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Changes of Colon Flora and Antitoxic Effect Induced by Zearalenone in Mice

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Authors' Contribution
HY, HF, XW and JZ designed the study. JZ, XW, YZ, YJ and YZ, performed the experimental work. HF and HY analyzed the data. JW, YF, RW, RL provided some help for this experiment. HY and HF wrote the article.

Key words
Zearalenone, Intestinal microflora, *Lactobacillus*, Mucosal immunity, Antitoxic effect

ABSTRACT

Although mounting evidence has shown that mycotoxins can cause intestinal dysfunction and mucosal immune disorders, the effect of zearalenone (ZEA) on intestinal mucosal function remains controversial. We aimed to explore the effects of short-term ZEA exposure on the mucosal barrier function in the colon. We found that the morphology of colonic mucosa in mice was normal after ZEA was administered by gavage for one week, the mRNA expression levels of mucosal Mucin-1, Mucin-2, regenerating islet-derived protein 3 gamma (Reg3γ), and tumor necrosis factor (TNF) were significantly downregulated. The mRNA expression levels of mucosal β-defensin, regenerating islet-derived protein 3 alpha (Reg3a), regenerating islet-derived protein 3 beta (Reg3β) and secretory immunoglobulin A (sIgA) levels were significantly increased. The interleukin-1beta (IL-1β) level was decreased. 16s RNA sequencing further indicated that the colonic microflora had changed, especially the *Lactobacillus* had increased significantly. Our study showed that short-term ZEA exposure led to resistive increase of colonic probiotics in mice, especially the increase of *Lactobacillus* can reduce intestinal mucosal immunotoxicity.

INTRODUCTION

According to previous reports, zearalenone (ZEA) is one of the most threatening environmental toxins in the world and it was associated with many fungal diseases in farm animals (Fink-Gremmels and Malekinejad, 2007). Nowadays, we often concern that ZEA induces reproductive toxicity in mammals. There are few studies on the toxic effects of ZEA on intestinal mucosal barriers in animal or human. Studies have revealed that ZEA impairs the small intestinal mucosal barrier (Liu *et al*., 2014), and we have previously demonstrated that ZEA not only disrupts the jejunal microecological balance but also causes inflammation of the jejunal mucosa (Wang *et al*., 2018). But we cannot ignore that colon contains large number of intestinal microbes and its microecological stability is more vulnerable to interference from external substances. Increasing evidence suggests that the incidence of colitis and colon cancer is closely related to environmental pollutants, especially mycotoxins (Wild and Gong, 2010; Seonghwan *et al*., 2010; Maresca and Fantini, 2010). Therefore, it is necessary to investigate the effects of ZEA exposure on the colonic mucosal barrier and microecological stability. As we all know, the intestinal mucosa is the first line of defense of the intestinal barrier. Notably, the intestinal epithelial cells, intestinal flora, mucus, sIgA and antimicrobial peptides (AMPs) in the intestinal mucosa constitute the "firewalls" of intestinal mucosal immunity, which protect the body from pathogenic bacteria, virus and environmental pollutants (Macpherson *et al*., 2009). Recent studies have shown that most mycotoxins can induce intestinal epithelial cell inflammation by destroying intestinal mucosal barrier (Seonghwan *et al*., 2010). Indeed, The intestinal flora plays a role in regulating intestinal immunity, and it acts as an adjuvant to the entire immune system (Molloy *et al*., 2012; Belkaid and Hand, 2014). However, there are few studies on the toxic effects of ZEA on the intestinal microflora and colonic mucosal barrier in mammals, and its mechanism is still unclear (Gajęcka *et al*., 2016).

Therefore, we systematically investigated the effects of ZEA on colonic microorganisms and mucosal barriers in mice by 16S rRNA sequencing technology, and explored the potential molecular mechanisms of reduced immunotoxicity, which provided theoretical basis for prevention and treatment of ZEA exposure.

MATERIALS AND METHODS

ZEA (purity > 98%) was purchased from Sigma-Aldrich (St Louis, MO, USA). It was dissolved in ethanol

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and made into 100 mg/ml. Then the storage solution was dissolved in olive oil at 10mg/ml products before use.

Twenty-four immature male BALB/C mice (Laboratory Animal Center of Jilin University, China), were individually divided into two groups, each group had three replicates, each containing four mice. After a weekend for a trial period, all mice were reared under standard test conditions, and were fed by basal diet (Liaoning Changsheng Biological Company) and sterile drinking water. The recommended dose of ZEA induces a physiological disorder in mice of 20 mg/(kg. bw) (~1% LD_{50}) (Zinedine *et al*., 2007). We adopted this recommended dose and conducted many tests on mice, we found it was indeed effective. Therefore, the experimental group (ZEA group) was administered ZEA by oral gavage at a dosage of 20 mg/kg body weight (~1% LD_{50}) once daily for one week, and the control group (CON group) was given an equal volume of vector. All experiments were approved by the Institutional Animal Care and Use Committee of Jilin University.

At the end of the experiment, the mice were sacrificed by cervical dislocation. The colon was separated, and the intermediate segment (1-2 cm long) was taken and washed away with saline, and the tissue was fixed with 4% polyoxymethylene. The remaining colon and feces were carefully collected, quickly placed in liquid nitrogen, and then transferred to -80°C.

Intestinal mucosal morphology analysis

The intestinal tissues were fixed with 4% polyoxymethylene and embedded in petrolin, then sectioned into 2-3 μm sections. The sections were stained with hematoxylin first, counterstained with eosin, and finally observed under an optical microscope.

Composition and diversity of the bacterial communities

Total genomic bacterial DNA was extracted from the feces of colon in BALB/c mice. Then the qualified genomic DNA was used for 16S-rDNA V3-V4 region PCR amplification. The primer was 341F (5' ACTCCTACGGGAGGCAGCAG-3') and 806R (5' GGACTACHVGGGTWTCTAAT-3'). All the procedures were performed according to the manufacturer's protocol (BGI, Shengzhen, China). Two hypervariable regions of 16S rDNA, the V3 and V4 regions, were used to identify the vast majority of the bacteria based on the 16S rDNA sequencing. The qualified PCR products were purified by 16S V3-V4 amplification, and the DNA library was constructed. The qualified DNA library was sequenced using an Illumina MiSeq 2*300, and information was acquired for the bioinformatic analysis.

The raw data were filtered to eliminate adapter pollution and low quality to obtain clean reads. Then, the paired-end reads with overlapping regions were merged to raw tags with FLASH software (v1.2.11) (Magoč and Salzberg, 2011). Paired-end reads without overlaps were removed. Following removal of the primer sequences, the forward and reverse amplification primers were mapped to the two ends of the tags. The tags were clustered to the operational taxonomic unit (OUT) at 97% sequence similarity by scripts using the software USEARCH (v7.0.1090) (RC, 2013). The PCR-generated chimaera from the OTU representation sequence was removed, and effective tags were obtained by UCHIME software (v4.2.40) (Edgar *et al*., 2011). The database used for species annotation was Greengene (V201305) (Desantis *et al*., 2006).

Determination of the relative expression of mRNA in intestinal mucosal barrie by real-time PCR

Total RNA was extracted from colon using TriPure (Roche, Basel, Switzerland) and the cDNA was synthesized using Reverse Transcription System (Promega, Madison, USA) and SYBR Green Mix Kit (Trans, Beijing, China) was used for real-time PCR. Primer design as shown in Supplementary Table I.

Detection of sIgA in the colon

Equal amounts of colonic feces were dissolved with 0.01 M PBS, and broken down at low temperature, centrifuged for 10 min at 3000 r, then the supernatant was collected. The total protein concentration of the feces was detected by BCA. Then the absorbance was detected at 562 nm according to the instructions of mouse sIgA ELISA kit (eBioscience, California, USA), and the sIgA content of feces was calculated (ng/ml).

Detection of cytokine in the colon

Colon was homogenized with 0.01 M PBS to make 10% tissue homogenate. Then at low temperature, the homogenate was centrifuged for 10 min at 3000 r. The supernatant was used for estimation of total protein according to the instructions of IL-1β, IL-10, and TNF (Naniing Jiancheng Bioengineering Institute, Nanjing, China). The cytokine levels were calculated as ng/g prot.

Statistical analysis

All the data were analysed using SPSS statistical software (version 20.0). The variation between the CON group and the ZEA group was analysed by a t-test, and the results are expressed as the mean ± SEM. The differences were judged as statistically significant at P values < 0.05.

RESULTS

Effect of ZEA on mucosal morphology

To objectively assess the effects of ZEA on intestinal

mucosa, the histological sections of colon mucosal morphology was observed, as shown in Figures 1A, 1B. We found that there was no significant pathological change between ZEA group and control group, but the intestinal cavity of the ZEA group became significantly larger (Fig. 1C).

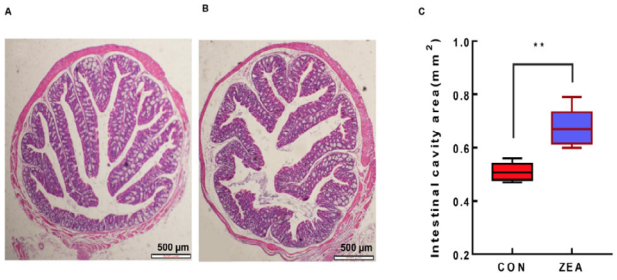

Fig. 1. The effect of ZEA on the intestinal mucosal morphology of the colon in BALB/C mice. A: The colon of the CON group. B: The colon of the ZEA group. C: The area of intestinal cavity. **: P value<0.01.

Table I. Effect of ZEA on bacterial community diversity in the colonic digesta.

Sample name	Tag number	OTU number	Length(bp)	ace	simpson
CON	25379	286	415	304.4	0.057
ZEA	24024	295	416	318.0	0.035

ZEA changes the balance of the intestinal flora

In order to study the effect of ZEA on intestine microflora in the mice, a total of 148209 effective tags were obtained by 16S-rRNA sequencing, with an average of 24702 effective tags for each sample and an average length of 416 bp (Table I). Using the ACE index (Table I) to assess bacterial richness, short-term ZEA exposure led to a tendency to increase the richness of the colonic microflora. At the same time, the Simpson index was used to evaluate changes in the intestinal microbial diversity (Table I). In the ZEA group, the bacterial diversity was almost similar to that of the CON group, but the diversity of the colonic microflora showed a tendency to increase. At 97% similarity, the colonic bacterial community of the CON and ZEA groups shared 325 OTUs (Fig. 2A). The flattening of the rarefaction curve based on the values of observed species (Fig. 2B) indicated that our data volume covered all species of the community in the colon samples. At phylum level (Fig. 2C), compared with the CON group, the abundance of Proteobacteria in the ZEA group was reduced more than 2-fold ($P < 0.001$). The abundance of Actinobacteria in the ZEA group was increased ($P < 0.01$). At genus levels (Fig. 2C), *Lachnoclostridium* and *Alloprevotella* were highly abundant. Compared to the CON group, ZEA reduced *Lachnoclostridium* from 16.7% to 9.8% ($P > 0.05$) and reduced *Alloprevotella* from 9.6% to 4.9% ($P > 0.05$). The abundance of *Desulfovibrio* was reduced from 3.1% to 1.5% ($P > 0.05$). Compared with CON group, the abundance of *Anaeroplasma, Flavonifractor, Helicobacter, Prevotella* and *Ruminiclostridium* in the ZEA group was decreased ($P > 0.05$). Interestingly, the *Lactobacillus* was upregulated more than 4-fold ($P < 0.05$). In addition, we found that short-term ZEA exposure led the emergence of *Akkermansia* in colon.

Effect of ZEA on intestinal mucosal barrier in mice

We further detected the effect of ZEA on colonic mucosal barrier. We examined the mRNA expression of key genes that play the first line of defense in the colonic mucosa (Fig. 3). The mRNA expression of Mucin 1, Mucin 2, and Reg3γ were significantly downregulated by ZEA exposure. Interestingly, ZEA increased mRNA expression of β-defensin, Reg3α, and Reg3β. In addition, the mRNA expression of inflammatory cytokines IL-1β and TNF were remarkably downregulated after ZEA exposure. There was no significant change in the mRNA level of IL-10.

The effect of ZEA on intestinal mucosal immunity

To assess the effect of ZEA on intestinal mucosal immunity, we firstly detect the faecal sIgA levels in the colon. The results showed that short-term ZEA exposure induced the mucosal sIgA ($P < 0.001$) and the faecal IgA in the ZEA group increased more than 3-fold compared to that in the CON group (Fig. 4A). At the same time, we investigated the effects of ZEA on cytokine secretion involved in the inflammatory response in the colon. Interestingly, ZEA inhibited the secretion of IL-1β in the colon, but there was no change in TNF and IL-10 levels (Fig. 4B).

DISCUSSION

Mycotoxin is a class of environmental pollutant that easily causes an intestinal ecological imbalance and induces some intestinal diseases (Maresca and Fantini, 2010; Choung and Iii, 2011; Compare and Nardone, 2014). We found that the structure of colonic microbial communities was altered by short-term ZEA exposure. Changes in some of the dominant bacteria in the gut indicated that our ZEA exposure induced disturbances in the intestinal microecology. We found that Proteobacteria which are associated with inflammation was decreased (Carvalho *et al.*, 2012). At the same time, *Lachnoclostridium* and *Alloprevotella*, *Desulfovibrio*,

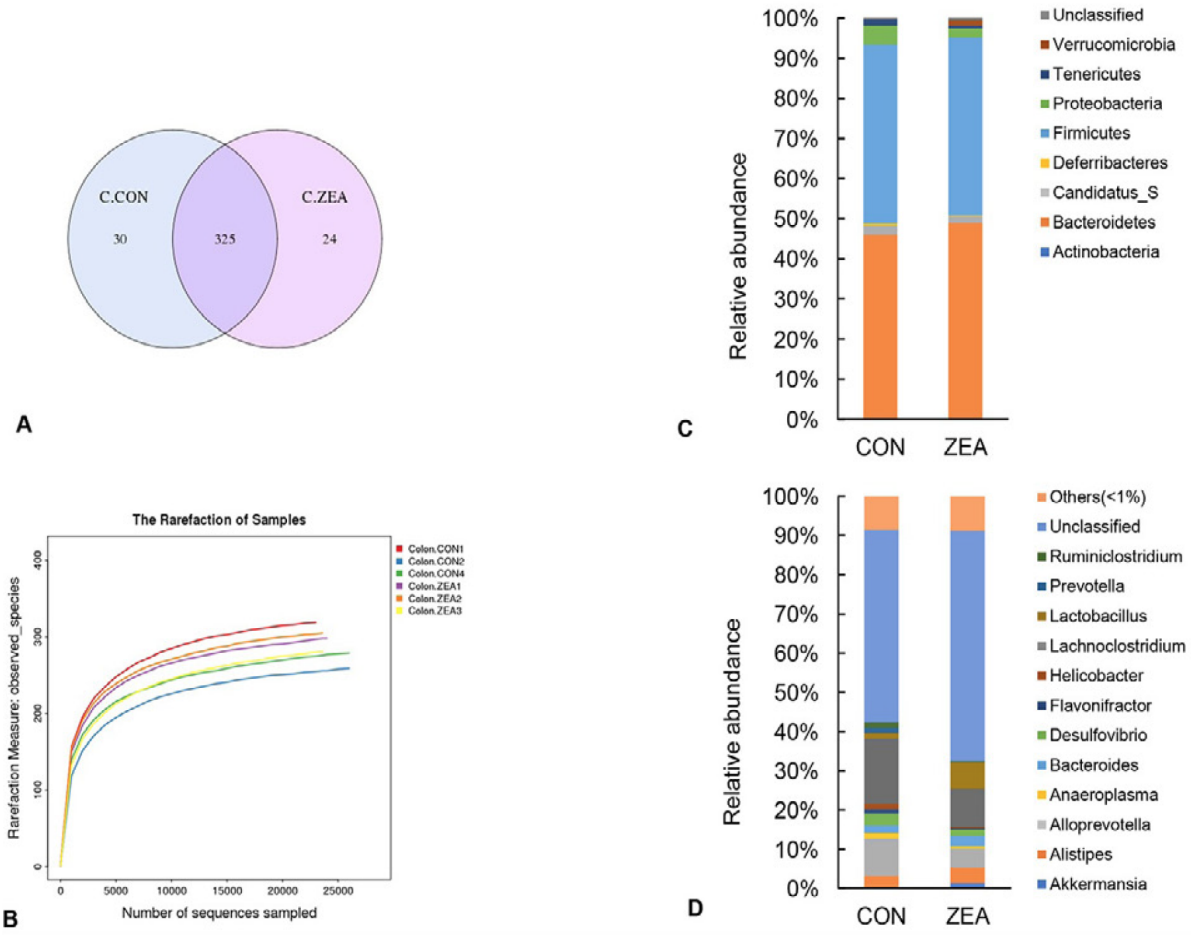

Fig. 2. Effect of ZEA on the intestinal microflora. (A), comparison of OTU. (B), rarefaction curves based on the observed species values. (C), relative abundance of different bacterial phylum. (D), relative abundance of different bacterial genus.

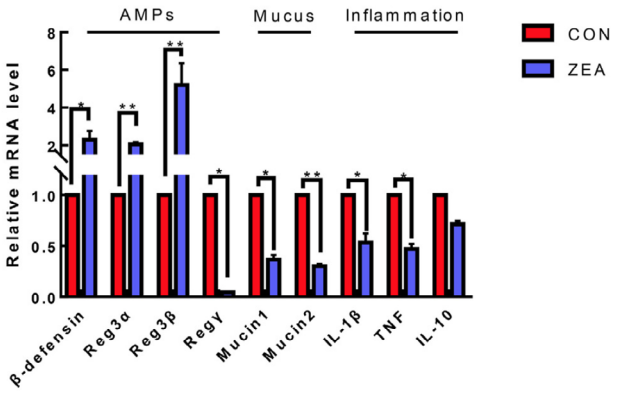

Fig. 3. Changes in the immune gene expression in the colonic mucosa of the mice. All the data are expressed as the means ± SEM. *: P value<0.05, **: P value<0.01.

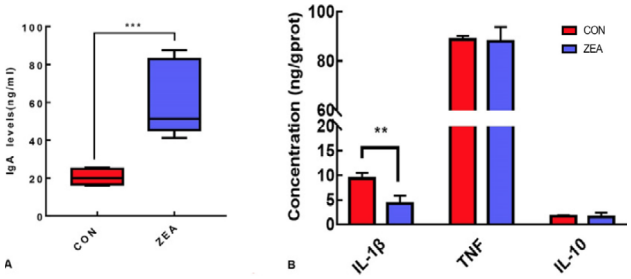

Fig. 4. (A), Changes in the sIgA levels in the colonic faeces. (B), Changes in the cytokines in the colon. All the data are expressed as the means ± SEM. **: P value<0.01, ***: P value<0.001.

Anaeroplasma, Flavonifractor, Helicobacter, Prevotella and *Ruminiclostridium* were decreased. Previous studies have shown that *Clostridium leptum, Lachnospiraceae, and Prevotellaceae* were reduced in inflammatory bowel

disease patients (Swidsinski *et al*., 2002). Changes in these micrbiotes suggest instability of the intestinal flora or sensitivity of the intestines. It is worth noting that ZEA increased the *Lactobacillus*. Piotrowska *et al*. (2014) argued that long-term exposure to low doses of ZEA caused an increase in the *Lactobacillus* in the colon, but the change was not significant. Reddy *et al*. (2018) found that the ZEA-contaminated feed significantly affected the colon microbiota, especially *Lactobacillus*, which was increased by 2.7%. They speculate that *Lactobacillus* plays a major role in detoxification of these mycotoxins. Indeed, *Lactobacillus* have been evaluated in studies in animals and humans with respect to antibiotic-associated diarrhoea, travellers' diarrhoea, pediatric diarrhoea, inflammatory bowel disease, and irritable bowel syndrome. *Lactobacillus* is not only beneficial to intestinal health (Sonnenburg *et al*., 2005) but also combined with mycotoxins to remove mycotoxins (Elnezami *et al*., 2002). The decrease of Proteobacteria and the increase of *Lactobacillus* seem to indicate that damage to the colon by ZEA has been controlled, as evidenced by normal colon morphology. We suspect that the increase of the intestinal cavity area may also be due to the changes in intestinal flora.

Further studies found that ZEA exposure significantly reduced the mRNA expression of Mucin 1, Mucin 2 and Reg3γ in colonic mucosa. Current studies suggest that ZEA increases the secretion of mucin in Caco-2/HT29-MTX cells (Wan *et al*., 2014). Reg3γ and mucins are used to prevent microbial contact with the epithelial layer (Turner, 2009; Sanchez *et al*., 2014; Stefania *et al*., 2015). The decrease of these mRNA expressions indicates the decrease of mucosal defense. Interestingly, ZEA increased the mRNA expression of colonic mucosal β-defensin, Reg3α and Reg3β. In addition, the sIgA in the colon was also increased. Indeed, β-defensin Reg3α/β and sIgA play an essential role in the immune defense of the intestinal mucosa (Wang *et al*., 2015).

ZEA has been described as either an inductor or a suppressor of pro-inflammatory cytokines (Salah-Abbès *et al*., 2010; Marin *et al*., 2011). Our study found that ZEA exposure inhibited the IL-1β and the mRNA expression of TNF was downregulated. Fan *et al*. (2017) suggested that ZEA leads to increased levels of IL-1β in colon tissue of mice. It contradicts our findings. However, Liu *et al*. (2014) revealed that different doses of ZEA showed a downward trend of IL-1β and TNF in the jejunum of rats. Braicu *et al*. (2016) found that exposure to ZEA caused a decrease in the IL-1β and TNF in animal which is still waiting for us to further study.

Current study suggests that ZEA is absorbed through the small intestine and some of it is degraded through the circulation in the liver and intestine (Fan *et al*., 2017) while some combines with intestinal microbes. Eventually, only a small amount of ZEA and its degradation products act on the large intestine (Kollarczik *et al*., 2010). On one hand, a low dose of ZEA is considered to be immunosuppressant (Bondy and Pestka, 2000) and may inhibit the inflammatory response. On the other hand, the increase of colonic *Lactobacillus* only remove mycotoxins by combining with mycotoxins but also regulate intestinal mucosal immunity by producing short chain fatty acid (SCFA) (Elnezami *et al*., 2002; Martín *et al*., 2013). Thereby mucosal damage is alleviated to some extent. But the specific reasons are still waiting to be explored.

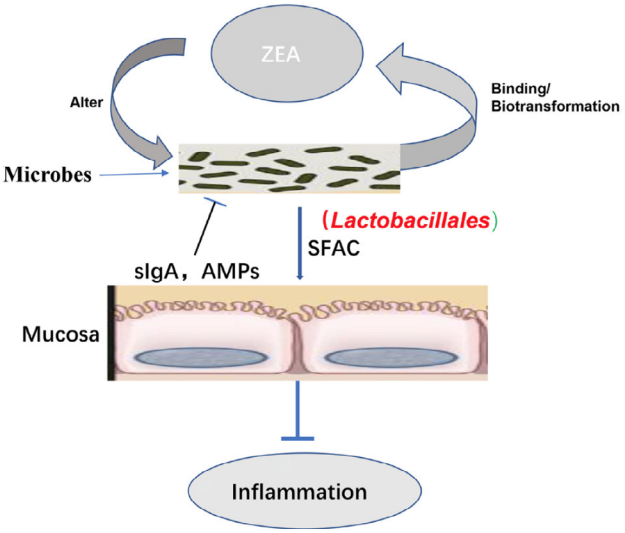

Fig. 5. Schematic diagram of ZEA acting on colonic mucosa.

CONCLUSION

Our study found that ZEA enhanced mucosal immunity in the colon and decreased the colonic *Proteobacteria*. It is worth noting that the intestinal *Lactobacillus* was increased. We believe that low doses of ZEA enter the colon and stimulate the growth of beneficial bacteria in the colon (Fig. 5), which indirectly activates the mucosal defense system. Ultimately the toxicity of ZEA to the intestinal mucosa was alleviated. All of this credit comes from the special role of *Lactobacillus,* which play a key role in regulating intestinal mucosal immunity and promoting colon health. Thus, the colonic mucosa can protect against a certain degree of toxins such as ZEA. This appears to provide us with a new way of thinking about controlling mycotoxins. We believe that intestinal *Lactobacillus* play an essential role in the intestinal mucosal immune defense against environmental toxins such as mycotoxins.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190115060153

Statement of conflict of interest

The authors declare that there are no conflicts of interest.

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