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Enhancing the Quality of Total Mixed Ration Containing Cottonseed or Rapeseed Meal by Optimization of Fermentation Conditions

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Abstract: Cottonseed meal (CSM) and rapeseed meal (RSM) are protein sources in livestock feed. However, the applications of both ingredients are limited in diets due to the existence of anti-nutritional factors such as free gossypol and glucosinolate. The aim of this study was to determine the optimal fermentation conditions for reducing anti-nutritional factors and increasing the nutritional value of fermented total mixed rations containing cottonseed or rapeseed meal. An orthogonal design L9 (3⁴) was performed to optimize the fermentation conditions, including fermentation time, temperature, moisture content and microbial strain. Optimum fermentation conditions were performed using different fermentation times (48, 60, 72 h), fermentation temperatures (28 °C, 32 °C, 36 °C), moisture content (40%, 50%, 60%) and microbial inoculations (1 = *Bacillus clausii* with 1 × 10⁹ CFU/kg DM for CSM or 1 × 10¹⁰ CFU/kg DM for RSM; 2 = *Saccharomyces cariocanus* with 5 × 10⁹ CFU/kg DM; 3 = mixed strain (*B. clausii*:*S. cariocanus* ratio 1:1). The results show that the concentration of free gossypol content was reduced ($p < 0.05$), while the crude protein content was increased ($p < 0.05$) in CSM through optimum fermentation conditions: time 60 h; temperature 32 °C; moisture content 50% and inoculated with *B. clausii* (1 × 10⁹ CFU/kg DM) as well as *S. cariocanus* (5 × 10⁹ CFU/kg DM). Likewise, the concentration of glucosinolate was lowered ($p < 0.05$) and the crude protein was increased ($p < 0.05$) in RSM through optimum fermentation conditions: time 60 h; temperature 28 °C; moisture 50% and inoculated with *B. clausii* (1 × 10¹⁰ CFU/kg DM) as well as *S. cariocanus* (5 × 10⁹ CFU/kg DM). Our findings indicate that the optimal fermentation conditions of total mixed rations with cottonseed meal or rapeseed meal enhance the nutritional value, thereby making them viable and usable feedstuffs for potential use in livestock industries.

Keywords: cottonseed meal; rapeseed meal; total mixed ration; optimum fermentation conditions; orthogonal design

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1. Introduction

A growing demand for protein in the feed sector requires substituting other protein sources. As a result, interest in animal nutrition research for alternative plant proteins to replace soybean meal (SBM) has increased. We are interested in cottonseed meal (CSM) and rapeseed meal (RSM) since they are less expensive and more readily available locally (on a protein basis).

CSM is a by-product of cottonseed oil production that contains between 34–40% crude protein (CP), 11% crude fiber (CF), vitamin B, and organic phosphorus [1]. However, the use of CSM in animal diets is limited due to the presence of free gossypol (FG), a toxic pigment that may have a detrimental effect on the growth, reproduction and digestive

development of animals, as well as resulting in internal organ abnormalities [2,3]. RSM is also a by-product of oil manufacture in large quantities. It contains high levels of protein and has a well-balanced amino acid profile [4,5], making it appropriate for use in livestock feed. Nevertheless, its meal contains glucosinolates [6,7] and other anti-nutrient factors that could poison the animal and impair development performance [8,9]. Therefore, the use of RSM in livestock diets is limited.

Different approaches for CSM and RSM detoxifying have been formed, including biological [10,11], chemical [12,13] and physical [14,15] detoxification. However, chemical and physical detoxification have drawbacks such as high expense, environmental problems and huge nutrient loss. In contrast, microbial fermentation is the optimal solution for detoxification since microorganisms' rapid growth could release complex enzymes that degrade the meal's toxic substances. Microorganism-mediated fermentation is an alternative method for removing anti-nutritional components of feed while increasing their nutritional content [16]. Additionally, microbial fermentation is the best processing approach since it substantially improves animal performance and health [17,18].

Bacillus clausii is a Gram-positive spore-forming microorganism that, when administered in sufficient amounts, confers health advantages on the host [19]. Yeast is widespread and is easily cultured in large quantities [20]. *Saccharomyces* have been widely used to reduce the anti-nutritional factor content of diets [21]. Furthermore, yeast has been shown to increase the crude protein and mineral content of plant-based meals [22]. Although *S. cerevisiae* has been the potential approach for improving animal performance over the last two decades [23,24], a recent study showed that *S. cerevisiae* is limited to creating cell biomass during aerated glucose fermentation. *S. cerevisiae* ferments alcohol rather than producing biomass when grown in aerobic conditions [25]. This restricts animal access to beneficial yeast biomasses such as protein, essential amino acids and vitamins. As a result, it is vital to expand the research field and strengthen the investigation into additional yeast strains. However, little information is available on optimizing fermented total mixed rations with CSM or RSM using *B. clausii* or *S. cariocanus* strains. This study aimed to optimize the fermentation conditions of fermented total mixed rations containing cottonseed or rapeseed meal using *B. clausii* or *S. cariocanus* to reduce the content of anti-nutritional factors and to determine their nutritional value.

2. Materials and Methods

2.1. Experiment Time and Place

This experiment was conducted from July 2020 to April 2021 at the Institute of Feed Research, Chinese Academy of Agricultural Sciences, Beijing 100081, China.

2.2. Experimental Materials

B. clausii with 1×10^9 CFU/kg DM or 1×10^{10} CFU/kg DM and *S. cariocanus* with 5×10^9 CFU/kg DM used in this study were purchased from a local company (Gaotang Huanong Bioengineering Co. Ltd., Liaocheng, Shandong, China). The CSM ration and RSM ration used as the fermentation substrate were collected from a local feed manufacturer (Dadi Feed Company, Chengdu, Sichuan, China).

2.3. Preparation and Formulation of TMR with CSM/RSM

The formulation of TMR was mixed with CSM/RSM, corn, wheat bran, whole corn silage, corn stalk, urea, fat powder, and a premixed supplement. Total mixed ration with CSM or RSM was used as the fermentation substrate. TMR with CSM/RSM containing 200 g was mixed thoroughly according to a method described in [26].

2.4. Design and Optimization of the Fermentation Conditions

The orthogonal experiment design (4 factors $\times$ 3 levels) was used to obtain the optimum fermentation time, temperature, moisture content and microbial strain (Table 1). Experiments were conducted in plastic bags containing 200 g TMR with CSM/RSM that

was inoculated with *B. clausii* with 1×10^9 CFU/kg DM for CSM or 1×10^{10} CFU/kg DM for RSM, *S. cariocanus* with 5×10^9 CFU/kg DM and mixed strain *B. clausii* and *S. cariocanus* of ratio 1:1 throughout solid-state fermentation in three levels. Fermentation was performed using different fermentation periods (48, 60, 72 h), fermentation temperatures (28 °C, 32 °C, 36 °C), moisture content (40%, 50%, 60%) and microbial inoculation levels (1, 2, 3) according to a previously described approach [27]. The incubator settings controlled the fermentation period and temperature, which were maintained throughout the incubation period.

Table 1. Factors and levels selected for optimum fermentation conditions of orthogonal design.

Levels	Factors			
	Fermentation Time (A) (h)	Fermentation Temperature (B) (°C)	Moisture Content (C) (g/100g)	Microbial Strain (D) ¹
1	48	28	40	1
2	60	32	50	2
3	72	36	60	3

¹ Symbols 1, 2, and 3 of factor D; 1 = *Bacillus clausii* at 1×10^9 CFU/kg DM for total mixed ration with CSM or 1×10^{10} CFU/kg DM for total mixed ration with RSM; 2 = *Saccharomyces cariocanus* at 5×10^9 CFU/kg DM for the total mixed rations; 3 = mixed strain *B. clausii*:*S. cariocanus* at ratio 1:1 for total mixed rations. The arrangements of columns A, B, C and D were decided by orthogonal design for 4 (factor) $\times$ 3 (levels).

The influence of fermentation time, fermentation temperature, moisture content and microbial strain on chemical composition and anti-nutritional factors (free gossypol, glucosinolate degradation) were evaluated. Nine different fermentation treatments were performed, as shown in Table 2. Each run was carried out in triplicate.

Table 2. Scheme of orthogonal L9 (3⁴) design for optimum fermentation conditions.

Run	Factors			
	Fermentation Time (A) (h)	Fermentation Temperature (B) (°C)	Moisture Content (C) (g/100g)	Microbial Strain (D) ¹
1	48	28	40	1
2	48	32	50	2
3	48	36	60	3
4	60	28	50	3
5	60	32	60	1
6	60	36	40	2
7	72	28	60	2
8	72	32	40	3
9	72	36	50	1

¹ Symbols 1, 2, and 3 of factor D; 1 = *Bacillus clausii* at 1×10^9 CFU/kg DM for total mixed ration with CSM or 1×10^{10} CFU/kg DM for total mixed ration with RSM; 2 = *Saccharomyces cariocanus* at 5×10^9 CFU/kg DM for the total mixed rations; 3 = mixed strain *B. clausii*:*S. cariocanus* at ratio 1:1 for total mixed rations. The arrangements of column A, B, C and D were decided by orthogonal design for 4 (factor) $\times$ 9 (run number).

2.5. Measurements

2.5.1. Chemical Composition

After fermentation, TMR with CSM or RSM samples were dried at 65 °C for 48 h, ground and passed through a 1-mesh sieve. Samples were ground to pass through a 1-mm sieve size for analysis of dry matter (DM), crude protein (CP) and ether extract (EE) according to AOAC [28]. According to Van Soest et al. [29], neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined.

2.5.2. Analysis of Anti-Nutritional Factors

The free gossypol was determined using the official method of the American Oil Chemists Society [30]. The presence of 3-amino-1-propanol determined free gossypol, a mixture of isopropyl alcohol and n-hexane was used to extract free gossypol, and aniline was used to convert gossypol to aniline cotton phenol. The colorimetric determination was carried out at the maximum absorption of the spectrophotometer at the wavelength of 440 nm. Two grams of the TMR with CSM sample was put in a 250 mL Erlenmeyer flask

with a stopper, 20 glass beads and a pipette. The tube was filled with 50 mL of solvent, then the bottle was closed, put in the shaker and was oscillated for 1 h. A dry filter was used and was then covered with funnel glass to reduce the solvent volatilization. The first few drops of the filtrate were discarded, and the remainder was collected using a 100 mL Erlenmeyer flask with a stopper.

Calculation Formula (Equation (1)):

$$X = \frac{A \times 1250 \times 1000}{a \times m \times V} = \frac{A \times 1.25}{amV} \times 10^6 \quad (1)$$

In the formula:

X = free gossypol content, mg/kg;

A = absorbance;

M = sample quality, g;

V = the volume of filtrate for determination, mL;

a = mass absorption coefficient, free gossypol is $62.5 \text{ cm}^{-1} \text{ g}^{-1} \text{ L}$.

Correspondingly, glucosinolates of the TMR with RSM were determined using palladium chloride [31]. Briefly, 0.2 g of TMR with rapeseed meal was powdered in a mortar and added to a graduated test tube containing 10 mL boiling water. The mixtures were thoroughly shaken and heated for 30 min in a water bath before being diluted to 10 mL. Following centrifugation, 2 mL of TMR with rapeseed meal extract suspension was pipetted to a graduated tube containing 4 mL of 0.15% sodium carboxymethyl cellulose and shaken well. Then, 2 mL of 8 mmol/L palladium chloride color was added. After vigorous stirring, the mixed solutions were kept at $22 \pm 3^\circ \text{C}$ for 2 h. The absorption at 540 nm (A) was determined using sodium carboxymethyl cellulose as the reference material and a blank solution as the standard solution. The glucosinolate content was determined using absorbance A, proportional to the glucosinolate content as ascertained by the standard curve. Standard curve: $A = Kx + b$.

$$\text{Glucosinolate content (X)} = (A - b)/k \quad (2)$$

X = glucosinolate content; ($\mu\text{mol/g}$)

A = absorbance value;

K and b = fixed values.

2.6. Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS software (version 9.4, SAS Institute, Cary, NC, USA). The significance of differences between mean values was assessed using Tukey's multiple comparisons to determine the statistical difference between means. Differences among treatment groups were considered significant if $p < 0.05$.

3. Results

3.1. Effect of Fermentation Conditions on Chemical Compositions of TMR with CSM or RSM

As shown in Table 3, there were significant differences ($p < 0.05$) in the effects of time and temperature factor levels on CP. However, there was no significant difference in the effect of moisture and microbial strain factor levels on CP ($p > 0.05$). Thus, level two (32°C) fermentation temperature significantly increased CP, followed by fermentation time, moisture content and microbial strain.

Similarly, as shown in Table 4, factor levels of fermentation time, fermentation temperature, moisture content and microbial strain had a significantly different effect on increasing crude protein ($p < 0.05$). The fermentation temperature level one (28°C) had the greatest impact, followed by moisture content, microbial strain and fermentation time. Through the combined analyses, the composition of A3B1C2D3 was the best, i.e., the best composition of optimum fermentation conditions was fermentation time 72 h, fermentation temper-

ature 28 °C, moisture content 50% and mixed microbial strain (*B. clausii* with 1×10^9 : *S. cariocanus* with 5×10^9 CFU/kg DM for ratio 1:1).

Table 3. Effects of different factor levels on CP of TMR with CSM.

Level	Fermentation Time (A)	Fermentation Temperature (B)	Moisture Content (C)	Microbial Strain (D)
1	14.48 ^b	14.58 ^b	14.68	14.66
2	14.69 ^{ab}	15.07 ^a	14.52	14.57
3	14.72 ^a	14.25 ^c	14.69	14.67
Delta	0.25	0.83	0.17	0.10
Rank	2	1	3	4
<i>p</i> -value	0.022	<0.001	0.121	0.454

^{a,b,c} Means in the same column with different superscripts differed ($p < 0.05$). Delta: difference between largest to smallest numbers in the column. Rank: based on the outcome of delta result as ranking order 1, 2, 3 and 4. CP, crude protein; TMR, total mixed ration; CSM, cottonseed meal.

Table 4. Effects of different factor levels on CP of TMR with RSM.

Level	Fermentation Time (A)	Fermentation Temperature (B)	Moisture Content (C)	Microbial Strain (D)
1	13.73 ^b	13.99 ^a	13.66 ^b	13.82 ^a
2	13.81 ^{ab}	13.71 ^b	13.85 ^a	13.70 ^b
3	13.82 ^a	13.65 ^b	13.84 ^a	13.83 ^a
Delta	0.09	0.34	0.19	0.12
Rank	4	1	2	3
<i>p</i> -value	0.033	<0.001	<0.001	0.003

^{a,b} Means in the same column with different superscripts differed ($p < 0.05$). Delta: difference between largest to smallest numbers in the column. Rank: based on the outcome of delta result as ranking order 1, 2, 3 and 4. CP, crude protein; TMR, total mixed ration; RSM, rapeseed meal.

As indicated in Table 5, the degree of influence of each factor on the NDF of TMR with CSM was $C > D > B > A$. Likewise, there were significant differences ($p < 0.05$) among the various levels of each factor except factor (A). The fermentation temperature of level one showed the highest reduction in NDF compared with other levels. The moisture content of level one in factor C also displayed a lower reduction compared with other levels. In addition, the microbial strain of level one was a better reduction of NDF compared with others. According to these results, the optimal combination of the factors mentioned above were C1D1B1A2 (moisture content 40%; a microbial strain of *B. clausii* with 1×10^9 CFU/kg DM; fermentation temperature of 28 °C; fermentation time of 60 h).

Table 5. Effects of different factor levels on NDF of TMR with CSM.

Level	Fermentation Time (A)	Fermentation Temperature (B)	Moisture Content (C)	Microbial Strain (D)
1	31.78	31.31 ^b	29.66 ^c	30.65 ^b
2	31.62	32.04 ^{ab}	32.22 ^b	33.20 ^a
3	32.54	32.58 ^a	34.06 ^a	32.09 ^a
Delta	0.91	1.27	4.41	2.55
Rank	4	3	1	2
<i>p</i> -value	0.149	0.048	<0.0001	0.0002

^{a,b,c} Means in the same column with different superscripts differed ($p < 0.05$). Delta: difference between largest to smallest numbers in the column. Rank: based on the outcome of delta result as ranking order 1, 2, 3 and 4. NDF, neutral detergent fiber; TMR; total mixed ration; CSM, cottonseed meal.

Likewise, as presented in Table 6, factor levels of fermentation time, fermentation temperature and microbial strain did not significantly influence the optimum fermentation conditions for NDF ($p > 0.05$) of TMR with RSM. However, moisture content had a significant effect ($p < 0.05$) on NDF. Level one of moisture content (40%) had a significantly better reduction on NDF than other levels ($p < 0.05$).

Table 6. Effects of different factor levels on NDF of TMR with RSM.

Level	Fermentation Time (A)	Fermentation Temperature (B)	Moisture Content (C)	Microbial Strain (D)
1	29.50	31.52	28.87 ^b	30.64
2	31.34	29.65	31.10 ^a	30.81
3	31.17	30.84	32.04 ^a	30.56
Delta	1.84	1.87	3.18	0.25
Rank	3	2	1	4
<i>p</i> -value	0.083	0.114	0.005	0.956

^{a,b} Means in the same column with different superscripts differed ($p < 0.05$). Delta: difference between largest to smallest numbers in the column. Rank: based on the outcome of delta result as ranking order 1, 2, 3 and 4. NDF, neutral detergent fiber; TMR; total mixed ration; RSM, rapeseed meal.

3.2. Effect of Fermentation Conditions on Anti-Nutritional Factors

As presented in Table 7, factor levels of fermentation time, temperature and moisture content had no significant effect on reducing free gossypol ($p > 0.05$). In contrast, the microbial strain had a significant effect ($p < 0.01$). However, levels two and three of microbial strain showed a better FG reduction than level one. This result indicates that the single strain of *S. cariocanus* with 5×10^9 CFU/kg DM or mixed strain (*B. clausii* with 1×10^9 : *S. cariocanus* with 5×10^9 CFU/kg DM) had the most influence on the optimum fermentation conditions of detoxification of free gossypol of fermented TMR with CSM.

Table 7. Optimal fermentations of FG of TMR with CSM.

Level	Fermentation Time (A)	Fermentation Temperature (B)	Moisture Content (C)	Microbial Strain (D)
1	68.30	64.14	65.50	74.34 ^a
2	65.03	69.34	67.65	61.75 ^b
3	67.64	67.47	67.80	64.86 ^b
Delta	3.27	5.20	2.30	12.59
Rank	3	2	4	1
<i>p</i> -value	0.2731	0.0628	0.4753	<0.0001

^{a,b} Means in the same column with different superscripts differed ($p < 0.05$). Delta: difference between largest to smallest numbers in the column. Rank: based on the outcome of delta result as ranking order 1, 2, 3 and 4. FG, free gossypol; TMR; total mixed ration; CSM, cottonseed meal.

Based on the degree order of rank, as displayed in Table 8, the effect of the factors on the glucosinolate decreased in the following order: initial moisture content (C), fermentation temperature (B), microbial strain (D) and fermentation time (A). Therefore, based on the magnitude, the optimal combination was A3B2C3D3.

Table 8. Effects of different factor levels on glucosinolate of TMR with RSM.

Level	Fermentation Time (A)	Fermentation Temperature (B)	Moisture Content (C)	Microbial Strain (D)
1	4.320 ^a	4.716 ^a	5.730 ^a	4.261 ^a
2	3.872 ^{ab}	3.079 ^b	3.559 ^b	4.058 ^a
3	3.245 ^b	3.642 ^b	2.147 ^c	3.118 ^b
Delta	1.075	1.637	3.583	1.144
Rank	4	2	1	3
<i>p</i> -value	0.002	<0.001	<0.001	0.001

^{a,b,c} Means in the same column with different superscripts differed ($p < 0.05$). Delta: difference between largest to smallest numbers in the column. Rank: based on the outcome of delta result as ranking order 1, 2, 3 and 4. GL, glucosinolate, TMR; total mixed ration; RSM, rapeseed meal.

3.3. An Optimum Combination of Four-Factor Orthogonal Tests of TMR with CSM/RSM

The combination of four factors of orthogonal tests is presented in Table 9. There was a significant difference ($p < 0.01$) among the nine runs of experiments, as shown in Table 9. This indicates that the optimal combination of chemical compositions and anti-

nutritional factors of run 8 was better than other runs. According to these results, the optimal combination of the factors mentioned above was A3B2C1D3. However, we have selected A2B2C2D3 (time 60 h; temperature 32 °C; moisture 50% and mixed microbial strain (*B. clausii* with 1×10^9 :*S. cariocanus* with 5×10^9 CFU/kg DM for ratio of 1:1) for optimum fermentation conditions of the subsequent experiment of TMR with CSM since there was no statistical significance between levels two and three (A2 & A3) in Tables 3, 5 and 7. In addition, the effect of factor level of moisture content on other parameters such as CP and FG of fermented TMR with CSM were not significant except NDF under the moisture content of factor levels. Thus, level one of 40% showed the highest reduction of NDF, but NDF that is too low is not wide enough for the diet; thus, level two (32.22% of NDF) had a moisture content of 50%, and was given attention for subsequent experiments.

Table 9. Orthogonal results of fermentation test with multi factors of TMR with CSM.

Run	Time (A)	Temperature (B)	Moisture (C)	Microbial Strain (D)	Result		
					Chemical Composition		Anti-Nutritional
					CP	NDF	FG
1	1	1	1	1	14.50 ^{ab} ± 0.480	27.46 ^a ± 0.354	71.32 ^{ab} ± 3.143
2	1	2	2	2	14.74 ^{bc} ± 0.085	33.31 ^{cde} ± 0.303	66.09 ^{ab} ± 0.376
3	1	3	3	3	14.19 ^a ± 0.040	34.58 ^{de} ± 1.412	67.48 ^{ab} ± 4.898
4	2	1	2	3	14.57 ^{ab} ± 0.080	31.31 ^{bc} ± 0.597	60.73 ^a ± 5.406
5	2	2	3	1	15.21 ^c ± 0.157	32.43 ^{bcde} ± 1.628	75.55 ^b ± 2.463
6	2	3	1	2	14.30 ^{ab} ± 0.070	31.12 ^{bc} ± 0.731	58.79 ^a ± 5.007
7	3	1	3	2	14.66 ^{ab} ± 0.021	35.17 ^e ± 0.920	60.38 ^a ± 3.650
8	3	2	1	3	15.26 ^c ± 0.151	30.39 ^b ± 1.324	66.38 ^{ab} ± 4.545
9	3	3	2	1	14.25 ^{ab} ± 0.076	32.05 ^{bcd} ± 0.872	76.14 ^b ± 6.719
SEM					0.078	0.458	1.376
p-value					<0.001	<0.001	<0.001

a,b,c,d,e Means in the same column with different superscripts differed ($p < 0.05$). The arrangements of columns A, B, C and D were decided by orthogonal design for 4 (factor) $\times$ 9 (run number). CP, crude protein; NDF, neutral detergent fiber; FG, free gossypol; TMR; total mixed ration; CSM, cottonseed meal.

Nine runs of experiments were evaluated, and the experimental results of the orthogonal test were significantly different ($p < 0.05$) among the treatments as shown in Table 10. This demonstrates that the optimal fermentation conditions for chemical compositions and anti-nutritional factors of runs 4 and 7 had better results than other runs. According to these results, the optimal combination of the factors mentioned earlier in runs 4 and 7 were A2B1C2D3 and A3B1C3D2, respectively. However, we have selected A2B1C2D3 (time 60 h; temperature 28 °C; moisture 50% and mixed microbial strain (*B. clausii* with 1×10^9 :*S. cariocanus* with 5×10^9 CFU/kg DM) for optimum fermentation conditions of the subsequent experiment of TMR with RSM since there was no statistical significance difference between them.

Table 10. Orthogonal results of fermentation test with multi factors of TMR with RSM.

Run	Time (A)	Temperature (B)	Moisture (C)	Microbial Strain (D)	Result		
					Chemical Composition		Anti-Nutritional
					CP	NDF	GL
1	1	1	1	1	13.84 ^{bc} ± 0.061	28.51 ^{ab} ± 2.555	7.59 ^d ± 0.416
2	1	2	2	2	13.65 ^{ab} ± 0.025	29.05 ^{ab} ± 1.224	3.58 ^b ± 0.673
3	1	3	3	3	13.70 ^b ± 0.076	30.93 ^{ab} ± 2.134	1.79 ^a ± 0.462
4	2	1	2	3	14.12 ^d ± 0.010	32.51 ^{ab} ± 1.714	3.83 ^b ± 1.089
5	2	2	3	1	13.83 ^{bc} ± 0.095	31.66 ^{ab} ± 1.137	1.92 ^a ± 0.866
6	2	3	1	2	13.46 ^a ± 0.110	29.85 ^{ab} ± 2.966	5.86 ^c ± 0.282
7	3	1	3	2	14.00 ^{cd} ± 0.045	33.53 ^b ± 1.039	2.73 ^{ab} ± 0.110
8	3	2	1	3	13.66 ^{ab} ± 0.035	28.24 ^a ± 1.387	3.73 ^b ± 0.329
9	3	3	2	1	13.78 ^b ± 0.031	31.74 ^{ab} ± 0.852	3.27 ^{ab} ± 0.704
SEM					0.038	0.449	0.353
p-value					<0.001	<0.02	<0.001

a,b,c,d Means in the same column with different superscripts differed ($p < 0.05$). The arrangements of columns A, B, C and D were decided by orthogonal design for 4 (factor) $\times$ 9 (run number). CP, crude protein; NDF, neutral detergent fiber; GL, glucosinolates; TMR; total mixed ration; RSM, rapeseed meal.

4. Discussion

The purpose of this study was to determine the optimal fermentation conditions to reduce anti-nutritional factors in the fermented total mixed ration containing either cottonseed or rapeseed meal. Optimum selection and optimization of technical parameters such as the temperature, time, moisture content of the fermentation medium, and the inoculation strains are critical to improve the efficiency of solid-state fermentation [32].

In this study, protein enrichment was increased as the fermentation temperature slightly increased. The level two (32 °C) fermentation temperature (factor B) was the most significant one increasing the CP of fermented TMR with CSM. Similarly, the CP of fermented TMR with RSM increased when the fermentation temperature was at 28 °C. Proteins and enzymes are susceptible to denaturation when exposed to high temperatures. However, the beneficial effect of increased temperature on improved protein content can be associated with greater enzyme secretion by certain microbes [33,34]. Inoculated microbes enhanced the CP levels of both fermented TMR with CSM/RSM, and maintaining a steady temperature throughout fermentation was noted as necessary to keep the bacteria growing and active [35]. The optimal temperature for reducing the concentration of GL was 32 °C, indicating that this temperature is favorable for microbial growth in fermented TMR with rapeseed meal medium. The reduction percentages of GL in TMR with RSM were 83.31% and 80.26% at 32 °C and 36 °C, respectively. However, our result was consistent with a previous study showing that the degradation rate of GL increases to the highest value from 30 °C to 36 °C, and then decreases when further raising the temperature [26]. This may be due to the increased temperature having a detrimental influence on the metabolic activities of microorganisms, and numerous scientists have found that the metabolic activities of microorganisms become sluggish at lower temperatures [36]. Our research revealed that the temperature rise to 32 °C was optimum, above which the reduction of glucosinolates decreased. This may be due to the increase in fermentation temperature promoting microbes' growth and their activity in decreasing the amount of glucosinolates present in the fermented product.

Another significant element is the time needed for fermentation, which has been shown to increase the crude protein content in the fermented TMR with CSM/RSM. As the fermentation periods extended, the crude protein content increased. Our findings are consistent with a study showing that the degradation rate of glucosinolates increased rapidly from 24 to 72 h, reaching a maximum of 83.67% after 72 h of fermentation [26]. Reduced fermentation time combined with good optimal fermentation is our priority because it can increase time efficiency and lower costs.

The moisture content is also important in protein enrichment during the solid-state fermentation [37]. The requirements of moisture content for yeast and bacteria are different. Low moisture content decreases the solubility of nutrients, thereby limiting microbial growth. However, high moisture content could reduce the porosity of substrates and promote material adhesion, which may affect temperature and oxygen transfer [38,39]. This study's optimum moisture contents were 40% and 50% for TMR with CSM or RSM, respectively. The absorption of the substrate may be responsible for the difference in results. Correspondingly, our study showed that fermented TMR with rapeseed meals containing 50% and 60% moisture achieved the degradation rate of GI at levels of 80.71% and 88.36%, respectively. However, our study is higher than that reported by Shi et al. [40], who found that the substrate containing 60% moisture achieved the degradation rate of GI at levels of 76.89% of RSC. Therefore, the optimal moisture content for the degradation rate of GI appeared to be 50–60%.

Conversely, the factor levels of moisture content affecting other parameters such as CP and FG of fermented TMR with CSM were not significant except NDF under the moisture content of factor levels; thus level one of 40% of moisture content showed the highest reduction in NDF, but NDF that is too low is not wide enough for the diet. Thus, level two of moisture content of 50% showed a satisfying reduction in NDF (32.22%), which can be utilized in the subsequent study. However, Ma et al. [41] reported that diets

high in NFC/NDF increased microbial nitrogen (MN) and metabolizable N, the ratio of metabolizable N to digestible protein or N intake in lambs, and decreased urinary N output. Similarly, incorporating protein sources into high-fiber feed might improve the utilization of these feeds by ruminants because of improved ruminal conditions [42].

In this study, we used single-strain (*B. clausii* or *S. cariocanus*) and mixed-strain (*B. clausii*:*S. cariocanus* at a 1:1 ratio) fermented TMR with CSM/RSM, which not only decreased the content of anti-nutritional factors but also enhanced the protein content. In the present study, mixed strains increased CP content (from 12.49% to 14.67% and from 11.65% to 13.83%) through TMR optimization with CSM or RSM, respectively. Furthermore, fermented TMR with CSM/RSM enhanced the CP content, which agreed with the results found with fermented CSC [27], rapeseed meal [43] and fermentation of soy meal [44]. Similarly, crude protein increased from 49.8% to 51% when cottonseed meal was inoculated with *B.s subtilis* ST-141 and *Saccharomyces* N5 [45]. An increase in CP content resulting from fermented TMR with CSM/RSM could be due to the optimum fermentation conditions that allow/make it suitable for synthesizing the microorganisms' proteins [43]. The increased CP level in fermented TMR with CSM/RSM is beneficial because it reduces other dietary protein sources and feed costs.

Cottonseed and rapeseed meals contain anti-nutritional factors that may disrupt nutrient availability, end up causing toxicity and impair animal performance [46]. Fermentation conditions such as time, temperature, moisture content and microbial strain must be considered to optimize FG and glucosinolate degradation with the least amount of nutrient loss possible. In the present study, factor levels such as time, temperature and moisture content did not significantly differ in FG degradation. At the same time, the mixed microbial strain showed a higher reduction of FG than with *B. clausii*, though the single- and mixed-strains were not significantly different. Similarly, the mixed strain of *B. clausii* and *S. cariocanus*, at a ratio of 1:1, showed the higher optimal reduction of glucosinolate. These results indicate that the fermentation of the mixed strain enhanced the reduction of 36.74% and 83.10% of FG and glucosinolate in TMR with CSM/RSM, respectively. Yeasts and bacterial strains can grow in synergy with one another, and this capability has led to their widespread use as co-cultures to improve the quality of unconventional feeds [47]. However, our study, in line with some other studies, has shown that mixed strains have a more practical effect on fermentation than a single strain [48]. Similar to that, our findings are consistent with prior studies showing that the degradation abilities of all the combined strains were better than a single strain for the degradation of glucosinolates after fermentation [49]. Likewise, our result is in line with the microorganisms of *C. utilis* and *B. subtilis* combined to ferment *Moringa oleifera* leaf meal, which reduced anti-nutritional compounds and increased the protein content [50]. As a result, combining multiple enzymes from yeast and bacteria is required to remove anti-nutritional elements from cottonseed or rapeseed meal. Anti-nutritional factors may be difficult to degrade when using solid-state fermentation with a single strain of microbe.

5. Conclusions

In this study, increase in CP and decrease in NDF as well as reduction in anti-nutritional factors were observed during fermentation of both TMRs containing CSM/RSM. Consequently, the optimal fermentation conditions of fermented TMR containing CSM were as follows: time 60 h; temperature 32 °C; moisture 50% and mixed microbial strain (*B. clausii* with 1×10^9 :*S. cariocanus* with 5×10^9 CFU/kg DM). Likewise, the optimal fermentation conditions of fermented TMR containing RSM were as follows: time 60 h; temperature 28 °C; moisture 50% and mixed microbial strain (*B. clausii* with 1×10^{10} :*S. cariocanus* with 5×10^9 CFU/kg DM). In addition, this research provides insight into the use of CSM or RSM in TMR that could potentially replace SBM in ruminant production. The results of these experiments should be evaluated in vivo to evaluate their effect on animal performance.

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