

Ruminal Lesions in Dairy Cows Naturally Infected by *Calicophoron microbothrioides* (Trematoda: Paramphistomidae)

Lesiones ruminales en vacas infectadas naturalmente por *Calicophoron microbothrioides* (Trematoda: Paramphistomidae)

 José F. Coronado ¹  Teófilo Torrel ¹  Luis Vargas-Rocha ^{1*}

¹ Universidad Nacional de Cajamarca, Cajamarca, Perú

² Universidad Politécnica de Tulancingo. Hidalgo, Mexico.

* Corresponding Author: Luis Vargas-Rocha. Universidad Nacional de Cajamarca, Av. Atahualpa Km. 3, Cajamarca 06003, Cajamarca, Perú.
lvargasr17_1@unc.edu.pe

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Abstract: *Calicophoron microbothrioides* is a relatively new trematode species reported in domestic ruminants in the Cajamarca Valley (Peru). This species poses a significant endemic risk to domestic ruminants in the local livestock; however, histopathological aspects of the infection remain understudied. This study aims to describe the histological ruminal lesions induced by *C. microbothrioides* in naturally infected Holstein cows. Ten cows were sampled, collecting 1 cm³ of rumen tissue from affected areas (rough areas without ruminal papillae) in positive cases during *post-mortem* inspections. Control samples were taken from the rumen tissue of cows not infected with *C. microbothrioides*. The samples were processed using the paraffin embedding technique and stained with hematoxylin-eosin. The rumen flukes exhibited a pink and shiny color, with a curved conical shape (concave ventral region), and the anterior section slimmer than the posterior one (length 5.45 ± 2.50 mm and width 2.8 ± 0.58 mm). Histology revealed cellular degeneration, inflammation, and tissue necrosis in all affected animals. These findings highlight the impact of *C. microbothrioides* infection in adult cows, leading to marked histopathological alterations in the rumen wall. Given the importance of these results, there is a need for further studies on this parasite, considering its potential threat to the health and productivity of local ruminants.

Keywords: bovine, cell disruption, ruminal lesions, rumen fluke, trematode.

Resumen: el parásito *Calicophoron microbothrioides* es una especie de trematodo relativamente nueva reportada en rumiantes domésticos en el valle de Cajamarca (Perú). Esta especie representa un riesgo endémico importante para los rumiantes domésticos en la ganadería local; sin embargo, los aspectos histopatológicos de la infección siguen sin estudiarse. El objetivo de este estudio fue describir las lesiones histológicas ruminales inducidas por *C. microbothrioides* en vacas Holstein naturalmente infectadas. Se tomaron muestras de diez vacas y se tomó 1 cm³ de tejido de ruminal de las áreas afectadas (zonas rugosas sin papilas ruminales) de los casos positivos durante las inspecciones *post-mortem*. Además, se tomaron muestras de control del tejido ruminal de vacas no infectadas con *C. microbothrioides*. Las muestras se procesaron mediante la técnica de inclusión en parafina y se tiñeron con hematoxilina-eosina. Las duelas presentaron un color rosáceo y brillante, con una forma cónica arqueada (región ventral cóncava), con la sección anterior más esbelta que la posterior (largo $5,45 \pm 2,50$ mm y ancho $2,8 \pm 0,58$ mm). La histología reveló degeneración celular, inflamación y necrosis tisular en todos los animales afectados. Estos hallazgos destacan el impacto de la infección por *C. microbothrioides* en vacas adultas, la cual genera alteraciones histopatológicas notables en la pared del rumen. Dada la importancia de estos resultados, se enfatiza la necesidad de llevar a cabo estudios adicionales sobre este parásito, teniendo en cuenta que es una amenaza potencial para la salud y la productividad de los rumiantes de la zona.

Palabras clave: alteración celular, bovino, duela ruminal, lesiones ruminales, trematodo.

Introduction

The parasites hinder profitable livestock farming by reducing production and incurring financial losses associated with control measures, treatment, and mortality costs (Charlier et al., 2020; Rashid et al., 2019). These losses can vary depending on the parasite species' pathogenicity or the animals' location, as certain environments and seasons favor the development of specific parasites (Jaja & Wanga-Ungeviwa, 2022; Marskole et al., 2016). Tropical and subtropical regions provide climatic conditions that promote higher prevalence and development of parasites in livestock due to high humidity and warm temperatures (Marskole et al., 2016; Pinilla et al., 2019; Thanasuwan et al., 2021).

Rumen fluke is a rumen-dwelling trematode found worldwide within the Paramphistomidae family, with distribution predominantly in tropical and subtropical regions. Their prevalence increases in areas characterized by extensive grazing. Several environmental factors, including, but not limited to, temperature, humidity, and precipitation, facilitate the perpetuation of the parasite's life cycle. Additionally, inadequate herd management, along with the age and breed of animals exposed to contaminated pastures, contribute to their susceptibility to infection (Díaz et al., 2007; Hajipour et al., 2021; Titi et al., 2010).

Rumen flukes typically require freshwater snails as an intermediate host to complete their biological cycle, with the final host being the ruminant. Similar to *Fasciola hepatica*, it has been verified that *Galba truncatula* also serves as a host for certain rumen flukes, such as *Calicophoron daubneyi* (Jones et al., 2015). Ruminants become infected by ingesting the metacercaria (parasite cyst, the only infectious stage), primarily with fresh forage and water (Hajipour et al., 2021). Subsequently, the newly excysted juveniles in the duodenum migrate to the duodenal submucosa and along the gastrointestinal tract to the rumen, where they mature and reproduce (Huson et al., 2021).

According to some authors, juvenile paramphistomes such as *C. daubneyi* in the intestine cause severe pathological disturbances, including enteritis and anemia, with a high incidence in young cattle. In their adult phase, they reside in the rumen and reticulum folds, giving rise to acanthotic zones and ruminitis, which appear macroscopically as fibrous areas devoid of rugosity and without ruminal papillae (Fuertes et al., 2015; Zintl et al., 2014). This condition disrupts the animal's physiological processes due to the inadequate absorption of nutrients, which is hindered by the resulting lesions (Herdt, 2000). Another study suggests that *C. daubneyi* neither induces significant macroscopic pathologies in the rumen or reticulum nor consistently triggers acute or chronic inflammatory changes in the duodenum (Busin et al., 2023).

The trematode *Calicophoron microbothrioides* has been identified using the taxonomic keys of Eduardo (1983) and Jones (2005) as the causal agent of paramphistomosis in ruminant cattle in the Cajamarca Valley (Ortiz et al., 2010). This identification has been confirmed through molecular methods (Manrique et al., 2013). Furthermore, its presence has been documented in various districts within the Cajamarca region (Torrel et al., 2023). Despite its significant presence in cattle, it remains a relatively understudied parasite. To gain a deeper understanding of the pathophysiology of this trematode, this study was conducted to describe the histological lesions induced by *C. microbothrioides* in naturally parasitized cows in Cajamarca, Peru.

Materials and Methods

This study was conducted in Cajamarca (6°11'40.74" S, 78°35'59.57" W), a province and region in Cajamarca, Peru. A convenience sampling of 10 cattle was considered. The animals included in the study were selected at random. The sampled animals were Holstein cows aged between 4 and 6 years. Furthermore, these animals belonged to an extensive grazing herd in the Cajamarca Valley.

To observe normal histology, rumen tissue samples were collected from unaffected areas of negative cows. In contrast, samples were taken from rumen areas affected by *C. microbothrioides* from positive cows for histopathological observations. These affected areas were identified macroscopically during *post-mortem* examinations of cattle processed at the municipal abattoir of Cajamarca. In both cases, approximately 1 cm³ of rumen tissue was cut.

The presence of *C. microbothrioides* in cattle from Cajamarca has been identified using the taxonomic keys of Eduardo (1983) and Jones (2005), according to Ortiz et al. (2010). Other local studies have also described the morphological characteristics of this parasite. These have indicated that in the fresh state, they are reddish, more intense at the anterior and posterior ends, with a conical body that curves ventrally and measures 4 to 15 mm long by 2 to 5 mm wide (Coronado et al., 2023; Ortiz et al., 2010; Torrel, 2009).

Tissue samples were collected using a scalpel and placed in sterile glass containers for transportation and initial processing at the Laboratorio de Embriología e Histología of the Facultad de Ciencias Veterinarias, Universidad Nacional de Cajamarca. The samples were fixed in a 10 % buffered formaldehyde solution and then dehydrated using ethyl and absolute alcohol. Clearing of the samples was done using xylene through baths in Coplin jars. Following the clearing process, the samples were placed in solutions of increasing alcohol concentrations and subsequently embedded in paraffin to obtain paraffin blocks.

Once the paraffin blocks were firm and cool, they were mounted on a rotary microtome, producing histological sections ranging from 5 to 8 micrometers thick. These sections were then placed in a water bath (37–40 °C) with adhesive gelatin and air-dried. Typically, two to three sections were considered per sample, and the process continued with the best slide. Subsequently, staining was carried out using hematoxylin-eosin. Finally, the assembly was done by placing a drop of Canada balsam as an adhesive on glass slides, then positioning a glass coverslip and applying gentle pressure to prevent the formation of air bubbles. These processes were conducted at the Laboratory of the National Agrarian Health Service of Peru (SENASA) in Lima, Peru.

The percentages of animals positive for *C. microbothrioides* were calculated according to the anatomical region of the rumen that showed lesions. Additionally, 95 % confidence intervals were calculated using the Clopper-Pearson method. These calculations were made in MS Excel 2019.

Results and Discussion

Pinkish and lustrous *C. microbothrioides* were discerned, displaying an arcuate conical morphology (ventral aspect slightly concave and dorsal convex), with the anterior part being slenderer than the posterior. Measurements from a cohort of 20 adult parasites revealed lengths of 5.45 ± 2.50 mm and widths of 2.8 ± 0.58 mm. These trematodes were located within the rumen and reticulum, inducing the ruminal papillae depletion, manifesting as coarse regions (Figure 1).



Figure 1. Macroscopic View of Structures and *C. microbothrioides*. A. Adult parasites in the rumen, B. Area with few ruminal papillae, C. Rough area without ruminal papillae, and D (a, b). Adult parasites in the rumen and abomasum of the same animal. Bar: Scale at 1,000 μ m. Source: Authors' archives.

All cows infected with *C. microbothrioides* ($n = 5$) exhibited cellular degeneration, inflammation, and tissue necrosis. Similarly, they all showed lesions in the ventral sac and the ventrocaudal blind sac. Only two animals also had lesions in the dorsocaudal blind sac (Table 1, Figure 2).

Table 1. Percentage (%) and Count of Lesions According to the Anatomical Region of the Rumen in Ten Cattle Affected by *C. microbothrioides*

Rumen region	Negative		Positive		Alteration	
	<i>n</i>	% [95 % <i>CI</i>]	<i>n</i>	% [95 % <i>CI</i>]	Type	Count
Ventral sac	5	50 [18.71–81.29]	5	50 [18.71–81.29]	Cellular degeneration	5
					Tissue necrosis	5
					Inflammatory tissue	5
Dorsocaudal blind sac	8	80 [44.39–97.48]	2	20 [2.52–55.61]	Cellular degeneration	2
					Tissue necrosis	2
					Inflammatory tissue	2
Ventrocaudal blind sac	5	50 [18.71–81.29]	5	50 [18.71–81.29]	Cellular degeneration	5
					Tissue necrosis	5
					Inflammatory tissue	5

CI: Confidence interval

Source: Own elaboration.

**Figure 2.** Macroscopic View of *C. microbothrioides* in the Rumen: ventral sac area (A), dorsocaudal blind sac (B), and ventrocaudal blind sac (C). Bar: Scale at 1,000 μm .

Source: Authors' archives.

The histological structures of rumen tissue unaffected by *C. microbothrioides* displayed stratified squamous keratinized epithelium, an inner circular muscular layer, blood vessels, highly vascularized loose connective tissue, and basal and parabasal cells without visible alterations (Figure 3).

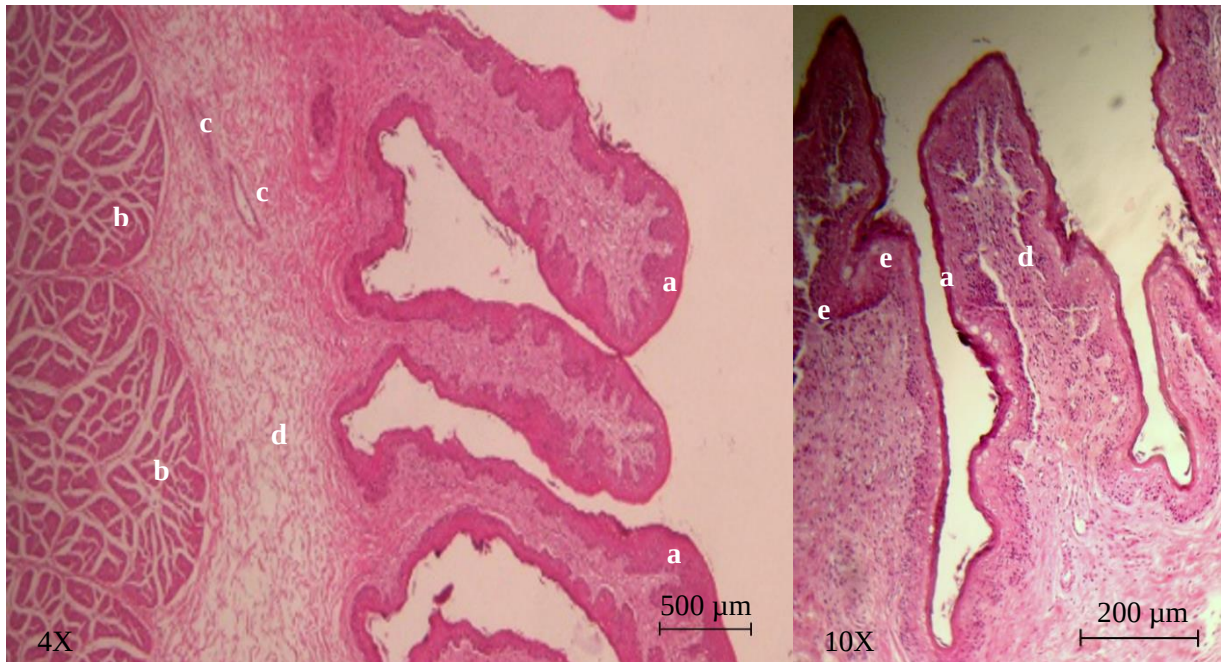


Figure 3. Histology of Healthy Rumen Tissue (standard sample). Histological section of rumen papillae and part of the rumen wall: keratinized stratified flat epithelium (a), inner circular muscular layer (b), blood vessels (c), lax connective tissue, very vascularized (d), and basal and parabasal cells (e) (hematoxylin-eosin).

Source: Authors' archives.

Histology of rumen papillae from non-infected cattle with *C. microbothrioides* revealed a lining of keratinized stratified squamous epithelium, consistent with findings from Arias et al. (1978), Bacha and Bacha (2012), and Dellmann (1971). Beneath this lining, the lamina propria of the submucosa exhibited vascularization alongside loose connective tissue and circularly arranged smooth muscle tissue. Above this layer, a longitudinal muscular layer facilitated rumen movements. The flattened cells of the rumen papillae displayed pyknotic nuclei, covered by superficial layers of thick dead cells with keratinized nuclei and cytoplasm. Polymorphic parabasal cells were observed in the intermediate zone, distributed in multiple overlapping layers. Deeper within, adjacent to the basal lamina, cuboidal basal cells were situated, characterized by minimal perinuclear cytoplasm. Fibroblast and collagen fibers contributed to the necessary resistance and support for the extracellular matrix's maintenance, where even polymorphonuclear leukocytes adhered.

Microscopically, the histological lesions observed in affected cows revealed focal, mild to moderate necrosis with trans-epithelial invasion of degenerated and lysed cells dispersed within

the lamina propria. Cells with hydropic degeneration were also noted, characterized by cellular swelling and cytoplasmic pallor (Figure 4).

While focal, mild to moderate necrosis was observed in the rumen, this phenomenon is not uncommon in the ruminal environment. However, the detachment of ruminal papillae is attributed to *C. microbothrioides*, potentially serving as a gateway for more consequential processes. In a toxic milieu, neutrophils rapidly degenerate, leading to karyolysis, cytoplasmic swelling or enzymatic vacuolization, eosinophilic staining, and loss of the cytoplasmic membrane. Furthermore, polymorphonuclear cells react to vascular disturbances and antigens produced by paramphistomes, potentially culminating in edema following the cellulolytic process (Anuracpreeda et al., 2013; Singh et al., 1984).

The inner and outer muscle layers and the serosa retained normal histological characteristics. This is because the ventral sucker of the paramphistomid only reaches the superficial layer and underlying tissues, i.e., the ruminal mucosa, where it attaches. The paramphistomid causes traumatic damage through suction, blood vessel alteration, and hemorrhages at the point of attachment and the surrounding areas, leading to cell degeneration, edema, mild to moderate multifocal necrosis, and subsequent immune reaction as an adaptive and compensatory response (Fuertes et al., 2015; Mason et al., 2012; Mavenyengwa et al., 2008). Undoubtedly, the severity and extent of structural changes depend on the number of migrating Paramphistomidae and the response of the animal's immune system (Rolfe et al., 1994).

The results obtained are different from those of Fuertes et al. (2015), who have noted that in natural infections, the adult parasite *C. daubneyi* generates acute inflammatory cells (eosinophils, globular leukocytes, intraepithelial lymphocytes, plasma cells, mast cells) at the site of the papillae to which they attach. This leads to a narrowing of the base and expansion of the apex of the papillae, giving them a fungiform appearance. Inflammatory cells such as eosinophils, globular leukocytes, intraepithelial lymphocytes, plasma cells, mast cells, and macrophages intervene, causing acanthosis characterized by hyperkeratosis and hyperpigmentation. This difference is possibly due to the size of the parasites, resulting in a larger acetabulum, which would confer a greater area of damage in the rumen. Adult *C. daubneyi* typically measures 1.5–2.0 cm in length (Huson et al., 2021), while a study indicated that the size of adult *C. microbothrioides* ranges from 0.588 to 0.668 cm (Cerdeira et al., 2019).

The damage is directly proportional to the number of parasites. Another study found that *Paramphistomum* spp causes atrophy of the ruminal papilla, along with mild to moderate infiltration of mononuclear cells and a few eosinophils in the lamina propria, in addition to hydropic degeneration in the rumen epithelial lamina (Ozdamar et al., 2010). Furthermore, older animals are expected to exhibit more severe lesions due to accumulated reinfections over their lifetimes, which increases the scar tissue in the rumen. Other studies have documented that older cattle are more affected by rumen flukes (Cueva-Rodríguez et al., 2024; Forstmaier et al., 2021).

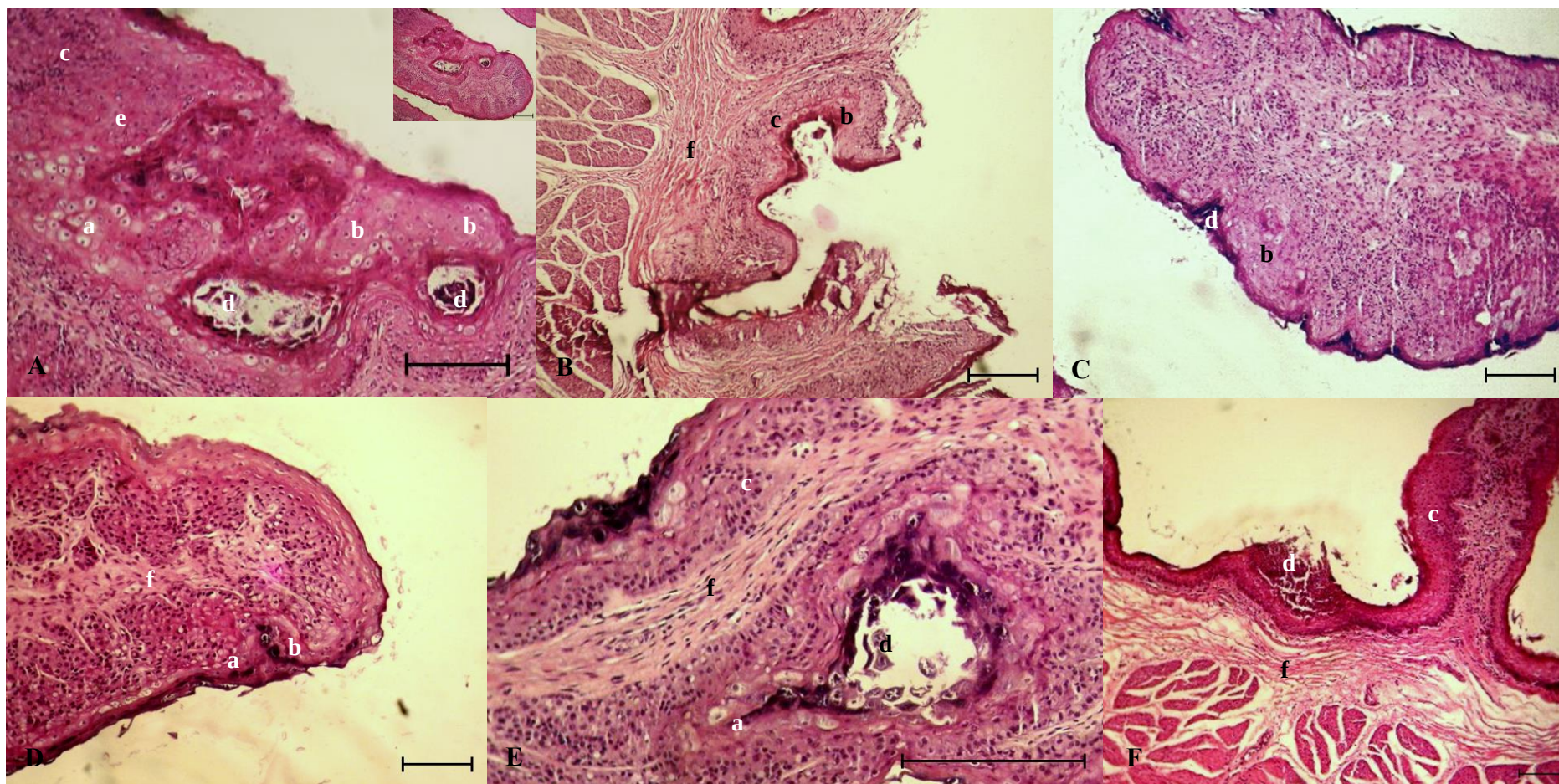


Figure 4. Histopathological Damage to the Rumen Papilla Caused by *C. microbothrioides*. A. Parasite attachment zone (10X, scale 200 μ m), B. Base of the papilla (4X, scale 500 μ m), C. Middle section of the papilla (10X, scale 200 μ m), D. Apex of the papilla (10X, scale 200 μ m), and E/F. Section of papilla detachment (10X, scale 200 μ m/4X, scale 500 μ m). Cells with hydropic degeneration (a), edema (b), hyperplasia (c), necrosis (d), polymorphonuclear cells (e), and lax connective tissue (f) (hematoxylin-eosin). Source: Authors' archives.

The limitations of this study must be considered, such as the small sample size and the lack of precise parasite identification due to the absence of molecular tests. However, these results serve as a starting point for future comprehensive studies that will incorporate both morphology and molecular identification of the parasite, a larger number of animals evaluated (increased statistical robustness), and additional analyses to assess the effects of *C. microbothrioides* more accurately in the pre-stomach compartments and even in the duodenum of cattle (juvenile parasite). Moreover, the parasitic load should be evaluated along with its relationship to the area of ruminal papillae-deprived zones caused by the parasite, as well as the nutrient absorption rate (VFA) and its impact on productive parameters in cattle, including weight gain, feed intake, milk production, growth rate.

Conclusions

Based on the objectives and results obtained, it can be concluded that adult *Calicophoron microbothrioides* cause alterations in the rumen wall. The histological lesions in affected cows revealed focal, mild to moderate necrosis with trans-epithelial invasion of degenerated and lysed cells.

Authors' Contributions

JFC: investigation, validation, data curation; TT: conceptualization, methodology, supervision; LV-R: writing - original draft, visualization. All authors approved the final manuscript (writing - review & editing).

Ethical Implications

This article has no ethical implications. The authors did not engage in the use of live animals for this study. Samples were obtained from animals slaughtered at the municipal abattoir from Cajamarca, which operates under the regulations of the Reglamento Sanitario del Faenado de Animales de Abasto del Perú (Supreme Decree No. 015-2012-AG).

Conflict of Interest

The authors declare no competing interests.

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