The diversity of lactic acid bacteria in oat samples obtained from Tibetan Plateau

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Abstract: Thirty-three representative strains of lactic acid bacteria (LAB) were isolated from oat samples including nonirrigated, wild and irrigated land, and soil samples gathered round the rhizosphere of oats in eleven areas of the Tibetan Plateau. They were identified on species and subspecies level by morphological and biochemical characters, and the phylogenetic affiliations of 16S rRNA gene sequences. The representative isolates were divided into eleven groups (A-K). Twenty-three representative strains belong to the four genus next: *Leuconostoc*, *Wesissella*, *Lactococcus* and *Enterococcus,* which were isolated from nonirrigated oat and soil samples in seven different regions. Five representative strains isolated from oat and soil samples in wild land of three areas belong to three genus including *Leuconostoc*, *Leuconostoc* and *Wesissella*. Moreover, five representative isolates from oat sample in irrigated land belong to two genus including *Lactobacillus* and *Leuconostoc*, whereas no strains were isolated from the soil sample of irrigated land. The study indicates that human cultivation practices caused some differences in the amount and species of LAB strains that isolated from oat samples. There is some correlation between the distribution of LAB and the human activities.
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1. Introduction

Tibetan Plateau is located in the northwest of China with a mean elevation of over 4000m. Oat (*Avena sativa*) is a popular crop and plays an important role in animal husbandry in the plateau. Until now, most of the studies about Tibetan Plateau oat focused on the nutritional value of the oatmeal (Kristina E. Andersson *et al*., 2012). As an important forage crop, oat plant just has been making hay instead of making silage in this region (J. Wallsten *et al*., 2012). Actually, the nutritional value of silage oat is much higher than the hay, and LAB play an important role in silage (E. Saarisalo *et al*., 2007), however few study have been reported about epiphytic microorganisms presented on oat or its rhizosphere soil. The epiphytic microorganism of oat should be influenced by human activities, such as watering, fertilizing and so on.

There is no report about the influence of human activities on the distribution of LAB in the Tibetan Plateau so far. This study was conducted to identify and detail the LAB on oats and its rhizosphere soil in the Tibetan Plateau using phenotypic and phylogenetic methods. Through analyzing the differences of LAB in samples from different sources, a preliminary result was concluded about the impact of human activities on the distribution of LAB under different sillage methods.

2. Materials and Methods

Samples and bacterial isolates

Oats and soil samples were collected from eleven areas in the Tibetan Plateau, Qinghai province, Northwest China (Fig. 1). There were three oat samples of wild land (OSWL), seven oat samples of nonirrigated land (OSNL) and one oat sample of irrigated land (OSIL). Likewise, there were three soil samples of wild land (SSWL), seven soil samples of nonirrigated land (SSNL) and one soil sample of irrigated land (SSIL). A total of 22 oats and soil samples were selected for LAB isolation.

Samples (10g) were blended with 90 ml sterilized water and were 10^{-1} to 10^{-5} serially diluted. The number of LAB, coliform, yeast and aerobic bacteria were measured by the plate count on different culture medium.

The number of LAB was measured in plates on MRS agar incubated at 30°C for 2-3 days under anaerobic conditions. Coliform bacteria were counted on blue light broth agar incubated at 30°C for 1-2days. Yeasts were counted on potato dextrose agar incubated at 30°C for 1-2 days under aerobic conditions. Bacilli and aerobic bacteria were counted on nutrient agar

incubated at 30°C for 1-2 days under aerobic conditions. Clostridia were counted on CLO agar.

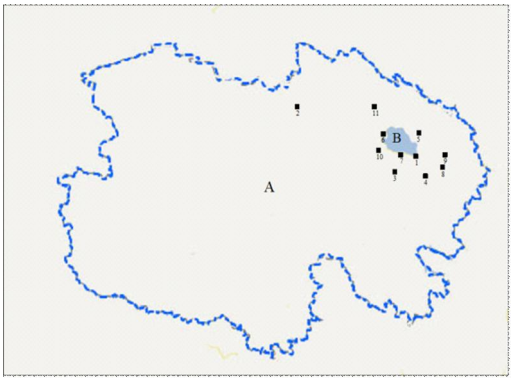

Fig.1. Location of sampling sites.

A, Qinghai province; B, Qinghai lake; 1, Qinghai lake scenic spot; 2, Delingha city; 3, Gonghe county; 4, Laji mountain of Guide county; 5, Jinyintan of Haiyan County; 6, Bird island in Qinghai lake; 7, Jiangxigou township side Qinghai lake; 8, Laji mountain of Huangzhong county; 9, Ants groove of Huangzhong county; 10, Dark horse river; 11, Gangcha county.

Every colony of LAB was purified three times by lineation on MRS agar. The colony purified were preserved at -80℃ in nutrient broth medium with 10% (v/v) dimethyl sulfoxide. Colonies were counted as numerable microorganisms (colony forming units (cfu) g^{-1} of fresh matter (FM)) (Zhongfang TAN *et al*., 2010; Huili Pang *et al*., 2012).

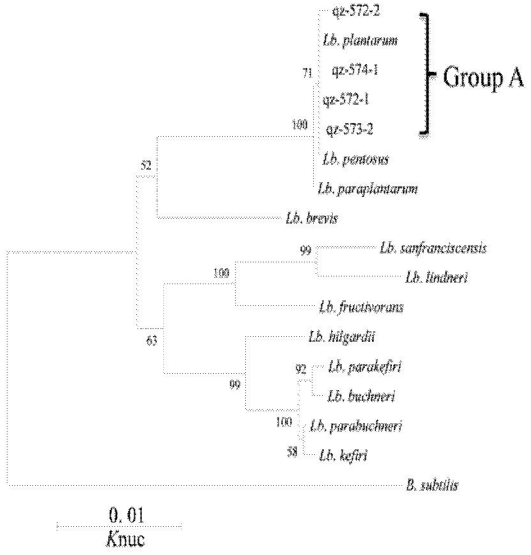

Fig. 2. The Phylogenetic tree of rod-shaped strains based on the 16S rRNA gene sequences.

Morphological, physiological, and biochemical tests

The Gram reaction type and morphological characteristics of LAB were identified after being cultured on MRS agar for 1-2 days. Gas production from glucose and catalase activity was determined as described (Kozaki M. *et al*. 1992).

The salt tolerance of LAB was tested in MRS broth with 3.0% and 6.5% NaCl included. Growth at different temperatures was detected in MRS broth as described. Growth at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 8.0, 9.0 and 10.0 was observed in MRS broth after incubation at 30°C for 7 days. Carbohydrate assimilation and fermentation were identified on analytical profile index 50 carbohydrate (API 50 CH) strips composed of 49 different compounds.

16S rRNA gene sequencing

Analysis of 16S rRNA gene was cultured on MRS agar for 1 day (Cai *et al.*, 1999). The 16S rRNA sequence coding region of single colony was amplified by polymerase chain reaction (PCR) and performed in a PCR Thermal Cycler and reagents from a Takara Fast PCR Master Mix (Takara biotechnology (Dalian) Co., Ltd.).Sequencing was completed by Beijing Genomics Institute (BGI).

16S rRNA gene sequence similarity was accomplished by using the BLAST program in the GenBank (http://www.ncbi.nlm.nih.gov/ genbank/) data library. The sequence data was introduced into the CLUSTAL W software program (Hitachi Software Engineering Co., Tokyo, Japan) for assembly and alignment. The 16S rRNA gene sequences of isolates were compared with sequences from the type strains collected in GenBank. Phylogenetic trees were constructed by the neighbor-joining method after calculating nucleotide substitution rates. *Bacillus subtilis* NCDO 1769^T was used as an outgroup organism. The topologies trees were evaluated by bootstrap analysis with CLUSTAL W software based on 1000 random resamplings.

The *rec*A multiplex-PCR analysis

The *rec*A multiplex-PCR analysis was accomplished with some modifications for further differentiation among *Lactobacillus pentosus*, *Lactobacillus plantarum* and *Lactobacillus paraplantarum*.

This *rec*A multiplex-PCR analysis adopted a reverse primer (pREV, 5'-TCGGGATTACCAAACATCAC-3') in combination with three species-specific forward primers (plan F, 5'-CCGT TTAT GCGG AACA CCTA-3'; pent F, 5'-CAGT GGCG CGGT TGAT ATC-3' and para F, 5'-GT CACAGGCAT TACG AAAAC-3') for distinguishing the closely related species of the *Lactobacillus plantaru*m group (Tohno *et al*., 2012; Omar *et al*., 2006).

A small quantity of cells from a single colony

were immediately transferred to a PCR reaction

Table 1. Micorbiological analysis of the samples and LAB strains used in this study

Samples	Collection site	Counts (cfu*/g^{-1} of FM*) of viable microorganisms						LAB strain
		LAB	Coliform bacteria	Aerobic bacteria	Yeast	Bacilli	Clostridium	
SSNL	1	1.5×10^{3}	2.5×10^{7}	6.5×10^{7}	2.5×10^{7}	2.4×10^{5}	ND	qz-69, qz-70
OSNL	1	1.4×10^{5}	0.9×10^{6}	2.1×10^{5}	1.0×10^{6}	5.1×10^{3}	ND	qz-71, qz-72, qz-73
SSNL	2	2.2×10^{1}	4.9×10^{5}	3.5×10^{5}	7.2×10^{5}	3.4×10^{4}	6.5×10^{2}	qz-104, qz-105, qz-106
OSNL	2	1.1×10^{5}	4.1×10^{7}	7.3×10^{7}	7.4×10^{7}	8.4×10^{4}	ND	qz-107
SSNL	3	ND	6.7×10^{6}	1.2×10^{7}	9.0×10^{3}	3.7×10^{5}	ND	——
OSNL	3	2.2×10^{4}	9.7×10^{4}	8.9×10^{5}	1.0×10^{3}	1.4×10^{5}	ND	qz-281, qz-282, qz-283
SSNL	4	ND	1.2×10^{6}	5.5×10^{7}	3.0×10^{3}	2.0×10^{3}	ND	——
OSNL	4	2.1×10^{7}	2.5×10^{8}	6.6×10^{3}	6.9×10^{4}	1.1×10^{5}	ND	qz-367, qz-368, qz-369
SSNL	5	ND	1.5×10^{5}	1.0×10^{7}	9.0×10^{3}	8.5×10^{4}	ND	——
OSNL	5	6.5×10^{5}	1.2×10^{6}	8.0×10^{7}	5.0×10^{2}	6.0×10^{3}	ND	qz-534
SSNL	6	ND	1.2×10^{7}	8.5×10^{7}	1.5×10^{7}	3.2×10^{4}	ND	——
OSNL	6	1.5×10^{5}	1.2×10^{5}	9.3×10^{5}	ND	2.5×10^{3}	ND	qz-539, qz-540, qz-541
SSNL	7	ND	8.0×10^{4}	1.2×10^{3}	ND	1.3×10^{5}	4.0×10^{4}	——
OSNL	7	3.1×10^{4}	1.4×10^{7}	3.1×10^{7}	ND	5.6×10^{4}	ND	qz-546, qz-547, qz-548, qz-549
SSWL	8	ND	3.8×10^{8}	5.3×10^{3}	0.9×10^{4}	3.3×10^{4}	5.0×10^{2}	——
OSWL	8	5.7×10^{5}	1.5×10^{8}	1.1×10^{9}	1.5×10^{4}	9.5×10^{4}	ND	qz-341
SSWL	9	ND	3.0×10^{6}	1.2×10^{7}	5.5×10^{3}	2.9×10^{4}	ND	——
OSWL	9	ND	5.3×10^{8}	1.1×10^{9}	2.4×10^{8}	3.7×104	ND	——
SSWL	10	4.2×10^{4}	5.5×10^{5}	3.0×10^{3}	2.2×10^{4}	2.8×10^{5}	ND	qz-484, qz-487, qz-488-2
OSWL	10	1.6×10^{5}	6.5×10^{7}	2.0×10^{9}	2.2×10^{4}	1.5×10^{4}	ND	qz-508
SSIL	11	ND	8.0×10^{5}	5.3×10^{9}	1.9×10^{4}	3.8×10^{3}	ND	——
OSIL	11	1.8×10^{5}	6.0×10^{5}	7.7×10^{5}	8.0×10^{6}	5.0×10^{4}	ND	qz-572-1, qz-572-2, qz-573-2, qz-574-1, qz-574-2

*ND: not detected; CFU: colony froming unit, FM: fresh matter. SSNL, soil sample of nonirrgated land; OSNL, oat sample of nonirrigated land; SSWL, soil sample ofwild land; OSWL, oat sample of wild land; SSIL, soil sample of irrigated land; OSIL, oat sample of irrigated land, ——,not detected lactic acid bacteria, 1, Qinghai lake scenic spot; 2, Delingha city; 3, Gonghe county; 4, Laji mountain of Guide county; 5, Jinyintan of Haiyan County. 6, Bird island in Qinghai lake; 7, Jiangxigou township side Qinghai lake; 8, Laji mountain of Huangzhong county; 9, Ants groove of Huangzhong county; 10, Dark horse river; 11, Gangcha county.

solution (25µl) including 1×SapphireAmp Fast PCR Master Mix, the primers paraF, pentF, pREV (0.25 mmol/L each) and 0.12mmol/L of the primer planF. The reaction solution was denatured (3 min at 94°C) and then subjected to 35 cycles of 5 s at 94°C, 5 s at 56°C, and 10 s at 72°C with Gene Amp® PCR System EDC-810 (Eastwin Life Sciences, Inc.).

3. Results

Counts of microorganisms

The counts of viable microorganisms in oat and soil samples are shown in Table 1. LAB was not detected from eight soil samples. Three soil samples have 10^{1} to 10^{4} cfu g^{-1} of LAB. All the oat samples have 10^{4} to 10^{7} cfu g^{-1} of LAB except one OSWL that was not detected. Overall 10^{4} to 10^{8} coliform bacteria, 0 to 10^{9} aerobic bacteria, 0 to 10^{6} yeast, 10^{3} to 10^{6} bacilli and 0 to 10^{4} clostridia were detected from the oat and soil samples.

Morphological, physiological, and biochemical properties

A total of 33 representative isolates were isolated from the oat samples and soil sample (Table 1). In accordance with the morphological, physiological, biochemical properties, and 16S rRNA gene sequence (Figs 2 and 3), the 33 representative strains were divided into eleven groups (A-K). The phenotypic and genotypic characteristics of these strains are shown in Table 3. All representative isolates were Gram-positive and catalase-negative bacteria. The representative strains of group A were rods, and had ability to grow at pH values from 3.0 to 10.0, in 3.0% and 6.5% NaCl, These representative isolates failed to produce gas from glucose and were homofermentative bacterium.

Representative strains qz-572-1, qz-572-2 and qz-573-2 in group A could grow at temperature of 45℃ whereas qz-574-1 at 5℃ and 10℃. Groups B to K belonged to cocci-shaped strains. The representative strains of groups B to G were heterofmentative coccus that produced gas from glucose, and groups H to K were homofermentative coccus, that did not produce gas from glucose.

Almost all cocci-shaped representative strains were able to grow at 4.0 to 9.0 pH values except qz-487 in group H and that it could only grew at pH values 8.0 in this study. Most representative strains were capable of growing in 3.0% but not in 6.5% NaCl, except qz-106 in group G and qz-548 in group K can

both grow well in 6.5% NaCl.

In the four temperatures circumstances, most representative strains were able to grow weakly, but group E strain qz-71 at 10℃ and group C included strains qz-539, qz-282 at 5℃ failed to grew. In addition, some representative strains showed the ability to grow at 45℃, such as groups C (qz-539), F (qz-508), G (qz-106, qz-70), I (qz-367), J (qz-69, qz-105) and K (qz-548).

API 50 CH fermentation patterns of representative strains obtained from the oat and soil samples are shown in Table 4. All representative strains produced acid from galactose, glucose, fructose, mannose, maltose, lactose and sucrose, but failed to produce acid from glycerol, erythritol, D-arabinose, L-xylose, adonitol, *β*-methyl-xyloside, sorbose, rhamnose, dulcitol, inositol, inulin, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ceto-gluconate, 5-ceto-gluconate.

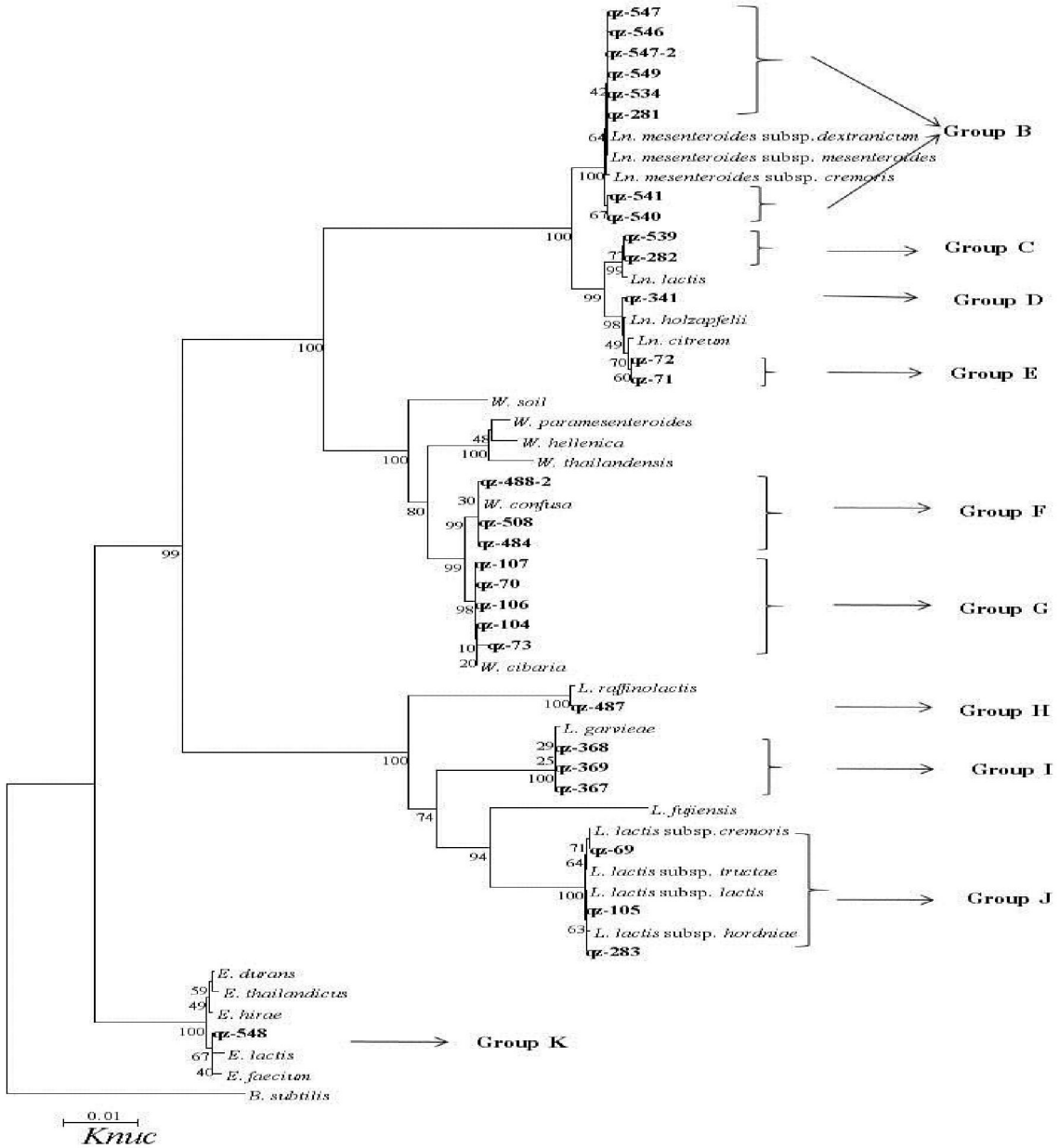

Fig. 3. The Phylogenetic tree of cocci-shaped strains based on the 16S rRNA gene sequences.
Numbers at nodes are bootstrap values based on a neighbor-joining bootstrap analysis with 1000 replications. Reference sequences of the Leuconostoc, Weissella, Lactococcus and Enterococcus species type strains from the GenBank/EMBL/DDBJ database are used for comparison. Ln., Leuconostoc, W., Weissella, L., Lactococcus, E., Enterococcus, B, Bacillus.

On the basis of Table 2 and 3, these representative strains revealed greatly diverse biochemical and physiological properties and API 50 CH fermentation patterns from the type strains. Accordingly, these representative strains could not be identified down to the species level on the basis of phenotypic characteristics.

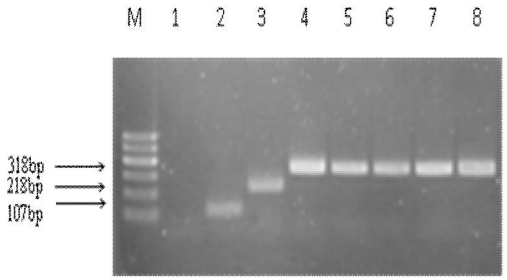

Fig. 4. Amplification products obtained from the *rec*A multiplex-PCR assay.

Lane M contained a 600 bp PLUS DNA ladder (Tiangen biotech Co, Ltd. , Beijing, China); Lanes 1,2,3 and 4, PCR amplification products from *Lb. casei* JCM 16167^T (negative control), *Lb. paraplantarum* JCM 12533^T, *Lb. pentosus* JCM 1558^T and *Lb. plantarum* JCM 1149^T, respectively; Lanes 5, 6, 7 and 8, PCR amplification products from qz-572-2, qz-574-1, qz-572-1 and qz-573-2, respectively.

16S rRNA gene sequence analysis

The 16S rRNA gene sequence of all the isolated representative strains were analyzed and determined for confirming the species by the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/). The phylogenetic trees of all representative strains from evolutionary distances were constructed using the neighbor-joining method as is shown in Figs 2 and 3.

According to the Fig 2, the phylogenetic tree of rod-shaped representative strains was shown the relative position of some species among *Lactobacillus* genus. Group A including qz-572-2, qz-574-1, qz-572-1 and qz-573-2 were clearly placed in the cluster of genus Lactobacillus, since it was divided into different groups according to the phylogenetic tree including *Lb. pentosus*, *Lb. plantarum* and *Lb paraplantarum* with 100% bootstrap values confirmed monophyly. The relative position of *Leuconostoc*, *Wesissella*, *Lactococcus* and *Enterococcus* is revealed from the phylogenetic tree of cocci-shaped strains (Fig. 3).

Numbers at nodes are bootstrap values based on a neighbor-joining bootstrap analysis with 1000 replications. Reference sequences of the Lactobacillus type strains from the GenBank/EMBL/DDBJ database are used for comparison. *Lb*., *Lactobacillus*.

Representative strains in group B presented maximum population of all groups were assigned to *Ln.mesenteroides*, These strains were identified to subspecies levels including *Ln. mesenteroides* subsp. *dextranicum*, *Ln. mesenteroides* subsp. *cremoris* and *Ln. mesenteroides* subsp. *mesenteroides*. In group C, qz-282 and qz-539 obviously belong to *Ln. lactis*, sustained with their 16S rRNA gene sequence similarity of over 99.8% to *Ln. lactis*.

Representative strain qz-341 of group D has got similar sequence to achieve 99.8% with the type strain *Ln. holzapfelii* LMG 23990^T, and clearly clustered with *Ln. holzapfelii* with high bootstrap values of 98%. Strains qz-71 and qz-72 in group E were placed in the cluster of *Ln. citreum* on the phylogenetic tree, moreover, displayed sequence similarity of 99.8% and 99.9% to *Ln. citreum*, respectively.

About group F, qz-488-2, qz-508 and qz-484 could definitely identify as *W. confusa* with bootstrap values of 99% and sequence similarity of more than 99.6% to *W. confusa*. Group G included five representative strains (qz-107, qz-70, qz-106, qz-104 and qz-73) were placed in the cluster of *W. cibaria*, which was supported by 98% bootstrap analysis in the phylogenetic tree. The representative strains of group H (qz-487) were identified as *L. raffinolactis* by forming a well-defined cluster (100% bootstrap) and 99.7% similarity in the 16S rRNA gene sequences. In group I, representative strains qz-368, qz369 and qz-367 were closely related to *L. garvieae* in the phylogenetic tree and each strain shown 99.9% homology to *L. garvieae*.

Representative strains qz-69, qz-105 and qz-283 of group J were attributed to *L. lactis* by forming a well-defined cluster in 100% of bootstrap analyses with four subspecies, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *tructae*, *L. lactis* subsp. *lactis*, and *L. lactis* subsp. *hordniae*. Representative strain qz-548 of group K was placed in the cluster of *E. lactis* with a sequence similarity of 99.7%.

Multiplex PCR results

The *rec*A multiplex-PCR products obtained from the *rec*A gene were displayed in Fig. 4. The negative control type strain *Lb. casei* JCM 16167^T did not produced amplicon. Type strain *Lb. paraplantarum* JCM 12533^T, *Lb. pentosus* JCM 1558^T and *Lb. plantarum* JCM 1149^T produced 107 bp, 218 bp and 318 bp *rec*A gene amplification products, respectively. Representative strains of Group A produced 318 bp amplification products, the same as type strain *Lb. plantarum* JCM 1149^T. Hence, in group A, strains qz-572-2, qz-574-1, qz-572-1 and qz-573-2 were definitely identified as *Lb. plantarum.*

Table 2. Characteristics of lactic acid bacteria strains isolated from the oat and soil samples

Character	Group A			Group B			Group C		Group D	Group E	Group F	Group G		Group H	Group I	Group J		Group K
	qz-572-1*	qz-574-1	qz-540	qz-281	qz-574-2	qz-534	qz-539	qz-282	qz-341	qz-71	qz-508	qz-106	qz-70	qz-487	qz-367	qz-69	qz-105	qz-283	qz-548
Shape	rod	rod	Coccus	Coccus	coccus	Coccus	coccus	Coccus	Coccus	Coccus	Coccus	coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gas from glucose	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Fermentation type	Homo	Homo	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Homo	Homo	Homo	Homo	Homo	Homo
Growth at temp(°C):																			
5	-	+	w	w	w	+	-	-	w	w	w	w	w	w	w	w	w	w	w
10	w	+	w	w	+	+	w	w	w	w	w	w	w	w	w	w	w	w	w
45	+	w	w	w	w	w	+	w	w	w	+	+	+	w	+	+	+	w	+
50	w	-	w	w	w	w	w	w	w	-	w	w	w	w	w	w	w	w	w
Growth in NaCl:																			
3.0%	+	+	+	+	+	+	w	+	+	+	+	+	+	+	+	+	w	+	+
6.5%	+	+	w	w	w	w	-	w	w	w	w	+	w	w	w	-	-	w	+
Growth at pH:																			
3	+	+	-	w	w	w	w	-	w	w	w	w	w	-	-	-	w	-	w
3.5	+	+	w	w	w	w	w	w	w	w	w	+	w	w	w	w	w	w	w
4	+	+	w	+	+	+	w	+	+	+	+	+	+	w	w	+	w	w	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+
5.5	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	w	+	+	+	+	+	w	+	+	+	w	+
10	+	+	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w

+, positive; -, negative; w, weakly positive; Homo, homofermentative; Hetero, heterofermentative; *, representative strain.

Table 3. API 50 CH fermentation patterns of lactic acid bacteria strains isolated from the oat and soil samples[a]

Substrate	GrupA				GrupB						GrupC		GrupD	GrupE	GrupF	GrupG		GrupH	GrupI	GrupJ			GrupK
	qs-572-1*	qs-574-1	qs-540	qs-281	qs-574-2	qs-541	qs-546	qs-547	qs-549	qs-534	qs-539	qs-282	qs-341	qs-71	qs-508	qs-106	qs-70	qs-487	qs-367	qs-69	qs-105	qs-283	qs-548
L-Arabinose	+	+	w	w	-	-	+	+	+	w	-	-	+	+	w	w	+	+	-	-	+	+	+
Ribose	+	+	w	w	-	-	+	-	-	-	w	-	-	-	w	-	w	-	+	+	+	+	+
D-xylose	-	-	-	+	+	-	+	w	w	+	w	-	-	w	w	w	+	+	-	+	+	+	+
Mannitol	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	+	+	w	+	+	+	+
Sorbitol	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D Mannoside	+	+	w	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w
α-Methyl-D Glucoside	-	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-
N-Acetyl Glucosamine	+	+	w	w	w	w	+	w	+	w	w	w	w	w	w	w	w	+	+	+	+	+	+
Amygdalin	+	+	+	+	-	-	+	-	-	-	-	-	w	w	-	-	+	+	+	+	w	+	+
Arbutin	+	+	+	+	-	-	+	-	-	+	-	-	+	w	-	-	w	+	+	+	+	+	+
Esculin	+	+	+	+	+	-	+	+	+	+	-	-	+	+	w	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	-	-	+	w	w	+	-	-	+	w	-	-	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	-	-	+	-	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+
Maltose	+	+	+	+	-	-	+	+	+	-	+	+	-	-	+	+	w	+	-	-	-	-	+
Trehalose	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+
Melezitose	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	w	+	-	w	-	-	-
Raffinose	+	+	+	+	-	-	+	+	+	-	-	+	-	-	-	-	+	+	-	w	-	-	-
Starch	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	w	-	w	w	w	w
Genichiose	w	w	w	w	-	-	w	-	-	-	-	-	w	w	w	-	w	+	+	+	w	+	w
D-Turanose	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	w	+	+	-	-	-
Gluconate	w	w	w	w	-	-	-	-	-	-	-	-	w	w	w	w	-	-	w	w	-	w	w

a, All strains produced acid from galactose, glucose, fructose, mannose, maltose, lactose and sucrose, but failed to produce acid from glycerol, erythritol, Dарabinose, L-xylose, adonitol, β-methyl-xyloside, sorbose, rhamnose, dulcitol, inositol, inulin, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-keto-gluconate, 5-keto-gluconate.

*, representative strain +, 90% or more of the strains positive; -, 90% or more of the strains negative; w, weakly positive.

4. Discussion

Oat is a popular forage crop on the widespread boundary of northwestern China especially in the alpine regions, as its resisting force to cold and arid conditions. In the Tibetan Plateau, despite oat hay is an important supplementary feed for pasturage dumb things, Serious feed shortage is still exists during the long, harsh winter season. Thus, silage oat may be one of the best means to improve the utilization of oat and relieve the feed shortage. The preservation of oat as silage depends on the activity of LAB under anaerobic conditions, meanwhile, undesirable epiphytic microorganisms should be inhibited. In Qinghai province, annual planting area of oat is more than 20000 hectares, including most un-irrigated, a few of irrigated and a very small amount of oat of wild land. In this research, all the oat samples have 10^4 to 10^7 cfu g^{-1} of LAB except one OSWL that was not detected. In this paper, the amounts of LAB on oat samples proved to be different under different cultivation practices through experiment results comparing analysis.

According to the analysis of 16S rRNA gene sequence and *rec*A multiplex-PCR, group A, C, D, E, F, G, H, I and K were identified on species level (Figs 2, 3 and 4). But, because the level of 16S rRNA gene sequences is extremely high (98–99%) similarities among three subspecies in group B of *Ln. mesenteroides* and four subspecies in group J of *L. lactis*, it is hard to identify by using 16S rRNA gene sequences homology approach (Conn et al. 1995; Schleifer 2009, Teuber 2009). In the present study, a slight disparity in carbohydrate fermentation patterns were observed among the different LAB strains despite the higher similarity of their genetic backgrounds. Thus the phenotypic characteristics of group B had a little difference in carbohydrate fermentation pattern compared with previously reports (Conn et al. 1995; Schleifer 2009). In group B, representative strains qz-541, qz-547, qz-549, qz-534 and qz-574-2 proved cellobiose negative as well as trehalose and sucrose positive reactions (Table 4), elucidating that these strains were *Ln. mesenteroides* subsp. *dextranicum*. Representative strains qz-546, qz-540 and qz-281 proved cellobiose, trehalose and sucrose positive reactions (Table 4), clarifying that these strains were *Ln. mesenteroides* subsp. *mesenteroides*. In addition, as shown in Table 4, Representative strains of group J, including qz-69, qz-105 and qz-283 produced acid from lactose, galactose, maltose and ribose as same as *L. lactis* subsp. *lactis* JCM 5805^T. The results shown that all the representative strains of group J were identified as *L. lactis* subsp. *lactis*.

As shown in the results, Three species including *Ln. citreum*, *W. cibaria* and *L. lactis* subsp. *lactis* were isolated from OSNL and SSNL of sites 1, 2, whereas, six species including *Ln. mesenteroides* subsp. *mesenteroides*, *L. lactis* subsp. *lactis*, *L. garvieae*, *Ln. mesenteroides* subsp. *dextranicum*, *Ln. lactis* and *E. lactis* were obtained from OSNL of sites 3 to 7 and no representative strain was isolated from the five SSNL, reason for this phenomenon may be that sites 1, 2 are both famous scenic spot, and there are more tourist people than sites 3 to 7. In addition, *Ln. holzapfelii* was isolated from OSWL of site 8, whereas *W. confuse* and *L. raffinolactis* were isolated from OSWL and SSWL from site 10 because of great influence of human activities close to water, but no strain was isolated from site 9. They were all cocci-shaped strains from samples of nonirrigated and wild land. It is possibly because the frequently fertigation by farmer. *Lb. plantarum* and *Ln. mesenteroides* subsp. *dextranicum* were isolated from the OSIL, whereas no representative strain was isolated from the SSIL from site 11.

In general, the impact of human activities on cultivation practices followed by OSIL, OSNL and OSWL. This study demonstrated that there were significant disparity among the count and species of LAB and different cultivation practices. However, further research to analyze the distribution of LAB from different sources and types is needed, in order to obtain exact conclusions.

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