The Isolation and Identification of *Lactobacillus* from Naturally Fermented Yoghurt

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Abstract. Lactic acid bacteria were isolated from natural fermented yoghurt by traditional isolation and culture method, and identified by gram staining and 16S rDNA sequencing analysis. The results showed that 5 strains of lactic acid bacteria were obtained, which were X22-1, X22-2, X22-3, X22-4 and X40-1. After 16S rDNA sequencing, X22-1, X22-3, X40-1 were *Lactobacillus fermentans*, X22-2 and X22-4 were *Lactobacillus delbrueckii* subsp. *Bulgaricus*.

1. Introduction

As a well-known dairy beverage, yogurt rich nutrition and various lactic acid bacteria are beneficial to our health, which is popular with people. In the industrial production of yoghurt, the added starter cultures come from the known fermentation strains. However, the natural fermentation yoghurt is formed under the effect of natural conditions and microorganisms without any food additives. It has a more viscous and delicate taste, and more importantly, it contains a greater variety of lactic acid bacteria [1]. In order to obtain fermenters with different characteristics and functions for industrial production, the unique microorganisms will be separated from naturally fermented yogurt. After cultivation, passage, mixing, identification and other ways, the lactic acid bacteria with different metabolism and physiological characteristics can be screened from the yoghurt [2], so as to satisfy the various needs of probiotics in the food and medicine industry.

Lactic acid bacteria ferment sugars to produce lactic acid, which is a gram-positive bacterium without spores [3]. The species of lactic acid bacteria are mainly divided into *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Pediococcus*, etc [3]. *Lactobacillus* is the most common probiotics in our daily life. It has good adhesion and colonization characteristics [4], and possess the abilities of regulating gastrointestinal flora, maintaining intestinal microecological balance, protecting intestinal mucosa [5], and regulating immunity [6]. Therefore, many researches showed *Lactobacillus* had a variety of physiological activities, such as anti-colitis, anti-arthritis, anti-obesity and so on [7]. It is generally believed that the prebiotic effects of *Lactobacillus* are realized by the metabolism of *Lactobacillus* and its fermentation products [8]. In this study, *Lactobacillus* were isolated and identified from natural fermented yoghurt, to obtain
excellent probiotic candidate strains. This investigation could enrich the probiotic resources and provide fundamental basis for the development of probiotics.

2. Experimental method

2.1. Yogurt sample

Yogurt sample X22 was collected from Siyeke Grassland, Kuoketiereke Township, Tekes County, Xinjiang, China. Yogurt sample X40 was collected from Balekesukaisi Grassland, Zhaosu County, Xinjiang, China.

2.2. Isolation and purification of lactic acid bacteria

After thawing natural fermented yoghurt samples, 1mL yoghurt samples were added into sterilized 12% skimmed milk, and then cultured in 37 ℃ biochemical incubator until the yoghurt was solidified. 1mL of rejuvenating yoghurt sample was added into 9 mL saline in test tube. Then, each sample group was respectively diluted 10^{-1}-10^{-6} concentration by gradient. 200 μL of 10^{-4}, 10^{-5}, 10^{-6} diluted bacteria solution was coated on MRS solid plate, and cultivated for 24h. The tablets with the most colony growth was selected, which were X22-1, X22-2, X22-3, and X22-4 from yogurt sample X22, and X40-2 and X40-1 from yogurt sample X40. The morphology of each colony was recorded.

Single colony was picked from the plate and cultured in MRS liquid medium. In the process, X40-2 grew weak and was discarded. The rest of the colonies were crossed on MRS agar plate with inoculation ring for several times until a single colony was obtained. Finally, X22-1 grew to 10 generations, X22-2 grew to 12 generations, X22-3 grew to 10 generations, X22-4 grew to 11 generations, and X40-1 grew to 11 generations.

2.3. Identification of lactic acid bacteria

2.3.1. Observation of strains. After centrifuged at 12000 rpm/min, the supernatant of bacterial liquid was poured out. According to the number of bacteria, ultra-pure water was added into residual strains. The mixed bacterial liquid was evenly coated on the burned slide. After drying, gram staining was carried out to observe the morphology of the strain. The purified single colony was stored in - 80 ℃.

2.3.2. 16S rDNA molecular identification of lactic acid bacteria. Total DNA of lactic acid bacteria was extracted according to the bacterial genomic DNA extraction kit. The total DNA was used as template for PCR amplification. The 16S rDNA universal primer was used, which is 27F: 5’-AGAGTTTGATCCTGGCTCA-3’, and 1495R: 5’-CTACGGCTACCTTGTTACGA-3’. 25μL DNA template, 1μL 27F forward primer and 1μL 1495R reverse primer were mixed, then 12.5μL 2xTaq PCR Master Mix was added. The reaction was proceeded under the condition: 90℃ for 5min, then 30 cycles of 94℃ for 40 s, 55℃ for 40 s, and 72℃ for 60 s, terminal extension 72℃ for 10 min.

PCR products were detected by gel electrophoresis. The gel solution was poured into the gel plate. After 30 minutes, the TAE buffer solution was poured into the electrophoresis tank, and then the gel plate that has completely solidified was put into the TAE buffer solution. Then PCR products was added to each empty tank in turn. Electrophoresis was performed for 45min at a voltage of 100V, in which D2000 DNA Marker was utilized. The positive PCR products were detected by electrophoresis were sent to Chengdu Qingke Biology Co., Ltd. for sequencing identification, and the sequencing results were retrieved in NCBI.

3. Result and analysis

3.1. Colony morphology and gram stain of lactic acid bacteria

5 single colonies were isolated. The colonies of X22-1, X22-3, X22-4 and X40-1 were round and white with a smooth and moist surface. The morphology of X22-2 colony was irregular shape of small
snowflakes. After gram staining, purplish red cell morphology was observed under the microscope, which was judged as gram-positive bacteria (G+). The specific single colony morphology and gram staining diagram are shown in Figure 1. The colony morphology is on the left and gram staining diagram is on the right respectively.

Figure 1 Single colony morphology of isolated strains and gram staining results

3.2. Sequence analysis of 16S rRNA of lactic acid bacteria

As shown in Figure 2, a clear and bright band was observed in agarose gel electrophoresis of the 16S rDNA gene amplification product, and there is no band in the negative control, indicating that the PCR process was not contaminated. The bands were located at 1000-2000 bp in accordance with the length
of the amplified fragment of *Lactobacillus*, which could be used for subsequent sequencing. In the figure, M represented D2000 DNA marker, 0 represented negative control, and 3-7 respectively represented X22-1, X22-2, X22-4, X22-3 and X40-1.

As shown in Table 1 the results of PCR amplification products were retrieved and analysed by BLAST. The homology was over 99%.

| Strain number | Strain type                     | Homology (%) |
|---------------|---------------------------------|--------------|
| X22-1         | *Lactobacillus fermentum*       | 99           |
| X22-2         | *Lactobacillus delbrueckii* subsp. bulgaricus | 99           |
| X22-3         | *Lactobacillus fermentum*       | 99           |
| X22-4         | *Lactobacillus delbrueckii* subsp. bulgaricus | 100          |
| X40-1         | *Lactobacillus fermentum*       | 100          |

4. Conclusion

5 strains of *Lactobacillus* were isolated and purified from natural fermented yoghurt. Among them, 3 strains were *Lactobacillus fermentum*, and other 2 strains were *Lactobacillus delbrueckii* subsp. *Bulgaricus*. The homology of the sequence was more than 99% by genebank database. This experiment provides a source for the subsequent screening of excellent probiotics, which can enrich the resources of lactic acid bacteria.

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References

[1] Jiang, H. Y., Chen, Z. L., Zhao, G. H., et al. (2014) Investigating the diversity of lactic acid bacteria in Tibetan traditional fermented dairy products by PCR-DGGE. Food Sci., 35: 167-173.

[2] Wu, J., Zhao, X. J., Chen, J. X., et al. (2013) Isolation and identification of lactic acid bacteria from natural yak yogurt in Tibet Plateau pastoral areas of Tibet and western Sichuan, Food Sci., 34: 150-155.

[3] Von Wright A., Axelsson L. (2011) Lactic acid bacteria: an introduction. CRC Press, London.

[4] Stanton, C., Ross, R. P., Fitzgerald, G. F., et al. (2005) Fermented functional foods based on probiotics and their biogenic metabolites. Curr. Opin. Biotechnol., 16: 198-203.

[5] Hou, Q., Ye, L., Liu, H., et al. (2018). Lactobacillus accelerates ISCs regeneration to protect the integrity of intestinal mucosa through activation of STAT3 signaling pathway induced by LPLs secretion of IL-22. Cell Death Differ. 25: 1657-1670.

[6] Baarlen, P. V., Wells, J. M., Kleerebezem, M. (2013) Regulation of intestinal homeostasis and immunity with probiotic lactobacilli. Trends Immunol. 34:208-215.

[7] Lee, H. S., Han, S. Y., Bae, E. A., et al. (2008) Lactic acid bacteria inhibit proinflammatory cytokine expression and bacterial glycosaminoglycan degradation activity in dextran sulfate sodium-induced colitic mice. Int. Immunopharmacol. 8:574-580.

[8] Valdes, A. M., Jens, W., Eran, S., et al. (2018) Role of the gut microbiota in nutrition and health. BMJ, 361: k2179.