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Dietary leucine supplementation enhances the health of early weaned Hu lambs

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ABSTRACT

This study investigated the effects of leucine supplementation on the performance of pre- and post-weaning of early weaned Hu lambs. Thirty lambs of five-day-old, with an average body weight of 3.60 ± 0.73 kg, were assigned randomly to the control or leucine group, with each group having 5 replicate pens. Lambs in the leucine group received 7.1 g/kg total feed dry matter leucine in milk replacer from 10 days of age onward. Blood samples were collected from one lamb of each replicate randomly to determine plasma metabolites, oxidative stress indicators and immunize variables when lambs were early weaned at 30 days of age. After weaning, one lamb of each replicate was kept in their same pen and fattened to 120 days old. These lambs (5 lambs per treatment) were slaughtered to determine the carcass traits and drip loss of muscle. Growth performance before weaning did not differ ($P > 0.05$) between the control and leucine group. Leucine supplementation increased ($P < 0.05$) plasma concentrations of asparagine, isoleucine and leucine, and total antioxidant capacity and glutathione peroxidase activity. Supplementation of leucine decreased ($P < 0.05$) concentrations of blood urea nitrogen, blood ammonia, and D-lactate. Compared to the control group, the leucine group achieved a higher dressing percentage ($P < 0.05$). Furthermore, leucine supplementation decreased ($P < 0.05$) the drip loss of mutton at both 24h and 48h. Collectively, these results indicate that leucine supplementation can enhance the antioxidant and immune status and help maintain the health of early weaned Hu lambs.

1. Introduction

Early weaning has many advantages, such as improved growth, feedlot performance (Myers et al., 1999a), and carcass quality (Myers et al., 1999b). However, stress associated with early weaning is an important challenge in ruminant feeding, which is reflected as decreased feed intake, body weight (BW) loss (Budzynska and Weary, 2008; Weary et al., 2008; de Passillé et al., 2011), diarrhea (Khan et al., 2007), and other adverse physiological responses. Early weaning was also shown to impact the expression of genes related to intestinal barrier function (Li et al., 2018). In our previous study (unpublished data), we found significantly higher (P = 0.002) plasma D-lactate concentrations in lambs at day 32 of age (1 d after early weaning, 1023 ng/ml) and day 35 of age (4 d

Abbreviations: AA, amino acid; ADF, acid detergent fiber; ADG, average daily gain; AOAC, Association of Official Agricultural Chemists; BUN, blood urea nitrogen; BW, body weight; CP, crude protein; DM, dry matter; GR, girth rib; GSH-PX, glutathione peroxidase; MR, milk replacer; NDF, neutral detergent fiber; T-AOD, total antioxidant capacity

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after early weaning, 1080 ng/ml) compared to lambs at day 30 of age (early weaning day, 920 ng/ml). D-lactate is negatively related to the intestinal permeability, and its concentration can increase when intestinal permeability is damaged due to weaning stress (Xiao et al., 2014). Therefore, stress caused by early weaning in lambs probably leads to intestinal barrier dysfunction and oxidative damage.

Leucine as a branched-chain amino acid (AA) is nutritionally essential for mammals. Recent studies have shown that leucine is not only a nutrient substrate for cellular metabolism but also a signal molecule, capable of mediating a range of biological processes, including protein metabolism, lipid decomposition, and insulin secretion (Hu et al., 2017; Pedroso et al., 2014; Liu et al., 2015). Furthermore, leucine has been recognized as a potent antioxidant able to scavenge nitrite radicals (Jin et al., 2015). Indeed, Hu et al. (2017) found that leucine supplementation decreased cellular reactive oxygen species levels. However, little information is available on the effect of leucine on the growth of early-weaned ruminants.

As compared to adult ruminants, suckling ruminants have underdeveloped forestomach, and their digestive tract is similar in function as monogastric animals. Thus they can directly use nutrients such as AA for their own growth and development more effectively. This study tested the hypothesis that leucine supplementation could alleviate the oxidative stress associated with early weaning, and improve the growth performance of early weaned lambs.

2. Materials and methods

2.1. Animals, feeds and experimental design

All experimental protocols involving animals were approved by the Animal Use and Care Committee, Zhejiang A & F University, and the experimental procedures used in this study were in accordance with the university's guidelines for animal research. The experiment was performed using Hu lambs at a breeding farm in Zhejiang Province of China.

Thirty healthy male Hu lambs with a mean BW of 3.60 ± 0.73 kg at the age of 5 days were randomly allotted to control (Control, no leucine supplementation) or leucine-supplemented groups (Leucine), with each group having 15 lambs. Three lambs with similar BW within each group were placed and housed in one pen (1.0 × 1.0 m), which was considered as an experimental replicate. Thus, there were five replicates in each treatment (n = 5). All the lambs were fed with a milk replacer (MR, Hangzhou Swot ASTCo., Ltd, China; Table 1) for 5 days before the designated feeding treatments. The amount of 150 g MR powder was diluted to 1000 ml water (39 °C) and was fed via baby bottles. From day 11–30, the lambs were fed either with the MR (Control, no leucine) or with the MR supplemented with leucine (7.1 g/kg total feed dry matter, DM) thrice daily. Leucine (99.8 g/100 g purity. King Technology Feed Co., Ltd, Hangzhou, China) was mixed into the MR fed only in the morning each day. The leucine dose was calculated based on the leucine percentage in dam's milk (Jandal, 1996; Rafiq et al., 2016) and the results of our preliminary study (unpublished data), which showed that oral administration of 14.2 g/kg total feed DM leucine had negative effect on daily BW gain among lambs aged 10–30 days. In addition to the MR, the lambs also had *ad libitum* access to pellets of a starter and hay of Chinese wild rye (*Aneurolepidium Chinese Kitagawa*) (Table 1) chopped into 6–8 cm lengths until weaning at d 30. To stimulate consumption of the starter and the Chinese wild rye hay, MR was given at 780 mL/lamb/day (based on consumption in the adaptation period) at d 11 and then reduced by 30 mL/lamb/day until reaching 210 mL/lamb/day at d 30 when the lambs were weaned. The amount of feed offered and refused, and the BW were recorded every ten days (day 10, 20 and 30 of age). Body weight of individual lamb was recorded before the morning feeding. Each measurement was recorded for two consecutive days.

After weaning, one weaned lamb was kept in their same pen for further fattening trial after other two lambs were removed. Lambs were (ad libitum) fed a concentrate feed, and a forages mixture of bamboo shoot shell silage and soybean straw (1 : 1 ratio). The chemical compositions of the concentrate feed, bamboo shoot shell silage, and soybean straw are shown in Table 1. The concentrate feed and the forages mixture were offered separately at the same time. Orts of the feeds were removed from bins and troughs at

Table 1
Chemical composition of the feeds (g/kg, DM basis).

Items[a]	Pre-weaning feed			Post-weaning feed		
	Milk replacer	Starter pellets[b]	Chinese wild rye hay	Concentrate Feed[c]	Bamboo shoot shell silage	Soybean straw
DM	945	914	929	882	966	889
Crude protein	184	174	84.9	151	102	82.1
NDF	36	169	659	396	701	610
ADF	9.0	73	384	219	364	478
Calcium	7.2	12.1	2.4	10.3	4.6	2.8
Phosphorus	6.3	5.9	1.0	4.5	2.7	1.5
Ash	41	73	60	92	88.5	80.3

[a] DM = dry matter; NDF = neutral detergent fiber, assayed without a heat stable amylase and expressed inclusive of residual ash; ADF = acid detergent fiber, expressed inclusive of residual ash.

[b] The starter pellets consisted of (per 100 g DM): 58.5 g corn, 10 g wheat meal, 27 g soybean meal, 1.1 g $CaHCO_3$, 1.4 g limestone, 1 g NaCl, and 1 g vitamin-mineral premix.

[c] The concentrate feed consisted of (per 100 g DM): 45 g corn grain, 20 g cottonseed cake, 15 g soybean meal, 15 g wheat bran, 2 g NaCl, 1 g $NaHCO_3$, and 2 g $CaHPO_4$.

0800 h each day, with new feed delivered three times daily at 0830, 1200 and 0430 h. All lambs had free access to water. For the fattening growth data, the amount of feed offered and refused and the BW were recorded every fifteen days.

At the end of the feeding trial, lambs in both groups were slaughtered at a local slaughterhouse to measure carcass weight and drip loss of meat. Briefly, lambs were stunned by captive bolt and exsanguinated according to the Animal protection law of the People's Republic of China (2009).

2.2. Feed sample collection and analysis

Feed samples were dried in a forced-air oven at 65 °C for 48 h and stored in sealed plastic containers at 4 °C until analysis. All feed samples were ground to allow passage through a 1-mm sieve (HK-08 A ground mill; Xu Lang Machinery, China) before analysis for DM (method 924.05; Association of Official Analytical Chemists (AOAC, 1990), CP (method 988.05; Association of Official Analytical Chemists (AOAC, 1990), acid detergent fiber (ADF expressed inclusive of residual ash, method 973.18; Association of Official Analytical Chemists (AOAC, 1990), ash (method 942.05; Association of Official Analytical Chemists (AOAC, 1990), calcium (method 927.02; Association of Official Analytical Chemists (AOAC, 1990), and phosphorus (Combs and Satter, 1992). Neutral detergent fiber (NDF assayed without a heat stable amylase and expressed inclusive of residual ash) was determined according to procedure described by Mertens (2002).

2.3. Blood sample collection and analysis

On the weaning day (d 30), one lamb per pen were selected randomly to collected blood samples. Approximately 10 ml blood samples were collected from the jugular vein into individual heparinized tubes (5 lambs per treatment). The blood samples were then centrifuged at 3, 000 × *g* at 4 °C for 15 min to obtain the plasma. Plasma samples were stored at −20 °C until analysis.

Concentrations of total protein, albumin, total glucose, total cholesterol, low density lipoprotein cholesterol, blood urea nitrogen (BUN), blood ammonia, total antioxidant capacity (T-AOC), malondialdehyde, and H_2O_2, and activities of superoxide dismutase, glutathione peroxidase (GSH-PX), and catalase were determined using commercial respective kits (Jiancheng, Nanjing, China). Concentrations of D-lactate, interleukin-6, cortisol, growth hormone, insulin-like growth factor, and tumor necrosis factor-α were measured using respective enzyme-linked immunosorbent assay kits (Bangyi, Shanghai, China).

Concentrations of AA in plasma samples were determined using reversed-phase HPLC after derivatization with o-phthaldialdehyde as described by Dai et al., (2014).

2.4. Analysis of carcass traits and drip loss

At the age of 120 days, the carcass traits and drip loss were evaluated according to the carcass and meat measurements (Fisher and de Boe, 1993) after lambs were slaughtered (5 lambs per treatment). The girth rib (GR) value (the depth of muscle and fat tissue from the surface of the carcass to the lateral surface of the twelfth rib 110 mm from the midline) was directly measured using a GR knife. Drip loss of a 2.5 cm thick meat sample was recorded after being kept at 4 °C for 24 h and 48 h in a plastic container with a double bottom (Russo et al., 2003).

2.5. Statistical analyses

The data were statistically analyzed as a completely randomized design. Each pen with 3 lambs was considered as one experimental unit for the analysis of feed intake (starter pellets and Chinese wild rye hay) and BW before weaning, while one lamb from each pen was regarded as one experimental unit for the analysis of feed intake (concentrate and forage mixture), BW after weaning, plasma variables, carcass traits, and drip loss. Data were analyzed using the PROC GLM procedure of SAS (1999). Statistical significance was declared at $P \leq 0.05$.

3. Results

3.1. Growth performance

All lambs showed no signs of illness or abnormal behavior irrespective of leucine supplementation (Table 2). Feed intake, BW, or daily weight gain did not differ ($P > 0.05$) between the control and the leucine groups at the three analyzed ages before weaning.

3.2. Concentrations of AA, other metabolites, oxidative stress indicators, and immunity variables in the plasma

In total, 17 A A were detected in the plasma sample, and leucine supplementation increased the concentrations of asparagine ($P < 0.05$), isoleucine ($P < 0.05$), and leucine ($P < 0.01$) by 11.7, 7.9, and 75.9%, respectively, while decreasing the concentration of glycine ($P < 0.01$), alanine ($P < 0.05$), and methionine ($P < 0.05$) by 9.6, 10.4, and 14.3%, respectively (Table 3). The plasma concentration of other AA (including threonine, serine, glutamine, cysteine, valine, tyrosine, phenylalanine, lysine, histidine, arginine, and proline) was not affected by the leucine supplementation.

Plasma concentration of total protein, albumin, total glucose, total cholesterol, or low-density lipoprotein cholesterol did not

Table 2
Effect of leucine supplementation on the growth of early weaned male Hu lambs before weaning.[a]

Variables	Treatments		SEM	*P*-value
	Control	Leucine		
Body weight, kg				
Day 10	4.66	4.70	0.259	0.913
Day 20	5.11	5.22	0.201	0.725
Day 30	6.03	5.90	0.146	0.652
Average daily gain, g/d				
Day11-20	45.4	51.7	9.20	0.645
Day21-30	91.4	67.7	10.5	0.150
Day11-30	68.4	59.7	10.7	0.582
Starter intake, g/d				
Day11-20	96.7	100	4.41	0.608
Day21-30	157	155	9.4	0.856
Chinese wild rye intake, g/d				
Day11-20	1.67	1.83	0.118	0.347
Day21-30	2.67	3.00	0.186	0.242

[a] Number of pens per treatment = 5, number of male Hu lambs per pen = 3.

Table 3
Effect of leucine supplementation on the plasma concentrations (μmol/L) of amino acids in early weaned male Hu lambs (day 30 of age).[a]

Variables	Treatments		SEM	P-value
	Control	Leucine		
Asparagine	72.0	80.4	1.85	0.013
Threonine	582	585	9.8	0.846
Serine	140	145	3.6	0.304
Glutamine	60.2	54.8	2.21	0.123
Glycine	794	718	8.7	< 0.001
Alanine	202	181	4.5	0.012
Cysteine	8.4	9.2	0.67	0.424
Valine	299	299	6.9	0.969
Methionine	48.8	41.8	1.88	0.030
Isoleucine	189	204	3.2	0.013
Leucine	374	658	15.1	< 0.001
Tyrosine	29.4	29.6	1.29	0.915
Phenylanaline	92.0	97.8	2.37	0.122
Lysine	30.6	28.6	1.73	0.439
Histidine	42.6	39.8	1.69	0.275
Arginine	347	337	6.5	0.277
Proline	120	92.5	14.5	0.220

[a] Number of male Hu lambs per treatment = 5.

differ ($P > 0.05$) between the control and leucine groups (Table 4). However, the concentrations of BUN and blood ammonia were lower ($P < 0.05$) in the leucine group than in the control group. The activities of T-AOC ($P < 0.05$) and GSH-PX ($P < 0.01$) of plasma were enhanced by leucine by 80 and 48%, respectively, while the plasma H_2O_2 concentration was lowered by 63%. The supplementation of leucine decreased ($P < 0.05$) D-lactate concentration but increased the concentration of interleukin-6 in the plasma. The other immune variables analyzed were not different ($P > 0.05$) between the two groups (Table 4).

3.3. Carcass traits and drip loss

No significant difference ($P > 0.05$) in the carcass weight was noted between the control and leucine groups (Table 5). However, dressing percent increased ($P < 0.05$) in the leucine group compared with the control group. Furthermore, the leucine supplementation decreased the drip loss of mutton both at 24h ($P < 0.05$) and 48 h ($P < 0.01$).

4. Discussion

As one of the essential branched-chain AAs, leucine can activate the target of rapamycin to stimulate protein synthesis and inhibit proteolysis in mammals (Wu, 2010). Several studies examined the effect of leucine supplementation on growth of nursery pigs (Edmonds and Baker, 1987) and finishing pigs (Hyun et al., 2003). When supplemented at 40 g/kg of DM, leucine did not affect the growth of nursery pigs, while at 60 g/kg of DM it depressed the growth of nursery pigs (Edmonds and Baker, 1987). In finishing pigs,

Table 4
Effect of leucine supplementation on the plasma concentrations of biochemical, oxidative stress indicators and immunity variables in early weaned male Hu lambs (day 30 of age).[a]

Variables[b]	Treatments		SEM	P-value
	Control	Leucine		
Biochemical				
T-CHO, mmol/L	2.12	1.42	0.446	0.303
TG, mmol/L	1.09	1.49	0.243	0.313
LDL-C, mmol/L	2.38	1.23	0.542	0.227
ALB, g/L	23.2	24.0	3.29	0.867
TP, g/L	38.0	38.9	4.68	0.892
BUN, mg/L	367	199	24.7	0.025
Blood ammonia, μmol/L	196	74.7	13.8	0.024
Antioxidant				
T-AOC, U/mL	3.95	7.13	0.495	0.030
SOD, U/mL	33.4	31.3	4.45	0.766
GSH-PX, U/mL	201	297	10.9	0.004
MDA, nmol/mL	4.55	3.38	0.683	0.262
CAT, U/mL	1.08	2.21	0.450	0.166
H_2O_2, U/mL	39.2	14.5	1.30	0.014
Immune parameters				
D -lactate, ng/mL	1024	967	14.5	0.050
Interleukin −6, pg/mL	143	155	3.0	0.024
Cortisol, ng/mL	33.2	33.3	1.64	0.992
Growth hormone, ng/mL	20.9	22.0	0.63	0.263
IGF, ng/ mL	90.4	86.1	4.43	0.503
TNF-α, ng/mL	307	318	6.1	0.251

[a] Number of male Hu lambs per treatment = 5.
[b] TP = total protein; ALB = albumin; TG = total glucose; T-CHO = total cholesterol; LDL-C = low density lipoprotein cholesterol; BUN = blood urea nitrogen; T-AOC = total antioxidant capacity; SOD = superoxide dismutase; GSH-PX = glutathione peroxidase; MDA = malondialdehyde; CAT = catalase; IGF = insulin-like growth factors; TNF-α = tumor necrosis factor -α.

Table 5
Effect of leucine supplementation on growth performance, carcass traits and meat drip loss of finishing male Hu lambs (day 120 of age) weaned early.[a]

Variables[b]	Treatments		SEM	P-value
	Control	Leucine		
Initial BW, kg	6.50	6.35	0.376	0.794
Final BW, kg	23.7	23.5	1.44	0.920
Feed intake, g/d				
Concentrate	585	580	20.9	0.879
Forage mixture	930	924	34.3	0.910
ADG, g/d	190	190	12.8	0.968
Carcass weight, kg	10.0	10.6	0.66	0.576
Dressing percent, kg/100kg	42.2	45.1	0.57	0.013
GR value, cm	1.08	1.00	0.073	0.490
Drip loss, g/100g				
24 h	7.55	5.51	0.425	0.015
48 h	9.08	6.94	0.292	0.008

[a] Number of male Hu lambs per treatment = 5.
[b] BW = body weight, ADG = average daily gain, GR = the depth of muscle and fat tissue from the surface of the carcass to the lateral surface of the twelfth rib 110 mm from the midline.

Hyun et al. (2003) showed that supplemental dietary leucine at 20 g/kg DM decreased BW gain though feed intake or feed: gain ratio was not affected. In our preliminary study (unpublished data), when supplemented at 14.2 g/kg total feed DM to 10 to 30-day-old lambs, leucine decreased daily BW gain. Thus, in the present study, we lowered the leucine dietary supplementation to 7.1 g/kg total feed DM. The lack of stimulatory effect of leucine supplementation on lamb growth is consistent with the observation on pigs. Given that neonatal lambs have similar digestive physiology as non-ruminants, these results are not surprising and suggest that excess leucine supplementation is not needed in neonatal lamb production. On the other hand, the outcome of leucine supplementation may also depend on the protein content of the basal diets. In pre-ruminant lambs, increasing the dietary leucine content of 23–106 or 126 g/kg DM in the adequate protein diet (240 g/kg of DM) led to a significant decrease in feed intake. Nevertheless, the depressing effect of leucine on feed intake was not worsened when pre-ruminant lambs were fed a low-protein diet (150 g/kg of DM) (Papet

et al., 1988).

Supplementation of leucine increased the plasma concentrations of leucine and isoleucine but did not affect valine concentration. Papet et al. (1988) showed that dietary supplementation of leucine led to an increase in plasma leucine concentration along with a decreased concentration of valine and isoleucine in pre-ruminant lambs. In another study on mice, leucine supplementation increased blood leucine concentration but decreased that of the other two branched-chain AAs (Nairizi et al., 2009). Sun et al. (2015) recently showed that leucine supplementation did not affect the plasma concentrations of isoleucine or valine in sucking piglets. The effect of leucine supplementation reported in the present study differed from those reported in either pre-ruminant lambs or non-ruminants. The discrepancies might reflect the differences in dietary intakes of AAs, supplemental doses of leucine, species, and ages of the animals. One positive effect of leucine supplementation observed in the present study was the decrease in the plasma concentration of BUN and blood ammonia. This may be due to the up-regulated expression of genes involved in AA transport in the small intestine (Sun et al., 2015) to increase AA utilization efficiency. Nevertheless, because higher BUN and blood ammonia reflect a poor balance of plasma AA and disarray of AA metabolism, leucine supplementation may positively affect AA metabolism and utilization.

Oxidative stress is defined as the imbalance between oxidation and anti-oxidation in living cells (Turner and Lysiak, 2008), and it is often caused by changes in external conditions, such as early weaning, heat stress, and other adverse impacts. In our previous study (unpublished data), early weaned lambs suffered oxidative damage as evidenced by higher levels of D-lactate concentration in plasma detected at 1d or 4d after lambs were early weaned at 30 days of age. A growing body of evidence has demonstrated that dietary supplementation of specific nutrients, such as vitamin C, vitamin E, and carotenoids, contributes to the attenuation of oxidative damage in mammals (Samoylenko et al., 2013). These findings indicate that antioxidant nutrient supplementation in the diet could be an approach to reduce potential damage caused by reactive oxygen species. In the present study, dietary leucine supplementation significantly increased the activities of T-AOC, GSH-PX while decreasing H_2O_2 in plasma, indicating that leucine can protect against oxidative damage caused by early weaning. Similar results were reported that dietary leucine at optimal concentrations could alleviate oxidative damage in piglets (Hu et al., 2017), blunt snout bream (*Megalobrama amblycephala*) (Liang et al., 2018), rohu (Giri et al., 2015), and grass carp (Deng et al., 2016). Mechanistically, leucine supplementation decreases cellular reactive oxygen species levels via an energy metabolism switch from oxidative phosphorylation towards glycolysis by activating the mTORHIF-1α pathway (Hu et al., 2017). On the other hand, Liang et al., (2018) believed that dietary leucine could improve plasma antioxidative enzyme activities of blunt snout bream and up-regulate their gene transcriptions of hepatopancreas through the Nrf2 signaling pathway. The molecular mechanism by which leucine protect against oxidative damage in the intestinal tract of early weaned lambs can be investigated in future studies using cell cultures.

Carcass traits, important indicators of animal production performance, are mainly affected by dietary nutrient levels. To our knowledge, it remains poorly understood if and how leucine affects the carcass traits and meat quality of lambs, though studies showed that dietary leucine had no effect on hot carcass weight, dressing percentage, carcass fat depths, longissimus color or drip loss of finishing pigs (Hyun et al., 2003) or pigs (Madeira et al., 2014). In the present study, dressing percent of lambs was increased, and drip loss of mutton was decreased by leucine supplementation. This may be due to the stimulating effect of leucine on protein synthesis and inhibitory effect on proteolysis (Rhoads and Wu, 2009), because water-holding capacity is centered in the protein and structures that bind and entrap water (Huff-Lonergan and Lonergan, 2005). On the other hand, leucine supplementation can enhance the antioxidation capability of lambs to alleviate the negative effect of early weaning, which may be beneficial for the fattening lambs.

5. Conclusion

Leucine supplementation to early weaned lambs is beneficial to their health as indicated by increased activity of T-AOC and GSH-PX, and decreased the concentration of H_2O_2 and D-lactate in plasma. These effects of leucine supplementation resulted in improved dressing percentage and water retention in the muscle of lambs. Leucine may be supplemented to lambs to improve health.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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