ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2020; 25(6): 2079-2085
doi: 10.25083/rbl/25.6/2079.2085

Received for publication, November, 30, 2019
Accepted, January, 6, 2020

Original paper

Distribution and antimicrobial activity of lactic acid bacteria associated with lychee fruits

LIN-HU NAN[1,3], YI-SHENG CHEN[1,3,*], HUI-CHUNG WU[1], YU-CHING SU[1], MIN-CHIN LIAO[1] and FUJITOSHI YANAGIDA[2]

[1]Department of Biotechnology, Ming Chuan University, No. 5, Deming Rd., Guishan Dist., Taoyuan City, Taiwan
[2]The Institute of Enology and Viticulture, University of Yamanashi, 1-13-1 Kitashin, Kofu, Yamanashi 400-0005, Japan

Abstract

Lychee is a popular fruit in China and southeastern Asia. Although it is very popular, the microbiota of lactic acid bacteria (LAB) associated with lychee remains poorly described. Lychee samples from seven different markets located in three cities in Taiwan were collected and a total of 104 LAB were isolated. Through RFLP analyses of 16S rDNA and *rpoA* genes for grouping and 16S rRNA gene sequencing, these isolates were finally divided into 6 groups (A to F). The most common genera of LAB in lychee samples were *Weissella* and *Leuconostoc*. *Weissella confusa* strain E was found to produce a bacteriocin active against *Listeria monocytogenes* and some other Gram-positive bacteria. Mass spectrometry analysis revealed the bacteriocin mass to be approximately 3426.77 Da, which is different to other known *Weissella* bacteriocins. In addition, strain MB7 included in the genus *Leuconostoc* was identified as potential novel species or subspecies on the basis of phylogenetic analysis of 16S rRNA, *rpoA* and *pheS* gene sequences. Thus, this is the first report describing the distribution and varieties of LAB associated with lychee fruits. In addition, one potential novel LAB species or subspecies and one potential novel bacteriocin were also reported in this study.

Keywords Lactic acid bacteria, lychee, bacteriocin, *Leuconostoc*, *Weissella*.

To cite this article: NAN L-H, CHEN Y-S, WU H-C, SU Y-C, LIAO, M-C YANAGIDA F. Distribution and antimicrobial activity of lactic acid bacteria associated with lychee fruits. *Rom Biotechnol Lett*. 2020; 25(6): 2079-2085. DOI: 10.25083/rbl/25.6/2079.2085

✉ *Corresponding author: YI-SHENG CHEN, Department of Biotechnology, Ming Chuan University, No. 5, Deming Rd., Guishan Dist., Taoyuan City, 333, Taiwan
Fax: +886-3-359-3878; Tel: +886-3-350-7001 ext. 3540
E-mail: yisheng@mail.mcu.edu.tw

Introduction

Lychee (*Litchi chinensis*) is a tropical fruit that has been widely cultivated in China and southeastern Asia for hundreds of years. Lychee fruits are harvested from May to August and usually eaten fresh. The lychee fruit is sweet with perfume-like flavor and is therefore popular in Taiwan. Although lychee is very popular, studies on lactic acid bacteria (LAB) distribution in lychee fruits remain scarce.

Diversity of LAB in various fruits, such as banana, ripe mulberries, watermelons and peaches have been previously studied in our laboratories (CHEN & al [1]; CHEN & al [2]; CHEN & al [3]; CHEN & al [4]). Various LAB species, included in genera *Lactobacillus*, *Weissella*, *Leuconostoc*, *Lactococcus* and *Enterococcus* were previously found in these fruits. In the genus *Leuconostoc*, species of *Leuc. mesenteroides* and *Leuc. pseudomesenteroides* were frequently found in these studies (CHEN & al [1]; CHEN & al [2]; CHEN & al [3]; CHEN & al [4]). When comparing the 16S rRNA gene sequences of the type strains of *Leuc. mesenteroides* ATCC 8293^T (KC429780) and *Leuc. Pseudomesenteroides* LMG 11482^T (HM443958), similarity approximately 99.57% was observed. It is therefore difficult to classify these two species in the initial step by using 16S rDNA RFLP analysis.

In the previous study of JANG & al. [5], *Leuconostoc*-specific PCR-RFLP method was developed to rapidly identifying *Leuconostoc* isolates from food samples. The only difference between species *Leuc. mesenteroides* and *Leuc. pseudomesenteroides* was observed at the digested patterns by restriction endonuclease *Tsp*509I (JANG & al [5]). Excepting DNA sequencing analysis and methods described by JANG & al. [5], a different method with similar identifying ability was expected.

In addition, bacteriocins produced by LAB can be applied to food preservation and are therefore attracting special interest. Besides isolation and identification, screening for bacteriocin-producing LAB strains always was another objective in our previous studies. A total of nine bacteriocins from *Weissella* have been previously reported which included those from *W. confusa* A3 (2706.68 Da), *W. confusa* MBF8-1 (3956.57, 2877.26, 2419.94 Da), *W. hellenica* QU 13 (4925.12, 4968.40 Da), *W. hellenica* 4-7 (3205.6 Da), *W. paramesenteroides* DX (4450 Da) and *Weissella cibaria* 110 (3487.8 Da) (SRIONNUAL & al [6]; PAPAGIANNI and PAPAMICHAEL [7]; MASUDA & al [8]; LEONG & al [9]; GOH and PHILIP [10]; MALIK & al [11]). On the whole, study on bacteriocin from species *W. confusa* remains few.

The three aims of this study were 1) to isolate and characterize LAB from lychee fruits; 2) to develop a different identification method for classifying *Leuc. mesenteroides* and *Leuc. pseudomesenteroides*; 3) to purify and characterize bacteriocin from lychee isolated strain.

Materials and Methods

Lychee fruits samples

A total of 7 lychee fruit samples were collected at seven traditional markets, located in the following three cities: Taoyuan City (S1-S4), Taipei City (S5 and S6) and Hsinchu County (S7) (Table 1). Each sample was collected aseptically in a sterile plastic bag and delivered to the laboratory within 24 h and immediately analyzed. Multiple lychee fruits collected from the same sampling point were crushed with hard shells and the mixtures were used for isolation.

Table 1. Analyses results and characteristics of isolates

		16S rDNA RFLP groups	**r1**	**r1**	**r2**	**r3**	**r4**	**r5**
		Final group classification	**A**	**B**	**C**	**D**	**E**	**F**
Sample	Location	Viable acid-producing cells (CFU g^{-1})	*Leuc. mesenteroides*	*Leuc. pseudomesenteroides*	*Leuconostoc* sp.	*W. confusa*	*W. cibaria*	*W. thailandensis*
S1	Taoyuan City	*ca.* 10^5				12		
S2	Taoyuan City	*ca.* 10^5	4	4		4		
S3	Taoyuan City	*ca.* 10^3				2	2	
S4	Taoyuan City	*ca.* 10^5	2	9	1			
S5	Taipei City	*ca.* 10^7	17	10		1		
S6	Taipei City	*ca.* 10^5	3	6			1	
S7	Hsinchu County	*ca.* 10^7	10	5		2	6	3
	Total		**36**	**34**	**1**	**21**	**9**	**3**

Abbreviations: Leuc., *Leuconostoc*; W., *Weissella*.

Isolation of LAB

To isolate LAB, dilutions of the resultant solution (10^{-1} to 10^{-6} fold) were spread directly onto the surface of Man Rogosa and Sharpe (MRS) (BD, Franklin Lakes, NJ, USA) agar plates and then incubated at 30°C for 3-5 days under anaerobic conditions (Mitsubishi AnaeroPak™ System, Pack-Anaero; *Mitsubishi* Gas Chemicals, Tokyo, Japan). To distinguish acid-producing bacteria from other bacteria, $CaCO_3$ with a final concentration of 1% was added to the MRS agar and the number of viable acid-producing cells were counted (CHEN & al [4]). Only colonies with a clear zone around them were randomly selected. The randomly selected colonies were streaked on new plates in order to purify the colonies for further characterization. Only Gram-positive, catalase-negative strains were kept and then stored at -80°C in 100 g L^{-1} skim milk broth.

Grouping and identifying of LAB isolates

Colony PCR method (SHEU & al [12]) was used for DNA amplification. PCR reactions were carried out using a Genomics *Taq* gene amplification PCR kit (Genomics, Taipei, Taiwan) and performed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The 16S rDNA RFLP was performed to cluster the LAB isolates into some genotypically related groups for subsequent identification. Three different restriction endonucleases described by Chen & al. [4], *Hae*III (GG/CC), *Alu*I (AG/CT) and *Msp*I (C/CGG), were used to generate the main groups. The isolates were identified to the species level by sequencing the 16S rRNA gene as described previously (CHEN & al [4]). DNA sequencing was performed using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were subjected to nucleotide-nucleotide BLAST using blastn in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/).

Differentiation of *Leuc. mesenteroides* and *Leuc. pseudomesenteroides*

It is found that the housekeeping gene *rpoA* similarity between *Leuc. mesenteroides* (AM711294) and *Leuc. pseudomesenteroides* (AM711315) is approximately 87.06% which much lower than that of 16S rRNA (99.57%). An approach from combination of *rpoA* gene and RFLP was therefore purposed. Amplification of *rpoA* gene was following the conditions described by NASER & al. [13]. Potential restriction enzymes were screened by using the simulation option of the Genetyx-Win program ver. 5.1 (Genetyx Co., Japan). Restriction fragments were visualized on a 2 % agarose gel in 1× TAE.

Screening for bacteriocin-producing strain

The agar spot test method described by SCHILLINGER and LÜCKE [14] was used to screen the antibacterial activities of isolates. For initial screening, antibacterial activity of colony was detected. *W. paramesenteroides* BCRC 14006^T was used as the indicator strain in this study. Antibacterial activity was further confirmed by pH adjustment and proteinase K treatment (SRIONNUAL & al [6]).

Purification of bacteriocin

The cell-free supernatant was initially loaded onto a strong cation-exchange column at 8 mL/min (TOYOPEARL GigaCap S-650M, Tokyo, Japan). Eluted fractions were collected and tested for activity against *W. paramesenteroides* BCRC 14006^T. Secondly, active fractions were collected then loaded onto a hydrophobic interaction column (TOYOPEARL Phenyl-650M, Tokyo, Japan). Eluted fractions with activities were collected then loaded onto a C_{18} cartridge (Sep-Pak C_{18}, Waters, Milford, MA, USA). The cartridge was initially washed with 5 column volumes of milli Q water and then eluted by using 20, 40, 60 and 100% acetonitrile/milli Q water (v/v) containing 0.1% TFA. Bacteriocin activity was determined with the same method described above.

The crude bacteriocin was further purified by reverse-phase HPLC on a HITACHI machine (5160, 5430; Tokyo, Japan) in a VP 260/10 NUCLEODUR C_{18} HTec column (5 µm, Macherey-Nagel, Germany), using gradients of (B) acetonitrile in (A) H_2O, both containing 0.1% TFA. Peptides were initially separated by the following gradient: 10-20% B over 10 min, then 20–100% B over 55 min and 100% B over 5 min. Antimicrobial activity of the peptide fractions was tested using the agar-spot test by using the *W. paramesenteroides* BCRC 14006^T as the indicator strain. Fraction showed inhibitory ability was repurified on the HPLC system using the same column. Conditions for the second purification were as follows: 10–51% B over 10 min, then 51-56% B over 50 min and 56-100% B over 5 min. The bacteriocin activity of each observed peak was determined with the same method described above; the corresponding fractions then were stored at -20°C until further study.

Mass spectrometry

MS analysis was performed on a mass spectrometer (Bruker Microflex, Bruker Daltonics, Bremen, Germany).

Inhibition spectrum of bacteriocin

The antibacterial activity of purified bacteriocin was tested against several Gram-positive and Gram-negative bacteria listed in Table 2. Duplicate tests were performed and average values were calculated.

Results

The number of viable acid-producing cells observed in each lychee fruit samples were showed in Table 1. A total of 104 acid-producing bacteria were isolated from the lychee fruit samples. The 104 isolates were temporarily classified into 5 groups (r1 to r5; Fig. 1A) according to cell morphology and the results of the 16S rDNA RFLP analysis. In detail, 70 were placed in Group r1, 1 in Group r2, 21 in Group r3, 9 in Group r4 and 3 in Group r5, according to similarities in RFLP patterns after DNA digestion with *Hae*III, *Alu*I and *Msp*I.

Representative strains in each group were randomly selected, and 16S rRNA gene sequencing analysis was performed. The results identified Group r1 isolates as *Leuc. mesenteroides* or *Leuc. pseudomesenteroides*, Group r2 as *Leuconostoc* sp., Group r3 as *Weissella confusa*, Group r4 as *Weissella cibaria* and Group r5 as *Weissella thailandensis*.

The simulation results from the Genetyx-Win program ver. 5.1 (Genetyx Co., Japan) showed that *Acc*II (CG/CG) digested fragments of the *rpoA* PCR product were useful for differentiating *Leuc. mesenteroides* from *Leuc. pseudomesenteroides*. Isolates in Group r1 were further verified based on *Acc*II digested fragments of their *rpoA* PCR product. A total of 36 strains were identified as *Leuc. mesenteroides* and re-classified into Group A (Fig. 1B, lane 1; Table 1). The remaining 34 strains were identified as *Leuc. pseudomesenteroides* and re-classified into Group B (Fig. 1B, lane 2; Table 1). Isolates in Groups r2 to r5 were therefore re-classified into Groups C to F (Table 1).

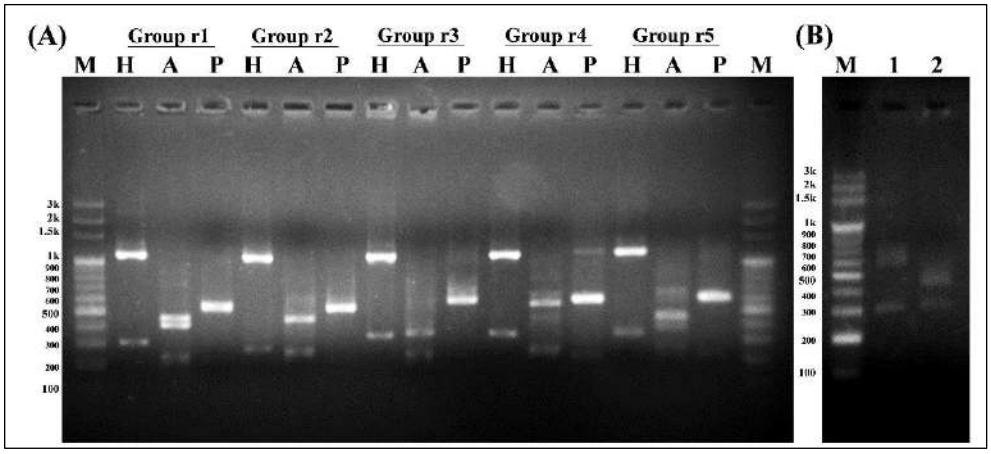

Figure 1. (**A**) 16S rDNA RFLP patterns of *Hae*III, *Alu*I and *Msp*I digests from Groups r1 to r5. Lane M, size marker; H, *Hae*III restriction pattern; A, *Alu*I restriction pattern; P, *Msp*I restriction pattern. (**B**) *Acc*II digested patterns of *rpoA* gene. Lane M, size marker; 1, *Leuc. mesenteroides* isolate; 2, *Leuc. pseudomesenteroides* isolate.

Strain MB7 in Group C showed the highest sequence homology, up to 99.25%, with the 16S rRNA gene sequences (LC259518), 90.07% with the *rpoA* gene sequences (LC259520) and 86.29% with the *pheS* gene sequences (LC259519) of *Leuconostoc mesenteroides* subsp. *suionicum* DSM 20241^T (CP015247, AM711330 and AM711232). Based on the results obtained in the current study, strain MB7 was identified as *Leuconostoc* sp.

Bacteriocin producing ability of all 104 strains were determined and only *W. confusa* strain E showed antibacterial activities against the indicator strain *W. paramesenteroides* BCRC 14006^T (Table 2). Bacteriocin from *W. confusa* strain E maintained its antibacterial activity after neutralization (pH 6.8); however, activity was completely lost after treatment with Proteinase K.

The supernatant from *W. confusa* strain E was serially purified by cation-exchange and hydrophobic interaction columns. These active fractions obtained from hydrophobic interaction column were desalted by using the C_{18} cartridges (Sep-Pak C_{18}, Waters, Milford, MA, USA) and then purified using reverse-phase HPLC. One well-separated peak (retention time: approximately 21.28 min) with bacteriocin activity was observed in this second reverse-phase chromatography step (Fig. 2).

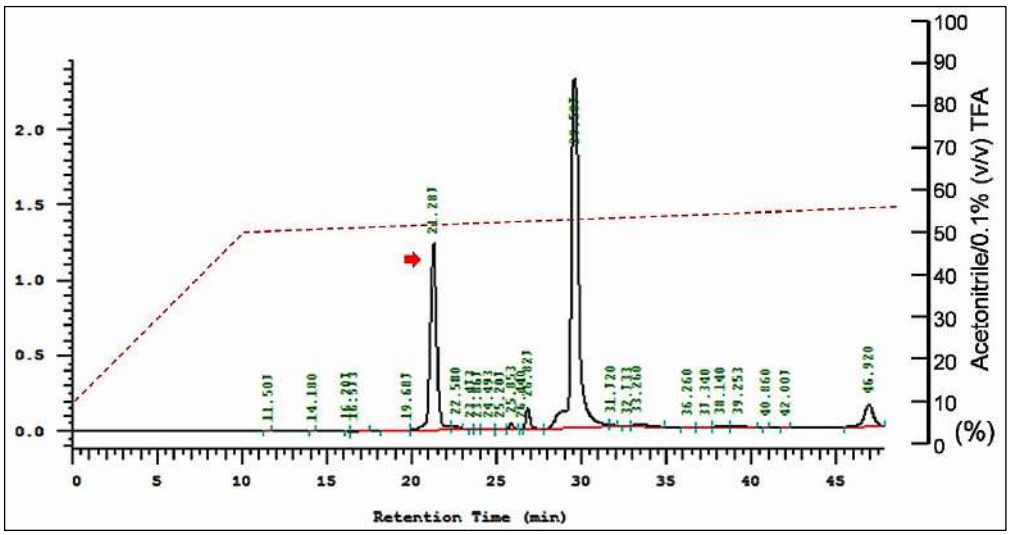

Figure 2. The second purification procedure of C_{18} reverse-phase chromatography profile of the bacteriocin from *W. confusa* strain E: the peak with bacteriocin activity was observed at 21.28 min, indicated by red arrow at top. The red dotted line indicated the concentration of acetonitrile.

The purified bacteriocin was also subjected to MALDI-TOF MS analysis in order to accurately determine its molecular mass, which was found to be 3426.77 Da (Fig. 3). The antagonistic effect of purified bacteriocin from *W. confusa* strain E on different Gram-positive and Gram-negative bacteria was tested (Table 2). The bacteriocin from *W. confusa* strain E showed inhibitory activity to growth of *Lis. monocytogenes* BCRC 14845 and some other LAB species as listed in Table 2.

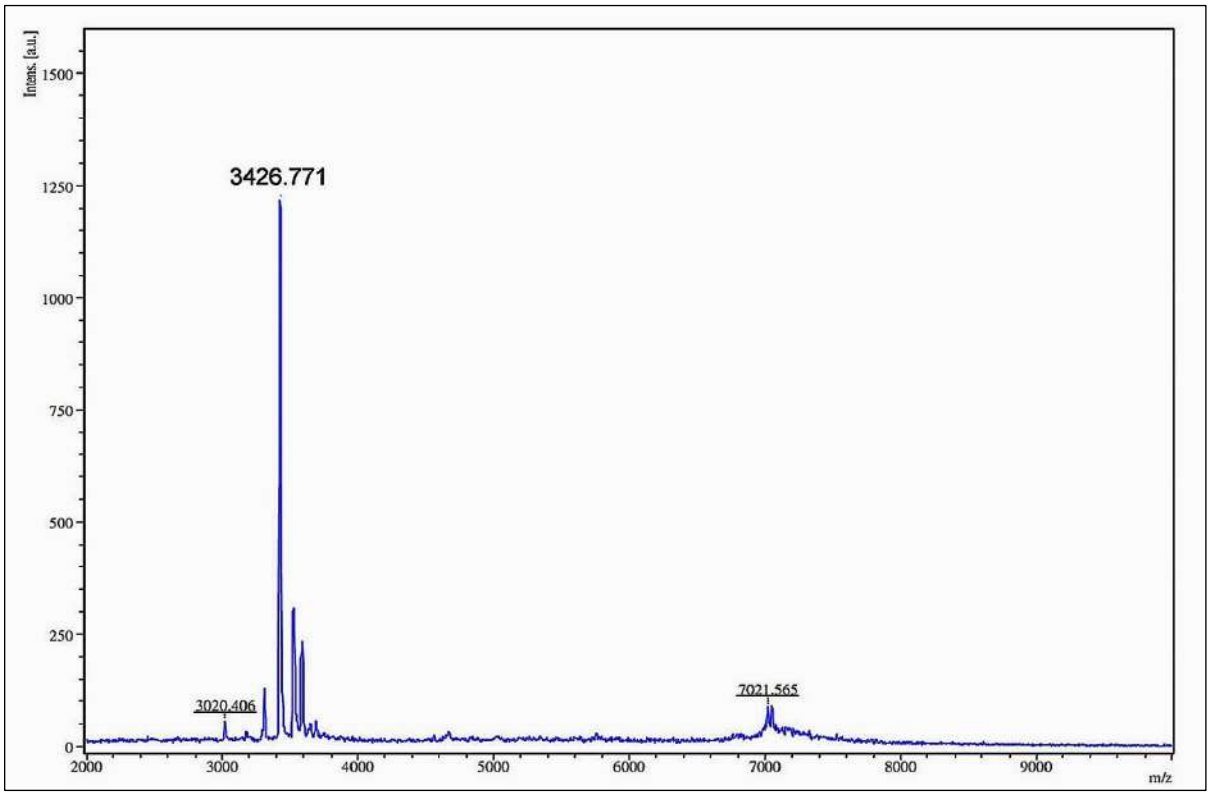

Figure 3. MALDI-TOF mass spectrum analysis of the purified bacteriocins from *W. confusa* strain E. Bacteriocin yielded a fragment with a mass (m/z) of 3426.77 Da.

Table 2 Inhibitory spectra of bacteriocin from *W. confusa* strain E.

Indicator strains	Medium	Incubation temp. (°C)[†]	Inhibitory ability [α]
Lis. monocytogenes BCRC 14845	BHI	37	+
L. sakei subsp. *sakei* JCM 1157^T	MRS	30	—
L. crustorum JCM 15951^T	MRS	30	—
L. farciminis BCRC 14043^T	MRS	30	—
L. formosensis S215^T	MRS	30	—
Lc. lactis subsp. *lactis* BCRC 12312^T	MRS	30	—
Lc. lactis subsp. *cremoris* BCRC 12586^T	MRS	30	—
Lc. lactis subsp. *hordniae* BCRC 80474^T	MRS	30	—
Lc. taiwanensis BCRC 80460^T	MRS	30	—
W. paramesenteroides BCRC 14006^T	MRS	30	+
W. viridescens BCRC 11650^T	MRS	30	+
Ent. pseudoavium NBRC 100491^T	MRS	30	+
Ent. gilvus NBRC 100696^T	MRS	30	+
Escherichia coli BCRC 11775	LB	37	—
Acinetobacter baumannii ATCC 10591^T	LB	37	—
B. thuringiensis BCRC 14616	LB	37	—
B. subtilis subsp. *subtilis* ATCC 6051^T	LB	37	—

Abbreviation: Lis., *Listeria*; L., *Lactobacillus*; Lc., *Lactococcus*; W., *Weissella*; Ent., *Enterococcus*; B., *Bacillus*.
[α]Determined using the agar spot test.
[†]The temperature used to grow the indicator strain during exposure to bacteriocin.
+, inhibitory zone observed; –, no inhibitory zone observed.

Discussion

In the current study, heterofermentive *Leuconostoc* and *Weissella* species were the most common genera observed in the 7 analyzed lychee samples. On the whole, *Leuc. mesenteroides* and *Leuc. pseudomesenteroides* were the dominant species found in the lychee samples (Table 1). They were found in 5 of 7 lychee samples, excepted lychee samples S1 and S3. Similar results were also observed at the distribution of *W. confusa*. In contrast to *Leuc. mesenteroides*, *Leuc. pseudomesenteroides* and *W. confusa*, the remaining LAB species were less found in these lychee samples. Although samples S1 to S4 were collected in the same city, differences in diversity were observed. On the other hand, samples S2 and S5 were collected in different cities, similar diversity was observed. In addition, LAB species found in this study, such as *Leuc. mesenteroides*, *Leuc. pseudomesenteroides*, and *W. cibaria*, have been also previously found in other Taiwanese fruits (CHEN & al [1]; CHEN & al [2]; CHEN & al [3]; CHEN & al [4]). Regional similarities and differences in diversity were observed in the current study.

In this study, *Acc*II digested fragments of the *rpoA* PCR product were used to classify species *Leuc. mesenteroides* and *Leuc. pseudomesenteroides*. A total of 70 *Leuconostoc* isolates were therefore exactly divided to the group which they belonged. To confirm its feasibility, more isolates in Groups A and B were selected and then checked by analyzing their *rpoA* gene sequences. The obtained results suggested that this method was feasible for classification. Nevertheless, a more efficient identification method for *Leuconostoc* species with high gene similarities, such as species-specific PCR, was still desired.

In addition, strain MB7 showed low similarities to the type strains in the results of *pheS* and *rpoA* gene analysis. The obtained results of genetic analyses suggest the possibility of being a potential novel species or subspecies of *Leuconostoc*. To clarify this, additional genetic and physiological information, such as GC content, saccharide fermentation ability and DNA relatedness values between strains and the closest type strains, are necessary.

Studies on bacteriocins from *W. confusa* remain scarce. The molecular size of bacteriocin from *W. confusa* strain E (3426.77 Da) was different to those produced by *W. confusa* A3 (2706.68 Da) and *W. confusa* MBF8-1 (3956.57, 2877.26, 2419.94 Da) (GOH and PHILIP [10]; MALIK & al [11]). Besides difference in molecular size, bacteriocin from *W. confusa* strain E showed inhibitory ability against *Listeria monocytogenes*, but bacteriocin from strain *W. confusa* A3 did not (GOH and PHILIP [10]) (Table 2). A contrasting result was observed at their inhibitory abilities against *Escherichia coli* (GOH and PHILIP [10]) (Table 2). Different characteristics between bacteriocins from *W. confusa* strains E and A3 were observed. Less information about bacterioicns from *W. confusa* MBF8-1 was available and it is therefore difficult to make comparison in the current study. No corresponding molecular size to other *Weissella* bacteriocins was found, this possibly indicating the presence of a novel bacteriocin. To confirm this, the amino acid sequence must be subjected to purification and identification analysis in the future.

In conclusion, this is the first report describing the distribution and varieties of LAB that associated with lychee fruits. A useful classification method for differentiating *Leuc. mesenteroides* from *Leuc. pseudomesenteroides* was purposed. One potential novel species or subspecies of *Leuconostoc* was found in the current study. One potential novel bacteriocin from *W. confusa* was also found in this study. The detailed characteristics of bacteriocin, including amino acid sequences, heat stability, and their sensitivity to enzymes, were not established in the current study. Future studies in our laboratory will characterize and identify the bacteriocin, and we anticipate it will be useful as food preservative.

Conflict of Interest

No conflict of interest declared.

The first two authors contributed equally to this work.

References

1. Y.S. CHEN, H.C. WU, F. YANAGIDA. Isolation and characteristics of lactic acid bacteria isolated from ripe mulberries in Taiwan. *Braz. J. Microbiol.* 41, 916-921 (2010).
2. Y.S. CHEN, H.C. WU, S.F. PAN, B.G. LIN, Y.H. LIN, W.C. TUNG, Y.L. LI, C.M. CHIANG, F. YANAGIDA. Isolation and characterization of lactic acid bacteria from *yan-taozih* (pickled peaches) in Taiwan. *Ann. Microbiol.* 63, 607-614 (2013).
3. Y.S. CHEN, H.C. WU, C.R. YU, Z.Y. CHEN, Y.C. LU, F. YANAGIDA. Isolation and characterization of lactic acid bacteria from *xi-gua-mian* (fermented watermelon), a traditional fermented food in Taiwan. *Ital. J. Food Sci.* 28, 9-14 (2016).
4. Y.S. CHEN, Y.J. LIAO, Y.S. LAN, H.C. WU, F. YANAGIDA. Diversity of Lactic Acid Bacteria Associated with Banana Fruits in Taiwan. *Curr. Microbiol.* 74, 484-490 (2017).
5. J. JANG. B. KIM, J. LEE, H. HAN. A rapid method for identification of typical *Leuconostoc* species by 16S rDNA PCR-RFLP analysis. *J. Microbiol. Methods* 55, 295-302 (2003).

6. S. SRIONNUAL, F. YANAGIDA, L.H. LIN, K.N. HSIAO, Y.S. CHEN. Weissellicin 110, a newly discovered bacteriocin from *Weissella cibaria* 110, isolated from *plaa-som*, a fermented fish product from Thailand. *Appl. Environ. Microbiol.* 73, 2247-2250 (2007).
7. M. PAPAGIANNI and E.M. PAPAMICHAEL. Purification, amino acid sequence and characterization of the class IIa bacteriocin weissellin A, produced by *Weissella paramesenteroides* DX. *Bioresour. Technol.* 102, 6730-6734 (2011).
8. Y. MASUDA, T. ZENDO, N. SAWA, R.H. PEREZ, J. NAKAYAMA, K. SONOMOTO. Characterization and identification of weissellicin Y and weissellicin M, novel bacteriocins produced by *Weissella hellenica* QU 13. *J. Appl. Microbiol.* 112, 99-108 (2012).
9. K.H. LEONG, Y.S. CHEN, Y.H. LIN, S.F. PAN, B. YU, H.C. WU, F. YANAGIDA. Weissellicin L, a novel bacteriocin from sian-sianzih-isolated *Weissella hellenica* 4-7. *J. Appl. Microbiol.* 115, 70-76 (2013).
10. H.F. GOH AND K. PHILIP. Purification and characterization of bacteriocin produced by *Weissella confusa* A3 of dairy origin. *PLoS One* 10, e0140434 (2015).
11. A. MALIK, S. SUMAYYAH, C.W. YEH, N.C. HENG. Identification and sequence analysis of pWcMBF8-1, a bacteriocin-encoding plasmid from the lactic acid bacterium *Weissella confusa*. *FEMS Microbiol. Lett.* 363, pii: fnw059 (2016).
12. D.S. SHEU, Y.T. WANG, C.Y. LEE. Rapid detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. *Microbiology* 146, 2019-2025 (2000).
13. S.M. NASER, F.L. THOMPSON, B. HOSTE, D. GEVERS, P. DAWYNDT, M. VANCANNEYT, J. SWINGS. Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. *Microbiology* 151, 2141-2150 (2005).
14. U. SCHILLINGER and F.K. LÜCKE. Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl. Environ. Microbiol.* 55, 1901-1906 (1989).