Binding of Aflatoxin B₁ to Lactobacillus paracasei SNP-2 and Stability of Bacteria-AFB₁ 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 B₁ (AFB₁) 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 10¹⁰ CFU/mL. Viable cells, the heated, and acid-killed cells were evaluated for their ability to bind AFB₁ in PBS pH 7.3. Stability of the L. paracasei SNP-2/AFB₁ complexes was evaluated by determining the amount of the released AFB₁ to the PBS following five times washing. The results showed that AFB₁ binding ability to heated-and acid-killed bacteria were higher than that of by viable cells. More than 70% of bound AFB₁ was released from viable bacteria after five times washing. However, the heated and acid-killed cell treatments significantly increased the complex stability of bacteria-AFB₁.

Keywords: Aflatoxin B₁, Lactobacillus paracasei SNP-2, binding of AFB₁, peptidoglycan

Introduction
Aflatoxins are fungal secondary metabolites produced by toxigenic strains of Aspergillus flavus, A. parasiticus, and A. nomius. Aflatoxin B₁ (AFB₁) 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; Oatley et al., 2000; Haskard et al., 2001; Lahtinen et al., 2004; Peltonen et al., 2001). 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 1,800 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 1,800 g for 20 min at 10°C, and supernatant liquid containing residual AFB$_1$ was collected and analyzed by HPLC.

**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 C18 column. The sample injection volume was set to 20 µL. AFB1 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 AFB1-bound cells or peptidoglycan was determined as the differences between the total AFB1 (control) and the amount of free AFB1 in the supernatant after treatment. The percentage of AFB1 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 AFB1 bound, AS is AFB1 peak area in the supernatant and AC is AFB1 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 AFB1 from the contaminated PBS solution in moderate amount (25.17%). Zinedine et al., (2005) reported that the *Lactobacillus* strains reduced the amount of AFB1 in MRS broth in the range of 2.14-44.89%. Dairy strains of lactic acid bacteria could bind AFB1 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 AFB1 bound. There were significantly increased in the percentages of AFB1 binding for both the heat-killed and acid-killed cells compared to the AFB1 binding to the viable cells. The percentage of AFB1 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 AFB1 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 AFB1 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 AFB1 content in liquid media as compared to viable bacteria. Removal of AFB1 from the contaminated solution by lactic acid bacteria has been suggested due to the binding of AFB1 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 AFB1 binding. However, sonication of cells did not

<table>
<thead>
<tr>
<th>L. acidophilus SNP-2</th>
<th>AFB1 binding</th>
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<tr>
<td></td>
<td>% AFB1 bound*</td>
</tr>
<tr>
<td>Viable cell</td>
<td>25.17±3.19 a</td>
</tr>
<tr>
<td>Non-viable cell (heat-treated)</td>
<td>56.85±2.00 b</td>
</tr>
<tr>
<td>Non-viable cell (acid-treated)</td>
<td>61.52±11.72 b</td>
</tr>
<tr>
<td>Cell fraction (sonication)</td>
<td>20.04±5.81 a</td>
</tr>
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*The percentage of AFB1-bound to cells was calculated as the differences between the total AFB1 and the amount of free AFB1 in the supernatant.

** Binding of AFB1 relative to AFB1 bound to viable cells.
Table 2. Binding of AFB$_1$ to Peptidoglycan

<table>
<thead>
<tr>
<th>Sample</th>
<th>AFB$_1$ binding</th>
<th>% relative to untreated peptidoglycan**</th>
</tr>
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<tbody>
<tr>
<td>Peptidoglycan</td>
<td>10.6 ± 4.26</td>
<td>100</td>
</tr>
<tr>
<td>Heat-treated peptidoglycan</td>
<td>17.5 ± 4.72</td>
<td>164.6</td>
</tr>
<tr>
<td>Acid-treated peptidoglycan</td>
<td>22.6 ± 5.48</td>
<td>212.9</td>
</tr>
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</table>

*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.

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 strain 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. amylovorus* CSCC 5160-AFB$_1$ and *L. amylovorus* CSCC 5197-AFB$_1$. Even after five washes, AFB$_1$ bound by *L. amylovorus* 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.

Heat 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.

References


