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Identification and Selection of Lactic Acid Bacteria Resistance to Acid and Bile Salt Isolated from Corn Silage

L. Li[1], N. Zhang [2], L. Kong[2], WC. Zhao[2], Y.N. Xiao[1], B.X. Li[1*] and X.R. Han[1]

[1]National Engineering Laboratory for Efficient Utilization of Soil and Fertilizer Resources, College of Land and Environment, Shenyang Agricultural University, Shenyang, Liaoning 110866, China.
[2]College of Biological Science & Technology, Shenyang Agricultural University, Shenyang, Liaoning 110866, China.

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The aim of the present study was to identification and selection of lactic acid bacteria resistance to acid and bile salt isolated from naturally fermented corn silage. Based on 16S rDNA gene sequence analysis, they were identified as *Lactobacillus plantarum* R1, *Lactobacillus plantarum* R2, *Lactobacillus plantarum* R3 and *Weissella paramesenteroides* ZH1. Growth curve, acid production rate, acid and bile salt tolerance of strains were determined. Isolates were cultured for 12h, and acid-production rate is significantly higher than other strains. In the silage fermentation process, isolates can be used as start strains. R1, R2, R3 have strong acid resistance, their relative OD_{600nm} values were 53.06%, 55.14%, 57.90%, respectively. ZH1 have no acid resistance (6.33%). At 0.03% (w/v) concentration of bile salt for 16h, isolates R1, R2, R3, ZH1 were resistant to bile salt and maintained the high population level, their relative OD_{600nm} values were 88.30%, 87.32%, 82.14%, 59.74%, respectively. At 0.06% (w/v) concentration of bile salt, the relative OD_{600nm} values of isolates were 84.91%, 70.47%, 68.61%, 19.87%, respectively. The results show that R1, R2, R3 exhibited resistance to low pH and tolerance to high concentrations of bile salts, and they could be used as silage additives.

Key words: lactic acid bacteria; acid and bile salt tolerance; 16S rDNA; silage.

Silage, the most commonly preserved forage crop in many countries [1], is based on anaerobic fermentation, whereby epiphytic lactic acid bacteria (LAB) utilize water-soluble carbohydrates (WSC) to produce organic acids, mainly lactic acid [2]. The primary acid is responsible for decreasing the pH to inhibit the undesirable microorganisms and the forage crop is preserved [3]. But natural populations of LAB on plant materials are often low in number [4]. In order to improve the quality of silage fermentation, various LAB inoculants have been used in silage processing, which stimulate lactic acid fermentation and decrease pH [5]. At the same time, LAB is probiotic group in the gastrointestinal tract of animals, which have antimicrobial activity and can exclude or inhibit pathogens, promote digestion and absorption of nutrients, modulate the host immune response and regulate intestinal flora balance, enhance the intestinal epithelial barrier, with a wide range of physiological functions [6-10]. It has important practical significance by using of probiotic LAB fermented feed for the promotion of animal healthy growth.

Nowadays, some of the commonly used probiotic LAB in silage inoculants include *Lactobacillus plantarum* [11], *Lactobacillus rhamnosus* [12], *Lactobacillus acidophilus* [13], *Pediococcus acidilactici* [14], *Enterococcus faecium*[5], *Lactobacillus pentosus* [15], *Lactobacillus buchneri*[16], *Pediococcus pentosaceus* [17],

* To whom all correspondence should be addressed.
Tel.: 024-88487155;
E-mail: libingxue1027@163.com

Lactobacillus xylosus [14], *Propionibacteria freudenreichii* [18, 19], which play an important role in silage fermentation. However, after the silage feeding ruminants, LAB of silage must be able to survive in the rumen, so that it may have an impact on rumen microbial flora, and thus stimulate the digestive tract epithelial tissue, promote the absorption of nutrients, effect on production performance and physiological status of ruminants [20]. To perform their effect in the intestine, probiotic LAB should be capable of surviving passage through the gastro intestinal tract. Thus, it is essential for the bacteria to have protection systems to withstand the low pH in the stomach and bile of the small intestine [21]. Tolerance to gastric acid and bile has thus become important selection criterion for probiotic strains [22, 23].

In this study, four strains of LAB isolated from naturally fermented corn silage. Based on 16S rDNA gene sequence analysis, they were identified as *L. plantarum* R10*L. plantarum* R20*L. plantarum* R3 and *W.paramesenteroides* ZH1. To screen excellent LAB strains for silage additive usage, strains were studied through growth curve, acid and bile salt resistant capacity, lay the foundation for research and application of silage additives.

MATERIALS AND METHODS

Collection of corn straw samples. Corn straw were collected from Faku County, Liaoning Province in China during the period from September 12th to 28th, 2013. The samples were collected aseptically into sterile ziplock bags, kept in an ice-box and transported to the laboratory for analysis.

Silage making. Samples were chopped into 1cm length. Sterilized distilled water was sprayed to the corn straw and the final moisture was approximately 70% of the mixtures. The mixtures were ensiled in 250g capacity plastic containers silos and were then fermented under anaerobic condition at 20°C for two months.

Isolation of LAB. Samples were removed from corn silages after 60d of fermentation. 5g of samples were mixed with 45 mL of sterilized distilled water, and were serially diluted 10^{-1} to 10^{-7} in sterilized water. For LAB isolations, appropriate dilution of each sample was plated on MRS culture agar plates, and then incubated anaerobically at 30°C for 2-3 days. A representative single colony was randomly selected from the agar plates and transferred to MRS broth for further identification. All isolates were initially examined by gram reaction, catalase reaction and cell morphology. Gram-positive, catalase-negative isolates were purified and stored until further examination. Detailed information on the reference strains is listed in Table 1.

16S rDNA gene sequence identification and phylogenetic analysis. Cells grown for 12h in MRS broth at 30°C were used for DNA extraction and purification, as described by Makimura [24]. The 16S rDNA gene were amplified using primers 7F (5-CAGAGTTTGATCCTGGCT-3) and 1540R (5-AGGAGGTGATCCAGCCGCA-3). The 16S rDNA gene sequence coding region was amplified by PCR performed in a PCR thermal cycler (TC-412 PCR, TECHNE). The PCR mixture was composed of 2×Taq MasterMix, which containing Taq DNA Polymerase, 2×Taq PCR Buffer, 3mM $MgCl_2$ and 400 µM dNTP mix (Beijing ComWin Biotech Co., Ltd. China), 10pmol of each primer, 10ng template bacterial DNA. PCRs were performed with the PCR system with initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 90s, and final extension at 72°C for 10 min. Reaction products were resolved by electrophoresis in 1.0% agarose gels and visualized by GoodView staining (SBS Genetech Co., Ltd). The PCR product of interest was isolated from the agarose gel using a Gel Extraction Kit (Sangon Biotech (Shanghai) Co., Ltd. China). The purified PCR fragments were used for sequencing by the corresponding sequencing primers. DNA sequencing was performed by Sangon Biotech (Shanghai) Co., Ltd. China. The sequences were analyzed and determined using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) and were submitted to the NCBI (http://www.blast.ncbi.nlm.nih.gov). Consensus sequences were imported into MEGA version 5.0 software, with which a sequence alignment and phylogenetic trees were created based on the neighbor-joining (NJ) method.

Screening of lactic acid bacteria for silage. To evaluate inoculum growth and decreases in pH value, all of the lactic acid bacteria were collected immediately after inoculation and after every 2h until 30h of fermentation. Growth was assessed by

turbidimetry in a spectrophotometer at 600nm (model SP-722E, Shanghai Spectrum Instruments Ltd. China),a pH Tester (model HI98103, HANNA, Sangon Biotech (Shanghai) Co., Ltd. China)was used to measure the pH. Each assay was conducted in triplicate. Results of LAB acid-production test were shown as average value.

Acid tolerance of strains was evaluated, according to the method described by Vijayakumar[25]. Briefly, the bacterial culture was inoculated into the sterilized MRS broth of various pH (2.0, 4.0, 6.0, 6.5 and 7.0) and then the broth was incubated at 37°C for 16 h. After the incubation, one tube of each culture medium and strain was removed, were used to measure optical density at 600nm.

The ability of the strains to grow in the presence of bile (w/v) was determined according to the method of García-Ruiz [26]. Each strain grown overnight was inoculated (3% v/v) into MRS broth with 0.03% and 0.06% (w/v) of bile. Cultures were incubated at 37°C for 16h, optical density at 600nm (OD_{600}) was measured and compared to a control culture (without bile salts). The results were expressed as the percentage of growth compared to the control. Assays were carried out in triplicate.

RESULTS

Isolation of LAB

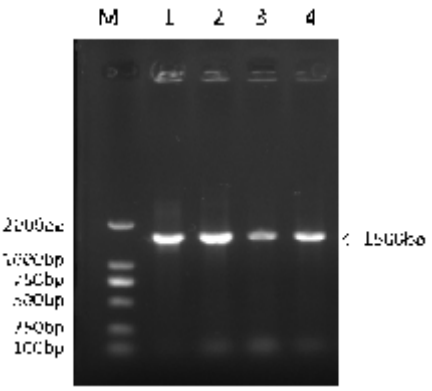
All the isolates were primarily confirmed as LAB since they manifested gram-positive status, absence of catalase and oxidase activity. Finally, a total of four LAB strains were isolated from MRS agar media. Three strains were rod-shaped LAB and one strains was cocci (Table 2).

16S rDNA gene sequence identification and phylogenetic analysis

To identify at the species level, the 16S rDNA gene sequences of the isolates were amplified and analyzed. It was found that the two selected primers (FA-7F and RA-1540R) were able to amplify the 16S rDNA gene fragment of each LAB isolate with an amplicon of approximately 1,500 bp (Fig.1).

Then the sequences were compared with related bacterial sequences in the GenBank and the sequence similarities were checked using the BLAST program (Table 3).

Phylogenetic tree analysis was performed to show the relationship of 16S rDNA gene sequences between the representative isolates and related type strains by using MEGA software (Fig. 2). Isolates R10R20R3 of group 1 were closely related to *Lactobacillus plantarum WCFS1* strain WCFS1 as it showed 100% homology to *Lactobacillus plantarum WCFS1* strain WCFS1. Isolate ZH1 of group 2 was placed in the cluster of *Weissella paramesenteroides* strain NRIC 1542 with a similarity of 100%. Based on 16S rDNA gene sequences analysis, 4 strains isolates from corn silage were named *L. plantarum* R1 (NCBI: KP296674), *L. plantarum* R2 (NCBI: KP296675), *L.*



Lanes: M, DL 2000 DNA Marker; 1, R1; 2, R2; 3, R3; 4, ZH1.

Fig. 1. Electrophoresis result of PCR-amplified 16S rDNA gene fragment from isolates

Table 1. Reference strains (China General Microbiological Culture Collection Center, CGMCC)

CGMCC number	Strain name
1.511	*Lactobacillus plantarum subsp. plantarum*
1.555	*Lactobacillus plantarum subsp. plantarum*
1.558	*Lactobacillus brevis*
1.1854	*Lactobacillus acidophilus*
1.1878	*Lactobacillus acidophilus*
1.1880	*Lactobacillus fermentum*
1.2435	*Lactobacillus casei*
1.2437	*Lactobacillus plantarum subsp. plantarum*
1.2439	*Lactobacillus pentosus*
1.2466	*Lactobacillus rhamnosus*
1.2469	*Lactobacillus plantarum subsp. plantarum*
1.3395	*Lactobacillus amylovorus*
1.3396	*Lactobacillus gasseri*

plantarum R3 (NCBI: KP296676), *W. paramesenteroides* ZH1,NCBI:KP296677.

Determination of growth curve

The adaptation periods of *L. fermentum* CGMCC 1.1880, *L. plantarum subsp. plantarum* CGMCC 1.555, *L. plantarum subsp. plantarum* CGMCC 1.511, *L.plantarum subsp. plantarum* CGMCC 1.2437, *L. rhamnosus* CGMCC 1.2466, *L. plantarum subsp. plantarum* CGMCC 1.2469, *L. casei* CGMCC 1.2435 were shorter, which entered logarithmic growth phase after 2h. And these strains reached a stable period after 10, 12, 14, 16, 18h, respectively. *W. paramesenteroides* ZH1, *L. plantarum* R1, *L. plantarum* R2, *L. plantarum* R3, *L. pentosus* CGMCC 1.2439 entered logarithmic growth phase after 4h and reached a stable period

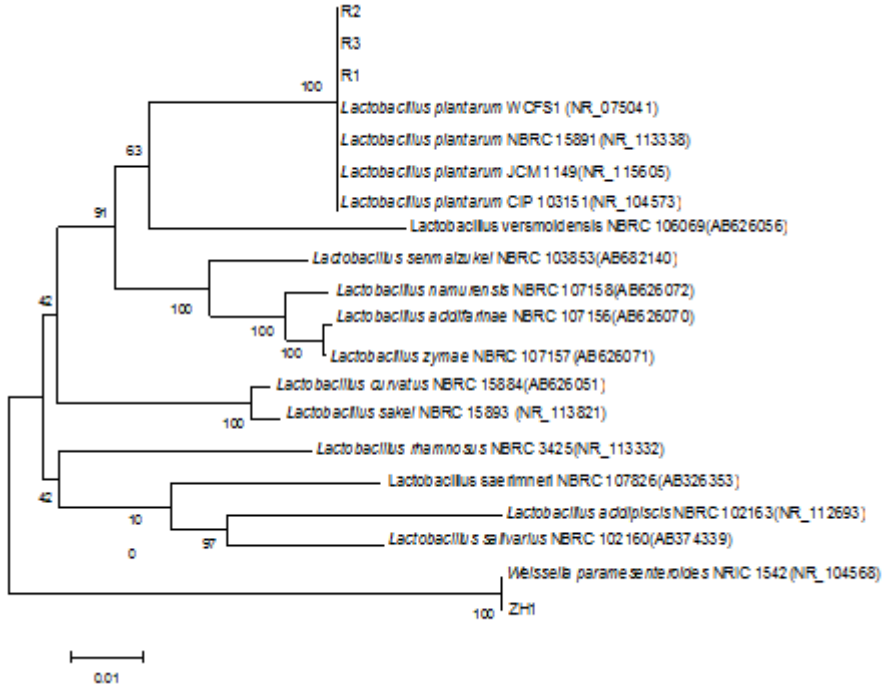


Fig. 2. Phylogenetic tree based on 16S rDNA gene sequence analyses, showing the phylogenetic placement of LAB strains isolated from naturally fermented corn silage.

Table 2. The morphological characteristics of isolates

Strain	colony characteristics						Microscopic characteristics	Dye	Catalase
	Colour	Status	Transparency	Size	Border	Form			
R1	Milky	Smooth	Opaque	middle	Neat	Round	Short Rod, Pairs or Short-chain	G^+	E^y
R2	Milky	Smooth	Opaque	middle	Neat	Round	Short Rod, Pairs or Short-chain	G^+	E^y
R3	White	Smooth	Opaque	Small	Neat	Round	Long rod, Single or Pairs	G^+	E^y
ZH1	Gray	Smooth	Opaque	Large	Neat	flat	Spherical or oval, Single or Pairs	G^+	E^y

Table 3. Isolates 16S rDNA sequence analysis results of BLAST

Stain	Reference sequence	Homology/%	The results of BLAST
R10	NR_075041.1	99	*Lactobacillus plantarum*
R2	NR_075041.1	99	*Lactobacillus plantarum*
R3	NR_075041.1	99	*Lactobacillus plantarum*
ZH1	NR_104568.1	99	*Weissella paramesenteroides*

after 14, 16h, respectively. These strains had no obvious lag phase in the fermentation process, which showed the metabolic activities were faster in MRS broth. Strains will soon enter cell division period, so that the number of cells was rapid growth and thus produced large amounts of lactic acid, the pH decreased quickly to inhibit the growth of yeast, mold and spoilage bacteria. These strains may have an advantage in the early silage. The adaptation period of remaining strains was longer, *L. brevis* CGMCC 1.558, *L. acidophilus* CGMCC 1.1854, *L. amylovorus* CGMCC 1.3395, *L. gasseri* CGMCC 1.3396 entered logarithmic growth phase after 6h and reached a stable period after 14, 16h, respectively. *L. acidophilus* CGMCC 1.1878 entered logarithmic growth phase after 10h and reached a stable period after 16h (Fig.3).

Acid-production test

Acid-production rate is an important feature of LAB with good vitality. There is a big difference between the different strains. The acid-production rate of the strains was determined respectively, which pH ranges are shown in Table 4. Then we found that the acid-production rate of *L. plantarum subsp. plantarum* CGMCC 1.2437 was fastest, the pH declined from 6.0 to around 4.2

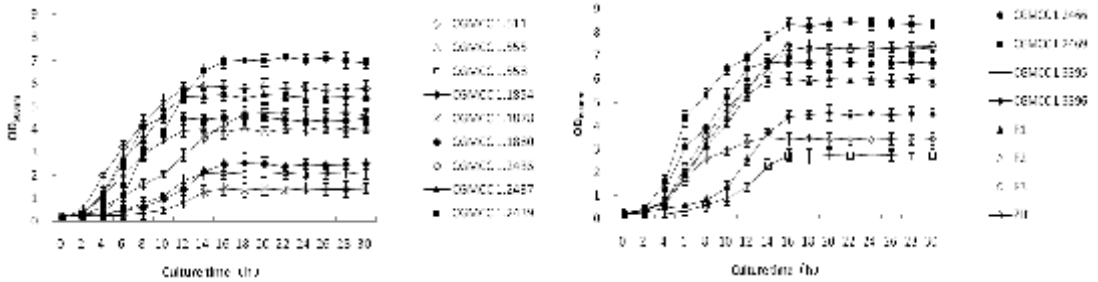


Fig. 3. The growth curve of LAB

Table 4. LAB acid-production test (pH)

strain	time(h)									
	0	2	4	6	8	10	12	24	36	48
L. plantarum subsp. plantarum CGMCC 1.511	6.00	5.49	4.87	4.41	4.15	3.92	3.80	3.59	3.53	3.46
L. plantarum subsp. plantarum CGMCC 1.555	6.00	5.59	5.06	4.58	4.31	4.19	4.09	3.90	3.65	3.49
L. brevis CGMCC 1.558	6.00	5.77	5.73	5.42	5.40	5.38	5.26	4.83	3.98	3.79
L. acidophilus CGMCC 1.1854	6.00	5.39	5.27	4.96	4.77	4.58	4.48	3.97	3.75	3.63
L. acidophilus CGMCC 1.1878	6.00	5.54	5.38	5.31	5.18	5.09	5.02	4.54	3.61	3.41
L. fermentum CGMCC 1.1880	6.00	5.45	5.20	4.79	4.35	4.24	4.06	3.84	3.76	3.62
L. casei CGMCC 1.2435	6.00	5.32	5.13	4.88	4.69	4.47	4.30	3.81	3.51	3.32
L. plantarum subsp. plantarum CGMCC 1.2437	6.00	5.20	4.78	4.19	3.98	3.81	3.72	3.56	3.49	3.34
L. pentosus CGMCC 1.2439	6.00	5.33	5.22	4.89	4.50	4.15	3.98	3.70	3.64	3.31
L. rhamnosus CGMCC 1.2466	6.00	5.56	4.97	4.54	4.26	4.09	3.97	3.83	3.77	3.64
L. plantarum subsp. plantarum CGMCC 1.2469	6.00	5.50	5.11	4.30	4.08	3.93	3.86	3.78	3.74	3.53
L. amylovorus CGMCC 1.3395	6.00	5.72	5.60	5.46	5.18	4.91	4.73	4.11	3.50	3.31
L. gasseri CGMCC 1.3396	6.00	5.64	5.43	5.26	5.03	4.80	4.62	4.02	3.60	3.48
L. plantarum R1	6.00	5.67	5.39	5.00	4.67	4.44	4.18	3.73	3.54	3.52
L. plantarum R2	6.00	5.66	5.46	5.10	4.66	4.38	4.17	3.72	3.51	3.50
L. plantarum R3	6.00	5.70	5.44	4.98	4.61	4.35	4.14	3.71	3.51	3.49
W. paramesenteroides ZH1	6.00	5.27	4.94	4.65	4.47	4.36	4.29	4.13	4.00	3.94

after 6 h, to around 3.3 after 48h. The pH of *L. plantarum subsp. plantarum* CGMCC 1.5110*L. rhamnosus* CGMCC 1.24660*L. plantarum subsp. plantarum* CGMCC 1.2469 declined to 4.2 after 8h, to 3.5 after 48h. The pH of *L. plantarum subsp. plantarum* CGMCC 1.5550*L. fermentum* CGMCC 1.18800*L. pentosus* CGMCC 1.2439 declined to 4.2 after 10h, to 3.5 after 48h. The pH of *L. plantarum* R10*L. plantarum* R20*L. plantarum* R30*W. paramesenteroides* ZH1 declined to 4.2 after 12h, to 3.5 after 48h. The pH value of *L. acidophilus* CGMCC 1.18540*L. casei* CGMCC 1.24350*L. amylovorus* CGMCC 1.33950*L. gasseri* CGMCC 1.3396 declined to 4.2 after 24h, to 3.5 after 48h. Above strains showed good acid production capacity, with potential capacity for silage.

Resistance to Acid

LAB for silage should pass through the highly acidic stomach in order to reach the intestine and create proper conditions for residence [27]. Therefore, a step in screening of LAB for silage is selecting those that are acid resistant. Figure 4 shows that OD_{600nm} values of some strains were the maximum at pH 6.0 MRS broth. The OD_{600nm} values of some strains were the maximum at pH 6.5. This shows that the optimal culture conditions of each strain was at about pH 6.0. Each strain does not grow at pH 2.0 for 16h. All strains was survived in the acidic conditions of pH 4.0 after 16h of incubation (Fig.5). Some of strains were resistant to acid in which acid resistance was between 31.5% and 64.76%, such as *L. plantarum subsp. plantarum* CGMCC 1.511 (48.82%)0*L. brevis* CGMCC 1.558 (31.91%), *L. casei* CGMCC 1.2435 (31.5%), *L.plantarum subsp. plantarum* CGMCC 1.2437 (64.76%), *L. pentosus* CGMCC 1.2439 (56.53%), *L. rhamnosus* CGMCC 1.2466 (45.98%), *L. plantarum subsp. plantarum* CGMCC 1.2469 (50.30%), *L. gasseri* CGMCC 1.3396 (52.72%), *L. plantarum* R1 (53.06%), *L. plantarum* R2 (55.14%), *L. plantarum* R3 (57.90%), these strains show higher acid resistance. But the remaining strains were resistant to acid in which acid resistance was between 3.98% and 28.70 %, such as *L. plantarum subsp. plantarum* CGMCC 1.555 (3.98%), *L. acidophilus* CGMCC 1.1854 (21.61%), *L. acidophilus* CGMCC 1.1878 (13.58%), *L. fermentum* CGMCC 1.1880 (18.06%), *L. amylovorus* CGMCC

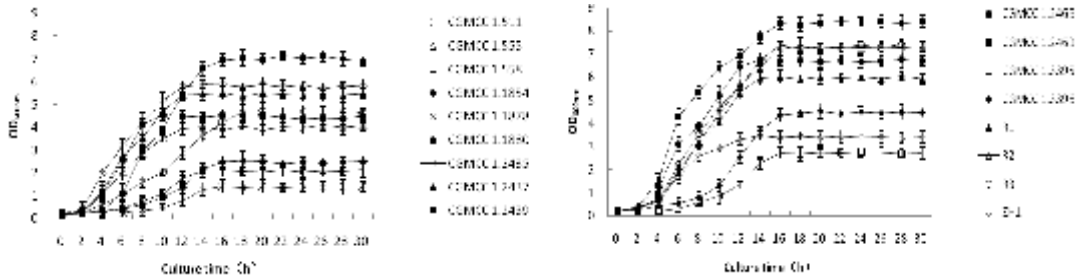


Fig. 4. The OD_{600nm} by the evaluated strains after 16h of culture in MRS with pH 2.0 ( ), pH 4.0 ( ), pH 6.0( ), pH 6.5 ( ) and pH 7.0 ( )

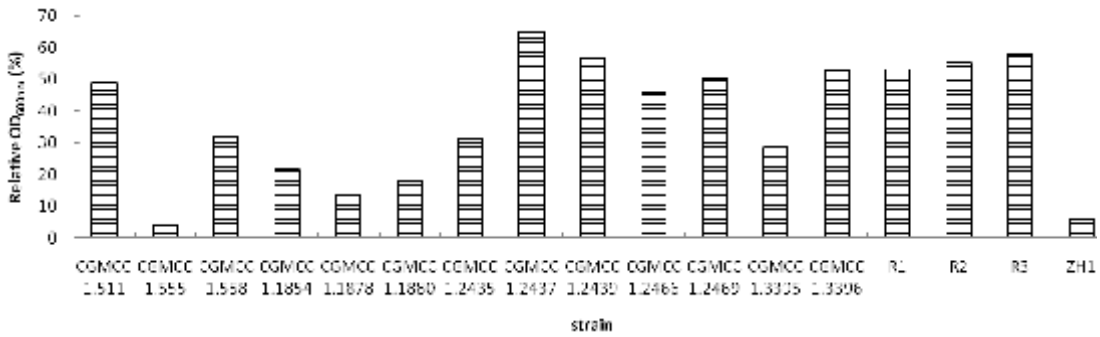


Fig. 5. The relative OD_{600nm} by the evaluated strains after 16h of culture in MRS with pH 4.0( )

1.3395, 28.70%, *W. paramesenteroides* ZH1 6.33%, these strains showed weaker acid resistance.

Bile salts tolerance

LAB for silage should not only be tolerant to acidic conditions of the stomach but also be resistant to animals' intestinal bile salt, so that they could survive in the digestive system [27]. To establish strains resistance to bile salts, a comparison of OD_{600nm} value obtained from the microorganism cultured in MRS and MRS-bile salts was conducted. It was noticed that for all of the strains, the OD_{600nm} value obtained in MRS-bile salt at any of the evaluated concentrations was lower than control culture (without bile salts) (Fig. 6). Four different strain growth profiles were observed with regard to bile salt concentration (Fig. 7). In a first group an intolerance bile model was observed. The concentration of bile salt was 0.03%, the relative OD_{600nm} values of *L. plantarum subsp. plantarum* CGMCC 1.555 and *L. acidophilus* CGMCC 1.1878 were 12.81%, 34%, respectively. The concentration of bile salt was 0.06%, their relative OD_{600nm} values were 3.31%, 14.33%, respectively. In a second group, some of strains only were resistance to low concentrations (0.03%) bile salt, such as *L. plantarum subsp. plantarum* CGMCC 1.511(79.53%), *L. fermentum* CGMCC 1.1880 (80.5%), *L. rhamnosus* CGMCC 1.2466 (89.02%), *L. gasseri* CGMCC 1.3396 (66.19%), *W. paramesenteroides* ZH1 (59.74%). But, these strains were resistant to 0.06% bile salt in which bile salt resistance was between 2.8% and 21.85%, which presented less resistance to bile salt. In a third group, strains proved to be certain resistant in the presence of 0.03% and 0.06% of the bile salt. In the 0.03% bile salt, the relative OD_{600nm} values of *L. brevis* CGMCC 1.558, *L. casei* 1.2435, *L. pentosus* CGMCC 1.2439, *L. plantarum subsp. Plantarum* CGMCC 1.2469 and *L. amylovorus* CGMCC 1.3395 were 57.31%, 53.4%, 53.66%, 50.66%, 75.46%, respectively. In the 0.06% bile salt, the relative OD_{600nm} values of them were 48.84%, 28.53%, 35.58%, 34.21%, 56.51%, respectively. Finally, for the fourth group, These strains were resistant to 0.03% and 0.06% bile salt and maintained the high population level through all tested concentrations, such as *L. acidophilus* CGMCC 1.1854 (88.74% for 0.03% bile salt, 61.94% for 0.06% bile salt), *L. plantarum subsp. plantarum* 1.2437 (87.4% for 0.03% bile salt, 82.17% for 0.06%

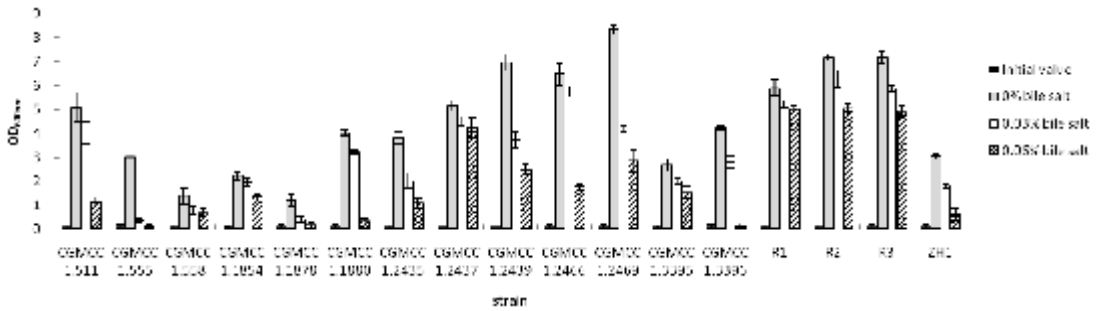


Fig. 6. The OD_{600nm} by the evaluated strains after 16h of culture in MRS ( ), MRS-bile salt 0.03% ( ) and MRS-bile salt 0.06% ( )

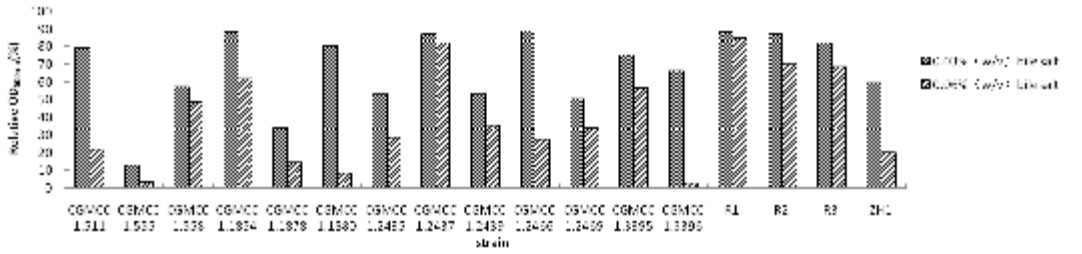


Fig. 7. The relative OD_{600nm} by the evaluated strains after 16h of culture in MRS-bile salt 0.03% ( ) and MRS-bile salt 0.06% ( ).

bile salt), *L. plantarum* R1 (88.30% for 0.03% bile salt, 84.91% for 0.06% bile salt), *L. plantarum* R2 (87.32% for 0.03% bile salt, 70.47% for 0.06% bile salt), *L. plantarum* R3 (82.14% for 0.03% bile salt, 68.61% for 0.06% bile salt).

DISCUSSION

Silages are produced using a traditional method of natural fermentation. This method preserves beneficial microorganisms. They attached plant surfaces, so they were more able to adapt to the environment of silage. Many strains of LAB isolated from silage, such as *Lactobacillus plantarum, Lactobacillus case* [28], *Lactococcus piscium, Weissella soli and Leuconostoc gelidum* [29], *Lactobacillus rhamnosus, Lactobacillus rapi, Pediococcus pentosaceus* [30], *Pediococcus lolii* [31], *Weissella cibaria, Weissella confuse, Leuconostoc citreum, Leuconostoc lactis, Leuconostoc pseudomesenteroides, Lactococcus lactis subsp. Lactis and Lactobacillus paraplantarum* [1]. Therefore, LAB isolated from silage is an efficient and fast way. In this study, four strains of LAB isolated from corn silage, which were gram-positive and catalase-negative bacteria. Based on 16S rDNA gene sequence analysis, they were identified as *L. plantarum* R1, *L. plantarum* R2, *L. plantarum* R3 and *W. paramesenteroides* ZH1.

In the process of ensiling, LAB converted WSC into organic acids. As a result, the pH decreases to 3.8-4.2 to inhibit the undesirable microorganisms and thus improve the fermentation quality [3]. Therefore, as silage additives, growth characteristics and acid-production capacity of lactic acid bacteria directly affect silage quality. In this study, screening lactic acid bacteria with potential fermentation characteristics were by growth curve and acid-production rate index. *L. plantarum subsp. plantarum* CGMCC 1.2437, *L. plantarum subsp. plantarum* CGMCC 1.511, *L. rhamnosus* CGMCC 1.2466, *L. plantarum subsp. plantarum* CGMCC 1.2469, *L. plantarum subsp. plantarum* CGMCC 1.555, *L. fermentum* CGMCC 1.1880, *L. pentosus* CGMCC 1.2439, *L. plantarum* R1, *L. plantarum* R2, *L. plantarum* R3, *W. paramesenteroides* ZH1 can be used as start strains in the silage fermentation process, because acid-production rates of these strains were significantly higher than other strains before 12 h.

However, acid-production rate of *L. acidophilus* CGMCC 1.1854, *L. casei* CGMCC 1.2435, *L. amylovorus* CGMCC 1.3395, *L. gasseri* CGMCC 1.3396, *L. brevis* CGMCC 1.558, *L. acidophilus* CGMCC 1.1878 were higher then others, when they were cultured for 24 and 36h, respectively. This characteristic plays an important role on subsequent fermentation of silage for the six strains.

LAB tolerated a low pH environment of the stomach, which may enter animal intestines for survival and proliferation, and play a prebiotic effect. All strains tested showed a steady loss in viability when exposed to acidic conditions. All strains hardly grow at pH 2. Each strain exhibited varying degrees of acid tolerance at pH 4. Acid resistance of *L. plantarum subsp. plantarum* CGMCC 1.2437 was the strongest. The relative OD_{600nm} of isolates *L. plantarum* R10R20R3 were 53.06%, 55.14%, 57.90%, respectively, and were higher than the rest of reference strains. It shows that the three isolates have strong acid resistance. The relative OD_{600nm} of isolate *W. paramesenteroides* ZH1 was low (6.33%), which did not have acid resistance. According to report, *W. paramesenteroides* plays an important role in the first phase of silage fermentation [32], but it did not improve silage quality and may cause some fermentation loss [33]. The acid resistance of LAB is the result of combined action of multiple mechanisms, including TCS (Two-component signal transduction system), outside and inside of the cell pH balance, the change of cell membrance, protein and DNA damage repair [34].

The relevant physiological concentration of animals' intestinal bile salt ranges from 0.03% to 0.5% [35, 36]. It has also been reported that good bile tolerance benefits the colonization in the host gastrointestinal tract [37]. Previous studies have shown that some lactic acid bacteria can tolerate a certain concentration of bile salt [38, 39]. In this study, at a concentration of 0.03% (w/v) bile salt, the relative OD_{600nm} of isolates *L. plantarum* R1, R2, R3 were 88.30%, 87.32%, 82.14%, respectively, and were higher than the rest of reference strains except *L. acidophilus* 1.1854 (88.74%) and *L. rhamnosus* 1.2466 (89.02%). The relative OD_{600nm} of isolate *W. paramesenteroides* ZH1 was 59.74%, which was resistance to low concentrations of bile salt. At a concentration of 0.06% (w/v) bile salt, the relative

OD_{600nm} of isolates *L. plantarum* R1, R2, R3 were 84.91%, 70.47%, 68.61%, respectively, with higher resistance to bile salt. Bile salt resistance of LAB is related to its bile salt hydrolase [40] and surface layer protein [41].

CONCLUSION

As good probiotic bacteria, LAB have important physiological function to animal, and LAB can survive in gastrointestinal is the basic premise of physiological function. Four strains of LAB were isolated from naturally fermented corn silage. Based on 16S rDNA gene sequence analysis, they were identified as *L. plantarum* R1, R2, R3 and *W. paramesenteroides* ZH1. In the silage fermentation process, isolates can be used as start strains because of good growth performance and acid-production rate. *L. plantarum* R1, R2, R3 exhibited resistance to low pH and tolerance to high concentrations of bile salt, and these stains had a good potential for silage, and could be used as silage additives.

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