Characterization of Lactic Acid Bacteria as Poultry Probiotic Candidates with Aflatoxin B1 Binding Activities

E Damayanti¹, L Istiqomah¹, J E Saragih², T Purwoko², Sardjono³

¹Research Unit for Natural Products Technology, Indonesian Institute of Sciences, (BPTBA-LIPI), Jl. Jogja-Wonosari Km. 31.5, Gading, Playen, Gunungkidul, 55861 D.I. Yogyakarta, Indonesia

²Faculty of Mathematics and Natural Sciences, Sebelas Maret University, Surakarta, Indonesia

³Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia

E-mail: emadamayanti80@gmail.com

Abstract. Our previous studies have selected lactic acid bacteria (LAB) with antifungal activities from traditional fermented foods made from cassava (G7) and silage feed palm leaf (PDS5 and PDS3). In this study we evaluated their ability to bind aflatoxin B1 (AFB1) and probiotic characteristic. The probiotic characteristic assays of LAB consisted of resistance to acidic conditions (pH 3), gastric juice and bile salts 0.3%. We also carried out an in vitro evaluation of LAB aflatoxin binding ability in viable and non-viable cell for 24 and 48 hours of incubation. The measurement of aflatoxin content was performed by ELISA method using AgraQuant Total Aflatoxin Assay kit. The results showed that all isolates were potential as probiotics and the G7 isolate had the highest viability among other isolates in pH 3 (92.61 %) and the bile salts assay (97.71 %). The percentage of aflatoxin reduction between viable and non-viable cell from each LAB isolate were different. The highest aflatoxin reduction in viable cell assay was performed by G7 isolate (69.11 %) whereas in non-viable cell assay was performed by PDS3 isolate (73.75 %) during incubation time 48 hours. In this study, G7 isolate performed the best probiotic characteristics with the highest viability in acid pH assay, bile salt 0.3% assay and percentage of aflatoxin B1 reduction in viable cell condition. Molecular identification using 16S rRNA sequence analysis showed that G7 isolate had homology with Lactobacillus plantarum (99.9%). It was concluded that Lactobacillus plantarum G7 was potential as probiotic with aflatoxin binding activities.

Keywords: aflatoxin B1, lactic acid bacteria, probiotic

1. Introduction

Local feed still have many limitations in quantity and quality. One of its limitation is high contamination of mold and mycotoxins in feed, especially feed-based grains which still dominates between 60-70% in the composition of the feed formula. Mycotoxin contamination in feed in Indonesia shows that more than 80% of corn contaminated with aflatoxin B1 (AFB1) and the average is above the threshold of tolerance (> 50 ppb) [1]. The other report also stated that loss of materials and fodder due to fungal contamination worldwide is estimated at 5-10 % [2], while the The Food and Agriculture Organization (FAO) estimates that around 25% of foodstuffs and animal feed contaminated with mycotoxins [3]. The accumulation of mycotoxin contaminant in the diet have a very fatal effect for livestock because it causes the immunosuppressive effect, unproductive livestock and even death. Mycotoxins also cause health problems in both humans and animals such as carcinogenic, mutagenic and teratogenic [4].
Physical handling for mycotoxin contamination such as heating and chemical handling such as the addition of certain toxin binders still causes the contamination of both fungi and mycotoxins [3]. The addition of toxin binders in feed need high costs and could not be guaranteed free mycotoxin. In addition, the use of chemicals in the feed could not be guaranteed safety because the residue in has an effect on livestock products. The use of microorganisms with mycotoxin binding activity and mycotoxin detoxification become a promising alternative. The use of microorganisms and natural materials is expected to be safe and ensures consumer protection with the production of healthy and safe-to-eat livestock products.

Several previous studies showed that some *Lactobacillus* strains have inhibitory activities on growth fungi and aflatoxin production [5]. *Lactobacillus casei* has ability as AFB1 binder and aflatoxin detoxification in foodstuffs [6]. The administration *Lactobacillus rhamnosus* GG in mice was also known to improve the excretion of aflatoxin in feces [7]. *Lactobacillus* strain has higher ability to bind AFB1 than the *Pediococcus* and *Leuconostoc* strains [8]. And the other study revealed that some isolates of *Lactobacillus* isolated from human and dairy product have ability to bind AFB1 [9].

A preliminary study of lactic acid bacteria (LAB) strains isolated from fermented food and silage feed resulted the selected isolates which have antifungal activities. In vitro assay showed that lactic acid bacteria had inhibitory effect on the growth of mycotoxin-producing fungal biomass. *Lactobacillus* sp. G7 inhibited growth of *Aspergillus flavus* FNCC 6002, *A. parasiticus* FNCC 6033, and *Penicillium citrinum* FNCC 6111 with biomass growth inhibition around 63.17% [10]. The other result showed that *Lactobacillus* sp. PDS3 and *Lactobacillus* sp. PDS5 isolated from silage feed have inhibition activities on growth of *A. flavus* FNCC 6002 [11]. Based on that previous studies it was important to determine the probiotic characteristic of selected LAB with aflatoxin binding activities.

2. Material and Method

2.1. Lactic acid bacteria isolates and culture conditions.

Three LAB strains (*Lactobacillus* sp. G7, *Lactobacillus* sp. PDS3 and *Lactobacillus* sp. PDS5) were used for this research for AFB1 binding activities. *Lactobacillus* sp. G7 was isolated from cassava fermentation whereas *Lactobacillus* PDS3 and *Lactobacillus* PDS5 were isolated from silage feed. LAB strains were maintained and collected in Microbiology Laboratory of Research Unit for Natural Product Technology, Indonesian Institute of Sciences (BPTBA LIPI) Yogyakarta, Indonesia. The isolates were identified by Gram staining, catalase tests, motility assay, morphological characteristic and grown on de Man Ragosa Sharpe Agar (MRSA) as a selective medium. LAB isolates were preserved on lyophilized cultures with skim milk as filler material.

2.2. Acid tolerant assay

Acid tolerance assay referred to modified method [12]. LAB cultures on MRS Broth were centrifuged at 4137 x g for 10 min at 4 °C. Pellets were washed two times by sterile phosphate buffered saline (PBS) and diluted in sterile PBS before being inoculated on MRS Broth (pH 2, with 1 M HCl addition). Cell viability was calculated by the total plate count (TPC) method on MRS Agar media on 0 and 90 min incubation times.

2.3. Gastric juice tolerant assay

Gastric juice tolerance was observed according to modified gastric juice simulation [12]. The selected LAB isolates were incubated on MRS Broth at 37 °C for 18 h. A total of 1 mL culture was centrifuged at 5000 g, 10 min, 4 °C then it was washed two times by using sterile PBS and diluted on 0.3 mL sterile PBS. A total of 0.2 mL dilution was taken and then mixed with 1 mL of artificial gastric juice. The mixture liquid was homogenized and incubated at 37 °C for 2 h and then sampled after 0, 1, and 2 h. Serial dilutions of samples was made on sterile PBS and then inoculated on MRS Agar media for
cell viability observation. The artificial gastric juice was made from pepsin (Sigma) (3 g/L) dilution at pH 2.

2.4. Bile salt tolerant assay
Bile salt tolerance was determined by modified method [12]. A total of 1 mL LAB culture was centrifuged at 4137 × g for 10 min at 4 °C and washed twice by using sterile PBS. The cells were diluted in 0.3 mL of PBS then mixed with 0.2 mL of dilution and 1 mL PBS containing 0.3% (w/v) bile salt (Merck). The mixture was incubated at 37 °C for 3 h and then was sampled after 0 and 2 h. The cell viability was calculated by using serial dilution and plated on MRS agar media. Three replications were used for each treatment.

2.5. Aflatoxin binding assay
Aflatoxin binding assay was determined by using method with modification [4]. Solid AFB1 was suspended in benzene–acetonitrile (97/3, vol/vol) to obtain an AFB1 concentration of 1000 mg/L. The benzene–acetonitrile was evaporated by nitrogen stream and AFB1 was resuspended in methanol. The working solution of 5 mg/L AFB1 was prepared in PBS at pH 7.0 by using a stock solution of AFB1 in methanol. The cultures of each strain were divided in two aliquots. In one aliquot, a volume of the culture broth corresponding to approximately 1×10¹⁰ bacteria was transferred into tubes and centrifuged (viable cells). The second aliquot (approx. 1×10¹⁰ bacteria in tubes) was autoclaved at 121 °C for 20 min (nonviable cells) and centrifuged. Supernatants were removed and bacterial pellets (viable or nonviable cells) were washed with deionized water and centrifuged again. Aflatoxin solutions without cells served as controls.

The experiments were driven as following conditions. To study the effect of incubation time on the reduction of mycotoxin concentration, samples were vortexed shortly (5 s) and incubated for 48 h with soft agitation. The binding assay was performed at pH 7.0 for AFB1 after incubation at 48 h. Bacterial pellets with bound AFB1 were washed by suspending in 2 mL PBS (pH 7.0) containing no AFB1, and incubated for 10 min at 37 °C. The bacteria were pelleted, and a sample of the supernatant was collected for the quantification of AFB1 from bacteria. This washing procedure was repeated another two times. Supernatant samples (500 μL) were collected by centrifugation after incubation for 0, 24 and 48 h, and analyzed by ELISA. The measurement of aflatoxin content was performed by ELISA method using AgraQuant Total Aflatoxin Assay procedure. They were compared against the control solution which were free of any bacteria but contained AFB1. The reductions in the quantities of AFB1 present in the test solutions was an indication of the capability of the bacterial strains in binding the AFB1 [13].

2.6. 16S rRNA sequence analysis
Genomic DNA were isolated from fresh culture on MRS Agar according to Genomic DNA Purification Kit (K0512) (Fermentas) protocol. DNA concentrations were measured using spectrophotometer (BioSpec-DNA/protein/Enzyme analyzer-Shimadzu) at λ260 nm and λ280 nm that was making a reaction formula for polymerase chain reaction (PCR). Primer used for PCR reaction was a universal primer 27f (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492r (5’-GGTTACCTTGTTCAGACTT-3’) [14, 15]. Reaction volume of PCR was 50 μl which consist of 20 ng/μl of DNA concentration, 25 μl of GoTaq Green Master Mix, 1 μl of 1492 r primer (10 pm) and 1 μl of 27f primer (10 pm). PCR reaction was performed on 35 cycles for 3 h at 25 min using PCR machine (Takara-Thermal Cycler). PCR condition was set as follows : 4 °C with 94 °C for 2 min of pre-denaturation, 94 °C for 2 min of denaturation, 48.5 °C for 1 min of annealing, 72 °C for 1 min of elongation and 72 °C for 1 min of final extention [14]. PCR product was analyzed using gel electrophoresis agarose 1% [16] using electrophoresis apparatus (BioCRAFT BE 520) and 1 kb DNA ladder (Gene Rule-Fermentas) as ladder. Sequencing of 16S rRNA sequence was conducted by First BASE Laboratories (Singapore). Sequence data was showed in *abi format and was edited by FinchTV program and was contiq analyzed by BioEdit program. Contiq sequence was then analyzed
using basic local alignment search tool (BLAST) program in Gene Bank of National Center for Biotechnology Information (NCBI) (website: www.ncbi.nlm.nih.gov).

2.7 Data analysis

The quantitative data were analyzed by using One-way analysis of variance (ANOVA) with post hoc test (Duncan multiple range test ($P<0.05$)) to distinguish the treatments means using CoSTAT statistical software [17]. The total of bacteria cell (cfu/mL) from viability test was converted to the logarithmic value before statistical analysis. Viability percentages was calculated by dividing a total of colonies in final incubation (log10 cfu/mL) with a total of colonies in initial incubation (log10 cfu/mL) and multiplied by 100%. Whereas the percentage of AFB1 binding for each bacteria was calculated by dividing the difference level AFB1 on certain and 0 minutes incubation time with the level AFB1 on 0 minutes incubation time and multiplied by 100%.

3. Result and Discussion

An important characteristic that must be possessed by lactic acid bacteria with probiotic ability were viability and survival ability on stress condition in digestive tract such as acid pH condition, gastric juice and bile salt exposure. Percentage viability of lactic acid bacteria on digestive tract simulation was shown in Table 1. The assay results on acid pH 3 conditions during 90 minutes incubation time showed that the viability of all isolates did not significant ($P > 0.05$). The viability in this result was lower than the previous results. *Pediococcus acidilactici* R01 and *P. acidilactici* R02 isolated from broiler proventriculus had 103.4 - 120.74 % of viability on acid pH [18]. Different result was reported by Istiqomah [19] that the viability of *L. plantarum* isolated from digestive tract of chicken on pH 3 condition were 41. 9 – 54.6%. The variation result of viability of each LAB strain showed that species of LAB probiotics was different from LAB which is commonly found in fermented foods [20].

| LAB isolates | Viability in simulation condition (%) |
|--------------|--------------------------------------|
|              | acid pH 3 (90 min) | gastric juice (45 min) | bile salt 0.3% (120 min) |
| G7           | 92.61 ± 7.87       | 99.60 ± 0.35           | 97.71 ± 1.64             |
| PDS3         | 88.55 ± 2.84       | 100.23 ± 2.99          | 91.95 ± 6.83             |
| PDS5         | 79.89 ± 10.91      | 98.97 ± 3.46           | 87.52 ± 2.14             |

Note: Means not significant ($P<0.05$)

In the gastric juice simulation test, the viability range were 98.97 - 100.23%. The viability of these isolates were higher than *P. pentosaceus* DB9 isolated from broiler chicken (75.66%) and *Lactobacillus* 172 isolated from the chicken intestine (81.76%) [21]. However, the viability of this study were lower than the isolates of *P. acidilactici* R01 and R02 isolated from broiler proventriculus of 100.35% - 102.71% and 100.02% - 102.65% respectively. While on the bile salt simulation assay for 1 - 2 hours incubation showed that G7, PDS3 and PDS5 isolates had higher viability than *P. acidilactici* R01 (59.69% - 76.53%) and *P. acidilactici* R02 (43.57% - 40.69%) [18].

The ability to survive on bile salt would be possible for LAB to deconjugate bile salt and would be effective to reduce serum cholesterol in broiler chicken. The high activity of bile salt hydrolyse in lumen of intestine could reduce bile salt conjugation ability to break down the lipid [12]. One of the essential characteristic of probiotic in order to give beneficial health for individual host was resistance to the gastrointestinal environment effects such as acid and bile salt in digestive tract [22]. This study showed that all LAB isolates had ability to survive on pH 3 after 90 minutes incubation. In the gastric juice tolerance assay, all LAB isolates also showed high viability after 45 minutes of incubation. Similar to the result in gastric juice tolerance assay, all LAB had high cell viability on 0.3% (b/v) bile salt at two hours of incubation. Based on the ability to survive on bile salt, gastric juice and low pH conditions, all LAB isolates had the same adaptation mechanism toward gastrointestinal stress condition.
Further assay for LAB isolates characterization was aflatoxin binding activity. The aflatoxin binding assay was performed using living bacterial cell (viable) and dead bacterial cell (non-viable). Based on previous research, it was known that aflatoxin binding of lactic acid bacteria was not based on detoxification mechanism by extracellular (enzymatic) metabolite. It was based on binding process by LAB cell wall. The results of aflatoxin binding ability assay were shown in Figure 1 for viable cell and Figure 2 for non-viable cell.

![Figure 1. The AFB1 percentage reduction by viable cell of lactic acid bacteria isolates.](image1)

![Figure 2. The AFB1 percentage reduction by non-viable cell of lactic acid bacteria isolates.](image2)

The results of aflatoxin binding assay occurred in strain-specific lactic acid bacteria and the longer incubation time had tendency to increase binding ability. The aflatoxin binding ability was performed by viable and non-viable lactic acid bacteria. Each bacteria had different ability to bind aflatoxin. Based on percentage of aflatoxin reduction, it was known that G7 isolate resulted the highest percentage to reduce AFB1 in viable cell condition (Figure 1) whereas PDS3 resulted the highest percentage to reduce AFB1 in non-viable cell conditions during incubation time 48 hours. As a mentioned in previous study, mycotoxin reduction by lactic acid bacteria was depend on specific strains [8]. The other study stated that the stability of the bacteria toxin complex was strain dependent and *L. casei* was a stronger binder of AFB1 compared with the other bacteria [6]. The AFB1 binding activity of G7 isolate at 48 hours incubation time was found to be higher (57.87%) than aflatoxin
reduction of *L. acidophilus* P022 and *L. fermentum* subsp. cellobiosus 408 (15.5% - 42.8%) at 60 minutes of incubation [23]. Similar results were stated by Fazeli et al. [6], that *L. casei*, *L. plantarum*, and *L. fermentum* isolated from traditional Iranian sourdough and dairy products are known to have AFB1 binding ability between 25 – 61 % during incubation time 24, 48 and 72 hours.

Lactic acid bacteria had the ability of AFB1 binding both from viable and non-viable cells, this was indicated that AFB1 removal were not depend on bacterial viability and that the metabolic conversion of the toxin did not occur in bacteria. It was known that the removal of toxins occurs via adhesion to cell wall components rather than covalent binding or metabolic degradation, since dead cells still display binding ability [23]. Even though the mechanism of AFB1 removal by lactic acid bacteria is still unknown, it has been suggested that aflatoxins molecules are bound to the bacterial cell wall components of bacteria. AFB1 is bound to the bacteria by weak noncovalent interactions, such as associating with hydrophobic pockets on the bacterial surface [24]. The binding process was reversible and AFB1 was released by repeated aqueous washes [8].

In this study, G7 LAB isolate performed the best characteristic as probiotic with highest viability in acid pH assay, bile salt 0.3% assay and aflatoxin reduction percentage in viable cell condition. One of important characteristic of probiotic was colonization ability of live cell probiotic in host digestive tract. The selected lactic acid bacteria (G7 isolate) which has probiotic characteristic and the highest aflatoxin binding ability was identified as *Lactobacillus plantarum*. The identification procedure was confirmed by molecular test with 16S rRNA. The PCR product of the 16S rRNA gene is about 1,500 bp. Using a 1,500 bp fragment from the LAB isolates, the 16S rRNA gene sequencing revealed 99.9% similarity with the species *Lactobacillus plantarum*. This result similar with biochemical characterization using API 50 CHL which have closed relation to *Lactobacillus plantarum* (99.3 %) [10]. Several studies have reported that *L. plantarum* strain has binding aflatoxin activities. *L. plantarum* strain completely inhibited aflatoxin B1 production by *A. parasiticus* NRRL2999 (98.9 – 99.99%) [5]. *Lactobacillus plantarum* isolated from dairy product had removal aflatoxin B1 ability approximately 56% [6]. *Lactobacillus plantarum* MON03 (LP) isolated from Tunisian butter was found to perform significant binding ability to AFB1 and AFM1 in PBS (i.e. 82 % and 89 %, respectively) within 24 h of incubation and able to tolerate gastric acidity [25]. Among all the investigated *L. plantarum*, strains *L. plantarum* C88 shown the strongest AFB1 binding capacity in vitro, and was orally administered to mice with liver oxidative damage induced by AFB1. The binding rates of different strains (viable and heat-killed bacteria) with AFB1 ranged from 20.88% to 59.44% [26].

**4. Conclusion**
In this study, G7 lactic acid bacteria isolate has the best characteristic as probiotic with high viability in acid pH assay (92.61 %), bile salt 0.3% assay (97.71 %) and aflatoxin reduction percentage in viable cell condition (69.11 %). The identification procedure using molecular test with 16S rRNA revealed that the G7 isolate was identified as *Lactobacillus plantarum* (99.9% of homology).

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