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Short communication

Growth performance and toxic response of broilers fed diets containing fermented or unfermented cottonseed meal

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ABSTRACT. The aim of the study was to determine the effects of solid-state fermentation of cottonseed meal (FCSM) with *Candida utilis* on broiler growth performance and toxicity in comparison to unfermented cottonseed meal (CSM) or soyabean meal (SBM, control). In total 150 one-day-old male Cobb 400 broilers were randomly assigned to 3 dietary treatments with 5 replications of 10 birds each. Animals were fed control diet with SBM, diet with CSM : SBM (1:1) and diet with FCSM : SBM (1:1) until 21 day of life. Fermentation decreased free gossypol content in CSM from 583.40 to 191.70 mg $\cdot$ kg^{-1}, and degraded total gossypol from 5830.19 to 3882.91 mg $\cdot$ kg^{-1} CSM. In comparison to the control diet, FCSM : SBM (1:1) diet did not affect ($P > 0.05$) the average daily growth and feed conversion ratio, while CSM : SBM (1:1) diet decreased ($P < 0.05$) these parameters. However, FCSM : SBM (1:1) diet, similarly to CSM : SBM (1:1) diet, increased ($P < 0.05$) the relative weight of liver and the serum activity of alanine aminotransferase, and decreased ($P < 0.05$) the relative weight of thymus in comparison to the control diet. In conclusion, residual gossypol or/and degradation products of gossypol in FCSM may still be hepatotoxic and immunotoxic for broilers when SBM is half-replaced by FCSM in diet (155 g $\cdot$ kg^{-1}), although the contents of free gossypol and total gossypol have been markedly reduced and no adverse effect on growth performance was observed.

Introduction

Cottonseed meal (CSM) is produced locally in considerable quantities in China. However, the amount of CSM used in animal diets has been partly limited by the relative low lysine level and nutrient bioavailability, and by the presence of toxic gossypol. The total gossypol (TG) found in CSM exists in both free and bound forms. Free gossypol (FG) has been recognised toxic to birds for a long time (Henry et al., 2001), whereas bound gossypol (BG) is usually thought to be physiologically inactive and nontoxic as it cannot be absorbed from digestive tract (Gadelha et al., 2014). Similarly, Willard et al. (1995) found that toxic effects of gossypol were much greater in non-ruminants than in ruminants due to the fact that FG is binding with soluble proteins or amino acids in the rumen. However, numerous studies have shown that BG may be released as FG during the digestion period and then can be absorbed by the digestive tract (Blackwelder et al., 1998; Mena et al., 2004). Furthermore, broiler chickens fed extruded CSM still show poor growth performance, even though the extrusion process greatly decreased FG but not TG concentration (Gadelha et al., 2014).

In addition, Mena et al. (2004) suggested that the amounts of TG besides FG should be considered when determining a safe dairy cow feeding with products containing available gossypol.

In order to extend the use of CSM in animal feed, many methods of eliminating the gossypol toxicity of CSM have been studied. Recently, Tang et al. (2012) and Gadelha et al. (2014) reported that solid-state fermentation could be an effective way to decrease the content of FG in CSM and increase the broiler growth performance. But still, a little information is available about changes between TG before and after fermentation and the toxicity of gossypol residual and degradation products in fermented cottonseed meal (FCSM).

Candida utilis is a GRAS (generally regarded as safe) fungus, often used as a food and feed additive. A strain of *Candida utilis* that grows well on a medium containing gossypol acetate as the sole carbon source and on basal substrates from CSM was screened by Hubei Key Laboratory of Animal Nutrition and Feed Science (Zhou et al., 2014).

The objective of this study was to verify the hypothesis that the reduced FG content and the improved growth performance were not the only marker factors to evaluate FCSM in broiler diets. Also, the toxic influence of CSM solid-state fermented by *Candida utilis* on young chickens less than 21-day of life still existed, when FCSM reached the highest level.

Material and methods

Preparation of FCSM

The solid-state fermentation process of CSM was performed according to the method of Zhou et al. (2014). Thus, a strain of *Candida utilis* approved officially as a feed ingredient, was collected in China General Microbiological Culture Collection Center (Beijing, China), and then screened by comparing the growth of different yeast strains on solid medium containing gossypol acetate without another carbon source. For the fermentation of CSM, the strain of *Candida utilis* was first cultured on Wort agar (BD-Difco, Detroit, MI, USA) at 37 °C for 48 h, and then the inoculum (one loop) was transferred into a 25-ml amber bottle containing 10 ml of yeast extract peptone dextrose (YPD) medium at 37 °C, and shook at 170 rpm for 24 h. The concentration of *Candida utilis* was determined using the dilution-plate method on Wort agar and found to be about 2.0×10^8 cfu · ml^{-1}.

Basal substrates (50 kg) from CSM were soaked with water in 1:1 ratio (one CSM part to one water part) and then inoculated with 50 g · kg^{-1} of *Candida utilis* culture (vol/wt, 0.1×10^8 cfu · g^{-1} CSM), mixed and fermented for 24 h at 30 °C. The resulting material was dried in an oven at 60 °C to provide approximately 900 g · kg^{-1} of dry matter; then was milled to pass through a 40 mesh screen (0.25 mm in particle size), weighed, and kept in sealed containers at a room temperature until analysed or used.

Birds and experimental design

In total 150 one-day-old male Cobb 400 broilers provided by a local commercial hatchery were randomly assigned to 3 dietary treatments. Soyabean meal (SBM) was the main protein ingredient used in the control diet. In the other two diets, 155 g · kg^{-1} of SBM was replaced by the equal amount of CSM or FCSM (1:1), respectively; isonitrogenous and isoenergetic levels were established, as well as equal lysine, by the addition of synthetic lysine (Table 1). The control diet was formulated to meet or exceed the NRC (1994) nutrient requirements. Each diet was self-fed to 5 replicates (cages) of 10 birds each. Each cage was 200 × 140 × 70 cm (length × width × height). Mash feed and fresh water were available *ad libitum* during the 3-week experiment. The lighting programme and temperature were maintained according to normal management practices. All practical procedures were approved by Hubei Province Institutional Animal Care and Use Committee (Wuhan, China).

Analyses of CSM and FCSM compositions

The proximate composition, trichloroacetic soluble protein (TCASP), amino acid, TG and FG contents in CSM and FCSM were determined in triplicates.

Growth performance

At 21 day of life, chickens were weighed after 12 h of feed withdrawal. Feed intake was recorded daily on a replicate basis. Average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR, feed-to-gain ratio) were calculated.

Serum enzyme activity

After weighing at 21 day of life, two birds per cage (10 chickens per treatment) with the closest weight to the average cage weight were selected and bled through a jugular vein. The blood samples were immediately placed on ice, transported to the laboratory and centrifuged at 2000 *g* for 15 min at about 4 °C in a refrigerated centrifuge within 3 h

Table 1. Ingredient composition and levels of nutrients and gossypol in diets

Indices	SBM (control)	CSM:SBM (1:1)	FCSM:SBM (1:1)
Ingredients, g · kg^{-1}			
maize	568.7	567.6	567.6
soyabean meal (SBM)	310.0	155.0	155.0
cottonseed meal (CSM)	0	155.0	0
fermented cottonseed meal (FCSM)	0	0	155.0
fish meal	40.0	40.0	40.0
soyabean oil	40.0	40.0	40.0
di-calcium phosphate	13.7	13.7	13.7
limestone	11.3	11.3	11.3
salt	1.5	1.5	1.5
sodium bicarbonate	1.5	1.5	1.5
DL-methionine (980 g · kg^{-1})	2.3	2.3	2.3
L-lysine (780 g · kg^{-1})	0	1.1	1.1
choline chloride (500 g · kg^{-1})	1.0	1.0	1.0
premix[1]	10.0	10.0	10.0
Analysed values, g · kg^{-1}			
ME[2], cal · kg^{-1}	3023	2983	-
crude protein	210.6	209.4	209.10
Ca	10.8	10.7	10.7
total P	7.7	8.4	8.5
available P	5.5	5.8	5.8
lysine	11.5	11.5	12.0
methionine	5.8	5.8	5.7
methionine + cysteine	9.1	9.1	9.1
arginine	14.0	16.0	15.6
Gossypol, mg · kg^{-1}			
free	ND[3]	90	30
total	ND	904	602
bound	ND	814	572

[1] provided per kg of diet: IU: vit. A 9,100, vit. D_3 1,750, vit. E 17.5; mg: vit. K_3 1.75, thiamine 3.5, riboflavin 8.75, pyridoxine 3.5, vit. B_{12} 0.035, calcium pantothenate 28, folate 1.05, niacin 35, Cu ($CuSO_4 \cdot 5H_2O$) 8, Fe ($FeSO_4 \cdot 7H_2O$) 96, Zn ($ZnSO_4 \cdot 7H_2O$) 96, Mn ($MnSO_4 \cdot H_2O$) 120, Se ($NaSeO_3$) 0.8, I (KI) 0.7, clopidol 125, colistin sulphate 10; [2] ME – metabolizable energy calculated according to NRC (1994); [3] ND – not detected

after collection. The serum was collected and stored at −80 °C until further analysis of enzyme activities.

The activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed by an automatic clinical biochemical analyser (Hitachi 7072, Tokyo, Japan), using clinical chemistry reagents (Randox, Crumlin, UK).

Organ weight

After collecting blood samples, the birds were euthanized by cervical dislocation and then eviscerated. The liver, pancreas, heart, thymus, spleen and bursa of Fabricius were removed, excised and weighed immediately. The relative organ weight was calculated as g per kg of body weight.

Statistical analysis

All data were statistically analysed by a one-way analysis of variance (ANOVA) using the GLM procedure of SAS software release 8.1 (SAS Institute Inc., Cary, NC, USA, 2000) as a completely randomized design to estimate the effect of treatments. Differences among means were compared using Duncan's multiple-range test. The level of significance was based on probability of 0.05 unless otherwise stated.

Results and discussion

Composition of FCSM and CSM

There was 9.76% substrate weight lost during the fermentation process (Table 2). In FCSM were indicated higher levels of crude protein, crude ash, total phosphorus, non-phytate phosphorus and calcium contents, and lower level of ether extract and lower crude fibre content than in CSM. The fermenting procedure led to an approximate 2.2-fold increase in TCASP which accounted for about 7.3% of the total protein. In FCSM the contents of threonine, valine, isoleucine, leucine, lysine and cysteine were higher of 7.20%, 13.49%, 11.18%, 10.66%, 14.54% and 11.84%, respectively, whereas contents of arginine and methionine were lower of 10.92% and 12.31%, respectively, than those of CSM. The concentrations of other amino acids were also slightly changed in the fermentation process. Fermentation resulted in 67.14% decrease in FG, 29.65% in BG and 33.40% in TG content.

The differences of the chemical compositions between FCSM and CSM were in line with previously obtained data (Zhou et al., 2014), and again demonstrated that the growth and metabolism of such microorganisms as *Candida utilis* converted substrates into microbial proteins and other biological compounds, and secreted proteases enzymes to hydrolyse protein from CSM into smaller molecular compounds (i.e. peptides and free amino acids). The decrease in TG content in CSM during fermentation shows that enzymes secreted by *Candida utilis* could degrade gossypol. However there are no reports on the chemical structure of the degradation products.

Growth performance

No mortality or clinical signs of gossypol intoxication were observed in any of the treatments during the experimental period. ADG (P = 0.004) and FCR

($P = 0.041$) from 1 to 21 day of life were affected by dietary treatments (Table 3). No difference in ADFI was shown among dietary treatments ($P > 0.05$). In birds fed CSM at a dose of 155 g · kg^{-1} of diet the ADG and FCR values were lower ($P < 0.05$) than in control animals. On the other hand, in chickens fed diet containing 155 g · kg^{-1} of FCSM, ADG and FCR were higher ($P < 0.05$) than in those fed control and CSM diets.

Incorporation of CSM into broiler diet has been studied for decades. It has been concluded that high level of FG in broiler feed from CSM resulted in growth depression, reduced feed intake and poor feed efficiency (Henry et al., 2001). However, the tolerance level of broilers to FG is very contradictory. According to the review of Gadelha et al. (2014), different research results are presented: FG at level above 160 mg · kg^{-1} of diet could affect poultry performance, the FG level up to 100 mg · kg^{-1} of diet was acceptable by broilers, and that the broiler performance was not significantly affected when FG content was lower than 200 mg · kg^{-1} of diet. In our study, there was significant negative effect of CSM on ADG and FCR of broilers from 1 to 21 day of life, when CSM at a dose of 155 g · kg^{-1} of diet replaced half of the SBM in the control diet (providing a diet containing only 90 mg FG· kg^{-1} diet). This may indicate that FG is not the only factor affecting growth performance of broilers. Among other factors may be dietary protein level, lysine concentration and essential amino acid availability, as well as TG source and content. Sterling et al. (2002) demonstrated that CSM could replace SBM in broiler grower diets at higher protein levels (260 g · kg^{-1} of diet) to achieve similar performance. Gamboa et al. (2001) indicated that CSM could be used successfully in chicken starter diets at levels up to 280 g · kg^{-1} diet for 3 weeks when diets were formulated on a digestible amino acid basis.

Several studies were conducted to evaluate the effects of FCSM on broilers. Tang et al. (2012) indicated that FCSM obtained with the use of *Bacillus subtilis* up to 120 g · kg^{-1} diet could be incorporated in yellow-feathered broiler diets, and replacement of SBM by 80 g · kg^{-1} of FCSM resulted in optimum growth performance based on body weight gain and feed intake. Similarly, Chen et al. (2009) concluded that supplementation of CSM fermented by *Bacillus subtilis* and *Saccharomyces cerevisiae* to feed enhanced body weight gain of 21- and 39-day-old broilers. Interestingly, in the present trial the ADG and FCR in broilers were improved (in comparison to CSM) by SBM replacement of FCSM (155 g · kg^{-1} diet) giving similar results to the control diet.

Table 2. Chemical composition of cottonseed meal (CSM) and fermented cottonseed meal (FCSM)

Indices	CSM	FCSM
Substrate weight, kg	50.00	45.12
Proximate composition, g · kg^{-1}		
dry matter	904.0 ± 2.1	906.0 ± 2.2
crude protein	435.0 ± 1.0	445.0 ± 1.1
ether extract	5.2 ± 1.3	5.0 ± 1.1
crude fibre	102.1 ± 10.5	93.2 ± 11.0
crude ash	60.2 ± 3.0	66.0 ± 3.2
total P	10.4 ± 2.0	11.3 ± 1.9
non-phytate P	3.6 ± 1.0	3.7 ± 0.9
Ca	2.6 ± 0.8	2.8 ± 1.0
Trichloroacetic acid soluble protein, g · kg^{-1}	10.2 ± 1.0	32.6 ± 2.1
Amino acids, g · kg^{-1}		
aspartic acid	30.2 ± 4.1	34.6 ± 3.6
threonine	12.5 ± 1.1	13.4 ± 1.2
serine	18.2 ± 1.3	18.0 ± 1.5
glutamic acid	61.0 ± 4.3	60.1 ± 3.2
glycine	16.1 ± 1.1	16.0 ± 1.1
alanine	17.0 ± 1.7	19.5 ± 1.3
valine	21.5 ± 2.3	24.4 ± 1.9
isoleucine	15.2 ± 2.0	16.9 ± 1.8
leucine	27.2 ± 2.3	30.1 ± 3.4
tyrosine	16.2 ± 2.4	15.7 ± 1.9
phenylalanine	23.2 ± 2.7	22.5 ± 2.8
histidine	13.0 ± 1.2	13.6 ± 1.6
lysine	22.0 ± 2.2	25.2 ± 2.3
arginine	48.5 ± 3.5	43.2 ± 3.4
proline	15.4 ± 1.8	16.4 ± 1.5
methionine	6.5 ± 0.8	5.7 ± 0.6
cysteine	7.6 ± 0.9	8.5 ± 1.0
Gossypol, mg · kg^{-1}		
free	583 ± 56	192 ± 32
total	5830 ± 120	3883 ± 111
bound	5247 ± 120	3691 ± 113

Analytical values are expressed as mean ± SD, n = 3

This may result from the decreased gossypol content and the improvement in nutrient contents such as crude protein, lysine, free amino acid and small peptides, after CSM was subjected to the fermentation process (Table 2).

It was also indicated that ADG in broilers was more sensitive to CSM or FCSM addition into diet than ADFI (Table 3). So, the observed changes in growth performance were not caused by the decrease in feed intake.

Organ weight and serum enzyme activity

The dietary treatments affected the relative weight of liver ($P < 0.001$) and thymus ($P = 0.023$), and the serum activity of ALT ($P = 0.007$) in broilers at 21 day of life (Table 4). The CSM addition into diet

Table 3. Effects of cottonseed meal (CSM) or fermented cottonseed meal (FCSM) as replacement of soyabean meal (SBM) on growth performance of broilers from 1 to 21 day of life

Indices	SBM (control)	CSM:SBM (1:1)	FCSM:SBM (1:1)	SEM	*P*-value
ADG, g	33.95[a]	30.81[b]	33.4[a]	0.34	0.004
ADFI, g	51.39	48.34	50.68	0.99	0.237
FCR, g · g^{-1}	1.51[b]	1.57[a]	1.52[b]	0.02	0.041

SEM – standard error of means; ADG – average daily gain; ADFI – average daily feed intake; FCR – feed conversion ratio = feed to gain ratio; [ab] – means with different superscripts within a row are significantly different at $P < 0.05$; each mean based on 5 replicates of 10 chickens per each treatment

Table 4. Effect of cottonseed meal (CSM) or fermented cottonseed meal (FCSM) as replacement of soyabean meal (SBM) on relative organ weight and serum enzyme activity of broilers at 21 day of life

Indices	SBM (control)	CSM:SBM (1:1)	FCSM:SBM (1:1)	SEM	*P*-value
Relative organ weight, g · kg^{-1}					
liver	24.14[b]	31.74[a]	31.70[a]	0.64	<0.001
thymus	4.00[a]	2.77[b]	3.15[b]	0.18	0.023
spleen	0.81	0.87	0.85	0.03	0.708
bursa of Fabricius	2.53	2.39	2.04	0.10	0.118
Serum enzyme activity, U · l^{-1}					
ALT	31.5[b]	40.7[a]	36.7[a]	1.7	0.007
AST	35.1	36.5	34.6	1.6	0.468

SEM – standard error of means; ALT – alanine aminotransferase; AST – aspartate aminotransferase; [ab] – means with different superscripts within a row are significantly different at $P < 0.05$; each mean based on 10 chickens per treatment

increased ($P < 0.05$) the relative weight of liver and the activity of serum ALT, but decreased ($P < 0.05$) the relative weight of thymus in comparison to the control group. The FCSM inclusion into diet caused the same changes as CSM. There were no differences ($P > 0.05$) in the relative weight of spleen and bursa of Fabricius and the serum activity of AST among dietary treatments. The relative weight of pancreas and heart were not significantly affected by the use of CSM or FCSM in the diets (data not shown).

As reported by previous authors, gossypol can exert hepatotoxic effect. Gadelha et al. (2014) reviewed that broilers which received a diet with 4 g · kg^{-1} of TG for 20 days had greater liver weight, and feeding with purified gossypol at a dose of 400 and 800 mg · kg^{-1} diet to chickens indicated a significant dose-related increase in the relative liver weight. The gossypol hepatotoxic effect was also shown in pigs by Fombad and Bryant (2004), who fed growing pigs (20–75 kg) with cottonseed cake at 150 g · kg^{-1} of diet and found increased liver weight. There are little data describing the precise mechanism of the gossypol effect on the liver. Gamboa et al. (2001) for 3 weeks fed broilers with CSM diets and indicated highest concentration of TG in the liver, followed by kidney, plasma and muscle. Moreover, Rincon et al. (1978) concluded that accumulation of gossypol in the pig organs was directly correlated with its dietary levels and the duration of ingestion. Thus, the increase of the relative liver weight in this present study might be a consequence of dietary gossypol accumulation.

In vivo and *in vitro* mouse experiments demonstrated that gossypol had immunosuppressive activity (Gadelha et al., 2014). There is little information on the alteration of the relative thymus weight. Mice that received gossypol had significantly decreased numbers of lymphocytes in the thymus (Deng et al., 2012).

The serum activities of ALT and AST are important indicators reflecting liver health. A significant increase in the serum activities of ALT and AST are usually associated with hepatic cellular damage or are thought to be a result of increased synthesis of γ-glutamyl transferase. Blevins et al. (2010) found that the level of serum γ-glutamyl transferase activity was elevated in chickens fed diet containing 1,000 mg · kg^{-1} of FG for 21 days. In the present study, the serum ALT activity on 21 day was significantly higher when broilers were reared on diets with more than 155 g · kg^{-1} of CSM or FCSM. This result indicates that not only CSM but also FCSM at high levels in broiler diets might cause liver damage. This conclusion was also supported by the significant increase in the relative liver weight. However, the lack of observed change in the serum AST activity might suggest that the toxicity of CSM and FCSM was not at the hepatocytes mitochondria level.

The most important finding of the presented study is that gossypol concentrations required to alter liver and immune organs are different (lower) from that required to reduce growth performance. This is consistent with findings of Henry et al. (2001) and Fombad and Bryant (2004). The latter authors reported that pigs fed toxic levels of gossypol might appear normal for a few weeks to a year, but then begin to gasp for breath and die within 2–6 days. Also showed histopathological changes in the liver due to the gossypol occurrence at levels lower than the levels that affect body weight. Similarly, Braga et al. (2012) found that in male sheep fed extruded cottonseed meal for 9 weeks there was no clinical signs of gossypol intoxication and no changes in growth and feed consumption but there was a decrease in lymphocyte counts indicating that cottonseed was not completely detoxified by the extrusion process and that gossypol residuals may be involved in lymphocyte deficiency.

Conclusions

Fermentation of cottonseed meal with *Candida utilis* resulted in a marked drop in free and total gossypol (FG and TG, respectively) contents. Although, fermented cottonseed meal (FCSM) could be acceptable to broiler diet in the amount of 155 g · kg^{-1} as half replacement of soyabean meal for the first 3 weeks of life, based on growth performance, but it may still be hepatotoxic and immunotoxic to broilers. Thus, in future studies evaluating FCSM usage in broilers feed, factors other than the effect of FG on growth performance should be considered.

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