Journal of Animal and Veterinary Advances 11 (14): 2417-2422, 2012
ISSN: 1680-5593

Removal of Zearalenone by Strains of *Lactobacillus* sp. Isolated from Rumen *in vitro*

[1,2]Miao Long, [1,2]Peng Li, [1]Wenkui Zhang, [1]Xiaobing Li, [1,2]Yi Zhang, [2]Zhe Wang and [2]Guowen Liu
[1]College of Animal Science and Veterinary Medicine, Shenyang Agricultural University,
120 Dongling Road, 110866 Shenyang, P.R. China
[2]College of Animal Science and Veterinary Medicine, Jilin University,
5333 Xi'an Road, 130062 Changchun, P.R. China

Abstract: Zearalenone (ZEN) can occur in food and feeds impairing farm animal performances and health. In this study, the ability to remove zearalenone by *Lactobacillus* sp. isolated from rumen was evaluated *in vitro*. Eight strains isolated from rumen were tested for their abilities to remove zearalenone in MRS liquid medium. The removal rate of these strains ranged from 25.64-69.33%. The highest removing ability of these strains was *L. mucosae* lm4208. Binding may the mechanism of *L. mucosae* lm4208 to remove ZEN as no toxin derivatives were observed and removal was not impaired in nonviable bacteria. The ability of removing ZEN by the strain was directly related to the bacterial cell wall structure and composition. Removal was affected by culture time, pH and temperature. Although, removal was decreased in simulated gastric/intestinal fluid, the stain still had the very strong elimination ability. These findings suggest that *Lactobacillus* sp. from rumen can be exploited as a potential approach of detoxification of ZEN.

Key words: Zearalenone, *Lactobacillus* sp., rumen isolated, stain, ability

INTRODUCTION

Mycotoxicosises impair animal health, welfare and productivity and cause important economic losses in animal husbandry (Glenn, 2007; Chukwuka *et al*., 2010; Abdel-Wahhab and Kholif, 2008; Iheshiulor *et al*., 2011; Tansakul *et al*., 2010). In addition, contaminated foods may also pose a health risk to humans directly or indirectly (through animal products) (Hussein and Brasel, 2001; Agbogidi *et al*., 2007; Adzitey *et al*., 2012). ZEN is one of the most widely distributed fusarium toxin biosynthesized through a polyketide pathway by a variety of fusarium fungi found in several food and feeds commodities worldwide (Zinedine *et al*., 2007). It is occasionally associated with hyperstrogenism and other reproductive disorders in pigs, sheep and other farm animals. Among farm animals, especially the pigs react quite sensitively to higher concentrations with hyperestrogenism and fertility disorders in the case of ZEN however, ruminants are regarded more resistant (Fink-Gremmels and Malekinejad, 2007).

Different physical and chemical methods have been recommended for the detoxification of mycotoxin contaminated in food and feeds. But these methods have some drawbacks such as decrease the value of nutrition in the food and feeds, cost much money on removing and disposing of the contaminated materials have potential toxicity to animals and human and cannot remove the mycotoxins *in vivo* when the mycotoxins enter the intestinel. These drawbacks have encouraged the recent study emphasis on biological methods.

So far, many data have actually shown that lactic acid bacteria can inhibit mould growth and that some of them have the potential to interact with mycotoxins (Dalie *et al*., 2010). A previous study indicated that certain strains of Lactic acid bacteria can bind mycotoxins such as aflatoxins (Shahin, 2007; Haskard *et al*., 2001), zearalenone (El-Nezami *et al*., 2002a), ochratoxin A (Piotrowska and Zakowska, 2005), fumonisins (Niderkorn *et al*., 2006) and trichothecenes (El-Nezami *et al*., 2002b). However, Lactic acid bacteria have a lot of kinds of genus, species and strains and the abilities of them from different origins to remove mycotoxins are significant difference. Screening the strains with the highest ability of removing mycotoxins are very necessary to apply them in food and feeds.

In this study, the ability of eight strains of *Lactobacillus* sp. isolated from rumen to remove ZEN in the liquid culture media was inve stigated. The effects of factors including pH, temperature, culture time, heat-treatment, acid-treatment and simulated gastric /intestinal environment treatment on the ability of the selected strain to remove ZEN were also examined.

Corresponding Author: Guowen Liu, College of Animal Science and Veterinary Medicine, Jilin University, 5333 Xi'an Road, 130062 Changchun, P.R. China

MATERIALS AND METHODS

Bacterial strains: The strains of *Lactobacillus* sp. used in this research were isolated from rumen of a dairy cows. These *Lactobacillus* sp. were identified by physiological, biochemical and 16S rDNA sequence analysis. The sequence had submitted to the GenBank. These *Lactobacillus* sp. were: *L. casei* lm41 (FJ171330), *L. casei* lm45 (FJ171331), *L. curvatus* lm9A (FJ171333), *L. curvatus* lm06 (FJ171328), *L. coryniformis* lm32 (FJ171332), *L. brevis* lm8828 (FJ171329), *L. mucosae* lm4208 (FJ171327), *L. mucosae* lm4209 (EU722291). Stock cultures were maintained at -80°C in 20% (v/v) glycerol.

Standard ZEN preparation: A stock solution of ZEN (purchased from Sigma-Aldrich) was prepared by dissolving the solid standard in acetonitrile (0.5 mL mL^{-1}). The ZEN standard solutions for HPLC calibration or spiking purposes were prepared daily by diluting the stock solutions in methanol. Concentration was determined by measuring the absorbance at 274 nm.

Screening of eight strains for ZEN removing capacity: All strains cultured in MRS broth at 37°C until the concentration of 10^{10} cfu mL^{-1}. Counting of viable bacteria was determined by turbidimetry at 600 nm (Niderkorn *et al*., 2006) with a spectrophotometer (DU640, Beckman Industries Inc., Fullerton, CA, USA). The strains (approx. 10^{10} cfu mL^{-1}) were centrifuged at 5000 rpm for 10 min and resuspended in 1.5 mL MRS (pH 6.5) contained with 10 μL mL^{-1} ZEN in tubes for 24 h at 37°C. The cells were again centrifuged and the samples of the supernatant fluid were analysed by HPLC. Positive controls containing no bacteria and a negative control for each genus of *Lactobacillus* sp. containing no toxin were included.

Effects of culture time, pH and temperature on removal of ZEN: After the initial experiments with the eight strains, the *L. mucosae* lm 4208, selected for its high removal rate was used in further experiments. The cells of *L. mucosae* lm 4208 were cultured in MRS broth at 37°C for 24 h then adjusted the concentration 10^{10} cfu mL^{-1}, centrifuged (5000 rpm for 15 min at 10°C) washed with 0.01 mol L^{-1} PBS for 3 times.

The supernatant was removed while the bacterial pellet was suspended and incubated in 1.5 mL of PBS containing with 10 μg mL^{-1} ZEN at 30°C for 0.5, 2, 4, 12 and 24 h, respectively. The supernatant was analyzed by HPLC after the cultures were centrifuged (5000 rpm for 15 min at 10°C). The effect of temperature on the reduction of ZEN was studied at 10, 20, 30 and 37°C. Determination of ZEN was done after 1 h of incubation. For the effect of pH, the pH of the medium was adjusted to 8.0, 7.0, 6.0, 5.0 and 4.0. ZEN was quantified after incubation for 1h at 30°C. All assays were performed in triplicate.

Effect of the strain treated with acid, heat or triton-100 on removal of ZEN: The *L. mucosae* lm4208 (10^{10}cfu mL^{-1}) was washed with 0.01 mol L^{-1} PBS after it was centrifuged (5000 rpm for 15 min at 10°C) and the supernatant was removed. The bacterial pellet was autoclaved at 121°C for 20 min (heat-treated bacteria) incubated in 3 mL of 2M HCL for 1 h (acid-treated bacteria) or incubated in 3 mL 5% triton 100 for 1 h (Triton 100-treated bacteria). All of the treated strains were centrifuged (4000 rpm for 15 min at 10°C) and the supernatants were removed. The pellets were washed twice with 3 mL of 0.01 mol L^{-1} PBS. After these treatments, the bacterial samples were centrifuged (4000 rpm for 15 min at 10°C) then the supernatant was removed. The bacterial pellet was suspended in 5 mL of PBS containing 10 μg mL^{-1} of ZEN. The mixture was incubated at 37°C for 30 min and the supernatant was analyzed by HPLC. All assays were performed in triplicate and both positive controls (PBS substituted for bacteria) and negative controls (PBS substituted for ZEN) were included.

Effect of artificial gastric/intestinal fluid: Artificial gastric fluid: 1000 U g^{-1} pepsin was dissolved in 0.01 mol L^{-1} PBS (pH 3.0), the final concentration was 2 g L^{-1} and pH to 3.0. Artificial intestinal fluid: 250 U g^{-1} pancreatin was dissolved in 0.01 mol L^{-1} PBS and the final concentration was 1 g L^{-1}, 4.5% bile salt of pig was added into this liquid, the final concentration of bile was 0.15% and the pH regulated to 8.0. The cells of lm 4208 were cultured with the artificial gastriointestinal fluid containing 10 μg mL^{-1} ZEN at 37°C for 1 h then centrifuged (4000 rpm for 15 min at 10°C), the supernatant was analyzed by HPLC. All assays were performed in triplicate and both positive controls (ZEN working solution substituted for bacteria) and negative controls (the artificial gastrointestina fluid substituted for ZEN) were included.

ZEN determination by HPLC: For analyzing ZEN and its metabolites, a C18 reversed-phase column (250×4.6 nm; particle size, 5 μm) was used for chromatographic separation. The mobile phase which consisted of acetonitrile-water (45:55(v/v)) was used at a flow rate of 1 mL min^{-1} detected by fluorescence at 235 (excitation) and 460 (emission) nm. The column was kept at 25°C. The

injection volume was 50 μL and the retention times were about 11 min for ZEN. The percentage of the ZEN removed was calculated by using the following equation: 100× (1- (peak area of ZEN in the supernatant/peak area of ZEN in the positive control)).

Statistical analysis: The Statistical Analysis System Software package (Version 8.1, 2000; SAS Institute Inc., Cary, NC, USA) was used for data analysis. One-way ANOVA was used to compare data sets with Duncan's multiple range test giving significant difference ($p<0.05$) among means. Data from triplicate assays are expressed as mean±standard deviation. The significant differences in the mean values are reported at $p<0.05$.

RESULTS AND DISCUSSION

Removal of ZEN from MRS medium: The removal rate of rumen strains of *Lactobacillus* sp. are shown in Table 1. The ability of *Lactobacillus* sp. to removing ZEN after fermentation ranged from 25.64-69.33%. *L. mucosae* lm 4208 removed more ZEN than other *Lactobacillus* sp. strains used in this study ($p<0.05$). After 24 h of culturing lm 4208 with ZEN, no degradation products were observed.

Effect of culture time, pH and reaction temperature: From the Fig. 1 the removal reaction was very fast for the removal rate achieved at 57.16% in 30 min. The removal rates were 60.12 and 66.14% after cultured 24 and 48 h, respectively. These results indicated that the detoxification process of lm4208 was a very rapid process (El-Nezami *et al*., 2002a, b). With the culture time extension, bacteria growth would be more slowly, the cell surface structure and electrification would be changed. When the bacteria were in a relatively strong vital force phase, it was difficult for nonpolar ZEN to bind to the adsorption sites on the cell surface. While the motion of the surface charged would rduced with the bacterian activity decreased, the nonpolar ZEN was relatively easy The speculation that the detoxification capacity was to bind the adsorption sites on the cell surface. The speculation that the detoxificapion capacity was enhanced with the culture time extension was needed further experimental verification.

Table 1: Removal of ZEN in culture medium by viable *Lactobacillus* sp.

Strains	Percentage of reduction±SD*
L. casei lm41	37.14±1.4[a]
L. casei lm45	35.28±1.2[a]
L. curvatus lm9A	46.24±0.6[b]
L. curvatus lm06	47.29±1.3[b]
L. coryniformis lm32	25.64±1.4[c]
L. brevis lm8828	31.07±1.3[d]
L. mucosae lm4208	69.33±0.4[e]
L. mucosae lm4230	67.44±0.9[e]

*Each Value is a mean of triplicate analyses±SD (Standard Deviation). Means shearing the same letter dot not differ significantly at $p<0.05$

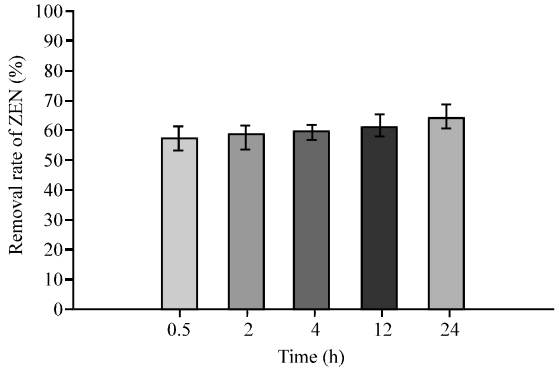

Fig. 1: Effect of culture time on removal rate of ZEN

The pH for the removal rate was another important factor which would directly affects the adsorption equilibrium constants and the electrification of the cells and ZEN. Figure 2 shows the effect of pH on the removal rate when pH decreased from 8.0-4.0 the removal rate increased from 12.04-43.75%. The result maybe because the state of ZEN in the solution was different at different pH. ZEN has two phenolic hydroxyl groups with an estimated $pKa_1 = 7.62$ (Lemke *et al*., 1998) when the pH is <7.62, ZEN mainly exist in the form of prototype. Because of its nonpolar hydrophobic effect, ZEN more easily absorbed into the nonpolar components of the cell wall or got into the internal nonpolar hole. When the initial pH increased, ZEN began to form into anions which make it difficult to combine hydrophilic polarity to the cell surface. Therefore, the lower the pH, the higher detoxification capacity.

Reaction temperature for the removal rate was an important factor which would affect the adsorption rate

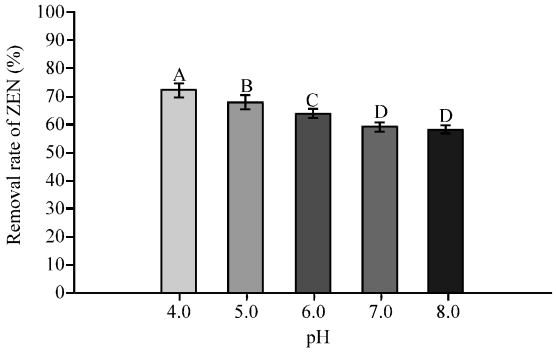

Fig. 2: Effects of pH value on removal rate of ZEN. Means with different letters were significant different according to Duncan's multiple range test ($p\leq0.05$) (N = 3)

and equilibrium constants. From Fig. 3 the removal rate and temperature relationship showed that the removal rate was increased with the temperature rised. But the rate was not significantly affected by the reaction temperature on the whole. There were not only no significant difference between 10 and 20°C but also no significant difference 30 and 37°C. Comprehensive view of the four reaction temperature, the 30°C of reaction temperature was better.

Effect of the strain treated with acid, heat and triton-100 on removal of ZEN: The removing capacity of the strain lm 4208 increased significantly ($p<0.01$) after heat treatment and acid treatment (Table 2). The ability of lm 4208 to remove ZEN increased after heat treatment and acid treatment was in agreement with previous studies. It indicated that binding at the bacterial cell wall was the mechanism on removal of mycotoxins by LAB (Haskard *et al*., 2000; El-Nezami *et al*., 2002a, b). The heat and acid treatments may change the original binding site of the viable bacteria and expose new binding sites (El-Nezami *et al*., 2004).

Although, the mechanism of binding of ZEN by bacterial cells is not well understood, it is thought that the primary cellular components involved are peptidoglycans and polysaccharides of lactic acid bacteria (Haskard *et al*., 2000). In this study, the triton-100 treatment on the ability of removing ZEN was also studied.

Table 2: Absorption rate of lm4208 to ZEN

	Percentage of reduction±SD*	
Treatments	Before treatment	After treatment
Heat treatment	69.96±0.81	77.21±0.43*
Acid treat	69.81±0.76	78.11±0.55*
Triton-100	69.12±0.43	73.31±0.73*
Attificial gastric fluid	69.76±0.41	46.04±0.47*
Artificial intestinal fluid	69.74±0.47	50.14±0.77*

*Significantly different ($p<0.01$)

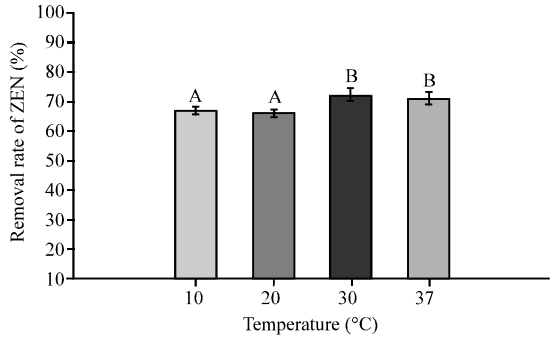

Fig. 3: Effect of temperature on removal rate of ZEN. Means with different letters were significant different according to Duncan's multiple range test ($p\leq0.05$) (N = 3)

The result showed that this treatment increased the removing rate up to 72.31%. The triton-100 treatment increased the toxin's removal maybe because:

- The aperture size of cell wall and the permeability of the cytomembrane were increasing, so it easily made ZEN bind with the cell wall
- The structure and electronic of the cell surface were changed and the vital movement of the cell surface were reduced after the treatment. It made ZEN more easily bind with cell surface and more difficult to separate them from cell surface. The increasing of removing ability by triton-100 treatment also demonstrated that the detoxification of ZEN by this strain was binding not degrading. It also, illustrated that the lipid is not the essential binding composition of ZEN because triton-100 is a nonionic surface active agent that can moderately dissolve the lipid

Effect of artificial gastric/intestinal fluid: It is very important to study the effect of simulated intestinal tract environment on the strains removing ZEN because it is a criterion to determine whether the strains can remove ZEN *in vivo*. The results showed that the ability of lm4208 to bind ZEN was decreased by 21.7% in the artificial gastric fluid and was decreased by 16.16% in the artificial intestinal fluid separately (Table 2). In the artificial gastric fluid low pH and the pepsinogen are two mainly influencing factors. The acid treatment of *L. rhamnosus* GG enhanced the capacity of removing ZEN and the capacity did not affect in pH from 2.5-8.5 (EI-Nezami *et al*., 2004). But the capacity of removing AFB1 was significantly decreased when the *L. rhamnosus* GG cell wall structure was destructed by protease E (Haskard *et al*., 2000). The ability of lm 4208 to remove ZEN decreased in the artificial gastric fluid maybe due to the pepsase effecting on the cell wall. Because pepsase destructed the peptide chain of the peptides or proteins and destroyed the integrity of cell structure resulting the removing capacity decreased.

Bile salt can significantly impact on the cell wall of the bacteria, especially phospholipids, proteins and cell surface structure (Maire *et al*., 2005). When the concentration of bile salt medium was at 0.05-0.15%, the lactobacillus gathered into groups cells from the smooth surface became to rough and vesicles can be observed in the cell wall structure (Bron *et al*., 2004). With the increased bile salt concentration, cell changes became obvious and the emergence of shrinkage can be seen (Bron *et al*., 2004). This bile salt induced cell aggregation

and destructed the phospholipids therefore, the structure of the cell may reduce the binding site of ZEN. However, Hernandez-Mendoza *et al.* (2009) results showed that the exposure of the bacterial cells to the bile significantly increased the binding ability of AFB1 maybe because the toxin and the bile concentration used were different.

CONCLUSION

This study is the first approach to examine the ability of *Lactobacillus* sp. isolated from rumen to reduce ZEN *in vitro*. The bacteria efficiently tested to bind ZEN at different conditions implicated that the strains could be used as feed additive to reduce ZEN. Further studies are needed to investigate the ability of selecting bacterial strains whether can efficiently reduce ZEN *in vivo*. The stability of the complex bacteria-ZEN in the gastro-tract of pigs is also essential for further study.

ACKNOWLEDGEMENTS

This researchers was financially supported by the National Natural Science Foundation of China (Grants No.: 31072178) and Liaoning Province Doctor Startup Fund of China (Grants No.: 20111086).

REFERENCES

Abdel-Wahhab, M.A. and A.M. Kholif, 2008. Mycotoxins in animal feeds and prevention strategies: A review. Asian J. Anim. Sci., 2: 7-25.

Adzitey, F., C.Y. Liew, A.P. Aronal and N. Huda, 2012. Isolation of *Escherichia coli* from ducks and duck related samples. Asian J. Anim. Vet. Adv., 7: 351-355.

Agbogidi, O.M., P.G. Eruotor, S.O. Akparobi and G.U. Nnaji, 2007. Heavy metal contents of maize (*Zea mays* L.) grown in soil contaminated with crude oil. Int. J. Bot., 3: 385-389.

Bron, P.A., M. Marco, S.M. Hoffer, E.V. Mullekom, W.M. de Vos and M. Kleerebezem, 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. J. Bacteriol., 186: 7829-7835.

Chukwuka, O.K., I.C. Okoli, M.N. Opara, A.A. Omede, I.P. Ogbuewu and O.O.M. Iheshiulor, 2010. The growing problems of mycotoxins in animal feed industry in west Africa: A review. Asian J. Poult. Sci., 4: 122-134.

Dalie, D.K.D., A.M. Deschamps and F. Richard-Forget, 2010. Lactic acid bacteria- Potential for control mould growth and mycotoxins. Food Control, 21: 370-380.

El-Nezami, H., N. Polychronaki, Y.K. Lee, C.A. Haskard, R. Juvonen, S. Salminen and H. Mykkanen, 2004. Chemical moieties and interactions involved in the binding of zearalenone to the surface of *Lactobacillus rhamnosus* strains GG. J. Agric. Food Chem., 52: 4577-4581.

El-Nezami, H.S., A. Chrevatidis, S. Auriola, S. Salminen and H. Mykkaen, 2002a. Removal of common Fusarium toxins in vitro by strains of Lactobacillus and Propionibacterium. Food Addit. Contam., 19: 680-686.

El-Nezami, H.S., N. Polychronaki, S. Salminen and H. Mykkaen, 2002b. Binding rather metabolism may explain the interaction of two food-grade *Lactobacillus* strains with zearalenone and its derivative α-zearalenol. Applied Environ. Microb., 68: 3545-3549.

Fink-Gremmels, J. and H. Malekinejad, 2007. Clinical effects and biochemical mechanisms associated with exposure to the mycoestrogen zearalenone. Anim. Feed Sci. Technol., 137: 326-341.

Glenn, A.E., 2007. Mycotoxigenic *Fusarium* species in animal feed. Anim. Feed Sci. Technol., 137: 213-240.

Haskard, C., C. Binnion and J. Ahokas, 2000. Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. Chem. Biol. Interact., 128: 39-49.

Haskard, C.A., H.S. El-Nezami, P.E. Kankaanpaa, S. Salminen and J.T. Ahokas, 2001. Surface binding of aflatoxin B1 by lactic acid bacteria. Applied Environ. Microbiol., 67: 3086-3091.

Hernandez-Mendoza, A., H.S. Garcia and J.L. Steele, 2009. Screening of Lactobacillus casei strains for their ability to bind aflatoxin B1. Food Chem. Toxicol., 47: 1064-1068.

Hussein, H.S. and J.M. Brasel, 2001. Toxicity, metabolism and impact of mycotoxins on humans and animals. Toxicology, 167: 101-134.

Iheshiulor, O.O.M., B.O. Esonu, O.K. Chuwuka, A.A. Omede, I.C. Okoli and I.P. Ogbuewu, 2011. Effects of mycotoxins in animal nutrition: A review. Asian J. Anim. Sci., 5: 19-33.

Lemke, S.L., P.G. Grant and T.D. Phillips, 1998. Adsorption of zearalenone by organophilic montmorillonite clay. J. Agric. Food Chem., 46: 3789-3796.

Maire, B., G.M. Cormac and H. Colin, 2005. The interaction between bacteria and bile. FEMS Microbiol. Rev., 29: 625-651.

Niderkorn, V., H. Boudra and D.P. Morgavi, 2006. Binding of *Fusarium* mycotoxins by fermentative bacteria *in vitro*. J. Applied Microbiol., 101: 849-856.

Piotrowska, M. and Z. Zakowska, 2005. The limitation of ochratoxin A by lactic acid bacteria strains. Pol. J. Microbiol., 54: 279-286.

Shahin, A.A.M., 2007. Removal of Aflatoxin B1 from contaminated liquid media by dairy lactic acid bacteria. Int. J. Agric. Biol., 9: 71-75.

Tansakul, N., N. Kusujarit, C. Kasorndorkbua, K. Witoonsatian and T. Songserm, 2012. The effect of low-dose ochratoxin: A fed in ducks on blood haematological profiles and histopathological alterations. Asian J. Anim. Vet. Adv., 7: 1021-1027.

Zinedine, A., J.M. Soriano, J.C. Molto and J. Manes, 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. Food Chem. Toxicol., 45: 1-18.