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EFFECT OF PROBIOTIC *LACTOBACILLUS PARACASEI* ON *ASPERGILLUS FLAVUS* GROWTH AND REDUCTION OF AFLATOXIN B1 IN VITRO

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ABSTRACT

This study was aimed to use of the probiotic *Lactobacillus paracasei* CNCMI-1572 to reduce the growth of the fungus *Aspergillus flavus* and determine its effect on the production or reduction of aflatoxin B1 through several treatments. All treatments (bacteria grown in MB and exposed to an ultrasonic device, bacterial filtrate exposed to ultrasonic, both treatments examined by FESEM to determine the final particle size, bacteria in MB only, bacterial filtrate only, all at concentrations of 1, 2, 3, 4, and 5%, and finally the treatment of solid MRS medium and bacteria with the fungus grown on it and incubated for seven days) showed significant differences in inhibiting fungal growth. The reduction of the toxin showed very high significant differences after measuring the concentration using HPLC technology. The last treatment was the best, showing no fungal growth with 100% inhibition and, consequently, no toxin production.

Key words: **adsorption, biocontrol, fesem, hplc, ultrasonic device.**

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INTRODUCTION

Mycotoxins are secondary metabolic products produced by a large number of fungal species belonging to several genera, the most notable of which are *Aspergillus*, *Penicillium*, and *Fusarium*. These metabolic products are toxic substances found in food and crops in small concentrations. They might not cause immediate death upon consumption due to their low concentrations, but if the concentration increases, they become very dangerous and can cause acute poisoning. The fungus *Aspergillus flavus* is considered one of the most important mycotoxins-producing fungi and a major disease-causing agent for humans and animals worldwide due to its ability to adapt to different environments. Additionally, it has many varieties that secrete different types of toxins on foodstuffs, causing their spoilage(Khalifa,2017; Mahmood and Al-Jaff,2021; Shams-Allah & Al-Sandooq,2020;Yassein,2020). There are many studies aimed at finding an effective method to reduce the growth of the fungus, its toxin production, or the reduction of the toxin in foodstuffs. These studies have arrived at various solutions, but the impact of environmental factors and the effects of these materials on it remain the most crucial concerns globally (Haider and Hussien,2020; Khalaf et al.2022). The bacteria *Lactobacillus spp*. is among the most important bacterial genera related to human health. These bacteria are also known as lactic acid bacteria because lactic acid is their primary metabolic product, which they produce by fermenting glucose and various carbohydrates. They can also produce other acids, such as acetic and succinic acids, but in very small amounts. These bacteria are naturally found in the mouth, gastrointestinal tract, urinary tract, and female reproductive system. Biological, medical, and environmental studies have been conducted on them, and many studies have addressed the importance of these bacteria in terms of reducing inflammation or their antagonistic resistance to various pathogenic bacteria(Rasheed et al., 2020) . The aim of this study was to determine the ability of the probiotic *Lactobacillus paracasei* CNCMI-1572 to influence the growth of the fungus *A. flavus* and its capacity to produce the toxin AFB1 through several methods and treatments.

MATERIALS AND METHODS

The probiotic used was the branded (L. casei DG®), containing a strain developed by the Italian company SOFAR of the bacterium *Lactobacillus paracasei* CNCMI-1572, which was isolated from human feces and deposited at the Pasteur Institute in Paris with the number (N. CNCMI1572). It was in the form of a powder encapsulated in water-soluble capsules, with each capsule containing 8×10^9 cells.

Preparation of the bacterial suspension

The liquid MRS medium (MB) from HIMEDIA was prepared according to the company's recommended method and sterilized by autoclaving. The bacterial suspension was prepared by adding three-quarters of the capsule to every 100 ml of MB at room temperature and shaking well until the medium changed color from clear golden to milky. Then, the medium was incubated in an anaerobic incubator at a temperature of 38 ±1°C for 72 h. Afterward, 5 ml of the medium was withdrawn and added to fresh MB medium, followed by incubation for 72 h. This process was repeated once more, but with an incubation period of 48 h. The purpose of repeating the inoculation was to activate the bacteria and enhance their vitality. After the third round, 5 glass volumetric bottles were prepared (for conducting experiments). To each bottle, 10 ml of the medium containing bacteria was added for every 200 ml of fresh medium with continuous shaking. The glass bottles were then anaerobically incubated at a temperature of 38 ±1°C for 48 h for conducting experiments. The bacterial counts were calculated from all prepared bottles using the Pour-Plate method as described by (Harrigan & McCance, 1976 ; Speak, 1984 ; Weber & Hekmat, 2013). Colony counts and bacterial counts were calculated per milliliter, and the count was 2×10^7 cfu ml^{-1}.

Isolation of *A. flavus*: An isolated strain of the fungus *A. flavus* was obtained from contaminated maize grains stored in grain stores in Babil Governorate. DNA was extracted through several extractions from Bioneer, a Korean company. PCR stages were conducted using forward primer (TCCGTAGGTGAACCTGCGG) and reverse primer (TCCTCCGCTTATTGA TATGC) , and the samples were sent to Bioneer for DNA sequencing. The fungal isolate was deposited in NCBI under the accession number (OR536429) with the code *A. flavus* isolate D88.

Efficiency testing treatments of *Lactobacillus paracasei*

MLU Treatment (MRS Broth + *Lactobacillus* + Ultrasonic): After 48 h of anaerobic incubation, the MB containing the bacteria was gently shaken five minutes, and then exposed to an Omni Sonic Ruptor 400 ultrasonic device at the Mycotoxin Laboratory in the College of Agricultural Engineering Sciences at the University of Baghdad. The bacterial suspension was subjected to ultrasound in four stages, each lasting 3 minutes, with a 20-minute interval between stages. Then, the suspension was allowed to cool down to room temperature. PDA medium was prepared and sterilized, left to cool to 40-43°C, and then the bacterial suspension was added to PDA at concentrations of 1%, 2%, 3%, 4%, and 5%. The mixture was poured into petri dishes and allowed to solidify.

MLMU Treatment (MRS Broth + *Lactobacillus* + Millipore + Ultrasonic):

After incubation, distributed into sterilized test tubes and centrifuged at 5000 RPM for 25 minutes to separate the bacterial cells from the metabolic substances produced by the bacteria in the MRS broth (Abbes et al., 2016; Cruz et al., 2020). the supernatant was slowly passed through 0.22 µm Millipore filters. The resulting extract was then subjected to ultrasonic treatment and allowed to cool. Subsequently, the bacterial metabolic extract was added to PDA medium using the same method and concentrations as the previous treatment .

MLM Treatment (MRS Broth + *Lactobacillus* + Millipore): The same procedures as treatment 2 were applied, but without using the ultrasonic device, and with the same concentrations.

ML Treatment (MRS Broth + *Lactobacillus*): After 48 h of anaerobic incubation of the fourth volumetric bottle, the MRS broth containing the bacteria was gently shaken five minutes. PDA medium was prepared, sterilized, and left to cool to 40-43°C. Then, the bacterial suspension was added to the PDA medium at the same concentrations of 1%, 2%, 3%, 4% and 5%, and the mixture was poured into petri dishes.

Mal Treatment (MRS Agar + *Lactobacillus*): MRS agar medium was prepared, sterilized, and cooled to 44°C. Then, it was poured into petri dishes. Next, 100 µl of bacterial suspension (grown in MB) was added to the MRS agar medium and spread evenly on each dish. The dishes were incubated anaerobically at 38 ±1°C for 48 h until complete growth of the bacterial layer. Then, sterilized PDA medium was poured slowly at 43°C (above bacterial growth) until the entire surface was covered with the bacterial film, and it was left to solidify. In all mentioned treatments, after solidification of the medium, the plates were inoculated with 50 µl of fungal suspension of *A. flavus* containing 5×10^6 spores in the center of each plate. They were then incubated in an aerobic incubator at 30°C for 7 days to measure fungal growth rates and diameters. After the incubation period, AFB1 toxin was extracted and its concentration was measured using HPLC technology. The particle size examination was conducted using Field Emission Scanning Electron Microscopy (FESEM), utilizing the MIRA III model device manufactured by Tescan, a Czech company, for treatments 1 and 2 to determine the size of the particles.

Statistical analysis: Statistical analysis was performed using GenStat Discovery Edition 4 software to analyze the study data through a Completely Randomized Design (CRD). Significant differences between means were compared using the Least Significant Difference (LSD) test at a significance level of 0.05.

RESULTS AND DISCUSSION

The results of the analysis by FESEM show that all medium materials formed a spherical structure with a diameter of 20.22-24.52 nanometers, scattered on the surface of the aggregated material. The (Figure 1) also indicates that the particles have separated from each other , indicating that the prepared materials will remain stable for a long period.

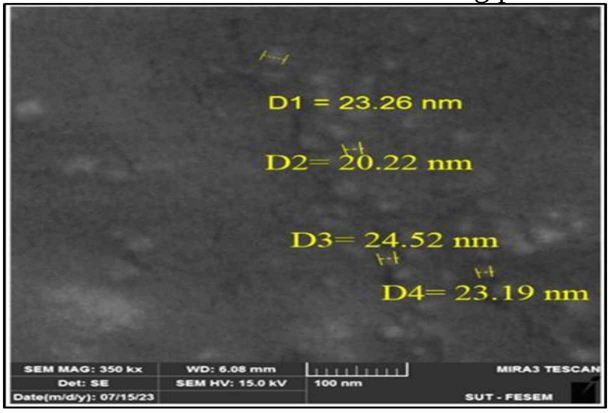

Figure 1. FESEM examination illustrating the particle size for treatment (MLU).

As for treatment MLMU, FESEM measurement was conducted as illustrated in (Figure 2). The measurement showed that the materials formed a spherical structure with a diameter of 12.62-16.09 nm. The measurement here indicated that the particles were smaller than those in the previous treatment. Examination revealed that the particles had separated from each other, indicating that the prepared materials would remain even more stable for a longer period.

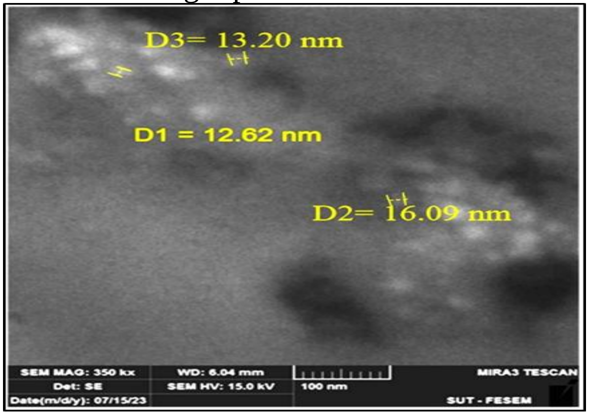

Figure 2. FESEM examination illustrating the particle size for treatment MLMU

In all treatments except for treatment MaL, radial fungal growth was somewhat good. However, when comparing the fungal colony density and spore production density for all treatments with the control treatment, the growth density in fungal colonies and spores was lower in the treatments compared to the control, as indicated by the results in (Figure 3) and (Table 1). All concentrations in the treatments showed significant reductions in fungal growth at different levels. The results of treatment MLU showed that all concentrations used were significant in reducing the growth rate of the *A. flavus* fungal colony. The best concentration recorded the lowest growth rate of the fungus over seven days of incubation, which was the 2% concentration with a rate of 6.791667 cm and an inhibition percentage of 11.79654% compared to the control treatment, which recorded a growth rate of 7.7 cm. All concentrations were characterized by inhibiting the fungus's ability to produce AFB1 toxin with high significance differences. The concentrations of 3%, 4%, and 5% showed no production of AFB1 toxin, with an inhibition percentage reaching 100% compared to the control treatment, which recorded a toxin concentration of 189 ppb. In treatment MLMU, the best concentration recorded the lowest growth rate for the fungal colony was the 1% concentration with a growth rate of 6.375 cm after seven days of incubation and an inhibition percentage reaching 17.20779% compared to the control treatment. Regarding the effect of the treatment on inhibiting fungal toxin production, all concentrations showed significant differences, but the best concentrations did not record any toxin production, namely 3% and 5%, with an inhibition rate reaching 100% compared to the control treatment. In treatment MLM, the aim was to assess the effect of the metabolic substances (without Ultrasonic treatment). The results showed that all concentrations had minor significant differences in fungal growth. The 3% concentration recorded the lowest growth rate for the fungal colony after seven days, with a rate of 6.958333 cm and an inhibition percentage of 9.632035% compared to the control treatment. Regarding its effect on toxin production, all concentrations showed high significant differences in inhibiting fungal toxin production. Concentrations of 4% and 5% showed no toxin production, with an inhibition percentage reaching 100% compared to the control treatment. In treatment ML, the aim was to assess the effect of live bacterial cells cultured in MB on inhibiting fungal growth and toxin production. In this treatment, the bacterial cell dimensions ranged from 1.6 to 3 µm in length and 0.5 to 1 µm in width. All concentrations were significant, and the best concentration recorded the lowest growth rate for the fungal colony after seven days of incubation, which was the 1% concentration with a growth rate of 5.916667 cm and an inhibition percentage of 23.16017% compared to the control treatment. All concentrations showed significant differences compared to the control treatment, and the 5% concentration recorded the best inhibition percentage, reaching 100% compared to the control treatment. In treatment MaL, the aim was to assess the efficiency of live and active bacterial cells cultured in solid medium and synchronize their growth with fungal inoculum growth. This treatment did not record any fungal growth, with a 100% inhibition rate of fungal spore growth after seven days of incubation, thereby inhibiting fungal growth and consequently no production of AFB1 toxin. Probiotics are living organisms with multiple benefits for humans when consumed, and they have other advantages as well. In terms of mycotoxins, they have the ability of bio adsorption, and sometimes they also possess biodegradation capabilities (although biodegradation often takes longer than bioadsorption). Biodegradation refers to the breakdown of some bonds and the structural change of the AFB1 toxin. This process may have undesirable effects and may potentially produce metabolites (perhaps) harmful to living organisms, such as Aflatoxicol , As for bio adsorption, it is considered the most efficient method for probiotics(Luo et al., 2018; Verheecke et al., 2016). Several hypotheses have been proposed by many studies regarding the mechanism by which bacteria inhibit toxins, Haskard et al. (Haskard et al., 2000) suggested that the mechanism of action of probiotics involves toxin reduction, as they observed that Aflatoxin toxins bind to the surface of bacterial cells through hydrophobic interactions. However, when the cell surface was treated with controlled heat, a decrease in hydrophobic particles was noted, while bioadsorption increased. This led to the interpretation that the interior of the bacterial cell may play a role in the bioadsorption process of AFB1 toxin. The outer surface of bacteria contains peptidoglycan, which plays a crucial role in aflatoxin binding to the cell. Additionally, electrostatic interactions and teichoic acid also contribute to the binding process. However, the presence of fatty acids on the surface was disproven to participate significantly in the binding process, as treatment with lipases did not significantly affect the AFB1 binding process. Another hypothesis is positive cooperativity in AFB1 bioadsorption, meaning that once one molecule of AFB1 is adsorbed onto the bacterial cell surface, it enhances the adsorption of a second molecule inside the cell. This evolved into the autoaggregation hypothesis, which suggests that when one molecule of AFB1 binds to the cell surface, another molecule of AFB1 that is not bound (free) interacts with the first molecule bound to the surface(Lahtinen et al., 2004; Liu et al., 2020; Sarlak et al., 2017).

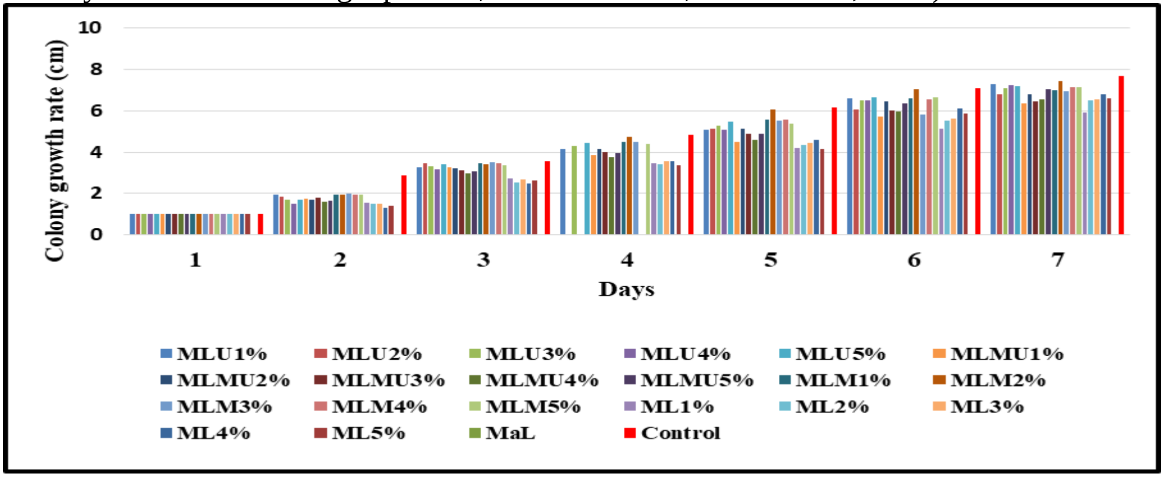

Figure 3. The effect of different concentrations of *Lactobacillus paracasei* CNCMI-1572 treatments on the growth rate of *A. flavus* fungus in PDA medium over seven days

Table 1. The effect of different concentrations of *Lactobacillus paracasei* CNCMI-1572 treatments on the growth rate of *A. flavus* fungus and the reduction of AFB1 toxin production

Treatments	Conc.	Colony growth rate (cm)	%Growth inhibition	Conc. AFB1(ppb)	% Inhibiting AFB1 production
MLU	%1	7.291667	5.30303	80.97	57.15873
	%2	6.791667	11.79654	41.22	78.19048
	%3	7.083333	8.008658	0	100
	%4	7.25	5.844156	0	100
	%5	7.208333	6.385281	0	100
MLMU	%1	6.375	17.20779	142.56	24.57143
	%2	6.791667	11.79654	80.11	57.61376
	%3	6.458333	16.12554	0	100
	%4	6.541667	15.04329	12.58	93.34392
	%5	7.05	8.441558	0	100
MLM	%1	7	9.090909	66.57	64.77778
	%2	7.416667	3.679654	20.51	89.14815
	%3	6.958333	9.632035	10.85	94.25926
	%4	7.125	7.467532	0	100
	%5	7.166667	6.926407	0	100
ML	%1	5.916667	23.16017	168.9	10.63492
	%2	6.5	15.58442	112.65	40.39683
	%3	6.5625	14.77273	80.65	57.32804
	%4	6.8	11.68831	44.9	76.24339
	%5	6.6	14.28571	0	100
MaL		0	100	0	100
Control		7.7	-	189	-
L.S.D.		2.501		36.73	

Subsequently, a newer hypothesis emerged, suggesting that lactic acid bacteria bind to metal ions present in the medium and then bind to AFB1 either through ions or in other sites. Regarding the mechanism of binding, it occurs through bioadsorption and can involve both live and dead (thermally killed or by other means) cells or cell wall components. *Lactobacillus casei* bacteria were found to play a significant role in AFB1 binding, not only through the bacterial cell wall but also through all cell components. However, the strength of binding depends on the toxin concentration, with live cells exhibiting higher binding efficiency (98%) compared to heat-killed cells. In general, several factors contribute to the bioadsorption process. As mentioned by Zhao et al. (Zhao et al., 2015), the incubation period is considered a crucial factor. Another factor, as noted by Serrano-Nino *et al.*, (Serrano-Nino et al., 2015), is the presence of teichoic acids on the peptidoglycan layer, which plays a significant role in the bioadsorption process. Another factor that increases bioadsorption capacity is the bacterial strain type, which is considered important due to the variation in chemical compounds present on the cell wall, differing from one bacterial strain to another (Liew et al., 2018). There are studies indicating that interactions between AFB1 and β-D-glucan of the bacterial cell wall may involve Van der Waals interactions and hydrogen bonds, leading to the bioadsorption process (Hernandez-Mendoza et al., 2009; Palomino et al., 2013). It has been found that more than 25 genes are involved in the biosynthetic process of aflatoxins. These genes include some involved in initiating the biosynthetic process and others activated later in the process. One of these genes is Omt-A (Omethyltransferase A), which is characterized by its involvement in the late stages of aflatoxin biosynthesis. It is capable of converting Sterigmatocystin to Omethyl sterigmatocystin and Dihydro sterigmatocystin to Dihydro-O-methyl sterigmatocystin. These bacteria contribute to the inhibition of the Omt-A gene through their antifungal compounds. It is believed that the inhibition of this gene contributes to reducing the regulation of mRNA levels for the rest of the genes involved in this pathway, thus inhibiting toxin production (Gomaa et al., 2017;J ahanshiri et al., 2015; Unnevehr & Grace, 2013). As mentioned earlier, the efficiency of binding varies depending on the bacterial species due to differences in the structural components of the cell wall and other associated structures. For example, it has been found that certain bacterial species such as *L. plantarum*, *L. acidophilus*, *L. reuteri*, *L. johnsonii*, *L. rhamnosus*, *L. bulgaricus, and L. casei* exhibit lower binding affinity to the toxin compared to *L. paracasei*. Isolates of *L. plantarum* 49 and *L. fermentum* have been noted for their higher efficiency in toxin binding (Corassin et al., 2013; Panwar et al., 2019). A study found that heating increased the interaction between bacterial cells and aflatoxin molecules in heat-killed bacteria (Elsanhoty et al., 2013). However, another study indicated that increased exposure to heat may lead to the destruction of bacterial cell wall components, especially proteins, reducing the bacterial cells' ability to bind toxins (Corassin et al., 2013). Generally, other studies have shown that live cells are more efficient than dead cells due to their secretion of certain metabolic substances upon binding with the toxin. These substances work to break down or alter the chemical composition of aflatoxins. Consequently, some experiments that rely on toxin recovery may not yield high recovery rates due to structural degradation and the breakdown of binding forces for aflatoxins (Corassin et al., 2013 ; Cruz et al., 2020 ; Elsanhoty et al., 2013 ; Panwar et al., 2019).

Studies have found that bacterial exudates contain various antifungal and anti-toxin production substances, preventing fungi from producing aflatoxins and inhibiting fungal growth in food materials. The inhibition of aflatoxins by certain *Lactobacillus* bacteria is likely attributed to the presence of low molecular weight bacterial metabolites spread on the cell membrane. These compounds are likely to be small peptides or acidic protein compounds capable of functioning even if the acidity of the environment changes. Generally, most antifungal substances are low molecular weight compounds, including organic acids like lactic and acetic acids, protein compounds, hydroxy fatty acids, rutinone, hydrogen peroxide, phenolic compounds, and other compounds such as 3,6-bis(2-methylpropyl)-2,5-piperazinedione (Dalie et al., 2010 ; Damayanti et al., 2014 ; Kim, 2005 ; Yang & Chang, 2010). Lactic acid bacteria species are distinguished by their significant antifungal properties due to their relatively large genome size. Consequently, they encode numerous enzymes that neutralize carbohydrate activity and biosynthesize secondary products such as amino acids and fatty acids. Additionally, these bacteria likely form a biofilm that limits oxygen availability to fungi, leading to an increase in phenolic compounds and a decrease in AFB1 toxin levels. It has been found that one of the inhibitory factors for fungal growth is the bacteria's ability to produce hydrogen peroxide in the presence of oxygen, which significantly contributes to fungal eradication (Ahlberg et al., 2017). Lactic acid bacteria are characterized by their high ability to produce organic acids and bacteriocins. They work to change the pH of the environment, converting bacteriocins from inactive to highly active compounds. Ultimately, these substances affect metabolic processes such as oxidation and the transportation of materials inside and outside the cell. The acid produced by bacteria can exist in two forms of solutions: dissociated and undissociated. The undissociated form is considered more harmful because it spreads to the cell membrane of living cells and then to the cytoplasm, causing the release of hydrogen ions and thus hindering cell functions. Additionally, bacteriocins and cytokines contain compounds known for their high affinity to bind to highly specialized cell receptors, leading to uncontrolled flow of amino acids and positively charged ions, resulting in membrane rupture and cell death (Saranya & Hemashenpagam, 2011). We can conclude that all these studies indicate the significant importance of these bacteria in the process of toxin absorption and reduction. This method (absorption) is considered the most preferred and popular mechanism of action against toxins. This study found that these bacteria and their derivatives have a significant inhibitory effect on toxin production, reaching up to 100% inhibition at certain concentrations. Therefore, these bacteria are considered one of the excellent solutions for toxin reduction, besides being environmentally and medically safe.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR/S DECLARATION

We confirm that all Figures and Tables in the manuscript are original to us. Additionally, any Figures and images that do not belong to us have been incorporated with the required permissions for re-publication, which are included with the manuscript.

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تأثير المعزز الحيوي *Lactobacillus paracasei* على نمو الفطر *Aspergillus flavus* و اختزال سم Aflatoxin B1 مختبريا

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المستخلص

هدفت هذه الدراسة الى استخدام المعزز الحيوي CNCMI–1572 *Lactobacillus paracasei* للحد من نمو الفطر *Aspergillus flavus* ومعرفة تأثيره على انتاج سم الافلاتوكسين B1 او اختزاله بعدة معاملات، اذ تميزت جميع المعاملات (معاملة البكتريا المنماة في MB وتعريضها لجهاز Ultrasonic، معاملة الراشح البكتيري وتعريضه ل جهاز Ultrasonic، كلا المعاملتين فحصت عبر FESEM لمعرفة حجم الدقائق النهائي، معاملة البكتريا في MB فقط ، معاملة الراشح البكتيري فقط وجميعها كانت بتراكيز 1و2و3و4و5% واخيرا معاملة وسط MRS الصلب والبكتريا، نمي الفطر عليها وحضنت لسبعة أيام). سجلت المعاملات فروق معنوية في تثبيط نمو الفطر أما اختزال السم فتميزت المعاملات بفروق معنوية عالية جدا بعد قياس التركيز بتقانة HPLC، و تميزت المعاملة الاخيرة كافضل معاملة بالدراسة حيث لم تسجل اي نمو للفطر وبنسبة تثبيط وصلت الى 100% وبالتالي لايوجد اي انتاج للسم.

الكلمات المفتاحية: الادمصاص، المقاومة الحيوية، جهاز الموجات الفوق الصوتية، fesem، hplc.