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Antifungal Activity of *Leuconostoc citreum* and *Weissella confusa* in Rice Cakes

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The antifungal activity of organic acids greatly improves the shelf life of bread and bakery products. However, little is known about the effect of lactic acid fermentation on fungal contamination in rice cakes. Here, we show that lactic acid fermentation in rice dough can greatly retard the growth of three fungal species when present in rice cakes, namely *Cladosporium* sp. YS1, *Neurospora* sp. YS3, and *Penicillium crustosum* YS2. The antifungal activity of the lactic acid bacteria against these fungi was much better than that of 0.3% calcium propionate. We found that organic acids including lactic and acetic acid, which are byproducts of lactic fermentation or can be artificially added, were the main antifungal substances. We also found that some *Leuconostoc citreum* and *Weissella confusa* strains could be good starter species for rice dough fermentation. These results imply that these lactic acid bacteria can be applicable to improve the preservation of rice cakes.

Keywords: lactic acid bacteria, antifungal activity, food safety, rice dough, rice cake

Introduction

Rice cakes are popular food items in Asia and are made from medium-grain ground white rice containing high proportion of amylopectin. Unlike bread, most rice cakes are steamed, not baked, and thus maintaining the moisture level of rice cakes is considered highly critical. Because rice cakes contain a relatively high moisture level and similar level of calories as bread, it is believed that fungal contamination of rice cakes is comparable to that of bread. However, unlike fungal contamination in bread, the nature of fungal contamination in rice cakes is largely unknown. Ji *et al.* (2007) reported that two *Penicillium* species, *P. citreoviride* and *P. citrinum* can contaminate Chinese traditional rice cake, consistent with the notion that among the spoilage fungi, *Penicillium* species including *P. roqueforti* are the most common spoilage organism found in contaminated bread. In bread, it is known that the common spoilage fungi belong to the genera, *Aspergillus*, *Cladosporium*, *Endomyces*, *Fusarium*, *Neurospora*, and *Penicillium* (Ryan *et al.*, 2008). In addition, a fungal group belonging to the genus *Aspergillus*, *Fursarium*, and *Penicillium* was found in contaminated rice samples (Park *et al.*, 2005), which suggests that the above-mentioned fungal species that contaminate bread might also contaminate rice cakes.

Chemical preservatives such as calcium propionate and sorbic acid, and sourdoughs containing natural antifungal products such as organic acids, fatty acids, and bioactive peptides from lactic acid bacteria are commonly used to extend the shelf life of bread. However, the effects of these preservatives and antifungal products on preventing and delaying fungal contamination in rice cakes have not yet been examined.

In this study, we showed that acetic and lactic acid produced by lactic acid bacteria such as *Leuconostoc citreum* and *Weissella confusa* were very effective in inhibiting the growth of the three fungal isolates, namely *Clostridium* sp. YS1, *Neurospora* sp. YS3, and *Penicillium crustosum* YS2. We also found that rice dough fermentation by lactic acid bacteria can increase the shelf life of rice cakes, indicating that the metabolites of lactic acid fermentation can effectively inhibit fungal growth in rice cakes.

Materials and Methods

Lactic acid bacterial strains

All lactic acid bacteria (LAB) with the exception of the type strains used in this study were previously isolated from different kimchi samples (Cho *et al.*, 2006), a fermented cabbage product, and cultured on MRS medium at 25°C unless indicated otherwise. Molecular identification of LAB was previously performed using 16S rRNA gene analysis (Jang *et al.*, 2002). All LAB used in this study were as follows: *L. citreum* KCTC 3524, KCTC 3526, IH22, I-11, I-12, II-3; *Lc. carnosum* D2-284; *Lc. gelidum* D2-185, D2-109, D2-182, DSM 5578; *Lc. gasicomitatum* D2-283, KCTC 3753, BCCM 18811, I-13, I-15, II-5; *Lc. mesenteroides* KCTC 3505, HO13, D2-221, KCCM 11325, I-22, I-23, I-24, II-14, II-8; *Lc. kimchii* D2-224, IH25, I-17, I-18, II-2; *Lc. lactis* D2-329, KCTC 3528, II-12, II-4; *Lc. inhae* D2-191, IH003; *Lactobacillus curvatus* KCTC 3767; *Lb. paraplantarum* SS-25, I-3; *Lb. pentosus* D2-44; *Lb. sakei* D2-212, KCTC 3603, DSM 20017, II-10; *Lb. plantarum* II-7, I-6; *Weissella cibaria* D2-120, KCTC 3807, II-13, II-9; *W. confusa* D2-96, HO24; *W. koreensis* D2-332, KCCM 41516, I-29, II-11.

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Isolation of fungal strains from rice cakes

Baek-sul-gi, a type of rice cake, was made by kneading 100 g of rice flour (33% water content), 10 g sugar, 1 g table salt, and 20 ml tap water. The rice dough was then steamed for 20 min in a steamer (Samwoo, Korea). Rice flour was made by grinding water-soaked medium-grain white rice two times with a roll mill (Samwoo, Korea). For later use, the rice flour was vacuum-packaged by a packaging machine (Tecnovac, Italy) and stored at -20°C. The steamed loaf was cooled at room temperature and sealed with wrap (LLD-PE Cleanwrap, Korea) containing 10 pores and then stored at room temperature until fungal growth was observed. Three different types of fungal strains were isolated and successively cultured on PDA (potato dextrose agar) media (Dickinson, USA). After identification, the fungal strains were named as follows: *Cladosporium* sp. YS1, *P. crustosum* YS2, *Neurospora* sp. YS3.

Molecular identification of lactic acid bacteria from rice dough

LAB strains from rice dough were cultured on MRS media and identified by 16S rRNA gene sequence analysis as previously described (Kim *et al*., 2000) or by REP-PCR (repetitive element-based PCR) according to the method reported by Versalovic *et al*. (1991). DNA extraction using bacterial culture on MRS broth was performed using the TEN-MINUTE DNA preparation method from yeast (Hoffman and Winston, 1987). Two degenerate primers used for REP-PCR were as follows: 1R (5′-IIINCGNCGNCATCNGGC-3′) and 2R (5′-N CGNCTTATCNGGCCTAC-3′), where I indicates inosine and N indicates A, G, T or Cs.

Phylogenetic analysis of fungal strains

For fungal identification, we analyzed both the rDNA and *β-tubulin* gene sequences. Amplification of rDNA using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATG-3′) produces partial sequence of the 18S and 28S rRNA, full sequence of 5.8S RNA gene, and internal transcribed sequence 1 & 2. Amplification of the *β-tubulin* gene using the primers Bt2a (5′-GGTAA CCAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCA GTGTAGTGACCCTTGGC-3′) produces short DNA fragment containing exon 3, 4, 5, and intron 3 and 4 of the *β-tubulin* gene (Sheir-Neiss *et al*., 1978). Polymerase chain reaction (PCR) was performed as previously described (Cho *et al*., 2006). The PCR products were sequenced by Solgent Co. (Korea). Gene sequences were compared with nucleotide sequence data in GenBank of the National Center for Biotechnology Information using the BLAST program. DNA sequences were deposited in GenBank database under accession numbers JF508483–JF508488. Multiple alignment of DNA sequences was performed using Clustal X software (Thompson *et al*., 1997) and phylogenetic analyses were performed using PHYLIP software (Felsenstein, 1989).

Preparation of antifungal fermentation supernatants and fungal spores

Lactic acid bacteria were cultivated on MRS medium at 25°C for 48 h. Supernatant collected after centrifugation of LAB cultures was used to examine antifungal activities. Fungal conidia were collected from 7-d old fungal cultures on PDA using 1% (v/v) Tween 80 and the concentration of conidia was determined by using a hemocytometer.

Rice dough fermentation and fungal growth on rice cakes

For rice dough fermentation, rice dough was made using the same protocol described for the preparation of regular rice cake, with the addition of 35 ml of tap water and 1×10^9 CFU of fresh LAB per g of dough. The rice dough was incubated at 25°C for 24 h. After cultivation in MRS, LAB cultures were washed once with 20 mM phosphate buffer (pH 7.0). For analysis of organic acids and pH, we prepared water-soluble extracts using fermented rice dough. 1 g of fermented dough was diluted in 10 ml of distilled water, vortexed for 10 min, kept at 4°C for 3 h, and centrifuged at 15,000×g for 10 min. The resulting supernatant was used for HPLC analysis or pH measurements. To measure the fungal growth on rice cakes made from fermented rice dough, rice dough inoculated with *L. citreum* or *W. confusa* was incubated at 30°C for 9 h or 24 h before the rice cakes were made. Fermented rice dough was steamed for 20 min, kneaded and used to make small disk rice cakes [50 mm (diameter) ×10 mm (thick)]. In contrast to sourdough bread, these rice cakes can be made from 100% fermented rice dough, because no yeast strains are added to make rice cakes. About 20 fungal spores were inoculated onto each cake, which was then incubated at 25°C for 24 h.

Organic acids

Organic acids in the supernatant of lactic acid bacterial cultures in the MRS broth or water-soluble extracts from the fermented rice dough were determined as previously described (Cho *et al*., 2006). Briefly, 500 µl of fermentation supernatant (FS) was mixed with 500 µl of 0.2% H_3PO_4 (Sigma). The mixture was centrifuged and then filtered through 0.2 µm filters. Organic acids were analyzed by high performance liquid chromatography. Acetic acid (70 mM), DL-lactic acid (70 mM), and organic acid analysis standard (containing oxalate, citrate, malate, succinate, formate, and acetate) (Bio-Rad) were used as standard acids. An Aminex HPX-87H column (Bio-Rad) was used to analyze FS (30 µl). HPLC analysis was performed at 60°C using 0.1% H_3PO_4 with a flow rate of 0.8 ml/min.

Antifungal tests

Microdilution tests were performed using 190 µl of FS and 10 µl of conidial suspension in 96 well plates (SPL life science, Korea). FSs were directly used without adding fresh medium in the microdilution tests, because these FSs are believed to contain sufficient amount of nutrients for further fungal growth. Indeed, normal fungal growth occurred when the pH of these FSs was adjusted to 8.0. All samples were placed in triplicate on the plates. The plates were incubated in a humid chamber at 25°C for 96 h and fungal growth was assessed by measuring the optical density (OD) at 595 nm using a spectrophotometer (SUNRISE, Tecan, Austria). Fungal growth inhibition was calculated by comparing the

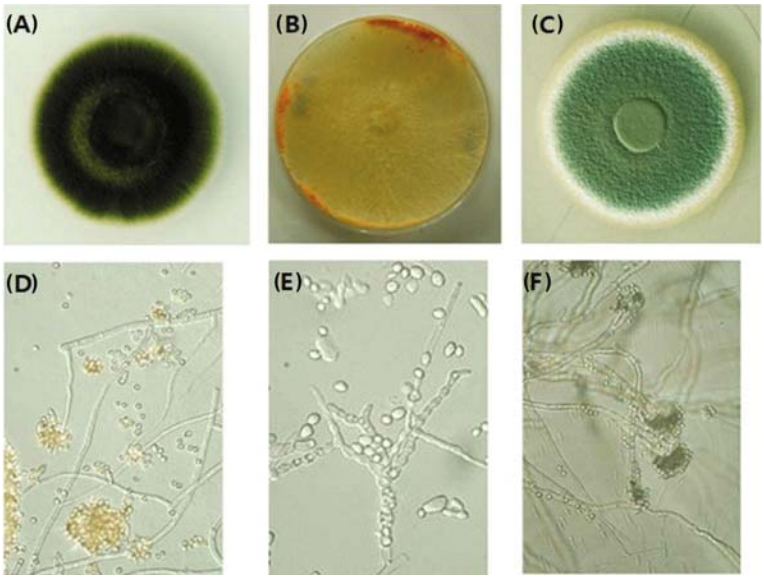

Fig. 1. Three newly isolated fungal species from rice cakes. (A–C) Colony morphology of fungal isolates on PDA agar. (D-F) Conidial morphology of fungal isolates (63× objective lens). (A & D) *Cladosporium* sp. YS1. (B & E) *Neurospora* sp. YS3. (C & F) *P. crustosum* YS2.

fungal growth on FSs or on MRS containing 0.3% calcium propionate with fungal growth on MRS broth.

The overlay method was carried out as follows: 5 μl of fresh LAB cultures on MRS medium was seeded on MRS plate and then incubated at 30°C for 48 h. After this incubation period, 10 ml of soft PDA agar (0.7%) containing fungal spores (10^6 spores/ml) was spread on the MRS agar (1.5%) plate. This plate was incubated at 25°C for 48 h (Magnusson and Schnurer, 2001).

Effects of pH and proteolysis on antifungal activity

To analyze the effect of pH on the antifungal activity of FS, the pH of FS was adjusted to pH 4.5, 5.0, 5.5, and 8.0 using 2 M NaOH, and the antifungal activity of the pH-adjusted supernatant was measured using the microdilution test. To adjust the pH of the fresh MRS medium, 252 μl of hydrochloric acid was added to 50 ml of the MRS medium and this mixture was sequentially diluted to adjust the pH (pH 4.6, 5.1, and 5.6).

The pH-adjusted FSs (pH 8.0) were treated with proteinase K (final concentration: 100 μg/ml) (Sigma) and incubated at 37°C for 1 h and then heat-inactivated at 92°C for 1 h. Fungal growth inhibition was measured at this adjusted pH or at the re-adjusted original pH in the microdilution tests.

Results and Discussion

Identification of three fungal strains isolated from rice cakes

Preventing fungal contamination in rice cakes is an important issue. However, the fungal species that frequently contaminate rice cakes are still largely unknown. To identify spoilage fungal species, we first isolated three morphologically different fungal strains from contaminated rice cakes. These three fungal strains, named YS1, YS2, and YS3, were determined to be *Cladosporium* sp., *Penicillium crustosum*, and *Neurospora* sp., respectively, by analyzing both rRNA gene related ITS (internal transcribed spacer) regions and *β-tubulin* gene. Strain YS1 appeared to be a relative of *Cladosporium sphaerospermum*. The ITS region of the YS1 strain (GenBank no. JF508486) was almost identical to that of *C. sphaerospermum* (99.6%; HQ248189) and very similar to those of other *Cladosporium* species (96%–97% identity). However, the *β-tubulin* gene of this strain (JF508483) was not highly similar to those of *C. sphaerospermum* (85% identity) or other *Cladosporium* species (80–85%), suggesting that YS1 may be a novel *Cladosporium* species that is close to *Mycospaerella hyperici* (data not shown). Colony morphology on MRS or PDA and coronate sporing structure were similar to those of some *Cladosporium* species (Figs. 1A and 1D).

Strain YS2 was identified as *Penicillium crustosum*. The ITS region of the YS2 strain (JF508487) was identical only to that of *P. crustosum* (100%; HQ026728), and similar to those of other *Penicillium* species (98.8–99.4%). The *β-tubulin* gene of this strain (JF508484) was also identical to that of *P. crustosum* (100%; AY674351), and similar to those of other *Penicillium* species (91–97.5%), which suggests that the YS2 is *P. crustosum*. The YS2 strain also had the typical colony morphology on PDA and terverticillate conidial structures of *P. crustosum*, supporting the molecular identification (Figs. 1C and 1F).

Strain YS3 belongs to the genus *Neurospora*. This strain had an identical ITS sequence (JF508488) to that of *Neurospora sitophila* (100%; AF388926), while showing only one mismatch with the ITS of *N. tetrasperma* (505/506; GU327631). *β-Tubulin* gene (JF508485) analysis showed that the YS3 strain was a close relative of *N. tetrasperma* (98.9%; AY681227). However, its relatedness to *N. sitophila* was not analyzed since the *β-tubulin* sequence of *N. sitophila* is still unknown. Therefore, we were unable to determine the identity of YS3. The YS3 strain showed typical morphological (red mold-like) and conidial pattern on PDA as the *Neurospora* species (Figs. 1B and 1E).

To our knowledge, no article describes fungal isolates from rice cakes. However, given similar nutritional aspects of

Table 1. Organic acid content and antifungal activity of fermentation products of some representative LAB strains

Strains	Concentration (mM)±SD[a]		pH	Fungal growth (%)±SD[a]		
	Lactic acid	Acetic acid		*Cladosporium* sp.	*Penicillium crustosu*	*Neurospora* sp.
MRS	-	36.1 ± 0.2	6.1[b]	100.0 ± 0.7	100.0 ± 0.8	100.0 ± 0.7
MRS+0.3% CP	-	36.7 ± 0.2	6.1[c]	36.1 ± 11.0	127.0 ± 30.3	213.1 ± 41.9
Lc. citreum IH22	55.8	44.4	4.3	27.6 ± 0.2	26.5 ± 0.4	36.4 ± 20.2
Lc. gelidum D2-185	67.1	50.94	4.4	25.3 ± 0.4	24.3 ± 0.8	25.1 ± 0.1
Lc. mesenteroides D2-221	56.6	37.4	4.5	25.1 ± 0.6	24.3 ± 0.1	25.3 ± 0.3
Lc. inhae IH003^T	50.1	74.8	4.5	25.4 ± 0.9	24.3 ± 0.1	25.1 ± 0.5
Lb. curvatus KCTC 3767^T	61.1	44.0	3.8	24.6 ± 0.1	23.5 ± 0.1	23.1 ± 2.4
Lb. plantarum I-6	53.7	35.0	4.4	25.3 ± 0.4	24.0 ± 1.0	25.1 ± 0.58
Lb. sakei D2-212	ND	ND	4.0	25.3 ± 0.2	24.3 ± 0.3	25.0 ± 0.3
W. cibaria II-9	56.4	38.6	4.4	25.9 ± 0.5	24.5 ± 0.3	34.4 ± 26.5
W. confusa D2-96	60.3	41.6	4.4	25.2 ± 0.1	24.2 ± 0.5	25.0 ± 0.6

[a] Percent fungal growth, which was calculated by comparing fungal growth on MRS in the presence of fermentation supernatants with fungal growth on MRS medium±standard deviation of quadruplicate experiments.
[b] MRS medium without any fermented supernatant was used for fungal growth.
[c] MRS medium containing 0.3% calcium propionate was used for fungal growth.
CP, calcium propionate; ND, not determined

rice to wheat, it is not surprising that genera *Cladosporium*, *Neurospora*, and *Penicillium* are commonly found from contaminated bakery and rice products.

Antifungal activity of lactic acid bacteria

To analyze the antifungal activity of lactic acid bacteria (LAB) against three fungal isolates, YS1, YS2, and YS3, we assessed inhibition of fungal growth by sixty-four different LAB, which were previously isolated from kimchi or a few type strains. We found that all 64 LAB showed strong and similar antifungal activity against the three fungal isolates. The antifungal activity of some representative strains is shown in Table 1. We hypothesized that this fungal growth inhibition mainly resulted from a high concentration of fermentation products such as lactic acid. Fermentation supernatant (FS) of LAB indeed contained a high concentration of lactic acid (50.1–78.0 mM) as well as acetic acid (0–38.7 mM), suggesting that an acidic environment (pH 3.8–4.5) or organic acids themselves might prevent fungi from rapidly growing on the FS (Table 1). To test this hypothesis, we measured fungal growth on pH-adjusted MRS medium, which was made by adding either acetic (AA) or lactic acid (LA). In these experiments, we found that either acetic (17.5–70 mM) or lactic acid (17.5–70 mM), or a mixture of these acids (AA 17.5–70 mM + LA 17.5–70 mM) could strongly inhibit the growth of all three fungal species, when the concentration of these acids were high enough to induce a mild acidic environment (pH<5.3) (Table 2). The inhibition effect of organic acid may be attributed to the hydrophobic nature of the undissociated form of organic acids. The dissociation constant of acetic acid (pK_a=4.76) is higher than that of lactic acid (pK_a=3.86) (Table 2). This may explain why the growth of *P. crustosum* YS2 on MRS containing 17.5 mM (pH 5.5) or 35 mM lactic acid (pH 5.0) was faster than the growth on MRS containing 17.5 mM (pH 5.6) or 35 mM additional acetic acid (pH 5.3), respectively (Table 2). Under these conditions, the total undissociated organic acids in MRS medium containing LA (pKa=3.86) is slightly lower than that in MRS containing the corresponding amount of AA (pKa=4.76). This effect of organic acid on fungal growth was also confirmed on pH-adjusted MRS media by hydrochloric acid. When the pH of the MRS media was lowered to 4.5 or 5.0, *Cladosporium* sp. YS1 was found to be highly sensitive to both pH conditions, while *P. crustosum* YS2 and *Neurospora* sp. YS3 showed slow growth at pH 5.0, but not at pH 4.5. This fungal growth inhibition may be attributed to the presence of acetic acid (36.7 mM) in the in MRS medium, which can exist primarily in the undissociated form (64.5% of total acetic acid) when pH is 4.5 (Table 1). The concentration of the undissociated form of acetic acid (about 6.8 mM) in the MRS medium at pH 5.4 was calculated to be almost equivalent to that in MRS medium containing an additional 17.5 mM acetic acid at pH 5.6. Indeed, similar fungal growth inhibition was observed under the two conditions, MRS+AA 17.5 (pH 5.6) and MRS+HCl (pH 5.1) (Table 2). When the pH of FS was adjusted to 7.0 or 8.0 by NaOH, normal fungal growth was observed (data not shown), suggesting that the remaining nutrients in FP was sufficient to support normal fungal growth.

This study demonstrated that natural organic acids produced by lactic acid bacteria (LAB), but not a common preservative calcium propionate (0.3%), were sufficient to inhibit normal growth of three new fungi isolated from rice cakes, *Cladosporium* sp. YS1, *P. crustosum* YS2, and *Neurospora* sp. YS3 (Tables 1 and 2). Both acetic and lactic acid were the main organic acids produced by heterofermentative lactic acid bacteria such as *L. citreum* and *W. confusa* in rice cakes or the *in vitro* system, while phenyllactic acid, another well-known organic acid showing strong antifungal activity (Lavermicocca *et al.*, 2000; Ndagano *et al.*, 2011), was not detected in this study. Lactic acid or acetic acid at concentrations of 17.5 mM or higher could retard the growth of both *Cladosporium* sp. YS1 and *P. crustosum* YS2, but not *Neurospora* sp. YS3, in a concentration-dependent manner.

Table 2. Antifungal activity of the organic acids added into the MRS medium

Medium	Organic acid added (mM)	pH	Fungal growth (%)		
			Cladosporium sp.	*P. crustosum*	*Neurospora* sp.
MRS	-	6.3	100.0	100.0	100.0
MRS+LA	70 LA	4.4	21.5 ± 0.6[b]	16.7 ± 3.0	16.6 ± 0.4
	35	5.0	19.4 ± 0.2	20.7 ± 1.4	38.1 ± 32.5
	17.5	5.5	19.1 ± 0.1	54.8 ± 7.7	143.7 ± 4.7
MRS+AA	70 AA	4.9	19.6 ± 0.3	15.1 ± 0.1	15.3 ± 0.2
	35	5.3	19.6 ± 0.1	14.9 ± 0.1	15.0 ± 0.1
	17.5	5.6	19.2 ± 0.1	38.2 ± 3.1	126.6 ± 6.1
MRS+AA+LA	140[a]	4.4	24.4 ± 0.9	19.1 ± 0.3	19.1 ± 0.5
	70	4.8	20.0 ± 0.2	15.4 ± 0.1	15.4 ± 0.1
	35	5.2	19.2 ± 0.2	16.8 ± 2.5	15.6 ± 1.9
	17.5	5.7	19.0 ± 0.1	50.6 ± 2.6	133.9 ± 3.1
MRS+HCl	-	4.6	19.1 ± 0.4	14.7 ± 0.2	14.0 ± 0.6
	-	5.1	18.5 ± 0.1	35.2 ± 3.1	124.7 ± 1.3
	-	5.6	24.2 ± 11.5	76.1 ± 2.3	135.3 ± 1.3

[a] A mixture of organic acids (70 mM AA+70 mM LA) was added into MRS medium; AA, acetic acid; LA, lactic acid.
[b] Percent fungal growth, which was calculated by comparing fungal growth on MRS medium containing additional organic acids with fungal growth on MRS medium ± standard deviation of triplicated experiments.

This result is consistent with several previous studies, which examined a mixture of organic acids including acetic, lactic acid and/or phenyllactic acid as antifungal agents in sourdough fermentation (Corsetti *et al.*, 1998; Lavermicocca *et al.*, 2003; Valerio *et al.*, 2009; Zhang *et al.*, 2010). Phenyllactic acid acts in high concentration (45 mM) as an antifungal agent and the addition of lactic acid was shown to further increase antifungal activity (Lavermicocca *et al.*, 2003). Given that all LAB tested in this study produced a high concentration of total organic acids in both fermented rice dough (pH 3.8–4.3) and *in vitro* system (53–89 mM; pH 3.8–4.5), it is not surprising that all fermentation products of the LAB culture displayed strong antifungal activity against the three fungal strains tested in this study.

The antifungal mechanism of organic acids is not yet fully understood. However, it is known that neutralized solutions of organic acids lose their antifungal activity. This suggests that either proton or the undissociated form of organic acids play a role in fungal inhibition. Our results favor the latter possibility. First, acetic acid showed higher antifungal activity than lactic acid (Table 2). Acetic acid showed a higher antifungal activity than lactic acid at the same concentration, indicating that the higher concentration of the undissociated form in the acetic acid (pK_a 4.76) than lactic acid (pK_a 3.86) might contribute to the stronger antifungal activity. This was assumed because the undissociated form of organic acids carry the hydrophobic property, which is believed to uncouple substrate transport and oxidative phosphorylation or acidify the cytoplasm of target organisms by crossing the cell membrane (Freese *et al.*, 1973). Second, when the commercial MRS medium containing sodium acetate (36.7 mM) was acidified by hydrochloric acid, fungal growth was greatly retarded (Table 2) in a pH-dependent manner, suggesting that the undissociated form of acetic acid in MRS may play a role in fungal inhibition. Meanwhile, the MRS medium containing calcium propionate (40 mM or 0.3%) at pH 6.1 did not show antifungal activity against the two fungal isolates, namely *P. crustosum* YS2 and *Neurospora* sp. YS3 (Table 1), possibly due to the very low level of the undissociated form of both acetic acid (1.6 mM out of 36.7 mM) and propionic acid (1.7 mM out of 40 mM). This finding is consistent with the previous studies, which reported that antifungal activity of calcium propionate (0.3%) was poor against most fungal species including *Penicillium roqueforti* (Lavermicocca *et al.*, 2000; Coda *et al.*, 2008). Our results are also in agreement with other reports (Arroyo *et al.*, 2005; Zhang *et al.*, 2010), which showed that 0.3% calcium propionate (pK_a=4.87) inhibited the growth of *Penicillium verrucosum* completely at pH 4.5, whereas normal fungal growth occurred at pH 6.0.

Proteinase K-resistant antifungal activity

It has been known that some protein substances can also inhibit fungal growth. To test this possibility, proteinase K was used to inactivate protein substances in LAB fermentation supernatants. We found that protein substances did not play a role in this fungal inhibition. Fungal growth retardation was not changed in proteinase K-treated and heat-treated FS, which suggest that antifungal substances are resistant to proteolysis and heat deactivation and therefore the antifungal substances could not be proteins (data not shown).

LAB starters for making rice cakes

We next tested whether fermented rice dough is also resistant to fungal contamination. We found that like sourdough, rice dough fermented at 25°C for 24 h was more resistant to fungal contamination than fresh dough (data not shown). This fungal growth inhibition in fermented rice dough was presumably due to the high concentration of organic acids produced by natural LAB species in rice dough. After 24 h

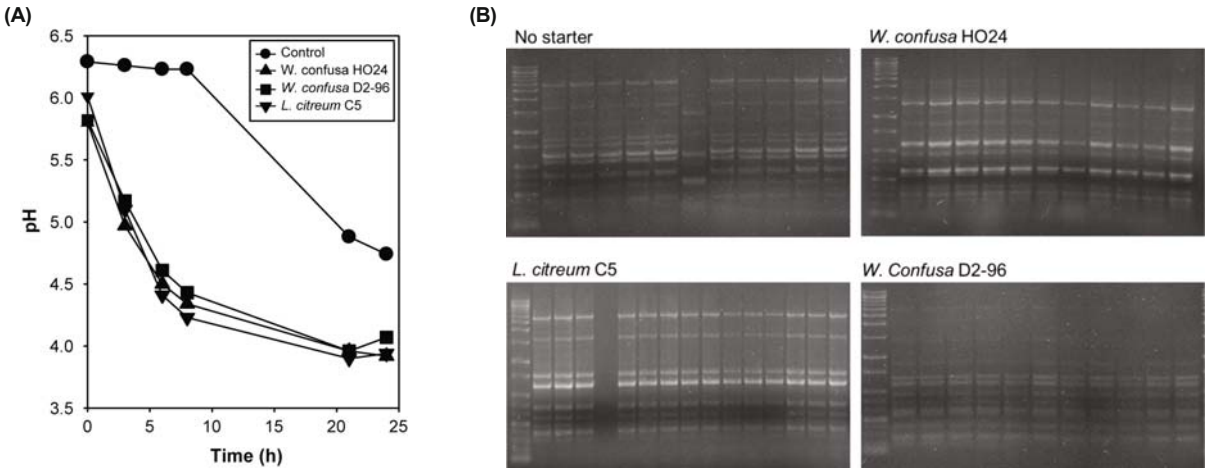

Fig. 2. Characterization of rice dough fermentation using starter strains. (A) Time course of changes in pH during rice dough fermentation at 25°C. The control is natural rice dough fermentation with no added starter strain. (B) REP-PCR patterns of lactic acid bacteria isolated from fermented rice dough with (*L. citreum* C5, *W. confusa* HO24, or *W. confusa* D2-96) or without starter.

of incubation, the pH of the rice dough dropped from 6.5 to 5.0 (Fig. 2A). Even though the kinetics and quantity of this pH drop was slightly different from the LAB-derived pH drop shown in MRS media, the fermentation of rice dough by LAB was surprisingly fast, considering the low water activity of the rice dough used in this study. To identify the LAB strains governing rice dough fermentation, forty seven LAB were isolated from four different fermented rice dough. In this analysis, we found that fermented rice dough contained various LAB such as *L. citreum* (26 *L. citreum* strains out of total 47 LAB isolates), *L. lactis* (1/47), *L. mesenteroides* (2/47) *L. pseudomesenteroides* (1/47), *Lactobacillus plantarum* (1/47), *Weissella cibaria* (3/47), *W. confusa* (11/47), and *W. paramesenteroides* (2/47). LAB identification using 16S rRNA gene and REP-PCR pattern analysis showed that two heterofermentative species, *L. citreum* and *W. confusa* were the predominant species in rice dough fermented at 25°C.

LAB starters might help expedite and control the fermentation of rice dough. To test this hypothesis, two rice dough isolates, *L. citreum* C5 and *W. confusa* HO24, and two kimchi isolates, *Lactobacillus plantarum* I-6 and *W. confusa* D2-96, were used for rice dough fermentation. We found that regardless of the LAB source, three heterofermentative strains were the predominant species during 24 h of rice dough fermentation (Fig. 2B). In contrast, *Lactobacillus plantarum* I-6 starter, a homofermentative species, was not isolated after 24 h of fermentation, because it was outcompeted by other autochthonous LAB such as *L. citreum*, *L. mesenteroides*, and *W. confusa* (data not shown). Unlike the control fermentation, the pH of the starter-added rice dough dropped quickly (Fig. 2A), due to the rapid accumulation of both acetic acid (69.5±14.7 μmol/g dough) and lactic acid (113.9±12.7 μmol/g dough), which were main fermentation byproducts. This amount of accumulated organic acids in rice dough appears to be almost equivalent to that in MRS media, but did not significantly harm the organoleptic taste of the rice cakes (data not shown). *Neurospora* sp. YS3 and *P. crustosum* YS2 grew slower on this fermented rice dough than on the control dough, while *Cladosporium* sp. YS1 did not grow, confirming that this species is the most sensitive to organic acids (Table 1).

It is known that during sourdough fermentation, *Lactobacilli*, *Leuconostoc*, and *Weissella* dominate the sourdough ecosystem. Since rice is a very similar cereal to wheat in term of its chemical components, it is not surprising that like sourdough, *Leuconostoc* species such as *L. citreum* and *Weissella* species such as *W. cibaria* and *W. confusa* dominate during the early stage of rice dough fermentation (De Vuyst and Neysens, 2005; Cho *et al*., 2006). These dominant lactic acid bacteria during the early stage of fermentation mostly contain the capacity to consume sucrose very easily (Cho *et al*., 2006), which is added during the preparation of rice dough. We also found that the milling equipment and storage condition for rice dough greatly influence the population diversity of the lactic acid bacterial community. To control the quality of rice dough, a starter culture can be used for fermenting rice dough and making rice cakes. In this study, we showed that *L. citreum* and *W. confusa* strains can be a good start culture for rice dough fermentation and the resulting fermented rice dough may have an extended shelf life without negatively impacting the organoleptic taste.

In summary, our results indicate that *L. citreum* and *W. confusa* as starter strains are the dominating species in rice dough fermentation and the dominance of this starter strain results in a delay of the rapid growth of fungal contaminants such as *Penicillium crustosum*, due to the high concentration of organic acids including acetic and lactic acid. Therefore, these lactic acid bacteria can be applicable to improve the quality control of rice cakes and preservation.

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