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ORIGINAL ARTICLE

Identification and phylogenetic characterization of novel *Lactobacillus plantarum* species and their metabolite profiles in grass silage

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Abstract Italian ryegrass (IRG), barley, and rye are types of forage that are difficult to ensile with an assurance of good quality. Therefore, the addition of lactic acid bacteria (LAB) is the best way to enhance the preservation of this silage. However, applications of LAB have been impeded due to its poor growth characteristics, the sudden decline in pH, and other non-beneficial microbial growth associated with its presence. To overcome these limitations, a new *Lactobacillus* sp. KCC-10 and KCC-19 strain was isolated from well-fermented IRG silage samples. Biochemical and physiological studies revealed that the strains were Gram-positive, catalase-negative, and produced gas from glucose, and produced more lactic acid in fermentation. The 16S rRNA gene-based phylogenetic affiliation was determined by using bioinformatic tools that identified *Lactobacillus* sp. KCC-10 and KCC-19 with 100 % sequence similarity to *Lactobacillus plantarum*. Novel *L. plantarum* strains were deposited in the Korean Collection for Type Cultures under the accession numbers KACC 91785P and KACC 91758P, respectively. The shake-flask cultivation of these new strains under aerobic, microaerobic, and anaerobic conditions showed a higher specific growth rate than that achieved using the well-studied *L. plantarum* KACC 91016 and KACC 91096 on MRS broth and grass juice. Lactic acid was detected as the dominant organic acid in IRG (78.45 mM), barley (51.28 mM), corn (16.28 mM), and rice paddy (11.05 mM), followed by acetic acid and succinic acid. The KCC-10 in the silage was observed to increase from 2.4×10^5 CFU/g per sample at day 0 to 0.58, 0.60, and 0.59×10^9 CFU/g at day 5 for IRG, barley, and rye, respectively. The growth of KCC-10 and KCC-19 in all the silages decreased, as the storage period increased from 5 to 50 days. Whereas, KCC-19 was noted to increase from 2.7×10^5 CFU/g per sample at day 0 to 0.71, 0.72, and 0.711×10^9 CFU/g at day 5 for IRG, barley, and rye. Among the total organic acids, lactic acid was detected as the dominant acid present in IRG, barley, and rye silages. From these results, we concluded that strains KCC-10 and KCC-19 can be used as appropriate inoculants to prolong the stability of silage and fermentation quality.

Keywords *L. plantarum* · 16S rRNA gene · Shake-flask cultivation · Lactic acid · Silage fermentation

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Introduction

Silages are now the most commonly preserved forage crops and grasses in many countries, including Korea, Japan, and China. Ensiling is a preservation method used for forage crops, as silage depends on the production of sufficient organic acid to inhibit the activity of undesirable microbial pathogens under anaerobic conditions. In this process, homo- and heterofermentative lactic acid bacteria (LAB)

convert water soluble carbohydrates into organic acids, mainly lactic acid, which is the primary acid responsible for decreasing the pH and inhibits the activities of plant enzymes and pathogenic or spoilage bacteria that could decrease the nutritive values of the silage (McDonald et al. 1991; Cai et al. 1999). Homolactic fermentation by *Lactobacillus* is more desirable than other types of fermentation because the theoretical recoveries of dry matter and energy are higher. The genus *Lactobacillus* is Gram-positive and mainly involved in the fermentation of dairy food products, food fermentation, and food preservation (Giraffa et al. 2010). *Lactobacillus* strains have been used for decades in food preservation as starters for dairy products, fermented vegetables, and fish. Natural populations of *Lactobacillus* strains on plant material are often low in number. Thus, the concept of using a microbial inoculant for silage involves adding fast-growing homofermentative *Lactobacillus* in order to dominate the fermentation, thereby producing higher-quality silage. Some of the commonly used homofermentative *Lactobacillus* in silage inoculants include *Lactobacillus plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. brevis*, *L. fermentum*, *L. reuteri*, *Pediococcus acidilactici*, and *Enterococcus faecium* (Avila et al. 2010). Commercially available microbial inoculants contain one or more of these bacteria that have been selected for their ability to dominate the fermentation process (Eitan et al. 2006).

Silage is an increasingly important source of animal feed in Korea, where Italian ryegrass (IRG) (*Lolium multiflorum* L.), corn (*Zea mays* L.), barley (*Hordeum vulgare* L.), paddy rice (*Oryza sativa* L.), and alfalfa (*Medicago sativa* L.) are popular forage crops used widely for ruminant feed production. IRG and alfalfa are forage crops rich in protein. However, they are difficult to ensile in a manner that ensures good quality due to their low fermentable carbohydrate content, high buffering capacity, and a tubular hollow stem that inhibits the complete removal of air during ensiling (McAllister et al. 1998). It is possible to use chemicals, enzymes, and inoculants containing *Lactobacillus* strains to overcome these problems in order to enhance the preservation of IRG, corn, barley, and rice silage (Cai et al. 1999; Winters et al. 2001; Rodrigues et al. 2001; Weinberg et al. 2003). Of these techniques, *Lactobacillus* strains are advantageous in that they are safe, noncorrosive to farm machinery, do not pollute the environment, and are less costly than enzyme preparations (Weinberg and Muck 1996). *L. plantarum* is the predominant species isolated from silage (Rodrigues et al. 2001). Many studies reported that the impacts of a pure culture of *L. plantarum* as a silage inoculant were not good because of its poor cell growth and poor fermentation metabolite production, and that *L. plantarum* used along with other species showed better performance (McAllister et al. 1998). However, *L. plantarum* is commonly found as part of the natural microflora of fermented foods. This LAB may also be added as a starter or adjunct culture, in both cases improving the organoleptic characteristics of the final products (De Angelis et al. 2008).

The objective of this study was to screen, isolate, and identify *L. plantarum* strains from silages prepared on dairy farms. *L. plantarum* KCC-10 and KCC-19 were selected based on morphological and biochemical properties, and identified by 16S rRNA gene sequences. The growth and fermentation metabolite profiles of *L. plantarum* KCC-10 and *L. plantarum* KCC-19 on forage crop juice and silage were also studied.

Materials and methods

Isolation of *Lactobacillus* strains

The strains KCC-10 and KCC-19 were isolated from Italian ryegrass forage silage. Briefly, the samples (10 g, wet weight) were mixed with 50 mL sterile water, homogenized by vortexing for 30 min, and centrifuged at 10,000 rpm for 20 min to remove the heavy particulate matter. The dilution was carried out for up to 10^{-5} dilutions. Aliquots (0.1 mL) of 10^{-3}, 10^{-3}, 10^{-4} , and 10^{-5} were spread on the isolation plates containing Man Rogosa and Sharpe agar medium (MRSA). The plates were incubated at 30 °C for 72 h, observed for bacterial growth, and then purified on MRSA. The pure cultures were grown on MRS agar at 30 °C for 24 h, and maintained at 4 °C for routine use. Alternatively, strains were re-suspended in 20 % glycerol and stored at −80 °C until further examination.

Characterization of *Lactobacillus* strains

Gram staining, morphological, and physiological characteristics were examined after 24 h incubation on MRS agar. Growth at different temperatures was observed in MRS broth after incubation at 20, 25, 30, 35, 40, 45, and 50 °C for 3 days. Growth of KCC-10 and KCC-19 at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0 was determined in MRS broth after incubation at 30 °C for 3 days. API 50CHB test kits were used to characterize phenotypes. The API test strips were prepared according to the manufacturer's instructions and scored after 48 h incubation at 30 °C.

Scanning electron microscopy (SEM) analysis of *Lactobacillus* strains

The strains KCC-10 and KCC-19 were grown exponentially in MRS medium under aerobic conditions, centrifuged at 8,000 rpm for 10 min, and the pellet was washed twice with 0.1 M phosphate buffer saline (PBS), pH 7.2. Then, the cells were re-suspended in a primary fixative solution containing glutaraldehyde (2.5 %) and paraformaldehyde (2.0 %)

buffered in 0.1 M sodium cacodylate (pH 7.2). Subsequently, the primary fixed cells were immobilized on a glass slide coated with poly-L-lysine. Secondary fixation was performed using potassium ferricyanide (1.5 %) and osmium tetroxide (1.0 %) in the sodium cacodylate buffer. Both primary and secondary fixation procedures were carried out for 3 h at 4 °C. After dehydration through a graded ethanol series (50, 60, 70, 80, 90, and 95 %), the fixed cells were dried twice with hexamethyldisilizane. Finally, the dehydrated cells were sputter coated with gold at 25 mA for 250 s and observed by SEM (JSM-6460 LV; JEOL, Tokyo, Japan) at 12 kV.

PCR amplification of 16S rRNA gene and sequencing

The DNA of the strains KCC-10 and KCC-19 were extracted using the kit method. The DNA isolation kit was purchased from Promega (Madison, WI, USA). Briefly, 24-h-old culture was used for the isolation of genomic DNA. The supernatant containing the DNA was used to amplify 16S ribosomal DNA fragments by polymerase chain reaction assay (PCR; Bio-Rad I cycler) using 27F (5′ AGA GTT TGA TCG TGG CTC AG 3′) and 1492R (3′ GGT TAC CTT GTT ACG ACT T 5′) primers (Arasu et al. 2013). The conditions for thermal cycling were as follows: initial denaturation of the target DNA at 95 °C for 10 min followed by 30 cycles of amplification, denaturation at 95 °C for 2 min, primer annealing at 58 °C for 1 min, and primer extension at 72 °C for 2 min. At the end of the cycle, the reaction mixture was held at 72 °C for 10 min and cooled at 4 °C. Amplified DNA was visualized at 100 V and 400 mA for 25 min on agarose gel (1 % (w/v) in TAE buffer 1×, 0.1 μL Ethidium Bromide solution). The concentration of DNA was determined by using a double-beam spectrophotometer (Bio-Rad). The amplified PCR products were purified by a QIAquick® PCR purification Kit (Qiagen Ltd., Crawley, UK). Then, 1,500 base pairs were sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystem, USA) in Solgent Co. Ltd. (16S rRNA, Seoul, Korea). The obtained sequences were subjected to a BLAST search at http://www.ncbinlm-nih.gov/ in the NCBI database. The evolutionary history was inferred using the neighbor-joining method. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

Nucleotide sequence and culture accession numbers

The 16S rRNA sequences of KCC-10 and KCC-19 were deposited in the NCBI nucleotide sequence database under accession number KC422325 and KC571201, respectively. The pure culture was deposited in the Korean Collection for Type Cultures, Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, Republic of South Korea, and given the accession number KACC 91785P and KACC 91758P, respectively.

Microorganism and culture conditions

The strains *L. plantarum* KACC 91016 and KACC 91067 were purchased from the microbial culture collection at the Rural Development Administration (RDA), Suwon, South Korea. In Korea, *L. plantarum* KACC 91016 and KACC 91067 were commercialized for the preparation of silages by Chung-Mi Bio Company (Korea). The strains were cultivated in MRS culture medium. In order to study the effects of water soluble components (WSC) from the grass powder, the water soluble extracts were collected from grass by soaking it in sterile filtered water at 50 % (w/v) with constant stirring at 30 °C overnight and filtered using 0.2-μm filters (Pall Corporation). The strains were cultured in 250 mL Erlenmeyer flasks containing 50 mL MRS broth or 50 mL of water extracts (only) individually at 30 °C on an orbital incubator shaker. The culture flasks were inoculated to 0.2 OD_{600} with freshly prepared cells grown in MRS medium under the same culture conditions. The flasks were plugged with oxygen-permeable cotton plugs and incubated at 200 rpm for aerobic conditions, whereas the flasks were plugged with oxygen-permeable cotton plugs, and incubated at 50 rpm for microaerobic conditions. The saline bottles were flushed with nitrogen gas (99.99 %) for 20 min to ensure that the bottles were completely deprived of oxygen. The autoclaved saline bottles were plugged with oxygen impermeable rubber stops and sealed with aluminum caps, and then incubated at 200 rpm for anaerobic conditions. After a 30-h incubation, the samples were withdrawn intermittently and analyzed for cell density (OD_{600}) and metabolites (acetic acid, lactic acid, and succinic acid).

Preparation and analysis of silages

One hundred grams of fresh IRG, barley, and rye were harvested at the flowering stage, chopped into 1.0–1.5-cm pieces, packed in an air-diffusible bag, and sterilized using ethylene oxide for 16 h (Person Medical, E.O Gas sterilizer, Person-E050). The samples were carefully transferred into the sterile, air-tight polypropylene bag in the laminar airflow. The lyophilized cells of *L. plantarum* KCC-10 and *L. plantarum* KCC-19 were individually dissolved in sterile water in separate sterile bottles and bacterial cells sprayed at the rate of 2.5 % of fresh grass, respectively, and then sealed to prevent air flow. The number of added strains of KCC-10 and KCC-19 were 2.4×10^5 and $2.7\ 10^5$ [colony forming units (CFU)/g sample], respectively. Each control (without addition of strains) and each of the samples with strains were prepared in triplicate. The samples were stored at 20±5 °C and opened at 5, 10, 20, 30, 40, and 50 days post-ensiling for microbial counting and metabolite analysis. For microbial counts, the silage samples (10 g wet weight) were transferred to 250-mL sterile flasks containing 90 mL sterile water. The suspension was kept in an

orbital incubator shaker at 150 rpm for 1 h. After incubation, ten-fold dilutions were prepared in sterile water and samples (0.1 mL) were placed on selective media. LAB was enumerated on MRS (Difco) and Bromocresol purple blue agar medium, and incubated under microaerobic conditions at 30 ±1 °C for 3 days. Yeasts and molds were enumerated on 3 M Petrifilm Yeast and Mould Count Plate (3 M Microbiology Products, St. Paul, USA), followed byaerobic incubation at 30 ±1 °C for 3 days. Coliforms (*Enterobacteriaceae*) were enumerated on McConkey agar (Merck) after aerobic incubation at 30±1 °C for 1 day. Fungi were enumerated on Potato Dextrose agar (PDA) [4 g/L of potato starch (Difco), 20 g/L of starch (Difco), and 20 g/L of agar (Difco)] following aerobic incubation at 30±1 °C for 4 days. Colonies were counted as viable numbers of microorganisms in CFU/g of fresh matter (FM). The pH of the supernatant was measured using a combination electrode. The fermentation byproduct content of lactic acid, acetic acid, and succinic acid was analysed by high-performance liquid chromatography (HPLC; HP1100 Agilent Co., USA).

Analytical methods

Cell concentrations were measured using a 96-well plate reader (Biorad; USA) at 600 nm. Concentrations of fermentation metabolites were determined by HPLC (HP1100 Agilent Co. USA). The supernatants, obtained by centrifugation of the culture samples at 10,000 rpm for 10 min, were filtered through an Acrodisc-syringe filter (PALL Life Sciences; USA) and eluted through a 300×7.8-mm Aminex HPX-87H (Bio-Rad; Hercules, CA, USA) column at 60 °C using 5.0 mM H_2SO_4. The HPLC analysis was carried out with a flow rate of 0.5 mL/min at a column wavelength of 220 nm. The injection sling was 10 μL. Quantification of the different organic acids was based on peak areas and calculated as equivalents of standard compounds.

Results

In the present study, two *Lactobacillus* strains were selected from the silage of Italian ryegrass. Gram staining confirmed that the strains were Gram-positive and rod shaped bacteria. The colonies of LAB strains were tiny, leathery, and viscous in nature. They are facultative anaerobes with a light cream color while growing on the MRS agar medium and a yellow color on the BCP agar medium. Cell shape and size were observed using a research microscope at 1,000X magnification and appeared to be short rod and motile in nature. The scanning electron microscope (SEM) photograph is shown in Fig. 1. These micromorphological properties strongly suggested that KCC-10 and KCC-19 belonged to the genus *Lactobacillus*. Biochemical characteristics of strains KCC-10 and KCC-19 were carried out using an API micro test kit (Table S1). Biochemical and physiological characterization revealed that the strains showed negative results for a catalase enzyme production test. Both strains showed similar characteristics, including their ability to utilize a wide range of carbon substrates (Table S2). The strains gave positive results for adonitol, methyl-D-xylopyranoside, D-galactose, D-glucose, D-fructose, dulcitol, D-mannitol, D-sorbitol, salicin, D-cellobiose, D-maltose, D-saccharose, D-trehalose, inulin, gentiobiose, D-turanose, and D-tagatose, but were negative for glycerol, erythritol, D-mannose, L-arabinose, D-ribose, L-xylose, D-mannose, L-sorbose, inositol, and amygdalin. The strains could grow at pH ranges from 2.0 to 8.0, at temperatures between 20 °C and 40 °C, and in 5.0 % (w/v) NaCl. Both strains were able to produce hydrogen sulphide. The molecular classification was determined by 16S rRNA sequence analysis and the NCBI BLAST program showed that the sequence data of KCC-10 and KCC-19 had high similarity (> 99 %) to those of *Lactobacillus plantarum*. The phylogenetic trees of KCC-10 and KCC-19 were constructed from evolutionary distances by the neighbor-joining method (Fig. 2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) (Tamura et al. 2011). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Growth characteristics and metabolite production profile

Aerobic conditions

The growth characteristics of the KCC-10, KCC-19, KACC 91016, and KACC 91096 strains were compared by growing them in Erlenmeyer flasks containing MRS medium, IRG, barley, corn, and rice juice under aerobic conditions. Results indicated that, compared to the standard *Lactobacillus* strains, KCC-10 and KCC-19 showed comparatively better growth (Fig. 3). The OD_{600} values recorded for the KCC-10, KCC-19, KACC 91016, and KACC 91096 strains after 24 h of cultivation in MRS medium were 2.8, 3.31, 2.59, and 3.0, respectively. All the strains grew well in the IRG juice compared to other juices. The maximum growth observed in the IRG juice was 2.1, 1.98, 1.25, and 1.65 respectively for KCC-10, KCC-19, KACC 91016, and KACC 91096 after 24 h of cultivation (Table 1). In barley, corn, and rice juice, the higher growth was observed at 12 and 24 h of cultivation. According to Fig. 3, KCC-10 and KCC-19 were able to grow well in the juice from the absorbance range of 0.2–2.3. The results revealed that after a 24-h cultivation, the pH of the fermented broth declined to 3.8–4.0. This clearly indicated that the strains were able to secrete organic acids. KCC-10 and KCC-19 produced the highest amount of lactic acid after 30 h of incubation under aerobic conditions (Fig. 4). They

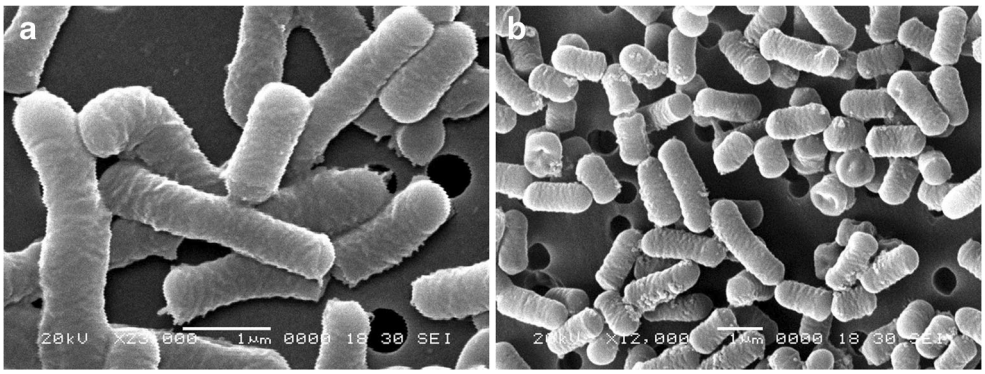


Fig. 1 Scanning electron microscopic image of strains. **a** *Lactobacillus plantarum* KCC-10, **b** *Lactobacillus plantarum* KCC-19

produced significant quantities of lactic acid and acetic acid in MRS medium and produced comparable amounts in the juices. These results indicated that the strains KCC-10 and KCC-19 can utilize the water extract of the grass and produce lactic acid and acetic acid.

Microaerobic conditions

Microaerobic cultivation was also carried out using MRS medium and juice individually. The maximum OD_{600} and μ_{max} values achieved by the strains were comparable in MRS medium, but the growth was poor in the juices. Shake-flask cultivation showed that the lactic acid production was faster in KCC-10 and KCC-19 than it was in KACC 91016 and KACC 91096. As the incubation progressed, lactic acid accumulation increased, while the acetate production decreased. The final lactic acid concentration at 30 h was slightly lower in the KCC-10 and KCC-19 strains than in the KACC 91016 and KACC 91016 strains cultured in MRS medium, whereas the lactic acid and acetic acid production profiles were comparable in cultures contained only juice. The final lactic acid concentrations at 30 h were slightly higher with KACC 91016 (84.0 mM) and KACC 91096 (81.0 mM) than with KCC-10 (77.45 mM) and KCC-19 (78.0 mM) in the IRG juice (Fig. 3). Among the juices, the organic acid production profile was comparatively less in the rice juice. Possible reasons for this decrease in lactic acid production could be the low availability of fermentable sugars, low nutrients, and/or limitations in one or more of the physiochemical parameters. In conclusion, the KCC-10 and KCC-19 can drop in pH from 6.0 to 3.7 in 12 h and accumulate a comparable amount of lactic acid in the MRS medium and other juices.

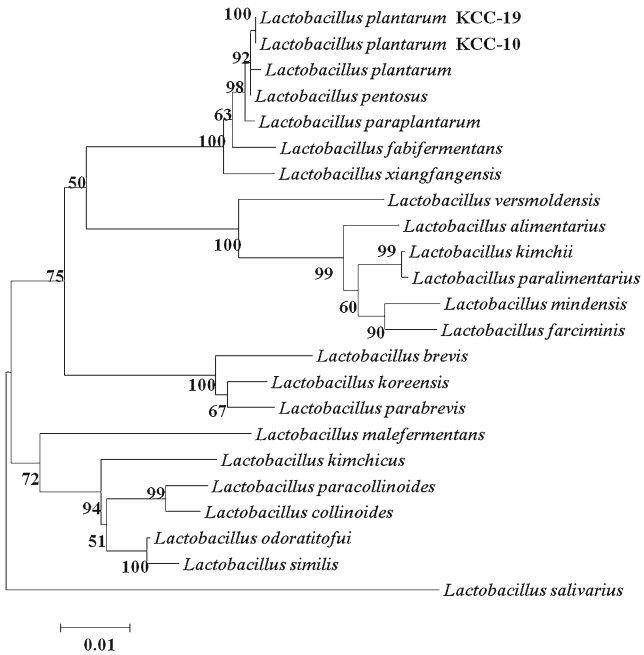


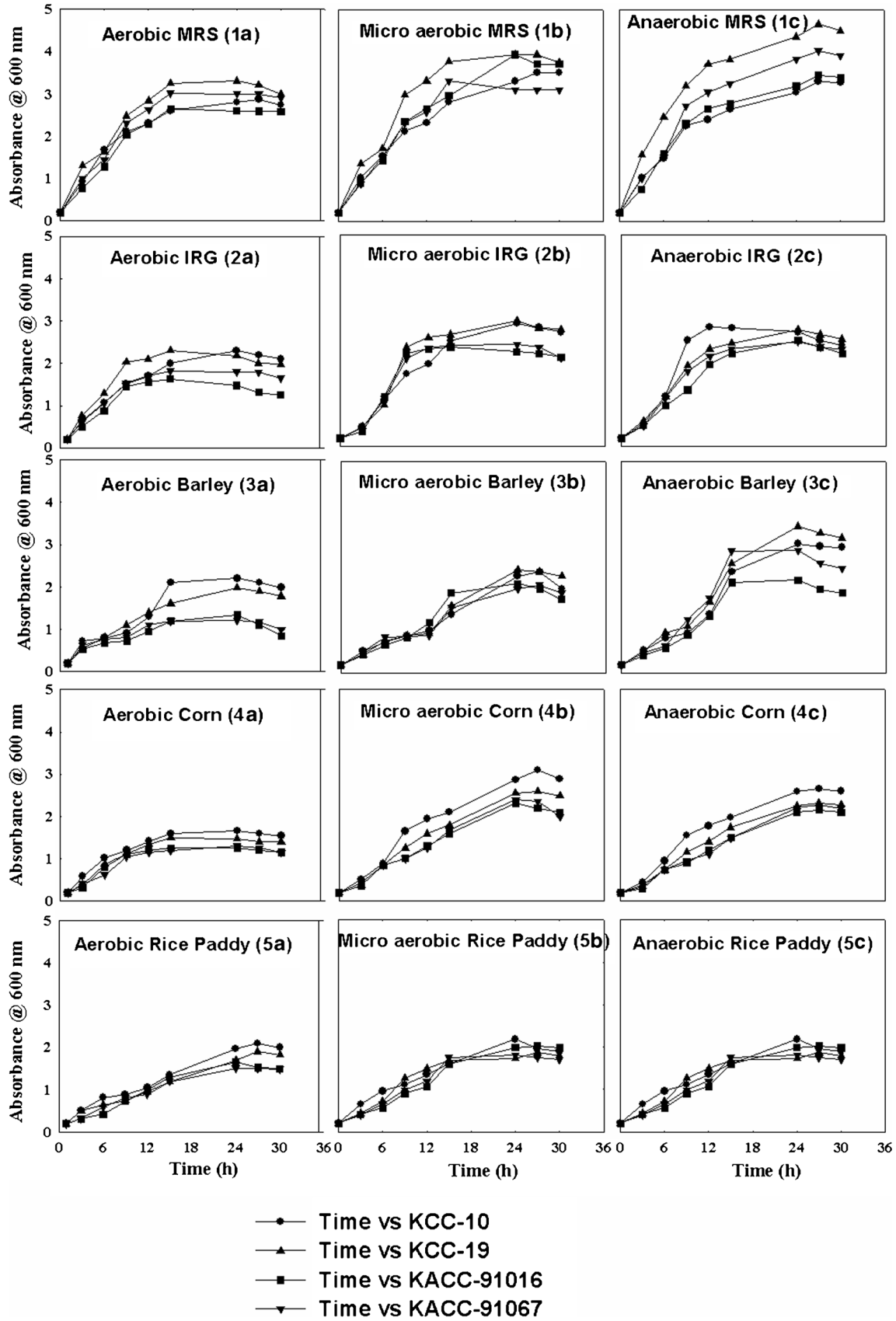
Fig. 2 Phylogenetic tree based on the 16S rRNA gene sequence showing the relationship between *Lactobacillus* sp. KCC-10, *Lactobacillus* sp. KCC-19, and other species belonging to the standard *Lactobacillus* strain 16S rRNA gene sequences obtained from the NCBI database. The 16S rRNA gene sequences originated from *Lactobacillus salivarius*. The tree was constructed using the neighbor-joining method

Anaerobic conditions

It was clear that the KCC-19 strain showed better growth under anaerobic conditions compared to that of KACC 91016 and KACC 91096 (Fig. 3). However, the cell growth measurement on MRS was accurate. In juice, the cells did not grow after 24 h and were either static or approaching a decline phase after 24 h. The maximum cell density of 1.75–3.47 was recorded at 24 h. Most of the consumed sugars led to the production of lactic acid and acetic acid (Fig. 4). This may have been due to the presence of large amounts of reductive equivalents generated under anaerobic conditions. The amount of lactic acid produced was higher in the KCC-10 and KCC-19 strains compared to that of the wild-type strain.

Effects of addition of KCC-10 and KCC-19 on IRG, barley and rye silage

The result on KCC-10 and KCC-19 counts in different silage is presented in Fig. 5. Our results indicated that after 5 days of


Aerobic MRS (1a)
Micro aerobic MRS (1b)
Anaerobic MRS (1c)
Aerobic IRG (2a)
Micro aerobic IRG (2b)
Anaerobic IRG (2c)
Aerobic Barley (3a)
Micro aerobic Barley (3b)
Anaerobic Barley (3c)
Aerobic Corn (4a)
Micro aerobic Corn (4b)
Anaerobic Corn (4c)
Aerobic Rice Paddy (5a)
Micro aerobic Rice Paddy (5b)
Anaerobic Rice Paddy (5c)
Absorbance @ 600 nm
Time (h)
Time vs KCC-10
Time vs KCC-19
Time vs KACC-91016
Time vs KACC-91067

◀ **Fig. 3** Growth profile of *Lactobacillus plantarum* KCC-10 and *Lactobacillus plantarum* KCC-19 under aerobic, microaerobic and anaerobic conditions. *1A* MRS medium aerobic condition; *1B* MRS medium microaerobic condition; *1C* MRS medium anaerobic condition; *2A* Only IRG juice aerobic condition; *2B* Only IRG juice microaerobic condition; *2C* Only IRG juice anaerobic condition; *3A* Only barley juice aerobic condition; *3B* Only barley juice microaerobic condition; *3C* Only barley juice anaerobic condition; *4A* Only corn juice aerobic condition; *4B* Only corn juice microaerobic condition; *4C* Only corn juice anaerobic condition; *5A* Only rice paddy juice aerobic condition; *5B* Only rice paddy juice microaerobic condition; *5C* Only rice paddy juice anaerobic condition; Symbols: *black circle*, KCC-10; *black triangle up*, KCC-19; *black square*, KACC 91016 and *black triangle down*, KACC 91096, respectively

incubation, KCC-10 counts were comparatively similar in IRG (0.58×10^9 CFU/g), barley (0.60×10^9 CFU/g), and rye (0.59×10^9 CFU/g), whereas KCC-19 counts were comparatively similar and slightly higher in the case of IRG (0.71×10^9 CFU/g), barley (0.72×10^9 CFU/g), and rye (0.711×10^9 CFU/g), respectively. KCC-10 and KCC-19 counts were increased when compared to the first day counts, whereas after prolonged incubation, the number of active colonies was decreased; this decrease in strain counts may be due to the reduction of pH with respect to an increase in acidity and the prolonging of ensilaging duration. The pH of silage was sharply decreased to 4.0 after 10 days of ensiling, and the accumulation of lactic acid and acetic acid amounted to 3.1 % (IRG), 3.31 % (barley), and 2.7 % (rye) in KCC-10 and 3.45 % (IRG), 3.21 % (barley), and 2.9 % (rye) in KCC-19. The organic acid composition of the IRG, barley, and rye silages treated with the strains for 50 days is shown in Fig. 6. Among the total fermentation acids, lactic acid was detected as the dominant acid in both barley and IRG silages, however, the barley silage contained a comparatively higher amount of lactic acid. The amount of acetic acids were found to be less in all the silages compared to lactic acid. Moreover, we predicted that the content of lactate, acetate, and total fermentation acid ratios were affecting the quality of silages. The amount of lactic acid did not change much between day 0 and day 30.

Discussion

Organic acids such as lactic acid, acetic acid, and succinic acid producing microorganisms such as *Lactobacillus* and other *Bacillus* species have been isolated from habitats such as fermented foods, rumen, and silages. Agriculture and silage environments contain a wide range of distinct LABs that are not present elsewhere. Though some reports are available on the presence of natural LAB in the food industry, the silage environment is still a potential source for new *Lactobacilli*, which can yield high secretions of lactic acid and fast fermentation rates with high cell-growing properties. The number of novel LAB isolated from silage environments has steadily decreased. To cope with the demand for the animal feed industry and to combat the undesirable pathogens in silage, researchers have been forced to look for novel microorganisms. Hence, there is an immense possibility to identify new *Lactobacillus* strains with high cell growth and other nutritive properties in silage samples. In our previous study, most of the *Lactobacillus* grew well on MRS and BCP media (Arasu et al. 2013), so MRS and BCP media supplemented with glucose were used for isolating *Lactobacillus* strains. During the isolation, *Lactobacillus* sp. KCC-10 and KCC-19 were identified and exhibited high growth rates in the shake-flask cultivation. *Lactobacillus* sp. KCC-10 and KCC-19 utilized most of the sugars that were provided, indicating a wide pattern of carbon assimilation. The morphological and biochemical characteristics are in agreement with our previous and present results, where we referred to carbohydrate fermentation patterns that showed ambiguity (Georgieva et al. 2008). However, they could not be identified at the species level based on the

Table 1 Growth profiles of *Lactobacillus* strains in different cultivation conditions

Condition	Aerobic				Microaerobic				Anaerobic			
	KCC-10	KCC-19	KACC 91016	KACC 91096	KCC-10	KCC-19	KACC 91016	KACC 91096	KCC-10	KCC-19	KACC 91016	KACC 91096
MRS	2.3	2.84	2.29	2.63	2.32	3.31	2.65	2.58	2.3	2.84	2.29	2.63
IRG	1.69	2.1	1.55	1.7	1.95	2.58	2.31	2.33	2.82	2.31	1.95	2.14
Barley	1.41	1.35	1.2	1.14	1.94	1.59	1.3	1.26	1.78	1.4	1.2	1.1
Corn	1.3	1.4	0.95	1.1	1.01	0.9445	1.2	0.9	1.4	1.68	1.354	1.78
Rice	1.05	0.95	1	0.9	1.35	1.49	1.066	1.2	1.066	1.2	1.21	1.287

Strains were grown in 250-mL Erlenmeyer flasks containing 50 mL of MRS broth, and in different juices at 30 °C in an orbital incubator shaker under the aerobic, microaerobic and anaerobic conditions. After 12 h of incubation, the samples were withdrawn for analysis of cell density

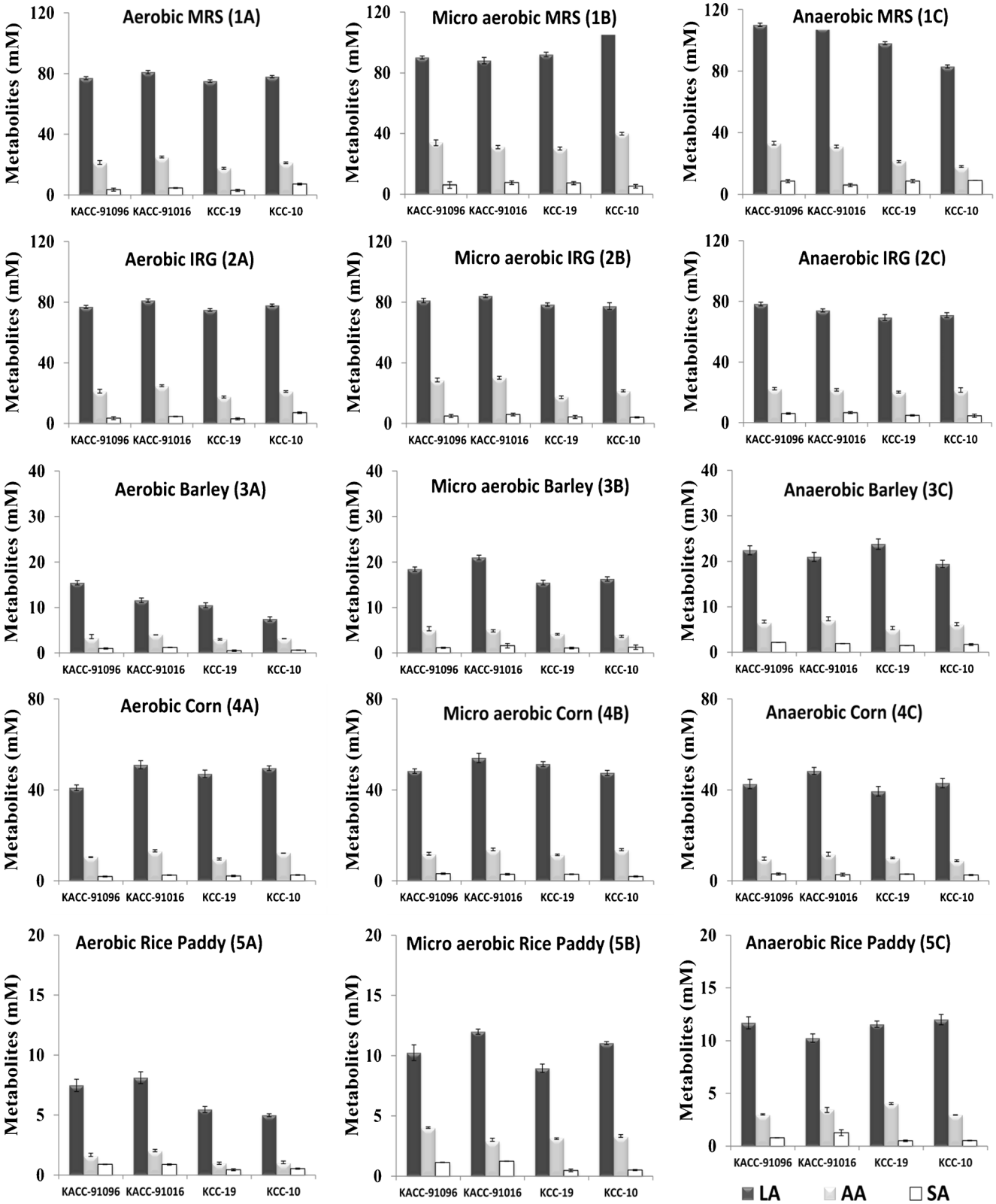


Fig. 4 Fermentation metabolite profiles of *Lactobacillus plantarum* KCC-10 and *Lactobacillus plantarum* KCC-19 under aerobic, microaerobic, and anaerobic conditions

biochemical, physiological and sugar fermentation analysis. Therefore, other phylogenetic analysis methods were required to distinguish these strains accurately. On 16S rRNA gene sequences, the strains KCC-10 and KCC-19 were clearly

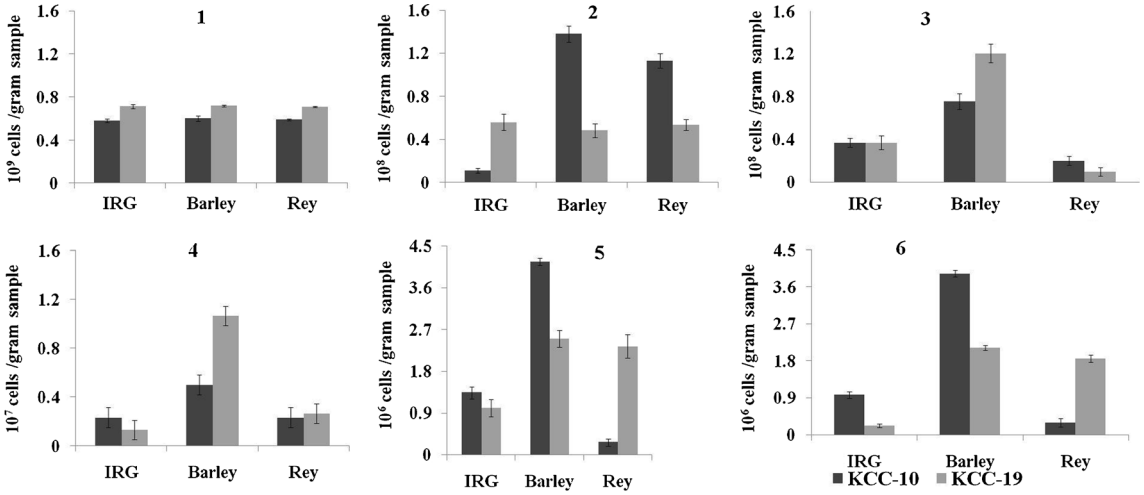


Fig. 5 Growth profile of *Lactobacillus plantarum* KCC-10 and *Lactobacillus plantarum* KCC-19 in different fresh grass silages under various time periods. *1* After 5 days of incubation; *2* after 10 days of incubation; *3* after 20 days of incubation; *4* after 30 days of incubation; *5* after 40 days of incubation; *6* after 50 days of incubation

assigned to the genus *Lactobacillus*, since they were grouped in the phylogenetic tree together with type strain *L. plantarum* by forming a very well-defined cluster.

An anaerobic or microaerobic environmental condition is the most important individual parameter that can enhance silage conservation (Woolford 1975). During conservation, most of the silages on individual farms are exposed to air, due to the permeability of plastic to air and difficulties in properly sealing the outer layer of the silage, or during the feed-out phase, due to an inadequate amount of silage being removed and to a poor management of the exposed silo surface (Ashbell et al. 1988). It has been well-documented that a large number of LAB are needed to ensure a rapid and vigorous fermentation that results in a rapid decrease in pH and a large amount of accumulated lactic acid and acetic acid, which are the key factors to ensuring good crop quality during the ensiling process (Ennahar et al. 2003). However, under field environmental conditions, numbers of LAB are not always large enough, especially in some low WSC content and high buffering capacity crops such as alfalfa, barley, corn, and rice (Nakui et al. 1988). Therefore, the addition of fast-growing inoculants such as *Lactobacillus* species to silage was an effective way to improve the silage quality. As shown in our results, after inoculation, the 2.4×10^5 of KCC-10 grew to 0.58×10^9 CFU/g (IRG), 0.60×10^9 CFU/g (barley), and 0.59×10^9 CFU/g (rye) by day 5. For KCC-19, the initial inoculation level was 2.7×10^5 CFU/g and the counts were comparatively increased at day 5. The increase in the total numbers of KCC-10 and KCC-19 at day 5 confirmed that the strains were able to use the carbohydrates present in the grasses and ferment them into lactic acid and acetic acid. The metabolite profiles of the silage extracts were found to be highly dependent on whether the grass was inoculated with a *Lactobacillus* strain.

Growth patterns revealed that the strains KCC-10 and KCC-19 showed better growth in IRG, barley, corn, and rice juices when compared to the control *L. plantarum* strain under aerobic, microaerobic and anaerobic conditions. Bacterial cell growth mainly depends upon the ability of the cells to form new protoplasms from nutrients available in the environment.

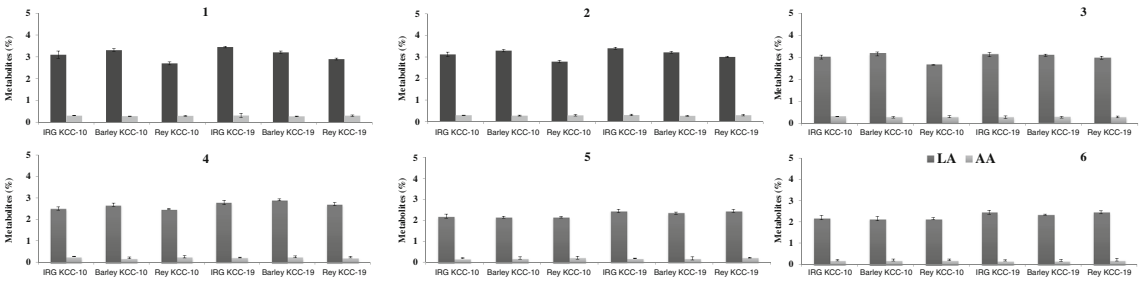


Fig. 6 Metabolite production profiles of *Lactobacillus plantarum* KCC-10 and *Lactobacillus plantarum* KCC-19 in different fresh grass silage under various time periods. *1* After 5 days of incubation; *2* after 10 days of incubation; *3* after 20 days of incubation; *4* after 30 days of incubation; *5* after 40 days of incubation; *6* after 50 days of incubation

Growth of the strains KCC-10 and KCC-19 was found to be better than the growth of the control; the cellular components of these new strains, such as ribosomes, chromosomes, and plasma membranes, are mainly involved in the growth of bacteria and might be active. Also, the active synthesis of complex cell wall components such as sugars, lipids and proteins might result in better growth. Our results indicated that the strains KCC-10 and KCC-19 exhibited more growth than the control strain under aerobic conditions, revealing that the strains might be more active in generating energy and cellular biosynthesis components than the control strain. Growth of bacteria depends upon the production of other metabolites such as phosphoenolpyruvate, which plays a main role in the synthesis of amino acids. In the absence of oxygen (O_2), the growth of bacteria (under anaerobic conditions) forces the bacterium not involved in the participation of the TCA cycle enzymes to undergo biosynthesis and generate energy in the form of ATP. Under these conditions, pyruvate, or acetyl-CoA derived from pyruvate, serves as the electron acceptor (reduced to lactate or ethanol, respectively) to maintain the redox balance.

The variation in temperature during silage fermentation is well-known. Generally, during fermentation, heating in the silage was consistently correlated with microorganism development and plant respiration. In the early stage of the ensiling process, temperature rises rapidly and reaches over 45 °C (Barnett 1954). In addition, the growth of some *Lactobacilli* would be inhibited by the high-temperature conditions. In our study, when stored at room temperature, silages inoculated with KCC-10 and KCC-19 were well-preserved. Among these two strains, strain KCC-10 showed an advanage as a probiotic strain because it grew well under low pH (3.0) and in the presence of bile salts, was able to produce extracellular enzymes, and was susceptible to commonly used antibiotics (Arasu et al. 2013). Therefore, the inoculation of silage with these strains may result in beneficial effects by promoting propagation and inhibiting the growth of other anaerobic and microaerobic toxic metabolite-producing bacteria, as well as by decreasing the amount of gas production. Our results revealed that during silage fermentation, strains KCC-10 and KCC-19 improved silage quality and fermented WSC to produce good amounts of lactic acid, allowing the pH value of the silage to fall no lower than 3.9.

The addition of LAB inoculants to ensiling is intended to ensure rapid and vigorous fermentation that results in the faster accumulation of lactic acid (McDonald et al. 1991). Many studies have shown the advantage of such LAB. Generally, moist dairy farm silage is based on natural lactic acid fermentation (Sebastian et al. 1996; McDonald et al. 1991). Among the LAB, *L. plantarum*, *L. buchneri* and *Enterococcus faecium*, and *Pediococcus* sp. are mainly present in forage samples (Lin et al. 1991, 1992). When LAB fail to produce sufficient lactic acid during fermentation to reduce the pH and inhibit the growth of Clostridia and other fungal spoilages, the resulting silage will be of poor quality. The growth of *Lactobacillus* strains in grass silage may hypothetically promote the production of antifungal substances other than the compounds isolated from liquid cultures. The addition of novel *Lactobacillus* strains to silages accelerated the ensiling fermentation because of the faster acid production by this microorganism during the initial stages of ensiling. In general, the application of *Lactobacillus* did not affect the composition of the final silages or their aerobic stability. The results of the current study are in agreement with those of Bach et al. (2002), who found similar fermentation profiles in barley silages.

The growth characteristics and lactic acid production capability of KCC-10 and KCC-19 in IRG, barley, corn, and rice could be used further in animal feed preparation. Recently, we reported that silage prepared using IRG enhances the nutritive value and positive adipogenesis of Hanoo steers when used as their fodder, and its expression levels were checked in adipogenic cell lines (Valan Arasu et al. 2014).

Conclusion

In this study, the *L. plantarum* KCC-10 and KCC-19 strains were isolated from silage samples and characterized based on biochemical, physiological, and 16S rRNA gene sequence analysis. KCC-10 and KCC-19 were able to grow in grass juices and produce organic acids such as lactic acid and acetic acid. These results confirmed that the inoculation of fresh grass with KCC-10 and KCC-19 strains increased the proportion of lactic acid in the silage and improving its overall quality. Consequently, trials are underway in our laboratory to develop a large volume of silage with different additive combinations according to field conditions.

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