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Suppression on the Aflatoxin-B Production and the Growth of *Aspergillus flavus* by Lactic Acid Bacteria (*Lactobacillus delbrueckii*, *Lactobacillus fermentum* and *Lactobacillus plantarum*)

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Abstract: This research was aimed to study the potency of lactic acid bacteria in the inhibition of *A. flavus* growth and the production of aflatoxin-B. Three species of the bacteria i.e., *Lactobacillus delbrueckii*, *L. fermentum* and *L. plantarum* were investigated for their ability in degradation of aflatoxin-B and suppressing its production as well as inhibiting the growth of *A. flavus*. The trial was designed in three variations by challenging the fungi with each lactic acid bacteria species, before (preceding), at the same time (simultaneous) and after (proceeding) fungal inoculation. It was found that all species of lactic acid bacteria are potential to inhibit fungal growth. Furthermore, aflatoxin-B was also able to be reduced in the medium by all the species. *L. fermentum* gave the lowest concentration of aflatoxin-B, followed by *L. delbrueckii* and *L. plantarum* during 15 days incubation with a total average concentration of 0.20, 0.31 and 0.45 mg L^{-1}, respectively, compared with control which produced 1.09 mg L^{-1}. In conclusion, this study was able to demonstrate that lactic acid bacteria are able to suppress the growth of *A. flavus* as well as to degrade the aflatoxin-B.

Key words: Lactic acid bacteria, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Aspergillus flavus*, aflatoxin-B

INTRODUCTION

Aflatoxin is a secondary metabolite which is produced by *Aspergillus flavus* and *A. parasiticus* (Mehan *et al*., 1991). It is considered as a toxigenic, mutagenic, teratogenic and carcinogenic substance. There are four groups of it i.e., aflatoxin-B1 (AFB1), aflatoxin-B2 (AFB2), aflatoxin-G1 (AFG1) and aflatoxin-G2 (AFG2). The most danger and abundant in nature is aflatoxin-B1 (AFB1). Therefore most research have been done is focused on aflatoxin-B1 (Coallier and Idzack, 1985).

Many cases of aflatoxin contamination on food and feed products have been reported to occur in Indonesia, particularly bean based products. Fardiaz (1996) showed that 80% of bean products which were sampled in the market were contaminated by *A. flavus* and contained aflatoxin-B1 in average of 30 ppm. Recent data reported by Research Institute Indonesia (LIPI) indicated that 47% of soy sauce distributed in Java are contaminated by aflatoxin.

Considering the significant negative effect caused by aflatoxin on health, some researches to overcome this problem have been done including the use of biological system by using potential microbes. Lactic acid bacteria have been reported in some literatures to be able to detoxify aflatoxin. For example, *Lactobacillus rhamnosus* and *Propionobacterium* have been reported were able to degrade aflatoxin-B in chicken intestine (El-Nezami *et al*., 2000).

This study aims to investigate the possibility of three species of lactic acid bacteria i.e., *L. delbrueckii*, *L. fermentum* and *L. plantarum* in degrading aflatoxin-B and inhibiting the growth of *Aspergillus flavus*, the aflatoxin-B producer.

MATERIALS AND METHODS

Pure culture of *A. flavus* and lactic acid bacteria (*L. delbrueckii*, *L. plantarum* and *L. fermentum*) were obtained from Microbiology Laboratory University of Gajah Mada, Yogyakarta. All lactic acid bacteria were grown on Lablemco Tripton Agar (LTA) and Lablemco Tripton Broth (LTB). At first, they were activated in LTA medium before the research was started.

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Inocula preparation: Inoculum for *A. flavus* was provided in conidia form. Pure culture of *A. flavus* containing conidia was taken 1 loop (Öse) and then stroke on slant LTA, before being incubated for four days at room temperature with no special light adjustment (room lighting at day time was using 40 W of Neon fluorescent light). The spores were harvested at day 4th (after obviously observed) by adding 10 mL of physiological solution (NaCI 0.85%) containing 0.1% Tween-80. The spores were dislodged carefully by using Öse while being shaken several times until the spores totally detached from agar medium. The conidia suspension was then used as inoculum after being enumerated and adjusted for their concentration about $1\text{-}5\times10^6$ conidia per mL.

At first, pure cultures of lactic acid bacteria from the provider were activated by three time sub-culturing in LTC medium. Each sub-culturing was incubated at room temperature for 12 h while shaking at 120 rpm. After the third sub-culturing, cell number were enumerated by measuring the Optical Density (OD) at 560 nm. From this enumeration data cell concentration was adjusted at OD value of ±0.5 by diluting with physiological solution (NaCl 0.85%).

After activation step, growth curve was then provided in order to find out the right age of the culture to be harvested as inoculum. The exponential growth phase of the culture was used as the right time for inoculum. The correlation of OD value with exact number of cell per mL was also figured out and made as a standard curve. The exact number of viable count was obtained by plating method. This standard curve was then used to find out the exact number of cell per mL after being measured with spectrophotometer. In amount of 10% v/v (20 mL) of LAB inoculum was inoculated into 500 L Erlenmeyer containing 180 mL LTC medium with or without *A. flavus* (according experiment design) before being incubated at room temperature over 15 days.

Experiment design: Three variation of treatment were conducted as follows. Group-1, *A. flavus* was grown first and then inoculated by each lactic acid bacteria separately (A1-L2). Group-2, each lactic acid bacteria was grown first and then inoculated by *A. flavus* afterwards (A2-L1). Group-3, both *A. flavus* and lactic acid bacteria were inoculated at the same time simultaneously (A1-L1). All experiments were conducted as duplo.

Extraction of Aflatoxin-B: During 15 day incubation the aflatoxin-B content in the medium was measured at day 3, 5, 7, 11 and 15 from five sets culture with two replications each. Mycelium of *A. flavus* grown on the medium was separated with tea strainer before grinding thoroughly with pestle and mortar. Ground mycelium was mixed back with the original medium. This mycelium suspension was then added with 50 mL chloroform and stirred well with electric stirrer over 20 min. Whatman paper No-1 was then used to separate the filtrate and the mycelium pasta. This pasta was added once again with 50 mL chloroform and stirred over 15 min for sec extraction. The first and the second filtrate were mixed together before being evaporated in water bath until concentrated extract was obtained (final volume of concentrated extract approximately 1 mL = 1/100 from original volume). The extract was placed in a glass vial and covered with aluminum foil for further analysis. All preparations were conducted in light-off room condition to avoid the degradation of aflatoxin-B before being measured (Coolier and Idzack 1985).

The analysis of aflatoxin-B: Qualitative analysis was using Thin Layer Chromatography (TLC) method with pure aflatoxin-B (Sigma) was used as a standard. The Retention Factor (Rf) value of the standard aflatoxin-B was compared with the Rf of the extract samples from this experiment. Aluminum plate coated with silica gel GF (20×20 cm) was used as stationary phase while chloroform-acetone (6:4) was used as dynamic phase (eluent). Concentrated extract provided from day 3, 5, 7, 11 and 15 of all treatments (A1-L2; A2L1; A1L1) were then spotted on the TLC plate with micro pipette in a concentration of 10 μL per spot, with 2 cm distance from one another. Last spot was used for aflatoxin-B standard. TLC plate was then developed in a developing chamber after being set saturated with eluent favour in advance. Development was stopped after the final migration of eluent reach the border line which was marked previously with a pencil. TLC plate was then dried out in the fume hood. Spots were then observed under UV light of 365 nm and marked for Rf calculation (Coolier and Idzack, 1985).

Quantitative measurement of the aflatoxin-B concentration was conducted by TLC Scanner (Camag-II) which equipped with IBM computer (CATS Software). The measurement result was calculated by using linier regression equation which was provided first by measuring different concentration of pure aflatoxin-B standard.

The suppression of *A. flavus* growth by lactic acid bacteria: The same design as previously was also applied for this experiment. Dry weight of *A. flavus* mycelium was measured as the parameter of growth suppression. The mycelium was strained with Whatman No.1 set-up in Buchner funnel and vacuum pump before being dried at 70°C out and weighed to obtain the dry weight. The measurement was conducted daily over 15 days.

RESULTS

The influence of Lactic Acid Bacteria on the Growth of *A. flavus*: The interaction between all LAB species with different time of inoculation relatively gave the same growth pattern on *A. flavus*. All treatments can inhibit the growth of *A. flavus* over 15 days significantly compared with control (Table 1). As can be seen on this table, the average result on *A. flavus* growth was most suppressed by *L. plantarum* with all variation and followed by *L. fermentum* and then *L. delbrueckii* over 15 days. However, all treatments are not statistically different from one another, except with control. Total average mycelial biomass which was produced by *A. flavus* after 15 day treatment with three species of LAB with all different variation of inoculation were: 0.52, 0.45 and 0.35 g for *L. delbrueckii*, *L. fermentum* and *L. plantarum* respectively, while for control was 0.62 g.

Growth pattern of *A. flavus* during 15 day incubation after treatment with lactic acid bacteria can be seen on Fig. 1-3 (samples for mycelial dry weight and aflatoxin-B analysis were taken at the same day). From the graph, it can be seen that the growth of *A. flavus* was suppressed from the beginning. Maximum growth of *A. flavus* with no treatment was achieved at day 12 with dry weight of 0.73 g. While, the maximum growth of *A. flavus* with lactic acid bacteria treatments was achieved between day 12 and 15 with maximum dry weight vary from 0.34 g (which was achieved by simultaneous inoculation of *L. plantarum*) until 0.59 g (which was achieved by advance inoculation

Table 1: *A. flavus* dry weight (g) mycelial biomass after treated with lactic acid bacteria over 15 days

	Biomass dry weight (g) after 15 days			
Treatments	A1L2	A2L1	A1L1	Average
A. flavus and *L. delbrueckii*	0.4986	0.5247	0.54	0.5211*
A. flavus and *L. fermentum*	0.4196	0.5024	0.4196	0.4472*
A. flavus and *L. plantarum*	0.3989	0.3254	0.3254	0.3499*
Control (*A. flavus* only)				0.6234

Note: A1L2: *A. flavus* grown first before inoculated with lactic acid bacteria A2L1: Lactic acid bacteria grown first before inoculated with *A. flavus* A1L1: Both *A. flavus* and lactic acid bacteria were inoculated at the same time * Indicates significantly different at 95% degree of confidence

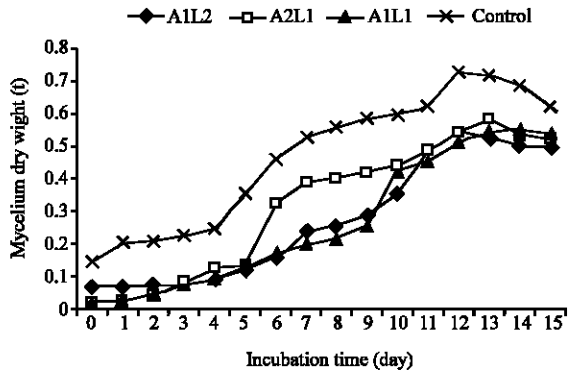

Fig. 1: Growth effect of lactic acid bacteria (*L. delbrueckii*) on *A. flavus* in LTU medium over 15 days

A1L2: *A. flavus* grown first before inoculated with lactic acid bacteria

A2L1: Lactic acid bacteria grown first before inoculated with *A. flavus*

A1L1: Both *A. flavus* and lactic acid bacteria were inoculated at the same time

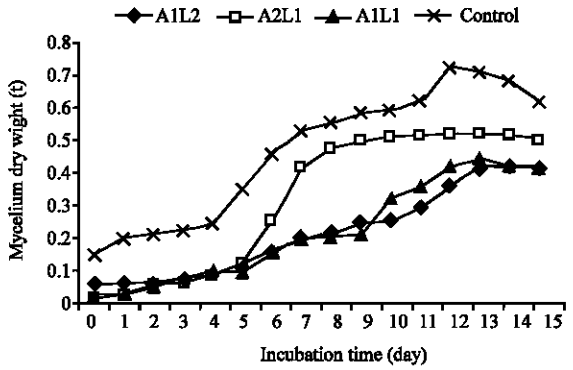

Fig. 2: Growth effect of lactic acid bacteria (*L. fermentum*) on *A. flavus* in LTU medium over 15 days

A1L2: *A. flavus* grown first before inoculated with lactic acid bacteria

A2L1: Lactic acid bacteria grown first before inoculated with *A. flavus*

A1L1: Both *A. flavus* and lactic acid bacteria were inoculated at the same time

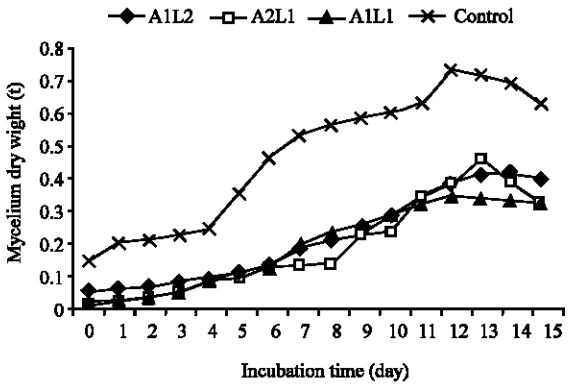

Fig. 3: Growth effect of lactic acid bacteria (*L. plantarum*) on *A. flavus* in LTU medium over 15 days

A1L2: *A. flavus* grown first before inoculated with lactic acid bacteria

A2L1: Lactic acid bacteria grown first before inoculated with *A. flavus*

A1L1: Both *A. flavus* and lactic acid bacteria were inoculated at the same time

with *L. delbrueckii*). Most variation of inoculation with lactic acid bacteria gave relatively the same pattern of inhibition on the growth of *A. flavus* except on the treatment with *L. delbrueckii* and *L. fermentum* which were inoculated with lactic acid bacteria first and then followed by *A. flavus*. This variation gave a bigger increase of *A. flavus* growth after 5 day, while other variations gave relatively small increment. Inhibiting factor produced by both species of lactic acid bacteria in advance inoculation apparently was able to be overcome by *A. flavus* resulting in a better growth compared with the situation of other treatment variations.

The influence of lactic acid bacteria on the production of aflatoxin-B by *A. flavus*: The growth interaction between *A. flavus* and lactic acid bacteria with three different variation of inoculation gave different respond on aflatoxin-B production by *A. flavus*. Overall result as can be seen on Table 2 indicates that all lactic acid bacteria species gave a significant reduction on aflatoxin-B production by *A. flavus* compared with control. The highest inhibition was achieved by treatment with *L. fermentum*, followed by *L. plantarum* and *L. delbrueckii* with total average aflatoxin-B production after 15 day incubation of 0.20, 0.31 and 0.45 mg L^{-1}, respectively, while control produced up to 1.09 mg L^{-1}.

The aflatoxin-B production during 15 day incubation with 5 times sampling (day 3, 5, 7, 11 and 15) was fluctuated from time to time (Fig. 4-6). Non lactic acid bacteria treated culture gave the highest aflatoxin-B production at day 11 with an amount of 1.49 mg L^{-1}, while culture with LAB treatment gave the highest aflatoxin-B production at day 3, 5 and 7. The lowest aflatoxin-B production by *A. flavus* was achieved by *L. fermentum* with simultaneous inoculation. This low concentration was constant from the beginning until the end of experiment (Fig. 5). The treatment with *L. plantarum*, particularly simultaneous and in advance inoculation also gave relatively constant result from the beginning until the end of experiment (Fig. 6).

Table 2: Aflatoxin-B concentration on LTB medium over 15 day incubation after treated with lactic acid bacteria

	Aflatoxin-B concentration (ppm) after 15 days			
Treatments	A1L2	A2L1	A1L1	Average
A. flavus and *L. delbrueckii*	0.746	0.1385	0.4545	0.4463*
A. flavus and *L. fermentum*	0.0699	0.332	0.184	0.1953*
A. flavus and *L. plantarum*	0.651	0.1415	0.1315	0.308*
Control (*A. flavus* only)				1.0901

Note: A1L2: *A. flavus* grown first before inoculated with lactic acid bacteria. A2L1: Lactic acid bacteria grown first before inoculated with *A. flavus*. A1L1: Both *A. flavus* and lactic acid bacteria were inoculated at the same time * Indicates significantly different at 95% degree of confidence

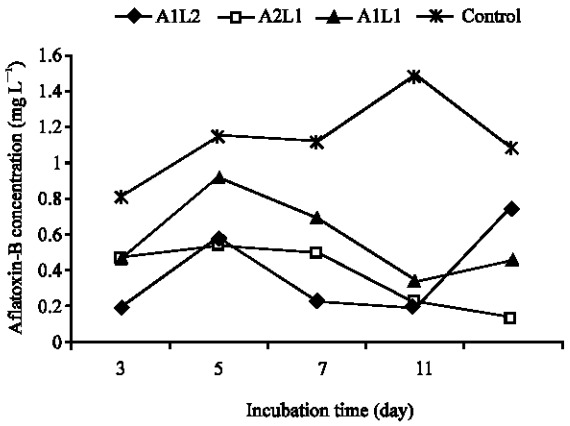

Fig. 4: Aflatoxin-B produced by *A. flavus* treated with lactic acid bacteria (*L. delbrueckii*) in LTB medium over 15 days (preceding initial means inoculated in advance while proceeding initial means inoculated later)

A1L2: *A. flavus* grown first before inoculated with lactic acid bacteria

A2L1: Lactic acid bacteria grown first before inoculated with *A. flavus*

A1L1: Both *A. flavus* and lactic acid bacteria were inoculated at the same time

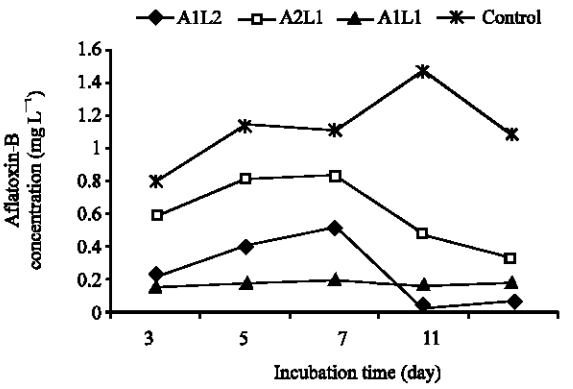

Fig. 5: Aflatoxin-B produced by *A. flavus* treated with lactic acid bacteria (*L. fermentum*) in LTB medium over 15 days (preceding initial means inoculated in advance while proceeding initial means inoculated later)

A1L2: *A. flavus* grown first before inoculated with lactic acid bacteria

A2L1: Lactic acid bacteria grown first before inoculated with *A. flavus*

A1L1: Both *A. flavus* and lactic acid bacteria were inoculated at the same time

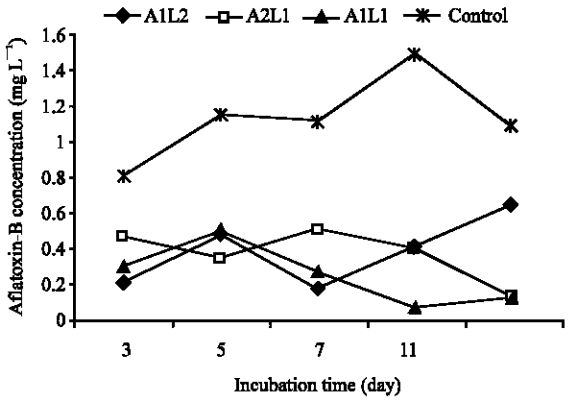

Fig. 6: Aflatoxin-B produced by *A. flavus* treated with lactic acid bacteria (*L. plantarum*) in LTB medium over 15 days (preceding initial means inoculated in advance while proceeding initial means inoculated later)

A1L2: *A. flavus* grown first before inoculated with lactic acid bacteria

A2L1: Lactic acid bacteria grown first before inoculated with *A. flavus*

A1L1: Both *A. flavus* and lactic acid bacteria were inoculated at the same time

The fluctuated phenomena of aflatoxin-B concentration at different time sampling indicate that the lactic acid bacteria culture were able to either suppress the production and to degrade the aflatoxin-B produced by *A. flavus*. As can bee seen from Fig. 4-6, that majority cases show a higher amount of aflatoxin-B at the beginning became lower at the end except for preceding inoculation with *L. delbrueckii* and *L. plantarum*.

DISCUSSION

Growth inhibition caused by lactic acid bacteria on *A. flavus* may be caused by several reasons as follows. Firstly, lactic acid bacteria can produce inhibition factors as was reported by Coallier and Idzack (1985). According to Jenie (1999), inhibition factors produced by lactic acid bacteria were generally heat resistant. Furthermore, it is said that the inhibition mechanism on target microorganisms by lactic acid bacteria is due to destabilization process of cytoplasm membrane. Secondly, lactic acid bacteria, whether they are homo or hetero fermentative group can convert nutrient into organic acids including lactic and acetic acids as well as carbon monoxide. The presence of lactic acid in the medium becomes an inhibition factor for the growth of *A. flavus*. Gourama and Bullerman (1995) stated, that the concentration of lactic acid between 0.75-1.5% was effective as an anti fungal agent. Inhibition mechanism caused by pH can be explained by chemiosmotic and pH homeostasis theory. The presence of lactic acid causes some molecules to dissociate into H^+ and anion. This situation increases trans-membrane proton leading to more proton movement into the cell. Ultimately, more energy is required to maintain the normal condition within the cell, in other words less energy is available for growing (Ray, 1996).

In contrary, Coallier and Idzack (1985) contradicted that lactic acid is not an inhibiting factor for *A. flavus* growth and aflatoxin-B production. Lactic acid is said to be a promoting factor for the growth of *A. flavus* as well as a cofactor substrate for aflatoxin-B production. This mechanism is based on the theory, that in excessive concentration of lactic acid, the cell will oxidize it into piruvic acid as an alternative source of energy for survival mechanism in an extreme environment condition (Moat and Foster, 1995). This point of explanation was also noticed during this study on the treatment with *L. delbrueckii* and *L. fermentum*. When both lactic acid bacteria were grown proceeding *A. flavus* a big increase on mycelial biomass occurred after day 5. These phenomena seem to be in line with Coallier and Idzack (1985) statement. Unfortunately, the lactic acid concentration was not measured during our experiment, therefore we can not confirm whether the increase of mycelial biomass due to the presence of excessive concentration of lactic acid.

Thirdly, competition between lactic acid bacteria and *A. flavus* in batch condition, which no substrate added during the incubation time, was won by lactic acid bacteria since bacteria are more simple living entity compared to fungi. Naturally (genetically), metabolic activity of bacteria is faster than fungi. Therefore, bacteria can utilize the original substrate earlier to produce more cell biomass, while fungi develop later after the nutrient became less available. These are common situation occurred during our study.

In term of aflatoxin-B production, most of the results in this study show a lower concentration of aflatoxin-B compared with control. Previous study also reported that *L. rhamnosus* was able to remove up to 80% of aflatoxin-B from liquid media (El-Nezami *et al*., 1998). This phenomenon can be explained by these following three reasons. Lactic acid bacteria are able to degrade aflatoxin-B enzymatically. Microbial interaction is a dynamic process of action and reaction between lactic acid bacteria and *A. flavus* within the system. A competition for accessing nutrient, toxic metabolic accumulation and any

other unfavorable environmental condition may induce certain enzymes of LAB to become active in order to remain sustained, including the use of aflatoxin-B as a nutrient.

Low concentration of aflatoxin-B in the medium is caused by the attachment of active ligands on lactic acid bacteria cell wall. Haskard *et al*. (2001) stated that a high concentration of acid in the medium induced the formation of attachment sites in the cell wall. Lactic acid bacterial cell wall which consists of polysaccharides and peptidoglycan is important components in the attachment process of aflatoxin-B. Acid condition of the medium may break down some glycosidic bonds on polysaccharides and amide bonds on peptides to produce monomers. These broken molecules loosen the cross linkages between cell wall components which ultimately facilitate the attachment mechanism for the aflatoxin-B. By five time water extraction, up to 71% of the total aflatoxin B_1 remained bound on the cell surface (Haskard *et al*., 2001). The same attachment mechanism by *L. rhamnosus* was also reported to occur on zearalenone, another mycotoxin produced by *Fusarium* (El-Nezami *et al*., 2002).

Another reason which causes the decrease of aflatoxin-B concentration in the medium is due to the less capability of *A. flavus* to produce aflatoxin-B. Meyers *et al*. (1998) and Chiou *et al*. (2002) reported that 18 enzymatic reactions involve in the synthesis of aflatoxin-B. It is said that Afi-J gene is responsible for the production of aflatoxin-B. If this gene is disturbed or become inactive, there will be no production of aflatoxin-B occur or the production may decrease significantly. Most cases in our study indicates that the production of aflatoxin-B after treatment with lactic acid bacteria did not give any significant increase from the beginning. This phenomenon indicates that the inhibition of aflatoxin-B production occurred since the beginning of the incubation time.

The last reason for low concentration of aflatoxin-B with treatment is due to the low of mycelial biomass formation. As already discussed previously, the treatment with lactic acid bacteria was significantly inhibit the growth of *A. flavus*. Growth inhibition is directly influence the production of aflatoxin-B due to low production of related enzymes. Although aflatoxin-B is a secondary metabolite which is not occur during the primary growth, but still the inhibition growth can reduce its production. It has been stated previously, there are many chemical reactions involved for aflatoxin-B production and these reactions are activated by many enzymes. These many enzymes are normally have direct or indirect correlation from one to another. Therefore, the inhibition of mycelial growth has a direct or indirect effect to aflatoxin-B production as well.

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