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RESEARCH ARTICLE

Biopreservative Potential of *Lactobacillus plantarum* YML007 and Efficacy as a Replacement for Chemical Preservatives in Animal Feed

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Abstract Antifungal and biopreservation potentials of *Lactobacillus plantarum* YML007 isolated from Korean kimchi were analyzed. The biopreservative efficacy of the bacterium was analyzed using maize grains. Maize was divided into 3 groups and treated with a 5× concentrated cell-free supernatant of *Lb. plantarum* YML007 (T3R3), organic acids (T2R2), and a control group (T1R1) lacking treatment. All groups were stored for 30 days. Maize was tested for moisture and crude fat contents, mold growth, and aflatoxin production. The moisture content, mold count, and toxin production were higher in the control maize. The YML007 and acid treated maize remained uninfected after 30 days. Animals fed with YML007 treated maize showed more weight gain and less feed consumption. YML007 can be used to preserve the nutritional value of stored grain and to ensure better quality feedstuffs that are necessary for improving animal health and performance.

Keywords: *Lactobacillus plantarum*, antifungal, *Aspergillus niger*, food preservative, food spoilage

Introduction

Cereal grains are the major source of food for most humans and domesticated animals (1). Maize is one of the top ranking cereal crops in terms of global productivity, second only to wheat in total production (2). Maize is the source of a large number of industrial products and use as a human food and an animal feed make it a cereal crop of increasing importance. A rapid increase in maize consumption by poultry is a major factor contributing to an increased use of maize for livestock feed (3). It is estimated that 5% to 10% of the world's food production is wasted due to fungal deterioration (4) and mold is an important reason for degradation of nutritional content of feed due to production of mycotoxins (5). Currently, more than 400 mycotoxins have been identified (6). Nevertheless, contamination is not limited to food and feed. Mycotoxin producing organisms also threaten human and animal health. Thus, for effective grain preservation it is crucial to keep mold contamination as low as possible.

Present strategies to destroy mycotoxins in food and feed include treatments with ammonia and radiation, screening, and heating. However, these methods are expensive, impractical for commercial applications, and/or destroy vital nutrients of the grain (7,8). The primary mode of control is use of chemical fungicides, but most fungicides have toxic effects. In addition, the stress or shock on the mold caused by the fungicide may cause increased mycotoxin production (9,10). Therefore, the nutritional and toxicological implications of microbiological changes in animal feed ingredients have become critical in animal nutrition.

Biofungicides can used as a viable alternative to chemical fungicides for a reduction in hazardous effects. Lactic acid bacteria (LAB) are a promising alternative to chemical preservatives (11). There are many previous reports on the antibacterial activities of LAB due to produced lactic acid, acetic acid, hydrogen peroxide, diacetyl, CO_2, and bacteriocin (12,13). However, reports on the use of LAB in biopreservation

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due to their antifungal activities are limited (14). In the present study, the antifungal activity of a cell-free supernatant of the kimchi isolate *Lactobacillus plantarum* YML007 was compared with commercially available preservatives to evaluate food spoilage due to molds and yeasts. The biopreservative potential of the bacterium was analyzed using maize grain for potential application as a commercial animal feed preservative.

Materials and Methods

Media and culture conditions *Lb. plantarum* YML007 was isolated from kimchi using de Man Rogosa Sharpe (MRS) agar (Difco Laboratories, Detroit, MI, USA) by incubation at 30°C for 24 h. Phylogenetic identification was done by comparing 16S rRNA gene sequencing results with GenBank sequences stored at the National Center for Biotechnology Information (NCBI) using the BLAST Program. Bacteria were stored at −80°C in MRS broth with 15%(v/v) glycerol. For production of a 5× concentrated cell-free supernatant, *Lb. plantarum* YML007 cultured in MRS broth at 30°C for 24 h was centrifuged with Mega 17R (Hanil Science Industrial, Incheon, Korea) at 10,000×*g* for 10 min, followed by a filtration through a 0.45-μm-pore size filter (Sartorius Stedim Biotech, Goettingen, Germany). The pH of the supernatant was adjusted to 6 using 1 M NaOH and was 5× concentrated using a vacuum concentrator (Modulspin 40; Hanil Science Industrial). The supernatant was stored at −20°C until use in further experiments. Molds were grown on PDA hard agar plates at 30°C for 7 days until sporulation occurred (15).

Antifungal activity analysis The antifungal spectrum of the 5× concentrated cell-free supernatant of *Lb. plantarum* YML007 was analysed using paper disk (11) and agar well diffusion assays (16). For the paper disk assay, 8 mm paper disks (Advantec Roshi Kaisha, Ltd., Tokyo, Japan) were used and for the agar well diffusion assay, cork borers with a 7 mm diameter (Cork Borer 12; Korea Ace, Seoul, Korea) were used. For both cases, 100 μL of sample was spotted on each paper disk/agar well for observation of the inhibition zone. The plates were incubated at 30°C, then examined after 24 h and 48 h for inhibition zones. MRS broth at pH 6 was used as a negative control in each assay. The antifungal activities of the two commonly used mold preservatives potassium sorbate and sodium benzoate were compared with the *Lb. plantarum* YML007 supernatant. Potassium sorbate and sodium benzoate were dissolved in 20 mM sodium acetate (pH 4.0) and adjusted to 0.1%(w/v) (7,11,17). All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). The filter-sterilized cell-free supernatant was concentrated 5× using 20 mM sodium acetate (pH 4.0). *Aspergillus niger* KCTC16683, *Fusarium oxysporum* KCTC16909, *Aspergillus flavus* KCTC16682, *Saccharomyces cerevisiae* KCTC12106, *Candida albicans* KCTC 17485, and *Pichia membranifaciens* KCTC 7628 were used as indicator organisms.

Biopreservation using *Lb. plantarum* YML007 To evaluate the preservative effect of *Lb. plantarum* YML007, fresh maize was purchased from a market in Daegu, Korea in September of 2011. Maize with an approximate 16% moisture content was used in the experiments and was stored for 30 days under environmental conditions favorable for mold growth. The maize was divided into three groups of a control group (T1R1), a group treated with organic acids (T2R2), and a group treated with a 5× concentration of the cell-free supernatant of *Lb. plantarum* YML007 (T3R3). An amount of 2 L per ton of organic acid containing a mixture of potassium sorbate and sodium benzoate was added to T2R2 maize samples at a concentration of 0.1%(w/v). Similarly, 2 L per ton of the *Lb. plantarum* YML007 supernatant was added to a T3R3 maize sample. The maize was mixed in a planetary mixer and stored under ambient temperatures for 30 days. The moisture and crude fat contents, the aflatoxin level, and the mold count were analyzed in all three groups on the 1st, 15th, and 30th days of storage.

Determination of aflatoxin levels in maize Aflatoxin was extracted using acetone, and then treated with cupric carbonate and ferric gel to eliminate fluorescent materials other than aflatoxin. Washing with acid and alkali followed, then extraction using chloroform followed by drying and redilution with chloroform. The resultant product was spotted on an activated thin layer chromatography (TLC) plate (Merck KGaA, Darmstadt, Deutschland, Germany) with standards. The concentration of aflatoxin was then ascertained using visual comparison in a UV viewing cabinet (Vilber Lourmat, Sud Torcy, Marne-la-Vallee, France) (18).

Estimation of maize moisture and crude fat contents The moisture content of maize was estimated using an infrared moisture determination balance (FD-610; KETT Electronic Laboratory, Tokyo, Japan). The total fat content of a 2.5 g maize sample was determined using an organic solvent extraction method with a Soxhlet extraction apparatus (Sigma-Aldrich) and petroleum ether-boiling ranging from 4°C to 60°C (19).

Animal trials Wister rats (weeks old) were purchased from Samtaco Bio Korea (Osan, Gyeonggi, Korea). Rats were acclimatized for 1 week after which veterinary health

checks were carried out on a select group of 12 young and healthy rats (male and female). The rats were then randomly divided into three groups for feeding. Experiments in each group were conducted in duplicate. The housing conditions were 19ºC to 25ºC with a 30% to 70% relative humidity and a 12 h light and 12 h dark cycle. Rats were allowed free access either to water and a control maize feed (T1R1), or to water and a treated maize feed (T2R2/T3R3) for 30 days. Maize consumption and body weight were recorded daily.

Statistical analysis All experiments were conducted in triplicate. Means and standard deviations were calculated. Differences in various parameters (mold count, aflatoxin production, and fat content), between the control and the T3R3 groups on the 30th day were tested for significance using the one way analysis of variance (ANOVA) procedure using a SAS software (version AS 9.2; SAS Institute Inc., Cary, NC, USA). Statistical significance was defined as $p<0.05$.

Results and Discussion

Antifungal activity of *Lb. plantarum* YML007 *Lb. plantarum* YML007 with a strong antifungal activity was identified using 16S rRNA gene sequencing. *Lb. plantarum* YML007 showed stronger antifungal activity against *A. niger*, *F. oxysproum*, *A. flavus*, *S. cerevisiae, C. albicans,* and *P. membranifaciens* than the commercial preservatives potassium sorbate and sodium benzoate ($p<0.01$) as shown in Table 1. Species of *Aspergillus* are more resistant to antifungal inhibition than *Fusarium* species, followed by *Penicillium* species. (15); however, a 5× concentration of the cell-free supernatant of *Lb. plantarum* YML007 strongly inhibited *Aspergillus* growth and spore formation, compared with other kimchi-isolated bacteria observed using a paper disk assay (Fig. 1). *Lb. plantarum* YML007 also exhibited better inhibition than potassium sorbate and sodium benzoate ($p<0.01$) against the indicator organisms (Table 1). *F. oxysporum* was highly resistant to both chemical preservatives; however, it was strongly inhibited by the *Lb. plantarum* YML007 supernatant. Compared to molds, *C. albicans* and *P. membranifaciens* yeasts exhibited increased resistance to all treatments (Table 1). The anti-fungal compound of *Lb. plantarum* YML007 was previously purified and identified as a low molecular weight peptide (20).

Fig. 1. Nos. 1-36, anti-fungal activities of bacteria isolated from kimchi; No. 6, growth inhibition of *Aspergillus niger* KCTC16683 *by Lb. plantarum* YML007. Each number indicates one paper disk.

Biopreservation using *Lb. plantarum* YML007 The moisture, crude fat, and aflatoxin contents, and the mold count, on the 1st, 15th, and 30th day of maize storage for the three groups T1R1, T2R2, and T3R3 are shown in Fig. 2. After 30 days, there was an increase in the mold count for the control maize group (T1R1), a slight change in moisture content, and a decrease in the total crude fat percentage ($p<0.05$), compared to the first day. Aflotoxin production was significantly less in the treated groups ($p<0.05$), compared to the control. There was almost no aflatoxin production in the acid-treated (T2R2) and *Lb.*

Table 1. Comparison of growth inhibition of *Lb. plantarum* YML007 and other chemical preservatives[1)]

Indicator strain	Inhibition zone (mm) by *Lb. plantarum* YML 007 (5× concentrated cell free supernatant)	Inhibition zone (mm) by potassium sorbate 0.1%(w/v)	Inhibition zone (mm) by sodium benzoate 0.1%(w/v)
Aspergillus niger	17.23±0.05^{a}	10.31±0.02^{a}	11.54±0.05^{a}
Fusarium oxysporum	16.38±0.06^{b}	2.57±0.02^{f}	4.42±0.09^{f}
Aspergillus flavus	15.3±0.1^{bc}	10.21±0.10^{ab}	10.63±0.2^{b}
Saccharomyces cerevisiae	12.66±0.89^{d}	8.24±0. 09^{c}	6.91±0.3^{e}
Candida albicans	10.91±0.23^{e}	6.34±0.06^{e}	8.21±0.92^{cd}
Pichia membranifaciens	10±0.04^{ef}	7.23±0.03^{d}	8.4±0.35^{c}

[1)]Each value is expressed as the mean diameter of the inhibition zone (mm)±standard deviation ($n=3$). Values followed by the same letter in each column are not signicantly different ($p<0.01$).

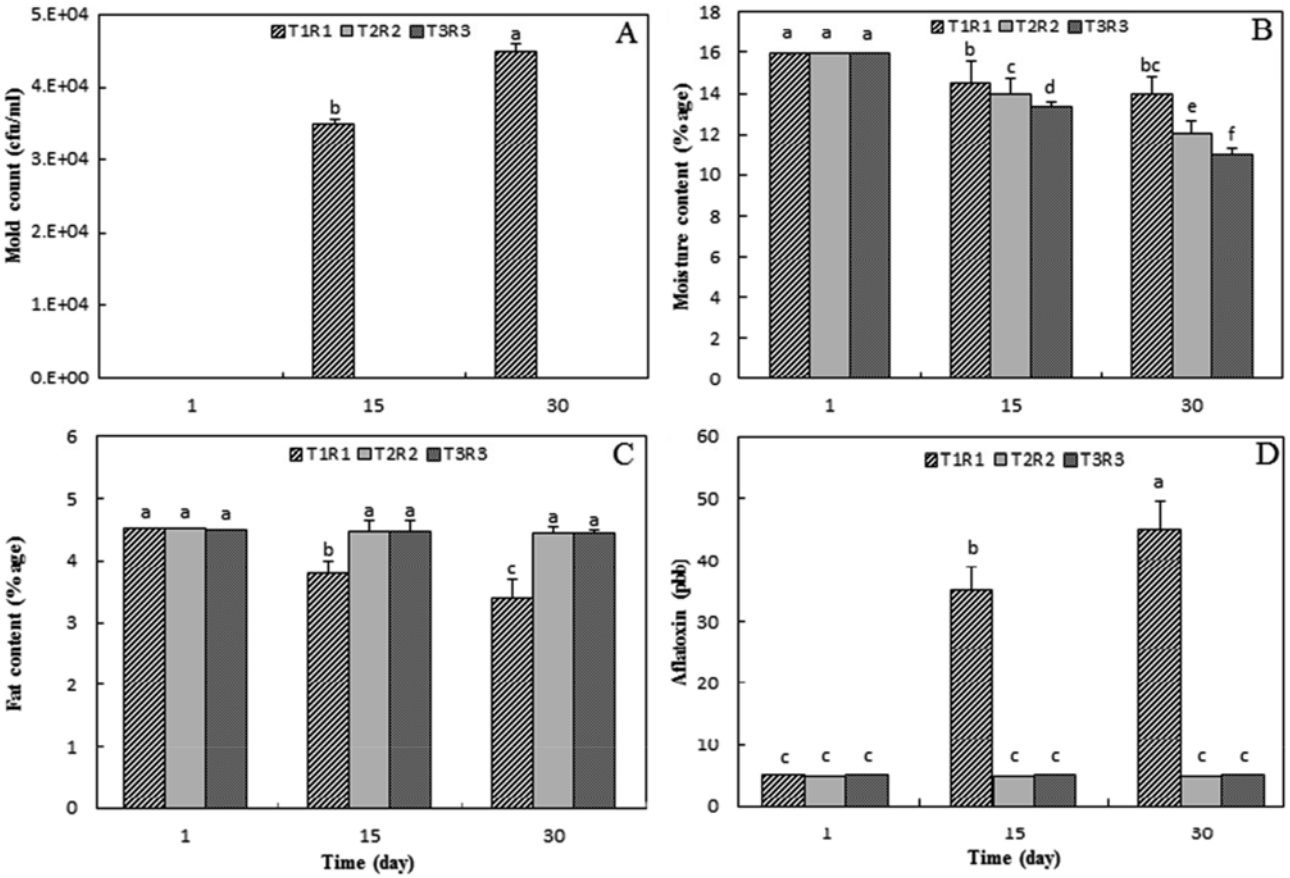

Fig.2. Effects of *Lb. plantarum* YML007 on (A) mold count, (B) moisture content, (C) fat, and (D) aflatoxin production in a maize sample during 30 days of storage. Each value is expressed as a mean±standard deviation (n=3). Different superscripts in each column indicate the signicant differences in the mean (p<0.05).

plantarum YML007 supernatant treated (T3R3) maize samples. High aflatoxin production, a high mold count, and a reduced crude fat content were observed in the control maize (T1R1), compared to the treatment groups (p<0.05). The average aflatoxin production in the T2R2 and T3R3 maize groups did not exceed 5 parts per billion (ppb) (Fig. 2). The aflatoxin tolerance for maize used for human food and animal feed is 20 ppb/kg, so the efficacy of the *Lb. plantarum* YML007 is better than acid preservatives, which have undesirable side effects on human and animal immune systems (21). A 16% maize moisture content, which is commonly found in maize stored in different regions of world, allowed proliferation of fungi and production of aflatoxins (Fig. 2). *Lb. plantarum* YML007 was effective in biopreservation of maize under long term storage conditions. As far as prevention of mold development is concerned, the results reported herein agree with the results of Sathe *et al.* (22), where good control of fungi growth was achieved using *Weissella paramesenteroides, Lb. plantarum* and *Lb. paracollinoides*. The ability to prevent mold growth on stored cereal grains is of significance with regards to human health and animal and agricultural economies.

Determination of feed consumption, body weight gain, and the feed consumption ratio in animals After 30 days, animals fed with control maize showed a smaller gain in body weight, compared with animals fed with *Lb. plantarum* YML007-treated maize (Fig. 3). The total mean body weight achieved by feeding with *Lb. plantarum* YML007-treated maize was 2.95 g, which was 1.1 g more than for control and acid-treated maize. The mean maize feed consumption ratio (FCR) of control maize was high, compared to treated maize (p<0.05). An overview of control maize and treated maize samples after 30 days of storage clearly shows the long-term storage efficacy of using *Lb. plantarum* YML007 (Fig. 4).

A 5× concentrated cell-free supernatant of *Lb. plantarum* YML007 isolated from kimchi was effective against mold growth and aflatoxin production in maize grains. Antifungal activity of LAB has been detected mostly under laboratory conditions. Only limited applications of antifungal bacterial strains have been reported in the food processing industry (23). LAB strains, which show inhibitory activity in agar spot assays, do not have the same effect in the context of food preservation. Addition of Nisaplin or nisin-producing LAB to bread dough showed no reduction of *Bacillus*

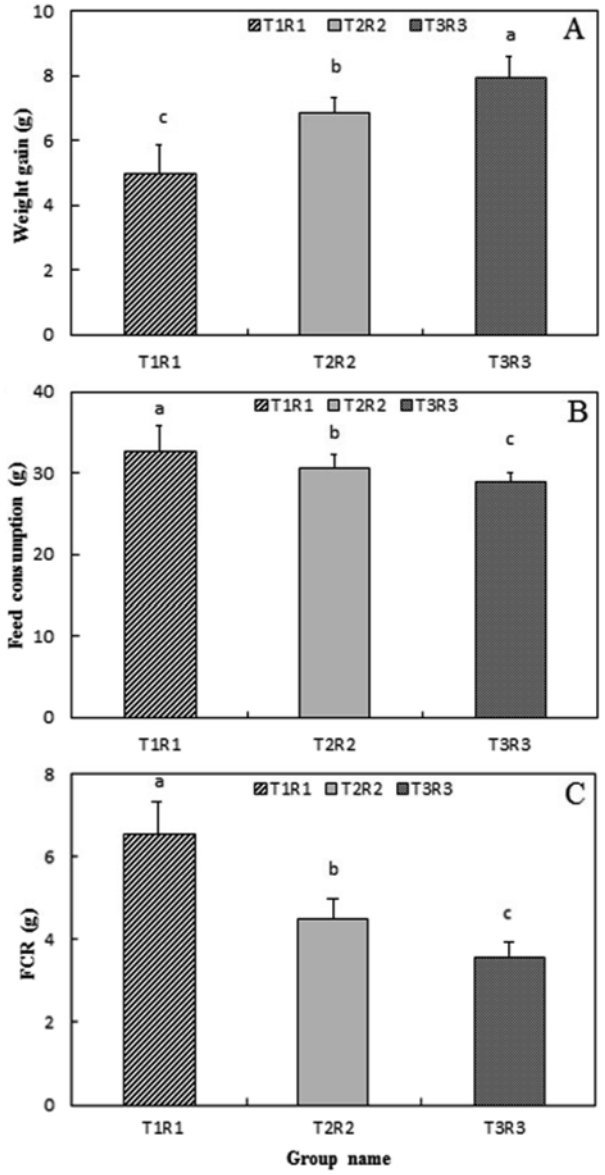

Fig. 3. Effect of *Lactobacillus plantarum* YML007 on (A) weight gain, (B) feed consumption, and (C) FCR in Wistar rats. Each value is expressed as a mean±standard deviation (n=3). Different superscripts in each column indicate the signicant differences in the mean (p<0.05).

counts, the effect of nisin was only observed in the well diffusion assay (24). The use of *Lb. plantarum* YML007 reduced microbial contamination in maize, compared to control. Use of YML007 preserves the nutritional value of stored grain and ensures a better quality of feedstuffs necessary for improving animal health and optimizing economic production. It is encouraging that aflatoxin production was severely affected by a 5× concentrated cell-free supernatant of *Lb. plantarum* YML007, even at low concentrations. There are several reports of aflatoxin biosynthesis inhibition by LAB, but the bacteria tested were unable to remove aflatoxins from the culture medium

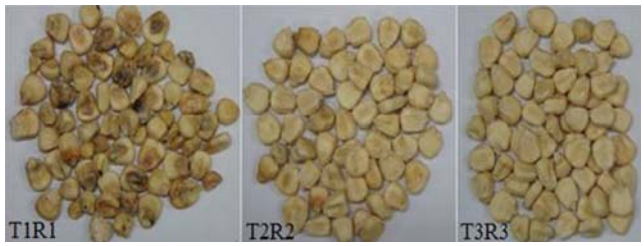

Fig. 4. An overview of control maize and treated maize samples after 30 days of storage. T1R1 (control maize without any treatment), T2R2 (sodium benzoate 0.1%[w/v] in 20 mM sodium acetate, pH 4.0, treated maize), and T3R3 (YML007 treated maize). Filter sterilized *Lactobacillus plantarum* YML007 was concentrated 5× in 20 mM sodium acetate at pH 4.0.

(25,26). This study confirms that a cell free supernant of *Lb. plantarum* YML007 inhibits fungal growth and, therefore, controls fungal toxin production in stored grains. A number of potential mechanisms have been reported for the inhibitory effects of LAB on fungal growth (27). Recently, Belal *et al.* (28) reported that both the cells and the supernatants of *Lb. fermentum* Te007, *P. pentosaceus* Te010, *Lb. pentosus* G004, and *Lb. paracasi* D5 could be used as biopreservatives in bakery products and other processed foods. Numerous studies have described antifungal components from LAB cultures (29), but limited applications of antifungal strains in the food industry have been reported. Firas (30) identified an antimicrobial compound from *Mentha longifolia L*. leaves, and Santosh and Sheela (31) isolated plantaricin LR14 from *Lb. plantarum* LR/14. Hamed *et al.* (32) showed that LAB reduced growth of *Fusarium oxysporum* on tomato seeds and improved root growth. However, only a few reports have been published regarding the antifungal and biopreservative applications of LAB. Species of LAB that produce antifungal compounds can be natural substitutes for chemical preservatives.

In summary, the lactic acid bacterium *Lb. plantarum* YML007 isolated from Korean kimchi was found to be effective in preventing mold growth and aflatoxin production in maize stored under ambient environmental conditions that were favorable for mold growth. There was no change in the physical properties of the stored maize, compared to control. Moreover, weight gain was observed in rats fed with *Lb. plantarum* YML007 maize, compared to control, untreated maize. *Lb. plantarum* YML007, with its strong inhibitory action against mold growth and aflatoxin production, can be used as an effective bio-preservative to replace chemical preservatives in food and feed.

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