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Effect of protected choline on productive performance and ruminal microbiome of growing lambs

Efecto de la colina protegida en el rendimiento productivo y microbioma ruminal de corderos en crecimiento

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ABSTRACT

Choline is an essential nutrient for ruminants and is involved in key metabolic functions. The objective of this study was to evaluate the effect of rumen-protected choline (RPC) supplementation on productive performance and the ruminal microbiome in lambs. Three male lambs (16 ± 0.45 kg BW) were assigned to a 3 × 3 Latin square design with two replicates. Each experimental period lasted 14 days. Animals were fed a basal diet formulated to supply 1.15 Mcal of net energy for gain (NEg) per kg of dry matter and 15% crude protein. Average daily gain (ADG), dry matter intake (DMI), feed conversion ratio (FCR), ruminal pH, ruminal ammonia nitrogen (NH_3-N), and bacterial concentration were evaluated. Supplementation with RPC increased ADG in a treatment-dependent manner and improved FCR without affecting DMI ($p \leq 0.05$). No significant changes in ruminal pH or bacterial populations were observed ($p > 0.05$), with both remaining within optimal physiological ranges, suggesting that RPC enhances productive performance without compromising ruminal fermentation.

Keywords: bypass B-complex; ruminal fermentation; nutraceutical; ruminant nutrition

RESUMEN

La colina es un nutriente esencial para los rumiantes, involucrada en funciones metabólicas clave. El objetivo del estudio fue evaluar el efecto de la suplementación con colina protegida de la degradación ruminal (CPDR) sobre el rendimiento productivo y el microbioma ruminal en corderos. Tres corderos machos (16 ± 0.45 kg PV) fueron distribuidos en un cuadro latino 3 × 3 con doble repetición. Cada periodo experimental duró 14 días. A los animales se les asignó una dieta basal formulada para aportar 1.15 Mcal de ENg/kg de MS y 15% de proteína cruda. Se evaluaron la ganancia diaria de peso (GDP), el consumo de materia seca (CMS), la conversión alimenticia (CA), el pH, el NH_3-N ruminal y la concentración de bacterias. La suplementación con CPDR incrementó la GDP de forma dependiente del tratamiento, y mejoró la CA sin afectar el CMS ($p \leq 0.05$). No se observaron cambios significativos ni en el pH ni en las poblaciones bacterianas ($p > 0.05$), y ambos permanecieron dentro de los rangos fisiológicos óptimos, lo que sugiere que la CPDR favorece el desempeño productivo sin comprometer la fermentación ruminal.

Palabras clave: complejo-B sobrepasante; fermentación ruminal; nutraceútico, nutrición de rumiantes

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1. INTRODUCTION

Choline is an essential nutrient involved in critical physiological functions, including maintaining cell membrane integrity, synthesizing neurotransmitters, regulating cell signaling, metabolizing lipids, and providing methyl groups (Hou et al., 2022). Its conversion into phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin highlights its central role in lipid homeostasis and proper cellular function (Kenny et al., 2025).

Although choline can be synthesized endogenously, this production is insufficient during periods of high metabolic demand, such as lactation, gestation, or rapid growth, thereby justifying dietary supplementation (Pinotti et al., 2004). In intensive production systems, where animals face increased metabolic challenges, such supplementation plays a strategic role in maintaining productivity (Ley-de Coss et al., 2024).

In ruminants, free choline and choline chloride are rapidly degraded in the rumen, limiting their post-ruminal availability (Holdorf et al., 2023). To overcome this limitation, rumen-protected choline (RPC) has been developed to bypass ruminal degradation and be released in the small intestine, thereby maximizing absorption (Arshad & Santos, 2024). In cattle, RPC supplementation has been shown to reduce hepatic triglyceride accumulation, improve dry matter intake, and enhance immune and antioxidant functions (Arshad et al., 2020).

In fattening animals, moderate doses of RPC promote average daily gain and feed efficiency without affecting feed intake; however, excessive levels may compromise carcass quality (Bindel et al., 2000; Kawas et al., 2020). These findings indicate that the effects of RPC depend on both dosage and the production system in which it is applied.

In parallel, phytogenic additives rich in phosphatidylcholine, such as BioCholine®, have been developed from plant sources including *Achyrantes aspera*, *Trachyspermum ammi*, *Azadirachta indica*, *Citrullus colocynthis*, and *Andrographis paniculata* (Mendoza-Martínez et al., 2024). These supplements provide secondary metabolites with antimicrobial, antioxidant, and immunostimulatory properties (Mendoza et al., 2019). In small ruminants, BioCholine® supplementation has improved overall health, average daily gain, and feed efficiency (Jin et al., 2023). Additionally, modulation of 5-hydroxymethylcytosine concentrations in blood has been reported, suggesting potential epigenetic effects (Roque-Jiménez et al., 2020).

Both synthetic choline and choline-based phytogenic additives have demonstrated their capacity to increase choline availability and improve productive parameters. In lambs, doses up to 2.5 g of RPC per kg of dry matter have been shown to increase weight gain without altering ruminal pH or total, cellulolytic, or lactic acid bacterial populations (Huang et al., 2023; Suarez-Suarez et al., 2023). In this context, there is a need to evaluate RPC supplementation under tropical conditions, where sheep production systems face nutritional and environmental constraints. This knowledge gap limits the practical application of RPC in tropical-adapted breeds such as Katahdin and highlights the importance of generating local evidence.

It was hypothesized that increasing RPC supplementation would enhance average daily gain and improve feed conversion in growing lambs by optimizing hepatic lipid metabolism without disrupting ruminal balance. Accordingly, the objective of this study was to evaluate the effect of RPC on average daily gain, feed efficiency, ruminal bacterial populations, ruminal pH, and ammonia nitrogen concentration in growing Katahdin lambs.

2. MATERIALS AND METHODS

Study area

The experiment was conducted from January to July 2024 at the Animal Health Laboratory facilities, equipped with metabolic cages for animal housing, located at the University Center for Technology Transfer (CUTT) "San Ramón," affiliated with the Faculty of Agronomic Sciences, Campus V of the Benemérita Universidad Autónoma de Chiapas, in Villaflores, Chiapas, Mexico (16°27′59″ N, 93°28′43″ W). The climate is classified as warm subhumid (AW1 (W)(i′) g) according to the Köppen–Thornthwaite classification (Yadav, 2024). The region has an average annual precipitation of 1200 mm, concentrated from June to November, a mean annual temperature of 22 °C, and an altitude of 591 m above sea level.

Animals and treatments

Three intact male Katahdin lambs with an initial average body weight of 16 ± 0.45 kg (mean ± SD) were used. Animals were housed individually in metabolic cages and managed in accordance with the Mexican Official Standard for the production, care, and use of laboratory animals (NOM-062-ZOO-1999).

Each lamb was randomly assigned to a 3 × 3 Latin square design with two temporal replicates separated by 30 days. Each experimental period lasted 14 days, consisting of 7 days of adaptation followed by 7 days of data and sample collection.

Three treatments were evaluated: basal diet (BD; control, without RPC), BD + 4 g animal^{-1} day^{-1} of RPC, and BD + 8 g animal^{-1} day^{-1} of RPC. The source of synthetic RPC was Excential Rumenpass CH® (25% choline chloride; ORFFA, Netherlands), which was mixed with the feed immediately before the morning (08:00 h) and afternoon (16:00 h) feedings. Water was provided ad libitum.

Diets were formulated according to NRC (2007) requirements for growing lambs to provide 1.15 Mcal of net energy for gain (NEg) per kg of dry matter and 15% crude protein. The basal diet (BD) consisted of 39% corn stover, 31% rolled yellow corn, 20% soybean meal (44%), 5% sugarcane molasses, 2% bypass fat, 2% commercial mineral mix Salmipro® (Ca 20%, P 3%, Mg 6.5%, S 0.5%, Se 500 ppm, Zn 2500 ppm, I 30 ppm, Co 30 ppm, Cr 500 ppm, Cu 200 ppm, Mn 2500 ppm, Fe 1500 ppm), and 1% NaCl (**Table 1**).

Prior to the experiment, animals were treated with 2% topical fipronil (1 mg kg^{-1} BW) for ectoparasite control.

Table 1.
Calculated nutritional composition of the diet

Nutrient	Concentration (%)
Dry matter (DM)	68.60
Crude protein (CP)	15.00

Ether extract (EE)	4.81
Neutral detergent fiber (NDF)	26.62
Acid detergent fiber (ADF)	15.09
Calcium (Ca)	0.91
Phosphorus (P)	0.38
Net energy for gain (Mcal/kg DM)	1.15

Measurement of productive variables

Dry matter intake (DMI; g day^{-1}) was determined daily during the sampling period as the difference between feed offered and refusals. Body weight was recorded at the beginning and end of each experimental period after a 2-hour partial fasting, using a calibrated electronic scale (±0.1 kg). Average daily gain (ADG; g day^{-1}) was calculated as the difference in body weight between sampling days divided by the duration of the period. Feed conversion ratio (FCR) was calculated as DMI/ADG for each animal.

Behavioral observations

To evaluate diet palatability and potential physiological effects of RPC, continuous behavioral observations were conducted on day 7 of each experimental period from 08:00 to 20:00 h. Observations were performed at 10-minute intervals, recording the time (min day^{-1}) spent eating, drinking, ruminating, resting, or moving within the cages, following the methodology described by Baumont et al. (2004). Body condition score (BCS; scale 1 to 5) was assessed at the beginning and end of each period (Kaler et al., 2009). Additionally, general health conditions were recorded, including coat condition, nasal discharge, ocular health, and overall behavior. Direct observations were complemented by high-resolution fixed-camera recordings to validate consistency and ensure data reliability.

Microbiological and fermentative parameters

In each experimental period, ruminal fluid samples were collected for 7 consecutive days at 10:00 h using an esophageal probe. Approximately 100 mL per animal was obtained and filtered through four layers of sterile gauze. Samples were processed to determine total bacteria (TB), cellulolytic bacteria (CB), and lactic acid bacteria (LAB).

For bacterial counts, 0.5 mL of ruminal fluid was serially diluted and inoculated in triplicate into sterile media: glucose–cellobiose–starch plus ruminal fluid (GCS+RF) for TB, cellulose + RF (C+RF) for CB, and MRS-based enriched medium for LAB. Each medium (4.5 mL) was dispensed into 13 × 100 mm tubes and sterilized at 121 °C and 15 psi for 15 min (All American 1925X autoclave, USA). Cultures were incubated at 38 °C for 24 h under anaerobic conditions with CO_2 flow, using the most probable number (MPN) technique (Cobos et al., 2011).

The GCS+RF medium was prepared using 0.06 g glucose, 0.06 g cellobiose, 0.06 g starch, 30 mL clarified ruminal fluid (centrifuged at 17,664 g for 15 min and sterilized at 121 °C and 15 psi for 20 min), mineral solutions I and II (**Table 2**), 8% Na_2CO_3, sulfide-cysteine reducing solution, tryptic peptone, and 0.1% resazurin as a redox indicator. In the C+RF medium, the GCS substrate was replaced with 0.1 g of Whatman filter paper. For LAB culture, a specific medium containing protease peptone, meat extract, yeast extract, glucose, sorbitan monooleate, KH_2PO_4, sodium acetate, sodium citrate, $MgSO_4·7H_2O$, and $MnSO_4·H_2O$ was used, adjusted to pH 6.35 and sterilized at 121 °C and 15 psi for 15 min.

Table 2.
Calculated nutritional composition of the diet

Reagents	Quantity (g) for 1.0 L of distilled water
Mineral solution I, 5.0 ml	
K_2HPO_4	6.0
Mineral solution II, 5.0 ml	
KH_2PO_4	6.0
$(NH_4)_2SO_4$	6.0
NaCl	12.0
$MgSO_4$	2.45
$CaCl_2·H_2O$	1.6

Ruminal pH was determined using a glass electrode and a digital potentiometer (Orion A250, Orion Research Inc., USA). Ammonia nitrogen (NH_3-N) concentration was determined using the phenol-hypochlorite colorimetric method described by Broderick and Kang (1980). Ten milliliters of filtered ruminal fluid were diluted 1:10 with distilled water, and to each 1.0 mL aliquot, 100 µL of phenol reagent, 100 µL of alkaline solution (NaOH 50 g/L), and 100 µL of 5% sodium hypochlorite were added. After incubation at 37 °C for 15 min, the indophenol complex was quantified at 630 nm using a UV-Vis spectrophotometer (METASH UV-6000, China) with a calibration curve prepared from NH_4Cl standards (0–50 mg/L; $R^2 > 0.99$).

Statistical analysis

The variables DMI, DWG, FI, ruminal pH, and NH_3-N were analyzed using the GLM procedure of SAS (SAS, 2011, version 9.3). The statistical model corresponded to a 3 × 3 Latin square design with two temporal replicates separated by 30 days:

$$Yijkl = \mu + Ti + Aj + Pk + Rl + \varepsilon ijkl$$

Where: Y_{ijkl} = observation of the dependent variable (DMI, DWG, FI, ruminal pH, and ammonia nitrogen), μ = overall mean, T_i = effect of the *i-th* treatment (CPDR dose), A_j = random effect of the *j-th* animal, P_k = effect of the *k-th* period, R_l = effect of the *l-th* temporal replicate, and ε_{ijkl} = random error, assuming a distribution of $N(0, \sigma^2)$.

The microbiological variables (TB, CB, and LAB) did not meet the assumptions of normality and were therefore analyzed using the Kruskal–Wallis test for non-parametric multiple comparisons with independent ranks (Wilcoxon). This test evaluates whether there are statistically significant differences in median values across treatments.

$$H = \frac{9}{n(n+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(n+1)$$

Where: H = Kruskal-Wallis statistic, n = total number of observations, k = number of treatments, R_i = sum of ranks of the *i-th* group, n_i = number of observations of the *i-th* group. Differences were considered significant with $p < 0.05$.

3. RESULTS AND DISCUSSION

Growth and Feed Efficiency

CPDR supplementation improved the productive performance of growing Katahdin lambs in a dose-dependent manner. Final live weight and average daily gain (ADG) increased significantly ($p < 0.05$) with supplementation, reaching the highest values with the 8 g CPDR/animal/day dose compared to the basal diet without affecting initial weight. Dry matter intake (DMI) remained similar between treatments ($p > 0.05$); feed conversion ratio (FCR) improved significantly with both CPDR supplementation levels, with no differences between them, indicating greater feed utilization efficiency in lean tissue (Huang et al., 2023) (**Table 3**).

These results suggest that supplementation with 8 g CPDR/animal/day resulted in the highest ADG without increasing DMI, corroborating a dose-dependent response consistent with that reported by Li et al. (2015) and Supriyati et al. (2016), who observed improvements in average daily gain (ADG) and feed conversion ratio (FCR) with moderate levels of CPDR. These findings support the idea that CPDR optimizes energy and protein balance by improving lipid transport and phospholipid synthesis, particularly phosphatidylcholine, which is essential for hepatic triglyceride export in very low-density lipoproteins (VLDL), thereby promoting productive performance (Shahsavari et al., 2016).

The absence of adverse effects on palatability and voluntary intake confirmed that up to 8 g/day of CPDR did not compromise feed intake (Lee et al., 2012). Overall, CPDR supplementation appears to be an effective nutritional strategy for improving feed efficiency and growth rate in finishing lambs, without affecting voluntary intake or metabolic health**.**

Table 3.

Effect of protected choline on daily weight gain, dry matter intake and feed conversion in Katahdin lambs.

Variable	Treatments			
	BD	BD+4	BD+8	EEM
Initial weight, kg	15.55^{a}	16.35^{a}	15.95^{a}	0.71
Final weight, kg	24.39^{b}	26.98^{ab}	28.78^{a}	0.23
Daily weight gain, g	157.81^{b}	189.82^{ab}	229.10^{a}	13.61
Dry matter intake, g	867.84^{a}	797.58^{b}	899.45^{a}	56.01
Feed conversion	5.49^{a}	4.20^{b}	3.92^{b}	0.96

BD = basal diet (control); BD+4 = BD + 4 g CPDR/animal/day; BD+8 = BD + 8 g CPDR/animal/day. SEM = standard error of the mean. Superscripts a, b indicate statistical differences ($p < 0.05$).

Feeding behavior and digestive activity. On average, all lambs spent between 4 and 4.5 h/day consuming dry matter, between 5 and 5.3 h/day ruminating, between 20 and 25 min/day drinking water, and between 9 and 10 h/day resting or moving. These activity patterns remained unchanged across the three treatments, indicating that the inclusion of CPDR did not affect palatability or voluntary intake, nor did it interfere with the rumination routine necessary for efficient fiber fermentation. These results are consistent with published data in lambs and steers, which showed that similar doses of CPDR did not alter feeding or rumination times (Huang et al., 2023).

Furthermore, the stable drinking time suggested that CPDR supplementation did not increase water requirements, while prolonged periods of rest or movement reflected comfort and well-being, with no signs of restlessness or gastrointestinal disturbances. Overall, it was confirmed that CPDR can be administered at up to 8 g/animal/day without altering feeding behavior or compromising rumen health.

Microbiological and fermentative variables. CPDR supplementation did not significantly affect rumen pH or the populations of total bacteria (TB), cellulolytic bacteria (CB), and lactic acid bacteria (LAB) ($p > 0.05$; **Table 4**), indicating that fermentative homeostasis remained stable. However, the treatment with 8 g/animal/day of CPDR showed a slight upward trend in TB and LAB counts, with no changes in CB populations. This pattern suggested that the higher CPDR dose may have created a slightly more favorable environment for certain microbial groups, without compromising fiber digestion. All bacterial populations remained within normal physiological ranges in healthy ruminants (Dehority, 2003), supporting the hypothesis that CPDR, when released post-ruminally, preserved the integrity of the ruminal ecosystem without inducing fermentative imbalances.

Table 4.
Effect of protected choline on the microbiome, pH, and ammonia nitrogen concentration in the rumen of Katahdin lambs.

Variable Ruminal	Treatments			
	BD	BD+4	BD+8	EEM
Total bacteria ($\times 10^{12}$ CFU mL^{-1})	15.55^{a}	16.35^{a}	15.95^{a}	0.71
Cellulolytic bacteria ($\times 10^{9}$ CFU mL^{-1})	24.39^{b}	26.98^{ab}	28.78^{a}	0.23
Lactic acid bacteria ($\times 10^{10}$ CFU mL^{-1})	157.81^{b}	189.82^{ab}	229.10^{a}	13.61
Ruminal pH (2 h postprandial)	867.84^{a}	797.58^{b}	899.45^{a}	56.01
Ammonia nitrogen (mg dL^{-1})	5.49^{a}	4.20^{b}	3.92^{b}	0.96

BD = basal diet (control); BD+4 = BD + 4 g CPDR/animal/day; BD+8 = BD + 8 g CPDR/animal/day. SEM = standard error of the mean. Superscripts a, b indicates statistical differences ($p < 0.05$).

Rumen pH and ammonia nitrogen (NH_3-N) concentration. Rumen pH ranged from 6.12 to 6.35 (2 h postprandial; **Table 4**), values above the critical threshold (pH < 6.0) associated with subclinical acidosis and consistent with optimal fiber fermentation (de Veth et al., 2016). These findings are consistent with previous studies showing that RPC did not alter basic fermentative parameters (Lee et al., 2012). Furthermore, it has been documented that unprotected choline is rapidly degraded by the rumen microbiota (de Veth et al., 2016), underscoring the use of CPDR to maximize intestinal absorption and systemic effects of choline (Kawas et al., 2020).

The recorded ammonia nitrogen (NH_3-N) concentrations (12.45 to 13.05 mg/dL; **Table 4**) were within the optimal range (10 to 20 mg/dL) for microbial protein synthesis in ruminants (Russell & Wilson, 1996). A slight, non-significant increase was observed with 8 g of CPDR ($p > 0.05$), indicating that supplementation did not alter the balance between protein degradation and fermentable energy availability.

These results were consistent with research on growing ruminants, in which CPDR did not significantly alter ruminal NH3-N concentrations. In older ruminants, Bryant et al. (1999) reported stable levels of 12.5-15.0 mg/dL across various CPDR doses, within the optimal range for microbial protein synthesis. Similarly, Bindel et al. (2000) observed a marginal increase in NH3-N (14.0 to 15.6 mg/dL) with 20 g of CPDR per animal/day, although still within physiological ranges. In growing lambs and fattening sheep, Li et al. (2015) reported concentrations of 11 to 13 mg/dL with moderate doses of CPDR (2.5 g/kg DM), while Supriyati et al. (2016) found levels of 12 to 14 mg/dL with no difference compared to the control.

Overall, the evidence suggests that in lambs, CPDR maintained NH_3-N concentrations within ranges compatible with efficient fermentation and without risk of toxic accumulation (Cobos et al., 2011). Overall, the inclusion of CPDR did not result in significant changes in bacterial counts, pH, or NH_3-N levels, demonstrating that RPC can be administered at up to 8.0 g/animal/day without compromising the stability of the rumen ecosystem. These results are consistent with previous literature showing that CPDR improves production parameters without affecting fermentation or the rumen microbiota (Supriyati et al., 2016; de Veth et al., 2016).

CONCLUSIONS

Supplementation with CPDR was associated with a significant increase in daily weight gain and improved feed conversion efficiency in growing Katahdin lambs, without altering voluntary dry matter intake. Furthermore, pH, NH3-N concentrations, and total, cellulolytic, and lactic acid bacterial counts remained within physiological ranges, indicating that CPDR does not interfere with measured indicators of ruminal fermentation or microbial ecology.

These results indicate that CPDR represents an effective nutritional strategy for optimizing ruminant growth under tropical conditions. Nevertheless, additional research is warranted to more thoroughly evaluate the dynamics of the ruminal microbiome and metabolic integration in breeds adapted to warm climates, such as Katahdin lambs.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this study.

AUTHOR CONTRIBUTIONS

Research: Alondra G. and Fátima V.

Methodology and Data Analysis: Alejandro, Alondra G., and Fátima V.

Validation: Reynerio B., Beatriz Z., Oziel M., and Cándido G.

Drafting – Original Draft: Alejandro.

Drafting – Review and Editing, Discussion of Results, and Manuscript Approval: Reynerio B., Beatriz Z., Oziel M., and Cándido G.

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