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Full Length Article

Biological Characteristics and Phylogenetic Analysis of Lactic Acid Bacteria Isolated from Free-Range Yaks (*Bos grunniens*) in Qinghai-Tibet Plateau

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Abstract

Lactobacillus is widely used probiotic lactic acid bacteria (LAB) all over the world. However, to date, there is limited information about LAB strains isolated from yaks. The present study was aimed to isolate and identify LAB from free ranging yaks to provide basic information for the prevention of bacterial infection in yaks. A total of four LAB strains were isolated and identified by biochemical, 16SrRNA sequence and phylogenetic analysis from yaks. The physical and chemical properties index, bile tolerance and auto-aggregation showed that Tibet-L5 and Tibet-L4 had a good physical and chemical properties as compared with the Tibet-L1 and Tibet-L3 isolates. The sequence analysis revealed that four lactic acid bacterium (Tibet-L1, L3, L4 and L5) exhibited approximately 93.4−98.6% similar identity of reference strains. The isolated Tibet-L1 and Tibet-L3 strains were most closely related to AB809572, AY862434 and KU991814 strains. This is the first study to examine free range yaks lactic acid flora using molecular methods, in high altitude Tibet, China. Four strains obtained from this study could be used in the industry as probiotic strains. © 2018 Friends Science Publishers

Keywords: Lactic acid bacteria; Yaks; Tibetan plateau; 16S rRNA

Introduction

Lactic acid bacteria (LAB) are gram-positive, non-spore forming, immobile, and catalase negative, which excrete lactic acid as major end product (Konings *et al*., 2000). They are also selected as probiotic and able to promote health and prevent infections against enteropathogenic bacteria and immunomodulation (Fernandez *et al*., 2003). At present, nearly 400 LAB species have been recognized (Euzeby, 1997). LAB are usually harbor carbohydrate-rich environments and found in various food products such as milk, plant, meat, intestinal mucosa of human, and animals (Fernandez *et al*., 2003), *Lactobacillus* is a gram-positive bacterium that is often used in dairy products as a probiotic strain and it can normally benefit the health of the host (Shida *et al*., 1998; Nagao *et al*., 2000). LAB are one of the normal and beneficial intestinal floras of human and animal, found in the oral cavity, stomach, small intestine and vagina (Hartemink *et al*., 1997). LAB are important part of the normal microbiota which help in fermentation to produce organic acids, special enzymes and bacterial surface composition, content that has antibacterial, enhance immunity, improved the gastrointestinal functions, and the maintenance of normal stability microbiota in the digestive tract (Nagao *et al*., 2000; Shioiri *et al*., 2006). Researchers showed that LAB could resist to *E. coli* and pullorosis salmonella infection in chickens (Ouwehand *et al*., 2014). The microbial additives can regulate the microbial in the bovine rumen that can improve rumen environment and animal production performance (Parmar *et al*., 2015). To date, LAB have been isolated from various animal species like chicken, cattle, sheep, pigs and others including humans. However, no study is performed about the LAB in Tibetan yaks. Yaks live in China and other elevated regions of central Asia, including Bhutan, Nepal and India. China has approximately 140 million yaks, accounting 90% of the total yak population (Li *et al*., 2014). The ancient domestic yak is used as a draught animal and providing meat and milk products in the remote areas of Qinghai-Tibetan Plateau (Qiu *et al*., 2012; Li *et al*., 2014).

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Yaks are economically important animals for Tibetans and yak diseases are publically and economically important for Tibetans (Han *et al*., 2013). Recently a large number of yaks died due to bacterial infections in different seasons, however, the meat and dairy products are necessities of the daily life in Tibetans and use of antibiotics will pose a health risk to local herdsmen. Hence, the present study was performed to isolate and identify the LAB species for the first time in Tibetan yaks that could be used as a reference for the prevention and control of bacterial infection in the Tibetan plateau, China.

Materials and Methods

Study Site and Sample Collection

The present study was carried out on the Tibetan Plateau, China that has an average height of 3100 meters, and the largest high elevation ecosystem (Zhang *et al*., 2017a, b). A total of 5 intestine samples from 2 years old yaks were collected randomly from local slaughterhouses during year 2016. After the collection, all intestines were stored and transported at 4°C for further analysis.

Physiological Tests and Bacterial Isolation, and Identification

Intestinal tissue samples were cut into 1 cm thickness and shake well for 60 sec with 90 mL sterile distilled water, and then serially diluted from 10^{-1} to 10^{-5} with sterile water. Ten grams of each sample was blended with 20 mL of distilled water, and pH was determined by pH meter (Metrohm 744, Herisau, Switzerland) (Panagou *et al*., 2008). Afterward the samples were incubated on MRS agar (Hangzhou microbial, China) at 30°C for 16−27 h in an anaerobic box. A total of four isolates were collected and determined to be LAB by gram staining. For further validation, physiological tests like catalase and lactic acid production (Table 1) were performed according to the previous studies (Kozaki *et al*., 1992). The bacterial species identification was performed by16S rRNA sequencing (Using universal primers) method as suggested by Collins and Pang (Collins *et al*., 1991; Kozaki *et al*., 1992; Pang *et al*., 2012).

LAB Extraction and PCR of 16S rRNA Gene

The bacterial chromosomal DNA was extracted using GenElute™ Mammalian Genomic DNA Miniprepkit as per manufacturer's instructions (Sigma-Aldrich (Shanghai) Trading Co., Ltd, Shanghai). The eluted DNA was stored at -20°C for further analysis.

A polymerase chain reaction (PCR) amplification approach was used to amplify a fragment of the 16S rRNA gene. The PCR forward: AGAGTTTGATCCTGGCTCAG and reverse AAGGAGGTGATCCAGCC primers were used with the following cycling parameter, 35 cycles at 95°C for 30 sec, and 72°C for 1.5 min, Annealing at 55°C for 40 sec; and final extension at 72°C for 5 min. PCR products were separated on Agarose gel (1.0%) with ethidium bromide (at the rate of 0.5 μg/mL) following electrophoresis was performed in 0.5×TBE buffer at 5 V/cm for 60 min. The products were purified using Agarose Gel DNA extraction kitVer.4.0 (Takara Biotechnology Co. Ltd. Dalian, China) according to manufacturer's instructions (Fig. 1). The positive products were sequenced by a commercial company (Quintara Biosciences, Wuhan, China).

The Physical and Chemical Properties Index of LAB

A total of 2% LAB were inoculated in MRS culture medium and cultured at 37°C, the OD_{600} value was detected after every 2 h for the growth curve. A total of 2% LAB were inoculated in MRS culture medium, and cultured in 10, 20, 30, 37, 40, 50, 60°C for 24 h, OD value was detected after 24 h for the sensitivity test of temperature. A total of 2% LAB were inoculated in MRS culture medium, and cultured in 6 h, 12 h, 18 h and 24 h. Then the pH value was detected and calculated by following the method; Acid production rate = the medium initial pH − pH at each time point.

Auto-aggregation Assay

The auto-aggregation ability was measured according to previous study (Del *et al*., 1998). Briefly, the samples were cultured on mediums at 37°C overnight, then centrifugation at 4, 000 × g for 20 min and washed two times with PBS. The samples were missed into PBS at a density of 108 CFU/mL, then the samples (5 mL) were cultured for 5 h, and the OD value was read at 600 nm wavelengths on micro plate reader. The results were based on OD value according to the formula:

$$\text{Self-aggregation (\%)} = 1-(A_t/A_0) \times 100$$

A_t was OD value in different hour, A_0 was OD value in 0 h.

Bile-tolerance Assay

Each strain was sub-cultured at least three times before the assay, and then the isolates were incubated in MRS broth added 20% (w/v) healthy pigs powder at 37°C. Bacterial growth was counted at 4 h, 8 h and 12 h.

Phylogenetic Tree and Sequence Analysis

The nucleotide sequences of the 16S rRNA gene of LAB were compared with previously reported LAB sequences available at NCBI database. Multiple alignments and phylogenetic analysis techniques were conducted using DNASTAR (Laser gene v7.1) and MEGA 6.0 software for windows. Phylogenetic tree was constructed using the neighbor-joining method.

Table 1: Biochemical tests used for LAB identification

Tests	Results	Tests	Results
Gelatin liquefaction	-	Indole production	-
Catalase test	-	Carbohydrate fermentation tests	+
Motility	-	Methyl red	+
Nitrate reduction	-	Vogesproskauer	-

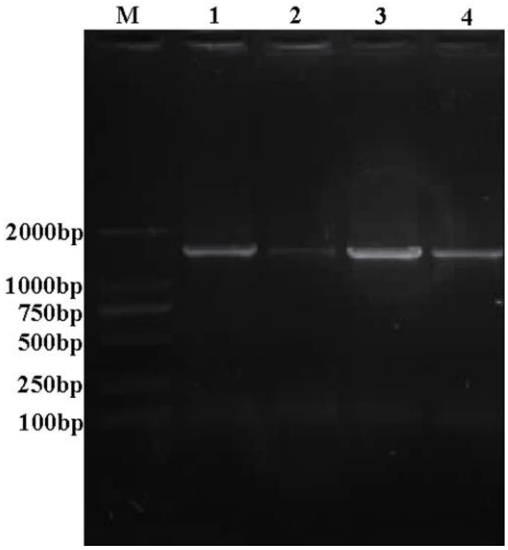

Fig. 1: The results of PCR amplification for the 16S rRNA gene of LAB. (M: marker; 1, 2, 3, 4: positive samples)

Results

This study was aimed to isolate the *Lactobacillus* strains from yak intestine sample, and a total four *Lactobacillus* strains were isolated from the samples. The growth curve showed a sharp increased before 14 h, and after that the growth become to slow; the sensitivity test of temperature showed a good growth with the temperature of 20°C to 50°C; the LAB showed a high acid production rate. Meanwhile, the Tibet-L4 and Tibet-L5 have good physical and chemical properties as compared with the Tibet-L1 and Tibet-L3 isolates (Fig. 2).

In our studies, we used an *in vitro* model and found the Tibet-L4 and Tibet-L5 have a good ability of auto-aggregation and bile tolerance as compared with the Tibet-L1 and Tibet-L3 isolates (Fig. 3). The product of four isolates were sequenced and analyzed at NCBI database. The sequence phylogenetic and homology analysis revealed that, these four Tibetan lactic acid bacterial isolates (Tibet-L1, 3, 4 and 5) showed 93.4−98.6% sequence similarity to reference strains (Fig. 5). The Tibet-L1 and Tibet-L3 strains were most closely related to AB809572, AY862434 and KU991814 representative strains (Fig. 4).

Discussion

Gut microbiota plays an important role in homeostasis of the body; they are considered influencing the immune and

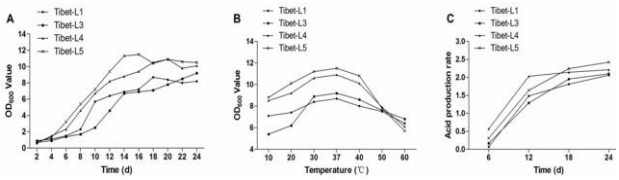

Fig. 2: The growth curve, sensitivity test of temperature and acid production rate of Lactic Acid Bacteria. (A: the growth curve of LAB; B: the sensitivity test of temperature of LAB; C: Acid production rate of LAB)

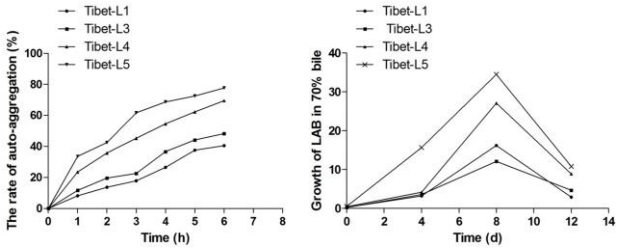

Fig. 3: The results of bile tolerance and auto-aggregation ability of LAB

nutritional system of the animal. Mainly participate in digestion and absorption of food (Pang *et al*., 2012). The intestinal microecological balance is relatively important to animal health (Shioiri *et al*., 2006; Pang *et al*., 2012); however, microbial imbalance can lead to disorders of gut ecosystem and multiple body issues. Intestinal ecosystem disturbance is usually due to the increase of harmful bacteria in the small intestine; particularly the overall increase of intestinal aerobe, mostly *Enterobacteriaceae*, *Streptococcus*, *Bifidobacterium* and *fusiform* buds arms *Bacillus*, may cause disorder of gut microbes (Bik and Relman, 2014). Previously, a high mortality of bacteriosisin Yaks was reported in China (Zhao *et al*., 2005). However, the Tibetan herdsmen are lacking the prevention awareness on this disease, and previous study found the overuse of antibiotics in some regions of Tibet (Henriksson *et al*., 1999; Zhang *et al*., 2017a, b). The presence of drug residues is a serious problem in yak meat and dairy products; therefore, screening a probiotic has the vital significance in this region.

Previous studies have demonstrated that the auto-aggregation is an important trait that contributes to the ability of LAB to colonize the intestinal tract (Del *et al*., 1998). In our study, we used an in vitro model and found the Tibet-L4 and Tibet-L5 have a good ability of auto-aggregation and bile tolerance as compared with the Tibet-L1 and Tibet-L3 isolates. The product of four isolates were sequenced and analyzed at NCBI database. The sequence phylogenetic and homology analysis revealed that, these four Tibetan lactic acid bacterial isolates (Tibet-L1, 3, 4 and 5) showed 93.4−98.6% sequence

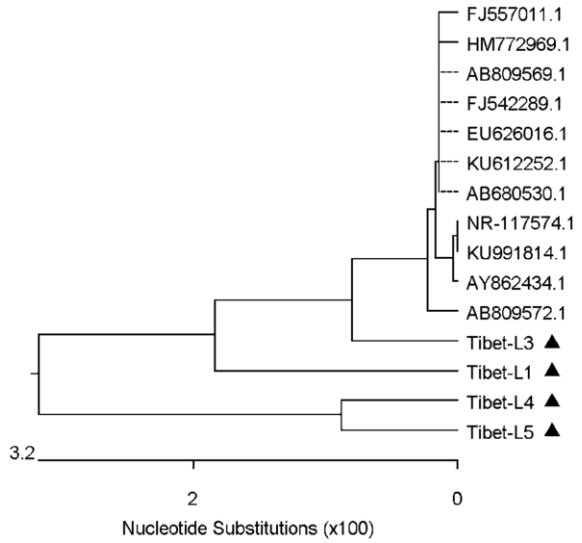

Fig. 4: Phylogenetic tree constructed by the neighbor joining method in DNASTAR (v7.1), using nucleotide sequences of the 16S rRNA gene

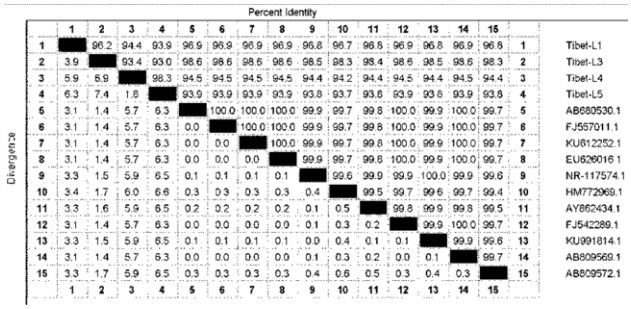

Fig. 5: Homology comparison of nucleotide sequence of isolates with other strains (100%)

similarity to reference strains. The Tibet-L1 and Tibet-L3 strains were most closely related to AB809572, AY862434 and KU991814 representative strains.

The present study performed first time to isolate and phylogenetic analysis of LAB from free-range yaks on the Tibetan Plateau of China. The special conditions on the Tibetan Plateau have unique microorganism communities as compared with other regions, this elevated region is more than 3100 m above sea level with a bad climate conditions (Henriksson *et al*., 1999; Zhang *et al*., 2017). It is also speculated that the survival and growth of Lactic acid bacteria have adapted in this region. Gram-positive bacteria generally considered as useful organisms used in fermentations, ubiquitous in nature and gastric commensalism in addition to beneficial probiotic bacteria. However, gastrointestinal infections can also cause by gram-positive pathogens (Bagga and Shenep, 2010). The lactobacilli identified in this study were markedly lower level in the natural fermentation in yaks, which showed the overuse of antibiotics in the study area of Tibet.

Conclusion

Four strains obtained from this study could be used in the industry as probiotic. The current study provides a reference for the development of strategies for the new probiotic products. These strains can protect the host from gut pathogen by maintaining the balance of gut microbiota during antibiotic treatment. However, additional studies are required to determine the efficiency of selected strains.

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