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Binding of Aflatoxin B_1 to *Lactobacillus paracasei* SNP-2 and Stability of Bacteria-AFB_1 Complex

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Abstract

The aim of this research was to study the binding ability of viable and non-viable of *Lactobacillus paracasei* SNP-2 to aflatoxin B_1 (AFB_1) in phosphate buffer saline (PBS) at pH 7.3. Bacterial cells were grown in MRS broth at 37 °C for 24 h. and then centrifuged at 1,800 g for 20 min at 10°C to get the pellet. Pellet was suspended in PBS pH 7.3 until the cell concentration was about 10^{10} CFU/mL. Viable cells, the heated, and acid-killed cells were evaluated for their ability to bind AFB_1 in PBS pH 7.3. Stability of the *L. paracasei* SNP-2/AFB_1 complexes was evaluated by determining the amount of the released AFB_1 to the PBS following five times washing. The results showed that AFB_1 binding ability to heated-and acid-killed bacteria were higher than that of by viable cells. More than 70% of bound AFB_1 was released from viable bacteria after five times washing. However, the heated and acid-killed cell treatments significantly increased the complex stability of bacteria-AFB_1.

Keywords: Aflatoxin B_1, *Lactobacillus paracasei* SNP-2, binding of AFB_1, peptidoglycan

Introduction

Aflatoxins are fungal secondary metabolites produced by toxigenic strains of *Aspergillus flavus, A. parasiticus,* and *A. nomius.* Aflatoxin B_1 (AFB_1) is regarded as the most prevalent form and also the most potent of these toxins. Aflatoxins commonly contaminate foods and feed including corn, peanuts, and tree nuts at any stage during growth, harvest, storage, and transportation (Rahayu et al., 2003; Dharmaputra et al., 2005). Aflatoxins are of great concern because of their detrimental effects on the health of humans and animals, including hepatotoxic, carcinogenic, immuno-suppressive and anti-nutritional effects (William et al., 2004).

Various attempts have been made to develop physical and chemical methods either to remove aflatoxins from contaminated foods and feeds or to degrade toxin present into less toxic compounds (Samarajeewa et al., 1990). Physical approaches to aflatoxin destruction have been done including treating with heat, UV light, or ionizing radiation. These methods are not very effective. Chemical degradations of aflatoxin are usually carried out by the addition of chlorinating, oxidizing or hydrolytic agents. Chemical treatments require expensive equipment and may result in losses of nutritional quality of treated commodities. In addition, the

undesirable health effects of such treatments have not been fully evaluated.

Lactic acid bacteria have been reported to remove AFB_1 from liquid solution (El-Nezami et al., 1998a; Oatley et al., 2000; Haskard et al., 2001; Lahtinen et al., 2004; Peltonen et al., 2001). Specific dairy strains of lactic acid bacteria have been shown to remove AFB_1 effectively from liquid media (El-Nezami et al., 1998a). Zinedine et al., (2005) described the reduction of AFB_1 by lactic acid bacteria strains isolated from Moroccan sourdough bread. Hernandez-Mendoza et al., (2009) investigated the ability of eight strains of *Lactobacillus casei* to bind AFB_1, and the strains exhibited different degrees of aflatoxin binding. Many of these studies have involved *Lactobacillus* strains and physical binding has been proposed as one mechanism of reduction of aflatoxins from the liquid media (Haskard et al., 2001; Lahtinen et al., 2004). Their results showed that the ability of removing AFB_1 from contaminated solution was strain specific. Lahtinen et al., (2004) reported that AFB_1 bound to the bacterial cell wall and suggested that peptidoglycan or other compounds related to peptidoglycan play important role in the AFB1 binding.

Peltonen et al., (2001) showed the ability of both strains of lactic acid bacteria and strains of *Bifidobacteria* to remove AFB_1 from contaminated solution. They found that the binding process was reversible and AFB_1 was released by repeated aqueous washes. El-Nezami et al., (1998b) also found that treatment of bacterial pellets with hydrochloric acid enhance the ability of lactic acid bacteria to remove aflatoxin from contaminated media. An enhancement of bacterial ability to bind aflatoxin B_1 was also observed when the bacterial pellets were subjected to heat treatment. Therefore, the removal of AFB_1 from contaminated liquid media was proposed to involve adsorption of the toxin to the bacteria rather than metabolic degradation. These findings need further investigation into the stability of acid and heat-treated bacteria-aflatoxin complexes both *in vitro* and *in vivo*.

Simanjuntak (2005) had examined the ability of selected strains of lactic acid bacteria to remove AFB_1 from liquid media. Strains of lactic acid bacteria were isolated from various Indonesian fermented foods and from a healthy infant fecal material. *L. paracasei* SNP-2, a candidate probiotic bacteria isolated from a healthy infant fecal material, was the most effective one. Furthermore, it is important to study the ability of viable and non-viable *L. paracasei* SNP-2 to reduce AFB_1 in the media and the stability of their complexes. The objectives of this study were to examine further the ability of the viable and non-viable *L. paracasei* SNP-2 to remove AFB_1 from contaminated liquid media, and to investigate the stability of AFB_1-bacteria binding complexes after washing of the bacterial surface. The binding of AFB_1 to peptidoglycan was also studied.

Materials and Methods

Bacteria

Lactobacillus paracasei SNP-2, which previously was stated as *L. acidophilus* SNP-2 was obtained from Food and Nutrition Culture Collection, Center for Food and Human Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. Stock culture was maintained at -40°C in 10% (v/v) glycerol-skim milk. Working cultures were prepared from frozen stocks by two transfers in MRS broth and incubation at 37 °C for 24 h.

For production of biomass, 10 mL of 24 h culture in MRS broth was inoculated into 100 mL of coconut water containing 5% of yeast extract and incubated at 37°C for 24 h. The culture was inoculated into 1 L of coconut water containing 5% yeast extract and incubated at the same temperature and time as the previous one. After incubation the

suspension was centrifuged at 1,800 g for 20 min at 10°C. The supernatant was discarded and bacterial pellet was re-suspended in phosphate buffer saline (PBS, pH 7.3) to get the viable cell of 10^{10} CFU/mL. Viable cell count was done by using dilution and plating method in peptone-glucose-yeast (PGA) agar.

Binding of AFB_1 to Viable and Non-Viable Lactic Acid Bacteria

Stock of AFB_1 (10 ppm) was obtained from Merck and diluted to 500 ppb with chloroform. It was kept at 4°C. Viable bacteria pellets (10^{10} CFU/mL) were suspended in 1.5 mL PBS pH 7.3 containing 0.25 µg AFB_1, and then incubated at 37°C for 24 h. Subsequently, cells were removed by centrifugation at 1800 g for 20 min at 10°C, and supernatant liquid containing residual AFB_1 was collected and analyzed by HPLC.

To study the binding of AFB_1 to non-viable cell, bacterial culture (10^{10} CFU) in 4 mL of PBS was heated at 121°C for 15 min (heat treated), or bacterial culture was incubated in 4 mL HCl 2 M for 1 h (acid treated). The suspension was centrifuged at 1800 g for 20 min at 10°C. The bacterial pellet was washed twice with 4 mL of PBS. The non-viable cells (heat treated and acid treated) were added with 0.25 µg AFB_1. All incubations were carried out at 37°C for 24 h. All bacterial samples were centrifuged at 1800 g for 20 min at 10°C and supernatant from each sample was collected for AFB_1 analyzes using HPLC. As a control, 0.25 µg AFB_1 in PBS (pH 7.3) was incubated at 37°C for 24 h and analyzed the AFB_1 content.

Binding of AFB_1 to Cell Fraction and Peptidoglycan

The binding of AFB_1 by cell fractions was evaluated by sonicating 10 mL of cell suspension in PBS using ultrasonic oscillator for 60 min at 4°C. Cell fraction was separated from supernatant by centrifugation at 1800 g for 20 min at 10°C and washed twice with PBS. Cell fraction pellet was then suspended in 1.5 mL PBS containing 0.25 µg of AFB_1 and incubated at 37°C for 24 h. Cell fractions were removed by centrifugation at 1800 g for 20 min at 4°C and supernatant containing residual AFB_1 was collected and analyzed by HPLC.

The isolated peptidoglycans from *Bacillus subtilis* (Fluka) were either untreated, heat treated (heated at 121°C for 15 min), or acid treated (incubated in 2 M HCl for 1 h). Each peptidoglycan (0.8 mg) was suspended in 1.5 mL PBS containing 0.25 µg of AFB_1 and incubated at 37°C for 24 h. Supernatants were separated from pellet by centrifugation at 1800 g for 20 min. The obtained supernatants were prepared for AFB_1 analysis.

Complex Stability

The stabilities of bacteria-AFB_1 complexes were evaluated by determining the amount of the remaining bound AFB_1 following five washes. Pellets of viable cell, non-viable cell, and cell fraction that bound AFB_1 were washed by suspending them in 1.5 mL PBS at 37°C for 10 min. After centrifugation, the supernatant was collected, the released AFB_1 was determined using HPLC, and the percentage of bound AFB_1 was calculated. This washing procedure was repeated for another four times. The stabilities of peptidoglycan-AFB_1 complexes, untreated, heat-treated, or acid-treated, were also tested using the same procedure as the bacteria-AFB_1 complexes.

Determination of AFB_1 using High Performance Liquid Chromatography

Samples of the supernatant containing free (un-bound) AFB_1 were analyzed for AFB_1 residues using HPLC method. The HPLC system consisted of pump solvent delivery system (model 110B), a scanning fluorescence

detector (model 474), and 250x4.6 mm Beckman ODS C_{18} column. The sample injection volume was set to 20 μL. AFB_1 was eluted with methanol:ionized water (60:40, v/v) as the mobile phase, at the flow rate of 0.5 mL/min. The detection wavelengths for excitation and emission were set at 365 nm and 418 nm, respectively. The AFB_1-bound cells or peptidoglycan was determined as the differences between the total AFB_1 (control) and the amount of free AFB_1 in the supernatant after treatment. The percentage of AFB_1 bound by bacterial or peptidoglycan suspension was calculated using the following formula:

$$AFB = \left(1 - \frac{AS}{AC}\right) \times 100\%$$

where AFB is percentage of AFB_1 bound, AS is AFB_1 peak area in the supernatant and AC is AFB_1 peak area in the positive control.

Results and Discussion

Table 1 shows the ability of both viable and non-viable cells of *L. paracasei* SNP-2. Viable *L. paracasei* SNP-2 removed AFB_1 from the contaminated PBS solution in moderate amount (25.17%). Zinedine et al., (2005) reported that the *Lactobacillus* strains reduced the amount of AFB_1 in MRS broth in the range of 2.14-44.89%. Dairy strains of lactic acid bacteria could bind AFB_1 in the contaminated solution in the range 5.6 and 59.7% (Peltonen et al., 2001).

This study also showed that heat and acid treatments have significant effect on the amount of AFB_1 bound. There were significantly increased in the percentages of AFB_1 binding for both the heat-killed and acid-killed cells compared to the AFB_1 binding to the viable cells. The percentage of AFB_1 bound to heat-and acid killed cells were more than twice to the viable cells. This is in agreement with research carried out by Haskard et al., (2001), which the AFB_1 binding ability of heat and acid treated of lactic acid bacteria increased 1.1-4.8 times compared to the one with viable cells. El-Nezami et al., (1998b) also stated that heat-killed bacteria significantly removed more AFB_1 from the contaminated solution compared to viable and freeze-dried bacteria. Vosough and Sani (2014) also reported that non-viable *L. rhamnosus* GG significantly reduced more AFB_1 content in liquid media as compared to viable bacteria. Removal of AFB_1 from the contaminated solution by lactic acid bacteria has been suggested due to the binding of AFB_1 to bacterial cell wall or to the cell wall components rather than metabolic degradation (El-Nezami et al., 1998b). Thus viable bacteria were not prerequisite to AFB_1 binding. However, sonication of cells did not

Table 1. Binding of AFB_1 to *Lactobacillus paracasei* SNP-2

L. acidophilus SNP-2	AFB_1 binding	
	% AFB_1 bound*	% relative to viable cell**
Viable cell	25.17±3.19 [a]	100
Non-viable cell (heat-treated)	56.85±2.00 [b]	225.9
Non-viable cell (acid-treated)	61.52±11.72 [b]	244.5
Cell fraction (sonication)	20.04±5.81 [a]	79.6

*The percentage of AFB_1-bound to cells was calculated as the differences between the total AFB_1 and the amount of free AFB1 in the supernatant.

** Binding of AFB_1 relative to AFB_1 bound to viable cells.

Table 2. Binding of AFB_1 to Peptidoglycan

Sample	AFB_1 binding	
	% AFB_1 bound*	% relative to untreated peptidoglycan**
Peptidoglycan	10.6 ± 4.26	100
Heat-treated peptidoglycan	17.5 ± 4.72	164.6
Acid-treated peptidoglycan	22.6 ± 5.48	212.9

*The percentage of AFB_1-bound to peptidoglycan was calculated as the differences between the total AFB_1 and the amount of free AFB_1 in the supernatant.

** Binding of AFB_1 relative to AFB_1 bound to untreated peptidoglycan.

significantly change the AFB1 binding ability of bacterial cells. Sonication destroyed bacterial cells to cell fractions or cell debris. Low density of intracellular materials might be removed during washing. Thus cell debris, which bound AFB_1 in the contaminated solution were cell wall components. Comparable AFB_1 binding ability between viable cells and cell fraction due to sonication indicated that cell wall components play an important role in the binding of AFB1 to bacterial cells.

Cell wall of gram positive bacteria consists of peptidoglycan, which is about 90% of the cell wall components. Therefore the ability of peptidoglycan to bind AFB_1 with or without heat and acid treatment was also studied. The results showed that peptidoglycan could bind AFB_1 (**Table 2**). The percentage of AFB1 bound to viable cells was greater than that of AFB_1 bound to peptidoglycan. It could be caused beside peptidoglycan, there were other cell components that involved in the binding of AFB_1. Protein and DNA can bind covalently with AFB_1 (Coulombe et al., 1993). Acid and heat treatments of peptidoglycan increased the binding of AFB_1 to peptidoglycan. Heat treatment may cause loss of bacterial cell permeability, thus it makes AFB_1 easier to bind to bacterial cell. Acid treatment may breakdown the structure of peptidoglycan. It is suggested that acid treatment may decrease the thickness of peptidoglycan layer, reduce the cross-links and increase pore size. This disturbance may allow AFB_1 to bind to cell wall easier than to viable cell. Our result also showed that non-viable cells due to sonication had comparable AFB_1 binding ability to the viable cells. This suggests that the bacterial ability to remove AFB_1 depends on bacterial cell wall structure. Heat- and acid-treatments may induce changes in the cell wall components, which enhance the ability of the bacteria to bind AFB_1. Lahtinen et al., (2004) studied the AFB_1 binding properties of *L. rhamnosus s*train GG, and suggested that cell wall peptidoglycan, or component that bound covalently to peptidoglycan are important for AFB_1 binding.

Complex Stability

Complex stability between bacterial cells and AFB_1, and between peptidoglycan and AFB_1 were determined from the amount of the released AFB_1 after successive washing. Variable amounts of AFB_1 bound with viable and non-viable bacterial cells were released back into the solution when the complexes of bacterial cells-AFB_1 were washed (**Fig. 1**). Washing of peptidoglycan-AFB_1 complexes also released some amount of AFB_1 (**Fig. 2**). The first washing resulted in the greatest released of AFB_1 from each complexes. Further washing did not significantly affect the binding stability the complexes. Complexes stability of viable cells-AFB_1 and untreated peptidoglycan-AFB_1 were very weak. After first washing, viable cells and untreated peptidoglycan retained 30.4% and

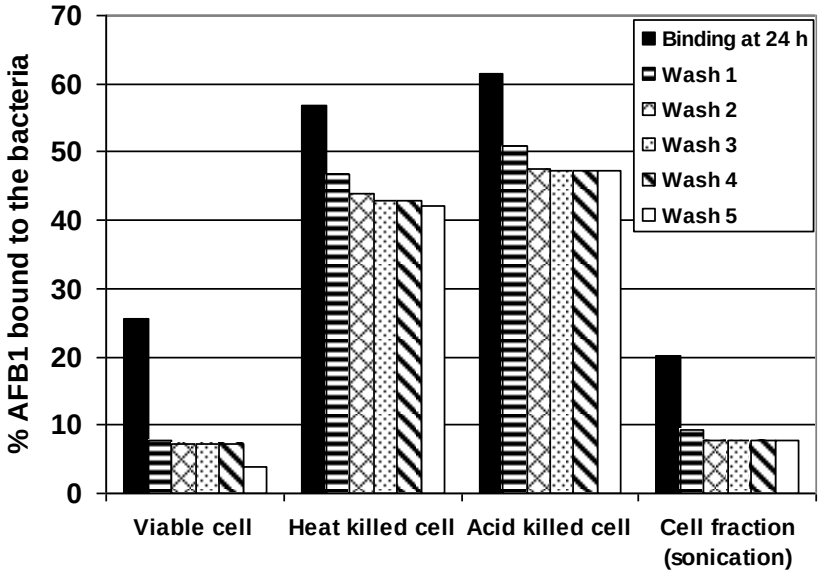

Fig. 1. The effect of washing to the complexes stability of *L. paracasei* SNP-2/AFB_1. Initial binding was determined after viable cell, heat-killed cell, acid-killed cell and cell fraction of *L. paracasei* SNP-2 and 1.5 mL PBS containing 0.25 µg of AFB_1 were incubated at 37°C for 24 h. The formed *L. paracasei* SNP-2/AFB_1 complexes were subjected to five washes with 1.5 mL PBS.

37.6% of the present AFB_1 in the complexes in the original solution, respectively. Sonication slightly increased the stability of cell fraction-AFB_1 complex.

It was observed in this study that AFB_1 was released from the bacterial cell or peptidoglycan-AFB_1 complexes by repeated washes. It means that the binding of AFB_1 to the bacterial cell or peptidoglycan were reversible. Peltonen et al., (2001) found the similar pattern for the complex stability of *L. rhamnosus* Lc 1/3-AFB_1. The release of AFB_1 was greatest during the first wash. Further washing did not significantly release AFB_1 from the complex. In contrast, further washing increase the release of AFB_1 from the complex of *L*. amy*lovorus* CSCC 5160-AFB_1 and *L. amylovorus* CSCC 5197-AFB_1. Even after five washes, AFB_1 bound by *L*. amy*lovorus* CSCC 5160 was completely released back into the solution. The release of AFB_1 from the cell-AFB_1 complexes back to the solution showed that the binding involved weak non-covalent interactions. The difference in the stability of bound AFB_1 to the bacterial cells is probably due to the different strength of non-covalent interactions. This also suggested that the stability of lactobacilli-AFB_1 complexes was also strain specific.

Heat and acid treatments to bacterial cells and peptidoglycan increased significantly their complexes stability. Only 25.8% and 23.1% of AFB_1 released back to the solution from heat and acid killed cells-AFB_1 complexes after five washes, respectively. Even relatively no AFB_1 released after washing of acid treated peptidoglycan–AFB_1 complex. Heat and acid treatments significantly increased not only the amount of AFB_1 bound but also the stability of bound AFB_1. More than 75% of bound AFB_1 still retained in the complexes of heat and acid killed bacterial cell-AFB_1 after five times washing. Heat and acid treatments significantly increased the stability of bound AFB_1 by a number of strains, including *L. acidophilus* LC1 and *L. acidophilus* ATCC 4356 (Haskard et al., 2001). After five times of washing, binding of AFB_1 to heat and acid

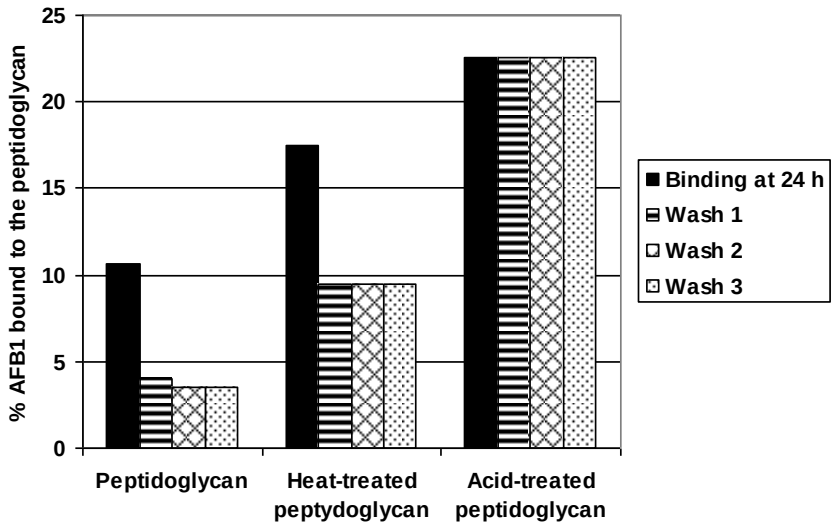

Fig. 2. The effect of washing on the complexes stability of peptidoglycan/AFB_1. Initial binding was determined after untreated, heated treated and acid treated peptidoglycans (0.8 mg) and 1.5 mL PBS containing 0.25 µg of AFB_1 were incubated at 37 °C for 24 h. The formed peptidoglycan/AFB1 complexes were subjected to three washes with 1.5 mL PBS.

treated *L. acidophilus* LC1 released 53.4 % and 0.32%, respectively, meanwhile binding of AFB_1 by heat and acid treated *L. acidophilus* ATCC 4356 released 49.1% and 0.35%, respectively. It seems that acid treatment has more impact on the complex stability. Acid treatment was more profound on the complex stability of peptidoglycan-AFB_1 compared to heat treatment. After three successive washes, almost no bound AFB_1 by acid treated peptidoglycan released back to the solution.

It is clear shown from this study that both viable and non-viable *L. paracasei* SNP-2 have the ability to reduce AFB_1 level in liquid media. *Lactobacillus paracasei* SNP-2 is a potent probiotic strain that can be used as a starter culture for fermented product. Further studies are required to study the ability of AFB_1 reduction ability in food system and the stability of complex under physico-chemical conditions similar to gastro-intestinal tract conditions.

Conclusion

L. paracasei SNP-2 had the ability to bind AFB_1 in PBS solution. The AFB_1 binding ability of heat and acid killed cells were significantly higher than that of viable cells. Cell fractions had comparable ability to bind AFB_1 to the viable cells. Peptidoglycan also has similar ability to bind AFB_1 to viable cells. The binding of AFB_1 to the bacterial cell or peptidoglycan were reversible, demonstrated by the release of bound AFB_1 back to the solution after washing. Heat and acid treatments significantly increased the complex stability of bacterial cell-AFB_1 and peptidoglycan-AFB_1. Even after the third wash, nearly no bound AFB_1 by acid treated peptidoglycan released back into the solution. This suggests that cell wall peptidoglycan is carbohydrate component involved in the binding process.

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