ISOLATION AND CHARACTERISTICS OF LACTIC ACID BACTERIA ISOLATED FROM RIPE MULBERRIES IN TAIWAN

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ABSTRACT

The objective of this study was to isolate, characterize, and identify lactic acid bacteria (LAB) from ripe mulberries collected in Taiwan. Ripe mulberry samples were collected at five mulberry farms, located in different counties of Taiwan. Eighty-eight acid-producing cultures were isolated from these samples, and isolates were divided into classes first by phenotype, then into groups by restriction fragment length polymorphism (RFLP) analysis and sequencing of 16S ribosomal DNA (rDNA). Phenotypic and biochemical characteristics led to identification of four bacterial groups (A to D). *Weissella cibaria* was the most abundant type of LAB distributed in four mulberry farms, and *Lactobacillus plantarum* was the most abundant LAB found in the remaining farm. Ten *W. cibaria* and one *Lactococcus lactis* subsp. *lactis* isolate produced bacteriocins against the indicator strain *Lactobacillus sakei* JCM 1157[T]. These results suggest that various LAB are distributed in ripe mulberries and *W. cibaria* was the most abundant LAB found in this study.

Key words: lactic acid bacteria, mulberry, *Weissella cibaria*, bacteriocin, Taiwan.

INTRODUCTION

Mulberry (*Morus australis*) has been widely cultivated in China and southeastern Asia for thousands of years. The leaves of mulberry trees represent indispensable food for silkworms and the ripe fruits are edible and have been widely used in juices, wines and jams (23). In Taiwan, mulberry trees are widespread and the ripe fruits are always harvested in early April. The ripe fruit is sweet with a very mild flavor and is therefore popular in Taiwan. Although mulberries are very popular, they have not been studied in detail.

Isolation of lactic acid bacteria (LAB) from fruits and vegetables have frequently been reported (2,3,7,14,18). However, studies on LAB associated with ripe mulberries remain scarce.

Bacteriocins produced by LAB have attracted special interest as potential alternative safe commercial food preservatives. LAB have been used as food and feed preservatives for centuries, and bacteriocin-producing LAB could replace chemical preservatives for the prevention of bacterial spoilage and the outgrowth of pathogenic bacteria in food products (6).

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The objectives of this study were to isolate LAB from ripe mulberries, identify the isolates at the species level, and screen them for antibacterial activity.

MATERIALS AND METHODS

Sampling

Samples for LAB isolation were collected from five mulberry farms (M1 to M5), which were respectively located in the following five counties in Taiwan: Tainan, Chiayi, Taichung, Miaoli and Taoyuan. The farms in these counties are listed from south to north and are 40-70 km apart. Three separate ripe fruit samples were randomly collected at each mulberry farm. The samples were taken aseptically and packaged into clean bags, then stored at 4°C. Samples were analyzed within 24 h of acquisition at the mulberry farms.

Isolation of LAB

MRS (de Man, Rogosa, and Sharpe)-agar plates were used for the isolation of LAB. To distinguish acid-producing bacteria from other bacteria, 1% $CaCO_3$ was added to the MRS-agar plates. Each mulberry sample was crushed and mixed with 0.75% NaCl solution. Dilutions of the mixed solution (10- to 1000-fold) were spread directly onto the surface of MRS-agar plates. Samples were incubated under anaerobic conditions (Mitsubishi AnaeroPak System, Pack-Anaero, Mitsubishi Gas Chemicals, Tokyo, Japan) at 30°C for 3 to 5 days. Colonies of acid-producing bacteria, identified by a clear zone around each colony, were randomly selected from MRS-agar plates and purified by replating on MRS-agar plates. Colonies were reselected and initially Gram stained and tested for production of catalase. Only Gram-positive, catalase-negative strains were selected. The selected strains were stored at –80°C in 10% skim milk broth.

Identification of isolates

Restriction fragment length polymorphism (RFLP) analysis and sequence analysis of 16S ribosomal DNA (rDNA) were used to classify and identify the bacterial isolates. DNA

was isolated by using the methods described by Kozaki *et al.* (12). Polymerase chain reaction (PCR) was carried out using a Takara Ex *Taq* gene amplification PCR kit (Takara Bio, Shiga, Japan) to prepare reaction mixtures and performed on a Gene Amp PCR System 9700 (PerkinElmer Corp., Boston, MA, USA) following the methods described by Chen *et al.* (4). RFLP analysis of 16S rDNA was carried out with the methods described by Chen *et al.* (4). In this study, three restriction enzymes, *Acc*II (CG/CG), *Hae*III (GG/CC) (16), and *Alu*I (AG/CT) (22) were used for grouping.

For sequence analysis of 16S rDNA, the PCR products were purified with a Clean/Gel Extraction Kit (BioKit, Miaoli, Taiwan) and then sequenced with the following primer: 5´-CTGCTGCCTCCCGTAG-3´ (27F). DNA sequencing was performed using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Partial sequencing, approx. 1000 bp ahead was performed and the sequence manually aligned with the software Genetyx-Win version 5.1. Sequence homologies were examined by comparing the sequences obtained with those in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) using FASTA. During sequence comparison, an initial threshold of 99% homology was required when comparing the raw sequencing result, while 100% homology was obligated for the sequencing data with high signal to noise ratios.

Detection of antibacterial activity

The agar-well diffusion method as described by Onda *et al.* (15) was used to detect and determine the antibacterial activities of isolates. *Lactobacillus sakei* JCM 1157[T], *Listeria monocytogenes* ATCC 19111 and *Helicobacter pylori* ATCC 43504[T] were used as the indicator strains. *L. monocytogenes* was propagated in PBN broth (pH 7.3) with the following components: 0.5% peptone, 0.3% beef extract, and 0.8% NaCl. *H. pylori* was propagated in a Tryptic soy agar (Difco, Sparks, MD, USA) with 5% defibrinated sheep blood added. To exclude the effect of lactic acid, antibacterial activity was later confirmed by pH adjustment (21).

Effect of enzymes on antimicrobial activity

The effect of various enzymes on antimicrobial activity was determined by incubating 500 μL of the neutralized and filter-sterilized cell-free supernatant with 20 μL of the following enzyme solutions at a final concentration of 3 mg/mL respectively: proteinase K (pH 7.0), trypsin (pH 7.0) and catalase (pH 7.0). Enzymes were purchased from Sigma Chemicals, St. Louis, MO, USA. After 5 h of incubation at 37 °C, antimicrobial activity was determined by using agar-well diffusion assay. Untreated samples were used as control and *L. sakei* JCM 1157[T] was used as the indicator strain.

RESULTS

A total of 88 acid-producing bacterial strains were isolated from the samples collected in mulberry farms of Taiwan. Of these, 13 strains were isolated from farm M1, 18 from farm M2, 20 from farm M3, 18 from farm M4, and 19 from farm M5 (Table 1). The 88 isolates were classified into four groups (A to D) based on cell morphology and the results of 16S rDNA RFLP analysis (Fig. 1). Of these isolated short-rod strains, 54 were placed in group A, 14 in group B, and 18 in group D, because digestion of their DNA with *Acc*II, *Hae*III, and *Alu*I gave the same RFLP patterns. The remaining coccal isolates were placed into group C.

To identify the isolates, representative strains were randomly selected from each group, and 16S rDNA sequencing analysis was carried out. The results identified group A isolates as *W. cibaria*, group B as *Leuconostoc pseudomesenteroides*, group C as *Lactococcus lactis* subsp. *lactis*, and group D as *Lactobacillus plantarum.* The sequences determined in this study have been deposited in the DDBJ database with sequential accession numbers AB510744 to AB510757.

Table 1. Isolates from ripe mulberries

| Mulberry farm	Strain No.	Specie	Mulberry farm	Strain No.	Specie	Mulberry farm	Strain No.	Specie
M1	H041703	*W. cibaria*		H042046	*L. pseudomesenteroides*		I042210	*W. cibaria*
	H041706	*W. cibaria*	M3	I041701	*L. plantarum*		I042211	*W. cibaria*
	H041710	*W. cibaria*		I041702	*L. plantarum*		I042212	*W. cibaria*
	H041711	*W. cibaria*		I041703	*L. plantarum*		I042213	*W. cibaria*
	H041713	*W. cibaria*		I041704	*W. cibaria*		I042214	*W. cibaria*
	H041715	*W. cibaria*		I041705	*W. cibaria*		I042215	*W. cibaria*
	H041716	*W. cibaria*		I041706	*L. plantarum*		I042217	*W. cibaria*
	H041717	*W. cibaria*		I041707	*L. plantarum*		I042218	*W. cibaria*
	H041718	*W. cibaria*		I041708	*L. plantarum*		I042219	*W. cibaria*
	H041719	*W. cibaria*		I041709	*L. plantarum*	M5	I042501	*L. pseudomesenteroides*
	H041720	*W. cibaria*		I041710	*L. plantarum*		I042502	*W. cibaria*
	H041721	*W. cibaria*		I041711	*L. plantarum*		I042503	*W. cibaria*
	H041722	*W. cibaria*		I041712	*L. plantarum*		I042504	*W. cibaria*
M2	H042001	*W. cibaria*		I041713	*L. plantarum*		I042505	*W. cibaria*
	H042002	*W. cibaria*		I041714	*L. plantarum*		I042506	*L. pseudomesenteroides*
	H042003	*W. cibaria*		I041715	*L. plantarum*		I042507	*L. pseudomesenteroides*
	H042004	*L. pseudomesenteroides*		I041716	*L. plantarum*		I042508	*W. cibaria*
	H042005	*L. pseudomesenteroides*		I041717	*L. plantarum*		I042509	*W. cibaria*
	H042006	*W. cibaria*		I041718	*L. plantarum*		I042510	*W. cibaria*
	H042007	*L. pseudomesenteroides*		I041719	*L. plantarum*		I042511	*L. lactis* subsp. *lactis*
	H042008	*W. cibaria*		I041720	*L. plantarum*		I042512	*L. pseudomesenteroides*
	H042009	*W. cibaria*	M4	I042201	*W. cibaria*		I042513	*L. lactis* subsp. *lactis*
	H042010	*W. cibaria*		I042202	*L. pseudomesenteroides*		I042514	*W. cibaria*
	H042011	*L. pseudomesenteroides*		I042203	*W. cibaria*		I042516	*W. cibaria*
	H042014	*L. pseudomesenteroides*		I042204	*W. cibaria*		I042517	*W. cibaria*
	H042015	*W. cibaria*		I042205	*W. cibaria*		I042518	*W. cibaria*
	H042016	*W. cibaria*		I042206	*W. cibaria*		I042519	*W. cibaria*
	H042017	*L. pseudomesenteroides*		I042207	*W. cibaria*		I042520	*W. cibaria*
	H042018	*W. cibaria*		I042208	*L. pseudomesenteroides*			
	H042021	*L. pseudomesenteroides*		I042209	*W. cibaria*			
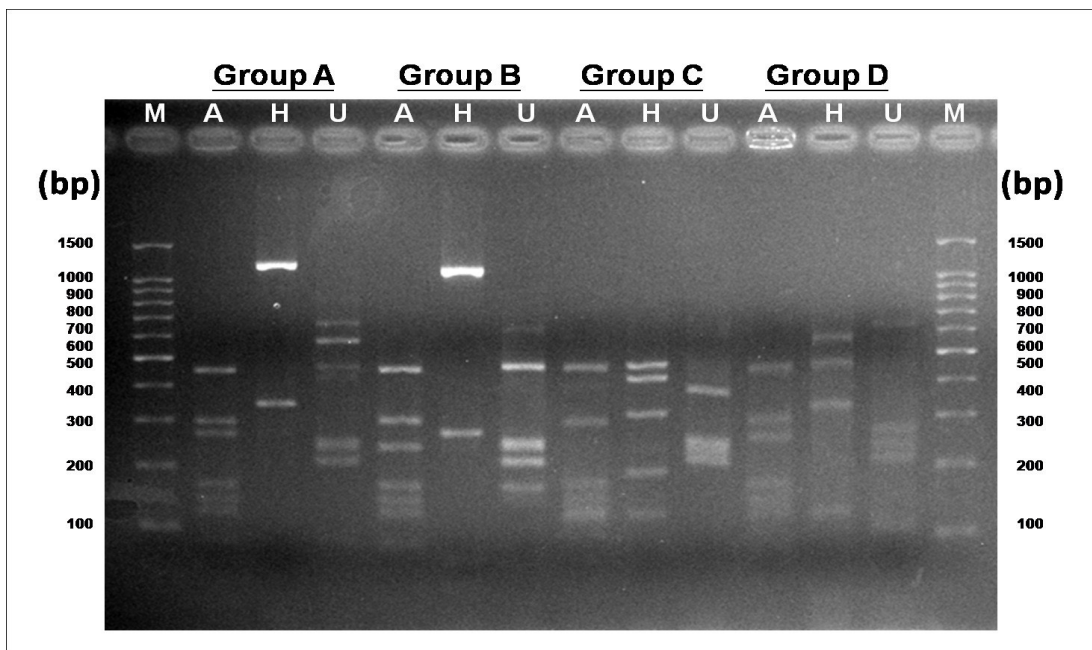
Figure 1. 16S rDNA RFLP patterns of *Acc*II, *Hae*III, and *Alu*I digests from groups A to D. Lane M, size marker; A, *Acc*II digestion patterns; H, *Hae*III digestion patterns; U, *Alu*I digestion patterns.

When assessing inhibitory activity against the indicator strain *L. sakei* JCM 1157[T], clear inhibition zones were observed for 10 *W. cibaria* and 1 *L. lactis* subsp. *lactis* isolates. However, only the same *L. lactis* subsp. *lactis* strain, I042513, showed inhibitory activity against *L. monocytogenes* ATCC 19111 and none of the isolates showed inhibitory activity against *H. pylori* ATCC 43504[T] (Table 2).

The effects of enzymes on the antimicrobial activity of the isolated substance from 10 *W. cibaria* and one *L. lactis* subsp.

lactis strains are shown in Table 2. All the antimicrobial substances were inactivated by proteinase K but not affected by treatment with catalase. However, trypsin inactivated the antimicrobial substance from 10 *W. cibaria* but showed no effect on the antimicrobial substance from *L. lactis* subsp. *lactis* strain. Protease sensitivity assays demonstrated that the antimicrobial substance produced by the strain listed in Table 2 can be described as bacteriocin since its inhibitory activity was completely eliminated by treatment with enzyme proteinase K (13).

Table 2. Inhibition spectra and effect of enzymes on the bacteriocin produced by lactic acid bacteria from ripe mulberries.

| Isolated strain | Indicator microorganisms | | | Enzyme treatments | | | |
| | *L. sakei* JCM 1157[T] | *L. monocytogenes* ATCC 19111 | *H. pylori* ATCC 43504[T] | Diameter of the zone of inhibition (mm) [a][β] | | | |
				Control	Proteinase K	Trypsin	Catalase
W. cibaria							
H041703	+	–	–	11	N	N	11
H041706	+	–	–	11	N	N	11
H041710	+	–	–	11	N	N	11
H041713	+	–	–	11	N	N	11
H041715	+	–	–	10	N	N	10
H041718	+	–	–	11	N	N	11
H041719	+	–	–	11	N	N	11
H041720	+	–	–	11	N	N	11
H041721	+	–	–	11	N	N	11
H041722	+	–	–	11	N	N	11
L. lactis subsp. *lactis*							
I042513	+	+	–	17	N	17	17

[a] Wells (8 mm diameter) were filled with 100 μL of culture supernatant.
[β] *L. sakei* JCM 1157[T] was used as the indicator strain.
Abbreviations: +, positive; -, negative; N, no inhibitory zone observed.

DISCUSSION

LAB cultures were isolated from ripe fruits of all five mulberry farm samples, with differences in abundance and diversity evident among the farms (Table 1). Although the mulberry farms are 40 to 70 km apart from each other, the same dominant species, *W. cibaria*, was found in all five farms.

In addition, *W. cibaria* was also the most abundant species of LAB found in the mulberry farms, except for farm M3. Instead of *W. cibaria*, *L. plantarum* was the most abundant species of LAB found in farm M3. *L. pseudomesenteroides* was found only at farms M2, M4 and M5, while *L. lactis* subsp. *lactis* was only found at farm M5.

Species such as *W. cibaria, L. plantarum* and *L. lactis* subsp. *lactis* have been frequently found in environments associated with plants (8,9,11,18). In a previous study, LAB associated with wine grapes was studied by Bae *et al.* (2). Differently from ripe mulberries, *L. lindneri* was the most abundant species of LAB found from wine grapes. In the case of tomato fruits, *L. plantarum* was the most abundant species of LAB found (8). Differences in LAB distribution, including not only species but also number, were observed among these fruits.

In this study, we focused not only on the distribution of LAB in mulberry farms, but also on the bacteriocin-producing ability of the isolates. A total of 11 strains, including 10 *W. cibaria* and 1 *L. lactis* subsp. *lactis*, showed inhibitory activities against the indicator strain *L. sakei* JCM 1157[T] (Table 2). However, only the same strain of *L. lactis* subsp. *lactis* showed inhibitory activity against *L. monocytogenes* ATCC 19111. Bacteriocins from *L. lactis* subsp. *lactis* (1,5,10,15) have been frequently studied. However, studies on bacteriocins from *W. cibaria* remain scarce (17).

The sensitivity of the substances to proteinase K or trypsin revealed their proteinaceous nature. In addition, no effect was observed with the catalase treatment, suggesting that the active agent is not involved in the H_2O_2 antimicrobial mechanism. The results obtained in this study were similar to the previous studies (13,17).

Furthermore, it is interesting that 10 bacteriocin-producing *W. cibaria* strains were all distributed in farm M1. It is difficult to interpret why *W. cibaria* strains found in other mulberry farms did not produce bacteriocin, and why some *W. cibaria* produced bacteriocin but some did not. In the previous study of Trias *et al.* (18), *W. cibaria* strain TM128 effectively decreased the infection level of fungi. A similar result was also reported by Valerio *et al.* (19). It is therefore suspected that these bacteriocin-producing *W. cibaria* may protect mulberries from other microorganisms. However, more studies are necessary to test this hypothesis.

Besides *W. cibaria* strains, *L. lactis* subsp. *lactis* I042513 was the other bacteriocin-producing LAB strain found in this study. An additional experiment was performed to find more information about this bacteriocin. Nisin-specific PCR primers, NISL: 5'-CGAGCATAATAAACGGC-3' and NISR: 5'-GGATAGTATCCATGTCTGAAC-3', were used for PCR amplification following the methods and conditions described by Villani *et al.* (20). The expected amplified band, located at 320 bp, was obtained. The PCR product was sequenced, and the DNA sequence was analyzed and found to encode a peptide containing an amino acid sequence with 100% coincidence to nisin Z (data not shown). However, more detailed protein analyses are necessary to support this result.

In conclusion, the results of this study suggest that various LAB are distributed in ripe mulberries. *W. cibaria* was the most abundant LAB in four mulberry farms, while *L. plantarum* was the most abundant LAB in a fifth mulberry farm. Many LAB isolated from ripe mulberries were found to produce bacteriocins. Future studies in our laboratory will characterize and identify the bacteriocins, and we anticipate that the bacteriocins from these LAB may be useful as biopreservatives. The authors hope that the results of this study will offer useful information for the improvement of mulberry cultivation.

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