



Article

Intestinal Microbiota Mediates the Beneficial Effects of γ -Polyglutamic Acid on Calcium Homeostasis and Bone Properties in Lambs

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Abstract

Optimizing calcium metabolism is crucial for skeletal development and overall productivity in growing ruminants. Twenty-four Sunite lambs were randomly assigned to four groups and fed 0, 0.6, 1.2, or 2.4 g/(d·head) of γ -PGA for 60 days. Growth performance, serum parameters, duodenal morphology and calcium transporter expression, bone microarchitecture, and duodenal microbiota were analyzed. Supplementation with 1.2 g/(d·head) of γ -PGA (the M group) yielded optimal results, significantly improving final body weight and size. It enhanced duodenal health, evidenced by increased villus height, crypt depth, and microvilli density. Crucially, this dose significantly upregulated the expression of key duodenal calcium transporters (TRPV5/6, CaBPD9k, PMCA, VDR, claudin-12) and altered systemic calcium-regulating hormones (elevated calcitriol, PTH, FGF23). Bone micro-CT analysis revealed changes in trabecular architecture indicative of active remodeling. 16S rRNA sequencing and weighted OTU co-expression network analysis (WOCNA) revealed that γ -PGA reshaped the duodenal microbiota and identified core microbial modules strongly associated with host phenotypes. Genera such as [Eubacterium]_ruminantium_group, Fusicatenibacter, and Prevotella emerged as central hubs. In conclusion, dietary γ -PGA at 1.2 g/(d·head) enhances calcium absorption and bone metabolism in lambs through a coordinated modulation of intestinal integrity and calcium transport, systemic endocrine responses, and the duodenal microbial community, with specific microbiota identified as potential key mediators associated with these effects.

Keywords: poly γ -glutamic acid; 16SrRNA; calcium absorption; WOCNA; bone microarchitecture



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1. Introduction

Calcium is a critical mineral for ruminant growth, development, and production. Unlike in monogastric animals, calcium homeostasis in ruminants relies on a synergistic

interaction between gastrointestinal absorption and dynamic bone mobilization [1,2], with renal excretion playing a minimal role [3]. Intestinal calcium absorption occurs via two primary pathways. The transcellular pathway involves apical entry through transient receptor potential vanilloid channels TRPV5 and TRPV6, cytosolic binding to calbindin-D9k, and basolateral extrusion via the sodium–calcium exchanger NCX1 and plasma membrane Ca^{2+} -ATPase PMCA1b. The paracellular pathway, mediated by tight junction proteins such as claudin-2 and claudin-12, allows passive diffusion down a concentration gradient [4].

Calcium homeostasis is regulated by calcitrophil hormones, including calcitriol (active vitamin D or 1,25-dihydroxy vitamin D, 1,25-(OH)₂-VitD₃), parathyroid hormone (PTH), and fibroblast growth factor 23 (FGF23) [5]. Intestinal calcium absorption is primarily regulated by 1,25-dihydroxy vitamin D, which binds to intestinal vitamin D receptors (VDRs) to activate gene transcription. This hormone increases the expression of TRPV6, calbindin-D9k, NCX1, and claudin-2/12, thereby enhancing both transcellular and paracellular calcium transport [6]. Calcitriol promotes bone resorption and calcium mobilization by enhancing osteoclast activity and supports bone mineralization by activating osteoblasts [7]. FGF23 reduces serum calcium, PTH, and calcitriol levels. PTH and calcitriol together stimulate osteoclast precursor maturation, facilitating bone resorption and calcium release into the blood. PTH also augments the vitamin D pathway, promoting calcium absorption via the gastrointestinal tract [8].

Despite the availability of various calcium supplements, limitations such as low absorption efficiency, gastrointestinal irritation, and inconsistent bioavailability persist. There is a clear need for novel, biocompatible supplements that can enhance calcium absorption without adverse effects. Poly- γ -glutamic acid (γ -PGA), a natural, anionic, biodegradable pseudopolypeptide, emerges as a promising candidate [9]. It is non-toxic, non-immunogenic, and widely used in food, feed, and pharmaceutical industries [10–14]. Crucially, γ -PGA can chelate calcium ions, improving their solubility and absorption. Studies in mice show that γ -PGA promotes intestinal calcium uptake, increases body calcium content, and supports bone development more effectively than inorganic calcium [15–17]. Furthermore, in sheep, γ -PGA has been shown to modulate rumen microbiota and function [18], suggesting a potential role in influencing the gastrointestinal environment to enhance nutrient utilization.

Intestinal microbes play a positive role in calcium absorption. Specific bacterial taxa (e.g., *Oscillibacter*, *Bacteroides*) and microbial metabolites like butyrate, derived from fiber fermentation, have been linked to enhanced calcium uptake [19,20]. Moreover, a growing body of evidence connects gut microbial ecology to bone health, indicating that microbes can influence bone mass and structure through immune and endocrine modulation [21–25]. Therefore, effective calcium supplements can not only act directly on the host's absorption pathway, but also indirectly exert their effects by shaping a beneficial gut microbiota.

Sunite sheep, a hardy indigenous breed from the Mongolian Plateau, are valued for their meat quality and adaptability [26]. However, the potential of γ -PGA as a calcium-enhancing supplement for this breed, and the underlying mechanisms, remain unexplored. Based on existing research, we hypothesized that γ -PGA can promote intestinal calcium absorption and bone utilization in sheep through mechanisms involving intestinal microbes and endocrine hormones.

To test this hypothesis, we conducted a dose–response study evaluating growth performance, calcium-regulating hormones, intestinal morphology and transporter expression, bone microarchitecture, and intestinal microbial community structure. This study provides the first comprehensive investigation into the role of γ -PGA in regulating calcium homeostasis via the gut–endocrine–bone axis in ruminants.

2. Results

2.1. Effects of γ -PGA on the Growth Performance of Sunite Lambs

The effects of dietary γ -PGA on growth performance were analyzed using linear mixed-effects models (Table 1). Significant treatment effects were observed for body height (BH, $p = 0.001$), body length (BL, $p = 0.024$), and chest circumference (CG, $p = 0.033$), while body weight (BW) showed a non-significant trend ($p = 0.065$). Time effects were highly significant for all traits ($p < 0.001$), reflecting normal growth. A significant group $\times$ time interaction was detected only for BH ($p < 0.001$), indicating that the effect of γ -PGA on height became more pronounced over time.

Table 1. Growth performance of Sunite lambs supplemented with different levels of γ -PGA.

Item	Time (d)	C (n = 6)	L (n = 6)	M (n = 6)	H (n = 6)	Fixed Effects (p-Value)		
						Treatment	Time	Treatment $\times$ Time
Body weight (kg)	0	25.1 (22.4–27.8)	28.5 (25.8–31.2)	28.3 (25.6–31.0)	28.1 (25.4–30.8)	0.065	<0.001	0.384
	30	27.4 (24.7–30.1)	32.0 (29.3–34.7)	32.0 (29.4–34.7)	30.5 (27.8–33.2)			
	60	31.0 (28.3–33.7)	35.3 (32.6–38.0)	36.6 (34.0–39.3)	34.0 (31.3–36.7)			
Body height (cm)	0	58.2 (55.9–60.4)	60.2 (57.9–62.4)	59.0 (56.7–61.3)	59.5 (57.2–61.8)	0.001	<0.001	<0.001
	30	58.0 (55.7–60.3)	63.3 (61.1–65.6)	64.7 (62.4–66.9)	61.3 (59.1–63.6)			
	60	60.4 (58.0–62.8)	70.6 (68.2–73.0)	66.8 (64.2–69.4)	62.8 (60.2–65.5)			
Body length (cm)	0	60.7 (57.1–64.3)	63.0 (59.4–66.6)	59.3 (55.7–62.9)	63.2 (59.6–66.8)	0.024	<0.001	0.403
	30	66.8 (63.2–70.4)	69.8 (66.2–73.4)	68.7 (65.1–72.3)	71.8 (68.2–75.4)			
	60	66.8 (62.3–71.2)	75.5 (71.9–79.1)	73.7 (70.1–77.3)	75.2 (71.2–79.2)			
Chest circumference (cm)	0	77.5 (73.9–81.1)	79.5 (75.9–83.1)	78.7 (75.1–82.3)	78.8 (75.2–82.4)	0.033	<0.001	0.106
	30	83.5 (79.9–87.1)	89.5 (85.9–93.1)	90.2 (86.6–93.8)	87.0 (83.4–90.6)			
	60	90.8 (87.2–94.4)	100.3 (96.7–103.9)	99.5 (95.7–103.4)	97.4 (93.1–101.6)			

At day 60, the L group (0.6 g/(d·head)) exhibited the greatest BH (70.6 cm, 95% CI: 68.2–73.0 cm), BL (75.5 cm, 95% CI: 71.9–79.1 cm), and CG (100.3 cm, 95% CI: 96.7–103.9 cm). The M group (1.2 g/(d·head)) showed the highest BW (36.6 kg, 95% CI: 34.0–39.3 kg), while the H group (2.4 g/(d·head)) generally showed intermediate values. Cubic polynomial regression of overall ADG identified 1.2 g/(d·head) as the optimal dose (Figure 1). Detailed statistical outputs of the mixed-effects models, including fixed-effect estimates, variance components, and ICCs, are provided in Table S1.

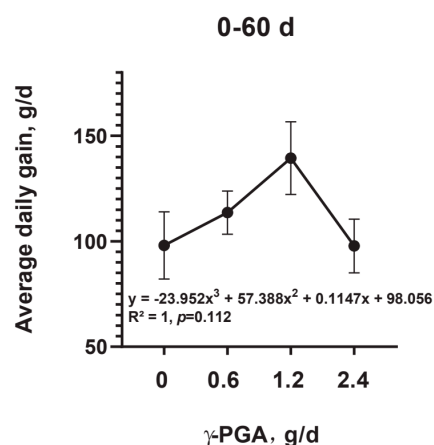


Figure 1. Cubic polynomial regression analysis of average daily gain (ADG) across γ -PGA supplementation levels. $n = 6$ per group. 0: C group; 0.6 g/(d·head): L group; 1.2 g/(d·head): M group; 2.4 g/(d·head): H group.

2.2. Serum Minerals, Hormones, and Antioxidant Status

Serum calcium and phosphorus concentrations remained stable and were not significantly affected by γ -PGA supplementation at either day 30 or day 60 (Figure 2). Likewise, no significant treatment effects were observed for serum biochemical indices related to protein and lipid metabolism (Table S2).

In contrast, the circulating levels of key calcium-regulating hormones exhibited marked dose-dependent changes by day 60 (Figure 2). Serum calcitriol, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and calcitonin (CT) all increased progressively with higher γ -PGA supplementation, showing significant linear and quadratic trends ($p < 0.001$). At day 60, the M (1.2 g/d) and H (2.4 g/d) groups generally displayed significantly higher hormone concentrations than the control group (indicated by asterisks in Figure 2). At day 30, only FGF23 responded significantly, with lower levels in the L and M groups compared to control ($p < 0.01$).

The activities of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH) were modulated by γ -PGA in a quadratic, dose-dependent manner. At both day 30 and day 60, SOD and GSH activities peaked in the M group and were significantly higher than in the control and H groups ($p < 0.05$). This pattern was confirmed by significant quadratic contrasts ($p < 0.01$) and the absence of linear trends.

2.3. Duodenal Morphology and Calcium Transport Protein Expression

The concentrations of calcium and phosphorus in the duodenal and ileal digesta were not significantly affected by dietary treatment (Table 2). However, ileal phosphorus concentration showed a linear and quadratic increasing trend across the γ -PGA dosage gradient ($p < 0.05$).

Table 2. Content of the levels of calcium and phosphorus of the duodenum and ileum in sunite sheep with different levels of γ -PGA supplementation (C, L, M and H group, $n = 6$).

Items	Content	C	L	M	H	SEM	<i>p</i> -Value		
							Treat	Linear	Quadratic
duodenum	Ca, mg/kg	1130.83	1640.00	1640.00	1587.00	180.87	0.168	0.177	0.105
	P, mg/kg	1036.00	901.17	887.17	781.67	100.627	0.382	0.089	0.228
ileum	Ca, mg/kg	4063.33	4861.67	4521.67	6384.00	808.386	0.724	0.311	0.605
	P, mg/kg	878.83	800.00	858.83	1347.33	166.330	0.107	0.034	0.044

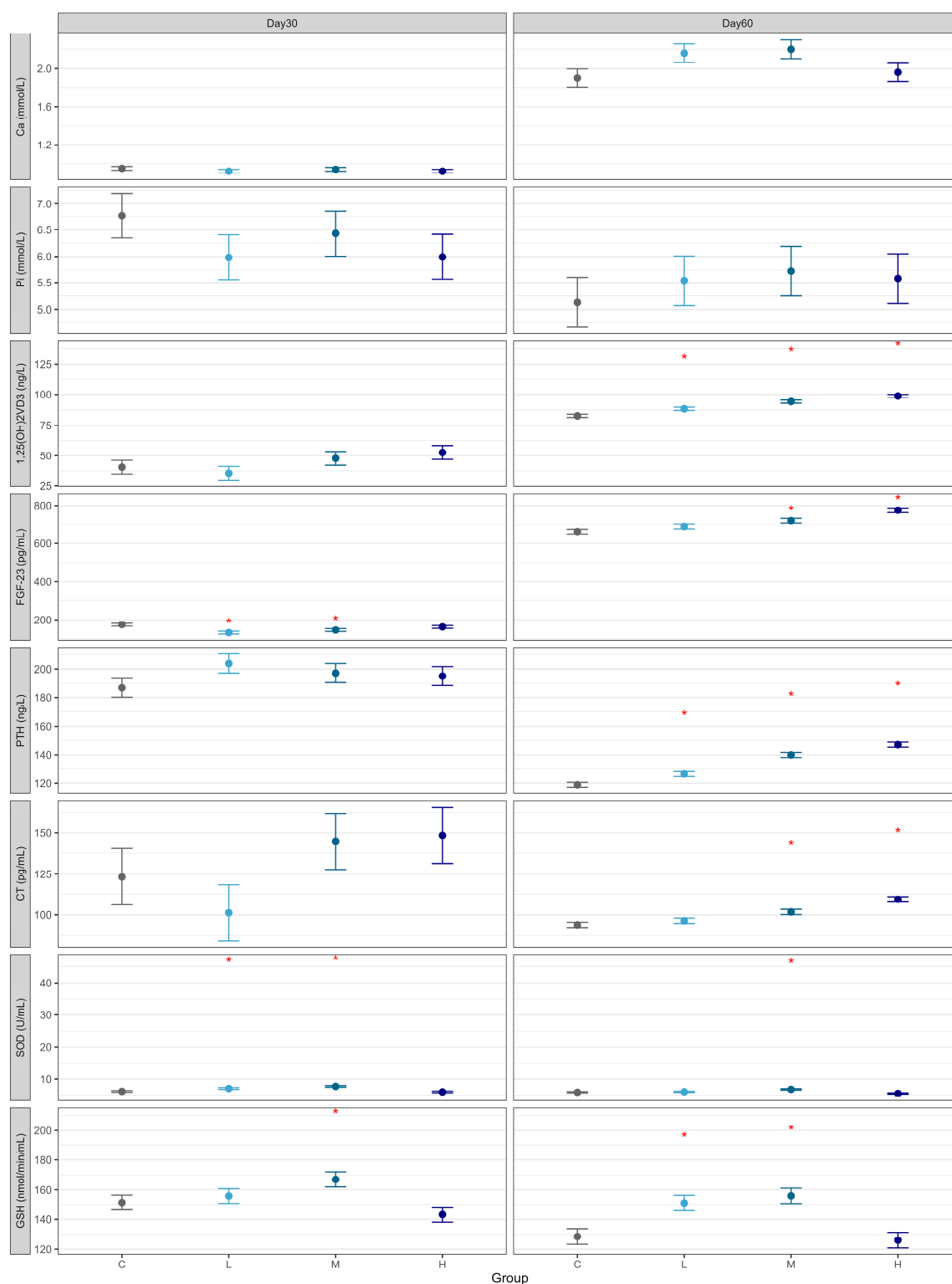


Figure 2. Serum calcium-regulating hormones and antioxidant enzymes in Sunite lambs supplemented with different levels of γ -PGA (C: 0, L: 0.6, M: 1.2, H: 2.4 g/(d·head)). Data are presented as mean $\pm$ SEM ($n = 6$ per group). Red asterisks indicate significant differences compared to the control group (C) within the same time point ($p < 0.05$, based on Tukey's test).

Although not reaching statistical significance (Table 3), duodenal villus height and crypt depth showed a clear increasing trend in the L and M groups compared to the C group, with the M group exhibiting the greatest numerical values. Representative hematoxylin and eosin (H&E) and Transmission electron microscopy (TEM) revealed that enterocytes

from the M group possessed more densely packed microvilli and a greater abundance of mitochondria compared to the control (Figures 3 and 4).

Table 3. Data of development of duodenal epithelium was observed by HE staining in sunite sheep with different levels of γ -PGA supplementation (C, L, M and H group, $n = 6$).

Items	Groups				SEM	<i>p</i> -Value		
	C	L	M	H		Treat	Linear	Quadratic
Duodenal muscle thickness	0.37	0.49	0.37	0.44	0.066	0.599	0.763	0.950
Duodenal crypt depth	0.22	0.36	0.48	0.19	0.092	0.236	0.107	0.793
Duodenal villus length	0.60	0.73	0.74	0.59	0.077	0.436	0.751	0.225

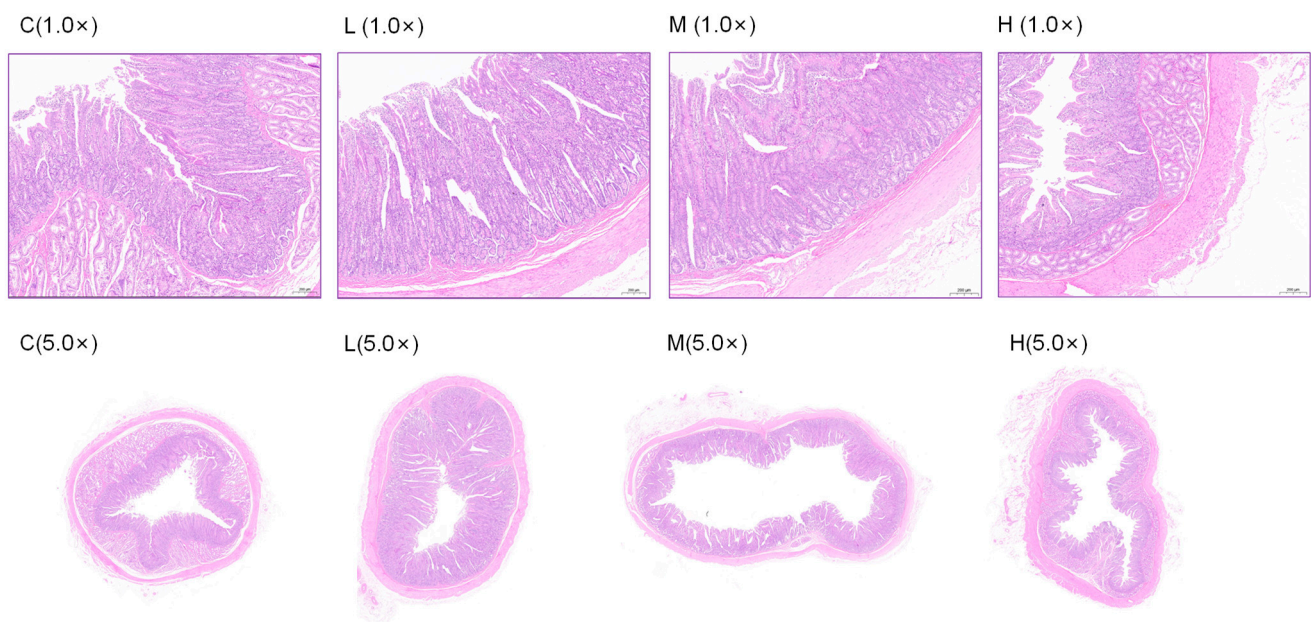


Figure 3. Representative hematoxylin and eosin (H&E) staining of duodenal sections from each group (1.0 $\times$ and 5.0 $\times$ magnification). (C, L, M and H group, $n = 6$).

Consistent with the morphological observations, the protein expression of key mediators involved in intestinal calcium absorption was significantly upregulated in the M group. This included proteins for both transcellular transport (TRPV5, TRPV6, calbindin-D9k, NCX1, PMCA, VDR) and paracellular transport (claudin-12). As shown in Figure 5, the M group exhibited increased protein levels of all measured transporters in both duodenal and ileal tissues compared to the C group.

2.4. Bone Microarchitecture

Micro-computed tomography analysis of the femoral metaphysis revealed that dietary γ -PGA supplementation altered several trabecular bone parameters (Table 4, Figure 6).

Several parameters indicative of trabecular architecture showed significant, dose-dependent changes. The structure model index (SMI) increased significantly with higher γ -PGA doses ($p = 0.023$, linear trend $p = 0.026$), rising from -0.87 in group C to 0.83 in group H. As SMI values approaching 0 and $+3$ indicate a transition from an ideal plate-like structure to a more rod-like architecture, this shift suggests a dose-related alteration in trabecular morphology. Consistent with this, the trabecular pattern factor (Tb.Pf), a measure of surface convexity where higher values indicate a more disconnected structure, also showed a significant dose-dependent increase ($p = 0.004$, linear trend $p = 0.005$), progressing from -4.68 mm^{-1} in group C to 1.23 mm^{-1} in group H. The bone surface to

bone volume ratio (BS/BV) trended higher with increasing dose ($p = 0.069$, linear trend $p = 0.042$), further implying a change towards more numerous, slender trabecular elements.

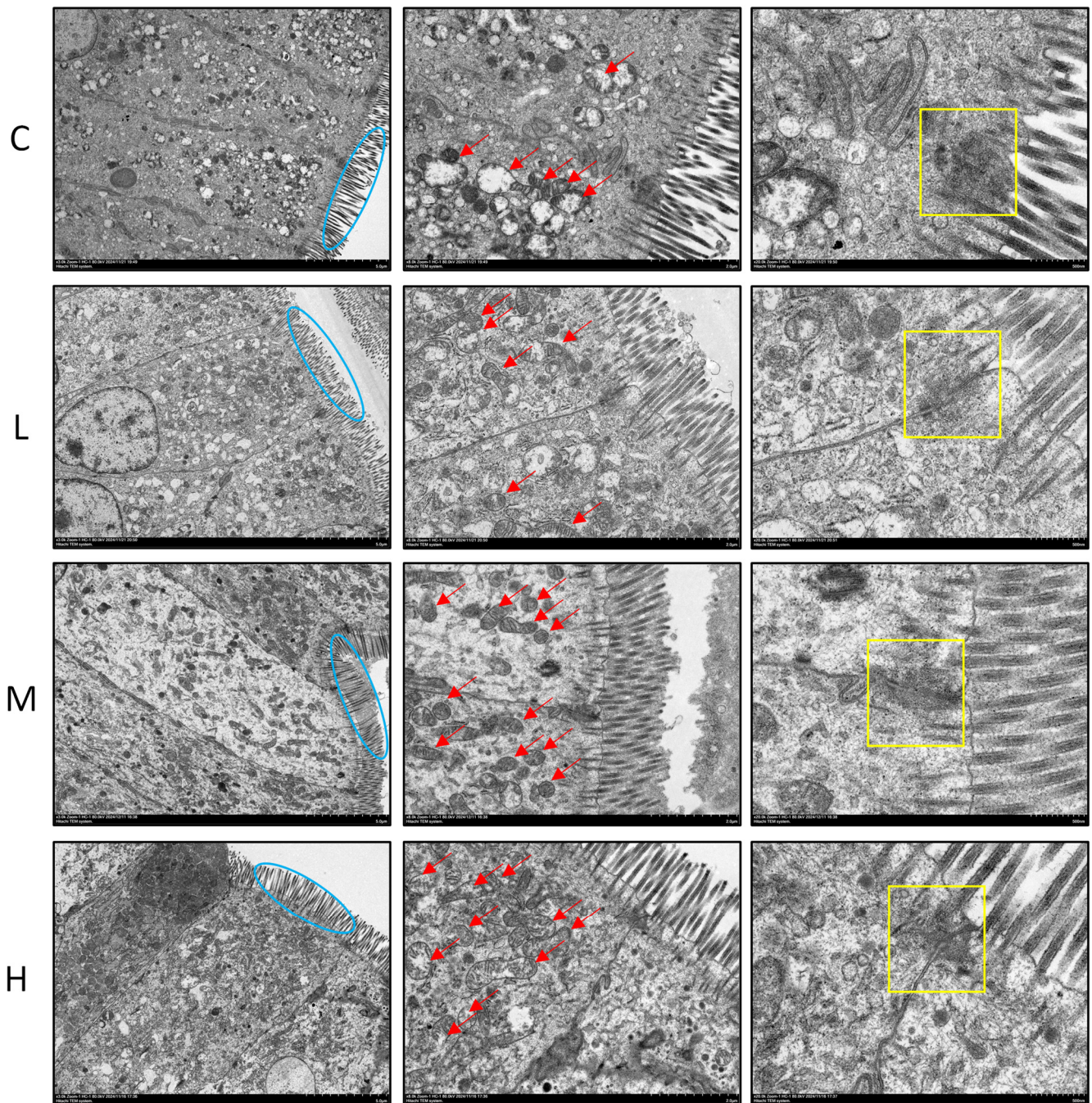


Figure 4. Transmission electron microscopy (TEM) images of duodenal epithelial cells with different levels of γ -PGA supplementation (C, L, M and H group, $n = 6$). Blue circles indicate microvilli, red arrows indicate mitochondria, and yellow boxes indicate tight junctions. Scale bar: 2 μm .

Interestingly, parameters reflecting connectivity and density presented a more complex, non-linear picture. Connectivity density (Conn.D), a direct measure of trabecular interconnectivity, tended to differ among groups ($p = 0.077$) and demonstrated a significant quadratic response ($p = 0.024$), with the highest value observed in the mid-dose M group (4.21 mm^{-3}) compared to controls (2.34 mm^{-3}). This suggests a potential biphasic effect on connectivity. Conversely, the CT attenuation value of the trabecular cavity (TbCav.CT_Value), a proxy for tissue mineral density within the marrow space, showed a decreasing linear trend with increasing γ -PGA ($p = 0.091$, linear $p = 0.044$). Consistently,

bone mineral density (BMD) also tended to decrease across groups ($p = 0.108$) with a significant linear trend ($p = 0.042$).

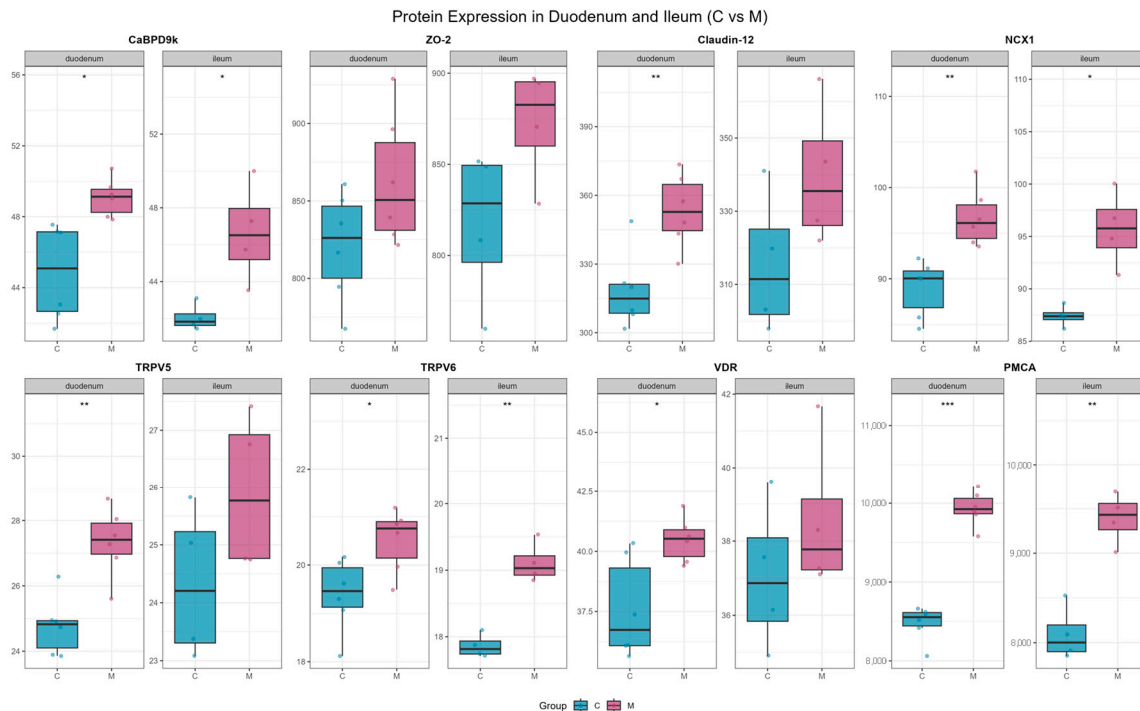


Figure 5. Protein expression of calcium transporters in the duodenum and ileum of Sunite lambs. Box plots show the expression levels of eight proteins in the duodenum and ileum for control (C, blue) and γ -PGA-supplemented (M, purple) groups, each circle represents an individual sample (data point). Asterisks indicate significant differences after FDR correction (* $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$).

Table 4. Data of Bone analysis parameters, bone trabecular analysis parameters in sunite sheep with different levels of γ -PGA supplementation (C, L, M and H group, $n = 6$).

Items	Groups				SEM	<i>p</i> -Value		
	C	L	M	H		Treat	Linear	Quadratic
BV, mm ³	1019.37	747.93	938.79	668.51	162.286	0.476	0.233	0.526
TV, mm ³	1831.64	1827.07	1845.48	1939.38	231.010	0.982	0.672	0.908
BV/TV	0.56	0.41	0.51	0.33	0.071	0.110	0.055	0.188
BS, mm ²	6569.58	6393.90	7824.08	5855.61	1239.518	0.730	0.747	0.666
BS/TV, mm ^{−1}	3.56	3.51	4.22	2.95	0.298	0.152	0.37	0.172
BS/BV, mm ^{−1}	6.39	8.52	8.41	9.01	0.499	0.069	0.042	0.050
Tb.Th average, mm	0.48	0.38	0.39	0.36	0.027	0.101	0.059	0.071
Tb.Th max, mm	0.99	0.78	0.90	0.81	0.089	0.427	0.328	0.559
Tb.Sp avg, mm	0.72	0.66	0.49	0.78	0.108	0.403	0.755	0.285
Tb.N, mm ^{−1}	0.85	0.96	1.13	0.89	0.078	0.187	0.779	0.107
Tb.Pf, mm ^{−1}	−4.68	−1.43	−2.83	1.23	0.481	0.004	0.005	0.031
SMI	−0.87	0.44	−0.04	0.83	0.225	0.023	0.026	0.083
BTC	4272.23	6340.28	7729.52	5973.50	1226.495	0.379	0.462	0.179
Conn.D, mm ^{−3}	2.34	3.42	4.21	3.00	0.349	0.077	0.546	0.024
DA	0.40	0.40	0.38	0.35	0.026	0.589	0.138	0.345
FD	2.46	2.51	2.51	2.49	0.050	0.890	0.773	0.740
TbCav.CT_Value, HU	1066.49	755.85	895.86	634.36	87.848	0.091	0.044	0.151
BMD, mg/cm ³	326.75	230.94	270.48	189.48	29.347	0.108	0.042	0.147
BMC, mg	596.29	416.23	503.24	379.82	88.830	0.421	0.187	0.431

Table 4. Cont.

Items	Groups				SEM	p-Value		
	C	L	M	H		Treat	Linear	Quadratic
Tb.CT_Value, HU	1611.46	1420.20	1407.56	1374.81	70.878	0.221	0.089	0.113
TMD, mg/cm ³	501.42	438.60	434.45	423.69	23.279	0.221	0.089	0.113
TMC, mg	510.15	326.68	411.53	290.40	81.661	0.357	0.159	0.379
Bone wet weight, kg	0.12	0.12	0.13	0.12	0.010	0.863	0.768	0.839
Bone dry weight, kg	0.08	0.08	0.09	0.08	0.007	0.859	0.930	0.800

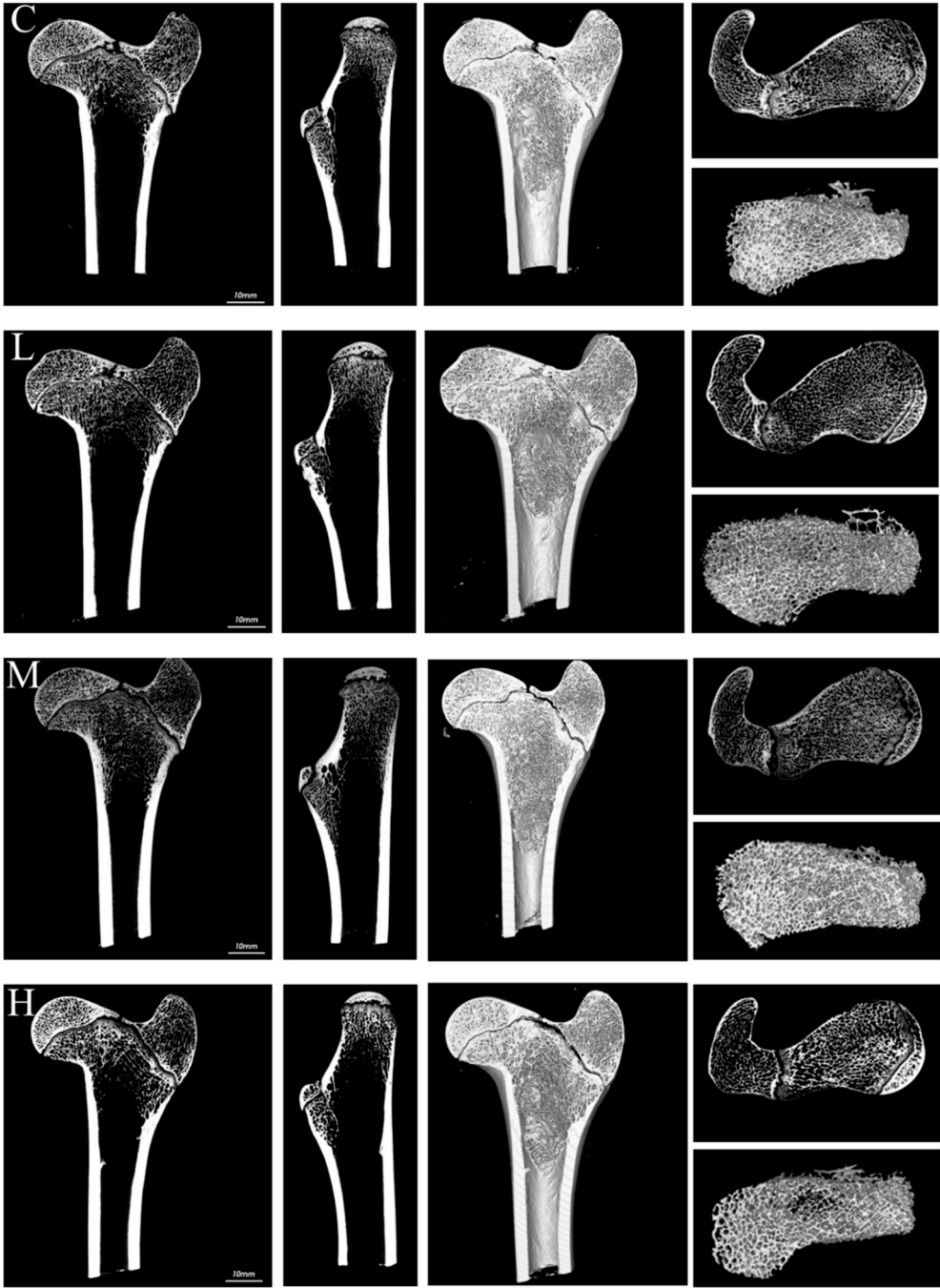


Figure 6. Three-dimensional micro-computed tomography (micro-CT) reconstructions of the proximal femoral metaphysis with different levels of γ -PGA supplementation (C, L, M and H group, $n = 6$). Scale bar: 5 mm.

No significant differences were observed in other static parameters including bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), or bone mineral content (BMC) ($p > 0.05$ for all). Bone wet weight and dry weight were also unaffected by treatment ($p > 0.05$).

In summary, γ -PGA supplementation did not increase bone mass or mineral density as measured by BV/TV and BMD. Instead, it induced significant architectural changes, characterized by a shift from plate-like to rod-like trabeculae (increased SMI and Tb.Pf) and a trend towards increased surface complexity (increased BS/BV). The trend towards reduced CT attenuation in the trabecular cavity, alongside the non-significant changes in BV/TV and BMD, suggests that the observed structural remodeling was not accompanied by enhanced net mineral deposition. The peak in Conn.D observed in the M group hints at a potential optimal dose for maintaining trabecular connectivity, despite the overall trend towards a more rod-like structure.

2.5. Duodenal Microbiota Composition and Diversity

To further analyze the effects of γ -PGA on intestinal microbial diversity in Sunite lambs, a total of 1,723,809 clean reads were obtained from duodenal samples (Table S3). The valid read ratio across the four groups was 94.5%. The species accumulation curve initially increased sharply, then stabilized, and the number of common species did not decrease with increasing sample size. The gradation–abundance curve showed that groups C and H had the highest richness and uniformity (Figure S2).

The intestinal microbiota (relative abundance) was classified and analyzed at the phylum, genus, and species levels and is displayed in bar charts. Core and dominant taxa, defined as those with more than 0.1% relative abundance across all four groups, were identified at the phylum (Figure 7A), genus (Figure 7B), and species (Figure 7C) levels. At the phylum level, Firmicutes, Actinobacteria, Bacteroidota, Proteobacteria, and Spirochaetota were predominant. Compared with group C, Firmicutes and Actinobacteria showed an increasing-then-decreasing trend with rising γ -PGA levels. Bacteroidota and Spirochaetota decreased initially and then increased ($p > 0.05$), while Proteobacteria displayed a significant trend of decreasing first and then increasing ($p < 0.05$) (Figure 7A). At the genus level, alpha diversity index results showed that the dominant genera included *Olsenella*, *Prevotella*, *Sharpea*, *Ruminococcus*, *Bifidobacterium*, *Roseburia*, *unclassified_[Eubacterium]_coprostanoligenes_group*, *Acetitomaculum*, *Lachnospiraceae_NK3A20_group*, and *Succinivibrio* (Figure 7B). At the species level, the most abundant taxa included *Parafannyhessea umbonata*, *Sharpea azabuensis*, *uncultured rumen bacterium*, *Prevotella ruminicola*, *Ruminococcus bromii*, *Rumenobacter*, *Prevotella bryantii*, *unclassified_[Eubacterium]_coprostanoligenes_group*, *Clostridiales bacterium JS109*, *Bifidobacterium pseudolongum*, and *Roseburia faecis* (Figure 7C). The comparison of different OTUs across groups was performed using a Venn diagram. A total of 101 species were shared across groups, with group-specific OTU counts of 1836 in group C, 1526 in group L, 1732 in group M, and 2038 in group H (Figure 7G). PCoA based on Bray Curtis distances showed a tendency for separation of duodenal microbial communities among the four group (Figure 7H). PERMANOVA confirmed a marginal effect of γ -PGA supplementation on community composition, explaining 17.8% of the variation ($R^2 = 0.178$, $p = 0.068$). However, the clustering did not differ significantly between treatment and control groups. A cross-distribution of microbial composition was observed among all groups (Figure 7I).

Alpha diversity index results are shown in Figure 7D–F. The Shannon diversity index demonstrated significant changes in richness and evenness with increasing γ -PGA supplementation. Group L had significantly lower diversity than group H ($p < 0.05$), while no significant differences were observed among groups M, H, and C ($p > 0.05$). The Chao1

estimator and Simpson index showed no significant differences in species richness and concentration among the four groups ($p > 0.05$).

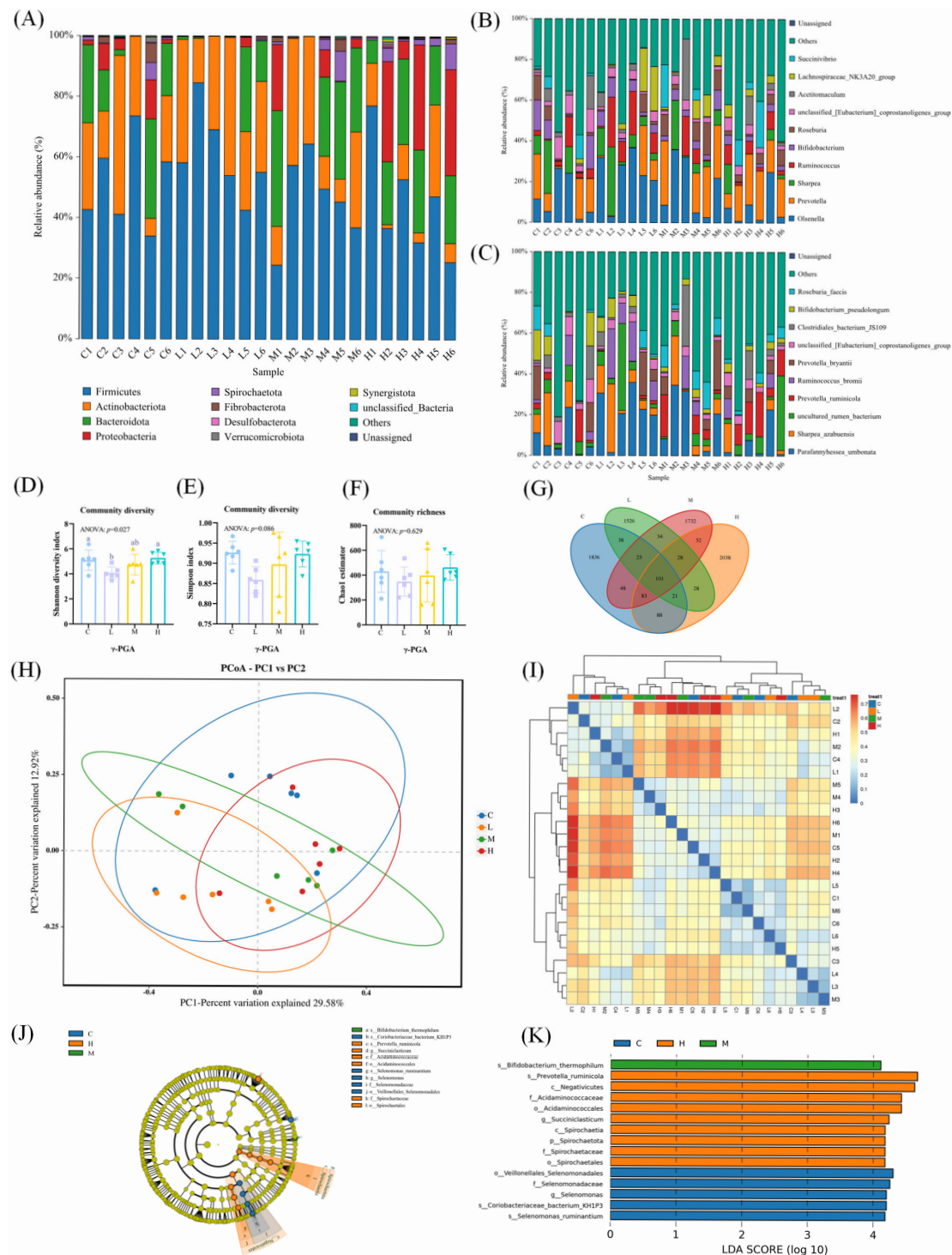


Figure 7. Duodenal microbiota composition and diversity in Sunite lambs supplemented with different levels of γ -PGA. Relative abundance at the phylum (A), genus (B), and species (C) levels (taxa with $\geq 0.1\%$ abundance). Alpha diversity in-dices: Shannon (D), Chao1 (E), and Simpson (F). p -values are derived from ANOVA testing for differences among groups (letters). (G) Venn diagram showing shared and unique OTUs among groups. (H) Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity. (I) Heatmap clustering of microbial communities at the OTU level. C: 0 g/(d-head); L: 0.6 g/(d-head); M: 1.2 g/(d-head); H: 2.4 g/(d-head). $n = 6$ per group. (J) Cladogram showing taxonomic representation across groups. (K) Histogram of LDA scores (log 10) showing differentially abundant taxa.

2.6. Differential Microbial Abundance

Linear discriminant analysis effect size (LEfSe) was used to identify taxa with significantly different abundances among the four groups, allowing for comparison of their relative contributions. LEfSe results showed that *Bifidobacterium thermophilum* in group M, and *Prevotella ruminicola*, *Negativicutes*, *Acidaminococcaceae*, *Succiniclaticum*, *Spirochaetia*, *Spirochaetaceae*, and *Spirochaetales* in group H were highly abundant under high γ -PGA supplementation. In contrast, *Veillonellales Selenomonadales*, *Selenomonadaceae*, *Coriobacteriaceae bacterium KH1P3*, and *Selenomonas ruminantium* were more abundant in group C (Figure 7J,K).

2.7. Microbial Co-Expression Network and Its Association with Host Phenotypes

Weighted OTU co-expression network analysis (WOCNA) of the duodenal microbiota identified seven distinct co-abundance modules (Figure 8A). Correlation analyses between module eigengenes and a comprehensive set of host phenotypic traits revealed several significant and biologically relevant associations (Figure 8B,C).

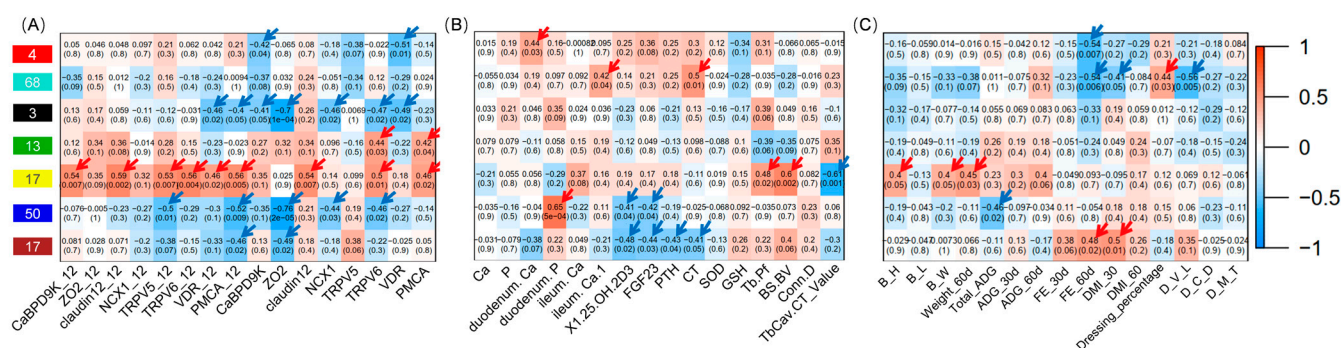


Figure 8. Weighted OTU co-expression network analysis (WOCNA) identifying microbial modules associated with host phenotypic traits. (A) Hierarchical clustering dendrogram showing seven distinct co-abundance modules (yellow, blue, brown, turquoise, etc.). (B) Heatmap of module–trait correlations, with red indicating positive and blue indicating negative correlations. (C) Detailed correlation matrix between module eigengenes and key phenotypic traits, including growth parameters, calcium transporters, and bone indices. Red arrows indicate a significant positive correlation, while blue arrows indicate a significant negative correlation ($p < 0.05$).

The yellow module exhibited the most notable associations with beneficial host outcomes. It was positively correlated with growth performance (body weight, height, and chest circumference: $r = 0.40$ – 0.45 , $p < 0.05$) and, crucially, with the protein expression of key duodenal calcium transporters and channels involved in both transcellular (TRPV5, TRPV6, CaBPD9k, PMCA, VDR) and paracellular (claudin-12) absorption ($r = 0.46$ – 0.59 , $p < 0.05$). This module was negatively correlated with serum calcitonin levels.

In contrast, the blue module and brown module showed significant negative correlations with serum hormones that promote calcium absorption and bone turnover, specifically calcitriol and FGF23 ($r = -0.41$ to -0.48 , $p < 0.05$). The brown module was also negatively correlated with PTH and feed efficiency.

Other modules were linked to specific traits: the turquoise module positively correlated with slaughter rate but negatively with feed efficiency and duodenal villus length; the red module negatively correlated with feed efficiency and positively with duodenal calcium content.

Network topology analysis within the most relevant modules identified several genera with high connectivity, suggesting their potential role as hub taxa. According to the network attribute parameters (Figure 9, Table 5), *Fusicatenibacter*, *Leuconostoc*, *Salipaludibacillus*, and *Staphylococcus* had the highest connectivity in the blue module. In the turquoise module,

Treponema, *Pyramidobacter*, *Rikenellaceae* RC9 gut group, and *Prevotella* were most connected. The brown module was dominated by the *Family XIII AD3011* group, and the yellow module showed high connectivity in *unclassified RF39*, *Methanobrevibacter*, *[Eubacterium] ruminantium* group, *unclassified Clostridia* UCG-014, and *Erysipelotrichaceae* UCG-006 (Table S4).

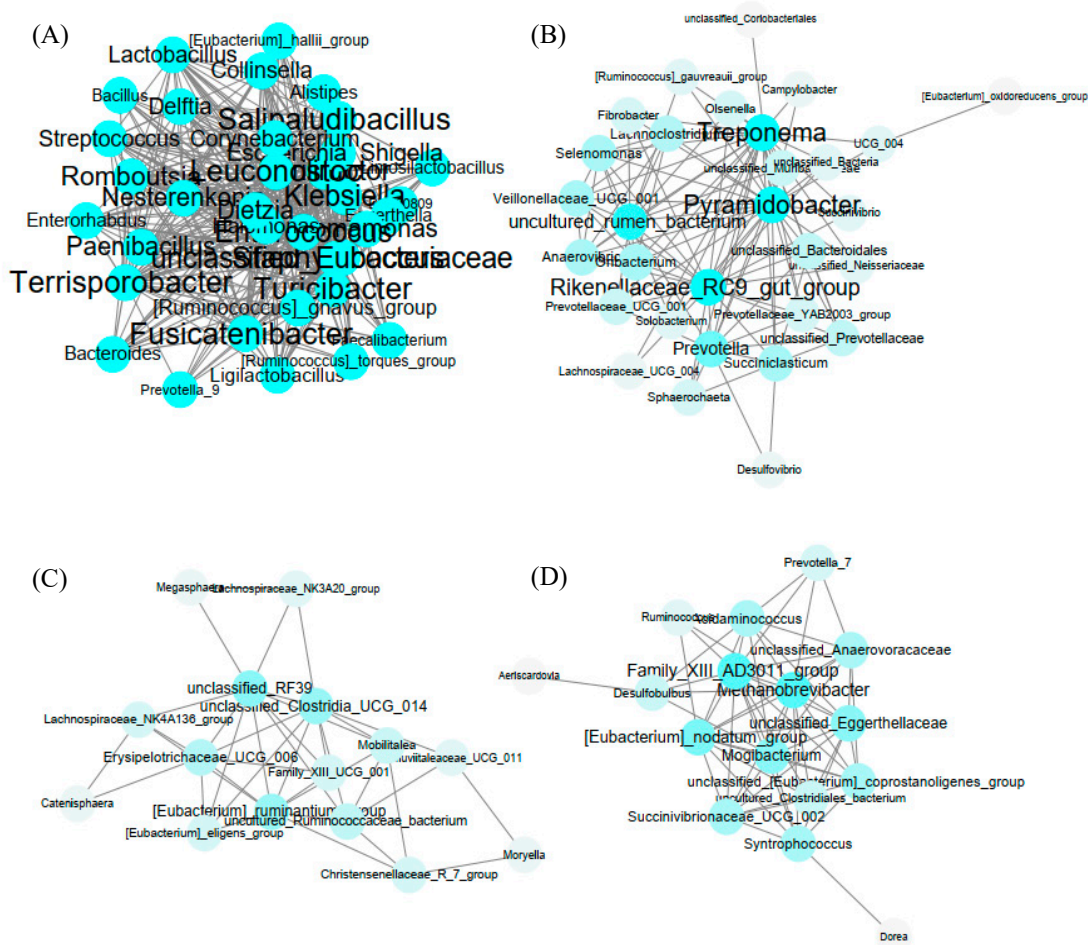


Figure 9. Network visualization of microbial co-expression modules significantly associated with host phenotypes. Node color intensity and label size correspond to degree of connectivity (darker color and larger label = higher connectivity). (A) Blue module, (B) Turquoise module, (C) Yellow module, (D) Brown module. Hub taxa with highest connectivity are labeled.

Table 5. Data of WOCNA parameters of duodenal intestinal genus level microorganisms with different levels of γ -PGA supplementation.

Microorganisms Name	AP	BC	Color	D	NC
Fusicatenibacter	1.00	0.04	blue	34	21.18
Leuconostoc	1.00	0.04	blue	34	21.18
Salipaludibacillus	1.00	0.04	blue	34	21.18
Staphylococcus	1.00	0.04	blue	34	21.18
Treponema	1.10	0.29	turquoise	26	6.92
Pyramidobacter	1.14	0.29	turquoise	25	7.04
Rikenellaceae_RC9_gut_group	1.31	0.13	turquoise	21	7.95
Family_XIII_AD3011_group	1.13	0.15	brown	13	7.77
Methanobrevibacter	1.13	0.15	brown	13	7.77
Prevotella	1.62	0.06	turquoise	12	10.58
[Eubacterium]_nodatum_group	1.33	0.05	brown	11	8.55
unclassified_RF39	1.36	0.24	yellow	10	5.30

Table 5. Cont.

Microorganisms Name	AP	BC	Color	D	NC
Mogibacterium	1.33	0.05	brown	10	9.20
[Eubacterium]_ruminantium_group	1.36	0.17	yellow	9	5.89
unclassified_Clostridia_UCG_014	1.36	0.19	yellow	9	5.67
Erysipelotrichaceae_UCG_006	1.57	0.11	yellow	7	6.00

3. Discussion

This study provides the first comprehensive evidence that dietary supplementation with poly- γ -glutamic acid (γ -PGA) is associated with changes in calcium metabolism and growth performance in growing ruminants via a coordinated gut–microbiota–endocrine–bone axis. Based on integrated analysis of growth, duodenal structure, bone microarchitecture, and microbial communities, 1.2 g/(d·head) was identified as the optimal dose for Sunite lambs.

Mixed-model analysis of growth performance revealed significant treatment effects on body height ($p = 0.001$), body length ($p = 0.024$), and chest circumference ($p = 0.033$). At day 60, the L group (0.6 g/d) exhibited the greatest values for these skeletal measures, while the M group (1.2 g/d) had the numerically highest body weight. The significant group $\times$ time interaction for body height ($p < 0.001$) indicates that the effect of γ -PGA on longitudinal growth became more pronounced over time, consistent with cubic polynomial regression identifying 1.2 g/d as the optimal dose for average daily gain (Figure 2).

The intestinal absorption process is particularly critical in ruminants. It involves the regulation of paracellular transport through claudin-2 and claudin-12 and active intracellular transport via TRPV6, calbindin-D9k, VDR, NCX1, PMCA1b, and calcitriol. In this study, protein levels of duodenal and ileal calcium-binding and tight-junction proteins were measured in group M. Transmission electron microscopy confirmed that γ -PGA not only enhanced intracellular calcium transport in duodenal cells but also improved intercellular absorption. In contrast, the ileum primarily supported intracellular calcium transport. Previous studies have shown that the intestinal epithelium contains a unique intercellular junction system, in which claudin-2 and claudin-12 form complementary pores to maintain calcium homeostasis [27–29]. When calcium concentration exceeds plasma levels, it can enter the intestine via paracellular pathways regulated by these junction proteins. Mice lacking both claudin-2 and claudin-12 exhibited reduced calcium uptake without changes in intestinal permeability [30].

On the apical membrane of ruminant intestinal cells, TRPV6 mediates calcium entry. Once in the cytoplasm, the calbindin-D9k complex transports calcium to the basolateral membrane. There, calcium is released from calbindin-D9k and extruded from the cell by NCX1 in an energy-independent manner. Although calcium also passes through paracellular pathways, recent studies suggest that vitamin D modulates claudin-2 and claudin-12, thereby influencing passive calcium transport from the intestinal lumen to the interstitial space. These proteins are widely expressed across species and play a regulatory role in calcium transport under the influence of calcitriol [27].

It is important to note that the biological activity of γ -PGA is known to be influenced by its molecular weight and purity. Previous studies have reported that high-molecular-weight γ -PGA (>2000 kDa) exhibits immunostimulatory properties [31] and can modulate lipid metabolism [32], while lower-molecular-weight forms may be more readily fermented by gut microbiota or exhibit different chelating capacities for minerals such as calcium. The γ -PGA used in this study had a molecular weight range of 15–20 kDa and a purity of 33.03%, which was selected based on prior work in sheep [18] and considerations of

practical application as a feed additive. In this study, γ -PGA did not significantly affect biochemical or lipid metabolism indexes, nor did it significantly influence total average daily gain, although a trend of initial increase followed by decline was observed. This difference in findings may be attributed to the unique gastrointestinal environment of sheep [33]. We found that γ -PGA did not directly alter plasma calcium or phosphorus levels, consistent with the known stability of blood calcium and phosphorus within a narrow range [34,35]. By comparing short- and long-term changes in antioxidant indexes, calcium and phosphorus levels, and calcium-regulating hormones, it was observed that γ -PGA inhibited calcitonin levels (Table S3) and enhanced antioxidant capacity over time.

Jong et al. reported that γ -PGA offers protective effects against oxidative damage in cells and probiotics, indicating antioxidant and cytoprotective properties with potential use as a food and feed additive [36]. γ -PGA exhibited strong in vitro antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl, hydroxyl radicals, and superoxide free radicals [37]. In this study, long-term γ -PGA supplementation significantly increased the levels of calcitriol, PTH, calcitonin, and FGF23. PTH, which is synthesized and secreted by parathyroid chief cells, raises blood calcium and lowers phosphorus levels. Its target organs include the bone and kidney, with indirect regulation of intestinal calcium absorption via calcitriol [38]. PTH increases calcium by stimulating osteoclast activity, whereas calcitonin inhibits osteoclasts. Elevated calcium and calcitriol suppress PTH secretion, while increased phosphorus levels stimulate it. Activated vitamin D enhances phosphorus absorption in both intestines and bones [8]. The absorption of calcium and phosphorus in the gastrointestinal tract is regulated by PTH, calcitriol, diet, and other factors. When calcium intake exceeds approximately 100 g/d, preduodenal net calcium absorption becomes positive [39]. Preduodenal calcium absorption increases blood calcium, which in turn inhibits PTH secretion and stimulates calcitriol synthesis, thus promoting active intestinal calcium absorption.

FGF23, a hormone derived from bone, plays a central role in phosphate (Pi) homeostasis in ruminants [40]. In bone tissue, FGF23 regulates plasma PTH by modulating 1α -hydroxylase activity, leading to increased calcitriol concentrations [41]. In this study, PTH decreased significantly from day 30 to day 60 in all groups, suggesting that long-term feeding favors calcium absorption. Overall, γ -PGA supports calcium absorption, and to maintain calcium–phosphorus homeostasis, hormonal responses operate in sequence and complement one another.

Our second objective was to investigate the mechanism by which γ -PGA facilitates calcium uptake in the gut and bone by analyzing intestinal microbial 16S rRNA and bone parameters. The species accumulation curve from Specaccum exhibited a sharp initial increase followed by stabilization, indicating that microbial composition remained unchanged as sample size increased (Figure S2A–D). The number of common species did not decline with additional samples, suggesting that the sequencing depth was adequate for downstream analysis. In this study, γ -PGA did not significantly alter bacterial diversity, but the gene expression concentration of the bacterial community was enhanced, and the dominant genera were mostly beneficial. Dominant bacteria included *Olsenella*, *Prevotella*, *Sharpea*, *Ruminococcus*, *Bifidobacterium*, *Roseburia*, unclassified [*Eubacterium*] *coprostanoligenes* group, *Acetitomaculum*, *Lachnospiraceae* NK3A20 group, and *Succinivibrio*. This profile was consistent with the findings of Wang et al. [42], who reported that *Prevotella*, *Lachnospiraceae* NK3A20 group, and *Olsenella* were dominant in the rumen of Plateau lambs, with substantial overlap observed.

Among these, the *Eubacterium coprostanoligenes* group was identified as a dominant taxon, known for promoting the digestion and absorption of butyric acid. *Acetitomaculum* acts as a butyrate-producing probiotic, while the *Lachnospiraceae* NK3A20 group participates in the metabolism of various carbohydrates. *Ruminococcus* produces propionic and acetic

acids and also contributes to cellulose and starch degradation [43]. *Olsenella* was negatively associated with feed conversion efficiency in sheep [44]. *Bifidobacterium* has been shown to improve bone health by regulating bone resorption via osteoclasts and bone formation via osteoblasts [45]. Animal studies in mice have also shown that γ -PGA can increase both the abundance and evenness of beneficial gut microbiota while inhibiting harmful bacterial growth [46].

The WOCNA R package classified genus-level expression in the intestinal microbiota into seven co-expressed gene clusters, and each module was analyzed for correlation with physiological traits. These association modules were imported into Cytoscape (version 3.6.1) software to calculate network properties and identify core regulatory microorganisms linked to specific traits. The core microorganisms of the brown module (Family XIII AD3011 group, *Methanobrevibacter*, and [*Eubacterium*] *nodatum* group) play key roles in the negative regulation of calcium- and phosphorus-related hormones and in the positive regulation of DMI and feed conversion rate. Previous studies have indicated that rumen microbial community structure is a major determinant of feed conversion efficiency in ruminants [47,48]. *Fusicatenibacter*, belonging to the phylum Firmicutes (butyric acid-producing bacteria), was identified as the core microorganism of the blue module. Butyric acid has known anti-inflammatory properties in the intestinal tract, enhances digestive enzyme activity and pancreatic secretion [49], promotes small intestine development in calves, and reduces diarrhea incidence [50]. The blue module was negatively correlated with total ADG, calcitriol, FGF23 levels, and calcium absorption proteins in the intestine, while positively correlated with duodenal calcium concentration. In conclusion, *Fusicatenibacter*, Family XIII AD3011 group, *Methanobrevibacter*, and related microorganisms contributed to improvements in duodenal calcium absorption, DMI, and feed conversion in Sunite lambs supplemented with γ -PGA.

Bone density in young animals increases with age, requiring calcium and phosphate (Pi) to be deposited as hydroxyapatite to strengthen the bone matrix. The extent to which calcium absorbed from the gastrointestinal tract is utilized depends on changes in bone parameters and structure. Some researchers have used femoral shaft weight and bone cortical index to indirectly evaluate bone calcium content [51]. Bone mineral density (BMD), trabecular structure, and related parameters are commonly measured to assess bone calcium deposition. Among these, BMD is the most frequently used index to evaluate calcium nutritional status, especially in regions rich in trabecular bone, which more accurately reflects calcium salt content in bone tissue. Intestinal physiology and microbial communities influence bone metabolism to varying degrees. In this study, γ -PGA supplementation significantly altered trabecular microarchitecture, as evidenced by increased trabecular pattern factor (Tb.Pf) and structure model index (SMI). These parameters indicate a shift toward a more rod-like trabecular structure, which in classical bone histomorphometry is often associated with reduced bone strength [52]. However, when interpreted in the context of elevated bone remodeling hormones (PTH, FGF23, calcitriol) and increased intestinal calcium absorption, these changes more likely reflect enhanced bone remodeling dynamics rather than unequivocal improvement or deterioration in bone quality. In rapidly growing lambs (5–6 months old), the skeleton undergoes continuous modeling and remodeling to adapt to increasing mechanical loads and mineral demands. The concurrent trends toward increased connectivity density (Conn.D, $p = 0.077$) and bone surface-to-volume ratio (BS/BV, $p = 0.069$) further support the interpretation of an active remodeling state, where both resorption and formation surfaces are expanded. This interpretation aligns with the observation that co-expressed microorganisms in the yellow module were positively correlated with body height, body weight, chest circumference, trabecular pattern factor, bone surface area, and bone volume ratio, as well as with intestinal calcium absorption

proteins. *Eubacterium ruminantium* is a beneficial bacterium that produces short-chain fatty acids (SCFAs) by fermenting cellulose and complex carbohydrates in the rumen. The SCFAs (acetic acid, propionic acid, and butyric acid) play important roles in nutrient metabolism and energy supply in ruminants [53]. They also contribute to intestinal health and enhance cellulose degradation [54]. Furthermore, one study reported that fecal microbiota transplantation from young to aged rats reversed intestinal dysbiosis and improved age-related osteoporosis, including bone volume, trabecular fraction, trabecular number, and trabecular thickness [55]. The gain in bone mineral density is consistent with previous findings showing that γ -PGA promotes BMD in mice [56]. These results highlight the potential role of beneficial microbiota in improving bone density and strength following γ -PGA supplementation.

A substantial body of research has shown that several probiotics enhance calcium absorption in the body [57]. Notably, in this study, *Prevotella* was not only the dominant genus but also the core microorganism within the turquoise module. Although the *Rikenellaceae* RC9 gut group did not differ significantly from the control group, its abundance differed significantly among γ -PGA treatment levels, and it also served as a core taxon of the turquoise module. The turquoise module was positively correlated with slaughter rate, ileal calcium concentration, and bone CT value, and negatively correlated with feed efficiency at day 60, dry matter intake at day 30, and duodenal villus length. *Prevotella* is known to metabolize complex nutrients such as cellulose and proteins and interacts synergistically with other microorganisms to promote host growth and development [58]. Previous studies have reported that *Rikenellaceae* RC9 is negatively correlated with nipple length and width, supporting the associations observed in this study [43]. Based on the duodenal histology and electron microscopy results, excessive γ -PGA was not beneficial to intestinal structure [59]. Considering all indicators, we propose 1.2 g/(d·head) as the recommended supplemental dose of γ -PGA. However, this study serves only as a starting point. Further investigations are needed to determine the optimal molecular weight range of γ -PGA, its precise absorption rate, and the mode of calcium chelation within the unique gastrointestinal environment of ruminants, which will require studies involving isotopic tracer techniques.

Several limitations of this study should be acknowledged. First, the sample size of six animals per group, while consistent with previous ruminant studies [18,42] and justified by ethical considerations (3R principles), may limit the statistical power to detect subtle phenotypic differences and could affect the robustness of network-based analyses such as WOCNA. Although WOCNA is designed to identify co-expression patterns based on correlation structures that are relatively robust to moderate sample sizes, larger cohorts would provide greater confidence in the stability and generalizability of the identified modules and hub taxa. Second, the cross-sectional nature of the post-slaughter sampling precludes assessment of dynamic changes in bone remodeling over time. Third, while we have identified strong correlations between specific microbial taxa and host phenotypes, these associations do not establish causality. Future studies employing larger sample sizes, longitudinal sampling, and intervention approaches such as fecal microbiota transplantation or gnotobiotic models would help to validate the causal roles of the identified core microorganisms in mediating the effects of γ -PGA on calcium metabolism and bone health.

4. Materials and Methods

4.1. Animal Ethics Statement

This study was approved by the Animal Ethics Committee of the Inner Mongolia Academy of Agricultural and Animal Husbandry Science (Approval No. [2024-0017]). All

procedures involving animals were conducted in strict accordance with the committee's guidelines for the care and use of laboratory animals.

4.2. Experimental Design, Animals, and Diets

A total of 24 healthy male Sunite lambs (5–6 months old; initial body weight 27.49 ± 3.68 kg, mean $\pm$ SD) were used in this 60-day experiment. After a 10-day adaptation period, lambs were randomly allocated into four dietary treatment groups ($n = 6$ per group). Control (C): Basal diet; Low γ -PGA (L): Basal diet + 0.6 g/(d·head) of γ -PGA; Medium γ -PGA (M): Basal diet + 1.2 g/(d·head) of γ -PGA; High γ -PGA (H): Basal diet + 2.4 g/(d·head) of γ -PGA (Figure 10).

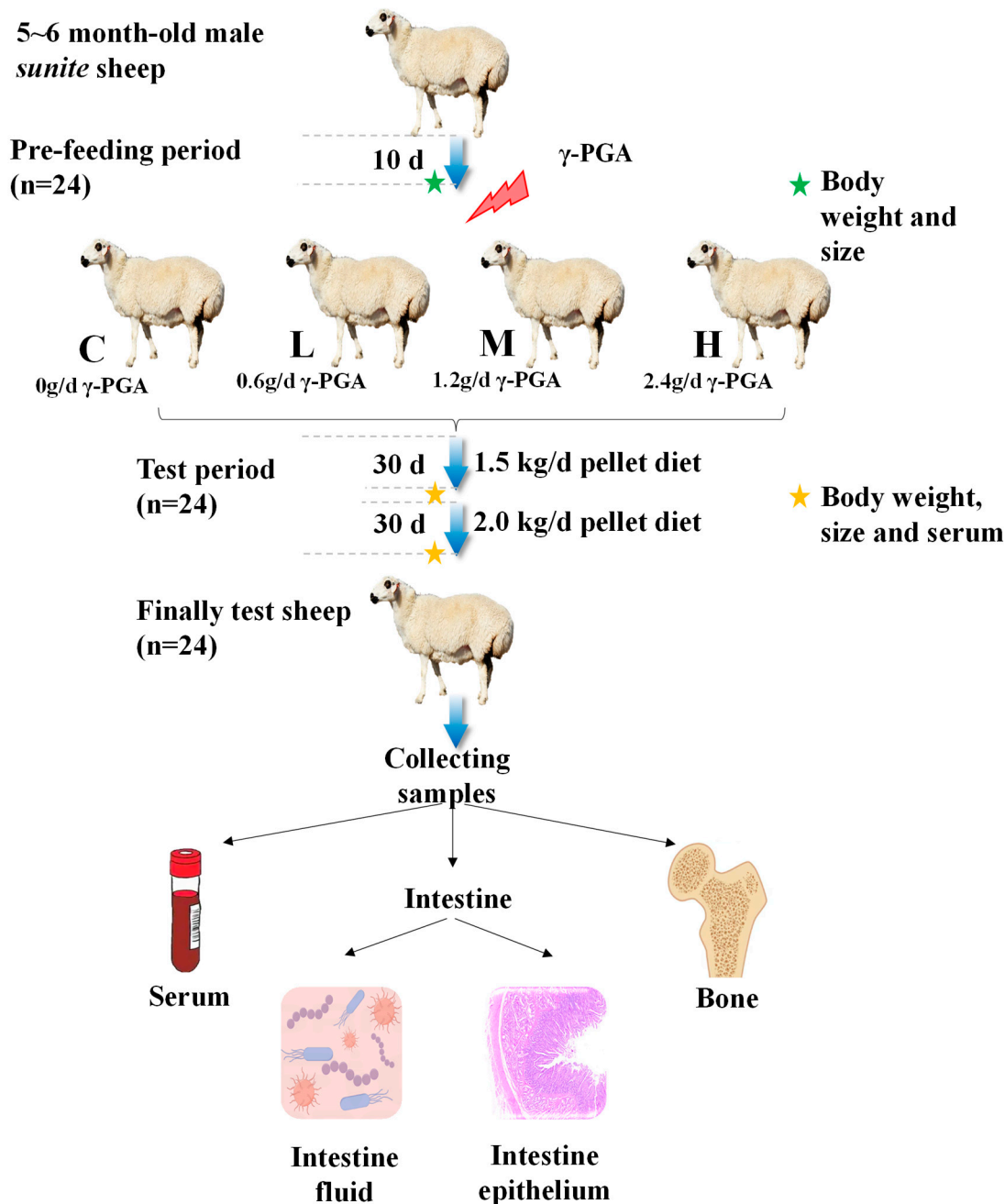


Figure 10. Experimental design overview and sample collection workflow. Lambs ($n = 24$) were randomly assigned to four groups (C, L, M, H) receiving 0, 0.6, 1.2, or 2.4 g/(d·head) γ -PGA for 60 days. Blood, duodenal tissue, digesta, and bone samples were collected at the indicated time points for analysis of growth performance, serum hormones, intestinal morphology and calcium transporters, bone microarchitecture, and duodenal microbiota.

The γ -PGA product (purity: 33.03%; molecular weight: 15–20 kDa; Nanjing Shineking Biotech Co., Ltd., Nanjing, China) contained approximately 33% active γ -PGA, with the remaining fraction consisting of fermentation by-products and corn flour carrier. The product was thoroughly mixed with additional corn flour to ensure uniform distribution in the concentrate, and the control group received an equivalent amount of corn flour without γ -PGA.

All doses are expressed as grams of the γ -PGA product (33.03% purity) per day per head. The corresponding active γ -PGA intake was approximately 0, 0.20, 0.40, and 0.79 g/(d·head) for groups C, L, M, and H, respectively.

The experiment was conducted at the Toktor Research Base (Hohhot, China; 40°30'03"–40°31'47" N, 111°23'14"–111°24'29" E) from 20 August to 21 October 2024. The lambs were individually housed in pens (2.5 × 1.0 m) under a natural mid-temperate continental monsoon climate (average temperature: 18.00 ± 6.06 °C; relative humidity: 60.00 ± 14.91%). All lambs were vaccinated and dewormed prior to the trial. They were fed twice daily (06:30 and 17:30) with free access to water. The basal diet (Table 6) was formulated to meet the nutrient requirements of lambs. Feed provision was 1.5 and 2.0 kg/d·head for the first and last 30 days, respectively. Orts were collected and weighed daily at 10:30 and 21:30 to calculate dry matter intake (DMI).

Table 6. Ingredients and chemical compositions of the feed (%DM basis).

Ingredients	Content	Nutrient Levels	Content
Corn grain	32.0	Nutrients of % DM	90.1
Wheat middings	10.0	Crude protein	15.0
Soybean meal	10.0	Ether extract	2.7
Corn germ meal	10.0	Crude fiber	14.0
Corn bran	5.0	Neutral detergent fiber	29.0
Soybean hulls	10.0	Acid detergent fiber	16.0
shells of melon seeds	10.0	Acid detergent lignin	3.2
Rice hull	5.0	Ca	1.2
Stone powder	2.4	P	0.34
Expanded urea	1.0	Digestible energy (Mcal/kg)	2.65
molasses	3.0	Net energy (Mcal/kg)	0.9
NaCl	0.6		
Premix	1		
Total	100		

4.3. Sample Collection

Blood samples were collected via jugular venipuncture on days 30 and 60 of the experiment. Serum was separated by centrifugation (3000 × g, 15 min, 4 °C) and stored at −80 °C for subsequent analysis.

Post-slaughter samples were collected from six randomly selected lambs per group at the end of the trial. Immediately after slaughter, segments of the duodenum and ileum were dissected. Luminal contents were collected aseptically, snap-frozen in liquid nitrogen, and stored at −80 °C for mineral and microbial analysis. Tissue segments from the duodenum were: (1) fixed in 4% paraformaldehyde for histomorphology; (2) fixed in 2.5% glutaraldehyde for transmission electron microscopy (TEM); and (3) snap-frozen for protein analysis.

Bone samples: Both left and right femurs were collected. The right femur was fixed for micro-computed tomography (micro-CT) analysis. The left femur was cleaned of soft tissue, weighed for wet weight, dried at 50 °C to constant weight, and re-weighed for dry weight determination.

4.4. Growth Performance and Carcass Measurement

Body weight (BW), body height (BH), body length (BL), and chest girth (CG) were measured at the start, on day 30, and at the end of the experiment. Average daily gain (ADG), DMI, and feed efficiency (FE = DMI/ADG) were calculated for the 0–30, 30–60, and overall 0–60 day periods.

4.5. Biochemical and Hormonal Assays

Serum concentrations of calcium, phosphorus, total protein, albumin, globulin, uric acid, glucose, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined using an automatic biochemical analyzer (Mindray BS-360E, Shenzhen, China).

Commercially available enzyme-linked immunosorbent assay (ELISA) kits (Jiancheng Bioengineering Institute, Nanjing, China) were used to measure serum levels of calcitriol (1,25-(OH)₂-VitD₃), parathyroid hormone (PTH), calcitonin (CT), and fibroblast growth factor 23 (FGF-23), according to the manufacturers' instructions. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in serum were also determined using commercial kits.

4.6. Calcium and Phosphorus Levels in Duodenal and Ileal Contents

The calcium and phosphorus concentrations in duodenal and ileal digesta were determined using inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 8000, PerkinElmer, Shelton, CT, USA). The content was calculated as:

$$W = [(C - C_0) \times V \times N] / m \quad (1)$$

where W is the content (mg/kg), C and C₀ are the sample and blank concentrations (mg/L), V is the constant volume (mL), N is the dilution factor, and m is the sample mass (g).

4.7. Duodenal Morphology and Ultrastructure

For light microscopy, paraffin-embedded duodenal sections (5 µm) were stained with hematoxylin and eosin (H&E). Villus height (from tip to crypt mouth), crypt depth, and muscularis thickness were measured using image analysis software (NIS-Elements BR 5.42.01, Nikon, Tokyo, Japan). At least 15 well-oriented, intact villi and crypts were measured per sample.

For TEM, duodenal tissues fixed in glutaraldehyde were post-fixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (HT7800, Hitachi, Tokyo, Japan). Microvilli density and mitochondrial morphology were assessed.

4.8. Bone Microarchitecture Analysis

The microarchitecture of the proximal femoral metaphysis was analyzed using a high-resolution micro-CT system (NMC-200, Midlife Medical Technology, Kunshan, China). Scans were performed with a 10 µm isotropic voxel size. Three-dimensional reconstructions and quantitative analyses of bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), trabecular pattern factor (Tb.Pf), structure model index (SMI), and connectivity density (Conn.D) were performed using the manufacturer's software (Cruiser and Avatar2.0, Pingsheng Medical Technology, Kunshan, China).

4.9. DNA Extraction, PCR Amplification, 16S rRNA Sequencing

Bacterial DNA from duodenal contents was extracted using the TGuide S96 magnetic bead fecal genomic DNA separation kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China; DP812) according to the manufacturer's protocol. The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using universal primers (338F: 50-ACTCCTACGGGAGGcAGcag–3', 806R: 5'-GGACTACHVGGGTWTCTAAT–3'). A 10 bp barcode sequence was added to the 5' end of each forward and reverse primer for each sample. The PCR mixture contained 5 µL of PCA-purified product from the target region, 2.5 µL of PMI-A, 2.5 µL of PMI-B, and 10 µL of 2× Q5 HF-MM. PCR products were visualized using 1.8% agarose gel electrophoresis and purified by column-based purification (OMEGA DNA). Sequencing was performed using the Illumina NovaSeq 6000 platform, San Diego, CA, USA. This phase of the study was conducted by Standard Life Sciences Co., Ltd. (Beijing, China).

4.10. Bioinformatics Analysis

Raw sequencing data were quality-filtered using Trimmomatic (version 0.33). Primers were identified and removed using Cutadapt (version 1.8.3). The DADA2 package in R was used for further quality control, which included double-end read merging and chimera removal. USearch (version 10) was applied to perform similarity-based clustering, with UCHIME (version 8.1) used for chimera detection, resulting in high-quality sequences for downstream analysis. For OTU analysis, sequences were clustered at 97% similarity using VSEARCH (version 2.4.3).

For species annotation, sequences were aligned to a reference database using BLASTN (version 2.9.0). Taxonomic classification was conducted using the lowest common ancestor (LCA) method implemented in the classify-sklearn plugin in QIIME.2 (version 2024.10), with a confidence threshold of 0.7. Sequences were first classified with BLASTN, and unmatched sequences were re-classified using classify-sklearn to improve accuracy. Beta diversity analysis was conducted using the FactoMineR package in R (version 4.2.0). To statistically evaluate differences in microbial community composition among groups, permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis2 function in the R package vegan (999 permutations) on the Bray Curtis distance matrix. Differential abundance was assessed using the LEfSeR package, and an LDA score > 4 was considered statistically significant. Venn diagrams were used to compare shared and unique microbiota across groups. All bar and line charts were created using GraphPad Prism 9.

Weighted OTU co-expression network analysis (WOCNA): The genus-level OTU table was normalized by $\log_{10}(\text{abundance} + 1)$ using the WOCNA package in R (version 3.2.5). A correlation matrix was generated, followed by the construction of an adjacency matrix. A soft threshold power of 4 and a correlation cutoff of 85% were applied. The topological overlap matrix was computed, and modules were identified. Growth performance, slaughter rate, and duodenal development parameters were used as trait data for weighted correlation analysis. Cytoscape (version 3.6.1) software was used to visualize the network. Network analysis was performed under undirected settings. Continuous color changes were applied to node labels to represent trait correlations. Each sheep was treated as a single experimental unit. The degree of correlation and node connectivity were calculated to identify key microorganisms, potential mechanisms, and candidate microbial markers.

4.11. Statistical Analysis

For longitudinal growth data (body weight, body height, body length, and chest circumference measured on days 0, 30, and 60), linear mixed-effects models were fitted using

the lme4 package in R (version 4.3.1). The models included treatment (C, L, M, H), time (day 0, 30, 60), and their interaction as fixed effects, with animal as a random intercept to account for within-subject correlation across repeated measurements. Denominator degrees of freedom were estimated using the Satterthwaite approximation via the lmerTest package. Least-squares means (LSMEANS) and their 95% confidence intervals were computed for each treatment group at each time point using the emmeans package, and pairwise comparisons were adjusted by Tukey's method.

For all other variables measured at a single time point (serum parameters, bone indices, antioxidant enzymes, hormones, duodenal morphology, and intestinal calcium/phosphorus content), one-way ANOVA within the general linear model framework was performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Differences among group means were adjusted using the Tukey–Kramer test. Orthogonal polynomial contrasts were used to examine linear and quadratic trends across γ -PGA supplementation levels.

Protein expression levels of calcium transporters in the duodenum and ileum were compared between the C and M groups using independent-sample *t*-tests. To account for multiple comparisons across the eight proteins, the Benjamini–Hochberg false discovery rate (FDR) correction was applied, and adjusted *p*-values (*q*-values) < 0.05 were considered statistically significant.

For all analyses, a significance threshold of $\alpha = 0.05$ was used, and exact *p*-values are reported throughout the manuscript. Effect sizes and 95% confidence intervals are presented where appropriate to facilitate interpretation.

5. Conclusions

In conclusion, this study demonstrates that dietary γ -PGA at an optimal dose of 1.2 g/(d·head) enhances calcium metabolism in Sunite lambs through a multifaceted mechanism involving the gut–microbiota–endocrine–bone axis. γ -PGA improves duodenal morphology and upregulates key calcium transport proteins. It concurrently induces a beneficial shift in the duodenal microbiota and stimulates a systemic endocrine profile that facilitates bone mineralization. *Fusicatenibacter*, *Prevotella*, *Rikenellaceae* RC9 gut group, Family XIII AD3011 group, and [*Eubacterium*] *ruminantium* group, which were identified as core genera in the intestinal microbial co-expression network, are strongly associated with duodenal calcium absorption and promoting bone mineral deposition. This study provides foundational evidence to support the development of amino acid-based feed additives for ruminants.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052373/s1>.

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