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Evaluation of rumen protective effectiveness of L-carnitine, *in vitro* and *in vivo*

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Abstract

This study aimed to evaluate the rumen protective effectiveness of L-carnitine through *in vitro* tests, rumen degradation tests and *in vivo* tests. Twelve rumen-fistulated crossbred rams with similar body weights of 55 ± 3.6 kg and ages of 3 ± 0.2 years old were divided into three treatment groups in a 3×3 Latin square design, G1 (basal diet with no additives), G2 (unprotected L-carnitine) or G3 (rumen-protected L-carnitine). Ruminal fluid and blood samples were collected before morning feeding on the last day of each experimental period (21 d). The percentage of L-carnitine remaining in the simulated rumen and abomasum and rumen increased with the increase in the wall material ratio ($P < 0.05$). L-carnitine supplementation decreased the plasma urea nitrogen concentration of the sheep ($P < 0.05$). G3 resulted in higher GSH-Px and SOD activities as well as T-AOC and lower MDA concentrations in plasma than G1, and the difference was significant among the groups ($P < 0.01$). Thus, L-carnitine in the rumen could be protected by encapsulation for a certain time. Unprotected and rumen-protected L-carnitine supplementation effectively enhanced the antioxidant capacity of sheep, and the antioxidant capacity of sheep supplemented with rumen-protected L-carnitine was higher than that of sheep supplemented with unprotected L-carnitine.

Keywords: L-carnitine; rumen protection; sheep; urea nitrogen; antioxidation

Introduction

Carnitine (β -hydroxy- γ -trimethylammonium butyrate) is a vitamin-like quaternary ammonium compound that exists widely in the animal, plant, and microbial kingdoms. Carnitine can be endogenously synthesized in the animal body by the catalysis of carnitine biosynthesis enzymes with lysine and methionine as substrates and has two configurations in nature, among which L-carnitine has biological activity (Vaz and Wanders, 2002). L-Carnitine mainly accumulates in the liver and muscle, and the primary biological function of L-carnitine is to facilitate the transfer of long-chain fatty acids into the mitochondrial matrix for β -oxidation (Fritz, 1959). L-Carnitine plays an important role in energy production (Stephens et al., 2007) and lipid metabolism (Hoppel, 2003). In addition, L-carnitine has been shown to have functions in the inhibition of oxidative damage (Kalaiselvi and Panneerselvam, 1998) and enhancement of body immunity (Athanasakis et al., 2001). Carnitine is widely used in humans and monogastric animals but rarely in ruminants. Studies on rumen-protected L-carnitine in ruminants have only been reported by White (2002), Walker (2005), Solhjoo (2014) and Olagaray (2018), but all of them were related to plasma metabolism and growth performance, and there were no related reports on antioxidant status and cytokine abundance.

Although most studies on ruminants indicated that the addition of L-carnitine, even in rumen-unprotected forms, increased the amount of L-carnitine in plasma, liver, muscle, and milk (Carlson et al., 2006; Carlson et al., 2007) and reduced hyperammonemia (Chapa et al., 1998; Chapa et al., 2001), it has been found in some studies on dairy cows (LaCount et al., 1995), growing and finishing steers (Greenwood et al., 2001) and lambs (Solhjoo et al., 2014) that L-carnitine administration was

ineffective at increasing milk production and improving growth performance. The reason may be related to the carnitine status in the rumen. In contrast to monogastric mammals, the special physiological environment of the rumen and the degradation of L-carnitine by ruminal microorganisms affect the efficacy of supplemental L-carnitine to a certain extent (Meadows and Wargo, 2015). The rumen contains a diverse microbial community, including anaerobic bacteria, archaea, ciliate protozoa and anaerobic fungi (Krause et al., 2013), which provides ideal environmental conditions for anaerobic fermentation. It has been reported that carnitine can be used as carbon (C), nitrogen (N), or energy for bacteria in different metabolic pathways (Ringseis et al., 2018).

Therefore, rumen-protected L-carnitine was independently developed by the research team, and the release efficiency in the simulated digestive tract and rumen degradation efficiency of the product were measured. The rumen fermentation parameters, plasma biochemical indices, antioxidant status and cytokine abundance in sheep supplemented with unprotected and rumen-protected L-carnitine were compared. The rumen protective effectiveness of L-carnitine was evaluated by *in vitro* tests, rumen degradation tests and *in vivo* tests.

Materials and methods

Materials

The unprotected L-carnitine used was a commercial product containing 50% L-carnitine that was obtained from Hubei Kangdeng Pharmaceutical Co., Ltd, Huanggang, China. Ethyl cellulose, carbomer, tributyl citrate and talc were obtained from Macklin, Shanghai, China.

Rumen-protected L-carnitine

Rumen-protected L-carnitine was an encapsulated product independently developed by the Safety and Green Feed Additive Pilot Center, Institute of Animal Nutrition and Feed Science, Jilin Academy of Agricultural Sciences, Gongzhuling, China, and the source material was unprotected L-carnitine. The preparation method was as follows: (1) L-carnitine pellets were prepared with particle sizes between 0.417 mm and 0.833 mm by a fluidized bed pelleting process; (2) a coating solution was prepared, and the L-carnitine pellets were encapsulated by the fluidized bed coating process; (3) the encapsulated L-carnitine was dried to yield the final product.

Coating materials included ethyl cellulose, carbomer, tributyl citrate and talc. Ten grams of carbomer and 90 g of ethyl cellulose were dispersed in 2000 mL of the solvent (water/ethanol = 3:7) and stirred with a magnetic agitator (TM300, Beijing Thmorgan Biotechnology Co., Ltd, Beijing, China) at 40 °C to complete dissolution. Thirty millilitres of tributyl citrate was then added to the solvent as a plasticizer. Finally, 40 g of talc was dispersed in the solvent as an anti-adhesion agent, and the coating solution was prepared by stirring evenly.

***In vitro* test**

The release of L-carnitine from rumen-protected L-carnitine was carried out at a rotation speed of 80 rpm/min in 500 mL of simulated ruminal fluid (citric acid 5.72 g, disodium hydrogen phosphate 52.09 g, constant volume in 1000 mL, pH 6.6) and abomasal fluid (citric acid 19.70 g, disodium hydrogen phosphate 4.44 g, constant volume in 1000 mL, pH 2.4) (Papay et al. 1984) at 39±0.5 °C using a dissolution tester (RC806D, Tianjin Tianda Tianfa Technology Co., Ltd, Tianjin, China). The samples (3

replicates) were removed after 2, 4 and 8 h, filtered with medium-speed qualitative filter paper and dried to constant weight at 65 °C. Then the concentration of L-carnitine in the samples was determined.

The selection of wall material and the appropriate ratio of core material to wall material are very important to the protective effect of the product. The rumen pH of ruminants is close to neutral, while the abomasum pH is acidic. Therefore, we selected the pH-dependent wall material carbomer to promote the appropriate protection in the rumen and the effective release in the abomasum of the product.

Rumen degradation test

The nylon bag technique was used to evaluate the rumen stability of rumen-protected L-carnitine in sheep. Twelve rumen-fistulated crossbred rams (Northeast fine-wool sheep × Dorper sheep) were randomly divided into four groups, each containing 3 rams. Products with different core material to wall material ratios (10:1, 8:1, 5:1) and unprotected L-carnitine equivalent to 5 g pure L-carnitine were placed into nylon bags. Nylon bags (3 replicates) were incubated in the rumen for 2, 4 and 8 h and then removed for the determination of L-carnitine concentration. The removed nylon bags were slowly washed in clean water for 5-10 min until the water flow was clear and dried in a 65 °C oven for 48 h, and then the concentration of L-carnitine in the samples was determined. Nylon bags without rumen treatment (0 h) were used as the control. The bags did not enter the rumen but were subjected to the same procedures as the other bags (washing and drying).

The residual percentages of unprotected and rumen-protected L-carnitine in simulated ruminal fluid, simulated abomasum fluid, and the rumen were calculated by the following equation:

$$C = A/B \times 100\%$$

where C is the residual percentage of L-carnitine in unprotected and rumen-protected L-carnitine. A ($\mu\text{g/mL}$) is the concentration of L-carnitine remaining in unprotected and rumen-protected L-carnitine in simulated ruminal fluid, simulated abomasum fluid, and the rumen. B ($\mu\text{g/mL}$) is the concentration of L-carnitine in unprotected and rumen-protected L-carnitine.

***In vivo* test**

The experiment was conducted from November to December 2019 at the Laboratory of Energy Metabolism, Institute of Animal Nutrition and Feed Science, Jilin Academy of Agricultural Sciences (JAAS), Gongzhuling, China. All research involving animals was reviewed and approved prior to beginning the research by the Jilin Provincial Experimental Animal Management Committee, and all research involving animals was conducted according to the Regulations on the Administration of Experimental Animals of Jilin Province.

Experimental design, diets, and feed management

Twelve rumen-fistulated crossbred rams (Northeast fine-wool sheep $\times$ Dorper sheep) with similar body weights of 55 ± 3.6 kg and ages of 3 ± 0.2 years old were randomly divided into three experimental periods according to the treatment sequence in a 3×3 Latin square design (4 replicates), with each experimental period of 21 days,

and all 4 animals in a pen stayed together in the same pen for all 3 periods. During each experimental period (21 d), the sheep were adapted to the diets for the first 20 days, and the samples were taken on the last day. To prevent potential carry-over effects, 7 d were left in between the experimental periods for administration of the basal diet. The dietary treatments were as follows: sheep in G1 were fed a basal diet with no additives, and sheep in G2 and G3 were fed a basal diet with unprotected L-carnitine and rumen-protected L-carnitine, respectively. The supplemental concentration of pure L-carnitine in G2 and G3 was 200 mg/kg basal diet (air-dried basis), and the dry matter intake of sheep in each treatment was 1.1 kg/ram/day on average. The basal diet was prepared to meet the nutrient requirements of adult rams (Agricultural Industry Standard of the People's Republic of China-sheep feeding standard; NY/T 816-2004) with a concentrate-to-forage ratio of 40:60, in which the concentration of L-carnitine was <5 mg/kg. The forage and the concentrate were offered free choice, and the dietary composition and nutritional levels are shown in Table 1. All sheep were fed twice daily at 0700 and 1700 in the same amount and had access to freshwater *ad libitum*.

Sampling procedures

On the last day of each experimental period, ruminal fluid and blood were collected before morning feeding to prevent the animals from drinking water before sampling. A sufficient amount of ruminal fluid (50 mL) from different sheep was pumped into a bottle through a rumen cannula by negative pressure; the ruminal fluid was filtered through four layers of gauze, and the pH was immediately determined by a pH metre (PHSJ-5, Shanghai INESA Scientific Instrument Co., Ltd, Shanghai, China). Five millilitres of fluid was added to 1 mL of 25% (wt/vol) metaphosphoric acid, and

another 20 mL of fluid was added to 4 mL of 1% (wt/vol) sulfuric acid. The mixtures were then stored at -20 °C for subsequent analyses. Ten millilitres of jugular blood was sampled into a heparinized vacutainer tube containing 200 USP units of sodium heparin. The blood was immediately centrifuged (TGL-16M, Hunan Cence Laboratory Instrument Development Co., Ltd, Changsha, China) at $3\,000 \times g$ and 4 °C for 15 min, and the plasma samples were collected and kept at -20 °C until subsequent analyses.

Chemical analysis

Feed samples were oven dried to a constant weight at 55 °C, ground to pass through a 1-mm screen, and stored in self-sealed bags. Ingredients were analysed for dry matter (DM; 930.15), crude protein (CP; as $6.25 \times N$; 990.03), P (946.06) and Ca (935.13) according to the procedures of AOAC (2005). Neutral detergent fibre (NDF, without heat-stable amylase and expressed inclusive of residual ash) and acid detergent fibre (ADF, expressed inclusive of residual ash) were determined according to Van Soest et al. (1991).

The concentration of L-carnitine in the basal diet, *in vitro* test and rumen degradation test was determined according to the procedures of NY/T 3321-2018 (Determination of L-carnitine in feeds of Agricultural Industry Standard of the People's Republic of China). Two grams of the sample was weighed (accurate to 0.01 g) and placed in a 100 mL volumetric flask, and then 80 mL of water was added. After mixing, the samples were ultrasonically extracted (KQ-500DB, Kunshan Beauty Ultrasonic Instrument Co., Ltd., Kunshan, China) for 30 min and allowed to stand for 10 min. After the constant volume reached 100 mL, the solution was filtered to remove the precipitate, diluted with water to between 2 µg/mL and 100 µg/mL, and then passed through a 0.45

µm filter membrane for testing. The concentration of L-carnitine was analysed by ion chromatography (Dionex™ Aquion™, Thermo Fisher, Waltham, Massachusetts, USA) using an uninhibited cation exchange column (CS16: 250 mm × 5 mm). The flow rate was 1.0 mL/min, the column temperature was 40 °C, and the sample size was 25 µL. The concentration of L-carnitine in the sample was expressed by mass fraction X_i , which was calculated according to Equations (1) and (2):

$$X_i = C_i \times V \times n \times 1000 / m / 1000 \times 0.815 \quad (1)$$

where X_i (µg/mL) is the concentration of L-carnitine in the sample solution. C_i was obtained from Equation (2) during single-point calibration and found from the standard curve during multipoint calibration. V (mL) is the total volume of the extract of the sample, n is the dilution ratio, m (g) is the mass of the sample, and 0.815 is the conversion relationship between L-carnitine and L-carnitine hydrochloride.

$$C_i = C_{si} \times A_i / A_{si} \quad (2)$$

where C_{si} (µg/mL) is the concentration of L-carnitine in the mixed standard working solution, A_i is the peak area of L-carnitine in the sample solution, and A_{si} is the peak area of L-carnitine in the mixed standard working solution.

Rumen fermentation parameters

The ruminal fluid with metaphosphoric acid was thawed and centrifuged at 10 000 × g and 4 °C for 15 min. Ruminal total volatile fatty acids (TVFAs) were analysed by GC-MS (ISQ 7000, Thermo Fisher, Waltham, Massachusetts, USA) using a capillary column (TG WAX: 30 m × 0.25 mm × 0.25 µm) according to the method of Zhou et al. (2018). The column oven was held at an initial temperature of 100 °C for 5 min, increased to 150 °C at a rate of 5 °C/min, then increased to 240 °C at a rate of 30

°C/min and held for 30 min. The carrier gas was helium with a flow rate of 50 mL/min. The ruminal fluid mixed with sulfuric acid was thawed and divided into two parts. One part was centrifuged at $3\,000 \times g$ for 10 min, and the ammonia nitrogen in the supernatant was determined colorimetrically (L9, Shanghai INESA Analytical Instrument Co., Ltd, Shanghai, China) according to Broderick and Kang (1980). The total nitrogen and microbial nitrogen in the other part was determined by the Kjeldahl method (as $6.25 \times N$, 990.03) according to AOAC (2005). The sample was prepared for microbial nitrogen determination according to Cotta et al. (1982), and microbial crude protein (MCP) was then calculated as the amount of $N \times 6.25$.

Antioxidant status, plasma biochemical indices, cytokine abundance and L-carnitine concentration

Plasma antioxidant indices, including glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activity, total antioxidant capacity (T-AOC) and the concentration of malondialdehyde (MDA), and plasma biochemical indices, including the concentration of total protein, albumin, and urea nitrogen, were determined using colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Cytokine abundance (TNF- α , IL-1 β , IL-6) in plasma was assayed using commercial ELISA kits for sheep (Fankew mall, Shanghai, China), and the product numbers for TNF- α , IL-1 β , and IL-6 were F72110-B, F6400-B and F3894-B, respectively. The L-carnitine concentration in plasma was assayed by commercial ELISA kits for sheep (Wuhan BOSK Bioengineering Co., Ltd, Wuhan, China, product number: BOS-47004). All these analyses were conducted according to the instructions provided by the manufacturer. The dilution linearity of the ELISA data for TNF- α , IL-1 β , IL-6 and L-

carnitine were $y = 0.003x + 0.0109$ ($R^2 = 0.9972$), $y = 0.0012x + 0.0007$ ($R^2 = 0.9983$), $y = 0.0028x - 0.0039$ ($R^2 = 0.9978$) and $y = 0.001x + 1.2141$ ($R^2 = 0.9984$), respectively. The coefficients of variation (CVs) of the ELISA data for TNF- α , IL-1 β , IL-6 and L-carnitine were 4.17, 10.00, 5.85, and 8.40, respectively. In the equation, x represents the concentration of the standard substance, and y represents the corresponding O.D. value. The dilution ratio of the sample for measuring the abundance of cytokines was 5 times, and the sample was not diluted for measuring the concentration of L-carnitine. The O.D. values measured by all samples were within the linear range and could be used with the standard curve equations to obtain the concentration after sample dilution and finally obtain the sample concentration.

Statistical analysis

The *in vitro* test and rumen degradation test data were analysed by Excel 2019. The ANOVA program of IBM SPSS statistics software version 25.0 was used for variance analysis. Duncan's method was used for multiple comparisons, and effects were considered significant when $P < 0.05$. The data were presented as the average value $\pm$ standard error.

The *in vivo* test data were subjected to analysis of variance using PROC MIXED in SAS software version 9.4 (SAS Institute Inc., North Carolina, US) according to the following model:

$$Y_{ijk} = \mu + S_i + T_j + P_k + E_{ijk}$$

where Y_{ijk} is the observation, μ is the overall mean, S_i is the random effect of the sheep (12), T_j is the fixed effect of treatment (G1, G2 or G3), P_k is the random effect of the period (1,2,3) and E_{ijk} is the residual error. Differences among the means were

identified using Duncan's procedure for multiple comparisons, and effects were considered significant when $P < 0.05$.

Results and discussion

***In vitro* release of rumen-protected L-carnitine**

The percentages of L-carnitine remaining in simulated ruminal fluid (pH 6.6) and abomasal fluid (pH 2.4) are shown in Tables 2 and 3. Due to the high solubility of L-carnitine, the percentages of L-carnitine remaining in unprotected L-carnitine in simulated ruminal fluid (8.48%) and abomasal fluid (6.48%) were less than 10% after 2 h. The percentage of L-carnitine remaining in simulated ruminal fluid and abomasal fluid increased with the increase in wall material ratio, and the percentage of the product with the core material to wall material ratio of 5:2 was significantly higher than that of other products ($P < 0.05$).

Efficacy of rumen protection

The percentage of L-carnitine remaining in the rumen increased with the increase in the ratio of wall material ($P < 0.05$, Table 4). The results showed that L-carnitine in the rumen could be protected by encapsulation for a certain time.

Rumen fermentation parameters

In view of the degradation of L-carnitine by rumen microorganisms, the effects of unprotected and rumen-protected L-carnitine supplementation on the rumen environment of sheep were compared. The ruminal pH value is a direct parameter for evaluating the rumen environment (Michael, 1997). It was found that the optimal pH for

cellulolytic microorganism growth ranged between 6.2 and 7.2, and maximum activity could be maintained at neutral pH (Van Soest, 1994). In this study, the sheep in different treatment groups had similar pH values (6.91 to 6.93) within a normal physiological range, and rumen acidosis was not induced ($P > 0.05$; Table 5). The TVFA concentration and individual acid molar ratios were not affected by the supplementation of L-carnitine, and there was no difference in total nitrogen, ammonia nitrogen, or microbial protein concentrations among treatments ($P > 0.05$; Table 5). Previous studies also confirmed that L-carnitine supplementation had no effect on rumen fermentation parameters (Bunting et al., 2002; LaCount et al., 1996). The insignificant difference in rumen fermentation parameters among treatments may be related to the feeding of the same basal diet in the study because the dietary nutrient composition has a great influence on rumen fermentation products.

Plasma biochemical indices and L-carnitine concentration

There was no significant difference in the concentrations of total protein, albumin and L-carnitine among treatments ($P > 0.05$; Table 6). Sheep fed L-carnitine had a reduced concentration of urea nitrogen in plasma ($P < 0.05$; Table 6). The results for plasma urea nitrogen concentration were different. In the experiments reported by Mehrez et al. (2015), Foroozandeh et al. (2014), and Chapa et al. (2001), the urea nitrogen concentration was not affected by L-carnitine supplementation. In accordance with the result of Cital et al. (2009), the plasma urea nitrogen concentration was reduced by L-carnitine supplementation. The primary biological function of L-carnitine is to facilitate the transfer of long-chain fatty acids into the mitochondrial matrix for β -oxidation. In addition, accelerated fatty acid oxidation can inhibit the activity of

branched-chain α -keto acid dehydrogenase (BCKDH) by increasing the concentrations of acetyl-CoA, NADH and ATP (Spydevole et al. 1981), thus reducing the oxidation of branched amino acids. In the study by Owen et al. (2001) on the effect of dietary L-carnitine on protein accretion in swine, it was found that the oxidation rate of fatty acids in liver and muscle increased in a dose-dependent manner with increasing carnitine dose. The PC flux and BCKDH flux in the liver and muscle could be increased and decreased, respectively, by L-carnitine supplementation, and the concentrations of some free amino acids continued to increase. In addition, the study by Li et al. (2020) pointed out that when amino acids were provided as a single nutrient, the synthesis of amino acids would not be promoted, while the degradation would be accelerated. Therefore, we inferred that the decrease in the plasma urea nitrogen concentration may be due to the promotion of lipid oxidative energy supply by L-carnitine supplementation, which changed the fluxes of BCKDH and PC, resulting in the inhibition of amino acid oxidative decomposition.

In this study, supplementation with unprotected and rumen-protected L-carnitine (the concentration of L-carnitine was 200 mg/kg basal diet) had no effect on plasma L-carnitine concentration ($P > 0.05$; Table 6). Contrary to the results in this study, Walker et al. (2005) reported that among different feeding dosages (0.125, 1.06, and 2.0 g/d), the plasma L-carnitine concentration of lambs was significantly increased when the rumen-protected L-carnitine was given at high feeding dosages (1.06 and 2.0 g/d). Since the dosage factor was not considered in this study, we hypothesized that the plausible reason for the insignificant difference in L-carnitine concentration among the different treatments may be related to the differences in feeding dosage and physiological state (sheep vs. lamb). Olagaray et al. (2018) confirmed that the delivery of bioavailable

carnitine to cattle could be achieved by the inhibition of L-carnitine degradation in the rumen, but the concentration of L-carnitine was not increased by rumen protection. Similar results were obtained in the present study, and we hypothesized that a low plasma L-carnitine concentration may be associated with excessive rumen protection. In addition, the results of the *in vitro* test also showed that the level of L-carnitine remaining in rumen-protected L-carnitine in the simulated abomasal fluid was not low.

Antioxidant status

L-Carnitine supplementation influenced antioxidant indices (Table 7). Plasma GSH-Px and SOD activities as well as T-AOC were increased and the MDA concentration was decreased by L-carnitine supplementation. Moreover, the indices of sheep supplemented with rumen-protected L-carnitine were significantly different from those of sheep supplemented with unprotected L-carnitine ($P < 0.01$).

As an antioxidant, L-carnitine has been shown to prevent oxidative stress and regulate nitric oxide, cellular respiration (Brown, 1999), and the activity of enzymes involved in defence against oxidative damage (Kremser et al., 1995). Previous studies demonstrated that L-carnitine can scavenge hydrogen peroxide and superoxide radicals (Gülçin, 2006) and is very effective in normalizing oxidative changes such as essential hypertension, atherosclerosis and ageing (Go'mez-Amores et al., 2007; Dayanandan et al., 2001; Kalaiselvi and Panneerselvam, 1998). It has been confirmed that the concentration of MDA and the activities of SOD and GSH-Px in the plasma of sows, broilers and seabreams were decreased and increased, respectively, by L-carnitine supplementation. In line with previous studies, the concentration of MDA and the activities of SOD and GSH-Px in the plasma of sheep were decreased and increased,

respectively, by L-carnitine supplementation. In particular, the sheep fed diets supplemented with rumen-protected L-carnitine showed stronger antioxidant capacity than that of the sheep fed diets supplemented with unprotected L-carnitine. The results of the rumen degradation test showed that encapsulation had a protective effect on L-carnitine for a certain time. Based on the fact that there was no difference in feeding dosage, animal species or nutrient composition, the improvement of antioxidant capacity may have been achieved through moderate rumen protection.

Cytokine abundance

There was no significant difference in plasma concentrations of IL-1 β , IL-6 and TNF- α among treatments ($P > 0.05$; Table 8). The antioxidant system is closely related to the immune system. Moreover, the nuclear factor kappa B (NF- κ B) signaling pathway is an important connection between the two systems. Most antioxidants not only play an antioxidant role *in vivo* but also play anti-inflammatory, antitumour, and anticancer physiological functions within the immune system. L-Carnitine has been shown to affect humoral immunity, inhibit apoptosis of immune cells (Famularo and De Simone, 1995), and act on glucocorticoid receptors (GRs) to play an anti-inflammatory role (Alesci et al. 2003). L-carnitine plays an inflammatory role by activating GR to regulate the target gene of glucocorticoids. Manoli et al. (2004) reported that L-carnitine increased the number of CD4⁺ and CD3⁺ T cells by inhibiting lymphocyte death and the production of TNF- α . Previous studies have reported that oral L-carnitine reduced the concentrations of serum cytokines such as TNF- α and IL-6 in mice with cancer (Liu et al. 2014). In this study, the reason why there was no significant difference in cytokine

abundance may have been related to the physiological state of the animals themselves or excessive protection.

We found that the antioxidant capacity of sheep was increased and the urea nitrogen concentration was decreased by L-carnitine supplementation, while there were no significant effects on rumen fermentation, plasma biochemical indices or cytokine abundance. Based on analysis of the *in vitro* test and plasma L-carnitine concentration, we concluded that the absence of differences in rumen fermentation parameters, plasma biochemical indices and cytokine abundance may have been related to lower plasma L-carnitine concentration, excessive encapsulation or the physiological state of the animals. A large number of studies have shown that supplementation with L-carnitine generally has no positive effect on the improvement of animal performance, while it can improve animal performance only when the animals are in a stressed state (pregnancy, lactation, high temperature, hyperammonemia) (Rehman et al., 2017; Güçlü et al., 2011; Wei et al., 2016, White et al., 2001). Another finding was that sheep supplemented with rumen-protected L-carnitine were superior to sheep supplemented with unprotected L-carnitine in resisting oxidative stress. Combined with the results of the rumen degradation test, we hypothesize that the significant difference in antioxidant status between sheep supplemented with rumen-protected L-carnitine and unprotected L-carnitine might be related to moderate rumen protection.

Conclusions

In conclusion, *in vitro* tests and rumen degradation tests showed that encapsulation protected L-carnitine. However, the L-carnitine level remaining in the rumen-protected L-carnitine in the simulated abomasal fluid was not low, and there was no effect on

most properties by L-carnitine supplementation. The protective effectiveness of L-carnitine was improved by encapsulation, and the digestion and absorption in animals was likely decreased. It is worth highlighting that the antioxidant capacity of sheep was improved by L-carnitine supplementation. In particular, the antioxidant capacity of sheep supplemented with rumen-protected L-carnitine was stronger than that of sheep supplemented with unprotected L-carnitine.

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Contributors' statement

All authors participated in the development of the study concept and design; B. W. and L. Z. were responsible for the execution of the study; L. Z., W. J., W. Y. and W. Z. were involved in the animal experiments; L. Z. and W. J. responsible for the data collection and statistical analysis; L. Z. wrote the draft of the manuscript; R. H., L. C. and B. W. reviewed and revised the manuscript.

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Data availability statement

All data underpinning the work are available.

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Table 1

Ingredients and chemical composition of the basal diet for sheep.

Ingredients	Percentage (%)	Nutrient compositions	
<i>Leymus chinensis</i>	60.00	Dry matter (%)	89.00
Ground corn	27.20	Metabolizable energy (ME) ² , MJ/kg	8.73
Soybean meal	8.50	Crude protein (%)	12.52
Cotton seed meal	0.80	Neutral detergent fiber (%)	57.99
Rapeseed meal	0.70	Acid detergent fiber (%)	22.56
Limestone	0.60	Calcium (%)	0.55
Salt	1.20	Phosphorus (%)	0.26
Premix ¹	1.00		
Total	100.00		

¹ Premix composition (per kilogram): Cu 15.0 mg, Fe 100.0 mg, Mn 60.0 mg, Zn 100.0 mg, I 0.9 mg, Se 0.3 mg, Co 0.2 mg, vitamin A 16000 IU, vitamin D 4000 IU, vitamin E 100 IU.

² Estimated from NY/T 816-2004.

Table 2

L-carnitine remaining of rumen-protected L-carnitine in simulated ruminal fluid (pH 6.6).

%

Times	Treatments ¹				
	Pure L-carnitine	10:1	8:1	5:1	5:2
2 h	8.48±0.71 ^{c2}	28.45±0.86 ^b	29.80±2.89 ^b	39.28±2.74 ^b	93.78±1.90 ^a
4 h	7.43±0.31 ^d	22.11±0.97 ^c	25.00±0.86 ^{bc}	29.78±2.13 ^b	85.51±2.97 ^a
8 h	6.18±0.34 ^d	16.34±0.97 ^c	23.28±0.86 ^b	28.72±1.07 ^b	73.03±2.69 ^a

^{a-d} Means in the same row with different superscripts are significantly different ($P < 0.05$).

¹ Treatments are the unprotected L-carnitine and rumen-protected products with different core material to wall material ratio (10:1, 8:1, 5:1 and 5:2).

² The means are the percentages of L-carnitine remaining at the specified times.

Table 3

L-carnitine remaining of rumen-protected L-carnitine in simulated abomasal fluid (pH 2.4). %

Times	Treatments ¹				
	Pure L-carnitine	10:1	8:1	5:1	5:2
2 h	6.48±0.71 ^{d2}	14.65±0.87 ^c	21.15±1.93 ^b	22.34±1.06 ^b	85.51±2.71 ^a
4 h	5.98±1.41 ^c	11.54±0.01 ^d	16.63±0.27 ^c	20.21±1.07 ^b	77.25±2.88 ^a
8 h	3.98±1.41 ^e	7.43±0.31 ^d	14.35±0.22 ^c	19.15±0.01 ^b	68.90±3.14 ^a

^{a-c} Means in the same row with different superscripts are significantly different ($P < 0.05$).

¹ Treatments are the unprotected L-carnitine and rumen-protected products with different core material to wall material ratio (10:1, 8:1, 5:1 and 5:2).

² The means are the percentages of L-carnitine remaining at the specified times.

Table 4

Rumen protective effectiveness of rumen-protected L-carnitine. %

Times	Treatments ¹			
	10:1	8:1	5:1	5:2
2 h	26.64±0.20 ^{d2}	27.65±0.29 ^c	28.83±0.12 ^b	90.70±0.34 ^a
4 h	20.93±0.25 ^d	24.23±0.58 ^c	26.85±0.88 ^b	80.46±0.30 ^a
8 h	16.08±0.37 ^d	21.79±0.69 ^c	25.75±1.00 ^b	56.13±0.13 ^a

^{a-d} Means in the same row with different superscripts are significantly different ($P < 0.05$).

¹ Treatments are the rumen-protected products with different core material to wall material ratio (10:1, 8:1, 5:1 and 5:2).

² The means are the percentages of L-carnitine remaining at the specified times.

Table 5

Effects of L-carnitine supplementation on ruminal fermentation parameters in sheep.

Item	Treatments ¹			SEM	P-value
	G1	G2	G3		
pH value	6.91	6.93	6.91	0.01	0.088
TVFAs (mmol/L)	75.17	75.46	75.81	0.45	0.908
Acetate (% of TVFAs)	55.13	55.45	53.87	1.27	0.142
Propionate (% of TVFAs)	24.17	23.08	25.31	0.50	0.378
Butyrate (% of TVFAs)	20.69	21.46	20.82	0.78	0.914
A/P ratio	2.28	2.40	2.13	0.10	0.403
Total nitrogen (%)	0.11	0.11	0.14	0.01	0.318
Ammonia nitrogen (mg/dL)	9.14	9.26	9.54	0.68	0.501
Microbial protein (mg/dL)	5.76	6.02	5.68	0.43	0.533

¹ Treatments are G1 (basal diet with no additives), G2 (unprotected L-carnitine) and G3 (rumen-protected L-carnitine) which the concentration of L-carnitine is 200 mg/kg basal diet.

TVFAs = total volatile fatty acids, SEM = standard error of the mean

Table 6
Effects of L-carnitine supplementation on plasma biochemical indexes and L-carnitine concentration in sheep.

Item	Treatments ¹			SEM	P-value
	G1	G2	G3		
Total protein (g/L)	125.5	126.4	132.3	3.06	0.512
Albumin (g/L)	40.85	42.08	42.78	1.93	0.803
Urea nitrogen (mmol/L)	6.32 ^a	5.14 ^b	4.78 ^b	0.12	0.001
L-carnitine (pmol/L)	1.05	1.18	1.24	0.31	0.065

^{a, b} Means in the same row with different superscripts are significantly different ($P < 0.05$).

¹ Treatments are G1 (basal diet with no additives), G2 (unprotected L-carnitine) and G3 (rumen-protected L-carnitine) which the concentration of L-carnitine is 200 mg/kg basal diet.

SEM = standard error of the mean.

Table 7

Effects of L-carnitine supplementation on antioxidant status in sheep.

Item	Treatments ¹			SEM	P-value
	G1	G2	G3		
GSH-Px (U/mL)	216.8 ^c	257.8 ^b	341.0 ^a	2.71	0.003
MDA (mmol/mL)	5.43 ^a	2.83 ^b	2.27 ^c	0.10	0.003
T-AOC (U/mL)	2.15 ^c	2.65 ^b	3.64 ^a	0.09	0.001
SOD (U/mL)	61.84 ^c	77.59 ^b	81.76 ^a	1.06	0.031

^{a-c} Means in the same row with different superscripts are significantly different ($P < 0.05$).

¹ Treatments are G1 (basal diet with no additives), G2 (unprotected L-carnitine) and G3 (rumen-protected L-carnitine) which the concentration of L-carnitine is 200 mg/kg basal diet.

GSH-Px = glutathione peroxidase, MDA = malondialdehyde, T-AOC = total antioxidative capability, SOD = superoxide dismutase, SEM = standard error of the mean.

Table 8
Effects of L-carnitine supplementation on the concentrations of cytokines in sheep.

Item	Treatments ¹			SEM	P-value
	G1	G2	G3		
TNF- α (pg/mL)	391.8	406.8	374.2	28.88	0.915
IL-1 β (pg/mL)	376.3	364.8	310.6	16.76	0.250
IL-6 (pg/mL)	362.3	357.3	324.8	9.46	0.223

¹ Treatments are G1 (basal diet with no additives), G2 (unprotected L-carnitine) and G3 (rumen-protected L-carnitine) which the concentration of L-carnitine is 200 mg/kg basal diet.

TNF- α = tumor necrosis factor- α , IL-1 β = interleukin-1 β , IL-6 = interleukin-6, SEM = standard error of the mean.