Comparative In Vitro Analysis of Protozoan Genus Responses to Antiprotozoal Agents from Extracts, *Albizia chinensis* Leaf Flour, and Commercial Saponin

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Abstract. The purpose of this study was to compare the in vitro antiprotozoal activity of *Albizia chinensis* leaf, *Albizia chinensis* extract, and commercial saponin against rumen protozoa. The process began with a six-hour incubation of Etawa crossbred goat rumen fluid under controlled anaerobic conditions. DNA was extracted and amplified using 18S rRNA primers, and amplicons were sequenced using Oxford Nanopore technology to identify the composition of prozoal genera. The analysis showed that the treatment inhibited several protozoan genera, with variations in spectrum and intensity. The strongest and broadest inhibition value was shown by commercial saponin and eliminated genera such as *Entamoeba* and *Isotricha*. *Albizia chinensis* leaf extract showed selective inhibition, while coarse flour provided a milder but broader effect, this could occur due to the synergism between saponins, flavonoids, and the fiber matrix. The results of the study inform that the level of processing will affect the specificity and potency of antiprotozoal activity. *Albizia chinensis* based products offer a sustainable plant-based alternative that can reduce protozoa and rumen methane production. This study recommends further research on bioactive compound profiles and in vivo validation to optimize the use of A. chinensis as a natural feed additive for ruminant microbial management.
Keywords: *Albizia chinensis*, Antiprotozoa, Saponin

1 Introduction

Ruminant livestock plays a vital role in the global food system as a major source of milk, meat, and fiber. They also contribute significantly to greenhouse gas emissions, particularly methane (CH_4) through enteric fermentation in the rumen. The rumen is a dynamic and complex microbial ecosystem of bacteria, archaea, fungi, and protozoa that collectively degrade feed and produce volatile fatty acids (VFA), microbial proteins, and gases [1]. Protozoa contribute 25 to 50% of the total microbial biomass as agents of fiber degradation and nitrogen metabolism [2]. However, protozoa also ingest bacteria, increasing ammonia production and reducing nitrogen efficiency in the host animal. Symbiotic associations with methanogenic archaea indirectly contribute to CH_4 formation [3]; [4]. Therefore, regulating protozoan populations in the rumen is a strategy to increase feed efficiency while reducing methane emissions.

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In recent years, the importance of using plant bioactive compounds to regulate rumen microbial ecology has become increasingly prominent. Saponins are steroidal or triterpenoid glycosides commonly found in tropical plants and are known for their antiprotozoan properties [5];[6]. The main mechanism of action of saponins is to form complexes with cholesterol in protozoan cell membranes. This increases cell membrane permeability and ultimately leads to cell lysis [4]. Adding saponin-rich additives can reduce the number of protozoa, alter the composition of volatile fatty acids (VFAs) to increase their propionic acid content, and reduce methane (CH_4) emissions [3];[6]. However, the extent and duration of these effects depend on the type and concentration of saponins, plant source, extraction method, and the adaptability of the rumen microbial community [7].

The tropical legume *Albizia chinensis* is widely cultivated in Southeast Asia due to its rapid growth and nitrogen-fixing ability. Its abundant leaf biomass makes it a potential source of unconventional feed. However, few studies have explored the saponin content in Albizia leaves and its effects on rumen protozoa. Previous studies on related species such as Albizia lebbeck and Albizia saman have shown that saponin-rich extracts can inhibit protozoan activity and regulate rumen fermentation [3]. Given the high price and complex composition of commercially available saponin preparations, it is of great scientific and practical significance to evaluate the protozoan activity of Albizia leaf powder and its ethanol extract compared with commercially available saponin preparations.

Due to differences in membrane sterol composition among different groups *Entodinium, Diplodinium, Isotricha*, and *Dasytricha*, protozoan genera also exhibit varying sensitivities to saponins [6]. Selective inhibition of certain genera can optimize rumen fermentation efficiency without leading to complete de-algaeization, thereby maintaining microbial balance. However, few comparative studies have systematically explored the effects of different saponin sources (from plant materials to extracts and commercial formulations) on protozoan genera at the same concentration and culture conditions [7].

Therefore, this study aimed to conduct an in vitro comparative analysis of the responses of different protozoan genera to antiprotozoan agents derived from Annona squamosa leaf powder, Annona squamosa leaf extract, and commercially available saponin products. Goat rumen fluid was used as an inoculum to simulate the natural rumen microbial environment. The results aim to gain a deeper understanding of the sensitivity of different genera to various saponin sources, the impact of extraction and formulation on antiprotozoan efficacy, and the potential of Annona squamosa leaf products as a sustainable natural feed additive in improving ruminant productivity and reducing methane emissions.

2 Materials and methods

2.1 Sample collection and DNA extraction

Rumen fluid samples were collected from Gadang goat slaughterhouses in Malang, Indonesia. Three treatment groups were prepared by adding 0.5 g of each material, *Albizia chinensis* leaf powder, *Albizia chinensis* leaf extract, and commercial saponin, to 10 mL of rumen fluid. Each mixture was supplemented with 40 mL of McDougall's buffer solution to maintain physiological conditions. The samples were then incubated for six hours under controlled laboratory conditions following a modified protocol described by [8].

Genomic DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, USA) with minor modifications to the manufacturer's protocol. Mechanical cell disruption was performed by bead-beating for 5 minutes using a BeadBug Microtube Homogenizer, with 30-second cooling intervals after every 1-minute cycle to prevent overheating. DNA concentration was determined using the Equalbit 1× dsDNA High-

Sensitivity Assay Kit (Vazyme, USA) and quantified with a Qubit Fluorometer (Thermo Fisher Scientific, USA). The extracted DNA yielded concentrations ranging from 30.4 to 33.2 ng/μL, indicating consistent recovery and purity suitable for downstream PCR analysis.

2.2 PCR amplification and sequensing

Amplification of the 18S rRNA gene (~1700 bp) was performed using SSUF and ITS-1 primers in combination with Promega GoTaq Green Master Mix (M712B; Promega, USA) on an Opentrons Flex GEN2 thermocycler. Each 25 μL reaction contained approximately 300 ng of DNA template. PCR products were separated by electrophoresis on a 1% (w/v) Smobio agarose gel prepared in 1× TBE buffer and stained with Smobio Nucleic Acid Gel Stain at a final concentration of 0.01% (v/v). Electrophoresis was conducted at 100 V for 30 minutes, and DNA bands were visualized alongside a molecular weight marker to confirm amplicon size integrity.

Sequencing was performed at the Integrated Genome Factory using the Oxford Nanopore PromethION platform. Library preparation was conducted with the SQK-NBD114.24 ligation-based sequencing kit (Oxford Nanopore Technologies, UK), following the manufacturer's protocol with minor adjustments. Each reaction contained 190 ng of genomic DNA diluted in 12.5 μL of nuclease-free water. The reaction mixture was incubated at 20 °C for 30 minutes, followed by 5 minutes at 65 °C, and adapter ligation at room temperature for 30 minutes. Basecalling was performed using Dorado v0.9.6 (Oxford Nanopore Technologies) with a minimum Q-score threshold of 10. Barcode and adapter sequences were automatically trimmed during the basecalling process to ensure high-quality reads for downstream analysis.

2.3 Bioinformatics and taxonomic analysis

Taxonomic classification and microbial community composition analyses were conducted using the EPI2ME v1.4.0 platform (Oxford Nanopore Technologies, UK), which integrates the Kraken2 software and the core_nt reference database for taxonomic assignment. Sequence reads ranging from 1,500 to 2,000 bp were retained for downstream analysis to ensure high-quality alignments and accurate genus-level identification. The analytical output included sequence summaries, relative abundance profiles, and diversity metrics generated according to the computational framework described by [9]. These data provided the basis for evaluating the differential responses of protozoan genera to the various saponin-based treatments.

3 Results and discussion

3.1 Comparative distribution of protozoan genus sensitivity based on venn diagrams analysis

The Venn diagram **Figure 1** demonstrates the comparative distribution of protozoan genera responding to antiprotozoal agents derived from *Albizia chinensis* leaf flour, its extract, and commercial saponin. The overlapping and distinct regions represent shared and unique protozoan genera influenced by each treatment.

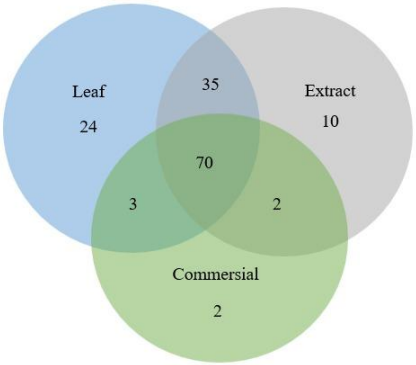

Fig 1. Distribution of Protozoan Genus

All treatment groups contained 70 different protozoan genera, suggesting a common inhibitory mechanism among the saponin-rich sources. These common protozoan groups are likely protozoa that are generally sensitive to saponins and other plant secondary metabolites that disrupt cell membrane integrity, reduce protozoan motility, and inhibit sterol-dependent processes [10]. This bioactivity is based on the amphiphilic nature of saponins, which allows them to form complexes with cell membrane cholesterol and create pores to lyse protozoa [11]. The leaf powder treatment group contained 24 different genera, while the leaf extract and commercially available saponins contained 10 and 2 different genera, respectively. The relatively large number of different genera in the leaf powder treatment group is due to the presence of components such as cellulose, lignin, and other plant secondary metabolites in the crude matrix, which control microbial interactions and saponin release. [10] The crude flour, therefore, has a more complex but broader profile of inhibitory activity. The reverse is the case with the extract, which is more selectively active due to its high content of saponins that can target surfactant-sensitive protozoa more specifically and oxidative stress [5].

It is noteworthy that only two genera of protozoa were specifically inhibited by commercially available saponins, suggesting that although the purified saponins are highly active, their activity spectrum is narrow and may be ineffective against protozoa with physiological adaptations or detoxification capabilities [11]. The number of overlapping genera between Albizia powder and extract (35) was greater than that between extract and commercially available saponins (2), suggesting that the natural and semi purified forms of Albizia share more common bioactive components and exhibit similar inhibitory spectra against protozoa.

Overall, the data show that the degree of processing influences the antiprotozoal activity's specificity and intensity. While its extract has modest but more focused efficacy, *Albizia chinensis* leaf flour exhibits broader but weaker inhibition. Due to its refinement, the commercial saponin causes strong but restricted inhibition. These findings support earlier research showing that natural saponin sources may provide synergistic benefits from concomitant phenolics and flavonoids, while being less standardized [12]. Consequently, *Albizia chinensis* leaf derivatives show potential as eco-friendly, plant-based alternatives to synthetic protozoacidal agents in animal nutrition and environmental applications.

3.2 Quantitative PCR analysis of protozoa genus response to *Albizia chinensis* -based treatments

The analysis yielded quantitative data from various protozoa genera analyzed using samples: *Albizia chinensis* leaf flour, *Albizia chinensis* leaf extract, and commercial saponin. **Table 1**

presents the analysis results regarding the distribution and relevance of each genus in this study.

Table 1. Number of genera in 3 treatments

Genus	Flour	Extract	Commersial
Eudiplodinium	45	69	13
Armophorida_Incertae_sedis	67	144	13
Epidinium	68	37	13
Theileria	75	116	14
Toxoplasma	77	105	28
Entodiniomorphida_Incertae_sedis	103	100	21
Paraisotricha	109	168	28
Anoplodinium	147	288	34
Polyplastron	148	164	46
Ostracodinium	162	158	34
Spirotrichea_Incertae_sedis	175	236	55
Tintinnida_Incertae_sedis	242	237	58
Trachelocerca	272	437	67
Litostomatea_Incertae_sedis	290	528	35
Triadinium	408	287	43
Balantioides	464	795	123
Diplodinium	466	618	110
Ophryoscolecidae_Incertae_sedis	850	1017	193
Colpoda	1460	899	6009
Enoploplastron	2425	10237	18855
Entodinium	2868	4752	510
Ophryoscolex	3569	1260	35
Entamoeba	4836	1328	0
Ciliophora_Incertae_sedis	15466	26177	3693
Isotricha	35193	49277	173

Quantitative PCR (qPCR) data showed that there were significant differences in the responses of different protozoan genera after treatment with Albizia leaf powder, its extract and a commercially available saponin product. The commercially available saponin generally had a stronger inhibitory effect on most protozoan genera than the leaf powder and extract. This trend supports the hypothesis that purified saponins, due to their well-defined amphiphilic structure, can exert potential antiprotozoan activity by disrupting cell membranes and complexing with sterols [12]. Among the 25 genera detected, the reduction in the number of *Entamoeba, Isotricha* and *Ophryoscolex* was the most significant, with the relative bacterial counts of these genera decreasing by 99% to 100% after treatment with commercially available saponins compared to the leaf powder control group. Similarly, genera such as *Triadinium*, *Litostomatea incertae sedis*, and *Entodinium* also exhibited declines exceeding 80%. These results indicate that the standardized saponin formulation

effectively suppressed a broad spectrum of protozoan taxa, including ciliate and amoeboid groups. The potent inhibition of *Entamoeba* and *Isotricha* is particularly interesting since these genera are known to play significant roles in intestinal and ruminal microbial ecosystems [13].

In contrast, the responses to mulberry leaf extracts were more varied. Some genera (such as *Eudiplodinium, Theileria*, and *Entodinium*) showed 40%–65% higher activity than flour, while others (such as *Epidinium* and *Ophryoscolex*) showed decreased activity. This inconsistency may stem from the complexity of the crude extract composition, which contains saponins, flavonoids, tannins, and carbohydrates [14]. These additional metabolites may have synergistic or antagonistic effects, thereby enhancing the inhibitory effect on protozoa or providing substrates for their proliferation. The different responses at the genus level suggest that the biochemical heterogeneity of the crude extract leads to selective rather than broad-spectrum inhibition.

Additionally, under extract and commercial treatments, taxa like *Enoploplastron* and Ciliophora incertae sedis shown significantly higher abundance than flour. The observed rise (up to 320% for *Enoploplastron*) may be the result of compensatory overgrowth or species-level resistance after sensitive taxa diminish. These compensatory changes are typical in microbial communities under phytochemical-induced selective pressure [15]. As a result, even while the general quantity of protozoa may decline, certain resistant lineages may briefly take control, changing the makeup of the population as a whole..

Mechanistically, saponins are known to bind to sterols in protozoan cell membranes, creating pores that lead to leakage of cell contents and ultimately cell death [5]. The higher efficacy of commercially available products compared to plant-derived raw materials may be attributed to the higher purity and concentration of their triterpenoid or steroidal saponins. However, saponins undergo microbial deglycosylation in biological systems such as the rumen, thereby reducing their antiprotozoan activity over time. Therefore, while the extract may initially exhibit inhibitory effects, its stability and duration of action may be limited compared to standardized commercial formulations. Furthermore, differences between genera highlight species-specific sensitivities related to variations in cell membrane sterol composition and enzymatic detoxification capabilities. For instance, ruminal ciliates such as *Ophryoscolex* and *Entodinium* are typically more sensitive to saponins than amoeboid species such as *Entamoeba*, which can form cysts that confer resistance [5]. The nearly complete suppression of *Entamoeba* in this study underscores the potential of saponin-based interventions for controlling protozoal infections, provided that cytotoxicity to host tissues is also assessed.

Even though these findings show distinct patterns, more confirmation is required. DNA from nonviable cells may be included in the current data since it represents relative qPCR abundance. Future research using RNA-based quantification or PMA-qPCR may be able to differentiate between live and dead protozoa with greater accuracy [15]. Furthermore, it would be possible to determine which particular saponins or phenolic compounds are mainly responsible for the reported genus-specific effects by employing chromatographic and spectroscopic techniques (HPLC–MS) for chemical profiling of the *Albizia chinensis* extract. The substantial in vitro antiprotozoal potential of saponins produced from *Albizia chinensis* leaves or commercial formulations is confirmed by this comparative PCR research. Depending on genus susceptibility, the crude extract produced selective inhibition and occasionally stimulation, but the commercial preparation showed the most noticeable and consistent suppression across genera.These findings support the potential application of *Albizia chinensis* bioactives as natural antiprotozoal agents, yet also highlight the need for compositional standardization and mechanistic validation before practical implementation

4 Conclusion

Albizia chinensis saponins have strong in vitro antiprotozoal activity, according to the PCR study. In several genera, including *Entamoeba, Isotricha*, and *Ophryoscolex*, commercial saponin demonstrated the strongest inhibitory action, lowering protozoan population by about 80–100%. On the other hand, the flour form produced broader but lesser effects, whilst the *Albizia chinensis* leaf extract showed moderate, selective inhibition. These results show that the concentration and purity of saponins have a significant impact on their antiprotozoal efficacy, with commercial products having the maximum potency and crude extracts having more variable but biologically significant activity Overall, *Albizia chinensis* leaf derivatives hold promise as natural, eco-friendly alternatives to synthetic antiprotozoal compounds, pending further standardization and in vivo validation.

References

1. Newbold, C. J., De la Fuente, G., Belanche, A., Ramos-Morales, E., & McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. Frontiers in Microbiology, 6(NOV), 1–14. https://doi.org/10.3389/fmicb.2015.01313
2. Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., Branco, A. F., Moate, P. J., Deighton, M. H., Williams, S. R. O., Kindermann, M., & Duval, S. (2015). An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proceedings of the National Academy of Sciences of the United States of America, 112(34), 10663–10668. https://doi.org/10.1073/pnas.1504124112
3. Tan, C., Ramírez-Restrepo, C., Shah, A., Hu, R., Bell, M., Wang, Z., & McSweeney, C. (2020). The community structure and microbial linkage of rumen protozoa and methanogens in response to the addition of tea seed saponins in the diet of beef cattle. Journal of Animal Science and Biotechnology, 11. https://doi.org/10.1186/s40104-020-00491-w
4. Patra, A., Patra, A., Stiverson, J., & Yu, Z. (2012). Effects of quillaja and yucca saponins on communities and select populations of rumen bacteria and archaea, and fermentation in vitro. Journal of Applied Microbiology, 113. https://doi.org/10.1111/j.1365-2672.2012.05440.x
5. Kholif, A. (2023). A Review of Effect of Saponins on Ruminal Fermentation, Health and Performance of Ruminants. Veterinary Sciences, 10. https://doi.org/10.3390/vetsci10070450
6. Yanza, Y., Irawan, A., Jayanegara, A., Ramadhani, F., Respati, A., Fitri, A., Hidayat, C., Niderkorn, V., Cieślak, A., Szumacher-Strabel, M., Hidayat, R., & Tanuwiria, U. (2024). Saponin Extracts Utilization as Dietary Additive in Ruminant Nutrition: A Meta-Analysis of In Vivo Studies. Animals : An Open Access Journal from MDPI, 14. https://doi.org/10.3390/ani14081231
7. Rao, Z., Li, Y., Yang, X., Guo, Y., Zhang, W., & Wang, Z. (2024). Diet xylo-oligosaccharide supplementation improves growth performance, immune function, and intestinal health of broilers. Animal Nutrition, 17, 165–176. https://doi.org/10.1016/j.aninu.2024.01.004
8. Hu, W., Liu, J., Wu, Y., Guo, Y., & Ye, J. (2006). Effects of tea saponins on in vitro ruminal fermentation and growth performance in growing Boer goat. Archives of Animal Nutrition, 60(1), 89–97. https://doi.org/10.1080/17450390500353119
9. Ondov, B. D., Bergman, N. H., & Phillippy, A. M. (2011). Krona-385.pdf. BMC

Bioinformatics, 385(September).

10. Singh, I., Hundal, J. S., Wadhwa, M., & Lamba, J. S. (2018). Assessment of potential of some tannins and saponins containing herbs on digestibility of nutrients, fermentation kinetics and enteric methane production under different feeding systems: An in vitro study. Indian Journal of Animal Sciences, 88(4), 443–452. https://doi.org/10.56093/ijans.v88i4.78812
11. Ramírez-Restrepo, C. A., O'Neill, C. J., López-Villalobos, N., Padmanabha, J., Wang, J. K., & McSweeney, C. (2016). Effects of tea seed saponin supplementation on physiological changes associated with blood methane concentration in tropical Brahman cattle. Animal Production Science, 56(3), 457–465. https://doi.org/10.1071/AN15582
12. Ramos-Morales, E., De La Fuente, G., Duval, S., Wehrli, C., Bouillon, M., Lahmann, M., Preskett, D., Bragança, R., & Newbold, C. (2017). Antiprotozoal Effect of Saponins in the Rumen Can Be Enhanced by Chemical Modifications in Their Structure. Frontiers in Microbiology, 8. https://doi.org/10.3389/fmicb.2017.00399
13. Ramos-Morales, E., Lyons, L., Fuente, G., Bragança, R., & Newbold, C. (2019). Not all saponins have a greater antiprotozoal activity than their related sapogenins. FEMS Microbiology Letters, 366. https://doi.org/10.1093/femsle/fnz144
14. Wei, W., Zhen, Y., Wang, Y., Shahzad, K., & Wang, M. (2022). Advances of Rumen Functional Bacteria and the Application of Micro-Encapsulation Fermentation Technology in Ruminants: A Review. Fermentation, 8(10). https://doi.org/10.3390/fermentation8100564
15. Zhang, G., Guo, Z.-Y., Ke, Y., Li, H., Xiao, X., Lin, D., Lin, L., Wang, Y., Liu, J., Lu, H., Hong, H., & Yan, C. (2024). Comparative analysis of size-fractional eukaryotic microbes in subtropical riverine systems inferred from 18S rRNA gene V4 and V9 regions. The Science of the Total Environment, 175972. https://doi.org/10.1016/j.scitotenv.2024.175972