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Bacterial Composition of Composite Low-temperature Lactic Acid Bacteria and Microbial Diversity in their Fermentation System with Corn Stover

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A low-temperature lactic acid bacteria(LAB) LAC-1 consortium was screened for treating corn stover from silage at 10 °C . The bacterial composition and microbial diversity of fermentation systems were analyzed by cloning and sequencing of 16S rDNA library. The changes in feed composition after LAC-1 inoculation were also determined.The results showed that LAC-1 consisted of senven *Lactobacillus* strains. After LAC-1 was inoculated in corn stover, *Lactobacillus* dominated the fermentation system and the ration was 82.5% among microbial compostion. While harmful strains such as *Enterobacteriaceae,* etc. dominated in the control treatment,. Meanwhile, the contents of acetic acid, lactic acid, glycerol, and crude protein (CP) of feed were increased, and the contents of propionic acid, butyric acid, acid detergent fiber (ADF) and neutral detergent fiber (NDF) were decreased by inoculation. The inoculated-LAC-1 well colonized and dominated in the fermentation system, significantly inhibited the proliferation of harmful bacteria and improved the feed quality.

Key words: Low-temperature consortium, Microbial diversity, Inoculation, Corn stover, Feed.

According to FAO statistics, the world produces more than 2.9 billion tons of stalks annually (Zhu, 2003), which has the capability of maintaining 1.674 billion sheep (50 kg live weight). The theoretical annual output capacity of stalk resources in China is 840 million tons; among them approximately 215 million tons (31.3%), equivalent to 100 million tons of standard coal, were burnt due to the lack of reasonable use (Tian *et al*., 2011), which not only brought serious pollution to the environment, but also wasted a large amount of valuable resources.

Use of stalk as feed is one of the important ways to utilize surplus stalks and solve the pollution problems. Chinese Ministry of Agriculture requested that till 2010 the country's rate of stalk utilized as feed should reach 55% (Li *et al*., 2005), while the actual rate of feed from stalk then was only 31.9%. The major reason is that current technology is mainly to produce the green straw feed, and there is lack of microbial technology to treat the post-harvest dried yellow straw.

Dried yellow stalk contains low content of CP but high content of crude fiber, with coarse texture, poor palatability, and low digestibility, resulting in a poor metabolism and utilization with livestock. Therefore, to improve the palatability and feeding value of dried yellow stalk feed is the key to solve the low rate of stalk utilization in China. Microbial inoculation is an important way to improve the palatability and feeding value of stalk feed. Currently there are relatively more reports focusing on single strain inoculation, such as inoculation of homofermentative *Lactobacillus Plantarum* (*L. plantarum*), heterofermentative *L. buchneri*, or both together(Filya, 2003). However,

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inoculations of pure culture into non-sterile fermentation system often fail to achieve the desired effect. Recent studies found that targeted culture and screening of natural microbial populations may obtain desired powerful and stable microbial flora (Haruta *et al*., 2002). Heilongjiang province of China is located in a cold area; relatively low ambient temperature after the harvest became a limiting factor for microbial fermentation and processing of stalk feed in this region. Studies of the effects of LAB community used for miro storage under low temperature have seldom been reported, particularly in dried crop straws. Therefore, this study was conducted taking advantage of the synergic relations among microbes and the previously-screened and -obtained composite low-temperature LAB LAC-1, which is stable and can rapidly reduce the pH in the fermentation system of corn stover after inoculation, as well as can improve the palatability of the fermented feed. At the 6th day of fermentation with composite system at 10 °C, the pH and Water-Soluble Carbohydrates (WSC) were reduced to 4.0 and 44.3 g/kg respectively, and the number of LAB reached 8.2 lg cfu/mL. The pH of the fermentation system of dried corn stover dropped to 4.0 on 15th day after LAC-1 inoculation. The application of LAC-1 could promote the conversion of dried cornstalk without any additional sugar into miro storage and improve the quality of feed (Wang *et al*., 2013).

This study has also analyzed the diverse composition of LAC-1 and its effect on the microbial diversity in the corn stover fermentation system, using 16S rDNA clone library. Through analysis of the composition changes in the stalk feed after inoculation of LAC-1, we studied the effect of inoculation treatment and solved the problems in microbial treatment of corn stalk under low temperature by inoculation of composite strains LAC-1, which has the potential to produce a wider range of high-quality feed for the fodder-deficient areas.

MATERIALS AND METHODS

Materials

The composite low-temperature LAB LAC-1 was selected in our laboratory(Wang *et al*., 2013). The testing material, threshed corn stover, was harvested from the Test Base of Heilongjiang Bayi Agricultural University, with a pH value of 6.4 and dry matter (DM) of 90% (w/w).

EXPERIMENTAL

LAC-1, statically cultivated in MRS-S medium(Gao, *et al*., 2008) for 5 days at 10 °C in screw-cap test tube, was analyzed for microbial composition and diversity, and used as inocula for subsequent fermentations. The experiment included 2 groups as CK (without LAC-1 inoculation) and LAC-1-inoculated (LAC-1). In LAC-1 group, LAC-1 was inoculated at a ratio of 3% into the corn stover (crushed into 0.1-0.3 cm diameter, 1-2 cm long). The amount of bacteria was 1×10^9 cfu/g FM (Fresh matter, FM). The system was adjusted with distilled water to 70% moisture content, placed in a 100 mL flask with blue screw cap, and statically fermentated at 10 °C for 15 days. Sample was taken from CK group at 0 day to analyze for microbial diversity in the initial stage of the fermentation system. The microbial diversity of the system in the end stage of fermentation was analyzed with the samples harvested from CK and LAC-1 groups at 15th day of fermentation. For the sampling, 3 bottles of samples were taken from each group and mixed; 2 g of the mixture were used for DNA extraction. At days 0, 3, 5, 10, and 15 of fermentation, 3 bottles of samples were collected respectively to analyze for pH, number of LAB, and the contents of respective acetic acid, propionic acid, butyric acid, lactic acid, glycerol, CP, NDF, and ADF.

PCR amplification of 16S rDNA and construction of cloning library

Total genomic DNAs were extracted from LAC-1 fermentation system using the benzyl chloride method as described by (Zhu *et al*., 1993). The PCR reaction was as follows: 10× PCR buffer 5 mL, 2.5 µM dNTP mix 4 µL, 50 µM 27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R(5'-GGTTACCTTGTTACGACTT-3) 0.5 µL each, template DNA 1 mL, and rTaq DNA polymerase (5 units/mL, TAKARA) 0.5 µL; the volume was adjusted to 50 µL with ddH_2O. The PCR conditions were as the following: 1 cycle of initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min; and final elongation at 72 °C for 10 min. The PCR products were detected by

electrophoresis on 1% agarose gel.

Bacterial 16S rDNA clone library was constructed according to Hiroyuki *et al*. (2002). Briefly, 16S rDNA PCR products were purified and cloned into the vector pGEM-TEasy, which was then transformed into *E. coli* TOP10. Some transformants were randomly selected from LB plate. The insert in each clone was amplified by bacterial PCR method, using primers RV-M (5'-GAGCGGATAACAATT TCACACAGG-3') and M13-47 (5'-CGCCAGGGTTTTCCCAGTCACGAC-3'), under the conditions as following: 10 mM dNTP 0.5 μL, 25 mM $MgCl_2$ 2 μL, 10× reaction buffer 2.5 μL, primers RV-M and M13-47 0.25 μL each, Taq polymerase (5 units/μL) 0.3 μL, and template bacteria a little; the volume was adjusted to 25 μL. The PCR was run as follows: 1 cycle of initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 90 s; and final elongation at 72 °C for 10 min. The PCR products were detected by electrophoresis on 1% agarose gel, which was stained with ethidium bromide (2.0 μg/mL) for 20 min. The results were documented and analyzed.

Amplified rDNA restriction analysis (ARDRA), sequencing, and phylogenetic analysis

For ARDRA, the PCR products were digested with restriction enzymes *Msp* I and *Hinf* I, and the restriction fragments were separated on 2% agarose gels and grouped according to DNA fingerprints. The sequences of representative cloned fragments were determined by Shanghai BGI Company (Shanghai, China), and were blasted against NCBI database (http://www.ncbi.nlm.nih.gov/blast/). The phylogenetic trees were then constructed using neighbor-joining method with MEGA 4.0 software. The nucleotide sequences determined in this study were deposited into GenBank nucleotide sequence database (Accession No. KC787546-KC820081).

Assay methods

The fermented corn stover feed was diluted 10 times and incubated for 20 min after shaking; the pH of the supernatant was detected using HORIBA B-212 pH meter. The number of LAB was determined according to Yang *et al*. (2007).

To measure the contents of acetic acid, propionic acid, butyric acid, lactic acid, and glycerol, corn stover feed (0.5 g) was dissolved in sterile water and incubated at 4 °C for 24 h. The leach solution was centrifuged at 8,000 rpm, 4 °C for 20 min, and the supernatant was filtered through 0.22 μm filter. The filtrate (1 μL) was used as sample to load Agilent Technologies 6890N GC for the analysis of above-mentioned chemical contents under the conditions as follows: capillary column, 30 cm × *f* 0.25 mm × 0.25 μm; inlet temperature, 200 °C; flame ionization detector (FID) temperature, 240 °C; column temperature program: initial temperature 150 °C for 2 min, subsequently temperature ascending at 10 °C /min to 180 °C, maintaining for 2 min, ascending again at 10 °C /min to 200 °C, maintaining for 11 min, total time 20 min; inlet pressure, 100 Kpa; carrier gas, H_2; column flux, 40 mL/min; and inlet split ratio, 20:1 (v/v). Each sample was repeated 3 times.

Corn stover feed was ground with a particle size of 40 mesh using FZ102 micro plant grinding mill, and CP was determined using Kjeltec 2300 Analyzer Unit. NDF and ADF were determined using the semiautomatic ANKOM220 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) according to Guo *et al*. (2008).

All the data were analyzed by one-way ANOVA using statistical software SPSS19.0.

RESULTS AND DISCUSSION

Analysis of the strain composition of LAC-1

A total of 181 positive clones identified from the 200 randomly-selected clones were studied for diversity by ARDRA. Bacterial populations represented by each enzyme digestion pattern were designated as LAC-1-1 to 7, and one clone from each enzyme pattern was selected for sequencing. Sequencing results of LAC-1 components and the phylogenetic tree were observed in Fig. 1. The results indicate that the bacteria in composite LAC-1 all were *Lactobacillus* stains. Among them, LAC1-1, 2, and 3 accounted for 32.1%, 19.9%, and 29.8% clones in the library, respectively, while the proportion of the remaining 4 stains in LAC-1 together was 18.2%.

The coverage of LAC-1 16S rDNA library was analyzed using Rarefaction Curve Analysis Software (Fig. 2). The results showed that when the number of clones reached 80, the curve approached plateau, indicating that the 16S rDNA

clone library of composite LAC-1 has a relatively good coverage and thus can fully reflect the microbial diversity.

***L. nodensis* iz4b-1**

The homology between LAC1-1 and *L. nodensis* iz4b-1 was 98%. *L. nodensis* iz4b-1 was initially isolated from rice bran. This strain is anaerobic and homofermentative, and can produce lactic acid from glucose without producing gas (Kashiwagi *et al*., 2009).

***L. curvatus* CTSPL4**

The similarity between LAC1-2 and *L. curvatus* CTSPL4 was 99%. *L. curvatus* CTSPL4 is a facultative anaerobe, with the optimum growth pH of 6.2-6.8. It grows fast and has a strong acid-producing ability. It is also homofermentative, and can ferment various sugars, producing acetic acid, propionic acid, butyric acid, and lactic acid. It can

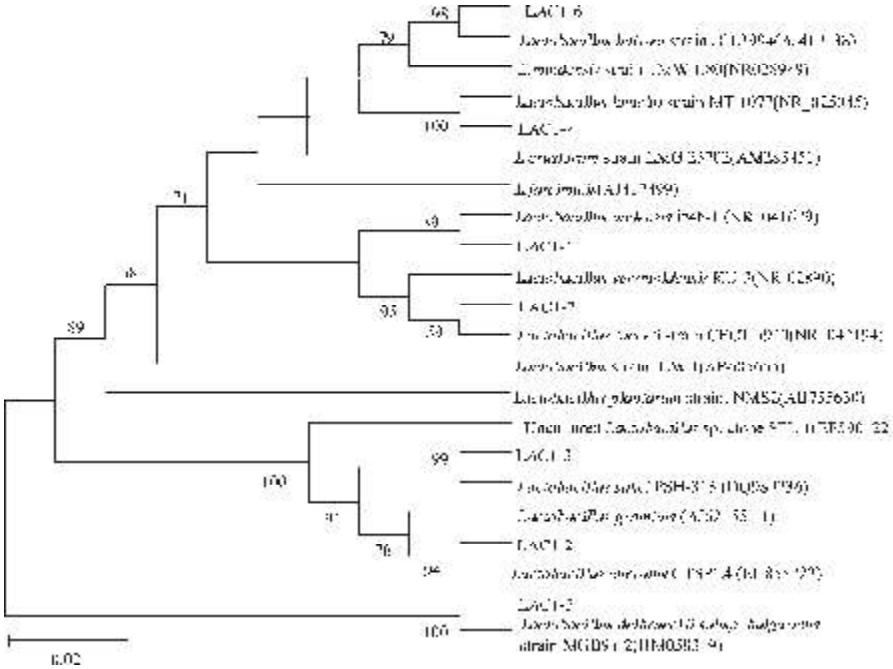

Fig. 1. Phylogenetic tree of 16S rDNA clones from the LAC-1 and their most similar Genbank sequences. Only bootstrap values above 50% were displayed. The same as below

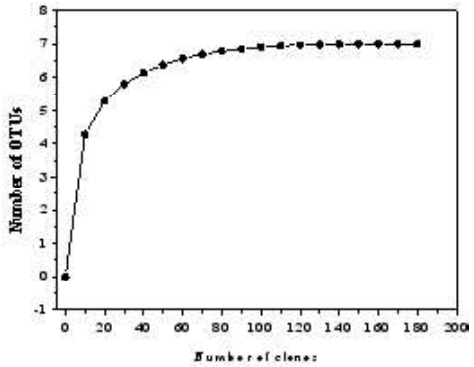

Fig. 2. Rarefaction curve for the different ARDRA patters of 16S rDNA clones

be found in relatively wide sources, such as air from the dairy warehouse, milk, meat, silage, etc. It can inhibit the growth of pathogenic bacteria such as *E. coli* and *Listeria*. Tanaka *et al*. (2000) showed that after inoculation to alfalfa and Italian ryegrass silages at 4 °C and 10 °C respectively, *L. curvatus* can quickly produce lactic acid and improve the feed quality.

***L. sakei* CECT 5920**

LAC1-3 showed 99% homology with anaerobic *L. sakei* CECT 5920. Stéphane *et al*. (2005) found that *L. sakei* grows well in cold environment, even at 4 °C. It produces sakacin A or P, which can strongly inhibit the foodborne pathogen *Listeria monocytogenes*. *L. sakei* is often found in fresh fish and meat, and in the fermentation process of some pickled products. It can use a variety of sugars to produce lactic acid (Lauret *et al*., 1996). It has been reported that *L. sakei* can be applied to low-temperature fermentation of rice straw feed, which can produce lactic acid, lower the pH, and improve the quality of straw feed (Yang *et al*., 2008).

***L. kimchii* MT-1077**

The homology between LAC1-4 and *L. kimchii* MT-1077 was 96%. The latter was isolated from Kimchi by Yoon *et al*. (2000). *L. kimchii* is facultative anaerobic and homofermentative (occasionally heterofermentative). It can use sugars to produce 96% L-lactic acid and 4% D-lactic acid. Meanwhile, *L. kimchii* produces bacteriocin to inhibit the growth of certain pathogenic bacteria. It is also reportedly a component of the composite strains AL2 that can promote the fermentation of rice straw feed (Gao *et al*., 2007).

***L. delbrueckii* subsp. *Bulgaricus* MGB94-2**

LAC1-5 showed 99% homology with *L. delbrueckii subsp. Bulgaricus* MGB94-2, which is facultative anaerobic, with strong acid producing ability. It can use glucose, fructose, and lactose to produce lactic acid by homofermentation (Hao *et al*., 2011). This bacterium is widely used as feed-related microbial agents. Zhu (2007) reportedly obtained good results in fermentation of fresh corn stover silage by inoculation of isolated *L. delbrueckii*.

***L. letivazi* JCL3994**

LAC1-6 and *L. letivazi* JCL3994 shared 96% similarity; the latter was isolated from Thalia clover, with unreported specific characteristics.

***L. tucceti* CECT592**

LAC1-7 showed 97% homology with *L. tucceti* CECT592, which was initially isolated from the sausage fermentation. This strain is homofermentative and can use L-rhamnose, D-mannitol, and L-trehalose to produce D-/L-lactic acid (Felis and Dellaglio, 2007).

The strains of *L. kimchii* 0*L. curvatus* and *L. delbrueckii* were reported in miro storage under mesophilic temperatures. However, the strains of *L. nodensis*0*L. letivazi* and L. tucceti have not been reported in miro storage.

Analysis of the microbial diversity in the initial stage of fermentation

A total of 230 bacterial clones were randomly selected and 205 positive clones were analyzed by ARDRA. The Ck-Q1 to 19 stands for different types of microbial species, represented by the digestion patterns of 16S rDNA clone library of corn stover feed in the initial stage of fermentation. The Phylogenetic tree of 16S rDNA clones is shown in Fig. 3.

Enterobacteriaceae

Ck-Q1 to 5 and Ck-Q15 belong to the family of *Enterobacteriaceae*. Ck-Q1 showed 99% homology with *Enterobacter ludwigii* K9, which is an endophytic bacterial strain from the acidic soil for Paraguay tea, the rhizosphere of corn stalks, the clinical samples, and the nomadic goats in Northeast Argentina. Its optimum growth pH was 4.2-4.5 and also has an antibacterial effect. The Ck-Q2 had 99% similarity with *Escherichia hermannii* T91, which belongs to the genus of *Escherichia* and family of *Enterobacteriaceae.* It is facultative anaerobic and can use lactose producing gas. It is a conditional pathogen, causing primary and secondary infections, such as diarrhea (*Gupta* et al., 2012). Ck-Q3 and *Klebsiella* sp. IS-J3 shared 99% similarity; the latter was isolated from the head of noctuid, sewage, soil, plant surfaces, and mucosal surfaces of certain mammals, with strong pathogenicity. It is able to grow in 10 °C environment. Some *Klebsiella* strains can use D-melezitose and L-sorbose to produce acid. *Klebsiella* is also a conditional pathogen, causing septicemia, pneumonia, urinary tract infections, and cartilaginous injury (Podschun *et al*., 1998). Ck-Q15 showed 99% homology with *Pantoea* sp. R4M-D, which belongs to the facultative anaerobic, chemoheterotrophic bacteria that can both metabolize and ferment. It can also use sugar to produce acid but without producing gas. It is a conditional pathogen too. Ck-Q4 and *Pantoea agglomerans* shared 99% similarity; the latter was derived from phosphorus-rich soil (Yang, *et al*., 2012). This bacterium is facultative anaerobic and chemoheterotrophic. It can both metabolize and ferment, and can use sugar to produce acid but no

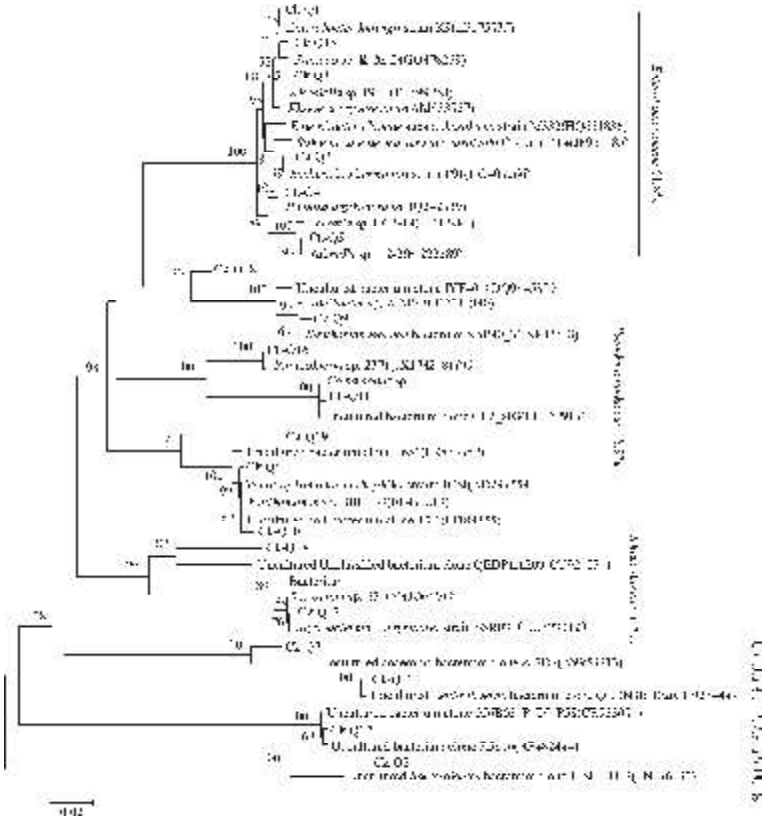

Fig. 3. Phylogenetic tree of 16S rDNA clones from the corn stover feed in the initial stage of fermentation and their most similar GenBank sequences

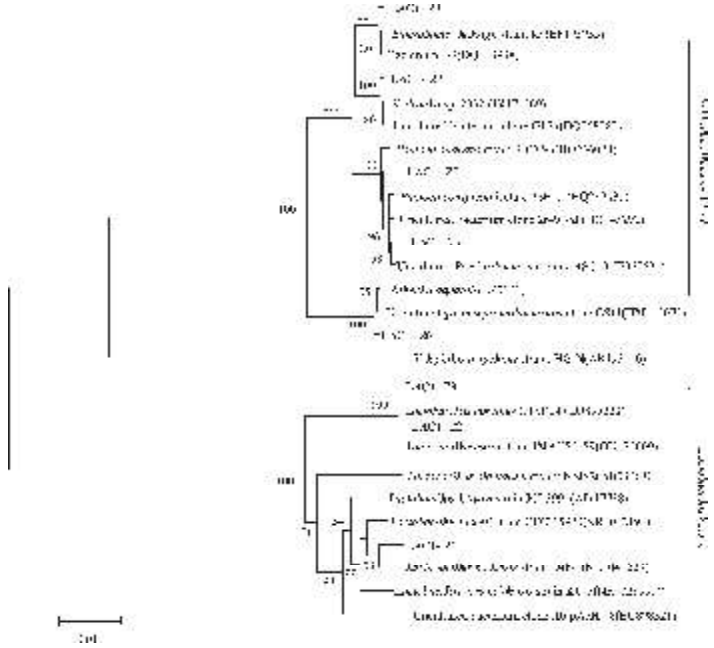

Fig. 4. Phylogenetic tree of 16S rDNA clones from the corn stover feed in the end stage of CK fermentation and their most similar GenBank sequences

gas. Ck-Q5 had 99% homology with *Rahnella* sp. N2-2. It is facultative anaerobic (Kaley, 2005), derived from wide sources such as blood, wound, freshwater fish, dairy products, fresh water, urine, feces, etc. It uses glucose to produce acid but without producing gas. It can grow at 4-10 °C, probably because that it has variants that can grow in the soil. It is also a pathogen and can use lipopolysaccharide to produce toxins and purulent matter.

Pseudomonadaceae

Stains Ck-Q6, 9-11, 16, 18, and 19 belong to the family of *Pseudomonadaceae*. Ck-Q6 had 99% homology with uncultured soil bacterium clone T7_1, which can degrade pyrene. Ck-Q9, 10, and 18 belonged to the genus of *Xanthomonas* and showed 99%, 99%, and 92% homologies with *Xanthomonadaceae* bacterium SAP40_3, *Xanthomonas* sp. BBCT38, and uncultured bacterium clone IYF40, respectively. They were found in the nectar of a plant from South Africa. These bacteria are chemoorganotrophic plant pathogens (Alvarez-Pérez *et al*., 2012). Ck-Q11 was 99% homologous with *Comamonas* sp. D1 that is identified from the rhizosphere of tobacco and is cold-resistant (Stéphane *et al*., 2003). *Comamonas* is widely distributed in the body of termite, soil, and compost samples (Nishino and Spain., 1995). Ck-Q19 showed 92% homology with *Pseudomonas* uncultured bacterium clone 1651 derived from the body of grass carp. In an anaerobic environment, it can ferment cellulose materials to produce short chain carbohydrates, such as glucose.

Rhizobiaceae

Ck-Q13 and 14 are bacteria of the family of *Rhizobiaceae.* Ck-Q13 showed 99% homology with *Agrobacterium larrymoorei* SSR03, which is aerobic and can use monosaccharides and

Table 1. The impact of LAC-1 inoculation on the composition of the fermentation system of corn stover (DM basis except pH and LAB)

Inspection items	Treatment	Culture time (d)				
		0	3	5	10	15
pH	Ck	6.1^{Ae}	5.9^{Ad}	5.7^{Ac}	5.3^{Ab}	4.9^{Aa}
	LAC-1	6.0^{Ab}	4.3^{Ba}	4.1^{Ba}	4.1^{Ba}	4.1^{Ba}
LAB (lg cfu/g FM)	Ck	6.1^{Aa}	6.8^{Bb}	6.8^{Bc}	7.5^{Bd}	8.1^{Be}
	LAC-1	6.2^{Aa}	8.9^{Ab}	9.0^{Aab}	9.0^{Aab}	9.2^{Ab}
Acetic acid (g/Kg)	Ck	5.9^{Aa}	6.3^{Bb}	9.0^{Bc}	10.8^{Bd}	12.0^{Be}
	LAC-1	6.2^{Aa}	8.3^{Ab}	11.2^{Ac}	13.9^{Ad}	16.4^{Ae}
Propionic acid (g/Kg)	Ck	3.0^{Aa}	3.7^{Ab}	4.6^{Ac}	6.0^{Ad}	7.1^{Ae}
	LAC-1	3.1^{Aa}	3.5^{Bb}	3.7^{Bbc}	4.3^{Bc}	5.0^{Bd}
Butyric acid (g/Kg)	Ck	1.5^{Aa}	2.1^{Ab}	3.5^{Ac}	4.3^{Ad}	6.2^{Ae}
	LAC-1	1.3^{Aa}	1.7^{Bb}	2.1^{Bc}	2.4^{Bcd}	3.2^{Bd}
Lactic acid (g/Kg)	Ck	8.3^{Aa}	8.4^{Ba}	8.9^{Bb}	10.0^{Bc}	12.5^{Bd}
	LAC-1	8.9^{Aa}	21.5^{Ab}	33.5^{Ac}	37.7^{Ad}	38.6^{Ae}
Glycerol (g/Kg)	Ck	1.1^{Aa}	1.8^{Bb}	1.9^{Bbc}	1.7^{Bb}	1.4^{Bab}
	LAC-1	1.4^{Aa}	3.7^{Ab}	5.6^{Ac}	8.4^{Ad}	8.9^{Ae}
CP(%)	Ck	7.5^{Aa}	7.5^{Ba}	8.2^{Bb}	8.7^{Bc}	9.7^{Bd}
	LAC-1	7.7^{Aa}	8.1^{Ab}	9.5^{Ac}	10.4^{Ad}	11.3^{Ae}
ADF(%)	Ck	33.7^{Ab}	33.4^{Ab}	33.3^{Ab}	31.9^{Aab}	31.0^{Aa}
	LAC-1	33.6^{Ad}	32.1^{Bc}	31.7^{Bb}	31.1^{Bab}	30.4^{Ba}
NDF(%)	Ck	61.9^{Ab}	61.6^{Ab}	61.4^{Ab}	59.5^{Aba}	58.8^{Aa}
	LAC-1	61.8^{Ad}	58.4^{Bbc}	57.5^{Bb}	57.2^{Bb}	55.1^{Ba}

Data are shown as mean(n=3). Different capital letters mean significant difference ($P<0.05$) among the treatments of the same inspection items, whereas different lower case mean significant difference ($P<0.05$) among the treatments of culture times.

disaccharides to produce acid but without producing gas. Ck-Q14 and uncultured unclassified bacterium clone QEDP1AE09 shared 93% similarity.

Uncultured bacteria

Ck-Q7, 8, 12, and 17 are uncultured bacteria. Ck-Q7 showed 99% homology with uncultured anaerobic bacterium clone A-2BS identified from anaerobic pig manure pool. Ck-Q8 shared 93% homology with uncultured *Bacteroidetes* bacterium clone LiM, which can break down glucose, lactose, and sucrose, to produce acetic acid, propionic acid, succinic acid, and isobutyric acid (Rotaru *et al*., 2012). This bacterium is pathogenic, mainly causing intracranial, abdominal, and pelvic infections. Ck-Q12 and Ck-Q17 shared 99% homology with uncultured *Lentisphaerae* bacterium clone QEDN3BH02 and uncultured bacterium clone R5p16 respectively; both were from the anaerobic digestion of sludge.

Analysis of the microbial diversity in the end stage of fermentation

A total of 260 and 240 bacterial clones were randomly selected and 225 and 198 positive clones were analyzed by ARDRA, respectively. The different types of microbial species represented by the digestion patterns of rDNA library derived from corn stover feed in the end stage of fermentation were designated as Ck-Z1 to 9 and LAC1-Z1 to 8, respectively. Their homology and phylogenetic tree were shown in Fig. 4 and 5.

Enterobacteriaceae

Ck-Z1, 2, and 7-9 from the late stage control fermentation system belonged to the family of *Enterobacteriaceae*. Among them, Ck-Z1 had 99% homology with *Pantoea* sp. R4M-E, which was found in a wide variety of sources, such as plants, soil, and pathogenic habitat in animals and humans containing these bacteria; it can grow at 4 °C (Liberto *et al*., 2009). Ck-Z2 shared 99% similarity with *Rahnella* sp. 0011 836, same as CK-Q5, while Ck-Z7 had 99% homology with *Enterobacter ludwigii* K9, like Ck-Q1. Ck-Z8 was 98% homologous with *Endophytic* bacterium GYPB16; it lives in plant tissues but nonpathogenic and can be found mainly in the flowers, fruit, leaves, roots, trunk, and seeds of various types of plants; it is resistant to plant pathogens and can fully utilize the carbon source and promote the growth of

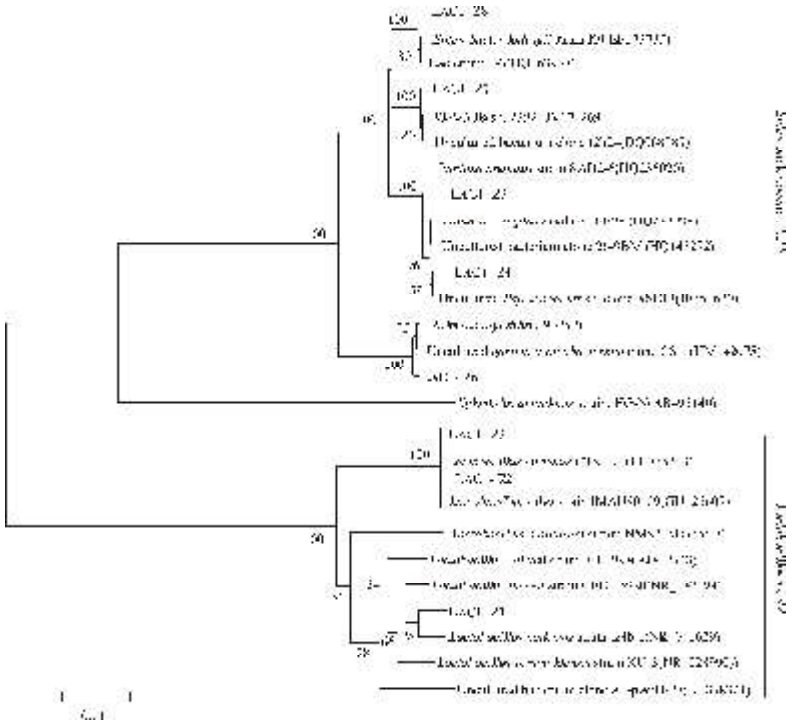

Fig. 5. Phylogenetic tree of 16S rDNA clones from the corn stover feed in the end stage of LAC-1-inoculated fermentation and their most similar GenBank sequences

plants and improve plant resistance to pathogens (Luo *et al*., 2011). Ck-Z9 showed 99% homology with bacterium TOPO-2YTclone3A.

In LAC-1-inoculated fermentation system, the LAC1-Z4 to 8 belonged to *Enterobacteriaceae*. LAC1-Z4 shared 99% homology with uncultured *Psychrobacter* sp. Clone ASC13, which belongs to uncultured psychrophilic bacteria that usually come from food, clinical samples, poultry, fish, Antarctic soil, water, glaciers, and krill. It can grow in a temperature range of 4-36 °C. Like Ck-Z4, it is anaerobic and can use soluble sugar to produce acid (Yoon *et al*., 2003). LAC1-Z5 had 99% homology with *Pantoea ananatis* SAD2-6, which was isolated from papaya, rice, tea, and other economic crops. It is a facultative anaerobic Gram-negative bacterium, which can use melibiose, sorbitol, glycerol, and inositol to produce acid. It was firstly isolated as apple pathogen in 1928 by Serrano. Similar with Ck-Q5, LAC1-Z6 showed 99% homology with *Rahnella aquatilis*. LAC1-Z7 was 99% homologous with *Klebsiella* sp. 2392, like Ck-Q3. LAC1-Z8 and *Enterobacter ludwigii* K9 shared 99% similarity, same as Ck-Q1. The reduction of *Enterobacter* numbers is a measure standard for the degree of acidification as well as a basis for assessing the quality of silage feed (Gordon, 1992).

Pseudomonadaceae

Ck-Z6 identified from the end-stage control fermentation system showed 99% homology with *Xanthomonas* sp. SPf, which is a Gram-negative onion pathogen and can utilize carbon and nitrogen sources such as glucose, sucrose, glycerol, and maltose (Roumagnac *et al*., 2000). This genus was not found in the end-stage composite LAC-1-inoculated fermentation system.

Lactobacillus

Ck-Z3 and 5 identified from the end-stage control fermentation system showed 97% homology with *L. versmoldensis* KU-3, which was initially identified from the raw fermented sausages in Germany. This strain is Gram-positive, rod-shaped, immobile, and facultative anaerobic, without the production of spores. It uses glucose, lactose, and maltose to produce lactic acid through homofermantation. It grows in the temperature range of 8-37 °C (Kröckel *et al*., 2003). Ck-Z5 shared 99% homology with *Leuconostoc mesenteroides* Ni109, which can be found in blood, urine tubes, vegetables and food, reportedly, *Lactobacillus*-fermented straw feed. It is facultative anaerobic and can utilize glucose to produce D-lactic acid and gas (Dimi, 2006).

LAC1-Z1 to 3 identified in the composite LAC-1-inoculated fermentation system were *Lactobacillus* bacteria. LAC1-Z1 shared 98% homology with *L. nodensis* iz4b-1, while LAC1-Z2 and 3 showed 99% homology with *L. sakei* IMAU80189 and *L. curvatus* CTSPL4, respectively.

Uncultured bacteria

Ck-Z4 from the control fermentation was uncultured bacterium, sharing 99% homology with uncultured *gamma proteobacterium* clone, which was not observed in LAC-1 fermentation system.

The impact of LAC-1 inoculation on the bacterial flora in corn stover fermentation system

As shown in Figure 6, in the initial stage of fermentation, the dominant strains were *Enterobacteriaceae,* accounting for 61.8% of the total clones in the library, followed by *Pseudomonas*, *Rhizobium*, *uncultured*

Fig. 6. Distribution of bacterial composition in corn stover fermentation system. a. the initial stage of fermentation. b. the end stage of CK fermentation. c. the end stage of LAC-1-inoculated fermentation.

Bacteroides, etc. In the fermentation system, *Pseudomonas, Bacteroides, Clostridium, Leptospirosis,* etc. can convert the wood fiber materials into sugars, and subsequently produce lactic acid and volatile fatty acids. But meanwhile they are also pathogenic, producing a large amount of toxins, causing intracranial, abdominal, and pelvic infections among other diseases.

In the end stage of fermentation, *Enterobacteriaceae* remained the dominant flora in the CK system, accounting for 65.5% of the total clones in the library (even higher than their initial percentage), followed by *Lactobacillus, Xanthomonas,* and *Leuconostoc mesenteroides*. In the composite LAC-1-inoculated stover fermentation system, *Lactobacillus* became the dominant flora in the end stage of fermentation, accounting for 82.5% of the total clones in the library, followed by *Enterobacter* (17.5%). The percentage of *Enterobacter* bacteria was significantly lower than that in the initial stage of fermentation. From a functional point of view, *Lactobacilli* is homofermentative; it utilizes sugars to mainly produce lactic acid. Therefore, it is evident that inoculated low-temperature composite LAB LAC-1 can well colonize in the corn stover fermentation system and inhibit the proliferation of harmful bacteria such as *Enterobacteriaceae, Pseudomonas,* uncultured bacteria, etc.

In the inoculated fermented corn straws, *L. nodensis, L. curvatus* and *L. sakei,* which were the favored species in LAC-1, dominated the microbial community. This suggests that when the LAC-1 is inoculated into air-dried corn straws, its main bacteria could rapidly dominate the fermentation system. However the dominant microorganisms in the control were not detected in the inoculated straws. This was most probably due to the inhibitive effect of the LAC-1 on other microorganisms (Danner *et al*., 2003; Yang *et al*., 2006).

The impact of LAC-1 inoculation on the composition of the fermentation system of corn stover

As shown in Table 1, with the progress of fermentation, the pH and the contents of ADF and NDF in different treatment groups showed a trend of decline. While the pH decreased to 4.1 in the inoculation group in the 5th day of fermentation, the pH in CK group did not reach 4.1 (indicator of the good quality of stalk feed) even at the end of fermentation. ADF and NDF in the inoculation group had significantly reduced since the 3rd day of fermentation, while the reductions of ADF and NDF in the CK group became significant from the 15th and 10th days respectively. The number of LAB and the contents of acetic acid, propionic acid, butyric acid, lactic acid, glycerol, and CP showed an increasing trend. The number of LAB and the contents of acetic acid, propionic acid, butyric acid, and glycerol were significantly higher than those in the initial stage at the 3rd day of fermentation in both CK and LAC-1-treated groups. The increase in lactic acid and CP contents in CK group became significant at the 5th day of fermentation, while those in LAC-1-treated group were significantly elevated from the 3th day of fermentation.

Compared with CK, the pH and contents of ADF, NDF, propionic acid, and butyric acid in the LAC-1 inoculation group significantly declined, while the number of LAB and contents of acetic acid, lactic acid, glycerol, and CP significantly increased, after 3 days of fermentation. The pH is one of main factors that influence the progress of silage fermentation and silage quality, as a low pH ensures that the forage is retained in a stable form. The pH of the inoculation LAC-1 group decreased to 4.1 in the 5th day of fermentation, more rapidly than the CK. The spoilage microorganisms are inhibited more effectively at lower pH than at higher pH, which is closer to the optimum pH range for spoilage organisms(Danner *et al*., 2003). The lactic acid and acetic acid are beneficial to the pH reduction in microbial treatment, inhibiting the growth of contaminants such as yeast and mold, and improving the quality of stover feed. Glycerol can increase the flavor of fermented feed, thereby enhancing its palatability (Gao *et al*., 2007). Inoculating with LAC-1 increases the content of lactic acid and acetic. Furthermore, the content of butyric acid in inoculated treatments , which is usually not desired in silages (Danner *et al*., 2003), was lower than that in the control. The digestibility of feed is negatively correlated with ADF and NDF, but positively correlated with CP content. Therefore, the above results indicated that LAC-1 inoculation promoted the process of microbial treatment and improved the quality of stover feed.

CONCLUSIONS

A bacterial consortium LAC-1 was screened at 10 °C, and it consisted of seven *Lactobacillus* strains based on the analysis of 16s rDNA clone library. Inoculation of corn straw with LAC-1 resulted in a rapid decreasing of pH in fermentation system, inhibition on the growth of harmful microorganisms and improvement of feed quality. To overcome the adverse effects of low temperature after harvest in cold regions and increase corn straw fermentation efficiency, further researches will be focused on large-scale fermentation process, related equipments research and screening, and livestock feeding experiments.

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REFERENCES

1. Alvarez-Pérez, S., Herrera, C.M., de Vega, C. Zooming-in on floral nectar: a first exploration of nectar-associated bacteria in wild plant communities. *FEMS Microbiol. Ecol.*, 2012; **80**(3): 591-602.
2. Danner, H., Holzer, M., Mayrhuber, E., Braun, R. Acetic acid increases stability of silage under aerobic conditions. *Appl. Environ. Microbiol*., 2003; **69**: 562–567.
3. Dimi, G.R., Characteristics of the *Leuconostoc mesenteroides* subsp. *mesenteroides* strains from fresh vegetables. *BIBLID*, 2006; **37**: 3-11.
4. Felis, G.E, Dellaglio, F., Taxonomy of Lactobacilli and Bifidobacteria. *Curr. Issues Intest. Microbiol.*, 2007; **8**(2): 44-61.
5. Filya, I. The effect of Lactobacillus buchneri and Lactobacillus plantarum on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. *Journal of Dairy Science*. 2003; **86**(11): 3575-3581.
6. Gao, L.J., Wang, X.F., Yang, H.Y., Gao, X.Z., Lü, Y.C., Cui, Z.J. Effects of a lactic acid bacteria community SFC-2 treated on rice straw. *Environmental Sciences*, 2007; **28**(6): 1392-1396.
7. Gao, L.J., Yang, H.Y., Wang, X.F., Huang, Z.Y., Ishii, M., Igarashi, Y., Cui, Z.J. Rice straw fermentation using lactic acid bacteria. *Bioresour. Technol.*, 2008; **99**: 2742-2748.
8. Gordon, F.J. Improving the feeding value of silage through biological control. Birmingham: Proceedings of the All-tech European Lecture Tour, 1992; pp 2-17.
9. Guo, P., Wang, X.F., Zhu, W.B., Yang, H.Y., Cheng, X., Cui, Z.J. Degradation of corn stalk by the composite microbial system of MC1. *J. Environ. Sci.,* 2008; **20**: 109-114.
10. Gupta, A.K., Nayduch, D., Verma, P., Shah, B., Ghate, H.V., Patole, M.S., Shouche, Y.S., Phylogenetic characterization of bacteria in the gut of house flies (Musca domestica L.). *FEMS Microbiol. Ecol.*, 2012; **79**(3):581-593.
11. Hao, P., Zheng, H.J., Yu, Y., Ding, G.H., Gu, W.Y., Chen, S.T., Yu, Z.H., Ren, S.X., Oda, M., Konno, T., Wang, S.Y., Li, X., Ji, Z.S., Zhao, G.P. Complete sequencing and pan-genomic analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* reveal its genetic basis for industrial yogurt production. 2011; *PLoS One*, **6**(1): e15964.
12. Haruta, S., Cui, Z., Huang, Z., Li, M., Ishii, M., Igarashi, Y., Construction of a stable microbial community with high cellulosedegradation ability. *Appl. Microbiol. Biotechnol*. 2002; **59**: 529-534.
13. Hiroyuki, S., Masataka, W., Tadaatsu, N., Baohua, X., Hiroo, U., Succession of bacterial community structure along the Changjiang River determined by denaturing gradient gel electrophoresis and clone library analysis. *Appl. Environ. Microbiol*., 2002; **68**(10): 5142-5150.
14. Kashiwagi, T., Suzuki, T., Kamakura, T. Lactobacillus nodensis sp. nov., isolated from rice bran. *Int. J. Syst. Bacteriol*., 2009; **59**(1):83-86.
15. Kaley, T. *Rahnella aquatilis* bacteremia from a suspected urinary source. *J. Clin. Microbiol.*, 2005; **43**(5):2526-2528.
16. Kröckel, L., Schillinger, U., Franz, C.M.A.P, Bantleon, A., Ludwig, W. *Lactobacillus versmoldensis* sp. nov., isolated from raw fermented sausage. *Int. J. Syst. Evol. Microbiol.*, 2003; **53**(2):513-517.
17. Lauret, R., Morel-Deville, F., Berthier, F., Champomier-Verges, M., Postma, P., Ehrlich,

S. D., Zagorec, M., Carbohydrate utilization in lactobacillus sake. *Appl. Environ. Microbiol.*, 1996; 62(6):1922-1927.

18. Li, J.F., Hu, R.Q., Song, Y.Q., Shi, J.L., Bhattaeharya, S.C., Abdul Salam, P. Assessment of sustainable energy potential of non-plantation biomass resources in ChinaÿBiomass Bioenerg., 2005; **29**(3):167-177.
19. Liberto, M.C., Matera, G., Puccio, R., Lo Russo T., Colosimo, E., Focà, E. Six cases of sepsis caused by Pantoea agglomerans in a teaching hospital. *New Microbiol.*, 2009; **32**(1):119-123.
20. Luo, S.L., Wan, Y., Xiao, X., Guo, H.J., Chen, L., Xi, Q., Zeng, G.M., Liu, C.B., Chen, J.L. Isolation and characterization of endophytic bacterium LRE07 from cadmium hyperaccumulator *Solanum nigrum* L.and its potential for remediation. *Appl. Microbiol. Biotechnol.*, 2011; **89**:1637-1644.
21. Nishino, S.F., Spain, J.C. Oxidative pathway for the biodegradation of nitrobenzene by comamonas sp. Strain JS765. *Appl. Environ. Microbiol*., 1995; **61**(6): 2308-2313.
22. Podschun, R., Ullmann, U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin. Microbiol. Rev*., 1998; **11**(4):589-603.
23. Rotaru, A.E., Schauer, R., Probian, C., Mussmann, M., Harder, J. Visualization of candidate division OP3 cocci in limonene-degrading methanogenic cultures. *Journal of Microbiology and Biotechnology*. 2012; **2**2(4): 457-461.
24. Roumagnac, P., Gagnevin, L., Pruvost, O. Detection of *Xanthomonas* sp., the causal agent of onion bacterial blight, in onion seeds using a newly developed semi-selective isolation medium. *European Journal of Plant Pathology*, 2000 ; **106**(9) :867-877.
25. Stéphane, C., Marie-Christine, C.V., Monique, C., Anne-Marie, C.L.C, Anne-Marie, D., Véronique, M., Sophie, B., Emmanuelle, D.R., Robert, B., Valentin, L., Monique, Z. The complete genome sequence of the meat-borne lactic bacterium *Lactobacillus sakei* 23K. *Nat. Biotechnol*., 2005; **23**: 1527-1533.
26. Stéphane, U., Cathy, D., Aurélien, C., Miena, E., Carine, S., Annik, P., Phil, O., Denis, F., Yves, D. Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. *Microbiology*, 2003; **149**: 1981-1989.
27. Tanaka, O., Mori, K., Omomo, S. Effect of inoculation with *Lactobacillus curvatus* on ensiling. *Grassland Science*, 2000; **46**(2):148-152.
28. Tian, Y.S., Zhao, L.X., Sun, L.Y., Meng, H.B. Analysis and evaluation on agricultural biomass resources. *Engineering Sciences*, 2011; **13**(2): 24-28.
29. Wang, C., Hong, X.J., Li, Z.G., Chang, Y.Y., Zhang, H.Y., Liu, Q., Yan, L., Wang, Y.J., Wang, W.D. Construction and optimization the culture conditions for the micro storage of corn straw by lactic acid bacteria community at low temperature. *Microbiol.China*, 2013, **40**(7): 1202-1213.
30. Yang, H.Y., Wang, X.F., Gao, L.J., Haruta, S., Ishii, M., Igarashi, Y., Cui, Z.J. Development of an enrichment culture growing at low temperature used for ensiling rice straw. *J. Microbiol. Biotechn.*, 2008; **18**(4):711-717.
31. Yang, H.Y., Wang, X.F., Liu, J.B., Gao, L.J., Ishii, M., Igarashi, Y., Cui,Z.J., Effects of water-soluble carbohydrates content on silage fermentation of the wheat straw. J. Biosci. Bioeng. 2006; **101**:232–237.
32. Yang, H.Y., Gao, L.J., Wang, X.F., Wang, W.D., Cui, Z.J. Effects of cultivation conditions on the diversity of microbes involved in the conversion of rice straw to fodder. *J. Environ. Sci.*, 2007; **19**(1) :67-73.
33. Yang, P.X., Ma, L., Chen, M.H., Xi, J.Q., He, F., Duan, C.Q., Mo, M.H., Fang, D.H., Duan, Y.Q., Yang, F.X. Phosphate solubilizing ability and phylogenetic diversity of bacteria from p-rich soils around dianchi lake drainage area of China. *Pedosphere*, 2012 ; **22**(5) :707-716.
34. Yoon, J.H., Kang, K.H., Park, Y.H. Psychrobacter jeotgali sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int. J. Syst. Evol. Microbiol.* , 2003; **53**(2) : 449-454.
35. Yoon, J.H., Kang, S.S., Mheen, T.I., Ahn, J.S., Lee, H.J., Kim, T.K., Park, C.S., Kho, Y.H., Kang, K.H., Park, Y.H. *Lactobacillus kimchii* sp. nov., a new species from kimchi. *Int. J. Syst. Evol. Microbiol.*, 2000; **50**:1789-1795.
36. Zhu, H., Qu, F., Zhu, L.H. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Res*., 1993; **21**(22): 5278-5280.
37. Zhu, D.W. Analysis of the feasibility and limiting factors of using stalks as animal feed. *Cereal and Feed Industry.* 2003; **1**: 26-27.
38. Zhu, W.. Study of silage by adding lactic acid bacteria. Hefei:Anhui Agricultural University, 2007; pp: 29-39.