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Potential probiotic characterization of *Lactobacillus reuteri* from traditional Chinese highland barley wine and application for room-temperature-storage drinkable yogurt

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ABSTRACT

The aim of this study was to select probiotic strains that could be used in drinkable yogurt to yield viable cells following storage at room temperature (RT). The uniquely high altitude conditions in Tibet and the alcoholic environment of certain products, such as the highland barley wine homemade in Tibet, may induce unusual characteristics of microbial strains. A total of 27 lactic acid bacteria were isolated from homemade highland barley wines. One strain, *Lactobacillus reuteri* WHH1689, demonstrated no ability for lactose utilization, exhibited a high survival rate during storage at RT in drinkable yogurts, and produced very weak post-acidification. This strain showed great resistance to conditions simulating the gastrointestinal tract, including strong adherence to HT-29 cells and inhibitory activity against *Escherichia coli*, *Shigella flexneri*, *Salmonella paratyphi* β, and *Staphylococcus aureus*. A dextran sulfate sodium (DSS)–induced mouse model was used to evaluate the in vivo influence of *Lb. reuteri* WHH1689 on the intestinal flora and showed that strain WHH1689 increased viable counts of bifidobacteria in feces of mice. The probiotic strain selected in this study—with its high survival at RT and lack of serious post-acidification problems—may provide significant improvements for dairy industry products by extending the storage time of dairy products with living cells.

Key words: *Lactobacillus*, highland barley wine, lactose utilization, room temperature storage, post-acidification

INTRODUCTION

Probiotics are nonpathogenic microorganisms that exert a positive health benefit on the host when ingested in an adequate amount, according to the FAO/WHO (2006). This definition was recently revised and accepted after minor grammatical modifications as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Most probiotic bacteria are lactic acid bacteria (**LAB**) and, among them, *Lactobacillus* is one of the most common genera (Argyri et al., 2013). In recent years, probiotic LAB have received increasing attention because of their long history of safe use and potential therapeutic benefits for human health. Probiotics affect human health mainly by regulating the gastrointestinal microbiota (Saad et al., 2013), and the best vehicles for the delivery of viable probiotics to the human gut are dairy foods (Hill et al., 2017).

According to the scientific community, probiotic counts should exceed the minimum value of 6 log cfu/mL to confer potential health benefits (Nualkaekul et al., 2012). In the dairy industry, maintaining viability of probiotic bacteria in foods throughout storage is a constant challenge (Batista et al., 2015). Fermented products with living probiotic cells are normally transported and stored at 4 or 10°C because low temperatures inhibit the metabolism of bacteria and suppress post-acidification and, hence, product deterioration. Myriad organic acids are produced by the LAB, including those in yogurt, and the persistent production of acid leads to high post-acidification (Shah, 2000). Even post-acidification occurring during chilled storage results in a short shelf life and inhibits the viability of other probiotics, including *Bifidobacterium* (Wang et al., 2013). To delay post-acidification, inulin was found to be effective in sheep milk yogurt (Balthazar et al., 2016). Storage of fermented products at low temperature increases the cost of transport and limits the market economically. Moreover, refrigerated products are uncomfortable for most Asians to ingest because drinking refrigerated products is not common in Asian culture. Lactic acid bacteria that could survive at room temperature (**RT**) during transport and storage of

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fermented products without serious post-acidification would be a huge improvement for the dairy industry by extending the storage time of products with living cells.

The Tibetan Plateau, generally called "the roof of the world" because of its very high altitude, is characterized by its extreme environment. Highland barley, the main grain crop on the Tibetan Plateau, can be made into a variety of conventional foods, including fried noodles (i.e., *zanba*) and wines (Zhong et al., 2016). Homemade highland barley wine may spontaneously undergo a secondary malolactic fermentation step by LAB (Mesas et al., 2011). In the Shigatse area of Tibet, the average temperatures are above 10°C from May to October, and the daytime temperature can reach 20°C (Jiang et al., 2009). In this study, we isolated LAB from homemade highland barley wines collected in August from the Shigatse area, and *Lactobacillus reuteri* WHH1689 was screened for later experimentation. This strain was identified from among the isolated LAB. Because this strain lacked lactose utilization ability, it would not cause post-acidification during RT storage and thus was selected for further experiments. First, we tested the potential application of this strain in drinkable yogurt during storage at room temperature. Then, we evaluated the survivability of the strain in drinkable yogurt and extent of post-acidification. Because of the promising in vitro probiotic characteristics of this strain, we further examined the in vivo regulatory effect on intestinal flora in a mouse model.

MATERIALS AND METHODS

Microorganisms and Cell Line

Lactobacillus rhamnosus GG (**LGG**) and *Lactobacillus casei* strain Shirota (**LcS**) were isolated separately from commercial fermented milk products (Yili, Inner Mongolia, China; and Yakult, Shanghai, China). A human colon adenocarcinoma cell line (HT-29) was purchased from Cell Institute of Chinese Academy of Sciences (Shanghai).

Isolation of LAB from Highland Barley Wines

Four samples (100 g each) of homemade highland barley wine were collected from local homes, with the residents' permission, in the Shigatse area of Tibet. Initially, 10 g of each sample was suspended in 100 mL of sterile saline and mixed thoroughly. Serial dilutions (10^{-1} to 10^{-4}) were performed, and 100-μL aliquots from each dilution were spread-plated onto de Man, Rogosa, and Sharpe (**MRS**) agar (Oxoid Ltd., Basingstoke, UK) and incubated at 37°C for 48 to 72 h anaerobically. Six to 7 colonies with different morphologies were randomly isolated from MRS agar plates for each sample. These isolates were submitted to analyses including Gram staining, morphology, and catalase activity (Garcia et al., 2016). Gram-positive, catalase-negative colonies were further stored at −80°C in MRS broth (Oxoid Ltd.) containing 25% glycerol for further studies.

Selection of LAB Lacking Lactose Utilization Ability

To determine which LAB showed reduced levels of post-acidification in yogurt, 27 isolates were added to sterile 10% skim milk and incubated at 37°C for 3 mo. Milk curdled by fermentation was subsequently observed.

Identification and Phylogenetic Analysis of the LAB Isolate

Bacterial genomic DNA of LAB was extracted using a Genomic DNA extraction kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. For the detection of 16S rRNA gene sequences, the following primers were used: 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′, and 1492R: 5′-GGTTACCTTGTTACGACTT-3′. The PCR program was as follows: 94°C for 4 min, followed by 32 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min 20 s, with a final step at 72°C for 10 min (Guo et al., 2010; Garcia et al., 2016). The PCR product was subsequently sequenced (Sangon Biotech). The resulting sequence was aligned via the National Center for Biotechnology Information BLAST program (http://blast.ncbi.nlm.nih.gov) to choose closely related strains and to identify the 16S rRNA gene sequence similarities of phylogenetic neighbors. Multiple alignment of 16S rRNA nucleotide sequences from 13 species included in the analysis was generated using CLUSTAL_X (Larkin et al., 2007). The phylogenetic tree was constructed with Mega 5.0 (Tamura et al., 2011) using the neighbor-joining method and a bootstrap value of 1,000.

Stability of Lb. reuteri WHH1689 in Products During Storage

Lactobacillus reuteri WHH1689 was propagated in MRS broth overnight at 37°C followed by sub-culture and incubation for a further 18 h. All cultures were harvested by centrifugation (5,752 × *g*, 10 min, 8°C) and the pellets were washed once in PBS, pH 7.4. A 1% inoculum of culture was aseptically distributed into 50-mL portions of 2 commercial dairy products (UHT-