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RESEARCH ARTICLE

Impact of Enzymatic Hydrolyzed Protein Feeding on Rumen Microbial Population, Blood Metabolites and Performance Parameters of Lactating Dairy Cows

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ABSTRACT

The objective of the current experiment was to elucidate the effect of peptides enzymatic hydrolysis from cottonseed protein on rumen microbial population, and hence on milking performance of lactating dairy cows. A total of 50 Holstein cows were assigned into two groups, fed diets with or without enzyme hydrolyzed cottonseed protein (ECP) supplementation. The supplemented group received 0.4% ECP in the diet. Results of rumen microbial population showed that ECP supplementation had no effect on the overall microbe diversity but an increasing trend in the population of *Succiniclasticum spp.* was observed with ECP supplementation ($P>0.05$). The results of milk production and composition showed higher lactose and milk yields in ECP supplemented diet as compared to the control group ($P<0.05$). However, no significant difference was observed in milk protein, milk fat, and milk urea nitrogen between the two groups ($P>0.05$). Dry matter, neutral detergent fiber and acid detergent fiber digestibility was also observed to be higher in the ECP group ($P<0.05$), while CP digestibility was not affected by ECP supplementation ($P>0.05$). Furthermore, an increased blood total protein and glucose and a decreased blood urea nitrogen were observed in the ECP group ($P<0.05$). In conclusion, it is suggested that the ECP is a rich source of peptides that can be used as a feed supplement for rumen microbial proliferation, improvement of rumen fermentation, and increase in milk yield.

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INTRODUCTION

The shortage of protein feed resources is a major issue in animal production worldwide (Hernández-Castellano *et al.*, 2019). Improving the utilization rate of protein in feed not only alleviate the shortage of protein feed resources, but could reduce nitrogen emissions, which is of significance to environmental protection (Beauchemin *et al.*, 2020). It is generally believed that the protein is hydrolyzed into free amino acids in the digestive process and these free amino acids are absorbed and utilized by the animals (Eugenio *et al.*, 2022). However, recent studies have shown that the utilization of protein in animal body is not limited to the form of free amino acids, whereas most of them are absorbed in the form of peptides composed of two to three amino acids (Cant *et al.*, 2022). The absorption of peptides is characterized as fast transport speed, low energy consumption, and hard to be saturated as compared to amino acids. Providing animal with protein nutrition in the form of peptides could improve the utilization rate of protein, enhance animal immunity and capacity of resisting diseases and improve the quality of animal products, to better realize the production potential of animals (Kumar *et al.*, 2022; Liu *et al.*, 2022).

The digestion of feed and breakdown of nutrients in the rumen is facilitated by rumen microbes which secrete various hydrolyzing enzymes (Bhagat *et al.*, 2023). Microbial enzymes allow ruminants to utilize proteins, cellulose and hemicellulose. The enzymes synthesized by the microbes in the rumen are functional molecules built from different rumen-degradable protein (RDP) and different sources of RDP exhibit various degrees of efficiency in being incorporated into the microbial proteins (Monteiro *et al.*, 2021). In general, the most common nitrogen sources available to rumen microbes include peptides, amino acids, and ammonia. Therefore, it could be speculated that supplementation of enzymes hydrolyzed protein may interfere microbial population in rumen and influence the performance of the ruminants.

Bhagat *et al.* (2023) categorized rumen microbes into either cellulolytic or amylolytic groups based on their preference of energy sources. Cellulolytic microbes prefer to use ammonia as their main nitrogen source, while amylolytic microbes may utilize other peptides aside from amino acids and ammonia (Zanton and Hall, 2022). However, numerous studies showed that the growth rate of rumen microbes increases with amino acid or peptides supplementation regardless of grouping (Fessenden *et al.*, 2019; Jiang *et al.*, 2022). Furthermore, the rate of fiber digestion has been shown to increase with amino acid or peptide supplementation for both cellulolytic and amylolytic microbes (Griswold *et al.*, 1996). Studies have also found that cellulolytic microbes use less ammonia in cases where peptides are available (Kamau *et al.*, 2020; Bhardwaj *et al.*, 2021). A recent study revealed that peptides could improve rumen fermentation and microbial nitrogen production in both glucogenic and lipogenic diets (Zhou *et al.*, 2022).

Until now, most of the studies on the effect of peptides on rumen fermentation were mainly done in vitro. In addition, only a few studies have delved into the contribution of peptide supplementation on the milking performance of dairy cows. To bridge this knowledge gap, this study aims to elucidate the effect of enzymatically hydrolyzed peptides from cottonseed protein as a source of functional peptides on milk yield and composition, nutrients digestibility, blood index, and rumen fermentation of dairy cows.

MATERIALS AND METHODS

Experimental animals, design and treatments: Fifty multiparous Holstein cows in mid-late lactation (3rd lactation period and 6 months after calving) with an average of 36 kg/d of milk were selected in the current study. The cows were divided into two groups and fed two total mixed rations (TMR): a control group (CON) which was fed with the basal diet only, and a treatment group (ECP) which was fed with the same basal diet supplemented with 0.4% ECP. The ingredients and chemical composition are presented in Table 1. The ECP used in this study was Ruminon® (55% crude protein (CP), 36% acid-soluble protein of DM) which was produced from cottonseed protein via enzymatic hydrolysis (Mytech Biotech Co., Ltd, Chengdu City, China). The experiment lasted from late March 2019 to April 2019, with a 10-day adaptation period and a 31-day experiment period.

Sample collection and analysis: Cows were milked three times daily and milk production was recorded electronically at each milking period. Milk samples were collected at 0, 15, and 30d, after evening milking and stored at 4°C after preservative (potassium dichromate, 0.03 g/100 mL milk) addition. The samples are then immediately shipped to the DHI testing laboratory (Kunming Dairy Herd Improvement Center) for analysis. Milk samples were analyzed for milk fat percentage, protein concentration in percentage, lactose percentage, and concentration of milk urea nitrogen (MUN) in percentage by using milk composition analyzer (FOSS MilkoScan FT3 Denmark).

In the last five days of the experiment, feed samples of TMR and rectum feces were collected at each feeding schedule using the quartering method and immediately stored at -20°C for the determination of nutrient composition. The samples were oven-dried at 65°C for 48 h and were ground through a 40-mesh sieve (0.425 mm screen) before the analysis of CP, neutral detergent fiber (NDF) and acid detergent fiber (ADF). Dry matter and CP in feed and fecal samples were analyzed according to AOAC (2005). Meanwhile, the NDF and ADF contents of these samples were measured based on a previous study (Van Soest, 1963) using the Ankom® A200I fiber analyzer.

Blood samples were taken on 0 d, 15 d, and 30 d from the coccygeal vein before night feeding and blood serum and plasma samples were processed for biochemical indices including concentration of true protein (TP), concentration of glucose (GLU), and concentration of blood urea nitrogen were analyzed at -20°C using an automated clinical analyzer (Hitachi 7020, Japan).

Rumen fluid was collected from the rumen at the end of the experiment by an oral sampler to investigate the effect of ECP on rumen microbes. The fluid was filtered using a 4-ply gauze pad and was stored at -80°C until Real-Time PCR analysis.

DNA extraction and sequencing: Total DNA was extracted from each sample by using DNA extraction Kit (ZymoBIOMICS™ DNA Miniprep Kit Catalog Nos. D4300T, America) according to the Instruction of the Kit as descried in recent studies (Xia *et al.*, 2018; Qiu *et al.*, 2019; Qiu *et al.*, 2020). The original offline data obtained by sequencing was spliced and filtered to obtain a high-quality target sequence for subsequent analysis. Subsequent bioinformatics operations were completed using Usearch and QIIME4 as described in recent study (Chen *et al.*, 2020).

Statistical analysis: The data of dry matter intake (DMI), digestibility, milk performance parameters, blood metabolites and rumen microbial population are reported as means with SEM. Differences between experimental groups were analyzed by the independent two-sample t-test using the SPSS 17.0 and significance was considered at $P<0.05$.

RESULTS

Dry matter intake, nutrient digestibility, milk production and composition: The effects of ECP supplementation on dry matter intake, nutrient digestibility and milking performance are summarized in Table 2. Results showed that the compared with the control group, the digestibility of DM, NDF, and ADF in the ECP group were increased by 2.59, 4.9 and 5.4% respectively ($P<0.05$), while CP digestibility was unaffected by treatment ($P>0.05$). Similarly, DM intake was higher in the ECP group as compared to control ($P<0.05$). Enzymatic hydrolyzed cotton seed supplementation in the diet of cows significantly increased both milk yield by 0.94 kg per day and milk lactose content ($P<0.05$). Results also showed that milk fat, protein, and MUN contents were not affected by ECP treatment ($P>0.05$). The trend of milk yield after supplementation of ECP to cow during the trial is shown in Fig. 1. With the passage of time, the milk yield in ECP group becomes significantly higher than that in control group ($P<0.05$).

Table 1: Ingredients and chemical composition of basal diet fed during the experiment.

Ingredients	% of DM
Corn	19.25
Soybean meal	9.12
Soybean meal	1.65
Soybean hulls	3.96
Beet pulp	4.34
Whole cottonseed	3.25
Alfalfa hay	13.86
Corn silage	27.62
Oat hay	5.99
Brewers wet grain	4.08
Molasses	4.76
Fat powder	1.23
Sodium bicarbonate	0.45
Dicalcium phosphate	0.24
Mineral vitamin mix[1]	0.20
Chemical analysis[2]	
Crude protein, % of DMs	15.6
Ether extract, % of DM	6.2
Neutral detergent fiber, % of DM	31.9
Acid detergent fiber, % of DM	21.76
Net energy for lactation, Mcal/kg of DM	1.78

1 Contained a minimum of 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5000 mg of Cu/kg, 250 mg of I/kg, 40 mg of Co/kg, 150 mg of Se/kg, 2200 kIU of vitamin A/kg, 660 kIU of vitamin D3/kg, and 7,700 IU of vitamin E/kg.
2 The net energy for lactation was a calculated value, while the others were measured values.

Table 2: Effects of supplemented Holstein cows with or without ECP on dry mater intake, nutrient digestibility, milk production and composition.

Items	Treatment			*P*-value
	CON	ECP	SEM	
Nutrient Intake and Digestibility				
Dry matter intake (kg/d)	25.58	26.12	0.08	0.264
Dry matter (%)	80.42	83.01	0.38	<0.001
Crude protein (%)	81.14	82.06	0.453	0.321
Ether extract (%)	68.96	72.56	1.036	0.562
Neutral detergent fiber (%)	56.19	61.09	0.883	0.003
Acid detergent fiber (%)	60.95	66.35	0.900	0.001
Milk Production and Composition				
Milk yield (kg/d)	35.47	36.41	0.25	0.038
Milk fat (%)	3.94	4.19	0.12	0.482
Milk protein (%)	3.25	3.24	0.21	0.876
Milk lactose (%)	5.06	5.17	0.59	0.001
Milk urea nitrogen (%)	22.33	21.22	0.95	0.201

CON: control, ECP: enzyme hydrolyzed cottonseed protein, SEM: standard error of mean

Plasma biochemical parameters: Cows supplemented with ECP had higher blood TP and GLU levels (P<0.05), and lower BUN levels (P<0.05) compared to the cows in the control group (Table 3).

Rumen microbial abundances: Enzymes hydrolyzed cotton seed protein supplementation had no significant effect on the rumen microbe diversity in both groups based on the Simpson and Shannon index (P>0.05) (Table 4). However, an increasing trend in microbe abundance with ECP supplementation was observed based on the Chao 1 index (P=0.086; Table 4).

Quantities of rumen microbes: The top 10 most predominant microbial genera present in the rumen of the two groups are shown in Table 5. An increasing trend in *Succiniclasticum spp*. population can be observed with ECP supplementation (P=0.05). However, dietary supplementation of ECP in the diet of cows had no significant effect on other rumen microbial species (P>0.05).

Table 3: Effects of ECP supplementation on blood index of dairy cows.

Items	Treatment		SEM	*P*-value
	Treatment	SEM		
TP (mmol/L)	61.92	66.86	1.172	0.026
GLU (g/L)	2.33	2.60	0.056	0.007
BUN (mmol/L)	4.36	3.82	0.098	0.001

TP: total protein, GLU: glucose, BUN: blood urea nitrogen, SEM: standard error of mean.

Table 4: Effects of supplementation ECP on rumen microbe species of dairy cows.

Items	Treatment		SEM	*P*-value
	CON	ECP		
OTUs	824.67	838.24	11.32	0.187
ACE index	842.52	856.31	5.55	0.138
Chao1 index	845.25	862.37	4.10	0.087
Simpson	0.022	0.022	0.006	0.979
Shannon	5.24	5.38	0.135	0.856

ECP: enzymolyzed cottonseed protein, OTUs: operational taxonomic units, SEM: standard error of mean.

Table 5: Effects of supplementation ECP on quantities of rumen microbes (Genus Level).

Genus	Treatment		*SEM*	*P*-value
	CON	ECP		
Succiniclasticum	0.079	0.133	0.021	0.055
uncultured_bacterium_f_Muribaculaceae	0.063	0.077	0.008	0.283
Ruminococcaceae_UCG-014	0.026	0.031	0.003	0.348
uncultured_bacterium_f_F082	0.034	0.041	0.005	0.453
Christensenellaceae_R-7_group	0.048	0.035	0.010	0.464
Prevotella_1	0.182	0.156	0.022	0.502
Ruminococcaceae_NK4A214_group	0.104	0.084	0.021	0.577
Rikenellaceae_RC9_gut_group	0.043	0.047	0.005	0.657
Succinivibrionaceae_UCG-001	0.055	0.052	0.023	0.941
Ruminococcus_2	0.049	0.049	0.007	0.986

ECP: enzymolyzed cottonseed protein, SEM: standard error of mean.

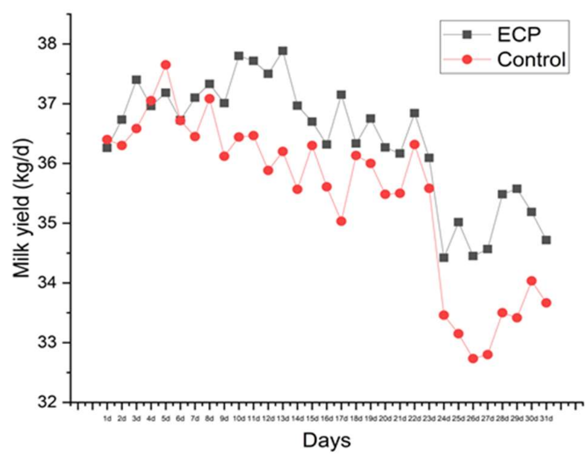

Fig. 1: Milk yield trend during the trial. (kg/d).

DISCUSSION

Cottonseed meal is a low-quality protein ingredient that is commonly used in ruminant rations because of its low price; however, it is not utilized in nonruminant diets due to its high gossypol content. Recent studies suggest that enzymatic hydrolysis of proteins to peptides could enhance the nutritional quality of the feeds and reduce the effects of anti-nutritional factors (Hou *et al.*, 2017). Hence, this process proves to be useful in increasing the quality of protein feeds like cottonseed meals. In mature ruminants, peptides can be utilized by rumen microorganisms for their proliferation, which in turn increase rumen fermentation (Cant *et al.*, 2022; Kumar *et al.*, 2022; Liu *et al.*, 2022) and fiber digestibility (Griswold *et al.*, 1996). Furthermore, it has been observed that peptides supplementation in the diet of ruminants could also stimulate the rumen microbial synthesis of various proteins (Tan *et al.*, 2021; Kumar *et al.*, 2022; Liu *et al.*, 2022).

The peptide supplementation often has little influences on DMI (Rastgoo *et al.*, 2020), which is consistent with this study. In our experiment, cows in the ECP group had a higher milk yield than in the control group. This is consistent with the findings in a separate study (Zhou *et al.*, 2022), where the results showed ECP supplementation led to a dramatic increase in milk yield. Like milk yield, the milk lactose content of cows in the ECP group was also higher than in the control group. Taking these results together, we hypothesized that ECP supplementation would enhance the fermentation of carbohydrates and increase the production of propionate, which is the precursor of GLU (Ríus, 2019). Our hypothesis was supported by the results of our experiments on nutrient digestibility. Specifically, cows with ECP supplementation had higher digestibility of dry matter, NDF, and ADF, which in turn produced more volatile fatty acids including propionate.

Enzymatic hydrolyzed cotton seed was found to be an effective peptide source in promoting microbial protein synthesis (Rehemujiang *et al.*, 2023). One study has found that the in vitro concentration of ammonia decreased linearly with peptides supplementation (Xie *et al.,* 2022). In our experiments, ECP supplementation lowered BUN, indicating the low ammonia concentration in the rumen when cows were supplemented with ECP. The lower BUN and the higher TP blood levels observed in this study lead us to conclude that ECP supplementation improved the nitrogen efficiency of cows, which is consistent with the findings in a previous study (Baumgard *et al.*, 2017). In a similar study, using yeast-derived microbial protein as a peptide source, the researchers found that the yeast-derived microbial protein treatment group was more efficient in nitrogen utilization compared to the control group which used the traditional protein source, soybean meal (Sabbia *et al.*, 2012).

Lactose is made from GLU and is the key factor that regulates the osmotic pressure of the mammary (Cai *et al.*, 2018). In one study, it was found that the blood GLU level in the mammary determined the milk lactose concentration since the mammary cells cannot synthesize GLU (Ward *et al.*, 2021). The results of higher milk lactose contents with the supplementation of ECP in the diet of dairy cows in the current study explored that ECP supplementation provided sufficient supply of GLU in the blood that resulted in higher lactose contents of milk. Taken together, we conclude that ECP supplementation is beneficial to the milking performance of dairy cows by improving nitrogen utilization and blood GLU supply.

A higher nutrient digestibility means a more effective rumen function. It is well-known that the rumen fermentation rate depends on the activities of numerous rumen microbes. In this study, we found that the rumen microbe diversity did not differ between the two groups, except for *Succiniclasticum spp*., which showed an increasing trend in population with ECP supplementation. The *Succiniclasticum spp.* are ruminal bacteria that convert succinate to propionate (Wang *et al.*, 2021). As previously discussed, propionate is an important gluconeogenesis material. These findings further proved that the cows supplemented with ECP had greater blood GLU supply and helped explain why milk lactose was higher in the ECP group than in the control group. Similar results were found in one study which found that milk yield and DMI were positively correlated with the abundance of *Succiniclasticum spp.* (Zhao *et al.*, 2021).

Conclusions: In this study, we found that dairy cows supplemented with 0.4% of ECP per day had reduced blood urea nitrogen and increased rumen microbes, NDF and ADF digestibility, blood GLU, and total protein. Overall, these factors result in a higher milk yield and better milk quality.

Authors contribution: Research was conceptualized by X.C and S.Y; experiment was carreied out by J.Q. and J.D. lab analysis and data analysis X.L. and H.X.; writing the original manuscript H.L. and Xl.C. It is further confirmed that all authors of the manuscript have reviewed and agreed the final draft and confirm it for publication.

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