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RESEARCH ARTICLE

Selection and characterisation of lactic acid bacteria isolated from different origins for ensiling *Robinia pseudoacacia* and *Morus alba* L. leaves

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Abstract

The objective of this study was to isolate lactic acid bacteria (LAB) strains from different origins and to select the best strains for ensiling *Robinia pseudoacacia* (RB) and *Morus alba* L. (MB) leaves. The LAB strains were inoculated into the extracted liquid obtained from RB and MB leaves to evaluate the fermentation products. 11 LAB strains were selected for further experiments based on the highest products of lactic or acetic acid, including 1 strain of *Weissella confusa*, 2 of *Lactobacillus reuteri* and 8 of *Lactobacillus plantarum*. The API 50 CH fermentation experiment indicated that all of the selected 11 LAB strains utilised most of the carbohydrates. All the strains grew at temperatures between 10 and 45°C and at a pH of 3.5 to 4.5; however, *L. reuteri* F7 and F8 tolerated a pH as low as 3.0. All 11 LAB strains showed antibacterial activity against *Listeria monocytogens*, *Escherichia coil*, *Salmonella* sp. and *Acetobacter pasteurianus*; however, after excluding the effect of organic acids, only F7 and F8 still exhibited antibacterial activity. The present study indicated that the selected 11 LAB strains could be used to prepare silages of RB and MB leaves, especially *L. reuteri* F7 and F8.

Keywords: lactic acid bacteria, *Morus alba* L., *Robinia pseudoacacia*, silage

1. Introduction

The demand for animal products is increasing notably in China and other developing countries. Therefore, it is now necessary to develop larger quantities of feedstuff for animals. *Robinia pseudoacacia* (RB) and *Morus alba* L. (MB) trees are fast-growing, drought-tolerant and adaptable to different growing climates and are cultivated in many parts of the world. Due to their high biomass yield, protein sources and digestibility, studies have been performed to explore their potential for animal feedstuff, indicating that they can be used in animal diets without negatively affecting animal products (Zhang *et al.* 2010). In addition, RB and MB trees are seasonally available and may require accumulating larger stocks for immediate use. Therefore, a method for preserving these two trees needs to be developed.

Ensiling has recently been considered the best way to preserve fresh forage crops and grasses with minimal losses. During the ensiling process, epiphytic lactic acid bacteria (LAB) ferments water-soluble carbohydrates under anaerobic conditions into organic acids, primarily lactic acid, which greatly and quickly reduces the pH (Cai *et al.*

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1998). To improve the silage quality, many LAB inoculants have been developed and are proven to be useful in forage and grass species (Ni *et al.* 2015). These could be used to solve problems in the variation in fermentation quality and microbial stability in the process of ensiling. Recently, some heterofermentative inoculants were also developed to inhibit aerobic spoilage, such as *Lactobacillus buchneri* and *Lactobacillus brevis* (Kleinschmit and Kung 2006). The characteristics of LAB are primarily associated with their physiological features, such as the utilisation of various substrates, and for their metabolic and antimicrobial capabilities (Wang *et al.* 2006). Several reports have demonstrated that inoculants with different strains belonging to the same species result in different effects on silage quality, which may indicate that the suitable inoculant candidates for ensiling should be selected at the strain level. Although numerous studies had been conducted on selecting LAB for inoculating corn, rice, alfalfa grass and other crops, limited information is available on what type of LAB strains are suitable for inoculating RB and MB trees.

The objective of this study was to select potentially suitable LAB strains to be used as inoculants for ensiling RB and MB trees. A large number of LAB, isolated from different origins, were evaluated by analysing their organic acid production, utilising substrate ability, tolerance to inhibitory conditions and antimicrobial activity.

2. Materials and methods

2.1. Materials

Corn (Zhengchaotian 2) at the dough-ripe stage, crop rice (*Oryza sativa* L.) at the milk ripe stage, alfalfa (*Medicago sativa*) at the flowering stage and pig feces were collected in Zhengzhou, Henan, China. RB and MB leaves were obtained at Luoyang, Henan, China. Dry matter (DM) and crude protein (CP) were analyzed by AOAC (1990) 934.01 and 967.05, respectively. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were analyzed according to the methods of Van Soest *et al* (1991). For RB, the DM was 34.8%; CP, ADF and NDF were 19.6, 30.2 and 44.6% of DM, respectively. For MB, the DM was 37.2%; CP, ADF and NDF were 12.3, 16.2 and 31.7% of DM. For the number of isolated strains, please see Table 1.

2.2. Strains

A total of 65 LAB strains were isolated from the employed materials and cultivated on lactobacilli de Man, Rogosa, Sharpe (MRS) agar incubated at 30°C for 2 days under anaerobic condition. Each colony was purified two times by streaking on the MRS agar, then transferred to nutrition broth (Difco Laboratories, USA) with dimethyl at a ratio 9:1 and stored as stock cultures at –80°C for further examination.

Table 1 The source of lactic acid bacteria (LAB) strains used in this study

Sources	Strains names
Corn	C1, C2, C3, ..., C13
Rice	R1, R2, R3, ..., R10
Alfalfa	A1, A2, A3, ..., A12
Pig feces	F1, F2, F3, ..., F15
Morus alba L. (MB)	MB1, MB2, MB3, ..., MB8
Robinia pseudoacacia (RB)	RB1, RB2, RB3, ..., RB7

2.3. Inoculation experiment

First, 65 LAB strains were cultivated in MRS broth for 24 h at 30°C. The broth of RB and MB was crushed out from 2 000×g of fresh materials mixed with 5 000 mL deionized water in a squeezer, separately, then filtered and sterilized (121°C, 15 min). After this period, the inoculum was standardized using a spectrophotometer (600 nm) at an optical density of 1.0. Subsequently, approximately 100 μL of each strain was inoculated into 30 mL of paddy rice broth, which was incubated at 30°C; two replicates were made for each treatment. After 48 h of fermentation, samples of cultures were taken to evaluate metabolite production by HPLC (1200 series; Agilent, USA). The HPLC system included column temperature 55°C, the speed of mobile solution 0.6 mL min^{-1} and UV detector 210 nm. Besides, 1 mL of fermented liquid were blended with 9 mL of sterilized water, and serially diluted from 10^{-1} to 10^{-5} in sterilized water. The number of LAB were measured by plate count on MRS agar incubated at 30°C for 48 h under anaerobic conditions (DG 250/min MACS; Don Whitley Science, England).

2.4. 16S rRNA gene sequencing

Cells grown at 30°C for 24 h in MRS agar were used for 16S rRNA gene sequence. The 16S rRNA gene sequence coding region was amplified by PCR in a PCR thermal cycler as described by Suzuki *et al.* (1996). The sequences of the PCR products were determined directly with a sequencing kit using the prokaryotic 16S ribosomal DNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Sequence similarity searches were performed using the DNA database of Japan (DDBJ) and the Basic Local Alignment Search Tool (BLAST).

2.5. Morphological, physiological and biochemical tests of LAB

Morphological, physiological and biochemical tests of LAB