Effects of *Lactobacillus casei* Shirota (LcS) supplementation on growth performance, intestinal histology, fecal AFB_1 and fecal bacterial profile of AFB_1-exposed rats

***Muhammad Firdhaus S*[1], *Mohd Redzwan S*[1], *Siti Raihanah S*[1], *Maizaton Atmadini A*[2], and *Fauzah AG*[2]**

[1]*Department of Nutrition, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia*

[2]*Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia*

INTRODUCTION

Aflatoxin B_1 (AFB_1) is known to be the most toxic mycotoxin that can contaminate food commodities. The International Agency for Research on Cancer (IARC) classified AFB_1 as a Group 1 carcinogen that is linked to liver cancer. This toxicity can either be acute or chronic, caused by exposure via contaminated-food consumption or respiratory tract. The use of probiotics as supplementation has been studied widely and known for its potential in enhancing gut bacteria proliferation, reducing colonization of pathogens in the intestine, stimulating immune response and preventing intestinal dysbiosis. *Lactobacillus* spp. and *Bifidobacterium* spp. are among the bacterial strains used as probiotics that can influence the adsorption and absorption of AFB_1, thus reducing its bioavailability in the body. Therefore, the focus of this study is to investigate the effects of *Lactobacillus casei* Shirota (LcS) supplementation on body weight, food intake, intestinal histomorphometry biomarkers, the composition of *Lactobacillus* spp. and *Bifidobacterium* spp. in feces, as well as the fecal AFB_1 in AFB_1-exposed rats.

METHODOLOGY

Male Sprague Dawley rats (n=32) divided into 4 groups; Control, AFB_1, AFB_1 +LcS & LcS

Baseline fecal samples collection & storage

*Analysis for <u>baseline</u> fecal *Lactobacillus* spp. & *Bifidobacterium* spp. counts

*Bacterial culture method using selective agar media (MRS agar, Bifidobacterium agar & lactitol-LBS vancomycin (LLV) agar

AFB_1 exposure (25 µg AFB_1/kg body weight) & LcS supplementation (2 × 10^9 CFU/mL) daily, for 4 weeks

End of exposure & supplementation

Fecal samples collection & storage

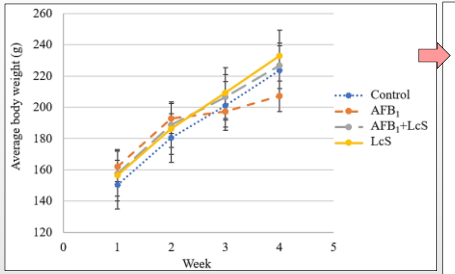
- *Analysis for <u>final</u> fecal *Lactobacillus* spp. & *Bifidobacterium* spp. counts*
- Fecal AFB_1 analysis (ELISA) & *fecal LcS counts

Duodenum, jejunum, ileum & colon collection & storage

H&E staining

Histomorphometric analysis

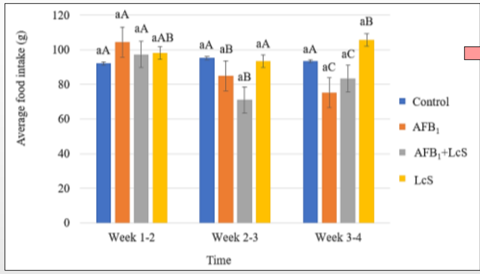
RESULTS & DISCUSSIONS



A. Average body weight of rats in four groups (n=8)

The **AFB_1 group showed no significant increment of body weight** (p>0.05) from Week 2 to 4, unlike other groups that showed significant increment throughout the 4-week study. In fact, **some rats in AFB_1 group** had **lost weight** during Week 2, 3 and 4 (Figure A).

The body weight reduction due to aflatoxin exposure can be described as the alteration in digestive enzymatic activities that cause nutrients malabsorption and changing metabolic processes such as tricarboxylic acid (TCA) cycle, glucose and fatty acid synthesis, mainly due to AFB_1 metabolites binding to DNA and proteins responsible for these processes (1).

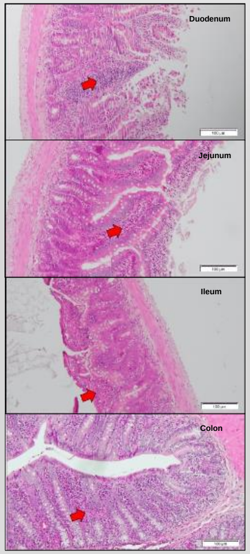


B. Average food intake of rats in four groups (n=8)

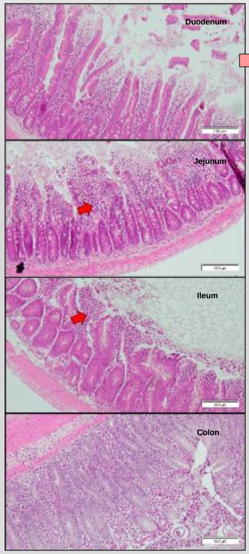
Food intake of **the AFB_1 and AFB_1+LcS group showed significant reduction** (p<0.05) throughout the 4-week treatment. However, the **AFB_1+LcS group** did show **significant increase** (p<0.05) in food intake at **Week 3 to 4** (Figure B).

AFB_1 was associated with its neurodegenerative effect, affecting the expressions of neuropeptides EM66 and its precursors, SgII, that previously reported to play an important role in regulating appetite in rats (2). Other than that, AFB_1 was also associated with alteration in protein synthesis, reducing enzyme activity required for digestion and absorption by reacting to amino groups of functional protein (3).

The improvement in body weight and food intake observed in the AFB_1+LcS group in comparison to AFB_1 group shows that the binding capacity of probiotics (1,4,5,6) would reduce the absorption of AFB_1, thus reducing its translocation (7), nutrients malabsorption and metabolism alteration (1).



C. H&E staining of duodenum, ileum, jejunum & colon of AFB_1 group



D. H&E staining of duodenum, ileum, jejunum & colon of AFB_1 +LcS group

The H&E staining showed a **mild to moderate inflammation in all parts of the intestine of AFB_1 group** (Figure C), while only **mild inflammation was observed in the jejunum and ileum of AFB_1+LcS group** (Figure D).

Previous study have shown the effects of AFB_1 exposure to the small intestine of animals. There was a prominent neutrophil infiltration and edema in the small intestine of broiler chicken (15), an increased of inflammatory cells in the ileum of ducks (18) and the occurrence of congested blood vessels in the intestine of rats (19). There was also a lymphocyte accumulation in the colon of rats exposed to AFB_1, even though there was no inflammation observed in the small intestine (21).

Other than that, an increase in pro-inflammatory biomarkers such as TLR4, NF-κB, TNF-α, IL-6, TXNIP, NLRP3, and IL-18 have been reported in regards to AFB_1 exposure (18).

In general, probiotic bacteria have the ability to reduce inflammation, especially in the intestine. The administration of probiotics has shown to have the ability to reduce the expression of pro-inflammatory cytokines via several pathways such as interfering the NF-κB pathway, activation of peroxisome proliferation-activated receptor gamma (PPAR-γ) nuclear receptor, and regulation of nitric oxide (NO) production by the inducible nitric oxide synthase (iNOS) (20).

E. Histomorphometric analysis of duodenum, jejunum, ileum and colon (n = 9).

	Mean ± SD			
	Villus height (µm)	Crypt depth (µm)	Villus width (µm)	Surface area (mm^2)
Duodenum				
Control	267.95 ± 27.16[a]	104.93 ± 10.16[a]	74.21 ± 4.54[a]	62.46 ± 7.34[a]
AFB_1	208.64 ± 23.18[b]	80.69 ± 10.28[b]	79.79 ± 11.84[a]	52.74 ± 12.62[a]
AFB_1+LcS	246.33 ± 26.73[a]	94.32 ± 16.52[ab]	72.30 ± 14.00[a]	56.16 ± 13.64[a]
LcS	250.31 ± 15.81[a]	79.48 ± 7.58[b]	67.11 ± 21.79[a]	53.40 ± 20.40[a]
Jejunum				
Control	203.33 ± 9.68[a]	79.38 ± 8.35[a]	67.69 ± 10.02[a]	43.19 ± 6.67[abc]
AFB_1	200.33 ± 14.71[a]	80.99 ± 5.99[a]	80.40 ± 9.67[b]	50.88 ± 9.25[a]
AFB_1+LcS	190.52 ± 7.78[a]	78.43 ± 11.61[a]	69.58 ± 6.44[ab]	41.61 ± 3.68[ab]
LcS	188.66 ± 16.50[a]	75.04 ± 10.47[a]	67.26 ± 11.18[a]	40.24 ± 9.35[bc]
Ileum				
Control	264.67 ± 49.02[a]	99.58 ± 14.78[acd]	73.52 ± 13.26[a]	61.28 ± 18.33[ab]
AFB_1	203.16 ± 15.43[b]	82.22 ± 13.99[b]	73.26 ± 9.61[a]	46.83 ± 5.45[b]
AFB_1+LcS	209.10 ± 32.19[b]	112.81 ± 8.77[ac]	75.54 ± 10.53[a]	49.64 ± 10.35[ab]
LcS	265.54 ± 14.84[a]	93.53 ± 6.92[bd]	78.07 ± 13.70[a]	65.20 ± 12.51[a]

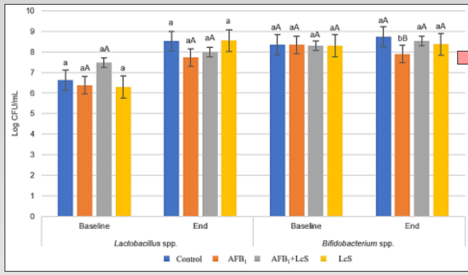
As shown in Table E, the histomorphometric analysis of **AFB_1 group showed a significantly lower** (p<0.05) **villus height in duodenum and ileum**, and **lower surface area in ileum** (p<0.05) **in comparison to LcS group**. Nonetheless, **the AFB_1+LcS group showed a higher duodenal and ileal villus height, and surface area of ileum**.

The atrophy of intestinal structure, especially the villus would affect the absorptive capability of the intestine (10), as it will influence the surface for absorption, overall nutrients transport system and the enzymes expression on brush border membrane (11).

The crypt depth is known to be the site for cell production as it houses stem cells. Increase in its measurement indicates the increasing rates of cell turnover (11,12).

The supplementation of probiotics in animal models have shown to improve the intestinal villus height, crypt depth, villus width and surface area (13,14).

The increase in villus height and crypt depth observed in AFB_1+LcS group as compared to AFB_1 group may indicates the cellular repairment after the damage done by the AFB_1 on the intestinal surface. However, previous studies have shown inconclusive results on the changes in intestinal morphology due to AFB_1 exposure (15,16,17).



F. *Lactobacillus* spp. & *Bifidobacterium* spp. counts in fecal samples

Fecal *Lactobacillus* spp. counts increased in all groups throughout the study, while ***Bifidobacterium* spp. counts showed increment in three groups, while AFB_1 group showed significant reduction** (p<0.05) from baseline to the end of the study (Figure F).

Similar to previous research, *Lactobacillus* spp. count did not show significant changes in mice given with three different AFB_1 concentration (8).

In contrast, *Bifidobacterium* spp. showed significant reduction in low-dose group (2.5 mg/L AFB_1) (8). *Bifidobacterium* spp. were previously reported to have negative correlation to serum cortisol, a biological stress indicator (22).

G. Fecal AFB_1

Groups	Mean ± SD (µg/L)	t-value	p-value
AFB_1+LcS	45.48 ± 0.23	77.319	0.000
AFB_1	32.21 ± 0.19		

H. Fecal LcS count

Groups	Mean ± SD (log CFU/g)	t-value	p-value
AFB_1+LcS	8.75 ± 0.05	5.092	0.007
LcS	7.79 ± 0.32		

The **fecal AFB_1 in AFB_1 group was significantly lower** (p<0.05) **than in AFB_1+LcS group** at the end of the study.

The detection of AFB_1 and its metabolites in fecal and urine samples of AFB_1-exposed animals, which indicates the level of unabsorbed AFB_1 (9). This result is supported by the **fecal LcS count, where AFB_1+LcS group had a significantly higher** (p<0.05) **LcS count than the LcS group**. This may be due to the ability of LcS to bind to AFB_1,and eventually excreted out in feces (4).

Other probiotics strain such as *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* LC-705, *Propionibacterium freudenreichii* spp. *shermanii* JS (5), *Lactobacillus casei* (L. casei) ATCC 334, L. casei L9, L. casei L30, L. casei 12A, L. casei 21/1, L. casei 7R1, and L. casei DPC 3968 (6) were also reported to have the ability to reduce the negative effects of aflatoxins.

CONCLUSIONS

Based on these results, it can be concluded that LcS supplementation of 2 × 10^9 CFU/mL per day can alleviate the adverse effects of AFB_1 exposure in terms of weight gained, intestinal histomorphometry, *Bifidobacterium* spp. counts in feces, as well as the AFB_1 excretion via fecal route. Further investigation on intestinal permeability and the analysis of AFB_1 in serum and urine is suggested to further understand the mechanism of AFB_1 excretion in presence of LcS.

FINANCIAL SUPPORT

This research is funded by the Ministry of Higher Education, Malaysia under Fundamental Research Grant Scheme (FRGS/1/2018/SKK06/UPM/02/2).

REFERENCES

1. Nikbakht Nasrabadi, E., Jamaluddin, R., Abdul Mutalib, M. S., Khaza'ai, H., Khalesi, S., & Mohd-Redzwan, S. (2013). Reduction of aflatoxin level in aflatoxin-induced rats by the activity of probiotic Lactobacillus casei strain Shirota. *Journal of Applied Microbiology*, *114*(5), 15071515. https://doi.org/10.1111/jam.12148
2. Trebak, F., Alaoui, A., Alexandre, D., El, S., Anouar, Y., Chartrel, N., & Magoul, R. (2015). NeuroToxicology Impact of aflatoxin B1 on hypothalamic neuropeptides regulating feeding behavior. Neurotoxicology,49,165–173. https://doi.org/10.1016/j.neuro.2015.06.008
3. Pandey
4. Liew, W.-P.-P., Nurul-Adilah, Z., Than, L. T. L., & Mohd-Redzwan, S. (2018). The Binding Efficiency and Interaction of Lactobacillus casei Shirota Toward Aflatoxin B1. Frontiers in Microbiology, 9(July), 1–12. https://doi.org/10.3389/fmicb.2018.01503
5. El-Nezami, H., Mykkanen, H., Kankaanpaa, P., Salminen, S., & Ahokas, J. (2000). Ability of Lactobacillus and Propionibacterium Strains to Remove Aflatoxin B1 from the Chicken Duodenum. Journal of Food Protection, 63(4), 549–552.
6. Hernandez-Mendoza, A., Garcia, H. S., & Steele, J. L. (2009). Screening of Lactobacillus casei strains for their ability to bind aflatoxin B1. Food and Chemical Toxicology, 47(6), 1064–1068. https://doi.org/10.1016/j.fct.2009.01.042
7. Schoultz, I., & Keita, Å. V. (2020). The Intestinal Barrier and Current Techniques for the Assessment of Gut Permeability. Cells, 9(8), 1–30. https://doi.org/10.3390/cells9081909
8. He, L., Liu, Y., Guo, Y., Xiao, N., & Tan, Z. (2018). Influences of Aflatoxin B1 on main intestinal bacteria communities and enzyme activities in mice. Toxin Reviews, 38(2), 121–126. https://doi.org/10.1080/15569543.2018.1426611
9. Gratz, S., Ta, M., Juvonen, R. O., Viluksela, M., Turner, P. C., & Mykka, H. (2006). Lactobacillus rhamnosus Strain GG Modulates Intestinal Absorption, Fecal Excretion, and Toxicity of Aflatoxin B1 in Rats. Applied and Environmental Microbiology, 72(11), 7398–7400. https://doi.org/10.1128/AEM.01348-06
10. Feng, G. D., He, J., Ao, X., & Chen, D. W. (2017). Effects of maize naturally contaminated with aflatoxin B1 on growth performance, intestinal morphology, and digestive physiology in ducks. Poultry Science, 96(6), 1948–1955. https://doi.org/10.3382/ps/pew420
11. Awad, W. A., Ghareeb, K., Abdel-Raheem, S., & Böhm, J. (2009). Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poultry Science, 88(1), 49–55. https://doi.org/10.3382/ps.2008-00244
12. Wang, M., Yang, C., Wang, Q. Y., Li, J. Z., Li, Y. L., Ding, X. Q., Yin, J., Yang, H. S., & Yin, Y. L. (2020). The growth performance, intestinal digestive and absorptive capabilities in piglets with different lengths of small intestines. 1196–1203. https://doi.org/10.1017/S175173111900288X
13. Galosi, L., Desantis, S., Roncarati, A., Robino, P., Bellato, A., Nebbia, P., Ferrocino, I., Santamaria, N., Biagini, L., Filoni, L., Attili, A. R., & Rossi, G. (2021). Positive Influence of a Probiotic Mixture on the Intestinal Morphology and Microbiota of Farmed Guinea Fowls (Numida meleagris). *Frontiers in Veterinary Science, 8*(October), 1–13. https://doi.org/10.3389/fvets.2021.743899
14. Sobolewska, A., Bogucka, J., Dankowiakowska, A., Elminowska-Wenda, G., Stadnicka, K., & Bednarczyk, M. (2017). The impact of synbiotic administration through in ovo technology on the microstructure of a broiler chicken small intestine tissue on the 1st and 42nd day of rearing. *Journal of Animal Science and Biotechnology, 8*(1), 1–8. https://doi.org/10.1186/s40104-017-0193-1
15. Poloni, V., Magnoli, A., Fochesato, A., Cristofolini, A., Caverzan, M., Merkis, C., Montenegro, M., & Cavaglieri, L. (2019). A Saccharomyces cerevisiae RC016-based feed additive reduces liver toxicity, residual aflatoxin B1 levels and positively influences intestinal morphology in broiler chickens fed chronic aflatoxin B1-contaminated diets. *Animal Nutrition, 6*(1), 31–38. https://doi.org/10.1016/j.aninu.2019.11.006
16. Jahanian, E., Mahdavi, A. H., Asgary, S., & Jahanian, R. (2016). Effect of dietary supplementation of mannanoligosaccharides on growth performance, ileal microbial counts, and jejunal morphology in broiler chicks exposed to aflatoxins. *Livestock Science*, 190, 123–130. https://doi.org/10.1016/j.livsci.2016.05.008
17. Applegate, T. J., Schatzmayr, G., Pricket, K., Troche, C., & Jiang, Z. (2009). Effect of aflatoxin culture on intestinal function and nutrient loss in laying hens. *Poultry Science*, 1235–1241. https://doi.org/10.3382/ps.2008-00494
18. Jin, S., Yang, H., Jiao, Y., Pang, Q., Wang, Y., Wang, M., Shan, A., & Feng, X. (2021). Dietary curcumin alleviated acute ileum damage of ducks (Anas platyrhynchos) induced by AFB1 through regulating Nrf2-ARE and NF-κB signaling pathways. *Foods, 10*(6). https://doi.org/10.3390/foods10061370
19. Akinrinde, A. S., Adebiyi, O. E., & Asekun, A. (2019). Amelioration of Aflatoxin B1-induced gastrointestinal injuries by Eucalyptus oil in rats. Journal of Complementary and Integrative Medicine, 17(1), 1–11. https://doi.org/10.1515/jcim-2019-0002
20. Toumi, R., Samer, A., Soufli, I., Rafa, H., & Touil-Boukoffa, C. (2021). Role of probiotics and their metabolites in Inflammatory Bowel Diseases (IBDs). Gastroenterology Insights, 12(1), 56–66. https://doi.org/10.3390/GASTROENT12010006
21. Nurul-Adilah, Z., Liew, W.-P.-P., Mohd-Redzwan, S. & Amin, I. (2018). Effect of High Protein Diet and Probiotic Lactobacillus casei Shirota Supplementation in Aflatoxin B1-induced Rats, 2018. https://doi.org/10.1155/2018/9568351
22. Aizawa, E., Tsuji, H., Asahara, T., Takahashi, T., Teraishi, T., Yoshida, S., Koga, N., Hattori, K., Ota, M., & Kunugi, H. (2019). Bifidobacterium and Lactobacillus counts in the gut microbiota of patients with bipolar disorder and healthy controls. Frontiers in Psychiatry, 10(JAN), 1–8. https://doi.org/10.3389/fpsyt.2018.00730