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Evaluation of the feeding value of microbially fermented cottonseed meal by *in vitro* method

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Abstract: In this study, different strains were used to ferment single/mixed cottonseed meal (cottonseed meal, corn flour, bran ratio of 7 : 2 : 1) substrates, and their feeding value was evaluated by an *in vitro* fermentation trial, to select suitable fermentation strains for cottonseed meal. For this purpose, two experiments (Exp.) were conducted. Exp. 1: Evaluation of the effect of fermentation of single cottonseed meal by different strains, which consisted of ten treatment groups: control (CON), *Saccharomyces* No. 1 (T1), *Saccharomyces Fubon* (T2), *Saccharomyces Lallemand* (T3), *Lactobacillus* (T4), *Bacillus licheniformis* (T5), *Bacillus subtilis* 10071 (T6), *Bacillus subtilis* 10089 (T7), *Aspergillus niger* (T8), and *Monascus purpureus* Went (T9). Exp. 2: Evaluation of the effect of fermentation of mixed cottonseed meal by different strains. The treatment groups were the same as in Exp. 1, numbered CON, M1, M2, M3, M4, M5, M6, M7, M8, and M9. The results showed that in both the single cottonseed meal group and the mixed cottonseed meal group free gossypol (FG) and methane (CH_4) were significantly reduced and the gas production, total volatile fatty acids (TVFA), and *in vitro* digestibility of nutrients ($P < 0.05$) significantly increased, with *Saccharomyces* No. 1 showing the optimal effect. This study provides a theoretical basis for screening the suitable strains for fermenting cottonseed meal.

Keywords: detoxification efficiency; free gossypol; *in vitro* gas production; microorganisms; rumen fermentation

Cottonseed meal is made from cottonseed that has been dehulled, heated, and flattened into flakes, which can be extracted and de-oiled to obtain by-products (Liu et al. 2024a). Cottonseed meal is rich in crude protein (CP) and amino acids, which can be used as a high-quality protein feed resource to alleviate the problem of insufficient protein feed resources in some large cotton-producing countries (Li et al. 2022a). However, cottonseed meal contains free gossypol (FG), a naturally occurring animal toxin that can limit the use of cottonseed meal in animal production, which affects growth

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performance and fertility in ruminants (Zhang et al. 2022a). Moreover, FG can also be transferred to meat, milk, and eggs (Gamboa et al. 2001; Wang et al. 2012; Gadelha et al. 2014; Yuan et al. 2014), causing harm to the human health.

For the methods of cottonseed meal detoxification, a lot of research has been done on the specific methods of chemical, physical, mixed solvent extraction, and microbial fermentation detoxification methods, etc. Among them, the most promising application is microbial solid-state fermentation. Microbial fermentation can effectively degrade FG, and the commonly used strains are *Saccharomyces*, *Aspergillus niger*, *Bacillus subtilis*, and *Lactobacillus*, etc. (Wang et al. 2020; Li et al. 2022b; Yusuf et al. 2022).

At present, most of the research on microbial fermentation of cottonseed meal has been reported on poultry and pigs (Gu et al. 2021; Niu et al. 2021), the relative research on the ruminants is limited, and the suitable strains for the fermentation of cottonseed meal have not been fully screened. Therefore, this study aimed to screen excellent microbial strains suitable for cottonseed meal fermentation, reduce free gossypol content, improve the feeding value of cottonseed meal for ruminants, and provide data support for the efficient and safe resource utilisation of cottonseed meal.

MATERIAL AND METHODS

Experimental materials

Fermentation substrate. The study included two experiments (Exp.). In Exp. 1, the fermentation substrate was single cottonseed meal. In Exp. 2, the substrate was a mixed formula mainly consisting of cottonseed meal, corn flour, and wheat bran at a ratio of 7 : 2 : 1. Specifically, wheat bran was selected as the bran raw material. All experimental ingredients were crushed, thoroughly mixed, and sieved through a 40-mesh screen before use. The nutritional composition of single and mixed cottonseed meal prior to fermentation is presented in Table 1.

Fermentation strains. At present, common strains used for fermenting cottonseed meal to degrade free gossypol include *Aspergillus niger*, *Bacillus subtilis*, *Lactobacillus*, *Saccharomyces*, and other probiotic strains (Sousa et al. 2022). Therefore, the following nine strains were used in both Experiment 1 and Experiment 2. Among them, *Aspergillus niger* (CICC 41481), *Monascus purpureus* Went (CICC 41606), *Bacillus licheniformis* (CICC 21112), and two types of *Bacillus subtilis* (CICC 10071; CICC 10089) were purchased from the China Centre of Industrial Culture Collection (CICC). *Lactobacillus* and *Saccharomyces* No. 1 were preserved in our laboratory. In addition, two yeast strains, namely *Saccharomyces Fubon* (mainly composed of active yeast cells) and *Saccharomyces Lallemand* (CNCM I-1077, milky-white globular granules, mainly composed of active yeast cells), were also included in this study.

Media. The medium used for the cultivation of *Saccharomyces* and *Aspergillus niger* was potato dextrose agar (PDA) medium, the medium used for the cultivation of *Lactobacillus* was de Man, Rogosa and Sharpe (MRS) medium, the medium composition of *Bacillus subtilis* and *Bacillus licheniformis* and the above medium composition are shown in Table 2. All of the above media need to be autoclaved at 121 °C for 20 min at the time of use.

An amount of 50 g cottonseed meal was placed in a brown glass bottle, 50 ml of water was added, and 5 ml of the above nine kinds of strains were added, each group has 6 parallels, natural pH value, the brown glass bottles were covered with a plastic wrap, and cultured in an incubator at 30 °C for 72 hours.

Experimental design

All procedures involving animals were performed with the approval of the Yanbian University

Table 1. The nutritional components of single cottonseed meal and mixed cottonseed meal before fermentation (DM basis)

Nutrient levels	Single cottonseed meal	Mixed cottonseed meal
DM (%)	95.3	95.0
Moisture (%)	4.70	5.04
EE (%)	5.66	5.15
CP (%)	44.0	38.0
ADF (%)	15.30	13.05
NDF (%)	40.0	37.0
Hemicellulose (%)	24.7	23.9
Ash (%)	7.89	6.36

ADF = acid detergent fibre; CP = crude protein; DM = dry matter; EE = ether extract; NDF = neutral detergent fibre

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Table 2. Medium component

Medium	Culture medium composition
Potato dextrose agar (PAD) medium	5 g potato starch, 20 g glucose, 0.100 g chloramphenicol
de Man, Rogosa and Sharpe (MRS) medium	10 g peptone, 10 g beef extract, 5 g yeast extract, 2 g K_2HPO_4, 2 g $C_6H_{14}N_2O_7$, 5 g CH_3COONa, 20 g glucose, 1 ml Tween 80, 0.500 g $MgSO_4$, 0.250 g $MnSO_4$, 15 g agar powder, 1 l distilled water
Wort medium	1 l 5°Bé wort, 15 g agar
Bacillus subtilis medium	10 g peptone, 10 g beef powder, 5 g NaCl, 20 g agar, 1 l distilled water
Bacillus licheniformis medium	5 g peptone, 3 g beef extract, 5 g NaCl, 15 g agar, 1 l distilled water

Institutional Animal Care and Use Committee. The study included two Exps.: Exp. 1 was an evaluation of the effect of fermentation of single cottonseed meal by different strains, and Exp. 2 was an evaluation of the effect of fermentation of mixed cottonseed meal by different strains.

Exp. 1 Evaluation of the effect of fermentation of single cottonseed meal by different strains. The experiment consisted of ten treatment groups: unfermented single cottonseed meal (CON); single cottonseed meal fermented by *Saccharomyces* No. 1 (T1); *Saccharomyces Fubon* (T2); *Saccharomyces Lallemand* (T3); *Lactobacillus* (T4); *Bacillus licheniformis* (T5); *Bacillus subtilis* 10071 (T6); *Bacillus subtilis* 10089 (T7); *Aspergillus niger* (T8); and *Monascus purpureus* Went (T9). Each treatment group had 3 replicates.

An *in vitro* fermentation trial was carried out according to Luan et al. (2023). Briefly, the rumen fluid for *in vitro* fermentation was collected before morning feeding from three ruminally fistulated Yanbian cattle. The rumen fluid was pooled and transferred into a pre-warmed insulated flask (with a temperature of about 39 °C) and immediately transported to the laboratory. *In vitro* fermentation trials included an *in vitro* gas production experiment and an *in vitro* digestibility experiment, and the specific experimental steps referred to Luan et al. (2023).

Analysis of indices. The gas volume was recorded at 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36, and 48 hours. The glass syringe was taken out, and the scale value (ml) at the bottom of the piston and the scale overlap of the syringe were recorded. The net gas production was calculated in each period: Net gas production (ml) = gas production in a period (ml) – blank gas production in this period (ml).

Based on the gas production model proposed by France et al. (2000) and the nonlinear regression program in SPSS v21.0 (SPSS Inc., Chicago, IL, USA) statistical software was used to estimate the gas production parameters (theoretical maximum gas production and gas production rate).

After 48 h of fermentation, the syringe was placed in an ice bath to terminate fermentation, and 500 µl of headspace gas was collected using a gas-tight injection needle from the gas-tight rubber pipe. Methane (CH_4) content was determined by using a gas chromatograph (GC-1120; Sunny Hengping Instrument, Shanghai, P.R. China) referring to the method of Luan et al. (2023).

After 48 h of fermentation, the rumen inoculum in the syringe was quickly discharged. The pH value of the rumen inoculum was measured by a rapid pH analyser (ST3100; Ohaus, NJ, USA). Volatile fatty acids (VFA) were determined by a gas chromatograph (GC-1120; Sunny Hengping Instrument, Shanghai, P.R. China). The rumen inoculum sample of 1 ml in volume was mixed with 0.2 ml of 25% (w/v) metaphosphoric acid solution containing 2-ethylbutyrate and centrifuged at 10 000 rpm/min for 15 min for VFA analysis. Ammonia nitrogen (NH_3-N) content in the rumen inoculum was determined by a spectrophotometer (721; Yoke Instrument, Shanghai, P.R. China) according to the method of Luan et al. (2023), and lactic acid content in the rumen inoculum was determined by a UV-visible spectrophotometer (UV759CRT; Yoke Instrument, Shanghai, P.R. China) according to the method of Luan et al. (2023).

After 48 h of fermentation, the *in vitro* digestibility culture tube was put into an ice water bath to terminate fermentation. The fibre filter bag (42 mm × 53 mm, pore size: 38–45 µm; First Beef Cattle Info & Tech Research Center, Beijing, P.R. China) was washed with distilled water and placed in the oven. Dry matter (DM) weight was obtained by drying at 105 °C for 12–24 h, which was used to calculate

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the *in vitro* digestibility of dry matter (IVDMD). The CP content was determined by an automatic Kjeltec nitrogen analyser (Kjeltec 8400; Foss, Denmark); neutral detergent fibre (NDF) and acid detergent fibre (ADF) content was determined according to the method of Luan et al. (2023). *In vitro* digestibility of DM, CP, NDF, and ADF were calculated according to Luan et al. (2023).

The FG in the sample was extracted with C_3H_8O-C_6H_{14} mixed solvent containing C_3H_9NO, and the gossypol was converted into aniline gossypol by aniline. The absorbance value was measured at 440 nm.

Exp. 2 Evaluation of the effect of fermentation of mixed cottonseed meal by different strains. The experiment was divided into ten treatment groups: CON, *Saccharomyces* No. 1 (M1), *Saccharomyces Fubon* (M2), *Saccharomyces Lallemand* (M3), *Lactobacillus* (M4), *Bacillus licheniformis* (M5), *Bacillus subtilis* 10071 (M6), *Bacillus subtilis* 10089 (M7), *Aspergillus niger* (M8), and *Monascus purpureus* Went (M9). Each treatment group had 3 replicates. The fermentation substrate was a mixture of cottonseed meal, corn meal, and bran (7 : 2 : 1). The mixed substrate was used to simulate practical feed formulations in production, making the test results more consistent with actual feeding scenarios of ruminants and improving the practical application value of this study. The experimental design and indices measured were the same as those in Exp. 1.

Data analysis

Data of this study were analysed by one-way analysis of variance using SPSS v21.0 (SPSS Inc., Chicago, IL, USA), and the Tukey–Kramer test was used to conduct multiple comparisons. $P < 0.05$ means a significant difference, while $P > 0.05$ means no significant difference.

RESULTS

Experiment 1

Gas production and gas production parameters. The effects of fermentation of single cottonseed meal by different strains on *in vitro* fermentation gas production are shown in Table 3.

Table 3. Effects of fermentation of single cottonseed meal by different strains on *in vitro* fermentation gas production (ml)

Items	Groups										SEM	*P*-value
	CON	T1	T2	T3	T4	T5	T6	T7	T8	T9		
2 h	8.12^{f}	9.35^{d}	9.75^{c}	9.15^{e}	9.75^{c}	9.80^{b}	9.85^{b}	9.85^{b}	9.90^{a}	9.95^{a}	0.102	0.001
4 h	18.2^{f}	18.6^{e}	18.9^{d}	18.6^{e}	21.6^{b}	19.0^{d}	19.8^{c}	22.4^{a}	19.0^{d}	19.8^{c}	0.299	0.001
6 h	24.7^{g}	25.5^{d}	25.5^{d}	24.9^{f}	28.5^{a}	25.1^{e}	26.3^{c}	28.5^{a}	25.5^{d}	26.5^{b}	0.302	0.001
8 h	28.3^{g}	29.2^{f}	29.2^{f}	29.6^{d}	30.5^{a}	29.3^{e}	30.2^{b}	29.8^{c}	29.7^{d}	30.2^{b}	0.135	0.001
10 h	31.6^{f}	32.5^{e}	32.6^{c}	32.5^{d}	32.8^{c}	32.7^{c}	33.2^{b}	33.4^{a}	32.8^{c}	33.3^{a}	1.50	0.001
12 h	34.6^{g}	35.2^{e}	35.7^{b}	35.1^{e}	35.8^{a}	35.1^{e}	34.8^{f}	35.5^{c}	35.2^{e}	35.4^{d}	0.082	0.001
16 h	37.8^{e}	39.4^{c}	39.0^{d}	38.9^{d}	39.4^{c}	39.0^{d}	39.3^{c}	39.6^{b}	39.7^{b}	40.0^{a}	0.128	0.001
20 h	40.8^{h}	43.1^{f}	43.8^{bc}	43.4^{d}	43.2^{e}	43.4^{d}	43.8^{b}	43.6^{c}	42.8^{g}	44.0^{a}	0.203	0.001
24 h	42.8^{e}	45.3^{c}	45.5^{b}	45.1^{d}	45.3^{c}	45.6^{b}	45.8^{a}	45.2^{c}	45.5^{b}	45.3^{c}	0.185	0.001
30 h	45.8^{a}	47.3^{b}	47.2^{b}	47.0^{b}	47.1^{b}	47.8^{b}	47.2^{b}	47.4^{b}	46.9^{b}	47.5^{b}	0.490	0.001
36 h	47.4^{g}	49.4^{d}	49.0^{f}	49.5^{d}	49.9^{b}	49.7^{c}	49.4^{d}	50.2^{a}	49.6^{c}	49.2^{e}	0.168	0.001
48 h	49.3^{g}	55.3^{a}	51.6^{f}	52.0^{c}	51.9^{d}	52.1^{c}	51.6^{e}	51.8^{d}	51.8^{d}	52.6^{b}	0.314	0.001

$^{a–h}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

CON = the control group; SEM = standard error of the means; T1 = the *Saccharomyces* No. 1 fermentation single cottonseed meal group; T2 = the *Saccharomyces Fubon* fermentation single cottonseed meal group; T3 = the *Saccharomyces Lallemand* fermentation single cottonseed meal group; T4 = the *Lactobacillus* fermentation single cottonseed meal group; T5 = the *Bacillus licheniformis* fermentation single cottonseed meal group; T6 = the *Bacillus subtilis* 10071 fermentation single cottonseed meal group; T7 = the *Bacillus subtilis* 10089 fermentation single cottonseed meal group; T8 = the *Aspergillus niger* fermentation single cottonseed meal group; T9 = the *Monascus purpureus* Went fermentation single cottonseed meal group

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The gas production of the single cottonseed meal substrate group after fermentation by different strains was significantly higher than that of the CON group ($P < 0.05$). The 48-h gas production of T1 was significantly higher than that of other groups ($P < 0.05$).

The effects of fermentation of single cottonseed meal by different strains on *in vitro* fermentation gas production parameters are shown in Table 4. The theoretical maximum gas production of each treatment group was significantly higher compared to the CON group ($P < 0.05$). In contrast, the gas production rate of the treatment groups did however not change significantly compared to the CON group ($P > 0.05$).

FG and CH_4 content. The effects of fermentation of single cottonseed meal substrate by different strains on CH_4 and FG are shown in Table 5. Compared with the CON group, the rumen fermentation of fermented single cottonseed meal substrate significantly reduced the content of FG ($P < 0.05$), when the FG content showed this order: T1 < T5 < T3 < T2 < T8 < T9 < T4 < T7 < T6. Meanwhile, the content of CH_4 also decreased to a different extent ($P < 0.05$), when the order of the CH_4 content was T1 < T9 < T3 < T4 < T8 < T7 < T2 < T6 < T5.

In vitro rumen fermentation parameters. The effects of fermentation of single cottonseed meal substrate by different strains on *in vitro* fermentation parameters are shown in Table 6. Compared

Table 4. Effects of fermentation of single cottonseed meal by different strains on *in vitro* fermentation gas production parameters

Items	Groups										SEM	*P*-value
	CON	T1	T2	T3	T4	T5	T6	T7	T8	T9		
Theoretical maximum gas production (ml)	47.7^{e}	49.8^{c}	49.7^{c}	49.8^{c}	49.8^{b}	49.3^{d}	49.3^{d}	49.4^{d}	49.9^{b}	50.0^{a}	0.302	0.001
Gas production rate (ml/h)	0.110	0.110	0.110	0.110	0.110	0.110	0.110	0.110	0.110	0.110	0.002	0.730

$^{a–e}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

CON = the control group; SEM = standard error of the means; T1 = the *Saccharomyces* No. 1 fermentation single cottonseed meal group; T2 = the *Saccharomyces Fubon* fermentation single cottonseed meal group; T3 = the *Saccharomyces Lallemand* fermentation single cottonseed meal group; T4 = the *Lactobacillus* fermentation single cottonseed meal group; T5 = the *Bacillus licheniformis* fermentation single cottonseed meal group; T6 = the *Bacillus subtilis* 10071 fermentation single cottonseed meal group; T7 = the *Bacillus subtilis* 10089 fermentation single cottonseed meal group; T8 = the *Aspergillus niger* fermentation single cottonseed meal group; T9 = the *Monascus purpureus* Went fermentation single cottonseed meal group

Table 5. Effects of fermentation of single cottonseed meal substrates by different strains on methane and free gossypol content

Items	Groups										SEM	*P*-value
	CON	T1	T2	T3	T4	T5	T6	T7	T8	T9		
FG (mg/kg)	215^{a}	105^{i}	135^{f}	134^{g}	139^{d}	132^{h}	173^{b}	167^{c}	136^{e}	138^{d}	6.60	0.01
CH_4 (%)	21.1^{a}	16.1^{i}	17.3^{d}	16.9^{g}	17.0^{f}	18.0^{b}	17.8^{c}	17.1^{e}	17.1^{e}	16.6^{h}	0.417	0.001

$^{a–i}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

CH_4 = methane; CON = the control group; FG = free gossypol; SEM = standard error of the means; T1 = the *Saccharomyces* No. 1 fermentation single cottonseed meal group; T2 = the *Saccharomyces Fubon* fermentation single cottonseed meal group; T3 = the *Saccharomyces Lallemand* fermentation single cottonseed meal group; T4 = the *Lactobacillus* fermentation single cottonseed meal group; T5 = the *Bacillus licheniformis* fermentation single cottonseed meal group; T6 = the *Bacillus subtilis* 10071 fermentation single cottonseed meal group; T7 = the *Bacillus subtilis* 10089 fermentation single cottonseed meal group; T8 = the *Aspergillus niger* fermentation single cottonseed meal group; T9 = the *Monascus purpureus* Went fermentation single cottonseed meal group

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Table 6. Effects of fermentation of single cottonseed meal substrates by different strains on *in vitro* fermentation parameters

Items	Groups										SEM	*P*-value
	CON	T1	T2	T3	T4	T5	T6	T7	T8	T9		
pH	6.66^{b}	6.61^{bc}	6.73^{a}	6.66^{b}	6.65^{b}	6.73^{a}	6.74^{a}	6.70^{a}	6.59^{c}	6.53^{c}	0.156	0.001
NH_3-N (mg/dl)	31.0	31.6	31.8	31.9	32.1	31.7	31.7	31.6	31.7	31.8	1.00	0.489
Lactic acid (mg/dl)	0.750^{bc}	0.720^{c}	0.750^{bc}	0.730^{c}	1.01^{a}	0.730^{c}	0.760^{c}	0.790^{b}	0.780^{b}	0.750^{b}	0.018	0.001
TVFA (mmol/l)	51.5^{g}	55.3^{e}	55.5^{f}	56.3^{d}	57.4^{a}	56.2^{e}	56.6^{c}	56.4^{d}	56.3^{d}	57.3^{b}	0.368	0.001
Acetic acid (%)	34.9^{a}	32.5^{f}	32.8^{e}	33.6^{d}	33.8^{c}	33.5^{a}	33.7^{d}	33.5^{d}	33.5^{d}	33.9^{b}	0.142	0.001
Propionic acid (%)	7.05^{b}	11.5^{d}	11.4^{e}	11.4^{d}	12.4^{a}	11.4^{e}	11.8^{b}	11.7^{c}	11.5^{d}	11.8^{b}	0.322	0.001
Isobutyric acid (%)	0.830^{d}	1.64^{a}	1.58^{b}	1.43^{c}	1.58^{b}	1.62^{a}	1.43^{c}	1.49^{c}	1.48^{c}	1.58^{b}	0.051	0.001
Butyric acid (%)	5.05^{d}	5.56^{b}	5.56^{b}	5.74^{a}	5.43^{c}	5.70^{a}	5.57^{b}	5.57^{b}	5.61^{b}	5.74^{a}	0.044	0.001
Isovaleric acid (%)	1.96^{c}	2.23^{b}	2.22^{b}	2.23^{b}	2.30^{a}	2.24^{b}	2.25^{b}	2.31^{a}	2.34^{a}	2.31^{a}	0.023	0.001
Valeric acid (%)	1.63^{d}	1.91^{b}	1.90^{b}	1.91^{b}	1.96^{a}	1.83^{c}	1.93^{ab}	1.87^{c}	1.91^{b}	1.92^{b}	0.021	0.001
A/P	4.95^{a}	2.83^{c}	2.89^{c}	2.94^{b}	2.73^{d}	2.95^{b}	2.86^{c}	2.86^{c}	2.92^{bc}	2.82^{c}	0.144	0.001

$^{a–g}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

A/P = the ratio of acetate to propionate; CON = the control group; NH_3-N = ammonia nitrogen; SEM = standard error of the means; T1 = the *Saccharomyces* No. 1 fermentation single cottonseed meal group; T2 = the *Saccharomyces Fubon* fermentation single cottonseed meal group; T3 = the *Saccharomyces Lallemand* fermentation single cottonseed meal group; T4 = the *Lactobacillus* fermentation single cottonseed meal group; T5 = the *Bacillus licheniformis* fermentation single cottonseed meal group; T6 = the *Bacillus subtilis* 10071 fermentation single cottonseed meal group; T7 = the *Bacillus subtilis* 10089 fermentation single cottonseed meal group; T8 = the *Aspergillus niger* fermentation single cottonseed meal group; T9 = the *Monascus purpureus* Went fermentation single cottonseed meal group; TVFA = total volatile fatty acids

with the CON group, the pH value of fermented single cottonseed meal substrate for rumen fermentation varied, when T2, T5, T6, and T7 could significantly increase the pH value ($P < 0.05$), and T8 and T9 could significantly decrease the pH value ($P < 0.05$), but T1, T3, and T4 did not have a significant effect on the pH value ($P > 0.05$). Compared with the CON group, different fermentation strains had different effects on lactic acid, when the T4 group could significantly increase lactic acid content ($P < 0.05$), while T1, T2, T3, T5, T6, T7, T8, and T9 did not have a significant effect on lactic acid ($P > 0.05$). The total volatile fatty acids (TVFA), propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid in each treatment group were significantly higher than those in the CON group ($P < 0.05$), while the ratio of acetate to propionate (A/P) was significantly lower than that in the CON group ($P < 0.05$). However, the effect on NH_3-N was not significant ($P > 0.05$).

In vitro digestibility of nutrients. The effects of fermentation of single cottonseed meal substrate by different strains on *in vitro* digestibility are shown in Table 7. Compared with the CON group, *in vitro* CP digestibility (IVCPD), *in vitro* NDF digestibility (IVNDFD), and *in vitro* ADF digestibility (IVADFD) were significantly increased in all treatment groups ($P < 0.05$), all treatment groups could significantly reduce the IVDMD ($P < 0.05$).

Experiment 2

Gas production and gas production parameters. The effects of fermentation of mixed cottonseed meal substrates by different strains on *in vitro* fermentation gas production are shown in Table 8. Compared with the CON group, the fermented mixed cottonseed meal substrate group was subjected to rumen fermentation, when all treatment groups could significantly increase the gas production within 48 h ($P < 0.05$).

The effects of fermentation of mixed cottonseed meal substrates by different strains on *in vitro* fermentation gas production parameters are shown in Table 9. Compared with the CON group, the *in vitro* fermentation of mixed cottonseed meal substrate has no significant effect on the gas production rate after fermentation ($P > 0.05$), but it can significantly increase the theoretical maximum gas

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Table 7. Effects of fermentation of single cottonseed meal substrates by different strains on *in vitro* digestibility

Items	Groups										SEM	*P*-value
	CON	T1	T2	T3	T4	T5	T6	T7	T8	T9		
IVDMD (%)	46.0^{a}	45.4^{c}	42.5^{i}	44.2^{f}	45.5^{b}	43.8^{g}	44.7^{d}	44.2^{e}	43.4^{h}	44.9^{d}	0.227	0.001
IVNDFD (%)	29.4^{g}	35.5^{a}	35.1^{c}	35.3^{a}	34.4^{e}	35.3^{b}	35.0^{d}	34.2^{e}	35.1^{cd}	33.3^{f}	0.195	0.001
IVADFD (%)	59.9^{g}	72.0^{d}	70.5^{e}	70.5^{e}	69.0^{f}	69.5^{f}	75.5^{b}	74.0^{c}	75.6^{a}	71.0^{e}	0.972	0.001
IVCPD (%)	69.1^{g}	79.7^{a}	75.9^{f}	79.1^{b}	77.6^{c}	77.6^{c}	76.4^{e}	77.0^{d}	79.0^{b}	77.5^{c}	0.655	0.001

$^{a–i}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

CON = the control group; IVADFD = *in vitro* acid detergent fibre digestibility; IVCPD = *in vitro* crude protein digestibility; IVDMD = *in vitro* dry matter digestibility; IVNDFD = *in vitro* neutral detergent fibre digestibility; SEM = standard error of the means; T1 = the *Saccharomyces* No. 1 fermentation single cottonseed meal group; T2 = the *Saccharomyces Fubon* fermentation single cottonseed meal group; T3 = the *Saccharomyces Lallemand* fermentation single cottonseed meal group; T4 = the *Lactobacillus* fermentation single cottonseed meal group; T5 = the *Bacillus licheniformis* fermentation single cottonseed meal group; T6 = the *Bacillus subtilis* 10071 fermentation single cottonseed meal group; T7 = the *Bacillus subtilis* 10089 fermentation single cottonseed meal group; T8 = the *Aspergillus niger* fermentation single cottonseed meal group; T9 = the *Monascus purpureus* Went fermentation single cottonseed meal group

Table 8. Effects of fermentation of mixed cottonseed meal substrates by different strains on gas production during *in vitro* fermentation (ml)

Items	Groups										SEM	*P*-value
	CON	M1	M2	M3	M4	M5	M6	M7	M8	M9		
2 h	9.20^{e}	9.45^{d}	9.40^{d}	9.75^{b}	9.80^{ab}	9.60^{c}	9.75^{b}	9.55^{cd}	9.90^{a}	9.65^{bc}	0.046	0.001
4 h	19.0^{f}	19.4^{e}	19.7^{c}	19.3^{e}	20.1^{a}	19.3^{e}	19.6^{d}	19.8^{b}	19.7^{c}	19.7^{c}	0.068	0.001
6 h	24.3^{e}	24.8^{c}	24.7^{cd}	24.6^{d}	24.7^{c}	24.9^{a}	24.8^{c}	25.0^{a}	24.9^{a}	24.8^{c}	0.045	0.001
8 h	29.2^{f}	30.3^{d}	30.4^{d}	30.1^{b}	33.5^{a}	30.0^{e}	30.1^{e}	30.5^{c}	30.7^{b}	30.1^{e}	0.088	0.001
10 h	32.2^{f}	33.0^{e}	33.4^{c}	33.4^{c}	40.0^{a}	33.3^{d}	33.7^{b}	33.7^{b}	33.3^{d}	33.4^{d}	0.476	0.001
12 h	38.0^{f}	39.3^{c}	39.1^{d}	39.3^{c}	42.4^{a}	39.3^{c}	39.8^{b}	39.7^{b}	36.3^{e}	39.8^{b}	0.334	0.001
16 h	40.5^{f}	42.0^{b}	42.0^{b}	41.6^{c}	47.5^{a}	41.0^{e}	41.0^{e}	40.9^{e}	41.6^{c}	41.3^{d}	0.440	0.001
20 h	43.5^{h}	44.3^{g}	44.5^{f}	44.9^{d}	50.3^{a}	45.2^{c}	45.3^{b}	44.7^{e}	44.9^{d}	45.0^{d}	0.400	0.001
24 h	45.5^{f}	46.6^{e}	46.8^{d}	46.6^{e}	52.5^{a}	47.0^{c}	47.3^{b}	46.8^{d}	46.8^{d}	46.8^{d}	0.414	0.001
30 h	50.1^{d}	50.8^{b}	50.8^{b}	51.3^{b}	55.0^{a}	50.4^{c}	50.5^{c}	50.8^{b}	50.8^{b}	50.8^{b}	0.305	0.001
36 h	52.2^{a}	53.5^{d}	53.8^{c}	53.2^{f}	57.0^{a}	53.3^{e}	53.8^{c}	54.3^{b}	53.4^{d}	53.1^{f}	0.274	0.001
48 h	56.6^{g}	58.1^{c}	58.0^{d}	58.0^{d}	59.5^{a}	57.0^{f}	57.1^{e}	58.0^{c}	59.4^{b}	59.4^{b}	0.225	0.001

$^{a–f}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

CON = the control group; SEM = standard error of the means; M1 = the *Saccharomyces* No. 1 fermentation mixed cottonseed meal group; M2 = the *Saccharomyces Fubon* fermentation mixed cottonseed meal group; M3 = the *Saccharomyces Lallemand* fermentation mixed cottonseed meal group; M4 = the *Lactobacillus* fermentation mixed cottonseed meal group; M5 = the *Bacillus licheniformis* fermentation mixed cottonseed meal group; M6 = the *Bacillus subtilis* 10071 fermentation mixed cottonseed meal group; M7 = the *Bacillus subtilis* 10089 fermentation mixed cottonseed meal group; M8 = the *Aspergillus niger* fermentation mixed cottonseed meal group; M9 = the *Monascus purpureus* Went fermentation mixed cottonseed meal group

production ($P < 0.05$), when the T8 group had the most significant effect on the increase of theoretical maximum gas production.

FG and CH_4 content. The effects of fermentation of mixed cottonseed meal substrates by different strains on CH_4 and FG are shown in Table 10. The

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Table 9. Effects of fermentation of mixed cottonseed meal substrates by different strains on gas production parameters during *in vitro* fermentation

Items	Groups										SEM	*P*-value
	CON	M1	M2	M3	M4	M5	M6	M7	M8	M9		
Theoretical maximum gas production (ml)	53.9^{h}	55.6^{b}	55.1^{f}	55.1^{f}	55.3^{d}	55.0^{f}	55.2^{e}	54.8^{g}	55.8^{a}	55.4^{c}	0.112	0.001
Gas production rate (ml/h)	0.090	0.110	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.002	0.462

$^{a–h}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

CON = the control group; SEM = standard error of the means; M1 = the *Saccharomyces* No. 1 fermentation mixed cottonseed meal group; M2 = the *Saccharomyces Fubon* fermentation mixed cottonseed meal group; M3 = the *Saccharomyces Lallemand* fermentation mixed cottonseed meal group; M4 = the *Lactobacillus* fermentation mixed cottonseed meal group; M5 = the *Bacillus licheniformis* fermentation mixed cottonseed meal group; M6 = the *Bacillus subtilis* 10071 fermentation mixed cottonseed meal group; M7 = the *Bacillus subtilis* 10089 fermentation mixed cottonseed meal group; M8 = the *Aspergillus niger* fermentation mixed cottonseed meal group; M9 = the *Monascus purpureus* Went fermentation mixed cottonseed meal group

Table 10. Effects of fermentation of mixed cottonseed meal substrates by different strains on methane and free gossypol content

Items	Groups										SEM	*P*-value
	CON	M1	M2	M3	M4	M5	M6	M7	M8	M9		
FG (mg/ kg)	181^{a}	84.5^{j}	91.3^{i}	97.0^{h}	106^{e}	112^{d}	120^{b}	115^{c}	98.5^{g}	102^{f}	5.90	0.001
CH_4 (%)	19.3^{a}	16.3^{f}	17.3^{c}	16.9^{d}	17.9^{b}	17.7^{b}	16.9^{d}	16.7^{e}	16.6^{e}	16.4^{f}	0.199	0.001

$^{a–j}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

CH_4 = methane; CON = the control group; FG = free gossypol; SEM = standard error of the means; M1 = the *Saccharomyces* No. 1 fermentation mixed cottonseed meal group; M2 = the *Saccharomyces Fubon* fermentation mixed cottonseed meal group; M3 = the *Saccharomyces Lallemand* fermentation mixed cottonseed meal group; M4 = the *Lactobacillus* fermentation mixed cottonseed meal group; M5 = the *Bacillus licheniformis* fermentation mixed cottonseed meal group; M6 = the *Bacillus subtilis* 10071 fermentation mixed cottonseed meal group; M7 = the *Bacillus subtilis* 10089 fermentation mixed cottonseed meal group; M8 = the *Aspergillus niger* fermentation mixed cottonseed meal group; M9 = the *Monascus purpureus* Went fermentation mixed cottonseed meal group

FG in each treatment group was significantly lower than that in the CON group ($P < 0.05$), when M1 < M2 < M3 < M8 < M9 < M4 < M5 < M7 < M6, which showed that the degradation of free cotton phenol by *Saccharomyces* No. 1 in group M1 was the most significant. The CH_4 was also significantly lower after fermentation than in the CON group ($P < 0.05$), when M1 < M9 < M8 < M7 < M6 = M3 < M2 < M5 < M4, which showed that the degradation of CH_4 by *Saccharomyces* No. 1 was the most effective.

In vitro rumen fermentation parameters. The effects of fermentation of mixed cottonseed meal substrates by different strains on in vitro fermentation parameters are shown in Table 11. Compared with the CON group, after the rumen fermentation of mixed cottonseed meal substrates, the pH value, NH_3-N, and valeric acid did not show any significant effects ($P > 0.05$), but the fermentation could significantly increase the content of TVFA, acetic acid, and propionic acid ($P < 0.05$), and could significantly decrease the A/P ratio in all treatment groups except M8 ($P < 0.05$).

In vitro digestibility of nutrients. The effects of fermentation of mixed cottonseed meal substrate by different strains on *in vitro* digestibility are shown in Table 12. Compared with the CON group, the fermented mixed cottonseed meal substrate group showed significantly increased IVCPD, IVNDFD, and IVADFD ($P < 0.05$). However, there was no significant effect on IVDMD ($P > 0.05$).

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Table 11. Effects of fermentation of mixed cottonseed meal substrates by different strains on *in vitro* fermentation parameters

Items	Groups										SEM	*P*-value
	CON	M1	M2	M3	M4	M5	M6	M7	M8	M9		
pH	6.71	6.69	6.68	6.69	6.67	6.71	6.67	6.69	6.72	6.71	0.004	0.068
NH_3-N (mg/dl)	33.1	33.4	33.6	33.6	33.6	33.3	33.2	33.6	33.6	33.3	0.961	0.205
Lactic acid (mg/dl)	0.760^{b}	0.750^{b}	0.750^{b}	0.750^{a}	0.830^{a}	0.810^{a}	0.770^{b}	0.810^{a}	0.730^{b}	0.760^{b}	0.808	0.014
TVFA (mmol/l)	55.7^{i}	62.1^{c}	61.0^{f}	60.9^{g}	61.8^{d}	60.2^{h}	63.8^{b}	61.3^{e}	65.7^{a}	61.8^{d}	0.558	0.001
Acetic acid (%)	33.2^{i}	35.8^{g}	36.7^{e}	36.4^{f}	37.5^{c}	35.6^{h}	38.5^{b}	37.2^{d}	40.2^{a}	37.5^{c}	0.401	0.001
Propionic acid (%)	10.3^{j}	12.6^{b}	12.7^{a}	12.6^{b}	12.4^{c}	12.5^{c}	12.1^{e}	12.6^{b}	12.2^{d}	11.9^{f}	0.151	0.001
Isobutyric acid (%)	1.06^{f}	1.01^{g}	1.00^{g}	1.11^{e}	1.17^{d}	1.44^{b}	1.59^{a}	1.25^{c}	1.15^{df}	1.21^{c}	0.040	0.006
Butyric acid (%)	6.52^{ef}	7.06^{b}	6.42^{f}	6.73^{d}	6.60^{e}	6.25^{g}	7.08^{b}	6.47^{f}	7.26^{a}	6.91^{c}	0.076	0.001
Isovaleric acid (%)	2.90^{b}	2.88^{b}	2.52^{d}	2.58^{d}	2.55^{d}	2.69^{c}	2.83^{b}	2.85^{b}	3.06^{a}	2.44^{e}	0.044	0.001
Valeric acid (%)	1.67	1.69	1.70	1.64	1.65	1.69	1.65	1.70	1.67	1.65	0.006	0.076
A/P	3.22^{a}	2.86^{b}	2.89^{d}	2.90^{d}	3.03^{c}	2.86^{d}	3.19^{b}	3.00^{c}	3.29^{a}	3.16^{b}	0.036	0.001

$^{a–j}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

A/P = the ratio of acetate to propionate; CON = the control group; NH_3-N = ammonia nitrogen; SEM = standard error of the means; M1 = the *Saccharomyces* No. 1 fermentation mixed cottonseed meal group; M2 = the *Saccharomyces Fubon* fermentation mixed cottonseed meal group; M3 = the *Saccharomyces Lallemand* fermentation mixed cottonseed meal group; M4 = the *Lactobacillus* fermentation mixed cottonseed meal group; M5 = the *Bacillus licheniformis* fermentation mixed cottonseed meal group; M6 = the *Bacillus subtilis* 10071 fermentation mixed cottonseed meal group; M7 = the *Bacillus subtilis* 10089 fermentation mixed cottonseed meal group; M8 = the *Aspergillus niger* fermentation mixed cottonseed meal group; M9 = the *Monascus purpureus* Went fermentation mixed cottonseed meal group; TVFA = total volatile fatty acids

Table 12. Effects of fermentation of mixed cottonseed meal substrates by different strains on *in vitro* digestibility

Items	Groups										SEM	*P*-value
	CON	M1	M2	M3	M4	M5	M6	M7	M8	M9		
IVDMD (%)	44.3	44.4	43.0	44.2	45.0	43.8	44.1	44.2	43.4	44.2	0.685	0.053
IVNDFD (%)	29.4^{g}	34.5^{a}	33.5^{b}	33.0^{c}	32.5^{f}	32.5^{f}	33.5^{b}	32.7^{e}	33.6^{b}	32.8^{d}	0.294	0.001
IVADFD (%)	66.6^{i}	76.0^{a}	75.3^{b}	73.4^{e}	72.9^{f}	74.3^{c}	72.1^{h}	73.3^{e}	74.1^{d}	72.7^{d}	0.559	0.001
IVCPD (%)	70.4^{f}	81.7^{b}	81.5^{c}	81.4^{c}	82.4^{a}	80.5^{e}	81.6^{b}	80.8^{d}	80.9^{d}	81.5^{c}	0.766	0.001

$^{a–i}$Means bearing different superscripts in the same row differ significantly (*P*-value < 0.05)

CON = the control group; IVADFD = *in vitro* acid detergent fibre digestibility; IVCPD = *in vitro* crude protein digestibility; IVDMD = *in vitro* dry matter digestibility; IVNDFD = *in vitro* neutral detergent fibre digestibility; SEM = standard error of the means; M1 = the *Saccharomyces* No. 1 fermentation mixed cottonseed meal group; M2 = the *Saccharomyces Fubon* fermentation mixed cottonseed meal group; M3 = the *Saccharomyces Lallemand* fermentation mixed cottonseed meal group; M4 = the *Lactobacillus* fermentation mixed cottonseed meal group; M5 = the *Bacillus licheniformis* fermentation mixed cottonseed meal group; M6 = the *Bacillus subtilis* 10071 fermentation mixed cottonseed meal group; M7 = the *Bacillus subtilis* 10089 fermentation mixed cottonseed meal group; M8 = the *Aspergillus niger* fermentation mixed cottonseed meal group; M9 = the *Monascus purpureus* Went fermentation mixed cottonseed meal group

DISCUSSION

Gas production is a reflection of the extent to which the fermentation substrate is utilised by rumen microorganisms and it can also be used to assess the effectiveness of rumen fermentation (He et al. 2024). *In vitro* fermentation of both single cottonseed meal and mixed cottonseed meal significantly increased gas production within 48 hours. The highest gas production in the single cottonseed

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meal group was due to *Saccharomyces* No. 1, and in the mixed cottonseed meal group it was caused by *Lactobacillus*. The reason for the increase in gas production may be the FG inhibition of the rumen fermentation effect (Wang et al. 2022), whereas the FG in the fermented cottonseed meal decreased in this experiment, which may be the reason for the significant increase in gas production in this experiment.

CH_4 is the second largest source of greenhouse gas emissions after carbon dioxide (CO_2), and its global warming potential (GWP) is about 28 times that of CO_2 (Zhao et al. 2020), so reducing methane emissions is an effective way to alleviate global warming. It has been shown that the addition of dry yeast to feeds has the effect of significantly reducing CH_4 (Phesatcha et al. 2021). This experiment showed that *Saccharomyces* No. 1 has the capacity to reduce CH_4, and the rest of the strains can also significantly reduce CH_4. In both Exps., *Saccharomyces* No. 1 was the most significant factor for the decrease of CH_4.

FG in cottonseed meal is toxic and may cause a decrease in growth performance, reproductive capability, liver, and immune dysfunction, when the reproductive failure can cause serious economic losses in the livestock industry (Gadelha et al. 2014). The toxicity of FG is mainly due to its active hydroxyl and aldehyde groups, which cause a variety of hazards (Wang et al. 2021). Both the fermented single cottonseed meal group and the mixed cottonseed meal group could significantly reduce FG in this experiment, and their detoxification effect was the best in the *Saccharomyces* No. 1 group. The significant decrease in FG in cottonseed meal after microbial fermentation may be due to the fact that microorganisms secrete proteins that bind to free cotton phenol to form bound cotton phenol or that microorganisms are able to secrete enzymes that degrade FG (Yusuf et al. 2022), making it less toxic.

The rumen pH value can comprehensively reflect the rumen microbial growth, metabolic balance, and fermentation degree (Deng et al. 2021). Normal rumen pH ranges from 5.5 to 7.0 (Yu et al. 2020) and the pH values of both experimental groups were within this range. This indicates that cottonseed meal fermented by different strains does not adversely affect the rumen environment.

TVFA are the main source of energy for growth and development in ruminants, and 70-80% of digestible energy in animals is provided by rumen TVFA (Yuan and Wan 2019). TVFA are the main metabolites of carbohydrate fermentation by rumen microorganisms, and TVFA reflect the degree of microbial substrate fermentation and represent the rumen fermentation activity (Liu et al. 2024b). The content of TVFA was significantly higher in both single and mixed cottonseed meal groups. Moreover, there was no effect on rumen pH, which suggests that the fermented cottonseed meal has a more desirable effect, as higher levels of VFA reduce rumen pH, which negatively affects rumen fermentation (Li et al. 2022b).

Propionate is the primary precursor for glucose synthesis in dairy cows and contributes as much as 60% to 74% of the carbon for gluconeogenesis (Zhang et al. 2015). The content of propionic acid was significantly higher and the A/P ratio was significantly lower in both Exp. 1 and Exp. 2, indicating that the conversion of rumen fermentation to propionic acid-based fermentation (Luan et al. 2023) improves feed efficiency and energy conversion (Zhang et al. 2022b).

Lactic acid is produced in the rumen and it is an intermediate product of carbohydrate fermentation, which accumulates after the intake of excessive amounts of cereals or other carbohydrate-rich feeds; the excessive lactic acid accumulation can lead to rumen acidosis, which can negatively affect animal health and productivity (Chen et al. 2019). Therefore, an effective decrease in lactic acid can reduce the chances of rumen acidosis. The lowest lactic acid content in the single cottonseed meal group was found in the *Saccharomyces* No. 1 group, which is similar to the findings of Liu et al. (2024b). The decrease in lactic acid in this experiment may be due to the fact that *Saccharomyces* can compete with other carbohydrate-utilising bacteria for its fermentation, thus preventing lactic acid from accumulating in the rumen (Patra 2012).

IVNDFD and IVADFD reflect the utilisation of NDF and ADF in feed. In this trial IVNDFD and IVADFD were significantly increased in both groups. The most significant increase in IVNDFD in both single and mixed cottonseed meal groups was caused by *Saccharomyces* No. 1, which is in agreement with the findings of Marlida et al. (2023); this may be attributed to the capacity of *Saccharomyces* to increase the number of total and cellulolytic bacteria in the rumen, which in turn increased the fibre digestibility (Ruiz et al. 2016). IVCPD was signifi-

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cantly higher in both single and mixed cottonseed meal groups, with the most significant increase in *Saccharomyces* No. 1 in the single cottonseed meal group and *Lactobacillus* in the mixed cottonseed meal group.

This is similar to the findings of Yusuf et al. (2022), who showed that the fermentation of mixed diets containing cottonseed meal or rapeseed meal using *Lactobacillus* and *Saccharomyces* reduced the FG of the diets and increased the CP content of the diets. This may be because the added strains can secrete a variety of enzymes, which can disrupt the structure of the cell wall, allowing for the rapid release of nutrients, and providing the rumen microorganisms with more substrate for fermentation, thus increasing the IVCPD (Sheperd and Kung 1996).

CONCLUSIONS

Under the conditions of this experiment, different groups of fermented cottonseed meal for rumen fermentation could significantly reduce the FG, with the most obvious effect of *Saccharomyces* No. 1. *In vitro* rumen fermentation of both single and mixed cottonseed meal substrates significantly reduced CH_4 and A/P, significantly increased IVCPD, TVFA, and total gas production, and *Saccharomyces* No. 1 had the most significant effect. Accordingly, *Saccharomyces* No. 1 was identified as the most suitable strain for cottonseed meal fermentation, as it effectively decreased free gossypol and enhanced the nutritional value of cottonseed meal.

Conflict of interest

The authors declare no conflict of interest.

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