***Bacillus licheniformis* CK1 alleviates the toxic effects of zearalenone in feed on weaned female Tibetan piglets**

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ABSTRACT: Zearalenone (ZEA) is widely present in feedstuffs and raw materials, causing reproductive disorders in animals. In this study, *Bacillus licheniformis* CK1 was used to detoxify ZEA in feed for alleviating its effect in Tibetan piglets. A total of 18 weaned female Tibetan piglets were randomly divided into 3 groups: control group (Control, ZEA-free basal diet); treatment group 1 (T1, ZEA-contaminated diet); and treatment group 2 (T2, ZEA-contaminated but pre-fermented by CK1 diet). There were no significant differences of average daily feed intake (ADFI), average daily gain (ADG), and feed efficiency (FE) among the 3 groups ($P > 0.05$). The T1 treatment significantly increased the vulva size and relative weight of the reproductive organ ($P < 0.05$), compared with the Control. However, the T2 treatment caused a significant reduction ($P < 0.05$) in vulva size and relative weight of the reproductive organ compared with the T1 group. The levels of luteinizing hormone (LH),

follicle-stimulating hormone (FSH), progesterone (P), and estradiol (E2) in the T1 group were significantly lower ($P < 0.05$) than those in the Control, while the levels of LH, P, and E2 in the T2 group were significantly greater ($P < 0.05$) than those in the T1 group. Zearalenone significantly increased ($P < 0.05$) the expression of estrogen receptor α in uterus and ovary and estrogen receptor β in vagina, while these indicators were not significant different ($P > 0.05$) between the T2 group and the Control group. In comparison with the Control group, ZEA significantly increased ($P < 0.05$) expression of several ATP-binding cassette (ABC) transporters: *ABCB1* and *ABCB4* in the vagina, *ABCA1* and *ABCB4* in the uterus, and *ABCB1*, *ABCB4*, *ABCD3*, and *ABCG2* in the ovary, while these transporters in the T2 group were significantly decreased ($P < 0.05$) compared with the T1 group. In conclusion, the present study demonstrates that *B. licheniformis* CK1 could alleviate the harmful effect of ZEA in Tibetan piglets.

Key words: *Bacillus licheniformis* CK1, estrogen receptors, Tibetan piglets, transporters, zearalenone

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INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by filamentous fungi (Savard, 2008). Recently, Taevernier et al. have proposed a more

detailed and concrete definition of mycotoxin as “something if and only if it is a secondary metabolite produced by microfungi, posing a health hazard to human and vertebrate animal species by exerting a toxic activity on human or vertebrate animal cells in vitro with 50% effectiveness levels $< 1000 \mu\text{M}$ ” (Taevernier et al., 2016). Mycotoxin contamination is regarded as an inevitable and unpredictable problem, even where

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good agriculture management, storage, and strict processing are implemented, posing a great risk for animal and human safety. Mycotoxins are difficult to be completely eliminated during feed or food processing such as milling, dehulling, cleaning, and cooking because of their stability against heat, physical, and chemical treatments (Marin et al., 2013). Mycotoxins can accumulate in crops during preharvest, transportation, and storage. After ingestion, the residues or metabolites of mycotoxins can also accumulate in animal-derived products, such as meat, milk, or eggs, leading to intake of mycotoxins by humans (Marin et al., 2013). Presently, 6 mycotoxins are regularly found in food and animal feedstuffs, including aflatoxins, ochratoxins, fumonisins, patulin, zearalenone (ZEA), and deoxynivalenol (Pereira et al., 2014). Of these, ZEA is one of the most important mycotoxins for its global incidence and toxicity (Schatzmayer and Streit, 2013).

Zearalenone is present globally in food and feed chain. For example, ZEA was highly prevalent in North Asia with 56% positive samples containing an average of 386 µg/kg of the mycotoxin (Schatzmayer and Streit, 2013). China is one of the most polluted countries of ZEA. Li et al. analyzed 55 feed ingredients and 76 complete swine feeds collected from 15 swine farms located in Beijing region of China and found that 95.42% of the samples were positive for ZEA and in particular 41% of distiller's dried grains with solubles samples had an average content of 883 µg/kg of ZEA (Li et al., 2014a). Additionally, Li et al. found that the incidence of ZEA was 27.6% in 76 cereal and oil products and the mean level of ZEA in the positive samples was 76.5 µg/kg, exceeding the legislation limit of China in food (≤ 60 µg/kg) (Li et al., 2014b).

Zearalenone, a macrocyclic acid lactone, has a low molecular weight and a good thermal stability. Due to the similarity of its structure to the naturally occurring estrogens, ZEA and its metabolites can competitively bind to estrogen receptors, thus affecting target gene transcription in many organs (Kowalska et al., 2016). Estrogen receptor α (ER α) and estrogen receptor β (ER β) are 2 subtypes of estrogen receptors, and the protein localization, and/or mRNA expression, of ER α and ER β have been studied in porcine reproductive organs (Slomczynska et al., 2001; Knapczyk-Stwora et al., 2011; Norrby et al., 2013). Zearalenone alters functions of reproductive organs, increases the embryo-lethal resorption, and decreases the fertility in laboratory and domestic animals (Zinedine et al., 2007). In addition, ZEA affects ATP-binding

cassette (ABC) transporters. It induced mRNA expressions of *ABCC1*, *ABCC2*, and *ABCG2* in human choriocarcinoma cell lines (Prouillac et al., 2009). Likewise, ZEA could modulate *Abcb1*, *Abcc4*, and *Abcc5* both in SerW3 Sertoli cells and the rat testis (Koraichi et al., 2013). Xiao et al. demonstrated that ZEA was a substrate of the placental ABCG2 transporter (Xiao et al., 2015).

ATP-binding cassette transporters are a family of membrane proteins that mediate diverse ATP-driven transport processes. They are found in organisms from prokaryotes to eukaryotes and responsible for the active transport of a wide variety of compounds including endogenous molecules, nutrients, drugs, and other xenobiotics across biological membranes (Koraichi et al., 2012). They can be subdivided into 7 subfamily labeled ABCA to ABCG, based on the dissimilarity in gene structure, order of domains, and amino acid sequence homologies in nucleotide-binding domains (NBD) and transmembrane domains (TMD) (Mamo and Pandi, 2017). ATP-binding cassette transporters pump transport substrates against a chemical gradient depending on ATP hydrolysis on the NBD, which drives conformational changes in the TMD, leading to alternating access from the interior and exterior of the cell for unidirectional transport across the lipid bilayer (Wilkins, 2015). Furthermore, several studies have found that gene expression of ABC transporters is regulated by hormones in vitro. Wang et al. reported that progesterone (P) could upregulate the gene expression of human *ABCG2* in human choriocarcinoma cells (Wang et al., 2008). Luteinizing hormone (LH) and P could upregulate the gene expression of *ABCB1* in granulosa cells isolated from porcine ovarian follicles (Fukuda et al., 2006).

To eliminate the mycotoxins from feed and reduce harmful effects of mycotoxins to animals, numerous physical, chemical, and biological detoxification methods have been tested. Among them, microbes or their enzymes are attracting more and more attention, due to their advantages of high specificity and high efficiency. Many microorganisms have been shown to bio-transform mycotoxins through biological reactions including acetylation, glucosylation, ring cleavage, hydrolysis, deamination, and decarboxylation (Hathout and Aly, 2014). However, the research for feeding animals with ZEA-contaminated diets degraded by microbes is still limited. Pigs are the most sensitive species to the toxicity of ZEA. Our previous study has demonstrated that *Bacillus licheniformis* CK1 degraded ZEA-contaminated diets, which could

alleviate the toxicity of ZEA to commercial piglets (Fu et al., 2016). The toxicity of ZEA could differ for different breeds of piglets, due to genetics and previous exposure to mycotoxins.

Tibetan pigs are a special Chinese indigenous pig breed, which are distributed in high-altitude areas of Qinghai-Tibet Plateau with long-term living in nonpolluting environment and raised mainly free-range. Therefore, there are much less chances for Tibetan pigs to get exposure to mycotoxins in comparison with commercial pigs raised in other parts of China. Thus, the aims of this study were to evaluate whether Tibetan piglets are more sensitive to ZEA and whether ZEA-contaminated diet degraded by *B. licheniformis* CK1 could alleviate the toxicity of ZEA to Tibetan piglets through determining the growth performance, vulva size, the serum hormone levels, and ZEA-related gene expression of piglets.

MATERIALS AND METHODS

All of the experimental procedures adhered to the Guide for the Care and Use of Laboratory Animals: Eighth Edition, ISBN-10: 0-309-15396-4, and all animal protocol was approved by the guidelines of Animal Use and Care Committee of Northwest Agriculture and Forestry (A&F) University (NWAFA1220).

Strain and Reagents

The strain, *B. licheniformis* CK1, was obtained from the National Taiwan University (Yi et al., 2011). Zearalenone and methanol [high-performance liquid chromatography (HPLC) grade] were purchased from Sigma-Aldrich (St. Louis, MO). The purity of ZEA used in the experiment was more than 99.0%. Water was purified by a Milli-Q Academic Water System (Darmstadt, Germany). All other reagents used were of analytical grade.

Diets Preparation

All diets (Table 1) used in the study were isocaloric and isonitrogenous. Nutrient concentrations met or exceeded nutrient requirements of NRC (1998) for swine. The feed for Tibetan piglets was prepared by the Yangling Experimental Farm, as in our previous study (Fu et al., 2016). Before preparing feedstuffs, all feed ingredients were checked for the presence of mycotoxins by the Yangling Experimental Farm. They were not detectable.

Table 1. Ingredients and compositions of the basic diet

Item	Amount
Ingredients, %	
Corn	53.00
Wheat middling	5.00
Whey powder	6.50
Soybean oil	2.50
Soybean meal	24.76
Fish meal	5.50
L-Lysine HCl	0.30
DL-Methionine	0.10
L-Threonine	0.04
Calcium phosphate	0.80
Limestone, pulverized	0.30
Sodium chloride	0.20
Premix ¹	1.00
Total	100
Nutrients, analyzed values	
Gross energy, MJ/kg	17.12
Crude protein, %	19.40
Calcium, %	0.84
Total phosphorus, %	0.73
Lysine, %	1.36
Methionine, %	0.46
Sulfur amino acid, %	0.79
Threonine, %	0.90
Tryptophan, %	0.25

¹Supplied per kg of diet: vitamin A, 3,300 IU; vitamin D₃, 330 IU; vitamin E, 24 IU; vitamin K₃, 0.75 mg; vitamin B₁, 1.50 mg; vitamin B₂, 5.25 mg; vitamin B₆, 2.25 mg; vitamin B₁₂, 0.02625 mg; pantothenic acid, 15.00 mg; niacin, 22.5 mg; biotin, 0.075 mg; folic acid, 0.45 mg; Mn, 6.00 mg; Fe, 150 mg; Zn, 150 mg; Cu, 9.00 mg; I, 0.21 mg; Se, 0.45 mg.

Zearalenone (90 mg) was dissolved in acetonitrile thoroughly and then mixed with an appropriate amount of talcum powder. The ZEA-containing talcum powder was blended with 6 kg of the basic diet (Table 1) and the mix was divided into 2 equal parts. One part was mixed with 42 kg of the basic diet as treatment 1 (T1), while the other part was fermented by *B. licheniformis* CK1 to degrade ZEA before mixing with 42 kg of the basic diet as treatment 2 (T2).

For the fermentation, batches of 300 g of autoclaved ZEA-containing diet were mixed with sterilized water in a 5-liter fermenter. The mixture was inoculated with an overnight bacterial culture of *B. licheniformis* CK1 and incubated for 36 h at 37 °C, 300 rpm. *Bacillus licheniformis* CK1 (1.2×10^{11} CFU/kg) was added to ferment ZEA-contaminated diet. The mixture was poured into a washbasin after the fermentation, and then the basic diet was slowly added in order to absorb the water. At the end, the mixture was naturally dried at

room temperature. A total of 3 kg ZEA-containing diet was fermented by *B. licheniformis* CK1. The amount of *B. licheniformis* CK1 in the diet of T2 group was 7.95×10^7 CFU/kg. All diets were prepared at the same time and stored in the covered containers before feeding.

Method of ZEA Analysis

Analysis of ZEA concentrations in feed was determined by HPLC. The HPLC system consists of a SIL-20A autosampler, a LC-20AT pump, a CBM-20A system controller, and a RF-20A fluorescence detector (Shimadzu, Kyoto, Japan). Zearalenone was separated by an Ascentis C18 HPLC column (5 μ m particle size, L $\times$ I.D. 250 mm $\times$ 4.6 mm; Sigma-Aldrich, Bellefonte, PA) at room temperature. Zearalenone was detected at excitation and emission wavelengths of 225 and 465 nm, respectively. The mobile phase was a mixture of methanol:water (80:20, v/v) and was delivered at a flow rate of 0.5 mL/min. Before the HPLC analysis, ZEA in the feed was extracted and cleaned up using the Romer Mycosep 226 column (Romer Labs Inc., Union, MO) according to the manufacturer's instructions, and 20 μ L was injected for HPLC analysis after filtration by 0.45 μ m syringe filter. Zearalenone was identified by retention time and quantified from peak area. Identified ZEA was quantified using the external standard. The standard curve was obtained by analyzing 6 standard solutions in different concentration for ZEA (0.1, 0.25, 0.5, 1.25, 2.5, and 5 μ g/mL) and each concentration was analyzed in triplicate. The regression equation for quantification of ZEA was $y = 734937x + 45373$ ($R^2 = 0.9995$).

Animals and Experimental Design

A total of 18 healthy female Tibetan piglets weaned at day 36 with an average body weight (BW) of 6.48 ± 0.74 (mean $\pm$ SE) kg were obtained from the farm of Tibet Agriculture and Animal Husbandry College, where all animals were fed the basic diet during a 10-d adaptation period after weaning. Piglets were randomly allocated to 3 treatments, with 6 piglets in each group according to BW. The diets and water were provided ad libitum throughout the study. Piglets were fed the basic diet (**Control**), treatment 1 (T1) or treatment 2 (T2) during a 21-d test period. Forty-five milligrams of ZEA were added to the diet for T1 group or T2 group before the fermentation. The actual levels of ZEA (analyzed) in feed were 0, 1.13 ± 0.03 ,

and 0.44 ± 0.02 mg/kg for the Control, T1, and T2, respectively. During the test period, all piglets were housed individually in metal pens at the room temperature. Body weights were measured weekly and feed intake of each treatment was recorded daily. Vulva length and width were measured on day 1, day 8, day 15, and day 22. The vulva area was calculated as (vulva length $\times$ vulva width)/2 according to Jiang et al. (2010).

Sample Collection

On the morning following a 12-h fasting at the end of experiment, approximately 10 mL blood sample was collected from anterior vena cava of each piglet. After centrifugation of blood at $1,500 \times g$ for 10 min at room temperature, the serum was separated and stored at -80°C until being analyzed for hormones as described below. All piglets were euthanized. Heart, liver, kidney, spleen, lung, and reproductive organs (vagina + uteri + ovary) were isolated and weighed. The organ relative weight equals the organ weight/the piglet's body weight (g:kg). Samples of vagina, uterus, and ovary tissue were kept at -80°C until extraction of total RNA for measuring gene expression.

Serum Hormone Determination

The concentrations of LH, follicle-stimulating hormone (FSH), P, estradiol (E2), and testosterone (T) were evaluated with radioimmunoassay (RIA) using commercial iodine [^{125}I] RIA kits (China Diagnostics Medical Corporations, Beijing, China) according to the manufacture's protocols as reported previously (Niswender, 1973; Van de Wiel et al., 1981; Diekman and Hoagland, 1983; Langhout et al., 1991; Biswas et al., 2001). Hormone concentration was tested according to each sample's level of radioactivity.

Total RNA Extraction and Real-Time PCR Analysis

Total RNA was extracted from frozen tissues using a total RNA kit (Omega Bio-Tek, Norcross, GA) according to the manufacture's instructions. For reverse transcription, 800 ng of RNA per sample was converted into cDNA using PrimeScript 1st Strand cDNA Synthesis kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Real-time PCR amplification was performed using the TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) with

Bio-Rad CFX96 qPCR system (Bio-Rad, CA). According to the GenBank, oligonucleotide primers were designed and shown in Table 2. Target genes expressions were normalized with glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) and the method of $2^{-\Delta\Delta Ct}$ was used to analyze the real-time PCR data expressed as the fold change relative to the average value of the Control group.

Statistical Analysis

Statistical significance of differences between the treatment groups was analyzed through 1-way analysis of variance (ANOVA) and Duncan's multiple range tests using the SPSS 16.0 statistical software (SPSS 16.0 Inc., Chicago, IL). Data were expressed as treatment means with their pooled SEM and differences were considered significant at P -value < 0.05 .

RESULTS

Growth Performance

During the 21-d feeding experiment, all the piglets appeared healthy without mortality. As shown in Table 3, no significant effect ($P > 0.05$) was noted on average daily feed intake (ADFI), average daily gain (ADG), and feed efficiency (FE) among 3 groups.

The Vulva Size

The vulva size of piglets at 4 points (day 1, day 8, day 15, and day 22) fed diets with different treatments was summarized in Fig. 1. There was no

significant difference ($P > 0.05$) in vulva size of piglets among the 3 groups at day 1. T1 group had a significantly greater ($P < 0.05$) vulva size than the Control and T2 groups at day 8, day 15, and day 22. In addition, significant difference ($P < 0.05$) was observed between the vulva sizes of the Control and those of the T2 group on day 22.

The Relative Weight of Organs

As summarized in Table 4, the relative weight of reproductive organs was significantly affected by the treatments. The relative weight of reproductive organs of piglets in T1 group and T2 group increased by 163.25% and 61.54%, respectively, compared with the Control group. Compared with the T1 group, the relative weight of reproductive organs of piglets was significantly decreased ($P < 0.05$) in the T2 group. On the other hand, there were no significant differences ($P > 0.05$) of relative weight of heart, liver, spleen, lung, and kidney among the 3 groups.

Changes of the Serum Hormones

The effects of different diets on the level of LH, FSH, P, E2, and T were shown in Table 5. The levels of LH and E2 were significantly lower ($P < 0.05$) in the T1 and T2 groups. The LH concentrations in the T1 and T2 groups decreased by 79.02% and 18.88%, respectively, compared with the Control. Similarly, the E2 concentrations in the T1 and T2 groups decreased by 21.78% and 14.84%, respectively, compared with the Control. The levels of FSH and P in the T1 group were lower ($P < 0.05$)

Table 2. Primers for quantitative PCR¹

Genes	Source	Primers sequence (5'→3')	Accession no.
<i>GAPDH</i>	Pig	F: CCTGGCCAAGGTCATCCATG R: CCACCACCCTGTTGCTGTAG	NM_214220.1
<i>ERα</i>	Pig	F: TTGCTTAATTCTGGAGGGTAC R: AGGTGGATCAAGGTGTCTGTG	EF195769.1
<i>ERβ</i>	Pig	F: GCTCAGCCTGTACGACCAAGTGC R: CCTTCATCCCTGTCCAGAACGAG	NM_001001533.1
<i>ABCA1</i>	Pig	F: CTCGTGCAGAAATAGCAGTAGC R: TCCTGATGAGGTGGAGATAGCG	NM_001317080.1
<i>ABCB1</i>	Pig	F: CATTCATCTATGGCTGGCAAC R: TCCTCCCGAGTCAAAGAAACAAC	NM_001308246
<i>ABCB4</i>	Pig	F: ACAACCTTTGATTGACAGCCACA R: CCACTGGACATTGAGTTTCTTTGC	EF067318
<i>ABCD3</i>	Pig	F: AAAGTGGTATCATTGGTCGTAGC R: GTCTGGATTAGCGATTCTGTG	NM_001244133
<i>ABCG2</i>	Pig	F: AGAGTTGGGTCTGGATAAAGTGG R: AAAGATGGAGTAACGAGGCTG	NM_214010

¹*GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse; *ERα*, estrogen receptor α; *ERβ*, estrogen receptor β; *ABC* (*A1*, *B1*, *b4*, *D3*, *G2*), ATP-binding cassette transporters.

Table 3. Growth performance of piglets fed different treatment diets for 3 wk¹

Item	Control	T1	T2	SEM	P-value
ADG, kg/d	0.09	0.07	0.08	0.006	0.267
ADFI, kg/d	0.27	0.25	0.27	0.004	0.422
FE ²	0.36	0.26	0.31	0.02	0.177

¹n = 6 per group; Control group, basal diets without addition of zearalenone or *B. licheniformis* CK1; T1 group, zearalenone-contaminated diets; T2 group, zearalenone-contaminated diets treated by *B. licheniformis* CK1.

²FE = feed efficiency is kilograms of gain/kilograms of feed intake.

Figure 1. Effect of the vulva size of piglets fed different treatment at 4 points (day 1, day 8, day 15, and day 22). Control group: basal diets without addition of ZEA or *B. licheniformis* CK1; T1 group: ZEA-contaminated diets; T2 group: ZEA-contaminated diets treated by *B. licheniformis* CK1. ^{a-c}Different letters at the same time point indicate significant differences ($P < 0.05$).

than those in the Control, while no significant differences ($P > 0.05$) were observed for FSH and P concentrations between the Control and the T2 groups. The T concentrations were not significantly different ($P > 0.05$) among the 3 groups.

The Expression Level of *ERα* and *ERβ* in Different Tissues of Tibetan Piglets

The relative gene expression levels were determined using the $2^{-\Delta\Delta C_t}$ method. As shown in Fig. 2, the T1 group significantly increased ($P < 0.05$) the mRNA expression of *ERα* in the uterus and ovary and *ERβ* expression in vagina, in comparison with the Control. On the other hand, there were no significant differences ($P > 0.05$) of *ERα* and *ERβ* mRNA expression in different tissues between the Control and the T2 groups.

The Expression Level of ABC Transporters in Different Tissue of Tibetan Piglets

In comparison with the Control group, T1 treatment significantly increased ($P < 0.05$) expression of

quite a few ABC transporters: *ABCB1* and *ABCB4* in the vagina, *ABCA1* and *ABCB4* in the uterus, and *ABCB1*, *ABCB4*, *ABCD3*, and *ABCG2* in the ovary (Fig. 3). Significant downregulation ($P < 0.05$) was observed for *ABCG2* mRNA in T1 and T2 group in vagina. Other than *ABCG2* in the vagina, there was no significant ($P > 0.05$) difference in terms of expression of the ABC transporters in the 3 tissues between the Control and T2 groups.

DISCUSSION

In this study, there was no significant difference in ADFI, ADG, and FE among the 3 groups similar to our previous study (Fu et al. 2016). Other studies also support our results. For example, Zhao et al. reported that there were no negative effects on the growth performance of gilts fed diet with final concentrations of 238.57 $\mu\text{g/kg}$ of ZEA (Zhao et al., 2015). Diet with or without ZEA (0.8 mg ZEA per kg diet) had no effect on growth performance of piglets (Denli et al., 2015).

The toxicity of ZEA is best reflected by swollen vulva (Alexopoulos, 2001). Our results showed that ZEA-contaminated diet significantly increased the vulva size of piglets similar to previous studies (Jiang et al., 2011; Oliver et al., 2012; Fu et al., 2016). However, Tibetan piglets seem more sensitive to ZEA than commercial piglets. The vulva size of Tibetan piglets in T1 group increased by 34.92% and 42.22% at the time of the day 8 and day 15, respectively, compared with the Control group. However, in our previous study, ZEA (1 mg ZEA per kg diet) increased the vulva size of commercial piglets by 19.07% and 32.26% at the time of day 8 and day 15, respectively (Fu et al., 2016). The toxicity of ZEA can also be observed by increase in the weight of reproductive organs. Denli et al. found that feeding ZEA-contaminated diets (0.8 ZEA per kg diet) significantly increased the weight of uterus and ovary in piglets (Denli et al., 2015). In our study, piglets fed ZEA-contaminated diets significantly increased the relative weight of reproductive organs, but had no effect on the relative weight of other organs (heart, liver, spleen, lung, and kidney). The reproductive organs index of Tibetan piglets in T1 group was 3 folds that of the Control group. However, the relative weight of reproductive organs in commercial piglets fed ZEA-contaminated diet (1 mg ZEA per kg diet) was only 1.4 folds that of the Control group (Fu et al., 2016). This again supports the notion that Tibetan piglets are more sensitive to ZEA. When piglets were fed the T2 diet, the vulva size and the relative weight of reproductive organs had significant decrease compared with

Table 4. The relative weight (g/kg of BW) of organs in piglets fed different treatment diets for 3 wk¹

Item	Control	T1	T2	SEM	P-value
Heart	4.23	4.96	5.47	0.29	0.250
Liver	28.24	27.52	30.29	0.59	0.119
Spleen	1.63	2.03	1.90	0.1	0.253
Lung	20.76	22.18	21.13	0.32	0.183
Kidney	4.64	5.18	5.14	0.21	0.568
Reproductive organs	1.17 ^a	3.08 ^b	1.89 ^c	0.22	<0.01

^{a-c}Within a row, means without a common superscript differ ($P < 0.05$).

¹ $n = 6$ per group; Control group, basal diets without addition of zearalenone or *B. licheniformis* CK1; T1 group, zearalenone-contaminated diets; T2 group, zearalenone-contaminated diets treated by *B. licheniformis* CK1.

Table 5. The hormone levels in serum of piglets fed different treatment diets for 3 wk¹

Item ²	Control	T1	T2	SEM	P-value
LH, IU/liter	1.43 ^c	0.30 ^a	1.16 ^b	0.13	<0.001
FSH, IU/liter	0.71 ^b	0.54 ^a	0.63 ^{ab}	0.02	0.006
P, ng/mL	1.70 ^b	1.40 ^a	1.59 ^b	0.04	0.001
E2, pg/mL	22.64 ^c	17.71 ^a	19.28 ^b	0.60	<0.001
T, ng/dL	31.11	31.00	31.38	0.50	0.957

^{a-c}Within a row, means without a common superscript differ ($P < 0.05$).

¹ $n = 6$ per group; Control group, basal diets without addition of zearalenone or *B. licheniformis* CK1; T1 group, zearalenone-contaminated diets; T2 group, zearalenone-contaminated diets treated by *B. licheniformis* CK1.

²LH, luteinizing hormone; FSH, follicle-stimulating hormone; P, progesterone; E2, estradiol; T, testosterone.

those in T1 group, indicating that CK1 detoxified ZEA in the diet and consequently reduced its estrogenic effect on the piglets.

Tibetan piglets fed with the T1 diet decreased the levels of serum hormone except testosterone in the present study. In our previous study, we only found that ZEA (1 mg ZEA per kg diet) significantly decreased the level of LH in commercial piglets (Fu et al., 2016). An earlier study demonstrated that ZEA at 4 and 8 mg/kg decreased the level of FSH and P in rats (Collins et al., 2006). Similarly, exposure to high dose ZEA (0.1 to 150 nM) in H295R cells decreased the levels of E2 and P (Frizzell et al., 2011). Arispe et al. reported FSH secretion was reduced in sheep pituitary cells cultured with ZEA (1 to 1,000 nM) (Arispe et al., 2013). These in vitro and in vivo results support the notion that ZEA led to downregulating the secretion of serum sex hormones. We hypothesize that ZEA, like estrogens, inhibits FSH and LH secretion through negative feedback. In our study, decreased secretion of LH, P, and E2 was ameliorated in T2 group compared with T1 group, probably due to reduced amounts of ZEA. Our results were in agreement with a study which reported that addition of 4 g/kg modified calcium montmorillonite

clay to ZEA-contaminated diets (1 mg ZEA per kg diet) increased serum P and E2 concentrations in female piglets (Jiang et al., 2012).

Tibetan piglets fed with the T1 diet had greater mRNA expression of *ERα* in uterus and ovary and *ERβ* in vagina. In our previous study with commercial piglets, the same T1 treatment upregulated the mRNA expression of *ERβ* in vagina, uterus, and ovary, but had no effect on mRNA expression of *ERα* (Fu et al., 2016). The differential effects of ZEA on *ERα* and *ERβ* expression between these 2 studies were interesting and need to be further studied. Zearalenone and its metabolites compete with 17β-estradiol for the specific binding sites of estrogen receptors which are the ligand-activated nuclear receptor and include 2 forms as *ERα* and *ERβ*, then binding the estrogen response element and eventually regulating the transcription of downstream genes (Adibnia et al., 2016).

ATP-binding cassette transporters exist widely in various tissues and utilize ATP to translocate very broad substrates such as endogenous molecules, nutrients, mycotoxins, and other xenobiotics. In our current study, ZEA significantly upregulated the mRNA expression of ABC transporters in piglets, and the transcription levels of ABC transporters were different in vagina, uterus, and ovary. Furthermore, T2 group adding *B. licheniformis* CK1 significantly decreased the mRNA expression of ABC transporters in piglets compared with T1 group. These results suggest that the piglets can use increased ABC transporters for removing ZEA from certain organs and tissues. Thus, increased ABC transporters could be a protective measure. Increased ABC transporters in the presence of ZEA were also observed by others. Zhang et al. reported that the pregnant rats fed ZEA-contaminated diet (100 mg ZEA per kg diet) improved the mRNA expression of *ABCB1* and *ABCG2* (Zhang et al., 2014). Koraichi et al. found that ZEA (1 mg/kg) significantly increased the expression of *ABCB1* and *ABCG2* in uterus of the pregnant rats

Figure 2. Effects of *B. licheniformis* CK1 to detoxify ZEA on the mRNA expression of *Era* (A) and *ERβ* (B) in the reproductive organs of the female weaned piglets. Control group: basal diets without addition of ZEA or *B. licheniformis* CK1; T1 group: ZEA-contaminated diets; T2 group: ZEA-contaminated diets treated by *B. licheniformis* CK1. ^{a,b}Different letters for each organ in each panel indicate significant differences ($P < 0.05$).

Figure 3. Effects of *B. licheniformis* CK1 to detoxify ZEA on the mRNA expression of ABC transporters in the different tissues [(A) vagina, (B) uterus, (C) ovary] of the female weaned piglets. Control group: basal diets without addition of ZEA or *B. licheniformis* CK1; T1 group: ZEA-contaminated diets; T2 group: ZEA-contaminated diets treated by *B. licheniformis* CK1. ^{a,b}Different letters for each ABC transporter in each panel indicate significant differences ($P < 0.05$).

(Koraichi et al., 2012). Some studies also reported that ZEA (10 μ M or 0.1 to 10 nM) could regulate mRNA expression of ABC transporter (Prouillac et al., 2009; Koraichi et al., 2013). In intestinal Caco2 cell line, *ABCC1–3* could directly transport ZEA (10 μ M) and its metabolites, suggesting that ABC transporters could regulate the biomass of ZEA (Videmann et al., 2009). Xiao et al. demonstrated that the environmental chemical ZEA could serve as a substrate for *ABCG2* in the placenta (Xiao et al., 2015). These results indicated that the increase of the expression of ABC transporters in the presence of ZEA could be a protective of organism.

Zearalenone contamination in foodstuffs and animal feeds is a global issue and could cause reproductive disorders and hyper-estrogenic syndromes in farm animals and humans due to its estrogenic effects (Zinedine et al., 2007). Results from our current study and the previous one demonstrate the efficacy of using microorganisms to detoxify ZEA (Fu et al., 2016). Among farm animals, pigs are the most sensitive species to ZEA, especially for pre-pubertal females. The main reason for ZEA sensitivity is that pigs convert ZEA to the

more estrogenically active α -zearalenol (α -ZEL) (Minervini and Dell'Aquila, 2008). Compared with the previous study (Fu et al., 2016), Tibetan pigs are more sensitive to ZEA, probably due to lack of or less previous exposure to ZEA and different genetic background. The climate in Tibet is not favorable for growth of fungi-producing ZEA. In addition, Tibetan piglets are mainly raised free-range, so they are not affected by ZEA-contaminated feed from other places.

In conclusion, Tibetan pigs were more sensitive to ZEA and more suitable as an animal model for studying the toxicity of ZEA to pigs. Moreover, *B. licheniformis* CK1 could degrade ZEA in feed and alleviate the adverse effect of ZEA on piglets.

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Conflict of interest statement. None declared.

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