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ORIGINAL RESEARCH PAPER

COMPOSITION OF LACTIC ACID BACTERIA IN DAIRY PRODUCTS AND THEIR EFFECT ON TOURISM DEVELOPMENT OF INNER MONGOLIA

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In recent years, the development of dairy industry in Inner Mongolia has accelerated its economic growth, and its grassland culture has become appealing to the public. As an important support industry for the economic development in tourism area of Inner Mongolia, dairy industry can create economic value for the development of tourism. In view of the importance of dairy products-the habitat of lactic acid bacteria, this study aims to reveal the composition of lactic acid bacteria in dairy products and isolate lactic acid bacteria resources. Firstly, we selected 60 traditional dairy product samples (from the pasture in scenic area of Inner Mongolia) as the research objects. Based on the 16S rRNA gene sequence analysis, lactic acid bacteria in the samples were isolated and identified; Real-time quantitative polymerase chain reaction (q-PCR) technology was applied to the comparative analysis on the population of dominant bacteria in samples. It was found that there were significant differences in the numbers of dominant bacteria in different dairy products. With the advantages of improving nutritional value and extending storage time of dairy products, lactic acid bacteria is contributive to the development of dairy industry, which further promotes the prosperity of economy and tourism. Therefore, it is of great importance to study the composition of lactic acid bacteria in dairy products.

Keywords: dairy products, lactic acid bacteria, 16S rDNA, q-PCR

Introduction

Given a long history and unique production technique, traditional fermented dairy products in China are well known for their characteristic taste and functions which benefit from the diversity in microorganism species that participate in the fermentation process (Pagan *et al.*, 2010). In recent years, a competitive industrial cluster (including dairy industry, coal and tourism

industry) has been formed in Inner Mongolia, which enhances its comprehensive economic strength, thus to provide a solid foundation for the development of tourism industry (Feng and Jia, 2009). While the tourist attractions are prospering in Inner Mongolia, more attention has been drawn to the modernization and industrialization in the production of traditional fermented dairy products as well as food safety issues. Accordingly, microbial classification has become a hot topic in the researches on fermentation process of traditional fermented dairy products. A lot of food (such as milk, soya bean milk, pickled cabbage, apples, lactic acid drinks) contains lactic acid bacteria, which can not only improve the nutritional value and flavor of food as well as increase its preservation duration and additional value, but also have unique biological activity and nutritional functions.

The biodiversity of lactic acid bacteria resources in China results from the vast territory, changeable climate and distinctive techniques of different nations in preparing traditional fermented dairy products (Khalaileh and Ajo, 2013). Endowed with temperate grassland pasture and vast land, Inner Mongolia is famous for producing high-quality dairy products which appeal to consumers. Owing to the reputation of dairy industry, Inner Mongolia is also developing its tourism industry and creating its tourism value (Mahrous *et al.*, 2013). At present, a lot of experts, scholars and research institutions have carried out relevant studies on the composition and diversity of lactic acid bacteria in dairy products.

Duolan et al. (2009) performed a detailed study on the diversity of lactic acid bacteria in naturally fermented milk from Chinese western minority areas, Mongolia pastoral areas and regions of Russia; by isolating and identifying the lactic acid bacteria from dairy products, they found that *Lactobacillus* was a kind of dominant fungus. Ichikawa et al. (2011) isolated *Streptococcus thermophilus* from traditional fermented yak milk products (collected from Inner Mongolia grassland in scenic region); through fermentation test, they detected its fermentation, sensory and aroma-producing characteristics; finally, they successfully screened bacterial strains with excellent fermentation characteristics.

In brief, as an essential strain in the production process of fermented dairy products, lactic acid bacteria is of high application value in food, health care, industry and agriculture for their unique biological functions (Arendt *et al.*, 2011). Dairy industry can promote the development of tourism industry in Inner Mongolia, thus to promote economic growth.

Materials and methods

Samples

The experimental samples included 60 traditional dairy products collected from scenic pasture of Inner Mongolia.

Table 1. Types and origin of 60 samples of traditional dairy products from Inner Mongolia

Type	Serial number	Origin
Yoghurt	1-34	Baiyanhua Town
Cream	35-44	Bayon Obo Sumu
Cheese	45-49	Ming'an Town
Fresh milk	50-60	Bailingmiao Town

Culture media and chemicals

The experimental reagents included bacterial growth media- MRS solid medium (Wuxi SAV Teconology Co., Ltd.) and MRS broth, galactococcus separation media— M17 solid medium annd M17 broth (Shanghai Hanni Biotechnology Co., Ltd.), 10% skim milk protective agent, 0.5 M ethylene diamine tetraacetic acid (EDTA), 10% sodium dodecyl sulfonate (SDS), 10 M cetyltrimethyl ammonium bromide (CTAB), agarose, isopropyl alcohol (Dalian Takara Technology Co., Ltd.), 10× TE buffer, proteinase K and NaAc buffer.

Sample collection

1.5 ml of fermented milk was mixed with 0.5 g of sterilized neutralizer (starch/$CaCO_3$, 50:1, M/M) in a sterile freezing tube; 5 g of solid sample was collected with a sterilized spoon and added into a sterile freezing tube. All the samples were labeled and sealed with sealing film; then, they were placed in a portable refrigerator at 4°C.

Isolation and purification of lactic acid bacteria

200µl of dilutions (10^{-5}, 10^{-6} and 10^{-7}) were respectively added into pre-prepared MRS and M17 solid media. The plate was placed in an anaerobic incubator and cultivated at 30°C for around 48 hours. Labeled single bacterial colony was selected with sterile inoculating loop and inoculated to the corresponding MRS or M17 broth; then, it was cultured at 30°C for 24-48 hours. From the selected colonies, Gram staining test and microscopic examination were performed. The strains were inoculated to MRS or M17 media for catalase test- colonies on solid media were placed in a clean test tube and 2 ml of hydrogen peroxide solution (3%) was added. If bubbles appeared within 30 seconds, the colonies were positive; otherwise, the colonies were negative.

Real time q-PCR analysis of dominant bacteria in dairy products

Genomic DNA extraction from the dairy products

1 ml of dairy product was placed in a sterile centrifuge tube containing 1g of acidized glass beads, and 3 ml of DNA extracting solution (composed of 100 mmol of Tris-Hcl, 25 mmol of EDTA, 500 mmol of NaCL and 1% SDS) was added; then, the tube was shaken to fully mix the liquid.

0.3 ml of SDS and 10 µl of proteinase K were added to the centrifuge tube which was then placed in an orbital shaker (at 50°C, 300 rpm) for 2h; afterwards, the tube was centrifuged at 12,000×g and 4°C for 10 min.

700 µl of chloroform was added to the collected supernatant; after mixing, they were centrifuged at 12,000×g and 4°C for 10 min; 80 µl of NaAc solution and 800 µl of ice isopropanol were added to the collected supernatant; after mixing, they were centrifuged at 12,000×g and 4°C for 5 min; the supernatant was removed and DNA sediment was obtained.

500 µl of ethanol was added to the DNA sediment; they were centrifuged at 8,000×g and 4°C for 5 min; the supernatant was removed, and the remaining was placed in the air for natural drying; 40 µl of TE solution was added and DNA was redissolved at room temperature. At last, using TE buffer, the extracted genomic DNA solution was diluted to a concentration of 100 ng/µl and preserved as standby.

Identification of lactic acid bacteria by q-PCR

ND-1000 spectrophotometer was used to determine the optical density (OD) and concentration of DNA solution; then, the DNA diluent (10^{-1}-10^{-8}) was used as template; specific primers (as shown in Table 2) were used for q-PCR amplification. The reaction system is shown in Table 3.

Reaction conditions of q-PCR amplification are as follows (Josefsen *et al.*, 2010; Pulford *et al.*, 2016): in the holding stage - 95°C, 20 s; in the cycling stage - 95°C, 5 s; T_m°C, 40 s; 72°C, 50 s (40 cycles); 95°C; 15 s; 75°C; 1 min (T_m is the annealing temperature). After amplification, the standard curve was drawn with software StepOiiev 2.3.

Table 2. Specific primers of q-PCR

Target bacteria	Primer pairs	Oligonucleotide sequence	Products size	Tm (°C)
Lb. plantarum	Lp-F	CAGAATTGAGCTGGTGGTGG	210	55
	Lp-R	TGTTACTTTCGCAACCAGAT		
L. lactis ssp. lactis	Lac-F	ATGCGTAAACTTGCAGGAC	262	56
	Lac-R	CAACCTTGAATGGTGGAG		
Leuc. mesenteroides	Leu-F	ATACAGGCGAACAGGGATTA	266	44
	Leu-R	GGGTGTAGTTTCTGGGTTTC		

PCR amplification of 16S rRNA genes

Genomic DNA was diluted to 100 ng/µl with TE buffer and used as PCR amplification template. Using the primers FA-27F and RA-1495R, PCR amplification was performed on a 50 µL reaction system. The primers used in this experiment were produced in Shanghai Alcott biotech Co., Ltd. Sequences of the primers are shown as follows:

FA-27F: 5'-gcagagttctcggagtcacgaAGAGTTTGATCCTGGCTCAG-3'

RA-1495R: 5'-agcggatcacttcacacaggaCTACGGCTACCTTGTTACGA-3' (Scarpellini *et al*., 2001).

The lowercase primer sequences were used for sequencing.

Table 3. Quantitative PCR amplification system

Components	Amount
SYBR	10 µl
50×ROX	0.4 µl
Primer F	0.4 µl
Primer R	0.4 µl
Template DNA (100ng/µl)	2 µl
ddH_2O	To 20 µl

Table 4. PCR amplification system of 16S rRNA genes

System components	Amount
10×PCR buffer	5 µl
dNTPmix (10mmol/L)	4 µl
Primer FA-27F (10pmol/µL)	1.5 µl
Primer RA-1495R(10pmol/µL)	1.5 µl
Tap DNA polymerase(5U/µL)	0.5 µl
Template DNA(100ng/µL)	2 µl
ddH_2O	To 50 µl

Results

Lactic acid bacteria viability

Table 5 shows the viability rate of lactic acid bacteria in 60 traditional dairy product samples collected from the pasture in Inner Mongolia scenic area.

As an important indicator, viable count reflects the freshness degree of dairy products. The higher the viable count, the fresher the dairy products. With high-concentration active bacteria, LAB products can play their roles more efficiently.

PCR amplification results of 16S rRNA genes of isolates

Primers FA-27F and FR-1495R were used for PCR amplification of 16S rRNA genes of the lactic acid bacteria isolates; DL 2000 DNA Markers were used for the agarose gel electrophoresis of the amplified products (the results are shown in Figure 1). It can be seen that a clear and bright stripe was located between 1000 bp and 2000 bp without trailing or evident nonspecific amplification;

accordingly, PCR amplified products of 16S rRNA gene of the isolates could meet the requirements of the subsequent sequencing test.

Table 5. Viability rate of lactic acid bacteria in dairy products

Sample number	Viable count ($\times10^{10}$ CFU/ML)	Sample number	Viable count ($\times10^{10}$ CFU/ML)	Sample number	Viable count ($\times10^{10}$ CFU/ML)
1	8.2±0.10	21	7.7±0.70	41	8.4±0.02
2	7.7±0.70	22	8.4±0.03	42	6.7±0.10
3	8.4±0.03	23	7.9±0.10	43	8.5±0.10
4	7.9±0.10	24	8.8±0.04	44	8.8±0.01
5	7.3±0.02	25	8.6±0.01	45	8.8±0.10
6	7.6±0.20	26	8.3±0.01	46	8.4±0.10
7	7.6±0.20	27	8.2±0.03	47	8.5±0.02
8	8.7±0.01	28	6.9±0.10	48	8.7±0.01
9	8.4±0.00	29	7.2±0.10	49	8.8±0.10
10	8.3±0.01	30	7.6±0.10	50	8.7±0.02
11	8.0±0.10	31	7.1±0.00	51	8.4±0.10
12	8.3±0.04	32	8.5±0.70	52	8.5±0.01
13	8.7±0.10	33	7.1±0.02	53	8.9±0.10
14	8.6±0.03	34	7.7±0.02	54	6.9±0.02
15	8.4±0.04	35	8.9±0.04	55	8.7±0.02
16	9.1±0.01	36	8.2±0.02	56	8.2±0.04
17	9.1±0.10	37	9.1±0.01	57	7.6±0.04
18	8.3±0.04	38	9.0±0.04	58	8.5±0.10
19	8.8±0.10	39	9.1±0.01	59	9.2±0.10
20	8.2±0.10	40	7.2±0.01	60	8.6±0.02

Quantitative real-time PCR analysis results of dominant bacteria in dairy samples

Standard curves

According to the test on OD values and the concentration of the standard DNA fluid, it was found that the concentrations of all the standard fluid ranged between 100 and 3000 ng/μL, and their purity was within the range of 1.8-2.0. Through ten-fold dilution on the DNA fluid extracted from the samples of *L. lactis ssp. lactis*, *Leuc. mesenteroides* and *Lb. plantarum*, the DNA diluent (10^{-1}~10^{-8}) was used as the template; specific primers (as shown in Table 2) were used for q-PCR amplification on the three kinds of standard bacteria. Figures 2-4 show the standard curves drawn with software StepOiiev 2.3.

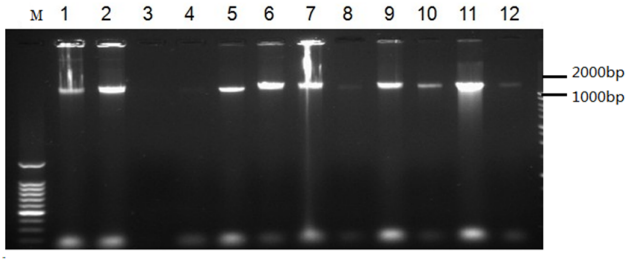

Figure 1. Agarose gel electrophoresis of PCR amplified products of 16S rRNA genes of lactic acid bacteria isolates

Note: 1~12 refer to the PCR products of 16S rRNA genes of lactic acid bacteria isolates; M refers to DL 2000 DNA Markers.

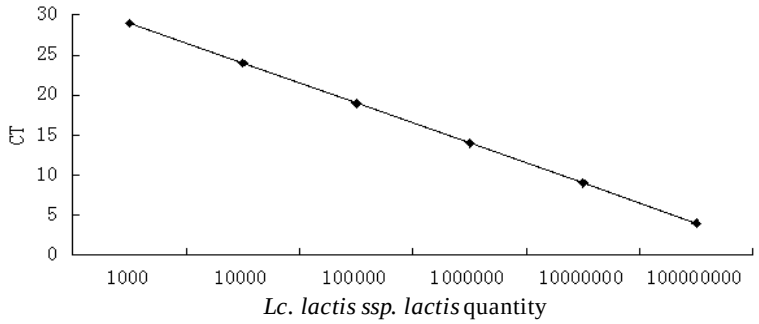

Figure 2. Quantitative PCR standard curve of lactic acid subspecies of *L. lactis ssp. lactis*

Note: Y= -4.83X + 42.81; correlation coefficient R^2=0.996

Quantitative PCR analysis of dominant bacteria in dairy products

Based on the standard curves, we calculated gene copies of *L. lactis ssp. lactis*, *Leuc. mesenteroides* and *Lb. plantarum* in yoghurt (samples 1-34), cream (samples 35-44) and cheese (samples 45-49). The average gene copies of *L. lactis ssp. lactis* were respectively $(9.4 \pm 0.3) \times 10^9$ copies/ML in yoghurt, $(8.3 \pm 0.1) \times 10^8$ copies/ML in cream and $(7.3 \pm 0.3) \times 10^7$ copies/ML in cheese; the average gene copies of *Leuc. mesenteroides* were respectively $(5.3 \pm 0.5) \times 10^5$ copies/ML in yoghurt, $(3.9 \pm 0.3) \times 10^3$ copies/ML in cream and $(3.2 \pm 0.1) \times 10^3$ copies/ML in cheese; the average gene copies of *Lb. plantarum* were respectively $(6.0 \pm 0.3) \times 10^5$ copies/ML in yoghurt, $(5.8 \pm 0.3) \times 10^5$ copies/ML in cream and $(4.2 \pm 0.3) \times 10^4$ copies/ML in cheese. Apparently, gene copies were significantly higher in yoghurt and cream samples than in cheese; there was no significant difference between yoghurt and cream.

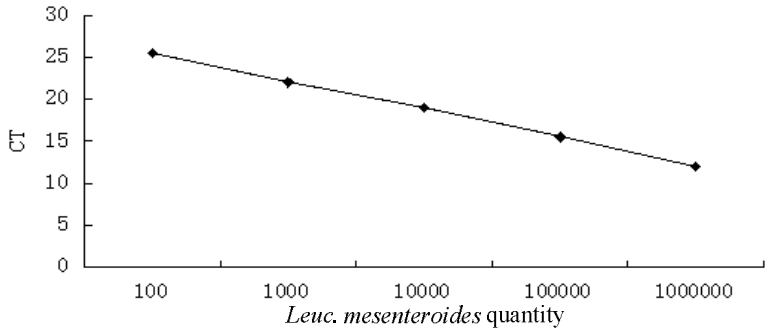

Figure 3. Quantitative PCR standard curve for *Leuc. mesenteroides*
Note: Y = -3.36X + 32.83; correlation coefficient R^2= 0.998

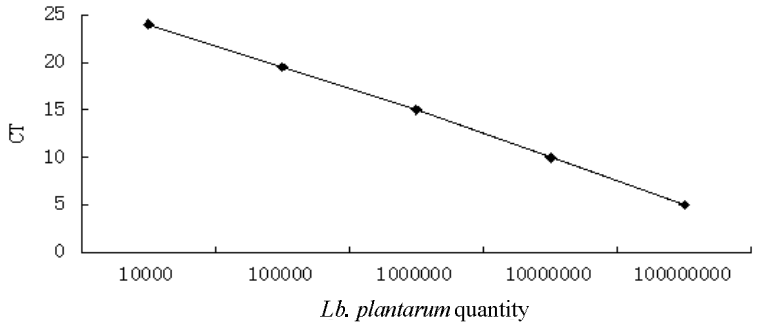

Figure 4. Quantitative PCR standard curve for *Lb. plantarum*
Note: Y = -4.68X + 47.26; correlation coefficient R^2= 0.998

In the study, cows were the milk sources of all the dairy products. The development of tourism in Inner Mongolia promotes its dairy consumption for the well-known high quality of dairy products; further, cattle rearing is boosted (Ashfield *et al.*, 2014; Giguet-Covex *et al.*, 2014). Meanwhile, the requirement of dairy products in tourism area also increases. Consequently, it is of great importance to improve the quality of dairy products and increase the production, which will enhance the tourism value of Inner Mongolia.

Discussion

The dairy industry in Inner Mongolia is well-developed for the abundant and varied lactic acid bacteria resources in dairy products (Mushtaq *et al.*, 2016; Stuart *et al.*, 2013). The types and amount of lactic acid bacteria in dairy products are closely associated with the living environment and isolation environment of lactic acid bacteria (Hirata *et al.*, 2015). Inner Mongolia is endowed with grassland resources and temperate steppe climate which are

conducive to the cultivation of dominant bacteria and development of dairy industry, thus to promote the double-development of local economy and tourism industry (Tálas, 2015; Zhou *et al.*, 2014).

In this study, 60 dairy products from Inner Mongolia scenic area were selected as the research samples; quantitative PCR technology was applied based on the theory of specific primers binding with fluorescent dye; amplification products which were formed during PCR reaction were given real-time measurement so that the microorganisms in samples were detected and quantified (Liu *et al.*, 2014). Currently, *Lb. plantarum, L. lactis ssp. lactis, Leuc. mesenteroides* have been widely applied in the production of a variety of fermented dairy products, providing solid guarantee for the rapid development of dairy industry.

Conclusions

There is a wide range of lactic acid bacteria in the dairy products of Inner Mongolia. The types and quantity of lactic acid bacteria in traditional dairy products are associated with the living environment and separation environment. Endowed with rich grassland resources, cattle rearing industry in Inner Mongolia is promoted; moreover, the yield and quality of dairy products are improved, which provides a material basis for the development of tourism value.

In summary, as an important member of probiotics family, lactic acid bacteria are widely applied in health-care food as well as medicine. Owing to the health care effects of the physiological activity, metabolites and bacterial components of lactic acid bacteria, the application of lactic acid bacteria is of great significance to the development of dairy industry, which in return promotes the development of economy and tourism industry in Inner Mongolia (Xie *et al.*, 2015).

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