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Effects of dietary protein levels and calcium salts of long-chain fatty acids on nitrogen mobilization, rumen microbiota and plasma fatty acid composition in Holstein bulls

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ABSTRACT

The aim of this study was to investigate the impact of dietary crude protein (CP) levels and calcium salts of long-chain fatty acids (CSFA) on the nutrient intake and digestibility, nitrogen deposition, rumen fermentation characteristic and microbiota, plasma biochemical indexes and fatty acid composition in the Holstein bulls. Eight Holstein bulls was used in a 4 × 4 Latin Square design with 2 × 2 factorial diets, including two levels of CP (133 or 112 g/kg dry matter), and with or without 2.32 g/kg CSFA. The high CP level diets increased the CP intake and the CP apparent digestibility and the urinary nitrogen excretion ($P = 0.001$). Dietary supplementation with CSFA promoted the apparent digestibility of organic matter ($P = 0.012$). The diets of CSFA and high CP level raised the rumen ammonia nitrogen concentration ($P = 0.009$), and enhanced the isovalerate and valerate concentrations, respectively. The high CP diets improved the abundance of *Butyrivibrio fibrisolvens* ($P = 0.037$) and *Megasphaera elsdenii* ($P = 0.023$), while the CSFA reduced the abundance of methanogens ($P = 0.047$). High CP increased urea concentration ($P = 0.008$). Dietary supplementation with CSFA increased the cholesterol and the low-density lipoprotein cholesterol concentration in the plasma, and the proportion of C16:0, C18:1n9c and Δ^9 desaturase C18, while it reduced the ratio of C21:1 and C22:0. The low CP diets reduced the protein waste and environmental pollution in the final stage of fattening Holstein bulls. Further study needs to be done to investigate the effect of CSFA on the CH_4 emission in terms of microbial mechanism in the rumen.

1. Introduction

In the process of fattening beef cattle, the gradual reduction of muscle protein deposition is helpful to increase the fat deposition and improve the beef quality. Recent studies have shown that administering diets with 140 g/kg crude protein (CP) levels in dry matter (DM) basis has no effects on the nitrogen retention and growth performance compared with 10 g/kg CP levels in finishing Nellore bulls at age of 20 months (Menezes et al., 2016), which means that high protein diets may lead to the protein waste and the

Abbreviations: CSFA, calcium salt of long-chain fatty acid; CP, crude protein; VFA, volatile fatty acid; DM, dry matter; OM, organic matter; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; NH_3-N, ammonia nitrogen; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol

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increase of feed cost. It is necessary to adjust beef cattle dietary protein levels to avoid protein waste and nitrogen emission in the final fattening stage. At the same time, appropriate feeding strategies should be used to increase the fat deposition by altering lipid metabolism.

Raising energy concentration and adding plant oil in the diets are always used to increase beef cattle fat deposition. But the excessive concentrate has the risk of rumen acidosis (Wetzels et al., 2017), and adding oil such as soybean oil is harmful to the rumen microorganisms (Gomez-Cortes et al., 2008) and the fiber digestibility (Manso et al., 2006). The calcium salts of long-chain fatty acids (CSFA) as one kind of rumen protecting fat, which has no negative effects on the fiber digestibility (Manso et al., 2006). However, the CSFA characteristics of rumen protection make scholars neglect the effect of it on the rumen microorganism, because fatty acid of calcium salts are not completely inert in the rumen (Vannevel and Demeyer, 1996). Besides, lipid metabolism firstly occurs in the liver and then through plasma transport to adipose tissue (Bell, 1979). Therefore, the plasma lipid index is an important indicator to reflect the fat metabolism.

The required protein level at fattening stage is dependent on cattle breeds and ages. Specialized beef breeds have persistent lean meat growth. while dairy breeds deposit fat in the early stage (Gallo et al., 2014; Spanghero et al., 2017). Consequently, the required protein level should be studied in the beef production of Holstein bulls. Previous studies have shown that the protein intake and digestibility have a linear dependence on the protein levels in dietary (Obeid et al., 2007; Menezes et al., 2016). However, in *vitro* study demonstrated a contrary result that diet protein levels have no effect on the protein disappearance (Amaral et al., 2016). The high protein diets can increase the concentration of rumen ammonia nitrogen and urea nitrogen (Da Silva et al., 2016), which are beneficial to microbial protein synthesis (Amaral et al., 2016). Dietary with high protein level also reduces the efficiency of protein utilization (Pina et al., 2009). However, there are no reports to reveal the effects of protein levels on the rumen microorganisms and plasma fatty acid profile.

Therefore, we hypothesized that the low protein diets have no effects on the animal feed nutrients intake and digestibility, and the CSFA is beneficial for the fat metabolism and rumen metabolism in the finishing Holstein bulls. The aim of the present experiment is to define the impacts of the CSFA and CP on the intake, apparent digestibility of nutrients, rumen fermentation characteristics, rumen microbiota populations, hematologic indexes and fatty acid composition in Holstein bulls.

2. Materials and methods

The experimental procedure was conducted by the Animal Welfare and Ethics Committee of China Agricultural University. Animal care and handling were followed the guidelines by the regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of P. R. China, 1988).

2.1. Experimental design and animal diets

In this study, 8 Holstein bulls (20 months old, and 485 ± 20 kg of body weight) were selected and randomly divided into 2 groups. The 4 bulls in each group received one of 4 experimental diets under the 4 × 4 Latin Square experimental design. Four diets are shown in Table 1, including LN: low CP diets with no CSFA; HN: high CP diets with no CSFA; LC: low CP supplemented with CSFA diets and HC: high CP level diet with CSFA supplement. These four diets had the same level of total digestible nutrients (750 g/kg) except for the added of CSFA, Dietary formulations were expected to average daily gain was 1.7 and 2.1 kg in low and high-protein diets according to the Nutrient Requirements of Beef Cattle (NASEM, 2016). The fatty acid composition of CSFA is shown in Supplementary Table 1. Each period contained 25 days, and the first 20 days were the adaptation, the other 5 days were the sampling time. The bulls were individually housed and fed twice per day at 7:30 and 17:00, offered fresh drinking water *ad libtum*. The feed intake was recorded daily.

2.2. Sampling and chemical analyses

The Orts and feed samples were collected on the 21st to 23rd days of each cycle, the rectal faeces were gathered at 6:00, 12:00, 18:00 and 24:00, respectively. About 10 mL blood was collected through the jugular vein using a vacuum blood tube containing sodium heparin at 7:00 on 24th day, and the plasma was separated by centrifugation 3000 × g for 10 min and stored at −20 °C. Representative samples of rumen contents were collected at 10:00 on the 25th day *via* esophageal tubing and by retaining particles attached to the metal strainer. The rumen liquid used for determination of volatile fatty acids (VFA) was separated by 3000 × g centrifugation 15 min. The 250 g/L partial phosphoric acid solution, which contained 2-ethylbutyric acid as the internal standard, was added into the rumen liquid in a concentration of 0.25 ml/mL rumen fluid before stored at −20 °C. While rumen contents for the rumen microorganism analysis were stored in liquid nitrogen. About 60 ml urine was gathered at 5:00 on 24th or 25th day, and 10 mL of 0.5 mol/L dilute sulfuric acid was added into the urine.

The neutral detergent fiber (aNDF; Van Soest et al., 1991), ether extract (EE; method 920.39), DM (method 934.01), organic matter (OM; method 942.05), acid detergent fiber (ADF, ; Van Soest et al., 1991) and CP (method 990.03) of feed, orts and feces were analyzed by the methods of AOAC (AOAC International, 2002). For the determination of EE in CSFA, it needed to treat with 0.5 v/v sulfuric acid for acid hydrolysis before extraction. The acid insoluble ash of the samples as the endogenous marker was determined by the method as before (VAN Keulen and Young, 1977). The apparent digestibility of the nutrients was calculated using the endogenous tracer method (Huhtanen et al., 1994). The urea nitrogen was measured by previous studies (Da Silva et al., 2016).

Table 1
Ingredients and nutrient compositions of the dietary treatments[1].

Item	HN	HC	LN	LC
Ingredient, (g/kg)				
corn grain	427	410	360	345
soybean meal	108	103	55.1	52.8
wheat grain	30.7	29.6	151	145
Leymus chinensis	420	420	420	420
Calcium salt of fatty acids	0	23.2	0	23.2
NaCl	5.80	5.80	5.80	5.80
Mineral-vitamin premix[2]	5.80	5.80	5.80	5.80
limestone	2.90	2.90	2.90	2.90
Chemical composition (g/kg DM)				
OM	945	940	946	941
CP	133	130	112	109
aNDF	466	472	475	477
ADF	186	184	184	183
EE	28.2	47.8	27.2	46.8
Main fatty acid profile (g/kg of total fatty acid)				
C16:0	170	286	181	295
C18:0	25.2	32	26.3	33.1
C18:1n9c	264	279	262	278
C18:2n6c	496	360	488	359
C20:1	34.5	26.9	29.4	25.7

CP: crude protein, CSFA: Calcium Salts of long-chain Fatty Acids, DM: dry matter, OM: organic matter, aNDF: neutral detergent fiber, ADF: acid detergent fiber.

[1] HN dietary treatment had high protein level with no calcium salts of fatty acid; HC dietary treatment had high protein level with calcium salts of fatty acids; LN dietary treatment had low protein level with no calcium salts of fatty acids; LC dietary treatment had low protein level with calcium salts of fatty acids.

[2] Every kilogram of mineral–vitamin premix contained: 1000000 IU Vitamin A, 350000 IU Vitamin D3, 5.7 g Zn, 4.2 g Fe, 5.72 g Mn, 2.5 g Cu, 85 mg I, 85 mg Se, and 30 mg Co.

2.3. Fatty acid composition and biochemical indexes of plasma

The long-chain fatty acid composition in the plasma and diets were detected by the gas chromatography (GC-2014 Shimadzu Corporation, Kyoto, Japan) according to the previous study (He et al., 2016). The mixed standard was purchased from the Sigma-Aldrich (product number:18919-1AMP, Sigma Chemical Co., Shanghai, China), which contains 37 kinds of methyl fatty acid from C4 to C24. The soluble C17:0 at 1 mg/mL hexane was used as an internal standard. Fat extraction and fatty acid methyl ester were performed by the following process: 1 mL plasma or about 0.3 g of feed sample was added into a 15 mL glass screw cap tube and freeze-drying at −40 °C, then added 4 mL mixed solution of ethyl chloride and methanol in 1:9 volume ratio, added 2 mL internal standard hexane solution. Then the tube was hot in water bath 2 h at 80 °C, after that it was cooled to room temperature and added 4 mL 100 g/L potassium chloride solution. The upper layer organic liquid was used in the gas chromatograph analysis. The pressure of carrier gas, nitrogen, was controlled at 358.2 K Pa. The sampling amount was 1 μL. The inlet temperature remained at 250 °C and the splitting ratio was controlled at 1:40. The initial oven temperature was set at 140 °C and kept 5 min; and after that the temperature were risen at 3.5 °C /min to 230 °C and kept 15 min. The detector temperature was kept at 280 °C. The fatty acid content was described as the proportion of total fatty acids from 14 to 24 carbons.

The biochemical indexes in the plasma were analyzed through the automatic biochemical analyzer (Hitachi 7020; Hitachi Co., Tokyo, Japan) using the corresponding indexes kits (Beijing Strong Bio-Technique Co., Beijing, China), included glucose (Item number GH121T), triglyceride (Item number GH111Z), cholesterol (Item number GH101Z), high-density lipoprotein cholesterol (HDL-C; Item number GH131Z), urea (Item number GH9311S), alanine transaminase (Item number GH001G), total protein (Item number GH0911G), albumin (Item number GH0921G), low-density lipoprotein cholesterol (LDL-C; Item number GH141Z), alkaline phosphatase (Item number GB040G), aspartate aminotransferase (Item number GH011G).

2.4. Rumen fermentation characteristics

The VFA in the rumen liquid were assayed by the gas chromatograph (GC-2014 Shimadzu Corporation, Kyoto, Japan). The flow rate of carrier gas, nitrogen, was controlled in the column at 46.3 cm/s. The sampling amount was 0.4 μL. The inlet temperature remained at 220 °C and the splitting ratio was controlled at 1:40. The initial oven temperature was set at 110 °C and kept 30 s; and after that the temperature were risen at 10 °C /min to 120 °C and kept 4 min; then the temperature was risen at 10 °C /min to 150 °C. The detector temperature was remained at 250 °C. The ammonia nitrogen (NH_3-N) was quantified using the ultraviolet spectrophotometer (UV-1700, Shimadzu Corporation, Kyoto, Japan) (Chaney and Marbach, 1962). PH value of the rumen liquid was measured by PH meter (205Testo Instrument Co. LTD., Lenzkirsh, Germany).

2.5. Relative abundance of rumen microbiota

The DNA of the rumen microorganisms were extracted and purified by the TIANamp Stool DNA Kit (Tiangen Biotech CO., LTD, Beijing, China). The DNA concentrations were measured and diluted the concentrations at the same level of 30 ng/μL. The real-time quantitative PCR of the rumen microorganisms were carried out by the Stratagene MX3000p (Agilent Technologies, Wilmington, DE, USA) in a 20 μL reaction volumes, which contained 10 μL of 2 × Faststart Universal SYBR Green Master (rox) (Roche Applied Science, Mannheim, Germany) and 2 μL DNA templates. The upstream and downstream primers concentrations were 400 nmol/L, which sequences are shown in Supplementary Table S2 and synthesized by Shenggong (Songon Biotech, Shanghai, China). The conditions of PCR amplification were set as 1 cycle at 95 °C for 10 min, then followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The relative abundance of the rumen microorganism was expressed as the relative amount to the total bacteria and calculated using the following formula (Niu et al., 2018): the relative amount of specific microorganisms $= 2^{-(\text{Ct specific microorganisms} - \text{Ct total bacteria})}$, where Ct corresponds to the cycle threshold of specific microorganisms and total bacteria.

2.6. Statistical analysis

All of the data were analyzed by the proc MIXED of SAS version 9.0 software (SAS Institute Inc., Cary, NC, USA) in the following model: $Y_{ijkl} = \mu + C_i + Dj + P_k + S_l + (PS)_{kl} + e_{ijkl}$, where Y_{ijkl} is the dependent variable, μ is the general mean, C_i is the random effect of period, D*j* is the random effect of cattles, P_k is the fixed effect of CP levls, S_l is the fixed effect of CSFA, $(PS)_{kl}$ is the interaction between CSFA and CP, and e_{ijkl} is the residual effect. Normality of distribution and homogeneity of variance for residuals were tested using proc UNIVARIATE.

3. Results

3.1. Nutrients intake and apparent digestibility

The effects of dietary protein level and CSFA on the nutrients intake of Holstein bulls are presented in Table 2. The high CP level diets significantly increased the CP intake ($P < 0.001$). Dietary supplement with CSFA increased the intake of EE ($P = 0.001$). The high CP level diets significantly increased the apparent digestibility of CP ($P = 0.001$). The CSFA remarkably enhanced the apparent digestibility of OM ($P = 0.012$), and tended to enhance the apparent digestibility of aNDF. The diets with high CP level significantly

Table 2
Effect of dietary treatments on nutrients intake and apparent digestibility in Holstein bulls.

Item	Dietary treatment				SEM	*P*-value		
	HN	HC	LN	LC		CP	CSFA	CP × CSFA
Feed intake (kg/d)								
DM	12.9	12.8	12.8	12.7	0.13	0.50	0.40	0.85
OM	12.2	12.0	12.1	11.9	0.10	0.68	0.37	0.74
CP	1.72	1.66	1.43	1.39	0.02	< 0.001	0.22	0.57
aNDF	6.01	6.03	6.08	6.06	0.07	0.39	0.75	0.36
ADF	2.40	2.35	2.35	2.33	0.02	0.47	0.59	0.78
EE	0.36	0.61	0.35	0.59	0.01	0.89	0.001	0.81
Apparent digestibility (g/kg)								
DM	731	745	731	738	1.54	0.68	0.30	0.14
CP	725	762	678	683	1.96	0.001	0.12	0.49
aNDF	561	590	556	556	1.59	0.092	0.055	0.28
ADF	483	497	498	474	1.79	0.93	0.99	0.30
EE	706[a]	688[ab]	673[b]	685[ab]	1.52	0.087	0.784	0.047
OM	757	776	756	771	1.46	0.79	0.012	0.96
N metabolism (g/d)								
N intake	202	207	158	154	15. 7	< 0.001	0.18	0.15
Fecal N	62.6	57.3	53.8	50.4	3.81	0.094	0.27	0.45
Urinary N	99.9	105	67.7	65. 9	7.43	0.016	0.63	0.21
N retention	40.1	44.6	36.8	37.6	3.17	0.10	0.31	0.55
N utilization	0.203	0.214	0.231	0.242	0.04	0.077	0.26	0.89

HN dietary treatment had high protein level with no calcium salts of fatty acid; HC dietary treatment had high protein level with calcium salts of fatty acids; LN dietary treatment had low protein level with no calcium salts of fatty acids; LC dietary treatment had low protein level with calcium salts of fatty acids.
Means with different superscript letters in the same row are significantly different ($P < 0.05$).
CP: crude protein, DM: dry matter, OM: organic matter, aNDF: neutral detergent fiber, ADF: acid detergent fiber.
N utilization was the rate of N retention to N intake.
The *P*-value of CP, CASF and CP × CSFA were the factorial analysis of crude protein levels, calcium salts of long-chain fatty acids and their interaction.

Table 3
Effects of dietary treatments on rumen fermentation characteristics in Holstein bulls.

Item	Dietary treatment				SEM	*P*-value		
	HN	HC	LN	LC		CP	CSFA	CP × CSFA
pH	6.13	6.81[b]	6.25[a]	6.01[a]	0.08	0.92	0.67	0.34
NH_3-N (mg/dL)	5.30	7.07[b]	5.42[a]	5.43[a]	0.20	0.009	0.003	0.64
VFA(mmol/L)								
Acetate	48.3	49.5	49.6	50.9	2.71	0.76	0.78	0.99
Propionate	11.1	11.1	11.5	11.3	0.56	0.75	0.93	0.90
Isobutyrate	0.352	0.397	0.374	0.375	0.01	0.99	0.23	0.47
Butyrate	9.60	8.28	8.14	8.55	0.41	0.44	0.56	0.27
Isovalerate	0.78	0.92	0.78	0.94	0.04	0.87	0.029	0.84
Valerate	0.51	0.53	0.43	0.47	0.02	0.015	0.31	0.73
TVFA	70.7	70.8	70.8	72.5	3.65	0.87	0.88	0.89
Acetate/Propionate	4.37	4.42	4.27	4.43	0.08	0.71	0.43	0.65

HN dietary treatment had high protein level with no calcium salts of fatty acid; HC dietary treatment had high protein level with calcium salts of fatty acids; LN dietary treatment had low protein level with no calcium salts of fatty acids; LC dietary treatment had low protein level with calcium salts of fatty acids.
The *P*-value of CP, CASF and CP × CSFA were the factorial analysis of crude protein levels, calcium salts of long-chain fatty acids and their interaction.
TVFA was the sum of acetate, propionate, isobutyrate, butyrate, isovalerate and valerate.

increased the N intake ($P < 0.001$) and urinary N ($P = 0.016$), tended to reduce the N utilization. The two factors of the dietary treatments had an interaction in the apparent digestibility of EE ($P = 0.047$), the EE apparent digestibility of HN group was significantly higher than that in LN group.

3.2. Rumen fermentation characteristics

In this study, the pH value and the NH_3-N concentration of HC group were significantly higher than those of the other dietary treatments as shown in Table 3. NH_3-N was affected by the diet composition, as high CP diets and CSFA significantly increased its concentration in the rumen fluid ($P = 0.009$). Acetate, propionate and butyrate, which are the main compositions of volatile fatty acids, were unaffected by the experimental factors, as well as the concentration of isobutyrate. The CSFA remarkably enhanced the concentration of isovalerate in the rumen fluid ($P = 0.029$). The diets with high levels CP remarkably improved the valerate concentration in the rumen ($P = 0.015$).

3.3. Rumen microbial flora

Effects of the dietary CP levels and CSFA on the rumen microorganisms are shown in Table 4. There was no significant difference among the relative abundance of the total fungi, *Streptococcus bovis*, *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Prevotella bryantii*. The diets with low CP level significantly decreased the abundance of *Butyrivibrio fibrisolvens* ($P = 0.037$) and *Megasphaera*

Table 4
Effects of dietary treatments on rumen microbial population in Holstein bulls.

Item	Dietary treatment				SEM	*P*-value		
	HN	HC	LN	LC		CP	CSFA	CP × CSFA
Butyrivibrio fibrisolvens ($\times 10^{-3}$)	2.93	1.95	1.46	1.31[c]	0.26	0.037	0.24	0.14
Fibrobacter succinogenes ($\times 10^{-3}$)	6.97	4.31	7.38	7.29	1.00	0.43	0.51	0.44
Total fungi ($\times 10^{-4}$)	0.763	1.09	2.42	1.28	0.36	0.21	0.58	0.70
Streptococcus bovis ($\times 10^{-4}$)	1.37	1.33	1.42	1.26	0.31	0.30	0.20	0.41
Ruminococcus.flavefaciens ($\times 10^{-4}$)	1.69	1.51	1.07	0.774	0.73	0.19	0.40	0.65
Ruminobacter amylophilus ($\times 10^{-4}$)	5.09	6.22	12.7	11.1	2.03	0.063	0.65	0.21
Ruminococcus.albus ($\times 10^{-4}$)	1.46	1.26	0.847	3.16	0.46	0.52	0.26	0.14
Protozo, ($\times 10^{-2}$)	2.62	2.42	4.61	2.13	0.6	0.49	0.29	0.23
Prevotella bryantii ($\times 10^{-3}$)	1.54	1.79	1.57	1.34	0.49	0.46	0.26	0.60
Methanogens ($\times 10^{-4}$)	3.44	1.59	2.03	1.68	0.30	0.21	0.047	0.33
Megasphaera elsdenii ($\times 10^{-7}$)	4.16	12.6	2.43	3.24	1.99	0.023	0.16	0.10

HN dietary treatment had high protein level with no calcium salts of fatty acid; HC dietary treatment had high protein level with calcium salts of fatty acids; LN dietary treatment had low protein level with no calcium salts of fatty acids; LC dietary treatment had low protein level with calcium salts of fatty acids.
The *P*-value of CP, CASF and CP × CSFA were the factorial analysis of crude protein levels, calcium salts of long-chain fatty acids and their interaction.

Table 5
Effects of dietary treatments on plasma biochemical indexes in Holstein bulls.

Item	Dietary treatment				SEM	*P*-value		
	HN	HC	LN	LC		CP	CSFA	CP × CSFA
Glucose (mmol/L)	3.78	4.02	3.48	4.01	0.29	0.78	0.51	0.80
Triglyceride (mmol/L)	0.114	0.121	0.128	0.172	0.01	0.14	0.25	0.42
Cholesterol (mmol/L)	2.22	2.72	2.24	2.64	0.11	0.77	0.027	0.72
Alanine transaminase (U/L)	19.8	17.9	23.0	21.0	1.71	0.25	0.46	0.99
Total protein (g/L)	48.0	43.4	50.1	40.7	3.69	0.96	0.22	0.67
Albumin (g/L)	25.4	23.2	26.3	23.3	1.80	0.86	0.32	0.87
Urea(mmol/L)	2.99	2.93	2.37	2.09	0.20	0.008	0.51	0.66
HDL-C(mmol/L)	1.30	1.40	1.32	1.49	0.09	0.67	0.32	0.77
LDL-C(mmol/L)	1.30	1.83	1.26	1.79	0.10	0.81	0.019	0.99
Alkaline phosphatase (U/L)	155	155	178	153	6.59	0.25	0.17	0.18
Aspartate aminotransferase (U/L)	0.147	0.115	0.139	0.103	0.01	0.58	0.083	0.92

HN dietary treatment had high protein level with no calcium salts of fatty acid; HC dietary treatment had high protein level with calcium salts of fatty acids; LN dietary treatment had low protein level with no calcium salts of fatty acids; LC dietary treatment had low protein level with calcium salts of fatty acids.
HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, SEM: standard error of the mean.
The *P*-value of CP, CASF and CP × CSFA were the factorial analysis of crude protein levels, calcium salts of long-chain fatty acids and their interaction.

elsdenii ($P = 0.023$), and tended to reduce the relative abundance of *Ruminobacter amylophilus*. The CSFA significantly decreased the relative abundance of methanogens in the rumen ($P = 0.047$).

3.4. Plasma biochemical indexes

The effects of diets on biochemical indexes are presented in Table 5. Dietary supplement with CSFA significantly improved the concentration of cholesterol ($P = 0.027$) and LDL-C in plasma ($P = 0.019$), and tended to reduce the activity of aspartate aminotransferase. Diets with low CP reduced the concentration of urea in plasma ($P = 0.008$). However, the other measured biochemical parameters, such as the glucose, triglyceride, alanine transaminase, total protein, albumin, HDL-C and alkaline phosphatase were not affected by the protein levels and the CSFA.

3.5. Fatty acid composition in plasma

The results of the fatty acid profile of plasma are presented in Table 6, which depicted that dietary protein levels had no effects on the plasma fatty acids composition. Dietary supplement with CSFA remarkably improved the percentage of C18:1n9c ($P = 0.005$), C18:3n6 ($P = 0.034$), and index of Δ^9 desaturase C18 ($P < 0.001$). It also tended to reduce the percentage of C14:0, C15:0, C18:0, C18:2n6t and C21:0, and tended to increase the percentage of C20:3n6 and n-6 to n-3 ratio. However, it also remarkably decreased the percentage of C20:1 ($P = 0.023$) and C22:0 ($P = 0.001$). There was an interaction effect between the CP level and the CSFA on the plasma C15:1. The proportion of C15:1 in HN and LC groups was remarkably lower than that in LN group.

4. Discussion

The high CP diets improved the apparent digestibility of CP, which was similar to the result of Menezes (Menezes et al., 2016), who suggested that the apparent digestibility of CP had a linear relationship with the protein level (100, 120 and 140 g/kgDM), and the apparent digestibility of DM, OM, EE and aNDF were not affected by the diet CP levels. Similar effects are also reported for CP levels at 90, 110, 130, and 150 g/kg dry matter (Obeid et al., 2006). Previous studies have shown that the high ammonia in the rumen can promote the growth of microorganisms, then the microorganisms can increase the protein degradation of diets, resulting in increasing the apparent digestibility of protein and the concentration of rumen ammonia nitrogen (Hristov et al., 2002). High ammonia nitrogen also promoted the excretion of urine nitrogen (He et al., 2018). Therefore, there were positive correlations among dietary protein level, rumen urea nitrogen and urinary nitrogen by the increased microbial growth and the apparent digestibility of proteins. The CSFA improved the apparent digestibility of OM because dietary supplementation with the CSFA increased the proportion of digestible organics and the possibly appropriate crude proteins metabolism in this study. Meanwhile, the tendency of CSFA to improve the apparent digestibility of aNDF also supported the above conclusion, because the aNDF is a part of the OM in the feed. The CSFA can slow down the rate of feeding through the rumen (Ngidi et al., 1990), resulting in the increased apparent digestibility of the OM. The absorption and transport of fatty acid in intestinal tract needs lipoprotein to mobilize, and dietary added with CSFA requires large amount of amino acids to synthesize the lipoproteins transport system in mobilization (Garcia Bojalil et al., 1998). Therefore, there were interaction effects between the CP level and the CSFA on the EE apparent digestibility and fecal N in this study.

Urea nitrogen is the main end-product of protein metabolism in the liver (Eisemann and Tedeschi, 2016). High CP intake does not result in a buildup or storage in the body as the unutilized protein will be excreted out of the body in the form of urine nitrogen,

Table 6
Effects of dietary treatments on the proportion of fatty acid in plasma in Holstein bulls (g/100 g total fatty acids).

Item	Dietary treatment				SEM	*P*-value		
	HN	HC	LN	LC		CP	CSFA	CP × CSFA
C14:0	0.398	0.364	0.448	0.354	0.03	0.57	0.084	0.95
C14:1	0.452	0.365	0.605	0.444	0.05	0.60	0.20	0.90
C15:0	0.338	0.217	0.423	0.274	0.04	0.99	0.085	0.70
C15:1	0.219[a]	0.487[ab]	0.665[b]	0.318[a]	0.05	0.24	0.19	0.002
C16:0	9.09	10.00	9.42	9.61	0.39	0.75	0.51	0.84
C16:1	0.458	0.388	0.558	0.390	0.05	0.94	0.10	0.87
C17:1	0.172	0.180	0.283	0.113	0.04	0.88	0.17	0.62
C18:0	19.0	19.1	20.2	17.3	0.40	0.89	0.074	0.057
C18:1n9t	0.496	0.432	0.361	0.270	0.04	0.12	0.26	0.51
C18:1n9c	7.81	9.61	8.36	8.79	0.24	0.91	0.005	0.34
C18:2n6t	0.532	0.325	0.436	0.333	0.05	0.38	0.062	0.53
C18:2n6c	49.2	49.1	47.7	51.1	0.82	0.80	0.15	0.50
C20:0	0.287	0.185	0.218	0.155	0.06	0.34	0.37	0.75
C18:3n6	0.069	0.115	0.174	0.221	0.03	0.066	0.034	0.68
C20:1	2.65	2.26	2.47	2.15	0.15	0.18	0.023	0.66
C18:3n3	3.04	3.14	3.23	3.01	0.01	0.31	0.33	0.32
C21:0	0.151	0.000	0.112	0.024	0.03	0.61	0.059	0.41
C20:2	0.160	0.098	0.207	0.049	0.05	0.19	0.11	0.86
C22:0	0.374	0.198	0.422	0.258	0.03	0.25	0.001	0.70
C20:3n6	0.734	1.21	0.812	0.807	0.12	0.29	0.050	0.12
C22:1n9	1.33	1.22	1.39	1.32	0.05	0.31	0.20	0.98
C20:3n3	0.165	0.116	0.163	0.158	0.03	0.65	0.46	0.86
C22:2	0	0	0.081	0	0.02	0.34	0.36	0.33
C24:0	1.46	0.219	0.222	0.675	0.23	0.51	0.38	0.13
C20:5n3	0.440	0.238	0.329	0.340	0.05	0.99	0.18	0.26
C24:1	0.300	0.201	0.215	0.224	0.04	0.66	0.35	0.34
C22:6n3	0.691	0.179	0.421	1.31	0.29	0.23	0.72	0.72
ΣSFA	31.0	30.3	31.4	28.6	0.68	0.60	0.22	0.57
ΣMUFA	14.0	15.1	15.0	14.0	0.28	0.57	0.76	0.40
ΣPUFA	55.0	54.5	53.6	57.4	0.85	0.55	0.27	0.48
ΣUFA	69.0	69.7	68.6	71.4	0.68	0.60	0.22	0.57
ΣMUFA/ΣSFA	0.456	0.501	0.482	0.498	0.01	0.77	0.28	0.99
ΣPUFA/ΣSFA	1.80	1.81	1.75	2.09	0.06	0.40	0.25	0.44
Σn-3	4.43	3.63	4.01	4.91	0.32	0.23	0.55	0.60
Σn-6	50.5	50.8	49.2	52.5	0.86	0.86	0.13	0.59
Σn-6/Σn-3	12.0	14.2	12.9	12.5	0.60	0.40	0.096	0.43
Δ9 desaturase 16	4.77	3.87	5.42	4.83	0.49	0.63	0.31	0.69
Δ9 desaturase 18	30.5	34.5	30.0	34.3	0.71	0.64	< 0.001	0.32
Elongase	68.6	68.4	68.8	67.4	0.53	0.62	0.35	0.26

HN dietary treatment had high protein level with no calcium salts of fatty acid; HC dietary treatment had high protein level with calcium salts of fatty acids; LN dietary treatment had low protein level with no calcium salts of fatty acids; LC dietary treatment had low protein level with calcium salts of fatty acids.

The *P*-value of CP, CASF and CP × CSFA were the factorial analysis of crude protein levels, calcium salts of long-chain fatty acids and their interaction.

ΣSFA was the sum of saturated fatty acid; ΣMUFA was the sum of monounsaturated fatty acid; ΣPUFA was the sum of polyunsaturated fatty acid; Σn-3 was the sum of (C18:3n3 + C20:3n3 + C20:5n3 + C22:6n3); Σn-6 was the sum of (C18:2n6t + C18:2n6c + C18:3n6 + C20:3n6); Δ^9 desaturase C16 was calculated by 100 × [(C16:1n9c) / (C16:1n9c + C16:0)]; Δ^9 desaturase C18: 100 × [(C18:1n9c) / (C18:1n9c + C18:0)]; Elongase was calculated by 100 × [(C18:0 + C18:1n9c) / (C16:0 + C16:1n9c + C18:0 + C18:1n9c)].

resulting in protein waste and environmental pollution. Urinary N excretion increases when the concentration of ammonia in the rumen exceeds the amount required by rumen microorganisms to synthesize the microbial protein (Russell et al., 1992). However, ruminants can control the quantity of urine nitrogen and nitrogen circulating into the rumen to achieve a sustained nitrogen supply for microbial growth (Eisemann and Tedeschi, 2016). The urinary N and the plasma urea had the same trend with the increase of dietary protein level in this study, which verifies the proposition that the blood urine nitrogen content is the main factor that influences the urine nitrogen (Harmeyer and Martens, 1980). Nitrogen retention was not reduced when feeds dietary with lower protein levels were administered. Therefore, we suggest a reduction in the diet protein levels in fattening Holstein bulls to conserve protein resources and reduce the environmental pollution.

The present studies show that the dietary protein levels and CSFA have no significant effect on the rumen pH, because the long-chain fatty acids released from the incomplete inert CSFA did not change the pH in the rumen fluid. Ammonia in the rumen is used by microorganisms to synthetic proteins, and the normal concentration of ammonia in the rumen varies from 5.0 to 30.0 mg/dL (Mehrez et al., 1977). The ammonia deficiency can inhibit microbial growth and fermentation. Usually, improved rumen ammonia denotes that an adequate part of proteins had been degraded to satisfy the requirement of microorganism growth (Pina et al., 2009). In

general, the increased concentration of ammonia in the rumen increases the pH of the rumen. The HC diet had the highest ammonia nitrogen concentration among the four groups, resulting in a significantly higher pH value compared with other groups. In the current study, the high CP diets increased the concentration of NH_3-N in the rumen. When considered the CP level in diet had no effect on N retention that can arrive at an inference that the low CP diets can provide sufficient ammonia for rumen microorganisms, which also indicated that there was nitrogen waste in the high CP diets. Dietary supplementation with CSFA increased the concentration of NH_3-N in this study, and this can be attributed to CSFA's ability to improve the microbial activity of the rumen and enhance the fermentation efficiency, because it also increases the OM and aNDF apparent digestibility (Broudiscou et al., 1994). Previous studies have found that when the ratio of total digestible nutrients to protein is less than 7, indicating that the dietary protein can meet the requirements of microorganisms (Moore et al., 1999). The apparent digestibility of CP will increase if the ratio is raised, resulting in increasing the NH_3-N concentration. The supplementation of CSFA can increase the total digestible nutrients of diets to raise the ratio in the high protein diets, which leaded the increase of the NH_3-N concentration and the pH in the rumen.

In the present study, the dietary protein levels and supplemental CSFA had no significant effects on the main volatile fatty acids (acetate, propionate, isobutyrate, butyrate, TVFA and acetate to propionate ratio) in the rumen, and these results were consistent with the previous reports (Da Silva et al., 2016; Ngidi et al., 1990). This suggested that diet treatments did not alter the main fermentation characteristics of the rumen. It also indicated that the CSFA was less released in the rumen and were not degraded by the rumen microbe. Ammonia stimulates activity of the rumen bacterial strains of *Megasphaera elsdenii*, which can produce butyrate and valerate by degrading lactate and glucose, resulted in elevating the concentrations of butyrate and valerate (Leeuw et al., 2016). However, this experiment only observed that the high CP diets can significantly increase the valerate concentration.

As one of the main bacterial flora in the rumen, *Butyrivibrio fibrisolvens* plays an important role in the hydrolysis of lignocelluloses (Forster et al., 1997) and bio-hydrogenation of unsaturated fatty acids (Maia et al., 2010). Isolated and cultured *Butyrivibrio fibrisolvens* showed that the linoleic acid inhibited the growth of it, but this inhibition was related to the concentration of linoleic acid and growth status (Kim et al., 2000). In this study, supplemental CSFA did not inhibit *Butyrivibrio fibrisolvens* growth, indicating that CSFA were able to resist the biological hydrogenation of rumen microorganisms. The high CP diets were beneficial for the *Butyrivibrio fibrisolvens* growth because of the greater level of ammonia in the rumen.

The CH_4 release is not only harmful to the environment, but also a waste of energy by relating to carbon loss in the rumen (Johnson and Johnson, 1995). Methanogens are positively correlated with the CH_4 production (Patra et al., 2017), and in this experiment, supplemental CSFA reduced the relative number of methanogens in the rumen, suggesting that it may reduce CH_4 emissions. This also shown that dietary CP levels have no effect on the production of CH_4 in the rumen.

The *Streptococcus bovis* has been associated with ruminal acidosis as a major pathogenic factor, because of its rapid generation on the starch-based substrate, producing the lactic acid as a primary fermentation product (Klieve et al., 2003). In the present study, the relative quantities of *Streptococcus bovis* in each treatment group were not different, and the rumen pH was also within the normal range, indicating that dietary protein levels and CSFA did not induce abnormal fermentation of the rumen that causes acidosis.

Fatty acids and ammonia are transported to bloodstream in duodenum and rumen epithelium, respectively. The plasma ammonia passes through the hepatic portal vein into the liver where it was converted into urea (Lobley et al., 1995). Previously, we have explained that the increase in urinary nitrogen is due to the elevated plasma urea (He et al., 2017). However, the increase of plasma urea had a strong correlation with the increase of ammonia concentration in the rumen (Hammon et al., 2005). Therefore, high protein level in diet can increase the amount of ammonia produced by rumen microbial degradation of proteins, and the ammonia can enter the liver to synthesize urea, resulting in increased concentration of urea nitrogen in plasma and urinary nitrogen excretion.

Dietary supplement with CSFA altered the lipid metabolism in bulls. Previous studies showed that dietary supplements of CSFA could increase the content of fatty acids and triglycerides in the liver (Do Prado et al., 2016). In consistent with our findings, Talavera et al. (1985) reported that other detected plasma biochemical parameters were not influenced by the dietary CSFA, except for the cholesterol and LDL-C. The fat metabolism in the liver is related to the dietary fat content. The increased cholesterol and LDL-C concentrations in plasma were the result of CSFA degradation in the abomasum after bypassing the rumen and absorbed in the small intestine.

The protein level in diets had no effects on the composition of plasma fatty acids.

There was no evidence that dietary protein levels have an impact on fat metabolism. Although diets supplementation with CSFA contained higher C16:0, it did not cause a significant increase in the proportion of C16:0 in the plasma. Supplemental CSFA increased the proportion of C18:1n9c and reduced the proportion of C20:1 in plasma, which was consistent with the dietary ingredients. Cooke et al. (2014) reported that calcium salts of soybean oil in *Bos indicus* beef cows have similar results in the plasma. At the same time, CSFA increased the Δ^9 desaturase C18, which could lead to the conversion of saturated fatty acids C18:0 into monounsaturated fatty acids C18:1n9c. Therefore, we proposed to use the CSFA, so that the bio-hydrogenation of the rumen tiny organisms can be reduced while the unsaturated fatty acids can be absorbed into the animal blood.

5. Conclusion

Based on the results of our observations, we suggested that the low protein diets reduced protein waste and environmental pollution in the final stage of fattening Holstein cattle at 20 months. However, high CP diets increased *Butyrivibrio fibrisolvens* and *Megasphaera elsdenii* population, which participate in bio-hydrogenation and cellulolytic. Dietary supplementation with CSFA enhanced the OM apparent digestibility and the isovalerate concentration. It also can partially improve the percentage of unsaturated fatty acids in plasma, but not influence the proportion of C16:0 and C18:2n6c. The CSFA inhibited the methanogens population. Further study needs to be done to investigate the microbial mechanism of the CSFA effect on the CH_4 emission in the rumen.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anifeedsci.2018.09.019.

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