isolates showed an increase with higher temperature and LAB was the most frequently found microorganism. The average number of total MAB was 5.32 logCFU/g at 20 °C, 6.09 logCFU/g at 30 °C and 6.29 logCFU/g at 37 °C, respectively, which is similar with that of the yeasts. The number of lactococcus was higher than that of lactobacillus.
Table 1 Mean counts (logCFU/mL) of microorganisms in the whey-based kefir beverages at different temperatures

|          | 20 °C        | 30 °C        | 37 °C        |
|----------|--------------|--------------|--------------|
| Total MAB| 5.32±0.14a   | 6.09±0.06b   | 6.29±0.05b   |
| LAB      | 7.42±0.06a   | 8.11±0.14b   | 8.44±0.09b   |
| Lactobacillus | 2.75±0.08a | 3.02±0.06b   | 3.18±0.12b   |
| Lactococcus | 3.72±0.04a | 4.01±0.03b   | 4.14±0.09b   |
| Acetobacter | 5.06±0.07a | 5.52±0.14b   | 6.01±0.06c   |
| Yeasts   | 4.92±0.12a   | 5.27±0.03b   | 5.87±0.11c   |

1) Values are expressed as means ± standard deviations; Different letters (a-c) in the same row indicate significant statistical differences (Duncan’s test, p<0.05).

The isolates were different under different fermentation time. A total of 225 isolates were obtained from the whey-based kefir beverages at 20 °C (Table 2). Among the isolates, 133 isolates were bacteria and 92 isolates were yeasts. The bacteria contained LAB (92 isolates) and acetic acid bacteria (41 isolates). The culture-dependent approach indicated that Lc. lactis represents the most commonly identified LAB isolates, with 43 of a total of 92 isolates, which was about making up about one-half of the LAB isolates. The yeast flora of the whey-based kefir beverages at 20 °C was dominated by the non-lactose-fermenting yeast (S. cerevisiae, 34 isolates; Klu. marxianus, 28 isolates; Kaz. exigua, 20 isolates) and the lactose-fermenting yeast (Klu. lactis) was only 10 isolates. For the samples at 30 °C, a total of 252 isolates were obtained. 148 isolates were bacteria and 104 isolates were yeasts. The bacteria contained LAB (101 isolates) and acetic acid bacteria (47 isolates). The culture-dependent approach indicated that Lc. lactis represents the largest and most commonly identified LAB isolates, with 59 of a total of 101 isolates, followed by Lb. parakefir (20 isolates), Lc. kefiri (17 isolates). Isolates of Lb. kefiranofaciens (5 isolates) was also sporadically identified. The only acetic acid specie, A. lovaniensis, was also identified (47 isolates). The lactose-fermenting yeasts (Klu. lactis) together with non-lactose-fermenting yeasts (S. cerevisiae and Kaz. exigua) were found in the whey-based kefir beverages at 30 °C. The yeast flora was dominated by lactose-negative strains. Among them, S. cerevisiae predominated, with 60 of a total of 104 isolates. A total of 273 isolates were obtained from the whey-based kefir beverages at 37 °C (Table 2). Among the isolates, 163 isolates were bacteria and 110 isolates were yeasts. The bacteria contained LAB (116 isolates) and acetic acid bacteria (47 isolates). The culture-dependent approach indicated that Lc. lactis (67 isolates) represents the largest and most commonly identified lactic acid bacteria isolates, followed by Lb. parakefir (20 isolates), Lb. kefiri (16 isolates) and Lb. kefiranofaciens (13 isolates). The only acetic acid specie, A. lovaniensis, was also identified (47 isolates). The yeast flora of the whey-based kefir beverages at 37 °C was dominated by lactose-negative strains. Among them, S. cerevisiae predominated, with 60 of a total of 110 isolates, followed by Klu. lactis (29 isolates) and Klu. marxianus (21 isolates). Under different fermentation temperature, Lc. lactis represents the largest and most commonly identified LAB isolates. Lb. kefir and Lb. parakefir were species-typical microorganism from kefir grains, which are beneficial for flavor development during whey beverage fermentation.
Table 2 Distribution of bacteria and yeasts isolated of the whey-based kefir beverages by the culture-dependent methods

| Closest relative    | Identity (%) | Accession          | 20 °C | 30 °C | 37 °C |
|---------------------|--------------|---------------------|-------|-------|-------|
| **Bacterium**       |              |                     |       |       |       |
| *Lb. kefiri*        | 100%         | AB366385.1          | +(23) | +(17) | +(26) |
| *Lb. kefiranofaciens* | 98%         | AB372208.1          | +(6)  | +(5)  | +(13) |
| *Lc. lactis*        | 99%          | NC002662.1          | +(43) | +(57) | +(67) |
| *Lb. parakefir*     | 98%          | AB447485.1          | +(20) | +(20) | +(20) |
| *A. lovaniensis*    | 99%          | AB308060.1          | +(41) | +(47) | +(47) |
| **Yeast**           |              |                     |       |       |       |
| *Klu. marxianus*    | 100%         | GQ179986.1          | +(28) | +(20) | +(21) |
| *Kaz. turicensis*   | 100%         | EU982208.1          | (*)(*)| (*)(*)| (*)(*)|
| *Klu. lactis*       | 99%          | AJ229069.1          | +(10) | +(30) | +(29) |
| *S. cerevisiae*     | 100%         | EU441887.1          | +(34) | +(54) | +(60) |
| *Kaz. exigua*       | 100%         | EU982209.1          | +(20) | (*)(*)| (*)(*)|

+, detected by PCR-DGGE and sequencing of the DNA fragment upon excision from the gel; * species not isolated by culturing methods; values in parentheses are numbers of colonies detected by culturing.

The groups of LAB cause rapid acidification of the whey by the mean of the production of organic acids, mainly lactic acid. Also, their production of acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes are also of importance [23]. *Lc. lactis* is an important bacterium found in the whey-based kefir beverages. Of all LAB, *Lc. lactis* is by far the most extensively studied organism. The relative simplicity of *Lc. lactis* metabolism that converts sugars via the glycolytic (homofermentative) pathway to pyruvate, generating energy mainly through substrate level phosphorylation [24]. It is widely used in cheese making and active yogurt beverage.

*Lc. Lactis* was the dominating species in the whey-based kefir beverages (Table 2). *Lc. Lactis* is the main constituent of dairy starter culture systems used worldwide for the production of numerous fermented dairy products. The predominant role of *Lc. lactis* is to produce lactic acid at a sufficient rate and contribute to the breakdown of milk proteins during fermentation, thus significantly contributing to the final products in terms of organoleptic properties and microbial qualities [13]. *Lb. kefiri* is also one of the key functional LAB in whey-based kefir beverages. Surface layers proteins from *Lb. kefiri* mediate the interaction of *Lb. kefiri* bacterial cells with yeasts present in kefir grains [22]. Besides, *Lb. kefiri* strains preserve a high percentage of viability after both spray-drying and freeze-drying procedures. All the mentioned properties show the potentiality of *Lb. kefiri* as probiotic microorganism [4]. In the whey-based kefir beverages, *Lb. kefiri* can produce NH₃ from arginine that could increase the pH value. *Lb. paracasei* isolates from the samples under different temperature was identical, which may due to metabolize citrate during fermentation, its mesophilic properties and antimicrobial properties [14, 27]. *Lb. kefiranofaciens* shows good adhesion, resistance to acidic pH values and bile acids, and inhibits some pathogenic bacteria. It produces a kefiran-related exopolysaccharide [28]. Kefiran also has interesting physicochemical properties and can be used as a thickener, stabilizer and emulsifier, film forming agent, fat substitute or gelling agent [29, 30]. Kefir grains increase their weight with subcultures in milk due to the increase in microorganism biomass together with the matrix that composed by protein and exopolysaccharide [31]. In a batch mixed culture of *S. cerevisiae* (also isolated in the whey-based kefir beverages) and *Lb. kefiranofaciens* could assimilate kefiran production rates of *Lb. kefiranofaciens* [7]. *A. lovaniensis* was the only acetic acid species identified in the WFB. *A. lovaniensis* species belongs to the *A. pasteurianus* group. Part of ethanol produced by yeasts may be converted to acetic acid by the genus *Acetobacter*, and then esterified with alcohols to form ethyl esters, which plays quite an important role in whey beverage flavor.

The main contribution of yeasts to whey beverage is utilizing lactic acid, removing the hydrogen
peroxide and producing compounds that stimulate the growth of other bacteria [33]. Meanwhile, the yeasts participate in the microbial metabolism and contribute to the formation of aroma precursors such as amino acids and fatty acids [15]. It is widely accepted that the acetate esters and higher alcohols produced during fermentation by yeast are particularly important for the food industry [12]. Further, aroma compounds are synthesized mainly by the S. cerevisiae species during food-related fermentations. S. cerevisiae was the dominant yeasts in the whey-based kefir beverages. The presence of S. cerevisiae contributes to enhancement of the organoleptic quality, promoting a strong and typically yeasty aroma as well as its refreshing, pungent taste. Acetate esters such as ethyl acetate, isoamyl acetate and isobutyl acetate are mainly synthesized by alcohol acetyltransferases from acetyl-CoA and ethanol or aliphatic or aromatic higher alcohols in S. cerevisiae [23]. Klu. lactis and Klu. marxianus also produce esters through alcoholysis of acyl-CoA and esterification of an organic acid with an alcohol, which impart fruity flavors in whey-based kefir beverages [34]. Yeasts belonging to the genus Kluyveromyces were shown to be able to utilize residual lactose as the sole source of carbon and energy and they are known for producing aroma compounds to fermented products [35]. The most widely-used species in the genus Kluyveromyces are Klu. lactis and Klu. marxianus, which were both isolated in the whey-based kefir beverages. In contrast to the others, these two species have the ability to utilize xylose, xylitol, cellobiose, lactose and arabinose, in both solid and liquid media. However, Klu. lactis and Klu. marxianus share DNA sequence identity of only 15-20% [35]. Ethyl acetate is by far the major ester. Kazachstania genus yeasts could be connected with the assimilation of some acids produced by lactic acid bacteria [36]. Kaz. turicensis and Kaz. exigua were appeared according to culture-independent method, however, Kazachstania exigua was not isolated with the culture-based way.

4. Conclusions
Using culture techniques and culture-independent methods, we have monitored the bacterial and yeasts communities in the whey-based beverage during Bodljong cheese-making. The dominant microbiota, as revealed by PCR-DGGE, was composed by yeast affiliated to Klu. marxianus, Klu. lactis, S. cerevisiae, and bacteria affiliated to Lc. lactis and A. lovaniensis. This application could provide an opportunity to better understand and control the transformation process during whey-based beverage. Moreover, the profiling of microbial populations can be useful to determine the technologically important strains employed as a suitable starter culture for whey utilization.

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