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In vitro Biocontrol of Aflatoxin B1 Through Reduction Using Lactic Acid Bacteria for Animal Heath

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Abstract

FURANO-COUMARIN compounds known as aflatoxins (AFs) are thought to be the most concentrated group of mycotoxin compounds, containing aflatoxin B1(AFB). Animal Feeds contaminated with AFB have a high risk for animal and human cancer. Therefore, it is essential to locate a microorganism or enzyme with extensive activity against the majority of mycotoxins. The study aims to impact the effect of LAB on broth medium by evaluating the potential efficacy of various spp of LAB for reducing AFB1. On the other hand, the safety tests and stages of LAB during the growth were done. Also, to evaluate the reducing ability of AFB1by LAB at 37°C and cold storage on in MRS. Lactic acid bacteria (LAB) have been broadly utilized in reduce aflatoxins in media it can reduce aflatoxins. The ability of five LAB in AFB reduction from broth MRS media greatly affected by several factors such as time of incubation, pH, temperature and inoculum size. 40°C was shown to be the ideal temperature for *Lactobacillus plantarum* to degrade AFB. Low deterioration was also attained at 4°C. The maximum reduction (58%) of AFB by *Lactobacillus plantarum* was obtained after incubation for 48 h. with inoculum size 10^9 and pH 5 at 40 ° C. Also, reduction of AFB recorded 47 % and 46% for *Lactobacillus rhamnosus* and *Lactobacillus bulgaricus* after 48 and 72h. while, 30 and 25% of AFB reduction by the culture supernatant of *Lactobacillus salivarius* and *Lactobacillus acidophilus* at 40 ° C and pH 5 and after 72h.

Key words: Aflatoxin B1 - Biocontrol - Reduction - Lactic Acid Bacteria - MRS broth media.

Introduction

Aflatoxins (AFs) are secondary metabolites of fungi generated by some Aspergillus species that cause serious harm when consumed by people or animals [1]. They are the most significant mycotoxins in terms of economic impact and public health [2]. According to Siahmoshteh et al. [3], the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* primarily produce the class of heterocyclics known as aflatoxins (AFs). *A. flavus* and *A. parasiticus* have a broad host range and cause aflatoxins (AFs), which contaminate rural harvests, feed, and food products [4]. AFB1 in particular has been shown to have hepatotoxic, carcinogenic, teratogenic, and immune-suppressive effects on both people and animals [5].

Whereas many physical and chemical techniques have been suggested to degrade [6], they have been made less attractive by limitations such as failure to provide the desired effectiveness, protection and nutrient retention along with cost requirements. Nevertheless, as useful alternatives to physicochemical approaches, biological degradation of AFs attracts considerable attention because of their added benefits, such as their minimal loss of product qualities, safety, efficiency, economic and ecofriendly nature [5].

The use of specific microorganisms to control postharvest disease and the toxification of mycotoxins has increased dramatically, making it an intriguing alternative method for removing toxins and guaranteeing the safety of food and feed. However, there are advantages to using bacteria for aflatoxin remediation, such as producing non-pigment and more removal in less time [7]. An illustration of the last strategy is the limiting of aflatoxins in the creature or human stomach by lactic acid bacteria (LAB) or yeast, especially *Saccharomyces cerevisiae* [8]. Many creators have detailed the defensive impact of LAB or different microorganisms [9], but no clear mechanism for this effect has been provided.

The aim of this study was to achieve detoxify aflatoxins by five LAB (*Lactobacillus salivarius, Lactobacillus bulgaricus, Lactobacillus plantarum,*

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Lactobacillus acidophilus and *Lactobacillus rhamnosus*) using ELISA test kit method in MRS broth media under optimum conditions and examine the best factor (incubation time, pH, temperature and inoculum size) affecting degradation of AFB.

Material and Methods

Bacteria strains and culture conditions.

Five dairy strains of lactic acid bacteria, *Lactobacillus bulgaricus, Lactobacillus salivarius, Lactobacillus plantarum, Lactobacillus acidophilus* and *Lactobacillus rhamnosus* from existing culture bank of our laboratory were used which were previously isolated form different sources and preserved. All the bacterial species gave code (Table 1) and analyzed for its ability to reduce the aflatoxins. Fifteen-milliliter overnight starter cultures were inoculated into 150 mL of growth media De Man–Rogosa–Sharpe agar (MRS) and incubated at 37º C for 20 h.

Preparation of the stock aflatoxin solution

Aflatoxin B1 [10] at 2.01 µg/mL was added to 5 mL of acetonitrile. The solution was placed in a water bath for 10 min at 70 °C to evaporate the acetonitrile. One milliliter of 0.1 M Phosphate buffered saline (PBS) pH 6.8 was added to the aflatoxin and the tube was shaken carefully to prepare the stock aflatoxin solution (10.10 µg/mL). Aflatoxins B1 of 100 µg/mL was prepared by introducing the toxins in the double strength specific broth media for each bacterium [10]

Preparation of lactic acid bacteria cultures

Active cultures of lactic acid bacteria were prepared by incubating them at 37 °C for 20 h in MRS broth media. The concentration of the bacterial cultures was determined by spectrophotometry at 600 nm (approximately 1×10^6 CFU/mL). Pellets were obtained by centrifuging the bacterial culture at 10,000 × *g* for 5 min at 4 °C. The bacteria pellets were resuspended in 0.1 M PBS (pH 7) before use [11].

Effects of incubation period, temperature, pH, and inoculum size on AFB reduction by LAB

The media with and without aflatoxins were examined for LAB growth. AFB methanol stock solution (100 µL) was added to (200 µL) pre-culture bacterial as a biomass roughly equal to 1×10^4 - 1×10^5 CFU/mL were introduced and incubated for 96 h vin a 5 mL MRS. The reaction mixtures were incubated in the dark at 37° C without shaking form 24, 48, 72 and 96 h, respectively. Bacterial cultivated in broth (MRS) before and after incubation were tested for AFB reduction. In the pH tests, initial pH value was obtained by adjusting pH to 3.0, 5.0, 0.9 and 9.0 with l.0 N HCl buffer, and to 7.0, 8.0 and 9.0 by 1.0 N NaOH buffer. To determine the effect of temperature, the mixtures were incubated at 4. 25, 40 and 70° C, respectively for 72 h. The effect of inoculum size of bacterial was checked at zero, 10^5, 10^7, 10^9 CFU/mL were introduced and incubated for 72 h vin a 5ml MRS at 40 °C. Negative and positive controls were set according to Guan et al., [12].

Determination of LAB growth

LAB concentration was determined by measuring optical density (OD) of LAB culture broth at the wavelength of 600 nm, then comparing to standard curves previously obtained for each LAB strain by relating colony forming units (CFU) per mL from plate counts and OD measurements [11].

Determination of aflatoxin levels

The reduction of aflatoxin detoxification in each experiment was based on measurements of the amount of AFB present before and after incubation [11]. Measurements were taken at 450 nm in an ELISA reader using an AFLA B1 ELISA test kit (Tecna Celer, MA 220). The sensitivity limit of the kit is rated at 1 ppb (µg/L). The results were calculated according to the manufacturer's instructions [10]. We calculated the percentage of AFB reduction by using the following formula:

$$AFB(\%) = 100X\frac{concentration\ of\ AFB\ in\ the\ medium\left(\frac{mg}{mL}\right)}{concentration\ of AFB\ in\ positive\ controt\left(\frac{mg}{mL}\right)}$$

Results and Discussion

An AFLA B1 ELISA test kit was used to measure the concentration of AFB in five lactic acid bacteria's detoxifying AFB solutions. Figure (1) depicts the aflatoxin B1 standard curve.

The results showed that the tested LAB reduce 34.5, 36.6, 22.2, 46.8 and 22.2 % of AFB by *Lactobacillus bulgaricus, Lactobacillus salivarius, Lactobacillus plantarum, Lactobacillus acidophilus* and *Lactobacillus rhamnosus* respectively (Figure 2). In terms of eliminating the toxins, LP performed better. Mohammad & Hashemi [13], who discovered that *Lactobacillus plantarum* were able to bind AFB at high percentages in a range of settings and discovered comparable outcomes.

According to Badji et al. 2023 [10], *Lactobacillus rhamnosus, Lactobacillus bulgaricus, Lactobacillus salivarius, Lactobacillus plantarum, Lactobacillus acidophilus*, and *Lactobacillus rhamnosus* had different binding potentials for AFB1. These changes might be caused by variations in each strain's size, shape, and surface area, genetic differences across strains. Mycotoxins are removed from contaminated medium by binding to the LAB cell wall, as evidenced by literature [14].

Table (2) shows the effect of AFB expansion in MRS media on the development of the tried LAB strains. Interestingly, MRS served as an ideal

medium for the expansion of all LAB. The addition of AFB to MRS media had no effect on any of the examined LAB strains. All strains entered log phase after 24 hours in either control or treated media, followed by 48 and 72 hours of cell decay, or lag phase. After 24 hours, both LS and LP showed the greatest growth in both control and treated medium. with (0.560 and 0.543) $x10^5$CFU mL and (0.40 and 0.422) $x10^5$CFU mL respectively. While, the lowest growth was recorded using LA, LB and LR in both control and treated medium (0.371 and 0.323) $x10^5$CFU mL, (0.341 and 0.306) $x10^5$CFU mL and (0.211and 0.191) $x10^5$CFU mL respectively).

Comparing this result to the control, the greatest growth was seen after one day of incubation, with gradual decay occurring after 48 and 72 h. Abdel-Shafi et al. [15] reported that treating media with AFB and using MRS media had no effect on the growth of any of the LAB strains.

About the effect of incubation time on reduction of AFB, we can observe a fast process and the AFB binding by L*P. LR and LB* increased from 4.4, 4 and 3.2% (0 h) to 50, 42 and 40% (48 h). while LA and LS reduced 25 and 23% after 48h. The reduction of AFB determination in treated media was recorded in Figure (3). Remarkably, around 5% of AFB content in MRS was adsorbed at 0 time by all bacteria inoculation. In the present work, all strains reduction AFB quickly at 24h, although the elimination remained the same during the first 48 hours of incubation.

These outcomes propose that the limiting of the poison happens rapidly, yet doesn't expand the evacuation significantly with the hatching time Topcu et al. [16]. Pizzolito et al. [17] found that *Ent. faecium* M74 and EF031 strains removed AFB at one hour, accounting for Throughout the course of the 48-hour incubation period, around 65% of the total AFB was eliminated.

In the current examination, it was seen that sums eliminated by LAB for AFB1 hatched in blend) relied upon the pH medium. Similar report by Dawlal et al who observation the decrease in pH from 7 to 6 caused higher mycotoxin reduction and the optimal pH for OTA removal was low [18]. In an acidic environment, the concentration of hydrogen ions (H^+) is higher. As a result, they might have an effect on the surface charge of the bacterial cell wall, which is what makes the interaction between negatively charged mycotoxin molecules and protonated binding sites better (Haskard et al., 2000). By now, it was hypothesized that the LAB strains bound mycotoxins via hydrogen bonds.

Figure (4) depicts the best efficiency and highest absorbance of the obtained results at pH 5. When compared to other applied pH, it was observed that lowering the pH value to 5 led to a significant increase in the reduction of AFB, reaching 53 and 46 percent for LP and LR, respectively. When compared to a pH that was neutral, a slight increase in reduction was also observed. It was exhibited that all applied pH showed different pattern on decrease of aflatoxin.

The ability of LAB strains to remove mycotoxins has been the subject of numerous studies. The efficacy of *E. faecium* in removing AFB1 from PBS solution under various pH and incubation time conditions was evaluated according to Badji et al. [10], to reduce the bioavailability of mycotoxins in the diets of humans and animals, the scientists proposed using these strains to produce fermented meals.

On the other hand, the reduction of AFB1 and OTA by cells of *Lactobacillus bulgaricus, Lactobacillus salivarius, Lactobacillus plantarum, Lactobacillus acidophilus,* and *Lactobacillus rhamnosus* at different pH levels was not significantly different

In a similar vein, Fernandez et al.'s [20] experiments revealed that pH had no effect on LAB strains' ability to detoxify mycotoxin, indicating that no cation-exchanging mechanism was in place.

The effects of five LAB strains on reduction AFB in broth MRS media at temperature (4. 25, 40 and 70 ° C) are shown in figure (5). The amount of AFB bound was hight at 25 and 40 ° C while at 4 and 70 ° C recorded low binding, that indicating the elimination of AFB is independent of bacterial viability, and bacteria are not involved in the toxin's metabolic conversion.

As a result, the total number of bacteria is an important factor which the bacterial cell wall contains that peptidoglycan, polysaccharides and teichoic acids as a principal parts of LAB cell wall ensnared in the limiting component of mycotoxins [16]. Heat inactivation affected these components, resulting in modifications to the bacterial cell wall (protein denaturation and pore aging) [19]. This may account for our study's highest binding efficiency with inactivated LAB cells.

Effects of different inoculum size on toxin removal by *LB, LS, LP, LA and LR* cells are shown in Figure (6). AFB elimination was saturable for both treatment groups and was reliant on toxin concentration. The amount of toxin removed increased with increasing bacterial cells concentration. Numerous studies have examined the factors that affect LAB's mycotoxin binding ability and found that they depend on a variety of parameters, including strain type and LAB cell density [22,23], LAB viability [10], mycotoxin concentration [24], pH medium [25], temperature [26] and incubation time [27]. This could have happened for one of two reasons: first, AFB1 didn't have identical binding sites, or second, the

concentration of mycotoxins used in this study wasn't high enough to cover all binding sites.

Conclusion

This study showed more than 58% degradation of AFB by *LP*. and *LR* might be an excellent candidate for bioremediation and detoxification of AFB from both media. The results reported in this study could contribute towards the development of food and feeds additives for the detoxification of AFs to improve, safeguard and ensure the quality of foods and feeds.

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TABLE 1. Lactic acid Bacteria and its code

Bacterial Strains	Code
Lactobacillus bulgaricus	LB
Lactobacillus salivarius,	LS
Lactobacillus plantarum	LP
Lactobacillus acidophilus	LA
Lactobacillus rhamnosus	*LR*
Positive control	CP
Negative control	CN

TABLE 2. Effect of AFB on LAB growth.

LAB code	10^5CFU mL									
	Zero time		24 h		48 h		72		96	
	Without AFB	With 10µg/L AFB	Without AFB	With 10µg/L AFB	Without AFB	With 10µg/L AFB	Without AFB	With 10µg/L AFB	Without AFB	With 10µg/L AFB
B	0.006	0.006	0.341	0.306	1.23	1.186	1.16	1.028	1.00	1.00
R	0.008	0.007	0.211	0.191	1.324	1.242	1.224	1.072	1.224	1.06
A	0.003	0.002	0.371	0.323	1.52	1.442	1.361	1.197	1.341	1.182
P	0.004	0.003	0.480	0.422	1.413	1.371	2.181	1.919	2.14	1.809
S	0.007	0.007	0.560	0.543	1.721	1.546	3.571	3.144	3.37	3.106

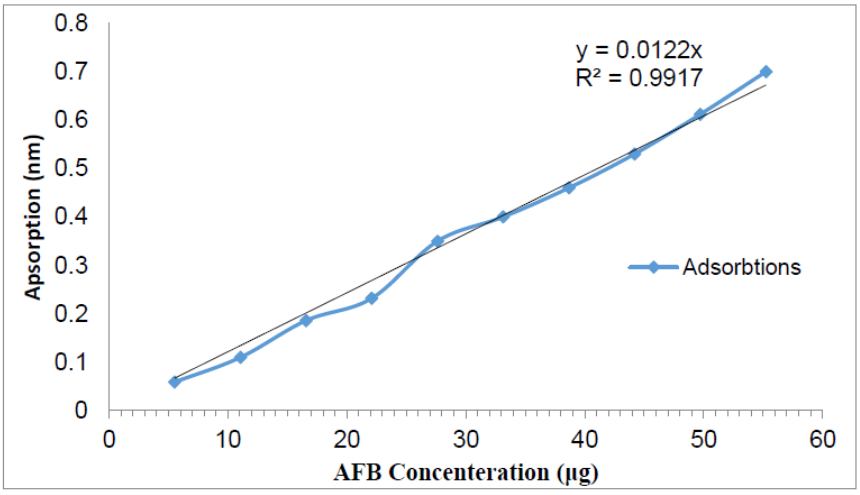

Fig.1. Standard curve Linear relationship between absorption of AFB and concentration

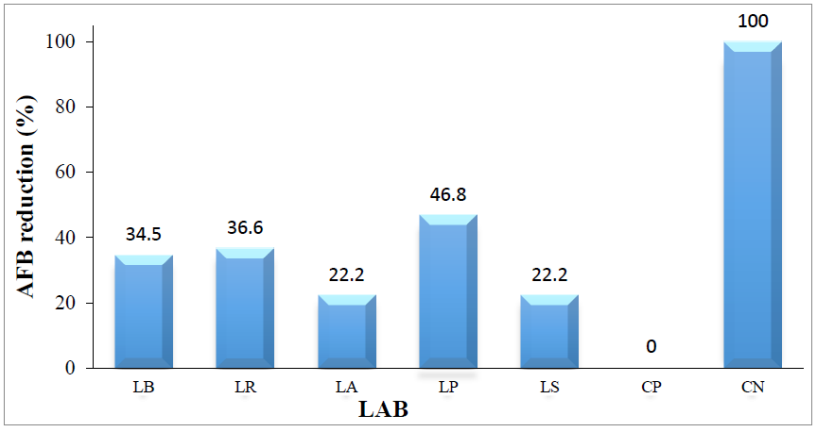

Fig. 2. Screening of different LAB reduced AFB in MRS liquid medium

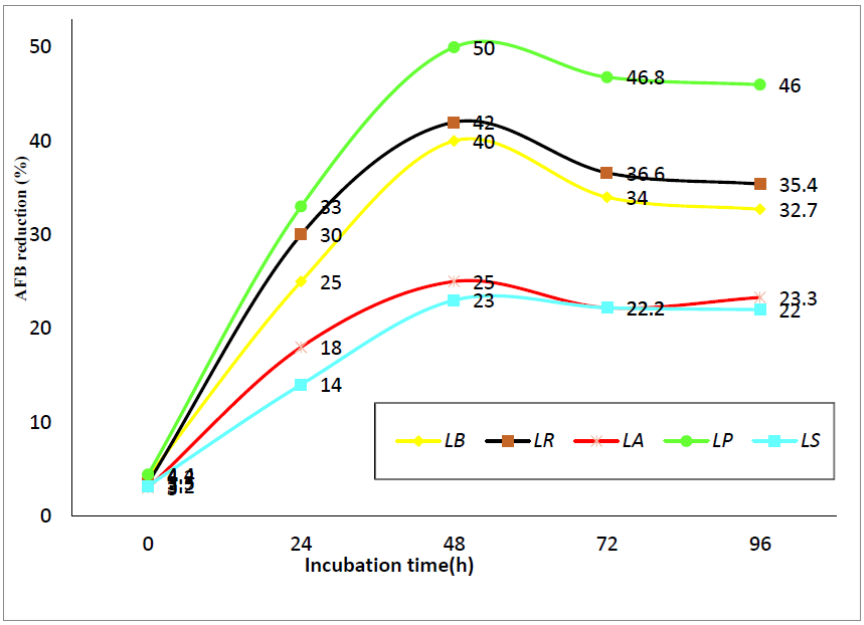

Fig. 3. Effect of LAB strains on AFB reduction at different incubation periods

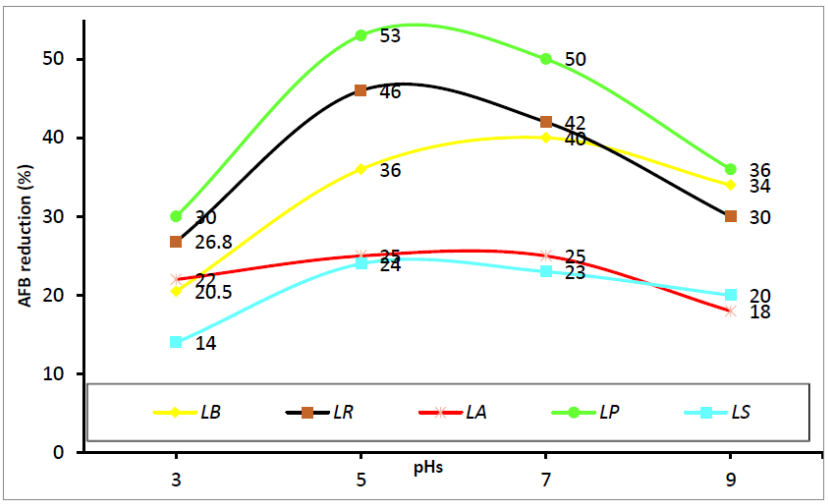

Fig. 4. Effect of different pH values for AFB1 reduction

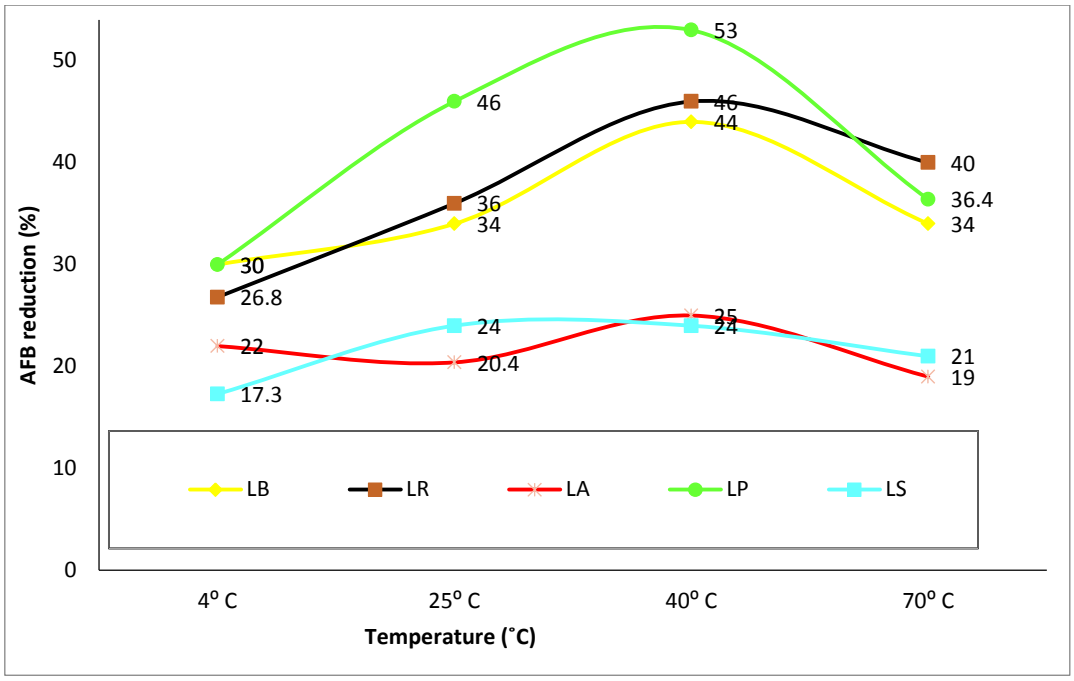

Fig. 5. Effect of different temperature on AFB reduction

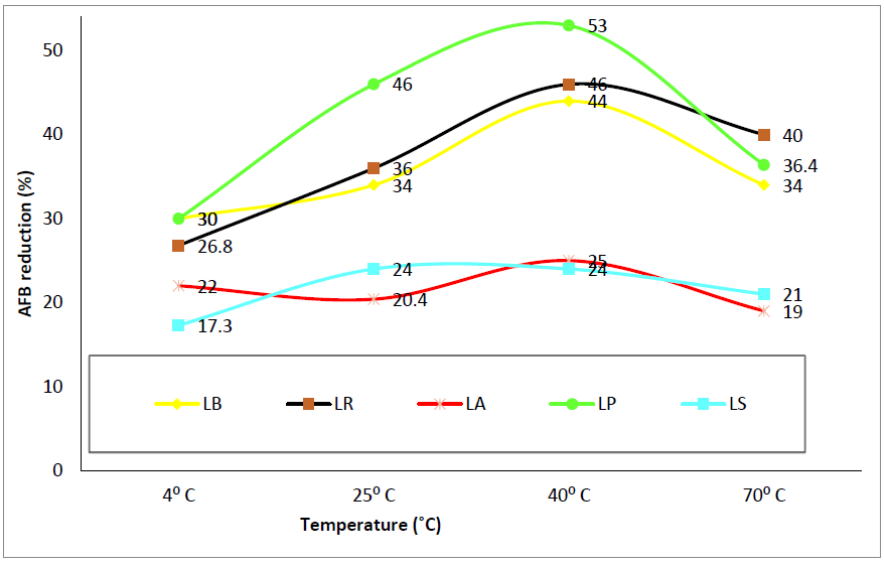

Fig. 6. Effect on deferent inoculum size on AFB reduction

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المكافحة الحيوية للأفلاتوكسين ب1 من خلال الاختزال باستخدام بكتيريا حمض اللاكتيك لتحسين صحة الحيوان

رقية صالح الحبيب

قسم الاحياء، كلية العلوم، جامعة القصيم، المملكة العربية السعودية.

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الملخص

يُعتقد أن مركبات فورانو كومارين المعروفة باسم الأفلاتوكسينات (AFs) هي المجموعة الأكثر تركيزًا من مركبات الميكوتوكسين، والتي تحتوي على الأفلاتوكسين B1 (AFB). تحتوي الأعلاف الحيوانية الملوثة بـ AFB على مخاطر عالية للإصابة بالسرطان لدى الحيوانات والبشر. لذلك، من الضروري تحديد ميكروب أو الإنزيم ذو قدرة عالية ضد السموم الفطرية. حيث تهدف الدراسة إلى معرفر تأثير لأنواع مختلفة من LAB على وسط المرق من خلال تقييم الفعالية المحتملة LAB لتقليل AFB1. من ناحية أخرى، تم إجراء اختبارات السلامة ومراحل LAB أثناء النمو. أيضًا، لتقييم قدرة AFB1 على الاختزال بواسطة LAB عند 37 درجة مئوية والتخزين البارد في MRS بيئة . تم استخدام بكتيريا حمض اللاكتيك (LAB) على نطاق واسع في تقليل الأفلاتوكسين في الوسائط التي يمكنها تقليل الأفلاتوكسين. إن قدرة خمسة بكتيريا لاكتوباسيلوس في تقليل AFB من وسط مرق MRS تتأثر بشكل كبير بعدة عوامل مثل وقت الحضانة ودرجة الحموضة ودرجة الحرارة وحجم العينة. وقد تبين أن 40 درجة مئوية هي درجة الحرارة المثالية لـ Lactobacillus plantarum لتحلل AFB. كما تم تحقيق من التدهور عند 4 درجات مئوية. تم الحصول على أقصى انخفاض (58٪) لـ AFB بواسطة Lactobacillus plantarum بعد التحضين لمدة 48 ساعة بحجم عينة 10^9 ودرجة حموضة 5 عند 40 درجة مئوية. كما سجل انخفاض 47 AFB٪ و 46٪ لـ Lactobacillus rhamnosus و Lactobacillus bulgaricus بعد 48 و 72 ساعة. بينما، 30 و 25% من التخفيض في AFB بواسطة المحلول العلوي لثقافة Lactobacillus salivarius و Lactobacillus acidophilus عند 40 درجة مئوية ودرجة حموضة 5 وبعد 72 ساعة.

الكلمات الافتتاحية: أفلاتوكسين ب1 - المكافحة الحيوية – الاختزال للسموم - بكتيريا حمض اللاكتيك - وسط مرق MRS