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The Binding of Aflatoxin B_1 Modulates the Adhesion Properties of *Lactobacillus casei* KCTC 3260 to a HT29 Colon Cancer Cell Line

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Abstract The 14 lactic acid bacteria (LAB) have been evaluated to determine the binding capacity to HT29 cell and Aflatoxin B_1 (AFB_1). The interaction of LAB to HT29 cells has been further investigated to identify the possibility of competing the binding sites with AFB_1. Of 14 LAB strains, *Lactobacillus casei* KCTC 3260 demonstrated the higher adhesiveness to HT29 and AFB_1 with the rate of 19.6% and 46.3%, respectively. In competitive analysis for binding sites, the adhesion of *L. casei* KCTC 3260 to HT29 cells was reduced with 100 nmol AFB_1 by 31.2%. The protoplast of *L. casei* KCTC 3260 showed no binding capacity to HT29 cells with increment of AFB_1 concentration, indicating that cell wall components might serve as a critical factor for the binding. To discriminate the major component influencing on *L. casei* KCTC 3260 binding to HT29 cells and AFB_1, four different pre-treatments (lipase, pronase E, sodium *m*-periodate, and urea) were employed. Of those, sodium *m*-periodate treatment caused the lower adhesion of *L. casei* KCTC 3260 to HT29 cells with the increment of AFB_1 concentration. These results indicated that carbohydrate moiety on the cell wall of *L. casei* KCTC 3260 might be the most critical component in binding to both HT29 cells and AFB_1.

Keywords: lactobacillus, HT29 cell, aflatoxin B_1, cell wall, adhesion

Introduction

As a probiotic, the selected strains of lactic acid bacteria (LAB) have been increasingly introduced into various food products. When consumed, these bacterial strains are claimed to exert a range of health promoting effects and to modulate intestinal immune system. In recent, a set of criteria has been proposed to select the potential probiotic strains. One of the important criteria for a good probiotic strain is its ability to adhere to mucosal surface of the human gastrointestinal tract (1). Adhesion of probiotic LAB has been reported to be species specific and temporarily colonize the intestine, which beneficially influence on the microbial balance of the host. For a last few years, some strains of LAB such as *L. acidophilus* LA1, *L. rhamnosus* GG, and *L. casei* Shirota have been proved to have an higher ability to adhere to *in vitro* intestinal cell lines, and their binding efficacy and characteristics have been defined in many studies (2, 3).

During the last two decades, several studies have suggested that LAB possess anticarcinogenic activity and are specially noted for their ability to bind to AFB_1 (4, 5). Aflatoxins are mycotoxins produced by *Aspergillus* molds and they have detrimental health effects, including mutagenic and carcinogenic effects in both humans and animals. Thus, the removal of AFB_1 is considered of industrial importance due to the economic losses, resulting from condemnation of contaminated crops, cheese defects, and impaired growth and feed efficiency of animals fed contaminated feeds. The recent studies have suggested that AFB_1 is bound noncovalently and extracellularly to LAB and that it may be released by the continual washing of the bacterial surface in the gastrointestinal tract (5). In the study of identifying physical characteristics of LAB bound to AFB_1, author has also suggested that cell wall polysaccharide and peptidoglycan are the two main elements responsible for the binding of mutagens to LAB. According to the facts, it has implicated that LAB may have common sites to interact to both intestinal mucosa cells and AFB_1.

Therefore, this study has focused on i) the selection of LAB with higher binding capacity to HT29 cells and AFB_1, ii) the competitive inhibition for LAB adhesion to HT29 by AFB_1 to identify the existence of common binding sites, and iii) the determination of bacterial components involved in binding to HT29 cells and AFB_1. A potential function of the selective strains may have a capacity to reduce the carcinogenic or toxic effect of AFB_1 by binding to them instead of binding to intestinal mucosa cells.

Materials and Methods

Bacterial strains, culture, and counts Fourteen LAB strains have been purchased from Korean Collection for Type Culture (KCTC) and used for *in vitro* adhesion to HT29 cells and AFB_1; *L. rhamnosus* GG as a postive control, *L. brevis* KCTC 3498, *L. brevis* 14L002, *L. casei* KCTC 3260, *L. casei* 01, *L. casei* KCTC 3109, *L. fermentum*, *L. bulgaricus* LB207, *L. acidophilus* LA5, *L. acidophilus* LA100, *L. acidophilus* KCTC 3111, *L. acidophilus* KCTC 3151, *L. helveticus* KCTC 3545, and *L.*

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johnsonii KCTC 3141. All strains were cultured for 16-24 hr in deMan-Rogosa-Sharpe (MRS) broth (Oxoid, Hampshire, United Kingdom) under aerobic conditions (37°C, 10% CO_2).

Bacterial counts were determined by flow cytometry using a FACS Calibur equipped with an air-cooled 488-nm argon-ion laser at 15 mV. Direct counts were enumerated by using Fluoresbrite beads (2.0 μm, Polysciences Inc.) as an internal calibration. Viability of bacterial population was assessed by using SYTOX green nucleic acid stain (Molecular Probes, S-7020) at 1 μM/10^6 - 10^7 bacteria to detect non-viable bacteria. A band pass filter of 525 nm was used to collect the emission for green SYTOX. To support the precise bacterial counts, the colony-forming unit (CFU) was determined by plating serial 10-fold dilutions of non-labeled bacterial suspension on MRS agar plates. All strains have been adjusted to 2 × 10^9 CFU/mL for assay, respectively. The optical value at OD_{600} has been simultaneously measured in equivalent with 2 × 10^9 CFU/mL to obtain the preliminary data for the radioactive labeled assay to avoid the contamination of the applied machines.

Preparation of bacterial protoplast Bacterial cells were harvested by centrifugation at 3,900 × *g* for 20 min and adjusted to 2 × 10^9 CFU/mL in phosphate buffered solution (PBS). Bacteria were incubated with 80 μL of lysozyme (50 mg/mL, Sigma, St Louis, MO, USA) for 40 min at 37°C. The bacterial protoplast was collected by centrifugation at 5,800 × *g* for 5 min, and the light microscope detection confirmed that more than 90% of protoplast was developed.

HT29 cell culture The HT29 cell line (ATCC HTB 39) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Macoy's 5A (Whitaker, USA) supplemented with 10% heat-inactivated (55°C, 30 min) fetal bovine serum (FBS, BioWhitaker, Walkersville, MD, USA), 2 mM L-glutamine (Sigma, St Louis, MO, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (BioWhitaker, Walkersville, MD, USA) at 37°C in an atmosphere of 5% CO_2. For adhesion assays, HT29 monolayers were prepared on 24-well tissue culture plates with a concentration of 5 × 10^5 cells per well to obtain confluence with Macoy's 5A medium containing 3 μM methotrexate (Sigma, St Louis, MO, USA) and maintained for 1 week prior to use in adhession assays. The cell culture medium was changed every other day and replaced by fresh non-supplemented Macoy's 5A at least 1 hr before the adhesion assay.

***In vitro* adhesion assay** To obtain the radioactive bacterial strains, 5 μL/mL of methyl-1,2,-[^{3}H]-thymidine (6.7 Ci mmol/ml, BMS, La Jolla, CA, USA) was added to 5 mL of bacterial culture that was inoculated from precultured bacteria at the exponential stage. After 4-hr incubation at 37°C with a shaking at 200 rpm, the bacterial cells were collected and washed with PBS containing 0.1% (w/v) NaN_3 to prevent bacterial growth and penetration into the HT29 cells. The adherence of bacterial strains to HT29 cell cultures was examined by adding 50 μL of a radiolabeled bacterial suspension to three wells containing the HT29 monolayer (1 mL of Macoy's 5A in the well). After incubation at 37°C for 1.5 hr, the HT29 cell cultures were washed three times with 1 mL of PBS and treated with 250 μL of solution containing 1% SDS and 0.9 M NaOH at 37°C for 1 hr to lyse the HT29 cells. The lysed cells were mixed with scintillation liquid and the radioactivity was measured by using Beckman LS 6500 scientillation counter. The radioactivity ratio (%) was calculated by comparing the radioactivity of the original bacterial suspension to the final counts from the lysed cells. All strains were tested at the same time in triplicate in three independent experiments.

AFB$_1$ binding assay AFB$_1$ (Sigma, St. Louis, MO, USA) was dissolved in benzene-acetonitrile (97:3). An amount equivalent to 5 mg AFB$_1$/mL PBS (pH7.4) was prepared and benzene-acetonitrile was evaporated by heating in a water-bath at 80°C for 10 min. Cells of the precultured strains (approx. 10 CFU/mL) were pelleted by centrifugation at 3,900 × *g* for 15 min and resuspended in 1.5 mL PBS containing AFB$_1$. Bacterial suspensions were incubated for 1 hr at 37°C. The cells were again pelleted by centrifugation and samples of the supernatant fluid were analyzed by HPLC.

Quantification of AFB$_1$ by HPLC The HPLC procedure used for the analysis of AFB$_1$ was similar to the previous study (6) with a slight modification. No extraction of AFB$_1$ from samples of supernatant fluid was required and 70 μL supernatant was directly injected into HPLC. The HPLC system (Waters, Milford, MA, USA) was fitted with a dual pump model 400 solvent delivery system, model 980 programmed fluorescence detector and a 220 × 4.6 mm, 5 μm ODS Spheri-5 Brownlee column fitted with C_{18} guard column. Water-acetonitrile-methanol (60: 30:10 by vol) was used as the mobile phase with a flow rate of 1 mL/min. Detection was performed by the excitation at 365 nm and the emission at 418 nm. The retention time was approximately 9.5 min. Chromatograms were recorded at chart speed of 0.3 cm/min and peak width of 0.4 min. The residue percentage was calculated using the formula: 100 × (peak area of AFB$_1$ in the supernatant / peak area of AFB$_1$ in the positive control).

Competitive inhibition for LAB adhesion to HT29 cells by AFB$_1$ Competitive inhibition was carried by adding a range of AFB$_1$ concentration from 1 to 120 nmol after radioactive-labeled *L. casei* KCTC 3260 (2 × 10^9 CFU/mL) were treated into HT29 cells. After 1-hr incubation at 37°C, HT29 cells bound to *L. casei* KCTC 3260 have been carefully washed three times with PBS and treated with lysis solution for 1 hr. The lysed cells were mixed with scintillation liquid and the radioactivity was measured by using Beckman LS 6500 scientillation counter.

Pretreatment of *L. casei* KCTC 3260 To change the structure of cell wall, 5 M HCl was treated with 2 × 10^9 CFU/mL of *L. casei* KCTC 3260 for 1 hr at room temperature. To determine the binding components of cell wall, a 0.5 mg/mL solution of pronase E (Sigma, St. Louis, MO, USA) and lipase was prepared in phosphate buffer (pH 7.6). A 10 mg/mL solution of sodium *m*-periodate

(Sigma, St. Louis, MO, USA) was prepared in acetate buffer (pH 4.5). An aqueous solution of 8 M urea was prepared in deionized water. Pronase E, lipase, sodium *m*-periodate, and urea were added to the *L. casei* KCTC 3260 and incubated at 37°C for 1 hr. After the centrifugation, pellets were washed three times with PBS and binding assay to HT29 cells was performed.

Statistical analysis The results of the bacterial adhesion and AFB_1 binding assay were subjected to Student's t-test for significant difference between strains. The results are only considered to be statistically different at $p<0.05$.

Results and Discussion

Adhesion of LAB to HT29 cells and AFB_1 Many studies have represented that binding capacity of LAB to intestinal mucosa is regarded as one of main properties to be a probiotic (1, 9). Although not many studies have been explored on a HT29 cell line, it has been known that *in vitro* binding capacity of LAB to Caco-2 cells is largely relied on pre-treatments such as composition of culture medium, pH, temperature, incubation time, and number of washing with PBS (5, 7). Of those, according to the report by Tuomola *et al.* (7), the number of bacteria bound to Caco-2 cell cultures was directly related to the number of bacteria, indicating that the concentration of bacteria added is one critical factor among pre-treatment conditions. Thus, the number of bacteria used in this study was 2×10^9 CFU/mL that was required to show the significant difference in removal of AFB_1 (10). To avoid the chance of AFB_1 to be metabolized in the bacteria, all strains have been treated with sodium azide. In this study, *L. rhamnosus* GG, which has shown to have an adhesive property in the previous reports (2), was used as a positive control. Of 14 LAB, *L. casei* KTCC 3260 and *L. brevis* KTCC 3498 demonstrated the higher adhesiveness to HT29 cells with rate of 19.6% and 16.8%, respectively (Table 1). In agreement with a report by Ouwehand *et al.* (2), *L. rhamnosus* GG also showed the relatively high binding capacity to HT29 cells with 15.3%, followed by *L. casei* KCTC 3109 with 14.6 %. However, both *L. casei* 01 and *L. helveticus* KCTC 3545 showed the lower adhesion to HT29 cells with the rate of less than 2%. Our data was obtained with slightly higher values in adhesive ratio than those from other reports. It may be due to the higher number of bacteria added in this study as compared to other reports that used almost 5-10 times less.

The binding of lactic acid bacteria to AFB_1 showed the similar trend as the effect in adhesion to HT29 cells with rates ranging from 2.4% to 48.9% (Table1). *L. casei* KCTC 3260 and *L. rhamnosus* GG were more effective strains to bind to AFB_1 with rate of 46.3% and 48.9%, respectively. These results indicate that the binding domain of *L. casei* KCTC 3260 to HT29 cells may be associated with its binding domain to AFB_1.

Modulation of *L. casei* KCTC 3260 binding to HT29 cells by AFB_1 *L. casei* KCTC 3260 and *L. rhamnosus* GG have been used for competitive adhesion assay. The radioactive-labeled bacteria have been used for competitive adhesion assay instead of measuring the level of

Table 1. The Binding Capacity of Various Lactobacillus Strains to HT29 Colon Cancer Cells and AFB_1

Strain	% bound to LAB	
	HT29 cells	AFB_1
L. rhamnosus GG	15.3 ± 0.32 [1)]	48.9 ± 2.89
L. brevis KCTC 3498	16.8 ± 1.12 [a]	42.1 ± 2.26 [a]
L. brevis 14L002	4.5 ± 1.43 [a]	3.8 ± 0.46 [a]
L. casei KCTC 3260	19.6 ± 1.09 [a]	46.3 ± 1.89 [a]
L. casei 01	1.7 ± 0.77 [a]	0.6 ± 0.44 [a]
L. casei KCTC 3109	14.6 ± 1.01 [a]	12.1 ± 1.35 [a]
L. fermentum	5.6 ± 2.35 [a]	1.7 ± 0.97 [a]
L. bulgaricus LB207	5.9 ± 0.97 [a]	2.4 ± 1.45 [a]
L. acidophilus LA5	14.8 ± 1.21	9.2 ± 0.67 [a]
L. acidophilus LA100	2.2 ± 0.56 [a]	6.4 ± 0.46 [a]
L. acidophilus KCTC 3111	7.4 ± 0.89 [a]	10.3 ± 1.67 [a]
L. acidophilus KCTC 3151	6.9 ± 1.73 [a]	14.5 ± 3.21 [a]
L. helveticus KCTC 3545	0.9 ± 0.54 [a]	2.9 ± 1.59 [a]
L. johnsonii KCTC 3141	4.3 ± 0.46 [a]	19.7 ± 2.76 [a]

[1)]The values represent the mean ± SD of triplicates.
[a]Data are significantly different compared to the value of *L. rhamnosus* GG at the level of $p<0.05$.

AFB_1, because released or unbound AFB_1 is hardly measured by HPLC due to the components of cell culture medium. To determine whether adhesion of LAB to HT29 cells could be inhibited by AFB_1, we tested the binding capacity of LAB to HT29 cells with increment of AFB_1 concentration (Fig. 1). Interestingly, the binding of LAB to HT29 cells was reduced by 31.2% at 100 nmol AFB_1. These results are in accordance with a report by Kankaanpaa *et al.* (13), in which *L. rhamnosus* GG reduced the adhesion to Caco-2 cells in the presence of AFB_1 by 30%, respectively.

Role of cell wall determined by acid-treated and protoplast of *L. casei* KCTC 3260 It has been assumed that the binding interaction is predominantly appeared to occur with polysaccharide residues on the bacterial cell wall by physical entrapment although its expression on surface is determined by genetic background rendering species specificity (8). Thus, to identify the role of cell

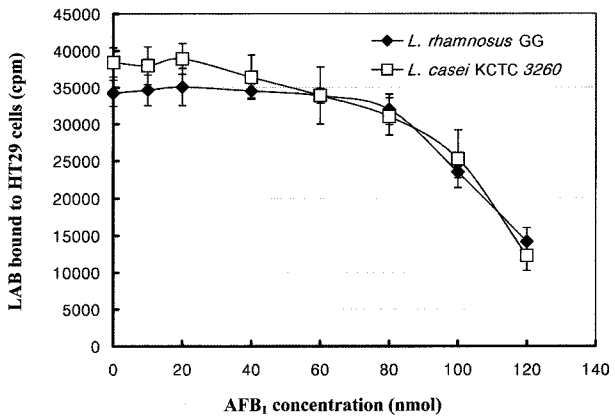

Fig. 1. Modulation of *L. casei* KCTC 3260 binding to HT29 cells by increment of AFB_1 concentration. All values are mean ± SD (n=3).

wall in LAB adhesion to HT29 and AFB_1, *L. casei* KCTC 3260 was treated with 5M HCl that alters the structure of bacterial cell wall, which could result in change of binding capacity. In addition, the protoplast of *L. casei* KCTC 3260 was tested for the binding capacity to HT29 cells with the increment of AFB_1 concentration. The binding of acid-treated *L. casei* KCTC 3260 to HT29 cells was inhibited by the increased concentration of AFB_1 compared to untreated control (Fig. 2). This result indicated that structural change of cell wall by acid treatment affected on binding capacity of *L. casei* KCTC 3260. The protoplast, which cell wall was removed, did not bind to HT29 cells through all range of AFB_1 concentrations. These results indicated that bacterial cell wall plays a critical role in binding to both HT29 and AFB_1, which supports the report by Haskard *et al.* (5).

Determination of cell wall components affecting the binding capacity The microbial adhesion process of LAB includes passive forces, electrostatic interactions, hydrophobic, steric forces, lipoteichoic acids and specific structures such as external appendages covered by lecitins. According to Rojas *et al.* (14), an adhesion-promoting protein with a molecular mass of 29 kDa, which is present on the cell surface of *L. fermentum* 104R and could be isolated from the culture supernatant fluid of the strain, has been isolated and characterized. Thus, we assumed that cell wall components such as carbohydrate, protein, and lipid might act as adhesion molecules for both cell lines and AFB_1. Four different pre-treatments (lipase, pronase E, sodium *m*-periodate, and urea) were employed to determine the cell wall components affecting the binding ability of *L. casei* KCTC 3260 to HT29 cells with the gradual increment of AFB_1 concentration. Treatment with lipase, which can remove lipids such as lipoteichoic acid from cell wall, did not cause a significant decrease in binding of *L. casei* KCTC 3260 to HT29 cells with the increasing AFB_1 concentration (Fig. 3). These results suggest that the involvement of lipids is unlikely and is supporting the data by Haskard *et al.* (5). Treatment with pronase E caused a modest decrease in binding to HT29 cells with the increment of AFB_1 concentration, suggesting that protein may play an important role in binding to HT29 cells and

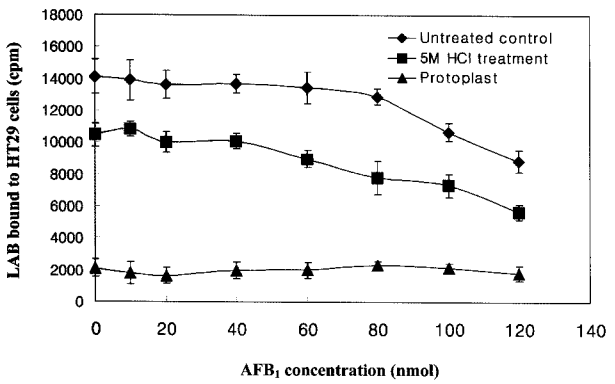

Fig. 2. The reduced binding capacity of *L. casei* KCTC 3260 with acid treatment and its protoplast to HT29 cells by increment of AFB_1 concentration. All values are mean ± SD (n=3).

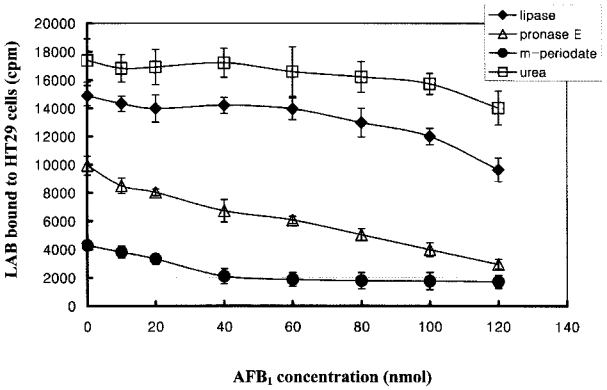

Fig. 3. The binding characteristics of *L. casei* KCTC 3260 to HT29 cells by increment of AFB_1 concentration after different pre-treatments for bacterial cell wall. All values are mean ± SD (n=3).

AFB_1. Compared to the treatment with pronase E, treatment with sodium *m*-periodate, which causes the oxidation of periodate-sensitive carbohydrate epitopes' cis-OH groups to aldehyde and carbon acid groups, led to the relatively lower adhesiveness of *L. casei* KCTC 3260 to HT29 cells with the increment of AFB_1 concentration. These results suggest that an adhesion occurs predominantly with carbohydrate components of the bacteria. Urea, which is an anti-hydrophobic agent, caused no difference in the binding of *L. casei* KCTC 3260 to HT29 by the increment of AFB_1 concentration. These results were not in consistent with a report by Haskard *et al.* (5), in which urea treatment caused a significant decrease, implying that hydrophobic interactions are involved in the binding mechanism. Unlike a report by Haskard *et al.* (5), in this study, HT29 cells could not be treated with high concentration of urea, which may cause toxic effects on cells. *L. casei* KCTC 3260 was pre-treated with urea and then used for binding assay to HT29 with different concentration of AFB_1. Thus, the cell wall hydrophobicity of *L. casei* KCTC 3260 may be recovered from urea treatment when the strain was added into cell medium. Urea can also denature the proteins, but this effect did not appear to be significant in this study. Many researchers have recently studied the physico-chemical properties of LAB to AFB_1 binding. For instance, it has been reported that the AFB_1 binding sites of these bacterial strains effectively compete with such proteins for AFB_1, at least in chickens, where they have been shown to inhibit the absorption of AFB_1 from the duodenal loop (6). In addition, in the study with heat- and acid-treated LAB, the binding ability of these LAB to AFB_1 was not decreased as compared to viable LAB (5), implicating that intercellular components or metabolism may not influence on the LAB binding to AFB_1; however, the bacterial ability to remove AFB_1 is dependent on bacterial cell wall structure. Based upon these results, carbohydrate on the cell wall of *L. casei* KCTC 3260 was the most critical component in binding to HT29 cells and AFB_1, implying that carbohydrate may be a non-specific binding molecule of *L. casei* KCTC 3260 for both HT29 cells and AFB_1. Protein on cell wall was partially involved in binding, but lipid was not a significant component in this study.

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