How to Cite:
AL-Masari, S. M. S., & Al-Obaidi, A. B. (2022). Using B.clausii to reduce the effects of aflatoxin B1. *International Journal of Health Sciences*, *6*(S4), 7702–7713.
https://doi.org/10.53730/ijhs.v6nS4.10867

Using B.clausii to reduce the effects of aflatoxin B_1

Salih M. S. AL-Masari
Department of Biology, Faculty of Science, University of Kufa, Iraq
Corresponding author email: salihmahdi782@gmail.com

Atheer B. Al-Obaidi
Department of Biology, Faculty of Science, University of Kufa, Iraq
Email: atheer.alobaidi@uokufa.edu.iq

***Abstract*---**In the experiment, a bacteria (B. clausii) was more efficient, with a percentage of inhibition of 58.03 percent, while a bacteria (B. subtilis) had a percentage of inhibition of (55.28 percent), so it was chosen in our study to test its efficiency as a probiotic to remove toxins in rats. So 12 rats were divided into four groups, each with three rats. The first group was the control group, which was given only food and water, and the second group was given (0.5) ml of aflatoxin poison for each rat. For each rat, the third group received (0.5) ml of B clausii suspension and the fourth group received (0.5) ml of B. clausii suspension + (0.5) ml of aflatoxin toxin) for 21 days. The findings suggest that B.subtilis bacteria have effects. By increasing the weights of male rats inoculated with B.subtilis alone and the group (B.clausii + aflatoxin toxin), B.subtilis bacteria were able to remove a large percentage of the negative effects of aflatoxin. In addition to the physiological study of blood, it revealed a significant increase in the rate of white blood cell count in animals given a suspension of B. clausii and (B. clausii + aflatoxin toxin) to 22,200 and 16,700 cells/mm3, respectively, compared to the 12,500 control treatment. While haemoglobin, hematocrit, and erythrocyte sedimentation (E.S.R.) rates were unaffected in all groups. In terms of the histological research, Microscopic examination of tissue sections taken from the small intestine, livers, and kidneys of male rats dosed with (0.3825 ug/ml) of aflatoxin toxin revealed significant differences when compared to the control treatment. Focused aggregates of inflammatory cells with necrosis in liver cells, blood vessel expansion and inflammatory cell accumulation in the liver, and a clear expansion and congestion of the liver's central blood vessel. The villi in the intestine were dwarfed, and their decomposition and shedding were increased when compared to the control treatment. The kidney tissue displayed histopathological changes such as thrombotic necrosis in the interstitial tissue of the kidney, necrosis of the renal tubules and glomeruli, haemorrhage in the renal interstitial tissue.

The glomeruli were damaged, and the blood vessel walls thickened with fibrosis, whereas histological examinations of the liver, kidneys, and small intestine of animals treated with doses containing only B.subtilis bacteria revealed no pathological effects and were comparable to the control group. It only showed slight changes in the tissues of animals treated with a mixture of aflatoxin and B.clausii bacteria, and the effect of probiotics on it was clear by treating toxins and reducing their effect.

***Keywords*---**B.clausii, reduce, aflatoxin B_1.

Introduction

Probiotics are live microbes that, when administered in sufficient quantities, provide health benefits to the host(Jayanthi,N and Ratna Sudha, 2015). Probiotic foods are becoming increasingly popular as consumers seek safe, functional foods with health-promoting properties and high nutritional value(KESENKAŞ et al., 2018)**.** *Bacillus species,* which produce spores, have been used as probiotics for the past five decades. Spore-forming probiotics have an advantage over non-spore formers such as Lactobacillus spp. in that they are heat stable and can be stored at room temperature without losing viability. Spore-forming bacteria are also resistant to stomach acid (low pH) and thus can survive the transit to the intestine (Barbosa et al., 2005). *Bacillus clausii* (*B.clausii*) spores and cells have been shown in experiments to adhere to the bowel wall and colonize the mucosa. Because *B.clausii* is extremely stable in acidic conditions, the entire dose of bacteria ingested makes it to the small intestine intact(Jayanthi,N and Ratna Sudha, 2015). *B.clausii* UBBC07(MTCC 5472) is a Gram-positive, spore-forming, motile, rod-shaped probiotic bacterium that is non-toxic and safe (Lakshmi et al., 2017). Spore probiotics are advantageous in this regard because they can survive and germinate in harsh GIT conditions(Casula & Cutting, 2002). It is critical to investigate spores' ability to survive and germinate, and a variety of in vitro GIT model systems reported their efficiencies when compared to in vivo data (Fois et al., 2019)**.**

Materials and Methods

Examine some fungal isolates' ability to produce aflatoxins B_1, B_2
Ammonia Vapor Test for Detection of Aflatoxin

Coconut Extract Agar The medium was made and poured into Petri dishes (9 cm). The dishes were inoculated with 5 mm discs grown on isolates of A. flavus at the age of (7) days and placed in the center of each dish after solidification. The process was repeated four times for each fungal species, then incubated at 25 ± 2 °C for a week, after which the dishes were treated with ammonia (20 %) by turning the dishes so that the base of the plate was upwards, placing a filter paper saturated with ammonia in the lid of each plate, then incubating the dishes in the incubator at 25 °C for (24) hours and watching for a change in the color of the colonies bases . The fungal color changes from transparent white to red,

indicating that the isolate can produce aflatoxins. (Saito & Machida, 1999; Belludi et al., 2022)**.**

Antagonism evaluation (in vitro)

***Bacillus* spp. isolates were tested for their ability to inhibit the growth of some fungi *A.flavus*.**

In this study, we looked at the antifungal potential of two Bacillus species (*B. clausii* and *B. subtilis*) in vitro and on (PDA) medium. 0.1 ml of the liquid medium in which each Bacillus isolate was grown was taken with a capillary tube and placed in a dish containing (PDA) medium (Ajmera et al., 2017) method. The dishes were then incubated for 72 hours at 1 ± 37 °C. The plates cultured with Bacillus isolates were then inoculated separately with a disc diameter of 5 mm from the radial growth of the fungus A. flavus and incubated at 30 °C for 6 days with a comparator treatment represented by plates that were not inoculated with any bacterial isolate but were inoculated with a disc of the fungus. Only after the incubation period has ended, The average radial growth diameters for each type of tested fungi were calculated by taking the average of two perpendicular diameters for developing colonies, and the percentage of inhibition was calculated using the Abbott equation (1925) as described in the pesticide book: The results of inhibition were then recorded, and the degree of inhibition was calculated separately for each isolate, and the isolate with the most effective inhibition was chosen based on the highest degree of inhibition.

Animals used in experiments (testing in vivo)

The current study included twelve male rats divided into four groups, each with three rats obtained from the animal house at the College of Science at the University of Kufa. The rats were between the ages of 16 weeks and 250-300 gm in weight. The experiment lasted three weeks, and the four groups were treated in the following order:

1- The Aflatoxin group: only this group received 0.5 ml of Aflatoxin (0.3825 ug/ml concentration of AfB1) via oral gavage.
2- the *Bacillus clausii* group: in this group, we only give them 0.5 mL of (*Bacillus clausii*) hanging by oral gavage administration.
3- the (*B.clausii* and Aflatoxin) group: in this group, we only give him 0.5 ml of (*B.clausii* and Aflatoxin) hanging by oral gavage administrator.
4- the control group: in this group, there was no dosing (only we give the normal food and water).

The rats were weighed after the experiment and then placed in a container containing chloroform. Following anesthesia, blood samples were taken to study the physiological parameters, as well as parts of the liver, kidney, and small intestine after death to make tissue sections from, as follows:

Physiological study

The following tests were performed as part of the physiological study: Hb,PCV,WBC and ESR.

Results and Discussion

Antagonism test (in vitro)

In this study, we evaluated the antifungal capability of two *Bacillus* spp .(*Bacillus clausii, Bacillus subtilis*) both in vitro and on (P.D.A) medium. In in vitro conditions, both strains were able to reduce the mycelial growth and they were able to degrade the four aflatoxins during the first seven days after inoculation. Both bacterial strains showed good antifungal activity that produced aflatoxin B 1 in vitro conditions and on (P.D.A) medium, But the strain of *(B.clausii*) showed more activity compared to that of (*B.subtilis*), where the percentage of inhibition was 58.03%, while the bacteria (*B.subtilis*) amounted to 55.28%.

The test results were as follows

Table 2
The table shows the antifungal activity of two Bacillus spp. in vitro and a medium (PDA)

Treatment	Percentage (%)
B.subtilis	55.28
B.clausii	58.03
Control	100

This result matches what he found(Devi et al., 2008) The study mentioned the possibility of *B.claussi* MB9 strain inhibiting many types of pathogenic fungi (Farzaneh et al., 2016) In this study, the treatment of pistachio nuts by *Bacillus subtilis* UTBSP1, a promising isolate to degrade aflatoxin B_1 (AFB_1), caused to reduce the growth of *Aspergillus flavus* R5 and AFB_1 et content on pistachio nuts, Also, the results of many studies are similar to the example of this study(Al-Hussaini, 2012) The research included studying the effect of *B.subtilis* bacteria with different concentrations in protecting wheat grains and plants from infection with the fungi *A.niger* and *A.flavus*. The results of the field experiment demonstrated the high ability of *B.subtilis* to protect plants by reducing the death rate of seedlings in the presence of *A.niger* 9.52% when treating grains with *B.subtilis* inoculum at a concentration of 50 ml/L. The results of a study also showed (2008) Abeysinghe, Efficiency of the *B.subtilis* CA32 bio preparation in inhibiting radioactive growth of R.solani on P.D.A culture media with an inhibition efficiency of 67%(Abeysinghe, 2009). The efficiency of some species belonging to the genus *Bacillus* spp. Its ability to produce antibiotics such as Bacillomycin D and Iturin A, and also its production of enzymes that break down chitin, which constitutes most fungal cell walls, which is the enzyme chitinase(Moyne et al., 2001)**.**

Physiological study of blood

In this study effect of diets contaminated with aflatoxin produced from *A.flavaus* on weights and rates of some physiological blood parameters of male laboratory white rats**,**represents the evaluation of the efficiency of the biological preparation of *B.clausii* in protecting rats from aflatoxins. The results of the statistical analysis of the average weights of male rats for the different treatments showed that there were significant differences between them, as the average weight of one rat that was fed a diet contaminated with *A.flavus* poison (Aflatoxin B_1) only (295) g / rat compared to the control treatment. The average weight per rat that was fed on diets contaminated with *B.clausii* was increased (648) g /rat. Table(4.8). There was no significant difference in the rates of haemoglobin concentration Hb and haemoglobin rate P.C.V in all treatments dosed at a concentration of (0.3825 ug /ml / 100 g) compared to the control treatment.

Histological study

Histopathological observations To clarify the protective effects of *B.clausii* and control damage in the liver tissues

Histopathological observations were performed. Histopathological results (Figure 1 A and B) revealed no hepatic lesions in the control and *B.clausii* alone-fed groups, which showed intact liver tissue, clear liver lobules, large and round nucleus, and visible hepatocyte cords. Rats exposed to AFB_1 showed disordered hepatic lobules, inconspicuous hepatocytes cord, necrotic and ruptured hepatocytes, and disappearance of cellular structure. The portal tracts also exhibited some degrees of congestion. Compared with the AFB_1-challenged group, the supplementation of *B.Clausii* to diet significantly ameliorated and restored AFB_1-induced liver damage. These findings suggested that *B.clausii* could protect structural integrity and inhibit AFB_1-induced liver impairment in Rats.This result is consistent with many studies that proved that many species belonging to these genera did not show any signs of disease or danger to the health of the tested animals.

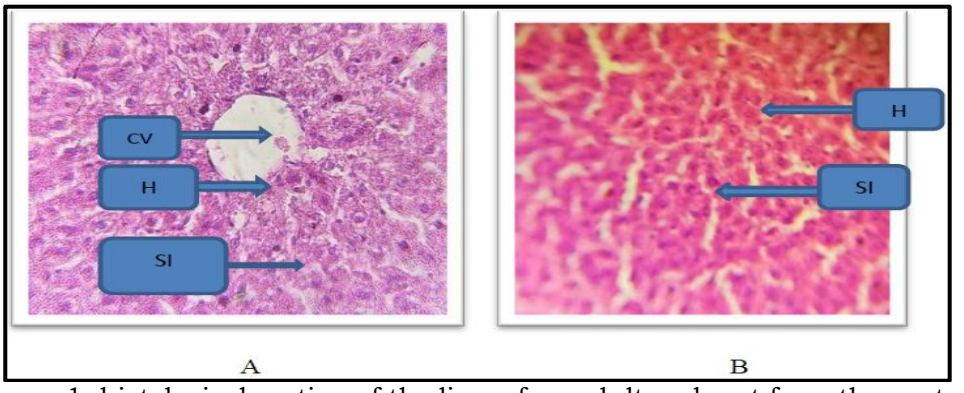

Figure 1. histological section of the liver of an adult male rat from the control group (A) and(B) treated with *B.Clausii*. Showing the cells normal hepatic (H) and central vein (CV). E&H staining 40X

Histopathological observations To clarify the effects of AFB$_1$-induced damage in the liver tissues

The necrosis in the liver may be caused by the effects of aflatoxin B_1 and other toxic metabolites, which affect the lipids of the plasma membrane, as it leads to a loss of the integrity of the membrane and thus cell lysis and the occurrence of necrosis. (Li et al., 2022) results showed obvious necrotic characteristics, including cell swelling, rupture of cell and mitochondrial membranes and inflammation in chicken livers. (Owumi et al., 2022) In this study, the liver and kidneys of AFB1-deficient mice showed evidence of focal congestion, inflammatory cell infiltration, mild water/balloon hepatocyte degeneration and moderate vesicular steatosis. (Papiya, 2005) indicated that the metabolic products of *A.fungus* .flavus led to the occurrence of vascular congestion in the liver.

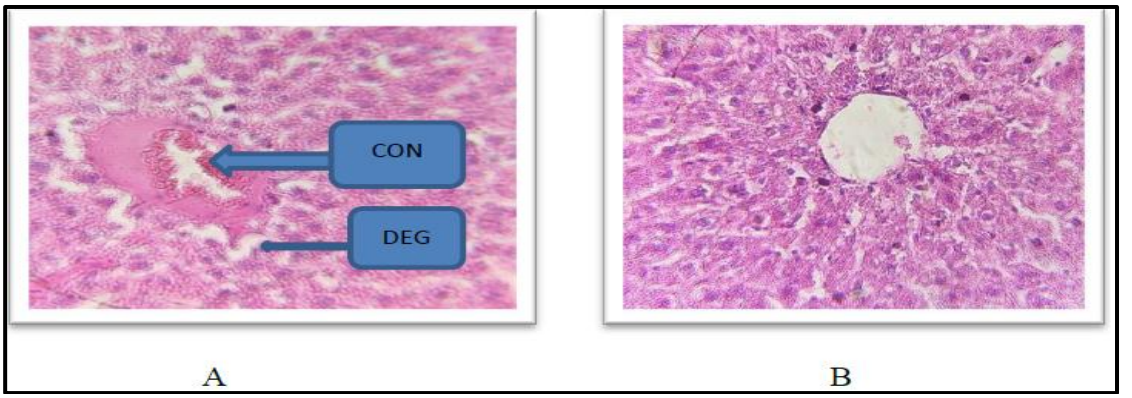

Figure 2. (A) The results of the microscopic examination of liver tissue in male rats treated with a concentration of 0.3825 ug/ml of aflatoxin B_1 for 21 days: To the presence of Coagulative necrosis (in the area around the central vein) with congestion in the central veins(CON).(B) Control showed normal

Histopathological observations To clarify the protective effects of *B.Clausii* against AFB$_1$ -induced damage in the liver tissues Figure (3)

Toxic effects of the studied *A.flavus* fungi, where the results of microscopic examination of tissue sections, whether of the liver or intestines of male rats treated with diets contaminated with the toxin of *A.flavus* fungi and to which the biological preparation was added at a concentration of (0.3825 ug/ml), showed the effect of bacteria *B.Clausii* on Reducing the toxic effects on those organs from any pathological changes. These results indicate that *B.Clausii* can protect the structural integrity and prevent AFB$_1$-induced liver damage in rats. I mentioned a study he did (Yalçın et al., 2012) Effect of water supplemented *B.clausii* on the liver biomarkers of broiler chicks Decreased level of ALT and AST in a group of birds that were treated with a higher concentration of probiotics might be due to its scavenging activities of free radicals produced during the metabolism at a liver site that causes hepatocellular damage and also plays role hepatic and muscles, protective agent. The reduced level of ALP in these groups might be due to the positive effect of probiotics on liver function and correlated organs. (Ognik and Krauze, 2016).

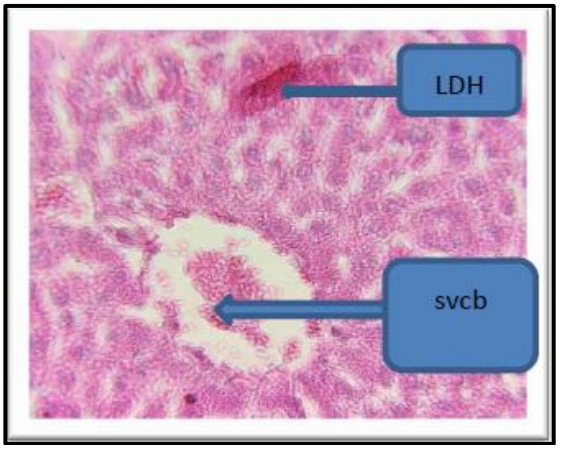

Figure 3. There are fewer degenerative changes to the cells Hepatocytes (LDH) and Slight vascular congestion bloody (svcb)

Histopathological observations To clarify the protective effects of *B.Clausii* and control damage in the kidney tissues

Cross-sections of the control group given a normal diet showed the normal shape of the glomerulus, proximal tubules, and distal tubules, as shown in Figure (4)(A). The cross-sections of the *B.clausii* group given *B.clausii* suspension show the normal shape of the glomerulus, proximal tubules, and distal tubules, as shown in (B).

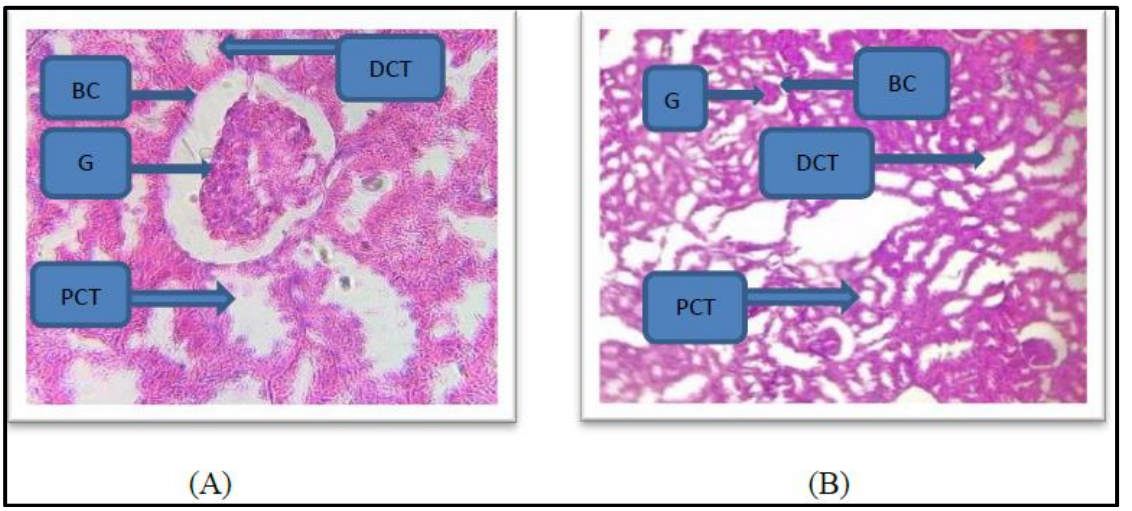

Figure 4. (A) Control (B) Treatment with *B.Clausii* H&E X40
(BC)Bomanes capsule.(G) glomerulus.(DCT) distal convoluted tubules
(PCT) proximal convoluted tubules

Histopathological observations to clarify the effects of AFB_1-induced damage in the kidney tissues Figure (5)

section of kidneys of the group (Treatment with AFB_1) showed damage of glomerulus (DG), and Pyaemia (PY) H&E X40

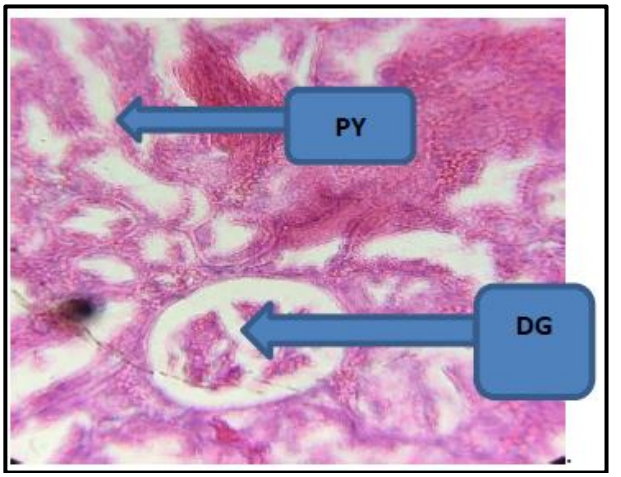

Figure 5. section of kidneys of the group (Treatment with AFB_1) showed damage to glomerulus (DG) and Pyaemia H&E X40

This result is consistent with many studies that proved that many species produce aflatoxinB_1cause damage to the liver and kidney for animals and humans. (Chen et al., 2019) It was reported that AFB_1 intoxication could increase mortality, liver and kidney pathology, and decrease body weight and feed intake for broilers.

Histopathological observations To clarify the protective effects of *B.Clausii* against AFB_1-induced damage in the kidney tissues Figure (6)

Toxic effects of the studied *A.flavus* fungi, where the results of microscopic examination of a tissue section of the kidney of male rats treated with diets contaminated with the toxin of *A.flavus* fungi, and to which the biological preparation was added at a concentration of(0.3825 ug /ml), showed the effect of bacteria *B.Clausii* on Reducing the toxic effects on that organ from any pathological changes. These results indicate that *B.Clausii* can protect the structural integrity and prevent AFB_1-induced kidney damage in mice. This matches most of the studies that studied the effect of probiotics on reducing the toxic effects in the body of a laboratory organism, including what it did(Deabes et al., 2021) The study was designed to reveal the efficiency of Bacillus subtilis NS4182-01 against the haematological, biochemical and histopathological alterations induced by AFB_1 in rats. Both AFB_1 and *B.subtilis* were studied on the experimental animals (rats) Groups 5&6 (AFB_1 + *B.subtilis* group) were orally treated with AFB_1 and then treated with *B.subtilis* at two studied doses respectively. It was noticed that the haematological measurements declined and the most biochemical measurements elevated significantly ($P \leq 0.05$) in AFB_1

treated group. *B.subtilis* restored all studied measurements towards the normal values.

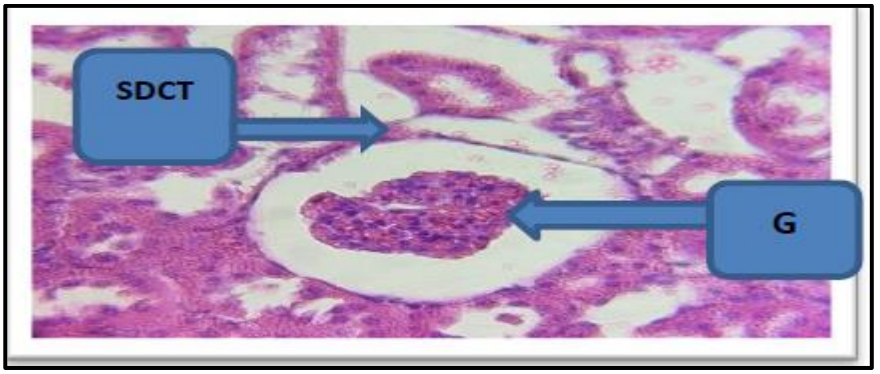

Figure 6. In this section there are a few degenerative changes in cells The lining of the renal tubules, slight damage to the glomerulus (G), and slightly present degeneration of convoluted tubules H&E X400

Histopathological observations To clarify the protective effects of *B.Clausii* and control damage in the small intestine tissues Figure (7)

Histopathological observations were made. Histopathology results Figure (A) and Figure (B) showed no intestinal lesions in the control group and groups fed with *B.clausii* bacteria.

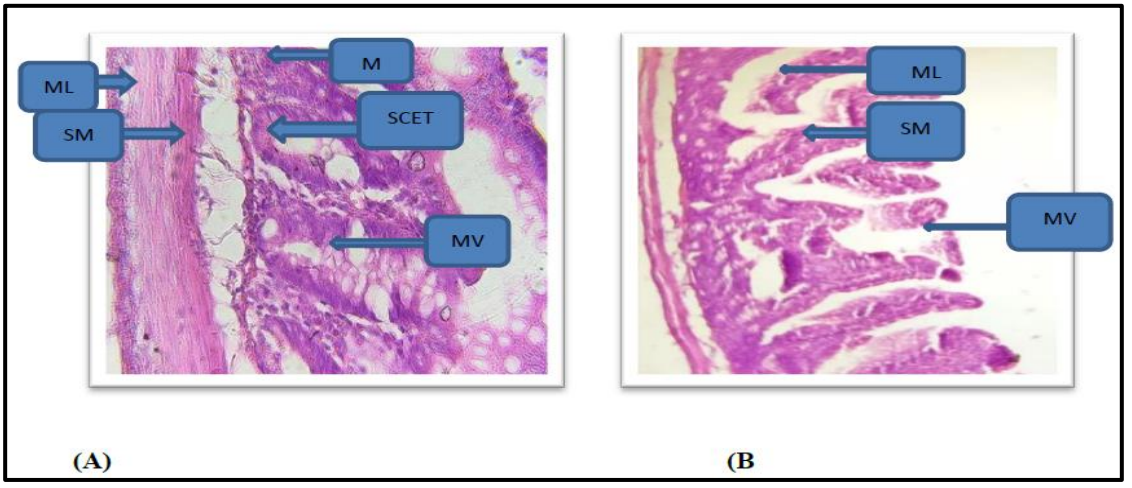

Figure 7. (A) Control (M) mucosa,(SM)submucosa,(ML) muscularis layer, (SCET)simple columaner epithelial tissue,(MV) microvilli. (B) Treated with *B.clausii* bacteria H&E X100

Histopathological observations To clarify the effects of AFB_1-induced damage in the small intestine tissues Figure (8A)

As for the sections taken from the small intestine that was treated with AFB_1 inoculation, the presence of sloughing of the intestinal mucosa as well as swelling

of the villi cells appeared compared to the control treatment. Also in the small intestine, shrinkage of the cytoplasmic content of the villi, a focal gathering of inflammatory cells inside the villi, dwarfing, decomposition, and falling off of the villi have appeared. This result is consistent with many studies that proved that many species belonging to these genera did not show any signs of disease or danger to the health of the tested animals (Al-Janabi, 2004).

Histopathological observations to clarify the protective effects of *B.Clausii* against AFB_1-induced damage in the small intestine tissues Figure (8B)

Toxic effects of *A.flavus* fungus studied, as the results of microscopic examination of small intestine tissue sections of male rats treated with *A.flavus* toxin, at aconcentration of(0.3825 µg/ml), with biological solution of *B.Clausii* showed the effect of *B.Clausii* bacteria in reducing the toxic effects On the member from any satisfactory changes.

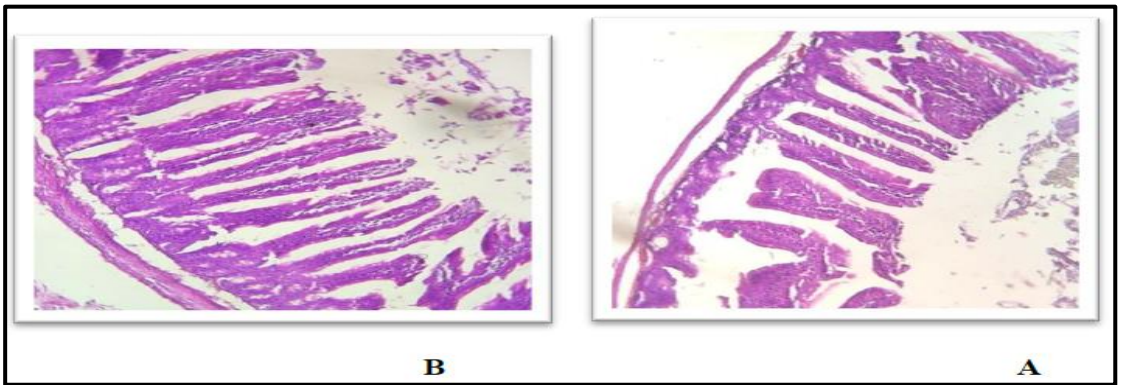

Figure 8. Histopathological observations to clarify the protective effects of *B.Clausii* against AFB_1-induced damage in the small intestine tissues

References

Abeysinghe, S. (2009). *Effect of combined use of Bacillus subtilis CA32 and Trichoderma harzianum RU01 on biological control of Rhizoctonia solani on Solanum melongena and Capsicum annuum.*

Ajmera, V., Perito, E. R., Bass, N. M., Terrault, N. A., Yates, K. P., Gill, R., Loomba, R., Diehl, A. M., & Aouizerat, B. E. (2017). Novel plasma biomarkers associated with liver disease severity in adults with nonalcoholic fatty liver disease. *Hepatology*, *65*(1), 65–77. https://doi.org/10.1002/hep.28776

Al-Husseini IM (2012). Use of Bacillus subtilis to protect wheat grains and plants from infection with Aspergillus flavus and Aspergillus niger in the field. Journal of University of Babylon, Issue 1, 64–80.

Al-Janabi, A. A. G. (2004). Studying the ability of CHAO fluorescens Pseudomonas strain to antagonize some pathogenic bacteria and some of their physiological, biochemical and histological effects in white female rabbits.

Barbosa, T. M., Serra, C. R., La Ragione, R. M., Woodward, M. J., & Henriques, A. O. (2005). Screening for Bacillus isolates in the broiler gastrointestinal tract. *Applied and Environmental Microbiology*, *71*(2). https://doi.org/10.1128/AEM.71.2.968-978.2005

Belludi, R., Sandhu, P. S., Sharma, P., & Sekhon, A. S. (2022). Diversity analysis of aflatoxigenic isolates of Aspergillus sp. from major groundnut growing states of India. *Indian Phytopathology*, 1–10.

Casula, G., & Cutting, S. M. (2002). Bacillus probiotics: spore germination in the gastrointestinal tract. *Applied and Environmental Microbiology*, *68*(5), 2344–2352.

Chen, Y., Li, R., Chang, Q., Dong, Z., Yang, H., & Xu, C. (2019). Lactobacillus bulgaricus or Lactobacillus rhamnosus suppresses NF-κB signaling pathway and protects against AFB1-induced hepatitis: a novel potential preventive strategy for aflatoxicosis? *Toxins*, *11*(1), 17.

Deabes, M. M., Aboulthana, W. M., Ahmed, K. A., & Naguib, K. M. (2021). Assessment of the Ameliorative Effect of Bacillus subtilis against the Toxicity Induced by Aflatoxin B1 in Rats. *Egyptian Journal of Chemistry*, *64*(4), 2141–2164.

Devi, N. K. A., Balakrishnan, K., Gopal, R., & Padmavathy, S. (2008). Bacillus clausii MB9 from the east coast regions of India: Isolation, biochemical characterization and antimicrobial potentials. *Current Science*, 627–636.

Farzaneh, M., Shi, Z. Q., Ahmadzadeh, M., Hu, L. Bin, & Ghassempour, A. (2016). Inhibition of the aspergillus flavus growth and aflatoxin B1 contamination on pistachio nut by fengycin and surfactin-producing bacillus subtilis UTBSP1. *Plant Pathology Journal*, *32*(3), 209–215. https://doi.org/10.5423/PPJ.OA.11.2015.0250.

Fois, C. A. M., Le, T. Y. L., Schindeler, A., Naficy, S., McClure, D. D., Read, M. N., Valtchev, P., Khademhosseini, A., & Dehghani, F. (2019). Models of the Gut for Analyzing the Impact of Food and Drugs. *Advanced Healthcare Materials*, *8*(21), 1900968.

Ghufran Flih. (2012). Employing the residues of cement plants in the manufacture of a biosynthesis from bacteria Bacillus subtilis. *Al-Kufa University Journal for Biology*, *4*(2).

Jayanthi,N and Ratna Sudha, M. (2015). Bacillus clausii - The Probiotic of Choice in the Treatment of Diarrhoea. *Journal of Yoga & Physical Therapy*, *05*(04). https://doi.org/10.4172/2157-7595.1000211

KESENKAŞ, H., KINIK, Ö., SEÇKİN, K., ERGÖNÜL, P. G., & Ecem, A. (2018). Keçi sütünden üretilen sinbiyotik beyaz peynirde Enterococcus faecium, Bifidobacterium longum ve Lactobacillus paracasei ssp. paracasei sayılarının değişimi. *Ege Üniversitesi Ziraat Fakültesi Dergisi*, *53*(1), 75–81.

Lakshmi, S. G., Jayanthi, N., Saravanan, M., & Ratna, M. S. (2017). Safety assesment of Bacillus clausii UBBC07, a spore forming probiotic. *Toxicology Reports*, *4*, 62–71.

Li, S., Liu, R., Xia, S., Wei, G., Ishfaq, M., Zhang, Y., & Zhang, X. (2022). Protective role of curcumin on aflatoxin B1-induced TLR4/RIPK pathway mediated-necroptosis and inflammation in chicken liver. *Ecotoxicology and Environmental Safety*, *233*, 113319.

Moyne, A., Shelby, R., Cleveland, T. E., & Tuzun, S. (2001). Bacillomycin D: an iturin with antifungal activity against Aspergillus flavus. *Journal of Applied Microbiology*, *90*(4), 622–629.

Ognik, K., & Krauze, M. (2016). The potential for using enzymatic assays to assess the health of turkeys. *World's Poultry Science Journal*, *72*(3), 535–550.

Owumi, S. E., Kazeem, A. I., Wu, B., Ishokare, L. O., Arunsi, U. O., & Oyelere, A. K. (2022). Apigeninidin-rich Sorghum bicolor (L. Moench) extracts suppress

A549 cells proliferation and ameliorate toxicity of aflatoxin B1-mediated liver and kidney derangement in rats. *Scientific Reports*, *12*(1), 1–19.

Papiya, M. (2005). *Pharmacological and toxicological studies of some mycotoxins in animals*.

Permana, A. T., Suroto, N. S., Parenrengi, M. A., Bajamal, A. H., Lestari, P., & Fauzi, A. A. (2022). Current update on stroke ischemic management: Stem cell as emerging therapy. *International Journal of Health & Medical Sciences*, *5*(1), 122-128. https://doi.org/10.21744/ijhms.v5n1.1851

Saito, M., & Machida, S. (1999). A rapid identification method for aflatoxin-producing strains of Aspergillus flavus and A. parasiticus by ammonia vapor. *Mycoscience*, *40*(2), 205–208. https://doi.org/https://doi.org/10.1007/BF02464300

Suryasa, I. W., Rodríguez-Gámez, M., & Koldoris, T. (2021). Get vaccinated when it is your turn and follow the local guidelines. *International Journal of Health Sciences*, *5*(3), x-xv. https://doi.org/10.53730/ijhs.v5n3.2938

Yalçın, S., Uzunoğlu, K., Duyum, H. M., & Eltan, Ö. (2012). Effects of dietary yeast autolysate (Saccharomyces cerevisiae) and black cumin seed (Nigella sativa L.) on performance, egg traits, some blood characteristics and antibody production of laying hens. *Livestock Science*, *145*(1–3), 13–20