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# Screening and Identification of Lactic Acid Bacteria from Airag for Antifungal Activity

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**Abstract:** The aims of this study were to isolate and identify antifungal Lactic Acid Bacteria (LAB) from Airag in Mongolia and Inner Mongolia of China and to evaluate their potential to prevent fungal spoilage of milk. A total of 520 strains were isolated from 20 Airag samples. Their antifungal activity against *Penicillium chrysogenum* was investigated using the overlay technique and 17 strains were found to have strong activity. The 17 strains were identified on the basis of morphological characteristics, biochemical properties and carbohydrate fermentation profiles through API50CH and 16S rDNA analyses. *Leuconostoc* (*Leu.*) *mesenteroides* 368 showed a broad spectrum of antifungal activity when its activity against eight molds was examined by the overlay technique. The antifungal activity was maximum (900 AU $mL^{-1}$) at 54-60 h of cultivation. LAB can be used to prolong the shelf life of dairy products. To the knowledge, this is the first time that *Leu. mesenteroides* which exhibits a broad spectrum of antifungal activity against a wide range of food spoilage fungi was isolated from Airag.

**Key words:** Airag, antifungal activity, *Leu. mesenteroides*, lactic acid bacteria, technique, milk

## INTRODUCTION

It is well known that molds and yeasts can lead to spoilage of foods such as fermented dairy products, cheese, bread, crops and silages (Bonestroo *et al*., 1993; Filtenborg *et al*., 1996). Such spoilage yields serious financial damage and causes public health problems as well (Legan, 1993). Biopreservation (the preservation of foods for a long period without any harmful effects by using plant/animal/microorganism-derived chemical compounds that can be consumed by humans as food or with food) is attracting attention as a solution to such problems (Stiles, 1996). The effects of Lactic Acid Bacteria (LAB) as demonstrated by biopreservation and the antibacterial compounds (Lindgren and Dobrogosz, 1990) produced by the bacteria are highly anticipated.

LAB have been used as a starter for food and feed for thousands of years. LAB are considered to be Generally Regarded As Safe (GRAS) (Stiles, 1996) and play the role of an antimicrobial by producing lactic acid, acetic acid, hydrogen peroxide and bacteriocin (Lindgren and Dobrogosz, 1990).

Although, there are reports on the antifungal activity of LAB separated from several fermented food and silages (Hassan and Bullerman, 2008; Magnusson *et al*., 2003; Sjogren *et al*., 2003; Lavermicocca *et al*., 2000; Laitila *et al*., 2002), the antifungal activity of Mongolian fermented food, particularly the traditional fermented mare's milk and Airag has been little reported. The Mongolians, over their long history have produced and consumed traditional fermented dairy products. In particular, Airag is fermented mare's milk produced by the joint fermentation of yeasts and LAB. It is a traditional drink well loved by the Mongolian people and is unique to the region of Mongolia where it plays an important role in the people's daily lives. This fermented mare's milk is produced by mixing non-pasteurized fresh mare's milk in the amount of approximately 30% (v/v) with leftover Airag from the previous day in a cowhide bag (called a huhuur) and by fermenting using the aerated fermentation method. Only a few studies on the use of microorganisms separated from Airag including *Lactobacillus*, *Lactococcus*, *Enterococcus* and *Leuconostoc* sp. have been conducted thus far (Baldorj *et al*., 2003; Ying *et al*., 2004; Ishii, 2001; Burentegusi *et al*., 2002; Uchida *et al*., 2007). In this study, the antifungal activity of LAB separated from Airag was investigated and identified using conventional microbiological techniques and modern molecular techniques. The strains that showed the strongest antifungal activity were selected.

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## MATERIALS AND METHODS

**Sampling:** Twenty samples of Airag used in the production of traditional fermented mare's milk were collected from nomadic families of Tuv aimag, Uvurkhangai aimag and Shilingool League in Mongolia and Inner Mongolia, China. The samples were brought to the laboratory in a cooler box (4-8°C) and kept refrigerated until analyzed.

**Bacterial strains and media:** Spoilage molds, *Cladosporium cladosporioides*, *A. oryzae*, *A. flavus var. flavus*, *A. niger*, *P. digitatum*, *P. citrinum*, *P. chrysogenum* JCM2056, *F. oxysporum* f. sp. *cucumerinum* and *R. oryzae* were obtained from Japan Collection of Microorganisms (JCM). The mold cultures were maintained on Potato Dextrose Agar (PDA) and glycerol-water (80% w/v) at -80°C as required.

**Isolation of LAB:** LAB strains were isolated from the twenty Airag samples collected from different regions of Mongolia and Inner Mongolia, China in 2008. Serial dilutions were made of each of the samples (0.5 mL) suspended in Ringer's solution (4.5 mL) and these were poured into plate count agar containing bromocresol purple (BCP agar, Nissui Pharmacy, Tokyo and Japan) and MRS agar (de Man Rogosa Sharpe agar, Oxoid, Hampshire and England) containing 10 ppm cycloheximide. Both BCP agar and MRS agar plates were incubated at 25 and 40°C for 3-5 days. Colonies of acid-forming bacteria were enumerated and then selected and purified on the basis of their morphological characteristics.

**Screening of bacteria for antifungal activity:** The LAB isolates were screened for antifungal activity by the overlay technique as described by Hassan and Bullerman (2008) using MRS agar plates with *P. chrysogenum* as an indicator with slight modifications. The plates were dried overnight before inoculating with LAB. Then, the inoculated plates were incubated for 24 h at 30°C in an inverted position. Plates that contained well-grown LAB colonies were overlaid with approximately 10 mL of soft PDA (0.8% agar) containing a final mold spore concentration of 1×104 spores $mL^{-1}$ and incubated at room temperature (ca. 23-25°C).

The incubation was carried out for 3-4 days and the zones of inhibition around the LAB colonies were recorded (+, weak inhibition <5.0 mm; ++, moderate inhibition 5.0-9.0 mm; +++, strong inhibition 10.0-15.0 mm and ++++, fairly strong inhibition >15.0 mm) on the 3rd day. These data were used for the selection of LAB isolates. Colonies that gave +++ inhibition were selected for further studies. Seventeen antifungal LAB were tested further for their ability to inhibit a wide range of molds as mentioned above by the overlay technique.

**Antifungal activity of selected LAB isolates:** The antifungal activities of the 17 selected LAB isolates against *C. cladosporioides*, *A. oryzae*, *A. flavus var. flavus*, *A. niger*, *P. digitatum*, *P. citrinum*, *F. oxysporum*, and *R. oryzae* were determined by the overlay technique. Clear zones of inhibition around the LAB circumference were recorded and compared for their potential to inhibit a wide range of spoilage fungi.

**Identification of antifungal bacterial isolates:** LAB isolates grown on MRS agar were examined for their morphology and motility under a phase contrast microscope (Olympus, Japan). The isolated strains were identified on the basis of their physiological and biochemical characteristics as described by Sneath *et al.* (1986) and Wood and Holzapfel (1995). Growth conditions were determined in MRS broth at different temperatures (10, 15 and 45°C), pH (4.5 and 9.6) and salt concentrations (NaCl) of 4.0 and 18% at 30°C for 48 h. Growth was determined in terms of turbidity. LAB isolates that showed the strongest inhibitory activity were identified using API 50 CH test strips (Biomerieux, Marcy l'Etoile, France). The isolates were matched to the species level on the basis of their carbohydrate fermentation patterns.

Genomic DNA was extracted and purified following the protocol of BioRad Co. using InstaGene Matrix (BioRad, CA, USA) and the DNA was used as the template for sequencing. About 500 bp of 16S ribosomal RNA (16S rDNA) was amplified by PCR using a set of universal primers (Endo and Okada, 2005). PCR was performed with a MicroSEQ 500 16S rDNA Bacterial Identification PCR kit (Applied Biosystems, CA, USA). The cycle sequence was performed with the MicroSEQ 500 16S rDNA Bacterial Identification Sequencing kit (Applied Biosystems). Thermal cycling was carried out with the GeneAmp PCR system 9600 (Applied Biosystems) and the products were analyzed on a model ABI Prism 3100 DNA sequencer (Applied Biosystems). Homology search was carried out by using BLAST and GenBank/DDBJ/EMBL database search programs (Altschul *et al.*, 1997). The phylogenic tree was constructed by using the neighbor joining method (Saitou and Nei, 1987).

Using the CLUSTAL W Program as reference (Thompson *et al.*, 1994; Altschul *et al.*, 1997), the evolutionary distance between the arrangements was calculated. Subsequently, the molecular genealogical tree of isolate 368 and the closest relative type strains was created using the neighborhood-joining method (Saitou and Nei, 1987).

**Characterization of antifungal substance produced by *Leu. mesenteroides* 368 in liquid medium:** *Leu. mesenteroides* 368 was examined for its ability to produce active antifungal substances in liquid medium (Sathe *et al*., 2007). An overnight culture (104 CFU $mL^{-1}$) was inoculated into (1% v/v) 500 mL of MRS broth and incubation was carried out under microaerobic conditions at 25°C for 72 h. Aliquots (50 μL) were withdrawn at 6 h intervals and examined for growth (OD600), pH and antifungal activity of the Cell-Free Supernatant (CFS) against *A. oryzae*. To obtain CFS, a 5 mL aliquot was centrifuged at 10,000 rpm for 20 min and the supernatant was passed through a sterile membrane filter (0.20 μm pore size, Millipore). The filtrate was designated as CFS. To determine the antifungal activity of CFS by the agar well diffusion technique, CFS (50 μL) was applied into wells prepared in MRS agar plates seeded with *A. oryzae* spores (104 $mL^{-1}$). The concentration of antifungal compound per mL of CFS was determined in terms of Arbitrary Units (AU) and is defined as the reciprocal of the highest dilution at which the fungus was inhibited (Wan *et al*., 1995). The above experiment was done in triplicate.

## RESULTS

**Screening of lactic acid bacteria for antifungal activity:** A total of 520 LAB were isolated on MRS agar and BCP agar from 20 Airag samples. Microscopy (Olympus, BH-2, Japan) revealed that 57% were rods and 43% were cocci. Screening for antifungal activity against *P. chrysogenum* with the overlay technique showed that 382 (73%) of the 520 isolates had no activity whereas 138 (27%) strains had variable activity. Seventeen isolates had fairly strong antifungal activity (inhibition zone >15.0 mm) (Fig. 1).

**Characterization and identification of lactic acid bacteria:** The 17 isolates showing fairly strong antifungal activity were subjected to species-level identification. Their phenotypic characteristics are shown in Table 1. Ten isolates were rods and the remaining were cocci. All isolates were Gram-positive, non-motile and catalase-negative and could grow at pH 4.5. Only one isolate could not grow at pH 9.6. In addition, all of them grew in 4.0% NaCl but not in 18% NaCl. For further identification, the *16S rDNA* gene sequences of these isolates were matched with the GenBank Database by BLAST (Table 1).

**Antifungal activity spectrum:** The spectrum of antifungal activity of the selected LAB strains against eight spoilage molds was determined with the overlay technique Table 2). Of the 17 isolates, strain no. 368 had fairly strong

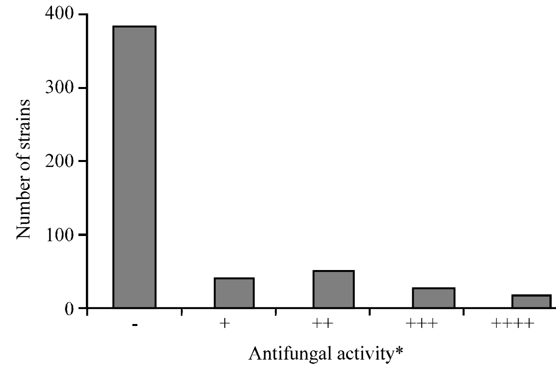


Fig. 1: Phylogenetic tree based on 16S rDNA sequences showing the positions of strain (strain No. 368) with representatives of some other related taxa. Scale bar 1% nucleotide sequence difference

Table 1: Characteristics of antifungal LAB isolates from Airag samples and their identification based on 16S rDNA sequences

| | | | | | | | Growth | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Temp (°C) | | | pH | NaCl (%) | | | | |
| Strain | Shape | Gram reaction | Catalase | Motility | Gas from glucose | $NH_3$ from arginine | 10 | 15 | 45 | 4.5 | 9.6 | 4.0 | 18 | Sequencing of 16S rDNA | Homology (%) |
| 60 | Rods | + | - | - | - | + | + | + | + | + | + | + | - | *L. plantarum* | 99.8 |
| 422 | Rods | + | - | - | - | - | + | + | + | + | + | + | - | - | 99.9 |
| 425 | Rods | + | - | - | - | - | + | + | + | + | + | + | - | - | 99.9 |
| 426 | Rods | + | - | - | - | - | + | + | + | + | + | + | - | - | 99.9 |
| 421 | Rods | + | - | - | - | - | + | + | + | + | + | + | - | *L. pentosus* | 99.8 |
| 423 | Rods | + | - | - | - | - | + | + | + | + | + | + | - | *L. brevis* | 98.8 |
| 430 | Rods | + | - | - | + | + | - | - | + | + | - | + | - | - | 99.9 |
| 447 | Rods | + | - | - | - | + | + | - | + | + | + | + | - | - | 99.9 |
| 470 | Rods | + | - | - | - | - | - | + | + | + | + | + | - | *L. gasseri* | 97.8 |
| 402 | Cocci | + | - | - | - | + | + | + | - | + | + | + | - | *Leu. mesenteroides* sp. *dextranicum* | 99.5 |
| 368 | Cocci | + | - | - | + | - | + | + | - | + | + | + | - | *Leu. mesenteroides* | 100.0 |
| 406 | Cocci | + | - | - | + | - | + | + | - | + | + | + | - | - | 100.0 |
| 369 | Cocci | + | - | - | - | - | + | + | - | + | + | + | - | *Lc. lactis* sp. *lactis* | 99.8 |
| 370 | Cocci | + | - | - | - | - | + | + | - | + | + | + | - | - | 100.0 |
| 371 | Cocci | + | - | - | - | - | + | + | + | + | + | + | - | - | 100.0 |
| 372 | Cocci | + | - | - | - | - | + | + | - | + | + | + | - | - | 100.0 |

+: Positive, -: Negative

Table 2: Antifungal activity of LAB isolates as determined by overlay technique

| | | Activity | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| LAB isolate | pH | *Aspergillus flavus* JCM10252a | *Aspergillus niger* JCM5546 | *Aspergillus oryzae* JCM2239 | *Penicillium citrinum* JCM5591 | *Penicillium digitatum* JCM9863 | *Rhizopus oryzae* JCM5557 | *Fusarium oxysporum* f. sp. *cucumerinum* JCM9284 | *Cladosporium cladosporioides* JCM3899 |
| 60 | 3.89 | + b | - | - | - | - | - | - | + |
| 368 | 4.18 | + | + | + | + | + | + | + | + |
| 369 | 4.17 | + | + | + | + | + | - | + | - |
| 370 | 4.19 | + | - | - | + | + | - | - | + |
| 371 | 4.19 | + | + | - | + | + | - | + | - |
| 372 | 4.22 | - | + | + | + | + | - | - | + |
| 402 | 4.22 | - | + | - | + | + | - | + | + |
| 406 | 4.21 | - | + | - | - | + | - | - | - |
| 421 | 3.92 | + | - | + | - | - | - | + | + |
| 422 | 3.94 | - | - | + | - | - | - | - | - |
| 423 | 4.01 | - | - | + | - | - | - | + | + |
| 425 | 4.01 | + | - | - | - | - | - | + | + |
| 426 | 3.98 | - | - | + | - | - | - | - | + |
| 427 | 3.94 | - | - | + | - | - | - | + | - |
| 430 | 4.61 | - | + | - | + | + | - | - | - |
| 447 | 4.62 | + | + | + | + | + | - | + | + |
| 470 | 4.00 | - | + | - | + | - | - | + | + |

aJCM: Japan Collection of Microorganisms. b+Inhibition; - no inhibition

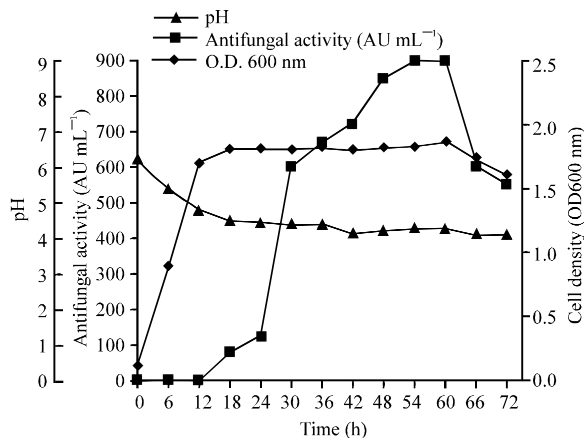


Fig. 2: Relationship among cell density, pH and antifungal activity during the growth of *Leuconostoc mesenteroides* 368 in MRS broth at 25°C over 72 h

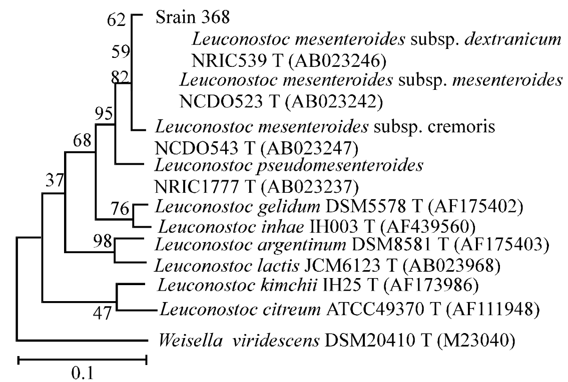


Fig. 3: Phylogenetic tree based on 16S rDNA sequences showing the positions of strain (strain No. 368) with representatives of some other related taxa. Scale bar 1% nucleotide sequence difference

activity against all the molds, revealing a broad spectrum. Meanwhile, the 16 isolates did not show any inhibitory activity against *R. oryzae* (Table 2).

**Production of antifungal substance:** The relationship among cell density, pH and antifungal activity during the growth of *Leu. mesenteroides* 368 in MRS broth at 25°C over 72 h was determined (Fig. 2). Antifungal activity was first detected at 18 h i.e., when the culture was in the early stationary phase. The antifungal activity was a maximum (900 AU $mL^{-1}$) when the culture was in the stationary phase (54 and 60 h) and remained high until the early stationary phase over 18 h. During the 72 h growth period, pH declined from 6.20-4.10. The antifungal titer was high when pH of the broth was in the range of 4.8-4.5.

However, the antifungal titer declined after 60 h and was only 550 AU $mL^{-1}$ at 72 h when the pH of the broth was 4.10.

**Identification and characterization of LAB:** The 16S rDNA sequence data were aligned to construct a phylogenetic tree and the phylogenetic positions of these strains were compared with related taxa in a dendrogram. As shown in Fig. 3 strain no. 368 was closest to *Leu. mesenteroides* subsp. *mesenteroides* NCDO523 as it showed 100% homology to strain NCDO523.

Based on the results of the 16S rDNA sequence analysis, the 17 strains that showed strong antifungal activity belong to *Lactobacillus*, *Leuconostoc* and *Lactococcus* sp.

According to detailed molecular system analysis, they were identified as *Leu. mesenteroides* sp. *dextranicum*, *Leu. mesenteroides*, *L. gasseri*, *L. brevis*, *L. pentosus*, *L. plantarum* and *Lc. lactis* sp. *lactis*.

## DISCUSSION

LAB have been used in the manufacture of fermented food products for centuries and even today, they play an important role in the preservation of dairy products and other food despite the fact that they are categorized as GRAS (Generally Recognized as Safe) (Stiles, 1996).

Traditional fermented products are a good source of autochthonous strains which are usually well adapted to a particular food environment and very useful to develop functional starters especially suited for small-scale fermentations (Holzapfel, 2002). Production of Airag and other fermented milk products has a very long tradition in Mongolian plateau.

At many places throughout the Inner Mongolia, Buryat mongola and Mongolia the regional traditional style of Airag manufacturing was retained. These Airag are producing at specific ecological localities such as Mongolian Plateau (over 900-1500 m above sea level), mountain's plateaus, river valleys, etc. and could be a valuable source of autochthonous LAB that inhabit different ecological niches.

The lactic acid bacteria with antibacterial activity (Rehaiem *et al*., 2010) are well documented while less attention has been paid to exploit its antifungal activity (Schnurer and Magnusson, 2005; Valerio *et al*., 2009; Sathe *et al*., 2007; Dal Bello *et al*., 2007). The lactic acid bacteria that produced bacteriocinses were separated from Airag (Batdorj *et al*., 2006). More recently, Hassan and Bullerman (2008) isolated antifungal LAB from sourdough bread culture. Only a few studies from scientific literature cite a wide spectrum antifungal activity for lactic acid bacteria isolates (Strom *et al*., 2002). The rest suggest a more strain specific antifungal activity against species of one or two major mold genera (Okkers *et al*., 1999; Niku-Paavola *et al*., 1999; Florianowicz, 2001; Gourama and Bullerman, 1997). The results show that *Leuconostoc mesenteroides* was able to inhibit closely related mold species in the different genus to a similar degree.

The study demonstrated that one of Mongolia's traditional fermented milk beverages, Airag (made from mare's milk that is naturally fermented by LAB) can be used as a source for the isolation of LAB due to its antagonistic properties toward microorganisms. In particular, *Leu. mesenteroides* which demonstrates a broad spectrum of antifungal activity was successfully isolated. Its application was examined using skim milk medium and valuable results were obtained. This is the first report presenting that *Leu. mesenteroides* separated from Airag demonstrates broad inhibitory activity against putrefying fungi. It is expected that the results of this study will provide new knowledge in the field of dairy production in terms of biopreservation and that the LAB isolated from Airag or their metabolites will be used in the biopreservation of dairy products which represent their natural habitat.

Very few reports deal with well-characterized antifungal compounds while the exploitation of antifungal lactic acid bacteria for dairy food preservation is scanty (Schnurer and Magnusson, 2005). The inference on antifungal activity of MRS media components like sodium acetate (Stiles *et al*., 2002) can be obliterated because of good growth of all fungi used in present studies on MRS agar.

## CONCLUSION

Study on the production of antifungal compounds in MRS broth by one of the isolates, *Leu. mesenteroides* indicate that antifungal activity was growth-dependent and was highest at the end of stationary phase of growth. Antifungal activity declines during the stationary phase and thereafter which hypothetically suggests that antifungal compound might be converted to other metabolites or degraded by autolytic enzymes (Sathe *et al*., 2007).

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