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Política editorial

La *Revista de la Facultad de Medicina Veterinaria y de Zootecnia* fue creada en 1929 por el doctor Doménico Geovine, decano de la Escuela Nacional de Medicina Veterinaria, hoy Facultad de Medicina Veterinaria y de Zootecnia. En el medio universitario y en el área pecuaria, es la revista del área de mayor antigüedad. Desde su creación su objetivo ha sido ofrecer un medio escrito de expresión para toda la comunidad académica interna y externa, en el cual exponer sus ideas, resultados de investigación, ensayos etc., en relación con el quehacer científico en el área de las Ciencias Animales y otras afines. Su filosofía ha sido tener un carácter abierto, decididamente transparente y democrático, no solo en la participación de los articulistas sino en los procedimientos internos de gestión. La Revista busca cumplir con sus objetivos de divulgar los trabajos de investigación, documentos críticos y de revisión técnico científica, permitiendo la difusión del conocimiento entre profesionales de las áreas pecuarias; siempre en la búsqueda de información pertinente y actualizada de temas relacionados con el sector y propendiendo a obtener reconocimiento en la comunidad en general, editando una revista que permita la interacción de la academia con el medio.
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Insectos en sistemas agroalimentarios: de la alimentación a la bioeconomía sostenible

En un contexto donde la seguridad alimentaria y la sostenibilidad son prioridades globales, el sistema agroalimentario enfrenta desafíos cada vez más complejos. La creciente demanda de proteína animal, la competencia por los recursos naturales y la necesidad de reducir el impacto ambiental de la producción agropecuaria han impulsado la búsqueda de alternativas más sostenibles. En este escenario, los insectos han emergido no solo como una fuente innovadora y eficiente de nutrición animal, sino también como una herramienta clave para la gestión de residuos, la optimización de sistemas productivos y el impulso de la bioeconomía. Su integración en sistemas agroalimentarios requiere un enfoque basado en la ciencia, la regulación y la adaptación a distintos contextos.

A nivel mundial, la investigación en este campo se ha centrado en la colecta, producción y transformación de un número limitado de especies con interés zootécnico, lo que ha permitido desarrollar protocolos estandarizados e integrarlos en sistemas agroalimentarios. Sin embargo, es crucial ampliar la perspectiva y reconocer los roles socioecológicos de los insectos, cuyo potencial va más allá de la producción animal. Estos organismos desempeñan funciones clave en los ecosistemas y pueden aportar soluciones innovadoras en la gestión de residuos, la seguridad alimentaria y la bioeconomía. No obstante, su aprovechamiento no puede seguir un modelo único; la selección de especies y el diseño de sistemas productivos se deben basar en un enfoque integral que contemple su viabilidad ambiental, económica y social, para asegurar estrategias sostenibles y adaptadas a cada contexto.

Para aprovechar plenamente el potencial de los insectos en el sistema agroalimentario y fortalecer su papel en la bioeconomía, es necesario, primero, avanzar en la bioprospección, mediante la identificación de sus servicios ecosistémicos y desafíos para explorar nuevas especies con aplicaciones más allá de la alimentación humana y animal. Segundo, es fundamental abordar el uso y la producción de insectos desde una perspectiva de escala, comprendiendo que sus objetivos e impactos varían según la región, el tipo de manejo y las necesidades locales. Estos aspectos se deben integrar de manera multidisciplinaria y colaborativa, al tomar en cuenta diversas perspectivas y mediante la adaptación a cada entorno. La selección de especies y el diseño de los sistemas productivos deben responder a un enfoque integral que garantice su viabilidad ecológica, económica y social, para asegurar estrategias efectivas y sostenibles en el marco de la bioeconomía.

La sostenibilidad en este campo se debe analizar con cuidado, ya que no es un concepto uniforme, sino que varía según el objetivo, el tipo de manejo y el contexto. Para que el uso de los insectos sea verdaderamente sostenible, es esencial equilibrar sus dimensiones ecológica, económica y social, considerando además su impacto en los ecosistemas, el bienestar animal y la seguridad alimentaria. En este sentido, el enfoque de One Health cobra relevancia al resaltar la interconexión entre la salud humana, animal y ambiental. Su integración en los distintos servicios ecosistémicos

que ofrecen los insectos, así como la gestión de los riesgos asociados y su contribución a la sostenibilidad, se debe basar en datos concretos y sistemas de evaluación rigurosos que permitan medir su impacto de manera integral y contextualizada.

Los desafíos normativos siguen siendo una barrera importante para la expansión de esta industria. La estandarización de los procesos de producción, la seguridad sanitaria y la aceptación regulatoria varían entre países y continentes, lo que dificulta el acceso a mercados y la implementación a gran escala. A nivel global, los marcos normativos evolucionan para garantizar la inocuidad y calidad de estos ingredientes, lo cual facilita su integración en la industria agropecuaria. Sin embargo, en América Latina aún es necesario fortalecer la formulación de políticas públicas que respalden su adopción. Desde la Red de Insectos de la Asociación Latinoamericana de Producción Animal (ALPA) impulsamos este proceso mediante la promoción del diálogo entre investigadores, productores y tomadores de decisiones, con el objetivo de avanzar en regulaciones que consoliden la industria de los insectos en la región.

El uso de insectos para abordar los desafíos del sistema agroalimentario ha avanzado significativamente en la última década, pero aún es necesario abordarlo bajo la perspectiva de uso y manejo sostenible. En el Centro de Investigación de Artrópodos Terrestres (CINAT), trabajamos en esta perspectiva mediante investigación aplicada, trabajo de campo y análisis de valor para fortalecer el papel de los insectos en sistemas productivos sostenibles. A través de iniciativas interdisciplinarias como Insectos por la Paz, EntoPro, BioInsectonomy, NutrInsecta e Insectonomy, en alianza con empresas e instituciones académicas nacionales e internacionales, promovemos soluciones innovadoras para el manejo de desechos, el empoderamiento comunitario y la exploración del potencial de los insectos desde su función ecosistémica y productiva. Estas iniciativas integran enfoques científicos, tecnológicos, sociales y económicos para desarrollar estrategias sostenibles adaptadas a distintos contextos.

En conclusión, la integración de los insectos en los sistemas agroalimentarios ofrece una oportunidad estratégica para mejorar la seguridad alimentaria, la sostenibilidad y la bioeconomía. La investigación aplicada y la colaboración interdisciplinaria han permitido desarrollar protocolos productivos eficientes y explorar el potencial de los insectos más allá de la nutrición animal, abordando desafíos en la gestión de residuos y la producción sostenible. Para consolidar este avance, es clave fortalecer los marcos regulatorios, adaptar las estrategias productivas a cada contexto y asegurar que el desarrollo de sistemas agroalimentarios sea sostenible e inclusivo. Además, en las carreras agropecuarias es necesario fortalecer esta nueva línea de trabajo e investigación para seguir explorando el potencial que la biodiversidad ofrece en el contexto agroalimentario.

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Insects in agri-food systems: from nutrition to a sustainable bioeconomy

In a global context where food security and sustainability are top priorities, the agri-food system faces increasingly complex challenges. The growing demand for animal protein, competition for natural resources, and the need to reduce the environmental impact of agricultural production have driven the search for more sustainable alternatives. In this scenario, insects have emerged not only as an innovative and efficient source of animal nutrition but also as a key tool for waste management, the optimization of production systems, and the advancement of the bioeconomy. Their integration into agri-food systems requires a science-based approach, appropriate regulatory frameworks, and adaptation to diverse contexts.

Globally, research in this field has focused on the collection, production, and processing of a limited number of species of zootechnical interest, enabling the development of standardized protocols and their integration into agri-food systems. However, it is crucial to broaden this perspective and recognize the socio-ecological roles of insects, whose potential extends beyond animal production. These organisms play key roles in ecosystems and can contribute to innovative solutions for waste management, food security, and the bioeconomy. Nevertheless, their utilization cannot follow a one-size-fits-all model; species selection and the design of production systems must be based on a comprehensive approach that considers environmental, economic, and social viability, ensuring sustainable and context-adapted strategies.

To fully harness the potential of insects in the agri-food system and strengthen their role in the bioeconomy, two key steps must be taken. First, bioprospecting efforts should be expanded to identify their ecosystem services and challenges, thereby enabling the exploration of new species with applications beyond human and animal nutrition. Second, the use and production of insects must be addressed from a scaling perspective, recognizing that objectives and impacts vary by region, management type, and local needs. These aspects must be integrated through multidisciplinary and collaborative approaches, considering diverse perspectives and adapting to specific environments. Species selection and system design should follow a holistic framework that ensures ecological, economic, and social feasibility, fostering effective and sustainable strategies within the bioeconomy.

Sustainability in this field must be carefully analyzed, as it is not a uniform concept but varies according to objectives, management types, and contexts. For insect utilization to be truly sustainable, it is essential to balance its ecological, economic, and social dimensions while also considering its impact on ecosystems, animal welfare, and food security. In this regard, the One Health approach is particularly relevant, as it emphasizes the interconnectedness of human, animal, and environmental health. The integration of insects into various ecosystem services—as well as the management of associated risks and their contribution to sustainability—must be based on concrete data and rigorous assessment systems that enable comprehensive and context-specific impact evaluation.

Regulatory challenges remain a significant barrier to the expansion of this industry. The standardization of production processes, sanitary safety, and regulatory acceptance vary across countries and continents, complicating market access and large-scale implementation. Globally, regulatory frameworks are evolving to ensure the safety and quality of these ingredients, facilitating their integration into the agricultural industry. However, in Latin America, further efforts are needed to strengthen public policies that support their adoption. The Insect Network of the Latin American Association of Animal Production (ALPA) is actively promoting this process by fostering dialogue among researchers, producers, and policymakers to advance regulations that consolidate the insect industry in the region.

The use of insects to address challenges in the agri-food system has advanced significantly over the past decade, yet it must continue to be guided by principles of sustainable use and management. At the Terrestrial Arthropod Research Center (CINAT), we work within this framework through applied research, fieldwork, and value chain analysis to strengthen the role of insects in sustainable production systems. Through interdisciplinary initiatives such as *Insectos por la Paz, EntoPro, BioInsectonomy, NutrInsecta*, and Insectonomy—in collaboration with national and international companies and academic institutions—we promote innovative solutions for waste management, community empowerment, and the exploration of insects' ecosystemic and productive potential. These initiatives integrate scientific, technological, social, and economic approaches to develop sustainable strategies tailored to different contexts.

In conclusion, the integration of insects into agri-food systems presents a strategic opportunity to enhance food security, sustainability, and the bioeconomy. Applied research and interdisciplinary collaboration have facilitated the development of efficient production protocols and expanded the potential of insects beyond animal nutrition, addressing challenges in waste management and sustainable production. To consolidate this progress, it is essential to strengthen regulatory frameworks, adapt production strategies to specific contexts, and ensure that the development of agri-food systems remains sustainable and inclusive. Moreover, agricultural science curricula must reinforce this emerging field of research and practice to further explore the potential that biodiversity offers within the agri-food context.

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Persistent ductus arteriosus in an elderly dog

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ABSTRACT

Patent ductus arteriosus (PDA) is characterized by the failure of complete closure of the communication between the aorta and the pulmonary trunk during the early stages of life and is frequently observed in young animals. This vascular connection originates from pulmonary non-functionality during the fetal period and should close shortly after birth. In the absence of complete closure, the patient becomes a carrier of PDA, often with hemodynamic consequences. The aim of the present case report is to describe a case of PDA in an elderly mixed-breed dog. A mixed-breed dog, approximately 12 years old, was referred for cardiological evaluation due to a history of excessive fatigue reported over the previous 60 days. Clinical and laboratory examinations revealed a continuous murmur localized over the pulmonary area. Additional diagnostic tests were requested for further investigation. On Doppler echocardiographic examination, the following findings were observed: continuous turbulent flow at the site of the ductus arteriosus in the pulmonary trunk (left-to-right shunting); a minimal ductal diameter ranging from approximately 4.3 to 5.7 mm; and no abnormalities in other cardiac structures. These findings confirmed the diagnosis of PDA. Given the presence of flow reversal, routine clinical monitoring was recommended.

Keywords: congenital heart disease, congenital defect, hemodynamics, ductal patency.

Persistência do canal arterial em cão idoso

RESUMO

A persistência do ducto arterioso (PDA) é caracterizada pela falha no fechamento completo da comunicação entre a artéria aorta e o tronco pulmonar durante os estágios iniciais de vida, sendo frequentemente observada em animais jovens. A ligação entre os vasos é derivada da afuncionalidade pulmonar no período fetal, que deveria ser ocluída logo após o nascimento e, na ausência de oclusão completa, o paciente se torna portador de PDA,

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geralmente com repercussão hemodinâmica. O objetivo do presente relato é descrever um caso de PDA em paciente sem raça definida idoso. Um cão, sem raça definida, com aproximadamente 12 anos de idade, foi encaminhado para avaliação cardiológica mediante histórico de cansaço excessivo, identificado há 60 dias. Ao exame clínico-laboratorial, constatou-se sopro contínuo em foco pulmonar. Exames complementares foram solicitados para maiores investigações. Ao exame ecodopplercardiográfico, notou-se: fluxo turbulento e contínuo em topografia do canal arterial no tronco pulmonar (direção esquerda-direita); diâmetro ductal mínimo de aproximadamente 4,3 – 5,7mm; demais estruturas sem alterações. Os achados foram conclusivos de PDA, e mediante a presença de reversão de fluxo, foi indicado apenas o monitoramento clínico rotineiro.
Palavras-chave: cardiopatia congênita, defeito congênito, hemodinâmica, patência ductal.

Introduction

Patent ductus arteriosus (PDA) is a congenital defect characterized by the failure of closure of the ductus arteriosus (Toom *et al.*, 2016). It is among the most common congenital heart diseases, with a worldwide prevalence ranging from 10% to 32% (Argenta *et al.*, 2018; Piantedosi *et al.*, 2019; Brambilla *et al.*, 2020). From an embryological perspective, the origin of the ductus arteriosus is partly explained by the absence of effective pulmonary function during fetal life, which facilitates blood circulation prior to birth (Toom *et al.*, 2016). In this context, pulmonary arterial pressure is higher than aortic pressure, resulting in blood flow from the pulmonary artery to the aorta (Toom *et al.*, 2016). After birth, with the establishment and development of pulmonary function, a reversal of the pressure gradient occurs, wherein aortic pressure exceeds pulmonary pressure. This change redirects blood flow from the aorta to the pulmonary artery, leading to the closure of the ductus arteriosus within the first week of life and the formation of the fibrous ligamentum arteriosum, a remnant of the ductus (Toom *et al.*, 2016).

In cases where the ductus fails to close or closes incompletely, the animal is considered to have a congenital condition and becomes susceptible to hemodynamic alterations. However, the extent of pulmonary communication determines the presence and severity of clinical signs. The most evident alterations include congestive heart failure (CHF) and signs such as pulmonary edema and congestion, concentric hypertrophy of the right ventricle, eccentric hypertrophy of the left ventricle, dilation of the atrioventricular and aortic trunks, and increased pulmonary blood flow (Toom *et al.*, 2016). In cases of flow reversal, animals may develop polycythemia and blood hyperviscosity (Toom *et al.*, 2016). The aim of the present case report is to describe the occurrence of PDA in an elderly mixed-breed dog.

Case description

A 12-year-old, 14 kg, neutered, dewormed, and vaccinated male mixed-breed dog was presented to a veterinary clinic in the metropolitan area of Belo Horizonte, Minas Gerais, Brazil, with a two-month history of progressive fatigue and lethargy. The animal was referred for a Doppler echocardiographic evaluation under

suspicion that the symptoms might be related to cardiovascular dysfunction. Physical examination revealed a heart rate (HR) of 132 bpm, a respiratory rate of 36 bpm, moist and normally pigmented mucous membranes, a rectal temperature of 38.1 °C, a muscle condition score of 3, and a body condition score of 6/9, according to the World Small Animal Veterinary Association (WSAVA) guidelines. Lymph nodes were normal in size, morphology, and dimension. The animal was clinically hydrated, with a negative jugular pulse, unremarkable abdominal palpation, and clear pulmonary auscultation, with no abnormalities detected in other examined regions. However, cardiac auscultation identified a continuous holosystolic murmur localized to the pulmonary area. Blood pressure measured by Doppler was 130 mmHg. Hematology, serum biochemistry (including urea, creatinine, alanine aminotransferase, aspartate aminotransferase, total proteins, and fractions), and urinalysis revealed no significant abnormalities. To complement the clinical evaluation and obtain a detailed cardiovascular morphofunctional analysis, electrocardiographic and Doppler echocardiographic examinations were recommended.

Electrocardiography showed sinus arrhythmia with a migrating pacemaker as the basal rhythm. The P-wave duration exceeded normal limits (0.46 ms), suggesting possible left atrial overload. Additionally, a T-wave amplitude exceeding 25% of the R-wave amplitude was observed, which may indicate nonspecific repolarization disturbances, potentially resulting from subtle myocardial oxygenation alterations and/or electrolyte or metabolic imbalances.

On echocardiographic evaluation, the animal, experiencing mild stress, had a heart rate (HR) of 100 bpm. Cardiac situs solitus with normal atrioventricular and ventriculoarterial concordance was confirmed. The left ventricle exhibited mild to moderate dilation, with a left ventricular diastolic internal diameter (LVIDd) of 42.0 mm and a normalized LVIDd of 1.9. Color Doppler imaging detected systolic mitral regurgitation. Aortic flow was laminar, though mild diastolic regurgitation was present, with a maximum velocity (Vmax) of 1.31 m/s and a pressure gradient (PG) of 6.83 mmHg, without apparent hemodynamic compromise. Pulmonary flow was laminar with mild diastolic regurgitation (Vmax = 1.12 m/s; PG = 5.02 mmHg), with minimal hemodynamic impact. The pulmonary artery trunk bifurcated normally but was dilated (pulmonary artery diameter: 19.6 mm; aorta: 16.0 mm; pulmonary-to-aortic ratio: 1.23) (figure 1).

Color doppler further revealed a continuous, turbulent flow within the pulmonary trunk, cranial to the pulmonary valve, predominantly directed left-to-right (aorta to pulmonary artery). The estimated Vmax of the turbulent flow was 4.82 m/s, with an approximate pressure gradient between the aorta and pulmonary artery of 93 mmHg. The minimum observed ductal diameter ranged from 4.3 to 5.7 mm, classified as medium to large according to Kittleson and Kienle (1998), although the sole clinical sign noted was fatigue.

The final echocardiographic diagnosis was a patent ductus arteriosus (PDA) with mild to moderate left ventricular overload. However, systolic and diastolic functional indices of the left ventricle remained within species-specific reference ranges. Therefore,

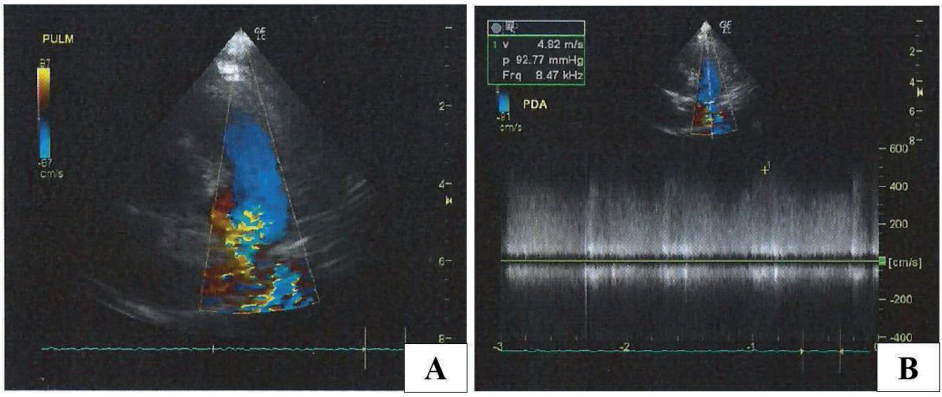

FIGURE 1. (A/B) Echocardiographic findings demonstrate the presence of a patent ductus arteriosus (PDA). Turbulent, continuous flow within the pulmonary trunk is observed, along with dilation of the pulmonary trunk and a continuous flow pattern characteristic of ductal patency.

Source: Own elaboration.

the animal was diagnosed with PDA at 12 years of age based on echocardiographic findings combined with clinical presentation (figure 1). Periodic follow-up examinations every 3-6 months were recommended. The attending clinicians informed the owner about the condition, emphasizing that surgical correction was not feasible due to flow reversal. Consequently, the owner was advised to continue monitoring the animal every 3-6 months, with a recommended reevaluation five days after the initial assessment to adjust pharmacological management if needed. However, the owner did not return for follow-up.

Discussion

The persistence of communication between the aorta and the pulmonary trunk is considered a congenital defect, primarily observed in young animals, and results from absent or incomplete closure between these vessels. According to Buchanan and Patterson (2003), normal closure of the ductus arteriosus requires the timely interaction of various morphological, biochemical, molecular, and environmental factors, many of which are influenced by gestational age and the consequent maturation of the ductus arteriosus and preparatory angiomalacia. Prior to the occlusion of the patent ductus arteriosus (PDA), blood flow occurs from the aorta to the pulmonary artery (left-to-right shunting) due to higher aortic pressure and the temporary non-functionality of the lungs (Toom et al., 2016). According to Greet et al. (2021), the direction of blood flow is influenced by factors associated with pulmonary and systemic resistance, favoring predominantly left-to-right shunting. In cases where patients present with concurrent pulmonary hypertension and PDA, blood flow may reverse from the pulmonary artery to the aorta (right-to-left shunting), leading to blood mixing and tissue oxygenation deficits (Toom *et al.,* 2016). The hemodynamic reasons for flow reversal are associated with increased pulmonary arterial pressure, changes in vascular resistance, and diversion of blood flow. Thus, in cases of PDA, either

unidirectional or bidirectional flow may be observed, depending on the presence of additional comorbidities (Toom et al., 2016). In the present case report, the patient was diagnosed with PDA via echocardiography following episodes of sudden fatigue and, at the time of evaluation, exhibited left-to-right flow (aorta to pulmonary artery).

PDA can be diagnosed at any age; however, it is most frequently identified in young animals (Toom *et al.*, 2016). Detection of PDA in older animals is less common compared to younger dogs (Toom *et al.*, 2016; Ro *et al.*, 2022), potentially due to the organism's compensatory capacity and the absence of classical clinical signs such as cyanosis and cough. The lack of evident clinical signs of PDA may be related to factors such as hemodynamic compensation, the size of the ductus, the animal's age, and overall health status, which could explain the absence of significant symptoms. Toom *et al.* (2016), in a study aimed at assessing the incidence, clinical presentation, and histopathology of PDA in dogs, reported that most diagnoses occurred in puppies, although a small proportion (n = 4) involved adult animals, indicating that late presentations are rare. In contrast to these findings, the animal in the present report was elderly at the time of diagnosis, which diverges from the data reported by Toom *et al.* (2016) and Ro *et al.* (2022), who primarily observed PDA in young animals. In this case, the defect was diagnosed at 12 years of age, and the delayed diagnosis may have resulted from the absence of adequate clinical evaluation during the animal's early life and the lack of obvious clinical signs.

Certain breeds, such as the Maltese, Chihuahua, and Pomeranian, are described as predisposed to PDA, although the defect may occur in any breed (Brambilla *et al.*, 2020; Grimes & Thieman Mankin, 2022). Congenital heart defects, particularly PDA, may be more frequently observed in mixed-breed dogs due to the blending of racial characteristics, potentially explaining its occurrence in non-pedigree dogs (Brambilla *et al.*, 2020). Data suggest that females are more commonly affected than males (Brambilla *et al.*, 2020; Grimes & Thieman Mankin, 2022), although the reason for this sex predilection remains unclear. Some studies have not reported significant sex-based differences in the occurrence of PDA (Toom *et al.*, 2016); however, in the present case, the affected patient was male, which contrasts with the findings of Brambilla *et al.* (2020) and Grimes and Thieman Mankin (2022). Furthermore, the animal was not from a breed traditionally predisposed to PDA, which aligns with Brambilla *et al.* (2020), who reported that approximately 43% of affected dogs were mixed breed.

According to Buchanan and Patterson (2003), changes observed in the wall of the ductus arteriosus in dogs with PDA may be a potential causal factor for the failure of ductal closure after birth. They noted that dogs may exhibit PDA sporadically, with morphological characteristics similar to those observed in hereditary cases, such as ductal hypoplasia, muscular asymmetry, and the presence of elastic tissue resembling that of the aorta within the ductus wall, which confers continued patency. The authors suggest that supposedly sporadic PDA may result from a genetic alteration of the ductal structure, partially resembling the presentation seen in Poodle breeds.

Ductus arteriosus occlusion is currently considered one of the most appropriate surgical procedures for the resolution of PDA, particularly to prevent complications such as congestion or cardiac

insufficiency. However, there is a specific window during which occlusion should be performed—namely, while the shunting remains left-to-right (Grimes & Thieman Mankin, 2022) and before any flow reversal occurs. If pressure overload leads to right-to-left shunting, occlusion should not be pursued; instead, the animal should undergo periodic monitoring and receive supportive medical management as needed. Cases of reversed PDA (right-to-left shunting) are not surgically manageable due to the high risk of cardiac failure and sudden death (Scurtu *et al.*, 2016). Although ductal occlusion remains the most frequently adopted approach, procedural complications such as ligature loosening or ductal rupture can occur (Grimes & Thieman Mankin, 2022). The use of more advanced therapeutic tools, such as Amplatzer vascular plugs and percutaneous closure (Bagardi *et al.*, 2022; Papa *et al.*, 2023; Wesselowski & Saunders, 2019), is not yet widely available in most veterinary centers in Brazil.

In the present case, the patient was not subjected to ductal occlusion due to the occurrence of flow reversal, although the procedure and its implications were thoroughly discussed. The decision to surgically intervene in PDA cases depends on multiple factors, including the patient's age, ductal size, and the presence of flow reversal; closure is contraindicated once right-to-left shunting has developed due to the associated elevated pulmonary pressure. Each case must therefore be evaluated individually. There is a hypothesis that the use of sildenafil may aid in the management of reversed PDA, although its efficacy remains controversial (Nakamura *et al.*, 2011; Greet *et al.*, 2021). Other pharmacological agents, such as prostacyclin analogs and endothelin receptor antagonists, have also been proposed, but their application remains poorly characterized in veterinary medicine. Given the delayed diagnosis, the clinical presentation (notably excessive fatigue over approximately 60 days), and the presence of flow reversal, periodic monitoring combined with supportive therapy, if necessary, was deemed the most appropriate management strategy.

The complications associated with PDA are highly variable and depend on numerous factors, including patient-specific characteristics, ductal diameter, hemodynamic status, and compensatory capacity. Some animals with PDA exhibit a range of clinical signs (Ro *et al.*, 2022), such as excessive fatigue, syncope, and coughing; however, asymptomatic cases are not uncommon, complicating early detection. Clinical manifestations associated with PDA vary based on intrinsic and extrinsic factors, including ventricular hypertrophy, aortic and pulmonary artery dilation, and cyanosis, among others (Toom *et al.*, 2016).

In the present report, the patient was referred for cardiological evaluation with a two-month history of excessive fatigue and a continuous murmur localized to the pulmonary area. More classical signs, such as coughing, cyanosis, and syncope, were not reported by the owner. The absence of overt cardiopulmonary symptoms may have delayed the suspicion and investigation of an underlying cardiovascular disorder, as owners typically seek veterinary evaluation only when noticeable clinical changes occur.

The echocardiographic findings reported herein are characteristic of PDA. In a case report by Ro *et al.* (2022), a 9-year-old Maltese female diagnosed with PDA exhibited severe left atrioventricular dilation, increased diastolic diameter, an elevated left atrium-to-aorta ratio, reduced

fractional shortening, mitral regurgitation, pulmonary artery dilation, elevated aortic, pulmonary, and transmitral flows, and severe diastolic dysfunction. The echocardiographic findings described by Ro *et al.* (2022) partially align with those in the present case. A notable difference was observed in the ductal diameter: Ro *et al.* (2022) reported a ductus diameter of 5.15 mm, whereas in the present case, measurements ranged from 4.3 to 5.7 mm. Based on these measurements and associated clinical findings, the ductus in this patient was classified as medium to large; however, it did not produce marked clinical repercussions. The right heart chambers in this patient maintained a pressure regime of approximately one-third to one-quarter that of the left ventricle.

The diameter of the ductus arteriosus partially explains why some animals maintain ductal patency into adulthood; hemodynamic complications are proportional to the volume of blood diverted from the physiological flow pathway (Pugliesi *et al.*, 2021). In the present case, the 12-year-old patient exhibited only mild clinical symptoms, suggesting that the persistent ductal diameter may have been relatively small or that incomplete closure occurred, which could account for the relatively mild clinical status despite the advanced age. However, these hypotheses remain speculative, as definitive imaging techniques, such as fluoroscopy, were not performed to precisely characterize the ductal morphology.

Routine clinical evaluation of puppies should be strongly encouraged, as it represents their first opportunity for assessment by trained professionals capable of detecting deviations from physiological norms. According to Pugliesi *et al.* (2021), presumptive diagnosis of PDA can often be made based on the auscultation of a strong, continuous "machinery" murmur, known as a Gibson murmur, especially in the left axillary region. Therefore, systematic clinical evaluation of puppies—ideally before initiating vaccination protocols (Vos & Szatmári, 2022)—is critical to detect and manage conditions that could adversely affect adult life. Furthermore, it is essential to inform prospective owners about the possibility of congenital defects when acquiring animals from certified breeders or adopting from shelters, including associated risks and available management options (Vos & Szatmári, 2022).

In the present case, suspicion of PDA could have arisen earlier through thorough veterinary examinations during routine auscultations, such as those performed at the time of primary vaccination. In such cases, early surgical correction might have been feasible following confirmation through Doppler echocardiography.

Conclusion

Patent ductus arteriosus (PDA) is a congenital condition characterized by persistent communication between the pulmonary trunk and the aorta. In the present case, the patient was diagnosed incidentally during an echocardiographic examination prompted by the onset of sudden fatigue. Due to the presence of flow reversal, surgical correction was deemed unfeasible, and routine monitoring was recommended instead. To date, the patient has not returned for follow-up clinical evaluations; however, at the time of the last assessment, the patient remained clinically stable.

An earlier diagnosis could have been achieved, for instance during the primary vaccination phase, had a thorough clinical evaluation been conducted. It is crucial that puppies undergo Doppler

echocardiographic assessment whenever abnormal sounds are detected during cardiopulmonary auscultation, to identify potential vascular communications. Early diagnosis may allow for timely and safe surgical intervention, ultimately promoting improved well-being and quality of life.

Conflict of interest

The authors declare no conflicts of interest.

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Use of artificial intelligence

No artificial intelligence tools were employed during the diagnostic or treatment processes, nor in the preparation of this manuscript.

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Influence of A1/A2 allelic variants of the CSN2 gene on milk composition and production in Holstein cows from Nariño, Colombia

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ABSTRACT

This study aimed to determine the allelic frequency and the effects of the A1 and A2 variants of the *CSN2* gene on milk production and quality in Holstein cows from five municipalities in Nariño, Colombia. Productive and compositional milk data were collected from 200 cows across 10 herds located in Pupiales, Pasto, Gualmatán, Guachucal, and Túquerres. The A1 and A2 variants of the *CSN2* gene were identified through allele-specific PCR using DNA extracted from blood samples. Associations between genotypes and milk production and composition traits were assessed using analysis of variance (ANOVA). The results showed allele frequencies of 0.46 for A1 and 0.54 for A2, and genotype frequencies of 0.23 (A1A1), 0.46 (A1A2), and 0.31 (A2A2). Observed heterozygosity (Ho) was 0.46, expected heterozygosity (He) was 0.47, and the polymorphic information content (PIC) was 0.37. Cows with the A1A1 genotype produced significantly more milk and total solids than A2A2 cows ($p < 0.05$). The A1 allele was associated with significantly higher yields of milk, fat, and protein ($p < 0.05$). A discriminant analysis revealed differentiation by municipality, suggesting that environmental factors influence variability in milk production and composition. It is concluded that the A1 allele is present at a moderately high frequency and is associated with enhanced productive traits in Holstein cows in the Department of Nariño. Therefore, the use of A2A2 genotype bulls with high genetic merit for productive traits is recommended, with the additional aim of promoting potential health benefits for milk consumers.

Keywords: β-casomorphin, β-casein, milk composition, molecular marker.

Influencia de las variantes alélicas A1/A2 del gen CSN2 sobre la composición y producción lechera en vacas Holstein en Nariño, Colombia

RESUMEN

El objetivo de este estudio fue determinar la frecuencia alélica y el efecto de las variantes A1/A2 del gen CSN2 sobre producción y calidad de la leche en vacas Holstein provenientes de

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cinco municipios de Nariño, Colombia. Se analizaron datos productivos y composicionales de la leche de 200 vacas de 10 hatos ubicados en Pupiales, Pasto, Gualmatán, Guachucal y Túquerres. Las variantes A1 y A2 del gen *CSN2* se identificaron mediante PCR alelo-específico a partir del ADN extraído de sangre. La asociación entre los genotipos y las características productivas y composicionales de la leche se evaluó mediante un análisis de varianza (ANOVA). Los resultados mostraron frecuencias alélicas de 0,46 para A1 y 0,54 para A2, y genotípicas de 0,23 (A1A1), 0.46 (A1A2) y 0,31 (A2A2), con valores de heterocigosidad observada (Ho) de 0,46, heterocigosidad esperada (He) de 0,47 y un contenido de información polimorfico (CIP) de 0,37. Las vacas con genotipo A1A1 produjeron significativamente más leche y sólidos totales que las vacas A2A2 ($p < 0,05$). El alelo A1 se asoció con mayores rendimientos ($p < 0,05$) de leche, grasa y proteína. Según un análisis discriminante, se observó diferenciación por municipio, sugiriendo que factores ambientales influyen en la variabilidad de la producción y composición de la leche. Se concluye que el alelo A1 está presente en una frecuencia moderadamente alta y además está asociado con aumento en las características productivas evaluadas en vacas Holstein del Departamento de Nariño, por lo que se sugiere usar toros con genotipo A2A2 de alto valor genético para características productivas, buscando, además, generar impactos positivos en la salud del consumidor de leche.

Palabras clave: β-casomorfina, β-caseína, composición láctea, marcador molecular.

INTRODUCTION

Colombia is among the leading milk producers in Latin America. Specifically, the department of Nariño contributes 6.03% of national milk production, with the Holstein breed being predominant in the region due to its high milk yield and favorable nutritional quality (Ministerio de Comercio, Industria y Turismo [MinCIT], 2021). Milk from cows contains approximately 45% β-casein among its protein content and provides essential nutrients such as carbohydrates, lipids, and micronutrients, including vitamins, calcium, and phosphorus (Padilla & Zambrano, 2021). The β-casein protein, encoded by the *CSN2* gene, exists in several allelic forms, of which A1 and A2 are the most common (Kamiński *et al.*, 2007; Padilla & Zambrano, 2021). These variants have different effects on milk composition, digestibility, and potential health impacts in humans (Laugesen and Elliott, 2003; Riaño and Narváez, 2015; Semwal *et al.*, 2022).

The β-casein variants differ in their amino acid sequences, particularly at position 67. In the A1 variant, the presence of histidine at this position allows for the release of β-casomorphin-7 (BCM-7) during digestion, whereas in the A2 variant, the presence of proline prevents this release (Küllenberg de Gaudry *et al.*, 2019; Padilla & Zambrano, 2021). BCM-7 is a bioactive peptide with opioid activity, released during the gastrointestinal hydrolysis of A1 β-casein in humans. Several studies have linked the consumption of A1 β-casein with health issues such as type 1 diabetes (Laugesen & Elliott, 2003), coronary heart disease (Küllenberg de Gaudry *et al.*, 2019),

ischemic heart disease (Laugesen & Elliott, 2003), atherosclerosis (Riaño & Narváez, 2015), and neurological disorders, including autism (Sokolov *et al.*, 2014) and schizophrenia (Küllenberg de Gaudry *et al.*, 2019). These associations are largely attributed to the ability of BCM-7 to cross the intestinal microvilli barrier, enter systemic circulation, and modulate the immune system (Kamiński *et al.*, 2007; Küllenberg de Gaudry *et al.*, 2019).

As a result, there has been increased consumer demand for A2 β-casein milk, especially in countries such as New Zealand, Australia, and the United Kingdom, where milk from cows producing only A2 β-casein is marketed as "A1-free milk," offering a more digestible and potentially safer alternative (Brooke-Taylor *et al.*, 2017). Nevertheless, in many countries, the A1 and A2 variants are not yet considered in genetic selection programs for cows and bulls, despite evidence linking these variants to productive traits such as increased yields of milk, fat, and protein (Winkelman & Wickham, 1996; Miluchová *et al.*, 2023; Olenski *et al.*, 2010).

Despite the relevance of β-casein variants, studies on the distribution and effects of A1/A2 in Colombia remain limited, particularly in the department of Nariño. Research on the *CSN2* gene in Colombian dairy herds is essential, as β-casein variants influence not only milk production and composition but also consumer health—especially among infants fed with formula milk. This study aimed to determine the frequency and effect of the A1/A2 allelic variants of the *CSN2* gene on milk production and quality in Holstein cows from five municipalities in the department of Nariño, Colombia.

MATERIALS AND METHODS

Population and sampling

A total of 200 adult Holstein cows were evaluated across 10 herds located in five municipalities within the dairy-producing zone of the Department of Nariño, Colombia. This region corresponds to a lower montane very humid forest zone (bmh-MB), situated at an altitude of 2,500–2,700 meters above sea level and characterized by an average temperature of 14 °C. A simple random sampling method was employed. In the municipality of Pasto, five herds were sampled; Guachucal had two herds; and Túquerres, Pupiales, and Gualmatán each had one herd. The average number of cows per herd was 20, ranging from 11 to 39.

Blood Sampling

Blood samples were collected using a chute to restrain the cows. Peripheral blood was drawn from the coccygeal vein, with 8 mL collected into Vacuette® tubes containing EDTA. Samples were kept refrigerated at 4 °C and transported to the Laboratory of the Research Group in Biochemistry and Genetic Studies (BIOGEN) at the University of Nariño.

DNA Extraction

For DNA extraction, 3 mL of blood were transferred into 15 mL Falcon® tubes and mixed with 6 mL of lysis buffer I, pH 7.6 (10 mM Tris-HCl, 320 mM sucrose, 5 mM $MgCl_2{\cdot}6H_2O$, and 1% Triton X-100). After vortexing and centrifugation at 4,000 rpm for 12 minutes, the supernatant was discarded, and the process was repeated using 8 mL of the buffer. The resulting pellet was resuspended in 5 mL of lysis buffer II, pH 8.2 (10 mM Tris-HCl, 400 mM

NaCl, and 2 mM Na_2EDTA), followed by the addition of 10 µL of proteinase K (CANVAX, Córdoba, Spain) at 2 mg/mL and 200 µL of 10% SDS (w/v). The mixture was incubated at 65 °C for 12 hours. Subsequently, 1.5 mL of saturated saline solution (6 M) was added, and the sample was centrifuged at 6,200 rpm for 10 minutes. DNA was precipitated using absolute ethanol at –20 °C, centrifuged at 4,000 rpm for 10 minutes, and the pellet was resuspended in 1 mL of TE buffer 1X (pH 8.0), then stored at –4 °C. DNA concentration and purity were assessed using a NanoDrop™ 2000 spectrophotometer, and integrity was verified by electrophoresis in 1% agarose gel (w/v).

Identification of CSN2 allelic variants via PCR-AS

The A1 and A2 alleles of the *CSN2* gene were identified using allele-specific PCR (PCR-AS), following the method of Ristanić *et al.* (2022) with slight modifications. Two separate PCR-AS reactions were performed using the following primers: IGBhF 5'-CTTCCCTGGGCCCATCCA-3' for the A1 allele, 5'-CTTCCCTGGGCCCATCCC-3' for the A2 allele, and the reverse primer IGBR 5'-AGACTGGAGCAGAGGCAAG-3'. Each PCR-AS reaction was carried out in a final volume of 20 µL, containing 75 ng of genomic DNA, 2 µL of 10X PCR buffer, 1 U of Green-Taq DNA polymerase (BrigthMAX™), 0.8 µL of 3 mM $MgCl_2$, 0.64 µL of 0.8 mM dNTPs, 0.75 µL of each primer at 5 pM, and ultrapure water. The thermal cycling conditions were as follows: initial denaturation at 94 °C for 5 min; 5 cycles of 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s; followed by 30 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s; with a final extension at 72 °C for 5 min. PCR-AS products were resolved on 1.5% agarose gels (w/v, BrigthMAX™) at 110 V for 60 minutes and visualized using an ENDURO™ GDS UV transilluminator, with a molecular weight marker ranging from 25 to 700 bp (BrigthMAX™).

Determination of allelic and genotypic frequencies

Allelic frequencies were calculated by summing the number of homozygotes and half the number of heterozygotes. Genotypic frequencies were determined by dividing the number of individuals with a given genotype by the total number of individuals evaluated. Genetic diversity was estimated using observed heterozygosity (Ho), expected heterozygosity (He), and their respective standard errors (SE). The polymorphic information content (PIC) was also calculated using GenAlEx version 6.5 (Peakall and Smouse, 2012).

Determination of productive traits

Milk samples were collected from the selected cows following teat disinfection with 70% alcohol and discarding of the first milk streams. A total of 50 mL of milk was collected from all four quarters into labeled plastic containers with barcodes. Samples were stored in cooler boxes with ice packs at 4 °C and transported to the AGROSAVIA milk quality laboratory. The percentages of fat (FAT), protein (PRO), and total solids (TS) were analyzed using infrared spectroscopy (AOAC Official Method 972.16-2005, 21st Ed. 2019). Daily milk yield (MY) per cow and 305-day adjusted milk yield (MY_305) were recorded, along with herd, municipality, age, and parity data provided by the Nariño Society of Farmers and Ranchers (SAGAN). Based on MY_305, PRO, FAT, and TS values, the 305-day adjusted production

of fat (FAT_305), protein (PRO_305), and total solids (TS_305) was calculated.

Association analysis

Normality and homogeneity of variance were tested using the Anderson–Darling and Levene tests, respectively. ANOVA was performed to evaluate associations between productive traits (MY, FAT, PRO, TS, MY_305, FAT_305, PRO_305, and TS_305) and fixed effects: parity (NPAR), herd (HAT), municipality (MUN), age, and genotype (GENT). Age was grouped into four categories: E3 (2–3 years), E4 (4 years), E5 (5 years), and E6 (6–7 years). Herds were grouped into four categories: NPAR1, NPAR2, NPAR3, and NPAR4 or more. The fixed effects also included the A1 and A2 alleles and the genotypes A1A1, A1A2, and A2A2. The linear model used was:

Where *Y* represents the dependent variables (FAT, PRO, TS, MY, MY_305, FAT_305, PRO_305, and TS_305), and the independent variables are: NPAR = parity, with four levels (i = 1…4); AGE, with four levels (j = 1…4); HAT = herd, with 10 levels (k = 1…10); MUN = municipality, with five levels (l = 1…5); GENT = genotype, with three levels (m = 1…3); and ALLELE, with two levels (n = 1, 2). For fixed effects that showed significant differences in ANOVA, Tukey's multiple comparison test was applied ($\alpha \leq 0.05$). Statistical analyses were performed using R software v4.3.0 (R Core Team, 2024).

Genetic structure analysis

Hardy–Weinberg equilibrium was assessed based on allelic and genotypic frequencies using GenAlEx 6.5 (Peakall and Smouse, 2012). A chi-square (χ^2) goodness-of-fit test was also performed to confirm Hardy–Weinberg equilibrium. The entire Nariño region was considered the total population, while municipalities were treated as subpopulations. The inbreeding coefficient (Fis) and the effective number of alleles (Ne) were calculated using standard equations. Analysis of molecular variance (AMOVA) was conducted to evaluate genetic variation within and between groups, with significance set at $\alpha \leq 0.05$. The polymorphic information content (PIC) was also estimated.

Discriminant analysis

Principal component analysis (PCA) was applied to the genetic data to address collinearity and high dimensionality in allelic frequencies. The most informative components were selected for discriminant analysis. The number of groups was determined using the Bayesian Information Criterion (BIC), which accounts for model complexity by applying a penalty. The contributions of individual alleles to the discriminant functions were assessed, along with the influence of each municipality. This approach enabled the evaluation of the independent effects of genetic and geographic factors on milk composition and yield traits.

Ethical considerations

This study was approved by the Research Ethics Committee of the University of Nariño under Approval Act No. 045, dated October 30, 2020.

RESULTS

The results of the allele-specific PCR (PCR-AS) for each genotype (figure 1) revealed allele frequencies of 0.46 for A1 and 0.54 for A2, with genotype frequencies of 0.23 for A1A1, 0.46 for A1A2, and 0.30 for A2A2. These findings offer insights into

the genetic distribution of the A1 and A2 variants in the Holstein cattle population sampled across the five municipalities of the Nariño department (table 1).

The Hardy–Weinberg equilibrium test, based on the chi-square statistic, revealed no significant deviation (χ^2 = 0.84; p = 0.36), indicating that the population is in equilibrium at the *CSN2 locus*. The effective number of alleles (Ne) was slightly lower, at 1.91 (SE = 0.068), compared to the total number of alleles (Na = 2) (table 1). Expected heterozygosity was 0.47 (SE = 0.021), while observed heterozygosity was slightly lower at 0.46 (SE = 0.066) (table 1), suggesting a minor deviation from expected genetic diversity. The inbreeding coefficient (Fis) was estimated at 0.03, indicating a low level of inbreeding within the population. Genetic differentiation among populations (Fst) was 0.05, and the polymorphic information content (PIC)

TABLE 1. Allelic and genotypic frequencies of A1/A2 variants and genetic structure in Holstein cows from the Nariño department

N	Alleles		Genotypes			Genetic structure						
	A1	A2	A1A1	A1A2	A2A2	Na	Ne	He	Ho	Fis	Fst	PIC
200	0.46	0.54	0.23	0.46	0.31	2.0	1.91	0.47	0.46	0.03	0.05	0.37

N: Sample size of Holstein cattle, Na: Number of different alleles, Ne: Effective number of alleles, Ho: Observed heterozygosity, He: Expected heterozygosity, Fis: Inbreeding coefficient within individuals, Fst: Fixation index, PIC: Polymorphic information content.

Source: own elaboration.

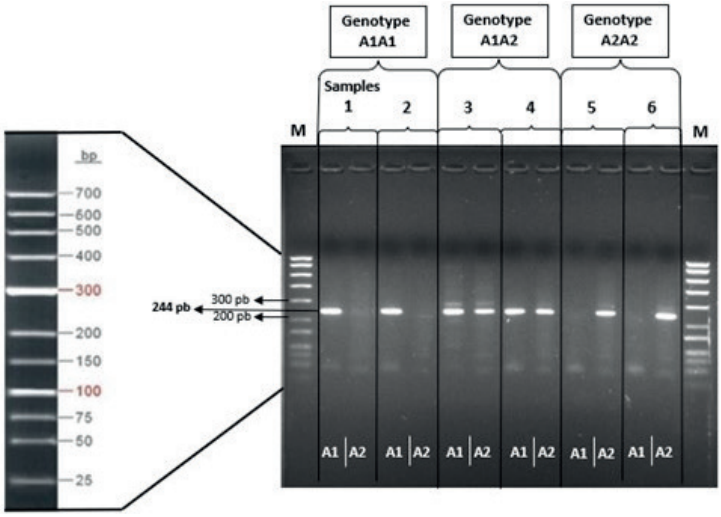

FIGURE 1. PCR amplification product obtained using the forward primer IGBhF (A1 allele), forward primer IGBpF (A2 allele), and reverse primer IGBR, from 200 Holstein cows.

Source: own elaboration.

was 0.37, both consistent with moderate genetic diversity at this locus in the studied population. Overall, these findings suggest that genetic diversity at the *CSN2* locus in Nariño is moderate.

The physicochemical analysis of milk revealed that the Holstein cows sampled across the five municipalities of the department of Nariño exhibited average values for productive and compositional traits that align with regional conditions, particularly given that these are not closed herds with animals of high genetic merit. On average, cows produced 13 ± 5.2 liters of milk per day, with a fat content of 3.4 ± 0.57%, protein content of 3.2 ± 0.27%, and total solids content of 11.3 ± 1.5%. Milk production adjusted to 305 days of lactation averaged 3,954 ± 1,594 liters, with protein yield at 134 ± 51.6 kg/lactation, fat yield at 133 ± 75 kg/lactation, and total solids yield at 454 ± 205 kg/lactation.

Additionally, ANOVA results showed a significant effect ($p < 0.05$) of both allele and genotype on milk composition and production traits. Cows with the A1A1 genotype produced 13.3% more milk per day, had a 13.5% higher 305-day milk yield, and 4.4% higher total solids content compared to cows with the A2A2 genotype ($p < 0.05$) (table 2). In the allelic comparison, cows carrying the A1 allele exhibited a 6.3% greater daily milk yield and a 6.6% greater 305-day milk yield than those carrying the A2 allele. Furthermore, A1 cows produced 8.5% more protein and 8.6% more total solids over 305 days than A2 cows, which showed lower yields across all production traits (table 2). These findings suggest that the A1 allele is associated with higher milk yield and improved compositional quality, particularly in fat and protein content.

The PCA plot of the overall genetic profile did not reveal clear separation

TABLE 2. Mean comparison test for milk composition and production traits in Holstein cattle.

		Composition			Production				
Genotypes	**N**	**FAT (%)**	**PRO (%)**	**TS (%)**	**MY (L/day)**	**MY_305 (L/lac)**	**FAT_305 (kg/lac)**	**PRO_305 (kg/lac)**	**TS_305 (kg/lac)**
A1A1	46	3.38	3.24	11.8^{a}	14.5^{a}	4423	148.2	153.1^{a}	523.6^{a}
A1A2	93	3.44	3.17	11.1^{b}	12.3^{b}	3762	127.2	127.3^{b}	424.6^{b}
A2A2	61	3.25	3.14	11.3^{ab}	12.8^{ab}	3895	129.4	130.4^{b}	445.2^{b}
Alleles	**N**	**FAT (%)**	**PRO (%)**	**TS (%)**	**MY (L/día)**	**MY_305 (L/lac)**	**FAT_305 (kg/lac)**	**PRO_305 (kg/lac)**	**TS_305 (kg/lac)**
A1	185	3.40	3.20	11.4	13.4^{a}	4090^{a}	138.7	140.1^{a}	473.8^{a}
A2	215	3.31	3.15	11.2	12.6^{b}	3837^{b}	128.5	129.1^{b}	436.3^{b}

N: Sample size. FAT (%): percentage of fat, PRO (%): percentage of protein, TS (%): percentage of total solids, MY: milk production per day, MY_305: Milk production per lactation of 305 days, FAT_305: Fat production per lactation of 305 days, PRO_305: Protein production of lactation of 305 days, TS_305: Production of total solids per lactation of 305 days. Different letters indicate significant differences between the means (p<0.05) according to the test of Tukey.

Source: own elaboration.

between groups based on genotype (figure 2A). Although considerable overlap was observed among genotypes, some distinction was noted for the A1A1 genotype, which exhibited a broader distribution along Dimension 1. Discriminant analysis based on genotype (figure 3) showed an improved ability to distinguish A1A1 (Area Under the Curve, AUC = 0.65) and A1A2 (AUC = 0.60) genotypes.

The A2A2 genotype was indistinguishable (AUC = 0.51) (figure 3). These results suggest that, while genetic constitution influences certain traits related to milk production and quality, its impact is particularly pronounced in cows with the A1A1 genotype.

On the other hand, substantial overlap was also observed among municipalities, although some differentiation was noted for Guachucal and Pupiales, which exhibited

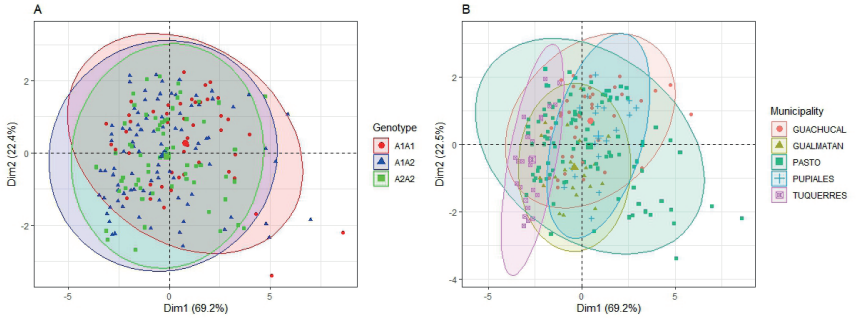

FIGURE 2. PCA score plots illustrate variation among genotypes and municipalities.

(A) Plot of the first two principal components (Dim1 and Dim2) showing the distribution of genotypes: A1A1 (red circles), A1A2 (blue triangles), and A2A2 (green squares). (B) Plot of municipalities: Guachucal (red circles), Gualmatán (yellow triangles), Pasto (green squares), Pupiales (purple diamonds), and Túquerres (pink squares). Ellipses represent 95% confidence intervals for each group. The first two dimensions explain 91.7% of the total variance, respectively.

Source: own elaboration.

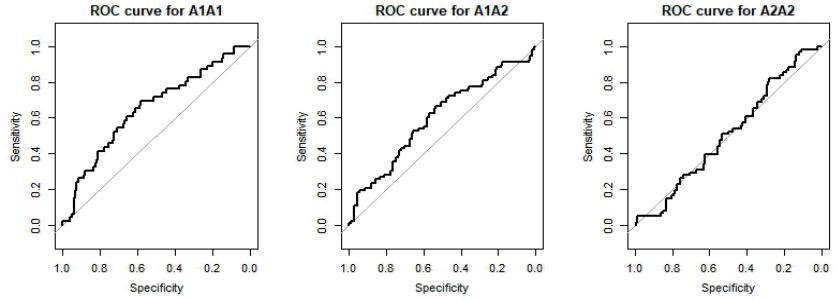

FIGURE 3. Receiver Operating Characteristic (ROC) curves for three different classes: A1A1, A1A2, and A2A2.

Each plot compares sensitivity (true positive rate) on the Y-axis against specificity (false positive rate) on the X-axis. The area under the curve (AUC) values were 0.65 for A1A1, 0.60 for A1A2, and 0.51 for A2A2.

Source: own elaboration.

broader distributions along Dimension 1 (figure 2B). Discriminant analysis based on municipality revealed a greater ability to distinguish Túquerres (AUC = 0.96) and Pupiales (AUC = 0.71), while it was less effective for Guachucal (AUC = 0.60), and indistinguishable for Gualmatán (AUC = 0.53) and Pasto (AUC = 0.44) (figure 4).

The most influential variables in the discriminant analysis were those with the highest contributions to Component 1, including protein yield (–0.65) and total solids (–0.49) at 305 days (table 3). For Component 2, the primary contributors were protein content (–0.97) and total solids (–0.26) (table 3). These results suggest that, in addition to genetic constitution, environmental or management-related factors may significantly contribute to the observed variability in milk composition and production across the different municipalities.

TABLE 3. Variables and their contributions to components 1 and 2 of the discriminant analysis.

Component 1		Component 2	
Variable	**Value**	**Variable**	**Value**
PRO_305	-0,65	PRO	-0,97
TS_305	-0,49	TS	-0,26
MY	-0,41		
MY_305	-0,41		
TS	-0,03		

Source: own elaboration.

DISCUSSION

The results indicate a balanced distribution of A1 and A2 alleles, with a slightly higher prevalence of the A2 allele. This balanced proportion suggests that there has been no strong historical directional selection favoring either allele of the *CSN2* gene in the study region (Padilla & Zambrano, 2021; Scott *et al.*, 2023).

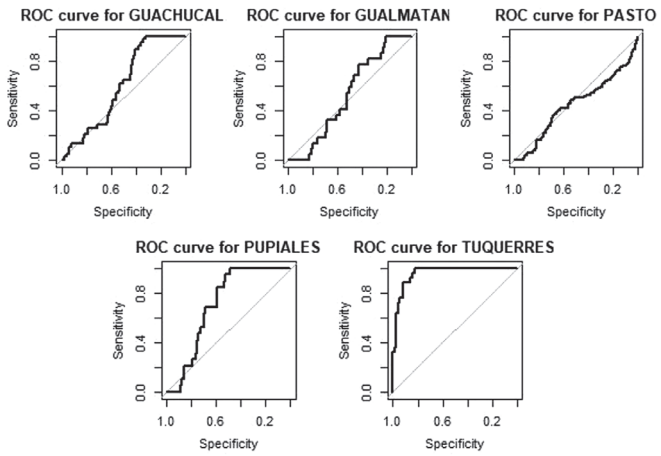

FIGURE 4. Receiver Operating Characteristic (ROC) curves for five municipalities: Guachucal, Gualmatán, Pasto, Pupiales, and Túquerres.

Each plot compares sensitivity (true positive rate) on the Y-axis with specificity (false positive rate) on the X-axis. The area under the curve (AUC) values were 0.60 for Guachucal, 0.53 for Gualmatán, 0.44 for Pasto, 0.71 for Pupiales, and 0.96 for Túquerres.

Source: own elaboration.

In several countries, a moderate predominance of the A2 allele has been reported in Holstein cattle. For example, studies in Latin American Holstein populations have shown allele frequencies similar to those observed in the present study. In the Peruvian Andes, Chaves (2023) reported an A2 allele frequency of approximately 0.57, compared to 0.43 for A1, closely aligning with the proportions found in Nariño. Similarly, research conducted in Mexico and other Latin American countries has reported a higher frequency of the A2 allele (around 0.60) compared to A1 (Manzano, 2017), further supporting our findings.

Globally, many dairy herds have increased the frequency of the A2 allele due to its potential advantages. A widespread preference for A2 β-casein has been documented in countries such as New Zealand (Winkelman & Wickham, 1996), various European nations (Barłowska *et al.*, 2022; Ladyka *et al.*, 2023), and several Asian countries, including China (Dai *et al.*, 2016) and India (Jawane *et al.*, 2018). In these regions, the frequency of the A2 allele ranges from 0.51 to 0.97, reflecting a general preference for A2 β-casein. For instance, studies in New Zealand reported an A2 frequency of 0.51 (Winkelman & Wickham, 1996), while in India, some populations showed frequencies as high as 0.97 (Jawane *et al.*, 2018), largely attributable to recent selection programs aimed at producing A1-free milk due to its perceived health benefits (Semwal *et al.*, 2022).

These values contrast with specific cases where the A1 allele is more prevalent. For example, in Pakistan, an A1 allele frequency of 0.67 has been reported—significantly higher than that of the A2 allele (Ayaz *et al.*, 2023). Such exceptions are often associated with the specific objectives of the dairy industry. In cheese production, for instance, the A1 β-casein variant may offer certain technological advantages, leading to a preference for A1-producing animals within those systems (Vigolo *et al.*, 2023). This underscores the complex relationship between genetic selection goals and the diverse demands of dairy production.

In Colombia, the findings of this study align with the international trend of coexistence between both β-casein variants. However, national studies on the distribution of A1/A2 alleles remain limited, particularly in Andean dairy regions such as Nariño. The concurrent presence of both alleles at similar frequencies suggests that local herds have been managed through mixed reproductive practices—such as the use of bulls or semen from diverse sources—without intentional selection for either β-casein variant.

From a public health perspective, the continued presence of a considerable frequency of the A1 allele implies that cows are still producing milk containing A1 β-casein—an important consideration given its potential health risks, particularly for newborns. Several studies have associated A1 β-casein consumption with an increased risk of chronic conditions, including type 1 diabetes (r = 0.92) (Laugesen & Elliott, 2003), coronary heart disease (Küllenberg de Gaudry *et al.*, 2019), ischemic heart disease (r = 0.86) (Laugesen & Elliott, 2003), atherosclerosis (Riaño & Narváez, 2015), sudden infant death syndrome (Wasilewska *et al.*, 2011), and neurological disorders such as autism (r = 0.85) (Sokolov *et al.*, 2014) and schizophrenia (Küllenberg de Gaudry *et al.*, 2019). Nonetheless, further research is needed to provide conclusive evidence supporting the hypothesis that consumption of A1-type milk increases the risk of these diseases

As interest in A2 milk continues to grow—driven by its potential health benefits, such as reduced gastrointestinal discomfort and a lower risk of chronic diseases—genetic improvement programs have increasingly prioritized the selection of the A2 allele (Dantas *et al.*, 2023; Żbik *et al.*, 2024). For dairy producers in Nariño, understanding the genetic composition of their herds and the advantages of A2-type milk may create new market opportunities, particularly in response to rising global demand and its positive implications for human health.

The genetic analysis of the Holstein cow population in Nariño enabled the determination of the effective number of alleles at the *CSN2* locus, revealing an allelic structure consistent with findings from other studies (Duifhuis *et al.*, 2014; Miluchová *et al.*, 2014; Ardicli *et al.*, 2024). However, the effective number of alleles in this population was slightly lower than that reported in countries such as Pakistan, suggesting a potential reduction in genetic diversity, possibly related to the indirect selection of phenotypic traits associated with production (Ayaz *et al.*, 2023). The observed and expected heterozygosity values indicate substantial genetic diversity within the population. The slight difference between these values, along with the polymorphism information content, suggests a minor deviation from Hardy–Weinberg equilibrium. This deviation may be attributed to evolutionary forces such as incipient inbreeding, natural selection acting against heterozygotes, or the influence of genetic drift in populations with a reduced effective size. Notably, the low inbreeding coefficient supports the hypothesis that inbreeding, while present, has not yet reached concerning levels. This finding is consistent with populations managed under semi-intensive systems or using assisted reproductive technologies, as reported in countries like Slovakia (Miluchová *et al.*, 2014) and Turkey (Ardicli *et al.*, 2024). Moreover, the effects of genetic drift may be linked to the limited effective population size, which can lead to random fluctuations in allele frequencies.

Analogous situations have been reported in Holstein herds managed under semi-intensive systems in Latin America and other regions, where the periodic introduction of external genetics and sire rotation help maintain genetic variability and prevent high levels of inbreeding (Manzano, 2017). For example, a study on Mexican cattle observed a similar genetic equilibrium at casein loci, attributing this stability to the use of imported semen from diverse sources (Duifhuis-Rivera *et al.*, 2014).

The impact of *CSN2* gene allelic variants on milk yield and quality remains a subject of ongoing debate within the scientific community. Some studies report that the A2 variant is associated with higher milk and protein yields, whereas the A1 variant is linked to increased fat percentages (Winkelman & Wickham, 1996; Miluchová *et al.*, 2023). Other research suggests that heterozygous A1A2 cows may outperform homozygous individuals in terms of milk and protein production, while some authors have found no significant association between these genetic polymorphisms and milk production traits (Manga & Dvorak, 2010). Although the present study identified associations between *CSN2* alleles and certain productive traits, no human intervention has been applied to select cows based on A1A1, A1A2, or A2A2 genotypes. Nevertheless, public health studies suggest a potential health risk associated with milk from both A1A1 and A2A2 cows (Küllenberg de Gaudry

et al., 2019; Borş *et al.*, 2024), underscoring the need for further research and cautious consideration in breeding strategies.

Efforts to promote the production of A2 milk—free of A1 β-casein—may lead to the implementation of rigorous breeding programs, similar to those established in New Zealand, which include strict genetic selection, separate grazing, and controlled feeding of A2A2 cattle. This study underscores the importance of using semen from bulls with high genetic merit for productive traits and confirmed A2A2 genotypes, with the goal of increasing the frequency of the A2 allele and delivering potential health benefits to consumers. Companies such as Genética Selecta, GENEX, SEMEX, and ABS Global offer semen from elite A2A2 bulls, supporting its adoption in Colombian dairy regions, including the Department of Nariño. As the Colombian dairy industry continues to advance alongside developments in animal genetics, the incorporation of A2A2 genotype animals into dairy herds is a strategic step toward promoting consumer health—particularly for newborns who consume infant formula.

With respect to the discriminant analysis, the results revealed a complex interaction between genetic and environmental factors influencing milk production and composition in Holstein cows from the municipalities of Nariño. Although some genetic distinctions were evident—particularly for the A1A1 genotype—the observed overlap among genotypes and municipalities suggests that genetic constitution alone does not fully account for the variability in productive traits. These findings align with previous studies indicating that milk yield and composition are polygenic traits influenced not only by genetic factors (Padilla & Zambrano, 2021) but also by environmental variables such as diet (AlSuwaiegh *et al.*, 2022), climate, and management practices (Gareli *et al.*, 2023).

The limited capacity of the analysis to clearly distinguish the A2A2 genotype and certain municipalities—despite local differences such as those observed in Túquerres—suggests that regional factors, including feeding and herd management practices, play a crucial role in trait expression and variation in milk yield (Padilla & Zambrano, 2021; AlSuwaiegh *et al.*, 2022). Moreover, the significant contribution of protein yield and total solids as discriminant variables highlights their importance in production environments, as these traits are highly responsive to nutritional inputs and management intensity (Gareli *et al.*, 2023). Their relevance also reinforces their utility in the genetic selection of cows and bulls. Overall, the results support the notion that optimizing milk production and quality requires a comprehensive approach that integrates both *CSN2* genotype and environmental influences.

It is important to note that in the present study, 200 individuals were randomly sampled from the municipalities with the highest milk production in the southern region of the Department of Nariño. However, environmental factors—such as management techniques, milking practices, and nutrition—were not evaluated. For future research, it is recommended to expand the sample size by including cattle from additional milk-producing municipalities. Additionally, complementing genotype determination with more advanced techniques, such as sequencing, and evaluating other alleles potentially associated with production traits would provide a more comprehensive understanding of the genetic influences on milk yield and quality.

CONCLUSIONS

The A1 genetic variant was associated with higher milk yield, protein content, and total solids compared to the A2 variant. Homozygous A1A1 animals exhibited the highest values across the productive parameters evaluated, while heterozygous A1A2 individuals showed only a weak association with these traits.

Genetic analysis of the *CSN2* locus in the Holstein cow sample revealed a moderate genetic structure, accompanied by indications of a slight decline in genetic diversity. While the observed heterozygosity reflects considerable genetic variation, a mild deviation from Hardy–Weinberg equilibrium was detected.

Based on the findings, it is recommended to increase the frequency of the A2 allele in the dairy region of the Department of Nariño through targeted improvement strategies, such as artificial insemination with elite A2A2 sires that possess high breeding values for productive traits. This approach aims to promote the production of milk with potential health benefits and a lower likelihood of causing dietary intolerance in consumers.

CONFLICTS OF INTEREST

No conflicts of interest were reported by the authors.

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DECLARATION ON THE USE OF ARTIFICIAL INTELLIGENCE

The authors declare that no artificial intelligence tools were used during the development of the research or in the preparation of the manuscript resulting from this study.

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Assessment of the anti-inflammatory, antioxidant, and wound-healing effects of methanolic soybean seed extract in an excision wound model in albino rats

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ABSTRACT

Wound healing is a complex physiological process influenced by oxidative stress and inflammation. This study assessed the antioxidant, anti-inflammatory, and wound-healing effects of a methanolic extract of soybean seeds using a full-thickness excision wound model in male albino rats. Fourteen rats were randomly divided into two groups (n = 7). Under anesthesia, full-thickness skin wounds were aseptically created in the thoraco-abdominal region. Group A received sterile water (placebo) topically, while Group B received the soybean seed extract daily for 21 days. Wound healing was evaluated by macroscopic examination, measurement of wound contraction, and analysis of inflammatory and oxidative stress markers.

By day 7, wound contraction was significantly higher in the control group (60.42 ± 6.65%) compared to the extract-treated group (43.96 ± 11.58%) ($p < 0.05$). No significant difference was observed in the neutrophil-to-lymphocyte ratio between the two groups. However, biochemical analyses showed elevated levels of serum malondialdehyde (MDA) and superoxide dismutase (SOD) activity in the treated group (MDA: 4.26 ± 0.39 µmol/mg; SOD: 1.43 ± 0.16 mg/mL) versus the control (MDA: 3.18 ± 0.51 µmol/mg; SOD: 1.01 ± 0.13 mg/mL) ($p < 0.05$).

In conclusion, topical application of soybean seed methanolic extract did not enhance wound healing but improved antioxidant markers, indicating its potential role in mitigating oxidative stress rather than directly accelerating tissue repair.

Keywords: antioxidant, oxidative stress, management, ethnopharmacology, ethnoveterinary medicine.

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Evaluación de los efectos antiinflamatorios, antioxidantes y cicatrizantes del extracto metanólico de semillas de soja en un modelo de herida por escisión en rata albina

RESUMEN

La cicatrización de heridas es un proceso fisiológico complejo influenciado por el estrés oxidativo y la inflamación. Este estudio evaluó los efectos antioxidantes, antiinflamatorios y cicatrizantes de un extracto metanólico de semillas de soya utilizando un modelo de herida por escisión de espesor completo en ratas albinas machos. Catorce ratas fueron divididas aleatoriamente en dos grupos (n = 7). Bajo anestesia, se indujeron heridas cutáneas de espesor completo de forma aséptica en la región toracoabdominal. El Grupo A recibió agua estéril tópica, mientras que el Grupo B fue tratado diariamente con el extracto de semillas de soya durante 21 días. La cicatrización fue evaluada mediante examen macroscópico, medición de la contracción de la herida y análisis de marcadores inflamatorios y de estrés oxidativo.

Para el día 7, la contracción de la herida fue significativamente mayor en el grupo control (60.42 ± 6.65%) en comparación con el grupo tratado con el extracto (43.96 ± 11.58%) ($p < 0.05$). No se observaron diferencias significativas en la relación neutrófilo-linfocito entre los grupos. Sin embargo, los análisis bioquímicos mostraron niveles elevados de malondialdehído (MDA) en suero y actividad de superóxido dismutasa (SOD) en el grupo tratado (MDA: 4.26 ± 0.39 µmol/mg; SOD: 1.43 ± 0.16 mg/mL) frente al control (MDA: 3.18 ± 0.51 µmol/mg; SOD: 1.01 ± 0.13 mg/mL) ($p < 0.05$).

En conclusión, la aplicación tópica del extracto metanólico de semillas de soya no mejoró la cicatrización de heridas, pero sí incrementó los marcadores antioxidantes, lo que sugiere un posible papel terapéutico en la mitigación del estrés oxidativo más que en la aceleración directa de la reparación tisular.

Palabras clave: antioxidante, estrés oxidativo, gestión, etnofarmacología, medicina etnoveterinaria.

INTRODUCTION

A cutaneous wound is defined as damage to the epithelial integrity of the skin's outer layer, resulting in the disruption of both the structure and function of the underlying tissue (Verma *et al.*, 2019). Wounds may result from various causes, including physical trauma, external factors (e.g., pressure, burns, and lacerations), or pathological conditions such as diabetes and vascular diseases (Tottoli *et al.*, 2020; Atala *et al.*, 2021). Wound healing (WH) is a complex, multi-phase biological process consisting of hemostasis, inflammation, proliferation, and tissue remodeling (Kilani *et al.*, 2025; Gushiken *et al.*, 2021; Rodrigues *et al.*, 2019). These stages involve inflammatory cells such as neutrophils, lymphocytes, and macrophages, which play key roles in the early phase by removing debris and pathogens to facilitate tissue regeneration (Berman *et al.*, 2017). The proliferative phase of WH includes angiogenesis, fibroblast proliferation, extracellular matrix deposition, and granulation tissue formation, while the remodeling phase is essential for restoring tissue strength (Plikus *et al.*, 2017; Liu *et al.*, 2020).

One of the primary barriers to effective wound healing is the persistence of oxidative stress and prolonged inflammation (Gushiken *et al.*, 2021). While reactive oxygen species (ROS) are vital for host defense and cell signaling at physiological levels, excessive ROS production induces oxidative damage, chronic inflammation, delayed tissue regeneration, and an increased risk of wound chronicity (Dunnill *et al.*, 2015; Sanchez *et al.*, 2018). Despite advances in wound care, managing chronic and complex wounds remains a challenge, largely due to persistent inflammation, oxidative stress, and microbial colonization (Kilani *et al.*, 2025; Gushiken *et al.*, 2021; Atala *et al.*, 2021). These challenges have spurred growing interest in natural antioxidants and bioactive compounds capable of modulating oxidative damage and promoting tissue repair (Mohsin *et al.*, 2022; Rahman *et al.*, 2023). Natural products are increasingly recognized as valuable sources of alternative medicine and bioactive compounds for the treatment of numerous conditions. Medicinal plants and traditional remedies used in wound care are often accessible, affordable, and, in some cases, freely available—particularly in regions with limited access to conventional medical treatments (Agyare *et al.*, 2009).

Soybean (*Glycine max*) seeds are rich in bioactive compounds, including isoflavones (genistein, daidzein), phenolic acids, flavonoids, saponins, and phytosterols (Lee *et al.*, 2008; Messina *et al.*, 2010). Recent studies have demonstrated that these compounds possess potent antioxidant and antimicrobial properties relevant to wound healing (Shen *et al.*, 2020; Kim *et al.*, 2021). Isoflavones, in particular, have been shown to scavenge free radicals, modulate cytokine expression, and enhance fibroblast activity, all of which are critical for tissue repair (Kim *et al.*, 2021; Shen *et al.*, 2020). Furthermore, soybean-derived saponins and phenolic compounds have been reported to stimulate angiogenesis and collagen synthesis, supporting their therapeutic potential in wound management (Zhang *et al.*, 2019).

However, there remains a lack of experimental data on the direct topical wound-healing effects of soybean seed methanolic extract, particularly regarding its impact on oxidative and inflammatory pathways during cutaneous repair. Therefore, this study aimed to evaluate the wound-healing efficacy of soybean seed methanolic extract, as well as its antioxidant and anti-inflammatory effects, using an experimental excision wound model in rats.

MATERIALS AND METHODS

Experimental animals

A preliminary power analysis was conducted to minimize the number of animals used, in alignment with the 3R principles (Replacement, Reduction, Refinement). The sample size was calculated using G*Power (version 3.1.9.7) for a one-way ANOVA (fixed effects, omnibus test), assuming a large effect size ($f = 0.8$), a significance level (α) of 0.05, and a statistical power ($1-\beta$) of 0.80. This analysis yielded a total sample size of fourteen (14) male albino rats. The animals, each weighing approximately 120 g and deemed apparently healthy, were sourced from a laboratory animal facility in Ibadan, Oyo State.

The rats were individually housed in metal cages at the Laboratory Animal Unit of the Veterinary Teaching Hospital, College of Veterinary Medicine (COLVET), Federal University of Agriculture, Abeokuta, Ogun State, where the study was conducted. The animals were allowed

a two-week acclimatization period to adapt to the laboratory environment. During this period and throughout the experiment, they were fed *ad libitum* with Growers Mash and had unrestricted access to clean drinking water provided hygienically.

All animals received appropriate care and humane handling in accordance with ethical standards and prior studies involving plant-based wound treatments in similar models (Zhang *et al.*, 2020; Kim *et al.*, 2021). This randomized experimental protocol was approved by the Research Ethics Committee of the College of Veterinary Medicine (COLVET), Federal University of Agriculture, Abeokuta, under approval number FUNAAB/COLVET/CREC/2024/05/02.

Crude extraction of soybean seed and phytochemical analysis

Soybean seeds were purchased from Sabo, Eleweran Market, Ogun State, and taxonomically identified as *Glycine max* (L.) Merr. (Fabaceae) at the Department of Botany, College of Biological Sciences, Federal University of Agriculture, Abeokuta. A voucher specimen was deposited under the number FHA-4337.

The seeds were thoroughly dried, ground into a fine powder, and approximately 650 g of the powder was soaked in 1.2 L of 90% methanol for 72 hours with intermittent stirring to enhance extraction. The resulting mixture was filtered using muslin cloth, and the filtrate was concentrated with a rotary evaporator at 45°C. The final crude extract, weighing approximately 21 g, was stored in an airtight glass container at 4°C until further use.

As shown in table 1, qualitative phytochemical screening was performed using standard tests to detect the presence of various classes of bioactive compounds, including phenolic compounds, flavonoids, saponins, alkaloids, steroids, and carbohydrates. Quantitative phytochemical analysis was also conducted to determine the concentrations of these compounds using established colorimetric and gravimetric methods, as previously described by Hashim *et al.* (2021).

TABLE 1. Phytochemical composition of the methanolic extract of soybean seeds used in the wound healing study

Phytochemicals	Test(s) performed	Soybean methanolic extract	% Yield (%w/w)
Saponins	Frothing test	++	0.80±0.00
Tannins	Ferric chloride test	+	2.23±0.01
Flavonoids	Ammonia/H2SO4 test	++	3.39±0.10
Cardiac glycosides	Keller-Killiani test	+	NA
Anthraquinones	Borntrager's test	+	NA
Terpenoids	Salkowski test	+	0.65±0.00
Steroids	Liebermann-Burchard test	++	NA
Alkaloids	Dragendorff's test	++	8.5±0.00
Phenol	Keller-Killiani test	++	2.07±0.00

++ = Abundant, + = Present, NA= Not Available.

Source: own elaboration.

Experimental wound creation

Prior to wound induction, all animals were fasted for 4 hours. Anesthesia was administered intramuscularly using 80 mg/kg of 5% ketamine hydrochloride (Rotexmedica, Trittau, Germany) and 10 mg/kg of 2% xylazine hydrochloride (Xylased, Bioveta, Ivanovice, Czech Republic). The dorsal thoraco-abdominal region was then aseptically prepared, and a full-thickness excisional skin wound measuring 2 × 2 cm was created on each rat. Following wound creation, the animals were returned to their individual cages lined with paper bedding.

Experimental design and wound management

Fourteen (14) clinically healthy male albino rats were randomly assigned to two groups (n = 7 per group), designated as Group A and Group B. Rats in Group A received a daily topical application of sterile water and served as the control group. Rats in Group B were treated topically with 100% (w/v) methanolic extract of soybean seeds.

WOUND HEALING EVALUATION

Gross wound assessment

Wounds were evaluated macroscopically throughout the study period for granulation tissue development, progression of epithelialization, exudate production, scab formation, wound bed appearance, presence of pus or necrotic tissue, as well as changes in color and odor.

Assessment of wound contraction

Wound contraction, expressed as the percentage of wound closure, was assessed using the wound tracing technique on days 0, 3, 7, 11, and 15. A transparent tracing sheet was gently placed over each wound to delineate the margins, which were then outlined using a fine-tip permanent marker. The traced area was measured using thread and a ruler. The percentage of wound contraction was calculated using Wilson's formula, considering the wound area on day 0 as the baseline (100%) (Chen *et al.*, 2015):

$$\% \text{ wound contraction} = \frac{Initial\ (0\ day) wound\ area - Wound\ area\ of\ specific\ day}{Initial\ (0\ day)\ wound\ area} \times 100$$

Neutrophil-to-Lymphocyte Ratio (NLR)

On day 9, blood samples were collected from five rats in each group into ethylenediaminetetraacetic acid (EDTA) tubes for neutrophil and lymphocyte counts, which were performed using an automated hematology analyzer (Biobase Auto-Hemoanalyzer, Shandong, China). The neutrophil-to-lymphocyte ratio (NLR) was subsequently calculated for each sample.

Evaluation of oxidative stress and antioxidant markers in serum and granulation tissue

On day 9, blood and granulation tissue samples were collected from five rats per group. Blood was drawn into plain tubes (without anticoagulant), and serum was separated by centrifugation at 15,000 × g for 5 minutes. Following blood collection, granulation tissue was excised from each rat and processed for antioxidant analysis.

Both serum and tissue samples were analyzed for oxidant and antioxidant markers, including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and the oxidative stress biomarker malondialdehyde (MDA), using previously established methods (Rahman *et al.*, 2017).

Statistical analysis

The Shapiro–Wilk test was performed to evaluate the normality of data distribution. All variables met the assumption of normality, allowing for parametric analysis. Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS), version 25. Results were expressed as mean ± standard deviation (SD). Differences in wound contraction and oxidative/antioxidant parameters between groups were assessed using an independent samples *t*-test. A p-value < 0.05 was considered statistically significant.

RESULTS

Qualitative and quantitative phytochemical analysis of soybean seed methanolic extract

The qualitative and quantitative phytochemical screening of the methanolic extract of soybean seeds revealed the presence of several bioactive compounds. These included alkaloids (8.5 ± 0.00 % w/w), flavonoids (3.39 ± 0.10 % w/w), tannins (2.23 ± 0.01 % w/w), phenols (2.07 ± 0.01 % w/w), saponins (0.80 ± 0.00 % w/w), and terpenoids (0.65 ± 0.00 % w/w), as presented in table 1.

Wound healing properties of soybean seed methanolic extract

Wounds in both groups assumed an irregular round shape, with coloration ranging from dark red to brown beginning on day 3 (figure 1). By day 7, a noticeable increase in wound contraction was observed (table 2), with the wound bed in Group

TABLE 2. Percentage of wound contraction measured during experimental wound healing in the male albino rat excision wound model

Days	Group	Mean ± SD (%)	Confidence Interval	*p*- value
Day 3	Group A	27.50±10.00	-3.98-20.48	0.161
	Group B	19.25±7.37		
Day 7	Group A	60.42±6.65	4.32-28.6	0.013*
	Group B	43.96±11.58		
Day 11	Group A	82.50±6.61	-3.44-53.44	0.068
	Group B	57.50±14.14		
Day 15	Group A	95.63±0.00	-254.47-268.22	0.795
	Group B	74.38±16.79		

*Value is significant, statistically at $p<0.05$

Source: own elaboration.

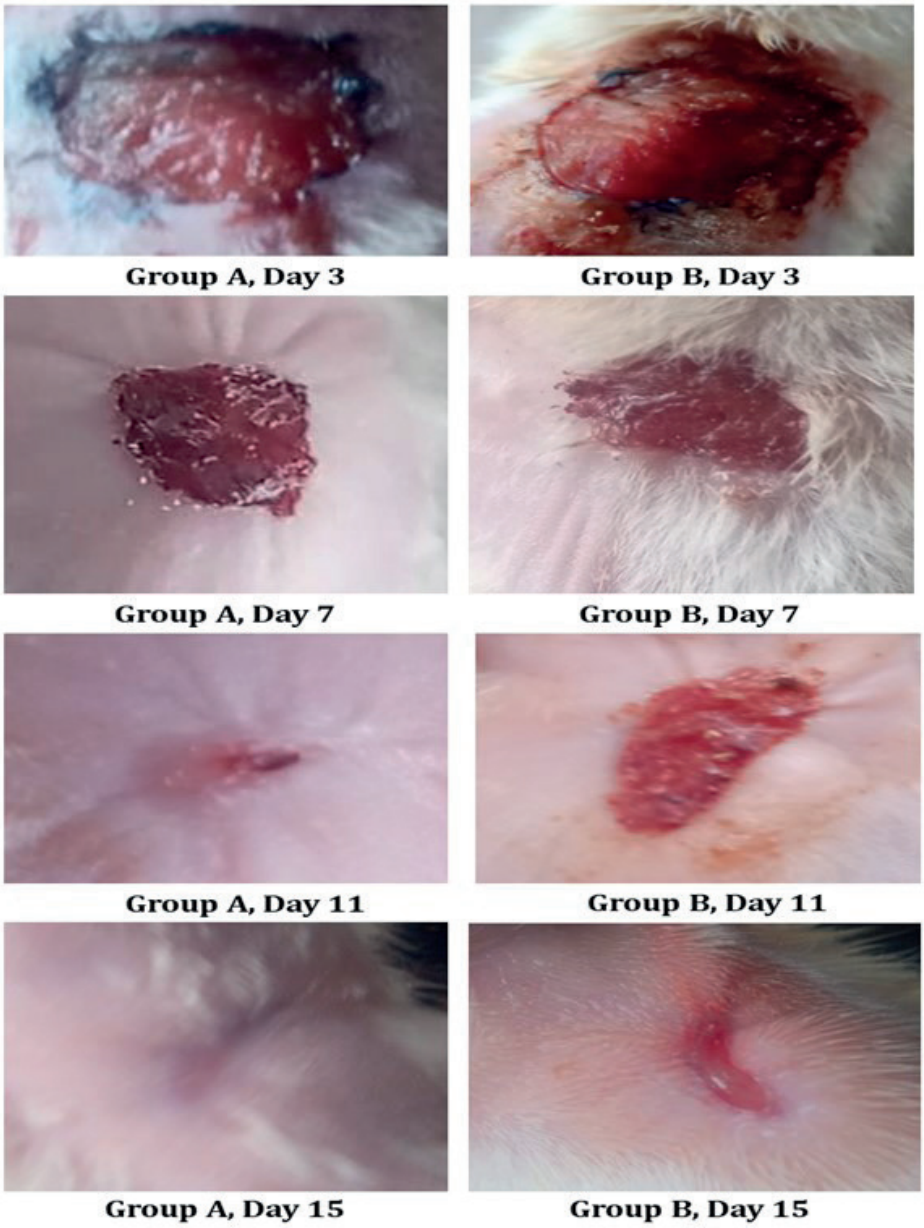

FIGURE 1. Gross appearance of excision wounds and percentage wound contraction in the male albino rat experimental wound healing model. A higher rate of epithelialization and wound contraction was observed on days 7 and 11 in Group A (treated with sterile water) compared to Group B (treated with soybean seed methanolic extract).

Source: own elaboration.

B appearing redder compared to Group A. However, the percentage of wound contraction in Group A (60.42 ± 6.65%) was significantly higher than that in Group B (43.96 ± 11.58%) ($p < 0.05$).

On day 11, wounds in Group A showed a more rapid rate of epithelialization and contraction, with the wound bed covered by a thin scab. Although the percentage of wound contraction in the control group (Group A) reached 82.50 ± 6.61%, compared to 57.50 ± 14.14% in Group B, the difference was not statistically significant.

By day 15, complete healing was observed in Group A, with a wound contraction rate of 95.63 ± 0.00%, whereas wounds in Group B had not yet fully healed, showing a contraction rate of 74.38 ± 16.79%.

Additionally, the neutrophil-to-lymphocyte ratio (NLR) was similar between the groups, with values of 0.55 in Group A and 0.53 in Group B, showing no statistically significant difference (figure 2).

Serum levels of malondialdehyde (MDA) and superoxide dismutase (SOD) (table 3) were significantly higher ($p < 0.05$) in the group treated with soybean seed methanolic extract compared to the group treated with sterile water. However, no significant differences were observed between the groups in serum levels of reduced glutathione (GSH) and catalase (CAT).

Although tissue concentrations of malondialdehyde and superoxide dismutase were not significantly different ($p > 0.05$), their values were higher in Group B than in Group A (table 4). Likewise, tissue levels of reduced glutathione and catalase showed no significant differences ($p > 0.05$) between the two groups.

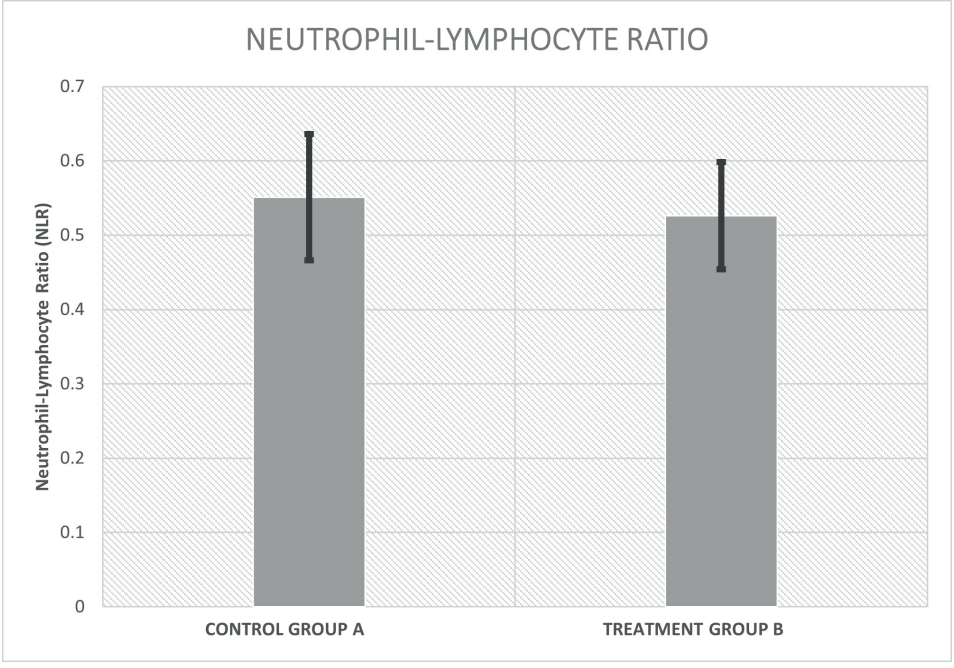

FIGURE 2. Neutrophil-to-lymphocyte ratio in the male albino rat excision wound healing model.

Source: own elaboration.

TABLE 3. Serum oxidant and antioxidant activities during experimental wound healing in rats treated with soybean seed methanolic extract

Parameters	Group	Mean ± SD	*p*- value
MDA (µmols/mg protein)	Group A	3.18± 0.51	0.04*
	Group B	4.26 ± 0.39	
SOD (mg/mL)	Group A	1.01 ± 0.13	0.02*
	Group B	1.43 ± 0.16	
GSH (µg/ml)	Group A	11.83± 0.40	0.979
	Group B	11.80± 1.10	
CAT (µg/ml)	Group A	0.22± 0.05	0.612
	Group B	0.27± 0.13	

*Value is significant, statistically at $p<0.05$

Source: own elaboration.

TABLE 4. Tissue oxidant and antioxidant activities during experimental wound healing in rats treated with soybean seed methanolic extract

Parameters	Group	Mean+SD	*p* -value
MDA (µmols/mg protein)	Group A	1.71± 0.59	0.401
	Group B	2.06± 0.26	
SOD (mg/mL)	Group A.	1.00± 0.25	0.249
	Group B.	1.59± 0.71	
GSH (µg/ml)	Group A	15.37± 0.55	0.160
	Group B	13.10± 2.21	
CAT (µg/ml)	Group A	0.35± 0.13	0.153
	Group B	0.21± 0.06	

Source: own elaboration.

DISCUSSION

The presence of saponins, tannins, flavonoids, cardiac glycosides, anthraquinones, terpenoids, steroids, alkaloids, and phenols in the methanolic extract of soybean seeds used in this study is consistent with previous reports (Hidayat *et al.*, 2018; Kumaran *et al.*, 2015; Prahastuti *et al.*, 2019). Flavonoids were identified as the second most abundant phytochemical class in the extract. This suggests a high content of isoflavones—flavonoid compounds structurally similar to endogenous 17β-estradiol—known for their diverse pharmacological activities (Vitale *et al.*, 2012). Kim *et al.* (2021) also reported that isoflavones such as genistein and daidzein exert protective effects against oxidative stress, inflammation, and related disorders.

In the present study, which aimed to evaluate the role of soybean seed methanolic extract in wound healing—a process characterized by inflammation and immune activation—we observed that the rate of healing in the treated group was slower than that of the control group, based on gross wound appearance and percentage wound contraction. Previous studies suggest that the phytoestrogenic activity of flavonoids, which are particularly abundant in soybeans, is modulated by sex and endogenous hormonal levels (Alwerdt *et al.*, 2019), potentially reducing the healing rate. Flavonoids may function either as weak estrogen agonists or antagonists depending on circulating estrogen levels and estrogen receptor expression (Kuiper *et al.*, 1998), potentially contributing to delayed wound healing in this context.

This dual behavior may explain the delayed healing observed in male albino rats treated with the soybean extract, where flavonoids could have exerted antagonistic effects. Estrogen receptors are distributed across various skin cell types—keratinocytes, sebaceous glands, hair follicles, dermal fibroblasts, and melanocytes—and are more prevalent in females than in males (Sagili *et al.*, 2021). As such, the pharmacological responsiveness to isoflavones may be attenuated in males.

Although Zhao *et al.* (2018) reported that dietary supplementation with low molecular weight soybean protein-derived peptides significantly ameliorated burn injury in rats by modulating systemic inflammatory markers (e.g., IFN-γ, MCP-1, and MCP-3) and suppressing the expression of muscle atrophy- and autophagy-related proteins (MuRF1, Atrogin-1, LC3, and Beclin-1), our findings—focused on the topical application of whole soybean extract—contradict these results in terms of macroscopic wound healing outcomes.

Similarly, in a related study by the same authors, Zhao *et al.* (2019) showed that dietary soybean peptides reduced inflammation, accelerated wound closure, and promoted tissue regeneration in a rat burn injury model. They reported decreased NF-κB signaling, reduced neutrophil and macrophage infiltration, and enhanced angiogenesis via increased CD31 expression. Again, these effects contrast with our findings, suggesting that the form of administration (dietary peptides vs. topical whole extract) and the wound model may significantly influence outcomes.

Draganidis *et al.* (2016) also noted that isoflavone-rich soy protein exerts antioxidant and anti-inflammatory effects, including modulation of NF-κB signaling, and reduces chronic inflammation and oxidative stress. While these findings support the potential of soybean-derived bioactives in tissue repair, our gross assessment revealed that topical treatment with the soybean extract did not outperform the control group treated with sterile water.

The neutrophil-to-lymphocyte ratio (NLR) is a recognized biomarker of inflammation and wound healing (Johan *et al.*, 2024). In this study, the inflammatory response was assessed during the proliferative phase, when inflammation is typically resolving. No significant difference in NLR was observed between groups. Neutrophils play an early role in host defense and debris clearance, while lymphocytes contribute to angiogenesis and extracellular matrix remodeling (Baht *et al.*, 2018; Johan *et al.*, 2024). Despite delayed healing in the treated group, the comparable NLR values may reflect the anti-inflammatory effects of flavonoids (Dower *et al.*, 2015; Esposito *et al.*, 2014).

This aligns with the findings of Zhang *et al.* (2018), who reported the immunomodulatory and anti-inflammatory effects of soybean protein (SBP) and soybean oligopeptides (SBO) in a mouse model of *Staphylococcus aureus*-induced epidermal trauma with negative nitrogen balance. They found that both SBP and SBO increased serum immunoglobulins (IgM, IgG, IgA) and downregulated inflammatory chemokines such as macrophage inflammatory protein-2 (MIP-2) and RANTES. Notably, SBO outperformed SBP in enhancing IgG levels and suppressing MIP-2, suggesting that low-molecular-weight soy peptides may provide greater immunological and healing benefits than intact proteins.

Furthermore, elevated serum and tissue levels of malondialdehyde (MDA), a key marker of oxidative stress, were observed in the treatment group, which may have contributed to delayed healing. Oxidative stress, resulting from excessive reactive oxygen species (ROS), is a critical factor in impaired wound repair (Sanchez *et al.*, 2018). Although increased serum SOD activity in the treatment group supports the antioxidant capacity of soybean isoflavones, this effect did not extend to tissue SOD levels, which were not significantly different. Previous studies emphasize that maintaining a balance between oxidants and antioxidants is essential for optimal tissue regeneration (Dunnill *et al.*, 2015).

CONCLUSION AND LIMITATION

In conclusion, the methanolic extract of soybean seeds did not enhance wound healing in male albino rats under the conditions of this study. Despite its documented anti-inflammatory properties, the extract was associated with a slower rate of wound recovery compared to the group treated with sterile water. Although it did not significantly reduce pro-inflammatory markers, the extract appeared to support antioxidant activity, suggesting that its therapeutic potential may lie more in mitigating oxidative stress than in directly modulating the inflammatory response.

A key limitation of this study was the absence of external positive controls for the quantitative phytochemical analyses. Due to resource constraints, only semi-quantitative methods were employed, which limited the precision and reproducibility of the findings. Future studies should incorporate validated positive controls to enable absolute quantification and more rigorous characterization of the extract's bioactive constituents.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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AI DECLARATION

No generative artificial intelligence tools were used in the writing, analysis, or interpretation of the data presented in this manuscript. All content is the original work of the authors.

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Production costs of golden button *Tithonia diversifolia* and corn *Zea mays* silage

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ABSTRACT

Currently, there is growing interest in the golden button plant, *Tithonia diversifolia* (Hemsl.) A. Gray (TD), a perennial shrub with a pantropical distribution that is native to and naturalized in Colombia. This species has demonstrated considerable potential as a forage plant and has been evaluated in silvopastoral systems and as silage for small ruminants. The objective of this study was to perform an economic analysis of the silage production process of *Tithonia diversifolia* (STD) in comparison with whole-plant maize silage (SMA). To estimate the production and ensiling costs of STD and SMA, a matrix was developed based on technical manuals from Agrosavia and price data from the National Administrative Department of Statistics (Dane, for its acronym in Spanish). The analysis was structured into three phases: crop establishment, silage production, and evaluation of productivity indicators. Financial metrics such as Net Present Value (NPV), Internal Rate of Return (IRR), and Return on Investment (ROI) were applied to assess profitability. The differential cost per ton was 85,461 COP for STD and 157,687 COP for SMA. The Total Annual Gross Income (TABI, for its abbreviation in Spanish) for STD exceeded that of SMA, as TD can yield up to five harvests per year, generating revenues of up to 45 million COP in its first year, whereas SMA, with only one or two harvests annually, generates approximately 29 million COP. Based on the productivity indicators for each crop, total annual profits were 22,789,510 COP·ha^{-1} for STD and 6,226,630 COP·ha^{-1} for SMA. From an economic standpoint, producing one ton of STD costs approximately 63% less than producing one ton of SMA.

Keywords: economic analysis, forage conservation, tropical forages, profitability.

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Costos de producción del ensilaje de botón de oro *Tithonia diversifolia* y maíz *Zea mays*

RESUMEN

En la actualidad se indaga de manera enfática en botón de oro *Tithonia diversifolia* (Hemsl.) A. Gray (TD), una hierba arbustiva perenne de expansión pantropical, propia y natural en Colombia, con probado potencial como planta forrajera, que se ha evaluado en sistemas silvopastoriles y como ensilaje para pequeños rumiantes. El objetivo fue desarrollar un análisis económico del proceso de ensilado de *Tithonia diversifolia* (STD) comparado con ensilado de Maíz (SMA) planta completa. Para estimar los costos de producción y ensilaje de STD y SMA, se elaboró una matriz basada en manuales técnicos de Agrosavia y datos de precios del Dane. El análisis se dividió en tres fases: establecimiento del cultivo, elaboración del ensilaje y evaluación de indicadores productivos. Finalmente, se aplicaron indicadores financieros como VPN, TIR y ROI para valorar la rentabilidad y, arrojó un costo diferencial de 85.461 y 157.687 COP$.ton^{-1} para STD y SMA respectivamente. El Total Ingreso Bruto Anual (TIBA) del STD es superior al de SMA, pues TD con cinco cosechas al año alcanza ingresos de hasta 45 millones COP en su primer año, mientras que SMA, con una o dos cosechas al año, apenas llega a 29 millones COP. Con base en los indicadores productivos que mostró cada cultivo, se logró una ganancia total anual de 22.789.51 y 6.226.630 COP·ha^{-1} para STD y SMA, respectivamente. En términos económicos, producir una tonelada de STD cuesta un 63% menos que de SMA.

Palabras clave: análisis económico, conservación de forrajes, forrajes tropicales, rentabilidad.

INTRODUCTION

In tropical regions such as Colombia, cattle feeding systems are primarily based on direct grazing. However, during the dry season, both the availability and quality of forages decline, leading to increased structural fiber content, reduced digestibility, and higher methane emissions (Morales & Ortiz, 2018; Vargas *et al.*, 2018; Partida *et al.*, 2019; Quintero *et al.*, 2021). Grazing systems depend heavily on soil fertility and climatic variability, both of which directly affect pasture availability (Angulo *et al.*, 2022). Consequently, the use of harvested forages and preservation methods, such as silage, has become essential to ensure a consistent feed supply for livestock (Guatusmal *et al.*, 2020; Miguel *et al.*, 2023).

Tithonia diversifolia (TD) is a pantropical shrub species commonly found in the coffee-growing region of Colombia. It has demonstrated high potential as a forage resource and has been evaluated in silvopastoral systems and as silage (Mejía, Mahecha, & Angulo, 2016). This plant is characterized by high biomass production, drought tolerance, resilience to frequent cutting, low fertilization requirements, and favorable nutritional composition. It contains up to 20% crude protein, high levels of soluble carbohydrates (25.7%), moderate fiber content (NDF: 33–45%; ADF: 26–29%), and secondary metabolites such as phenols and tannins, which contribute to reduced methane emissions (Holguín *et al.*, 2015; Mejía *et al.*, 2016; Meza *et al.*, 2021; Gallego *et al.*, 2016; Hernández *et al.*, 2022). TD thrives naturally at elevations of up to 2,000 m a.s.l. in the coffee-growing region of

Colombia, where it maintains efficient biomass production, persistence during the dry season, tolerance to frequent cutting, and consistently high nutritional value (Holguín *et al.*, 2015; Meza *et al.*, 2021; Vivas *et al.*, 2022).

Ensiling enhances the utilization of TD by enabling early harvest maturity, stabilizing forage availability, reducing production costs, and supporting diversification toward stall-fed livestock systems among small-scale coffee growers (Castaño *et al.*, 2023). In forage and livestock production systems, production costs are typically divided into fixed costs—such as land rental and infrastructure—and variable costs, which fluctuate with production volume and include inputs, labor, fertilization, and machinery (Báez *et al.*, 2023; de las Cuevas *et al.*, 2024). Based on this cost structure, unit costs can be calculated and expressed per ton of forage, allowing for the assessment of technical and financial efficiency within the evaluated systems (Báez *et al.*, 2022).

Economic analysis is crucial in tropical livestock production, as it enables the evaluation of the actual profitability of technologies or forage alternatives, considering their impact on net margins and overall system sustainability (Junca *et al.*, 2025; Murgueitio, 2023). Within this framework, *Tithonia diversifolia* has shown strong potential due to its ecological adaptability, favorable agronomic performance, relatively low production cost, and the added value it provides in animal feeding, whether used as fresh forage or silage (Mejía *et al.*, 2016). Based on these considerations, the present study compares the production costs of *Tithonia diversifolia* silage (STD) and maize silage (SMA) to assess their economic feasibility in tropical livestock systems.

MATERIALS AND METHODS

Study location

The biomass production of *Tithonia diversifolia* (TD) and maize for silage was carried out at the Experimental Center of the Universidad Nacional de Colombia, Palmira Campus (CEUNP, for its abbreviation in Spanish), located in Candelaria (Valle del Cauca) at 3°25'34″ N, 76°25′53″W, at an altitude of 951 m a.s.l. The site has average temperatures ranging from 23 to 27 °C, a relative humidity of 75%, and a mean annual precipitation of 1,000 mm, distributed across two rainy seasons (Vallecillo *et al.*, 2022).

Biological material

Tithonia diversifolia

An established one-year-old *Tithonia diversifolia* field at CEUNP was used, planted at a density of 10,000 shrubs·ha^{-1}. Based on prior experience, a uniformity cut (UC) was performed at a height of 40 cm, followed by a single irrigation to field capacity. Fertilization was applied in three installments—on days 5, 10, and 15 after the UC—using 340, 245, 70, and 30 kg·ha^{-1} of nitrogen (N), phosphorus (P), potassium (K), and micronutrients, respectively. To apply the fertilizer as a root drench (250–300 mL per shrub), a solution was prepared by diluting 7.6, 5.4, 1.6, and 0.66 kg of N, P, K, and micronutrients, respectively, in 200 liters of water (Castro, 2019; Holguín, 2016). The TD was harvested at 60 days of growth, during the pre-flowering stage, and cut at a height of 40 cm.

Maize (Zea mays)

As a control, a 6,400 m² plot was planted with Pioneer 30F35VYHR maize from

Corteva. For biosafety, conventional hybrid maize was sown in four rows surrounding the test plot. Conventional sowing practices were followed: soil preparation involved one pass of a plow and two passes of a harrow. Direct seeding was carried out with a Montana pneumatic planter–fertilizer machine, spacing rows 0.8 m apart and 7–8 plants·m^{-1}. The management plan was based on the guidelines provided by Corteva, Inc. and the technical manual for maize cultivation developed by Fenalce.

A pre-emergent herbicide (Prowl®, 2 L·ha^{-1}) was applied before sowing. Fertilization at planting included 20% of the nitrogen (Amidas) at 100 kg·ha^{-1}, 150 kg·ha^{-1} of monoammonium phosphate (MAP), and 50% of the potassium chloride (KCl) at 100 kg·ha^{-1}. At stages V4–V6, glyphosate herbicide (1.0 L·ha^{-1}) was applied, along with 40% of the nitrogen (200 kg·ha^{-1}) and the remaining 50% of KCl (100 kg·ha^{-1}). At V10–V12, the final 40% of nitrogen (200 kg·ha^{-1}) was applied. The irrigation system consisted of a 2-inch mainline drip system with 16 mm drip tape and 15 cm emitter spacing, operated for one hour per day depending on rainfall availability to avoid water stress. Maize was harvested at 90 days, during the R3 (milk) stage.

Silage Production

Tithonia diversifolia Silage (STD)

TD was manually harvested at 60 days, during the pre-flowering stage, and left in the field for 24 hours to reduce moisture content. Chopping to a length of 1 cm was performed using a manually fed forage harvester (IDEAGRO SIGMA 2002), followed by transportation. Under shelter, the chopped material was sprayed during baling with a 50:50 (v/v) molasses–water mixture and a microbial inoculant (Sil-All 4x4) at 10 g·t^{-1}, using a backpack sprayer. Baling was carried out with a stationary compactor (IDEAGRO SILO PACK J–402; 2 t·h^{-1}), and the material was packed into black polyethylene bags (gauge 6; 60 kg capacity), sealed with 0.4 mm polypropylene twine, labeled by date and treatment, and stored in a clean, dry, cool location.

Maize Silage (SMA)

Whole-plant maize was harvested at 90 days, during the R3 stage. Except for the field-wilting step (which was not performed for maize), the silage process was identical to that used for TD, employing the same equipment (IDEAGRO SIGMA 2002 and SILO PACK J–402) with continuous field processing and subsequent bagging.

ECONOMIC ANALYSIS

To estimate the production and silage costs associated with STD and SMA, a cost matrix was developed based on the *Manual de costos y análisis financiero para el sistema productivo de ganadería de ceba en la Orinoquía colombiana* and the *Guía de registro para costos de producción de pequeños y medianos agricultores de cultivos transitorios en el piedemonte llanero* (Agrosavia, 2020).

Agricultural input prices were obtained from updated data from the Agricultural Sector Price Information System (SIPSA, for its abbreviation in Spanish) of the National Administrative Department of Statistics (Dane, 2020) and from the 2020 Agricultural Cost Guide published by the Department of Rural Development, Agriculture, and Fisheries of the Government of Valle del Cauca.

The economic analysis was structured in three stages: (1) estimation of crop establishment costs, (2) estimation of silage processing costs, and (3) evaluation of financial profitability. First, cost types were identified and categorized. Fixed costs included those independent of production volume, such as land rental, while variable costs were directly associated with agricultural management and silage processing, including soil analysis, machinery use, fertilization, irrigation, labor, inputs, and storage. Unit costs were then calculated per ton of forage produced (COP·ton^{-1}) by dividing the total cost by the projected yield per hectare. This classification enabled a comparative analysis between the two crops, considering both absolute investment and relative efficiency per unit of product (Báez Quiñones *et al.*, 2023).

In the second stage, specific silage production costs for STD and SMA were integrated, encompassing the preparation of plant material and the associated use of machinery, labor, and preservation materials. Additionally, the productive parameters of each crop were characterized, including cycle duration, number of harvests per year, and yield (t·ha^{-1}).

Finally, a financial profitability analysis was conducted using three classical indicators proposed by Ross, Westerfield, and Jordan (2021):

- Net Present Value (NPV): estimates the present value of expected net returns, discounted at a 15% rate.
- Internal Rate of Return (IRR): reflects the annual effective return of the production system.
- Return on Investment (ROI): represents the ratio of cumulative net profit to initial investment.

The calculation of net cash flows and the subsequent analysis were carried out using Microsoft Excel 2016 spreadsheets. This methodological approach has been previously validated and applied in economic studies on silvopastoral systems and tropical forages such as *Tithonia diversifolia* (Báez Quiñones *et al.*, 2023; Junca Paredes *et al.*, 2025; Mejía-Díaz *et al.*, 2016).

RESULTS AND DISCUSSION

Establishment cost

The initial stage of establishing *Tithonia diversifolia* (TD) and maize is determined by the green forage yield of each crop. As shown in table 1, the difference between the two lies in the number of annual harvests and the yield per cut. TD outperformed maize, yielding 80 t·ha^{-1} per cut compared to 70 t·ha^{-1} for maize.

TABLE 1. Gross field yield for *Tithonia diversifolia* and whole-plant maize *Zea mays*

Characteristic	TD	MAIZE
Sowing to harvest time (days)	60	90
Yield (ton ha-¹)	80	70
Harvests per year	5	2
Where: TD : *Tithonia diversifolia*		

Source: own elaboration.

The total estimated costs were divided into variable and fixed components. For TD, these are presented in Table 2. Variable costs included machinery use, seed procurement, irrigation, fertilization, and labor, totaling a Total Variable Cost (TVC) of COP $9,601,908·ha^{-1}. Fixed costs comprised the monthly land lease, resulting in a Total Fixed Cost (TFC) of COP $440,000·ha^{-1}. Consequently, the Total Establishment Cost (TEC) for TD was COP $10,041,908·ha^{-1}.

For TD, the total establishment cost per hectare represents a one-time initial investment at the start of the project. As TD is a perennial species, the productive lifespan (PL) of a plantation was estimated at five years. To more accurately reflect the financial conditions faced by a producer, it was assumed that this investment was financed through a five-year consumer loan with fixed monthly payments of COP $245,306, calculated at an annual interest rate of 4.7% (Bancolombia). Accordingly, the establishment cost was amortized over the PL rather than being allocated entirely to the first year.

Regarding unit costs, the breakdown was as follows:

- Unit Fixed Cost (UFC): COP $5,500·$t^{-1}$
- Unit Variable Cost (UVC): COP $120,024·$t^{-1}$
- Total Unit Cost (TUC): COP $125,524·$t^{-1}$

Assuming five harvests annually throughout the PL and incorporating debt servicing (interest rate of 1.34%), the per-harvest establishment cost was estimated at COP $245,306·$ha^{-1}$·$cut^{-1}$. This estimate offers a more realistic long-term cost perspective and supports effective financial planning.

TABLE 2. Variable and fixed costs associated with the establishment of *Tithonia diversifolia* and maize

Item	TD (COP·ha^{-1})	Maize (COP·ha^{-1})
Variable costs		
Soil analysis	114,000	114,000
Machinery	166,666	318,266
Fertilization	1,898,900	3,401,500
Irrigation	972,342	298,950
Seeds	6,000,000	1,800,000
Labor	450,000	650,000
Total variable costs (TVC)	9,601,908	6,582,716
Fixed costs		
Land lease, infrastructure, etc.	440,000	660,000
Total fixed costs (TFC)	440,000	660,000
Total establishment cost (TC)	10,041,908	7,242,716
Plantation lifespan (years)	5	N/A
Estimated monthly installment (loan, 4.7%)	245,306	—

Note: The Total Cost (TC) corresponds to the sum of Total Variable Costs (TVC) and Total Fixed Costs (TFC) per hectare (TC = TVC + TFC).

Source: own elaboration adapted from Production Cost Recording Guide (Agrosavia, 2020) and Agricultural Sector Price Information System – SIPSA (Dane, 2025).

Table 2 also presents the establishment costs for maize. The TVC—including machinery, seed, irrigation, fertilization, herbicides, and labor—was COP $6,582,716·ha^{-1}. The TFC, corresponding to the monthly land lease, amounted to COP $660,000·ha^{-1}, resulting in a TEC of COP $7,242,716·ha^{-1}. With an estimated yield of 70 t·ha^{-1}, the unit costs were as follows:

- UFC: COP $9,429·t^{-1}
- UVC: COP $94,039·t^{-1}
- TUC: COP $103,467·t^{-1}

The relatively low UFC is attributed to the combination of affordable land leasing and high projected yields, which reduce the fixed cost per ton. When comparing total establishment costs per hectare between maize and TD, maize is approximately 7% less expensive. However, considering the higher yield potential of *Tithonia diversifolia* (80 t·ha^{-1}), the difference in total unit cost narrows to 5%.

Recent research on the "Altoandina" variety of oats showed that, although maize generally produces higher green and dry matter yields, its establishment costs exceed those of oats (Gallo *et al.*, 2022). Crops such as maize, which require extensive fertilization, weeding, and phytosanitary management to achieve optimal performance, tend to incur higher establishment costs (Villalobos *et al.*, 2013). Consequently, maize cultivation demands greater investment, increasing production costs and reducing profit margins. It is therefore advisable to balance input use to avoid excessive application rates (Castro & Huertas, 2018) and to select resistant genotypes or genetically modified hybrids (GMOs) that require fewer inputs while delivering better overall performance.

In contrast, although TD requires a larger initial investment, its high biomass yields help amortize these costs over time (García *et al.*, 2007). Implementing agronomic practices tailored to the crop's characteristics, together with the use of proven clonal material, can enhance adaptation and improve resource-use efficiency (Villalobos *et al.*, 2015). Consequently, shrubby forages such as TD may offer economic advantages over annual crops by lowering production costs through higher green forage yields and extended longevity (Báez *et al.*, 2023).

Silage production costs of Tithonia diversifolia and maize

The costs associated with the silage production of TD (STD) are detailed in table 3. These include:

- Machinery: COP $998,000·ha^{-1}
- Additives: COP $911,500·ha^{-1}
- Bagging: COP $1,920,400·ha^{-1}
- Labor: COP $600,000·ha^{-1}

In addition, the amortized establishment cost over five years was considered, corresponding to the 60-day growth cycle required for TD to reach harvest maturity. This cost amounted to COP $490,612·ha^{-1}.

Maintenance costs, including irrigation and fertilization, were also included, totaling COP $1,886,342·ha^{-1}. Summing all components, the total silage production cost for STD reached COP $6,836,854·ha^{-1}, equivalent to COP $85,461·t^{-1}.

For maize silage (SMA), machinery costs were equal to those for TD. Additional costs included:

- Additives: COP $887,000·ha^{-1}
- Bagging: COP $1,710,400·ha^{-1}
- Labor: COP $200,000·ha^{-1}

The total silage production cost for SMA was COP $3,795,400·ha^{-1}, or COP $54,220·t^{-1} based on yield.

The production costs of silage from *Tithonia diversifolia* include amortized

TABLE 3. Costs of silage production for STD and SMA

Description – bagged silage	STD	SMA
Machinery		
Harvest machinery (COP $·ha^{-1})	400,000	400,000
Silo pack ensiler (COP $·ha^{-1})	598,000	598,000
Machinery subtotal (COP $·ha^{-1})	998,000	998,000
Additives		
Molasses (COP $·ha^{-1})	810,000	810,000
Sil-All (COP $·ha^{-1})	101,500	77,000
Additives subtotal (COP $·ha^{-1})	911,500	887,000
Packing inputs		
Bags ($1,200/bag)	1,920,000	1,680,000
Polypropylene roll ($15,200/roll)	30,400	30,400
Packing subtotal (COP $·ha^{-1})	1,950,400	1,710,400
Labor – silage conditioning		
Cutting (COP $·ha^{-1})	200,000	
Transport (COP $·ha^{-1})	200,000	
Packing (COP $·ha^{-1})	200,000	200,000
Labor subtotal (COP $·ha^{-1})	600,000	200,000
Establishment cost (amortized over 5 years)		
Amortized cost per cut (COP $·ha^{-1})	490,612	
Establishment subtotal (COP $·ha^{-1})	490,612	
Maintenance		
Urea 46% (50 kg sack)	500,000	
Triple 15 (50 kg sack)	414,000	
Irrigation application	972,342	
Maintenance subtotal (COP $·ha^{-1})	**1,886,342**	
Total silage production cost (COP $·ha^{-1})	**6,836,854**	**3,795,400**
Total cost per ton of silage ($·ton^{-1})	**85,461**	**54,220**

Where: STD = *Tithonia diversifolia* silage; SMA = maize silage; Harvest machinery = Tractor with forage harvester header and transport trailer.

Source: own elaboration adapted from Production Cost Recording Guide (Agrosavia, 2020); the values for variable and fixed costs were taken from agricultural inputs reported on the Dane agricultural inputs website (2023).

establishment expenses, calculated by dividing the total establishment cost by the number of harvests expected over the plantation's productive lifespan. These are combined with per-harvest silage processing costs, debt servicing, maintenance (irrigation and fertilization), and labor (harvesting and transportation) (Beltrán & Lemus, 2015). The detailed costs for STD and SMA are presented in table 3.

TD harvesting is manual, whereas maize is mechanically harvested. Consequently, per-ton silage costs are:

- SMA: COP \$54,220·t^{-1} (production only) and COP \$157,687·t^{-1} (including establishment)
- STD: 47% lower than SMA when including establishment

This positions *Tithonia diversifolia* silage as a cost-effective alternative for specialized dairy systems, offering:

- Greater production efficiency.
- Improved profit margins.
- Demonstrated economic viability (Angulo *et al.*, 2022).

These results align with previous studies indicating that mixed silages (e.g., *Mucuna pruriens* with *Pennisetum purpureum*) can reduce production costs. In particular, maize–*Mucuna* silage has demonstrated superior biomass yield potential for bovine feeding (Beltrán & Lemus, 2015).

High-quality silages such as STD improve:

- Farm profitability through increased milk production and income (Villalobos *et al.*, 2015).
- Nutritional efficiency, enhancing microbial biomass synthesis.
- Nitrogen utilization, potentially reducing emissions (Bernardes & Rego, 2014; Pérez *et al.*, 2023).

Productive indicators of Tithonia diversifolia and maize

According to table 2, establishing *Tithonia diversifolia* (TD) requires an initial investment of COP \$10,041,908·ha^{-1}, amortized over five years through 60 fixed monthly payments of COP \$245,306. This corresponds to COP \$490,612·ha^{-1}·cut^{-1}. In contrast, maize establishment involves a one-time investment of COP \$7,242,716·ha^{-1} (table 4).

When considering silage production costs, the following values are observed:

- STD (*Tithonia diversifolia* silage): COP \$6,346,242·ha^{-1}
- SMA (maize silage): COP \$3,795,400·ha^{-1}

The combined costs of establishment and silage production result in:

- STD: COP \$6,836,854·ha^{-1}
- SMA: COP \$11,038,116·ha^{-1}

In terms of cost per ton of silage produced, the values are:

- STD: COP \$85,461·t^{-1}
- SMA: COP \$157,687·t^{-1}

These results indicate that, although TD has higher initial establishment costs, its perennial nature and greater biomass productivity substantially reduce the total cost per ton of silage. Specifically, the cost of STD is 46% lower than that of maize silage, representing a considerable economic advantage for medium- and long-term forage systems.

Total revenue

Maize silage (SMA) commands a higher unit price (COP \$210,250·ton^{-1}) than *Tithonia diversifolia* silage (STD; COP \$113,948·ton^{-1}). However, STD offsets this price difference through its higher annual yield, resulting from its perennial nature and harvesting frequency every

TABLE 4. Comparison of costs, revenues, and annual profitability per hectare between STD and SMA

Total costs	**STD**	**SMA**
Total establishment cost (COP $·ha^{-1})	490,612	7,242,716
Total silage cost (COP $·ha^{-1})	6,346,242	3,795,400
Establishment + silage process cost (COP $·ha^{-1})	6,836,854	11,038,116
Cost per ton ($·ton^{-1})	85,461	157,687
Total revenues		
Unit price (COP $·ton^{-1})	113,948	210,250
Total revenue per cut (COP $·ha^{-1})	9,115,805	14,717,488
Total annual revenue (COP $·ha^{-1})	45,579,027	29,434,976
Total profits		
Unit profit (COP $·ton^{-1})	28,487	52,562
Total profit per cut (COP $·ha^{-1})	2,278,951	3,679,372
Total annual profit (COP $·ha^{-1})	11,394,757	7,358,744

Source: own elaboration.

60 days (Mejía-Díaz *et al.*, 2016). Based on data from this study, annual revenue reaches COP $45.5 million·ha^{-1} for STD, compared with COP $29.4 million·ha^{-1} for SMA. The advantage of STD lies in its stable productivity and lower recurring costs: as a perennial crop, it avoids annual planting expenses, unlike maize, which requires reinvestment each cycle. This structural efficiency (Murgueitio, 2023; Báez Quiñones *et al.*, 2023) translates into higher annual profitability despite lower unit margins. Furthermore, STD can amortize its initial investment within one to two years and reduces reliance on external inputs (de las Cuevas-Milán *et al.*, 2024). As Ross *et al.* (2021) observe, systems characterized by frequent cash flows and low fixed costs tend to optimize net margins. Consequently, STD represents an attractive option for sustainable intensification, combining continuous production, reduced financial pressure, and medium-term stability.

Total profitability

Although maize silage (SMA) yields higher unit profits per ton, the *Tithonia diversifolia* system (STD) demonstrated greater annual profitability per hectare due to its high biomass yield and frequent harvesting (every 60 days). This intensive management exceeded the 37.9 t·ha^{-1} reported by Londoño *et al.* (2019), aligning with the findings of Guatusmal *et al.* (2020). Despite its higher initial cost—primarily driven by seed expenses (Padilla *et al.*, 2023)—the investment in STD, when amortized over five years and requiring minimal fertilizer and supplementation, can be recovered between the first and second year (Báez Quiñones *et al.*, 2023). Moreover, as highlighted by Buitrago and Vargas (2017), STD offers

stable and increasing profitability, unlike SMA, which depends on replanting. Consequently, STD emerges as the more profitable medium-term option, particularly for systems prioritizing continuous production, cost reduction, and financial sustainability.

Financial analysis

Table 5 summarizes the economic performance of the STD production system over a five-year period, including gross income, operating costs, net income, and key financial indicators. The analysis applies a conservative 10% technical adjustment to the fresh biomass yield per hectare, in line with recommendations for modeling forage systems under field conditions (Cenicaña, 2021; Gallo *et al.*, 2021), and assumes five harvests annually. This adjustment is intended to reflect realistic field scenarios, where climatic variability, management practices, and physiological exhaustion may reduce effective productivity.

The initial establishment of the STD system required an investment of COP \$10,041,908·ha^{-1} (Year 0), covering land preparation, planting, and infrastructure. Although this stage concentrated the highest financial risk, the project achieved rapid recovery: in the first year, it generated gross income of COP \$45.5 million·ha^{-1} (production: 80 t·ha^{-1}) and a net profit of COP \$11.4 million·ha^{-1}. Even as productivity declined to 40 t·ha^{-1} by year five, costs were reduced by 44% through management optimization. Financially, the project demonstrated strong performance, with a net present value (NPV) of COP \$30.7 million·ha^{-1} (at a 15% discount rate), an internal rate of return (IRR) of 115%, and a return on investment (ROI) of 510%. These indicators confirm that, despite the initial financial challenge, the STD model is profitable, sustainable, and efficient, with early positive cash flows that support adoption—particularly among producers with access to credit (Londoño & Pérez, 2019; Rodríguez *et al.*, 2019; FAO, 2021).

TABLE 5. Five-year projection for the SBO and SMA systems, considering an inflation rate of 8% and an interest rate of 10%

Discount rate 15%								
Item	**Year**	**Yield (ton·ha^{-1})**	**Total annual gross income (COP/year)**	**Total ensiling + maintenance costs (COP/year)**	**Total annual net income (COP/year)**	**NPV**	**IRR**	**ROI**
SBO	0			\$ 10,041,908	-\$ 10.041.908	\$ 30,772.49	115%	510%
	1	80	\$ 45,579.03	\$ 34,184.27	\$ 11,394.76			
	2	70	\$ 43,072.18	\$ 31,381.16	\$ 11,691.02			
	3	60	\$ 39,872.53	\$ 26,673.99	\$ 13,198.55			
	4	50	\$ 35,885.28	\$ 22,672.89	\$ 13,212.39			
	5	40	\$ 31,004.88	\$ 19,271.96	\$ 11,732.93			

Discount rate: 15%. IRR = Internal Rate of Return; NPV = Net Present Value; ROI = Return on Investment. All monetary values are expressed in Colombian Pesos (COP).

Source: own elaboration.

OUTLOOK

Botón de oro (Tithonia diversifolia) silage is projected as a valuable forage resource and a cost-effective alternative to other preserved forages, offering significant advantages in financial profitability. This potential underscores its suitability as a foundational component in the development of livestock systems in Colombia.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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DECLARATION ON THE USE OF ARTIFICIAL INTELLIGENCE

Generative artificial intelligence was used exclusively to enhance language economy and fluency.

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Preliminary design and evaluation of an RT-PCR assay for detecting *Reptarenavirus* in snakes (subfamily *Boinae* and families *Pythonidae* and *Colubridae*) from the Reserva Natural Bioparque Wakatá, Colombia

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ABSTRACT

Inclusion body disease (IBD) is a viral infection of snakes caused by *Reptarenavirus*, characterized primarily by neurological signs, although it can also be subclinical. This study preliminarily designed and evaluated an RT-PCR assay to detect the virus in snakes from the Wakatá Biopark (Colombia), using two sets of primers and without a positive control. Although the results were negative and the assay proved unviable for routine use, the study generated valuable information as a basis for future projects addressing this problem and provided recommendations for the differential diagnosis of the disease.

Keywords (MeSH): reverse transcriptase polymerase chain reaction, snakes, *Reptarenavirus*, wild animals.

Diseño y evaluación preliminar de una prueba de RT-PCR para la detección de *Reptarenavirus* en serpientes (subfamilia *Boinae* y familias *Pythonidae* y *Colubridae*) de la Reserva Natural Bioparque Wakatá, Colombia

RESUMEN

La enfermedad de cuerpos de inclusión (ECI) es una afección viral de serpientes causada por *Reptarenavirus*, con signos clínicos principalmente neurológicos, aunque puede ser subclínica. Este estudio diseñó y evaluó, de forma preliminar, una prueba RT-PCR en un intento de detectar el virus en serpientes del Bioparque Wakatá (Colombia), utilizando dos *sets* de *primers* y sin un control positivo. Aunque los resultados fueron negativos y la prueba no mostró ser viable para uso rutinario, se generó información valiosa como

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base para futuros proyectos acerca de este problema y se hacen recomendaciones acerca del diagnóstico diferencial de la enfermedad.

Palabras clave (DeCS): Reacción en cadena de la polimerasa de transcriptasa inversa, serpientes , *Reptarenavirus*, animales silvestres .

Introduction

Inclusion body disease (IBD) is a transmissible viral disorder of captive and wild snakes, caused by infection with viruses of the genus *Reptarenavirus* (Alfaro-Alarcón *et al.*, 2022). IBD primarily affects boas of the subfamily *Boinae* and pythons of the family *Pythonidae* (Thiele *et al.*, 2023). The clinical manifestations are predominantly neurological and include loss of righting reflex, opisthotonos (commonly referred to as "star-gazing" or "corkscrew posture"), head tremors, flaccid paralysis, disorientation, and regurgitation (Argenta *et al.*, 2020). However, in many cases the disease remains subclinical, with affected individuals serving as asymptomatic carriers for extended periods and presenting nonspecific signs such as progressive emaciation, secondary infections, and sudden death (Ossiboff, 2018; Simard *et al.*, 2020).

Transmission of the disease has been reported to occur both horizontally and vertically. In horizontal transmission, snakes shed viral RNA through the skin, feces, and urates (Dervas, 2024), whereas vertical transmission may occur from either parent (Alfaro-Alarcón *et al.*, 2022; Dervas, 2024). A potential vector under consideration is the cosmopolitan snake mite *Ophionyssus natricis*, commonly known as the reptile mite (Mendoza-Roldan *et al.*, 2023). In addition to causing irritation, anemia, and even death in severe infestations, this ectoparasite has been proposed as a possible vector of IBD, although conclusive evidence remains lacking (Chang & Jacobson, 2010; Dervas, 2024).

From a geographical perspective, IBD is considered a globally relevant disease, with cases reported worldwide. Several studies have documented its occurrence in snakes under human care in North America, Europe, Asia, and Australia. Currently, no cure is available, and euthanasia of infected animals is commonly practiced to protect the health of the remaining population (Alfaro-Alarcón *et al.*, 2022).

Regarding differential diagnoses, other viruses are also capable of producing inclusion bodies and may cause diseases with clinical signs similar to those of IBD, including *Herpesvirus*, *Adenovirus*, and *Paramyxovirus* (Marschang, 2014). Among these, *Paramyxoviruses* are the most relevant, as they also form intracytoplasmic inclusion bodies and produce neurological signs comparable to those observed in IBD.

According to the International Committee on Taxonomy of Viruses (ICTV), five species of *Reptarenavirus* are currently recognized: *R. giessenae*, *R. commune*, *R. californiae*, *R. aurei*, and *R. rotterdamense*. However, several additional unclassified species remain under provisional and non-official designations (Radoshitzky *et al.*, 2023). The *Reptarenavirus* genome consists of a bisegmented single-stranded RNA with an ambisense coding strategy (De la Torre, 2020; Hetzel *et al.*, 2021). The first segment, designated S (short), encodes the glycoprotein precursor and the nucleoprotein (NP), while the second

segment, designated L (long), encodes the zinc-finger matrix protein (ZP) and the RNA-dependent RNA polymerase (RdRp) (Lintala *et al.*, 2022b; Radoshitzky *et al.*, 2023).

Several authors have demonstrated that snakes affected by IBD frequently exhibit coinfections with multiple *Reptarenavirus* species, as evidenced by the presence of more than one type of S and L segment in a single individual (Argenta *et al.*, 2020). Moreover, higher levels of the S segment have been correlated with a greater abundance of inclusion bodies (Thiele *et al.*, 2023). These findings are consistent with previous studies characterizing the composition of inclusion bodies, which revealed that they are primarily composed of NP encoded by the S segment (Chang *et al.*, 2013; Hetzel *et al.*, 2021; Lintala *et al.*, 2022a; Thiele *et al.*, 2023).

In general, animals affected by IBD develop inclusion bodies (IBs) in most tissues. Consequently, the historical antemortem diagnostic method of choice has been the detection of IBs in samples such as blood smears and, most commonly, liver biopsies (Alfaro-Alarcón *et al.*, 2022). However, although IB detection is a rapid and cost-effective diagnostic tool, it is not fully reliable, as IB formation appears to vary both among individuals and across species (Hetzel *et al.*, 2021; Thiele *et al.*, 2023).

In a significant proportion of cases, snakes may develop chronic forms of the disease in which, despite harboring characteristic intracytoplasmic IBs, they exhibit no obvious clinical signs. Conversely, some individuals have been documented with clinical manifestations consistent with IBD but lacking detectable IBs (Alfaro-Alarcón *et al.*, 2022; Argenta *et al.*, 2020; Dervas, 2024; Lintala *et al.*, 2022b; Thiele *et al.*, 2023).

Other methods for detecting *Reptarenavirus* include immunohistochemistry targeting the NP protein, transmission electron microscopy for direct viral visualization, next-generation sequencing (NGS), Sanger sequencing, viral inoculation in cell cultures, ELISA, and Western blotting (Alfaro-Alarcón *et al.*, 2022; Chang *et al.*, 2013; Hetzel *et al.*, 2013; Ihász *et al.*, 2022; O'Rourke & Lertpiriyapong, 2015). Nevertheless, these approaches are not yet suitable for routine diagnosis, either because of their high cost or the limited accessibility of the required technologies.

Due to the genetic variability of the virus and the limited information available on the disease in Latin America, diagnosing and reporting its presence remain challenging, as the specific *Reptarenavirus* species and/or variants circulating within snake populations in Colombia are still unknown. According to records from the Animal Health Coordination of the RNBW, an ongoing issue has been observed since 2019, in which several snakes, including both boas and pythons, have exhibited clinical signs and postmortem lesions consistent with IBD (figure 1). In some of these cases, characteristic IBs of IBD have also been detected in blood and tissue samples (figure 1).

Given the suspicion of IBD, several individuals were euthanized due to compromised health status and as an epidemiological preventive measure against potential transmission. In light of these circumstances, the aim of this study was to design and preliminarily evaluate an RT-PCR assay to detect *Reptarenavirus* in snakes under professional care at the RNBW.

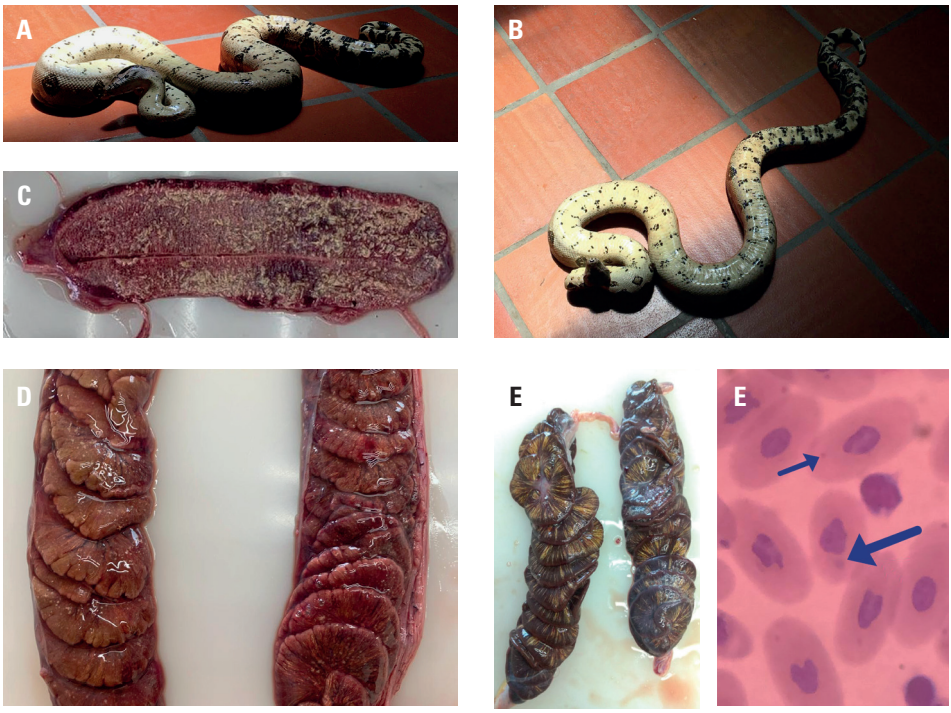

FIGURE 1. (A) Boa (*Boa constrictor*) exhibiting "star-gazing" posture. (B) Boa (*Boa constrictor*) showing delayed or absent righting reflex. (C) Lung of a boa (*Boa constrictor*) with pyogranulomatous material compatible with bacterial pneumonia, with IBs reported in the histopathological report. (D) Kidneys of a boa (*Boa constrictor*) showing multiple whitish pinpoint foci within the renal parenchyma, with IBs confirmed in the histopathological report. (E) Kidneys of another boa (*Boa constrictor*) with renal calcification and IBs identified in the histopathological report. (F) Hematoxylin and eosin–stained blood smear from one of the boas at the RNBW, showing several amphophilic intracytoplasmic IBs characteristic of IBD (arrows).
Source: RNBW, 2018-2020.

Materials and methods

Ethics committee

This study was approved by the Research, Ethics, and Bioethics Committee of the Fundación Parque Jaime Duque, as well as by the corresponding Subcommittee of Research, Ethics, and Bioethics of the RNBW, in accordance with the Research Policy of the Fundación Parque Jaime Duque (Approval Act No. 005, Project Code 0016, approved on December 6, 2023). In addition, it was approved by the Committee for the Care and Use of Animals in Research (CICUA) of Universidad de La Salle under reference FUA No. 102-2023 (version 03), approved on April 1, 2024.

Study design and location

The study was conducted at the RNBW facilities located within the Fundación Parque Jaime Duque, in the municipality of

Tocancipá, Cundinamarca, approximately 20 km north of Bogotá. Molecular analyses were performed at the José Joaquín Vargas Muñoz Laboratory of Universidad de La Salle and at the Institute of Genetics of the National University of Colombia, both in Bogotá.

Population and samples

The study population consisted of 15 snakes. Sampling was conducted opportunistically and based on convenience, prioritizing individuals that exhibited the greatest number of historical and/or current clinical signs consistent with IBD. This approach was selected because the remaining snakes appeared clinically healthy but were considered suspect cases due to their proximity and potential exposure. Sample collection was carried out between February and June 2024.

The population comprised individuals born at the biopark as well as snakes obtained through seizures and donations. However, the exact geographic origins and lineages of these individuals remain unknown. It is assumed that all snakes originated within the national territory, with the exception of non-native species (Pythonidae).

Blood samples were collected by venipuncture of the ventral coccygeal vein under physical restraint, following all RNBW protocols and parameters for handling, animal welfare, and sample collection established for the species (Divers & Stahl, 2019). A total of 14 individuals (93%) were successfully sampled. The distribution of individuals is presented in table 1.

It is noteworthy that one of the brown boas, BCH1 (*Epicrates maurus*), had to be euthanized, and tissue samples were collected from the liver, brain, heart, muscle, and from abnormal cysts observed in the musculature and skin of the cranial third of the body. No samples could be obtained from one *Boa constrictor* individual (BC6), which cohabited with two conspecifics that were successfully sampled; therefore, the results from the latter are theoretically extrapolatable to the former.

RT-PCR Design

Primer design

We sought a tool to computationally optimize primer selection targeting a conserved region shared among multiple

TABLE 1. Snakes sampled in the study and designation assigned to each individual

Species	Common name	Number of individuals	Study designation
Boa constrictor	Common boa	6	BC1, BC2, BC3, BC4, BC5 y BC6
Eunectes murinus	Green anaconda	1	A
Lampropeltis micropholis	Ecuadorian milksnake	1	FC
Epicrates maurus	Brown rainbow boa	2	BCH1 y BCH2
Python molurus	Indian python	3	P1, P2 y P3
Python curtus	Sumatran short-tailed python	1	PS
Python molurus bivittatus	Burmese python	1	PA

Source: own elaboration.

Reptarenavirus sequences. For this purpose, the ConsensusPrime tool was used (Collatz *et al.*, 2022). As input, we used *Reptarenavirus* S-segment sequences reported in GenBank. The tool was run according to the authors' instructions under default parameters (Collatz *et al.*, 2022). The candidate primers are listed in table 2.

RNA extraction

Blood samples were processed with various reagents for RNA extraction using the Invi-Sorb® Spin Universal Kit. All manufacturer's instructions for viral RNA extraction from liquid samples were followed, with the only modification being an extension of the incubation time of the lysis buffer, RNA carrier, proteinase K, and blood sample mixture from the recommended 10 minutes to 30 minutes. This adjustment was implemented to ensure more efficient lysis and RNA recovery. For tissue samples, all manufacturer's instructions for viral RNA extraction from biopsy tissues were strictly followed.

cDNA synthesis

Complementary DNA (cDNA) was synthesized from the RNA obtained in the previous step using random hexamer primers and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), following the manufacturer's protocol.

cDNA PCR

The cDNA product was subsequently subjected to PCR amplification using

TABLE 2. Primers used in this study, showing 100% identity and cases with 1-2 mismatches according to BLAST. UGV: University of Giessen Virus; ROUTV: Boa Arenavirus NL; RNI: unidentified *Reptarenavirus*

Primer	Sequence	Amplicon size (bp)	100% ID	1–2 mismatches
NP1F	ACATTGGATCAACTCCTCAT	252	• UGV-1	
			• UGV-2	
			• UGV-3	• RNI
NP1R	ATGTTGTCACCCTTTCAAAG		• ROUTV	
			• RNI	
NP2F	ATGTGTCCTGAGGAATTGAT	229	• UGV-1	
			• UGV-2	
			• UGV-3	• RNI
NP2R	GACCAAACAACCCAACATTA		• UGV-4	
			• RNI	

Source: own elaboration.

DreamTaq DNA Polymerase (Thermo Fisher Scientific). A temperature gradient was initially performed to determine the optimal annealing temperature, testing 50.0, 50.7, 52.0, 53.9, 56.3, 58.3, 59.4, and 60.0 °C. All reactions were carried out in a BIO-RAD® C1000 thermal cycler. The initial master mix formulation is shown in Table 3. Throughout the study, specific parameters of the master mix and PCR conditions were modified to optimize amplification, including increasing magnesium concentration, adjusting the amount of cDNA template, and testing different annealing temperatures.

Visualization of PCR products

For the visualization of results, BIO-RAD® Mini-Sub-Cell GT electrophoresis chambers were used. Initially, a 1% agarose gel was prepared and run at 100 V for 60 minutes. Subsequently, the agarose concentration was increased to 2% and the electrophoresis time extended to 75 minutes. The sizes of the amplified products visualized in the gel were then compared with the expected theoretical sizes for each primer set. For this purpose, a molecular size marker with fragments of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1300, and 2500 base pairs (bp) was employed. Additionally, Red Gel stain, which intercalates into DNA and fluoresces under UV light, was used together with a UV transilluminator to visualize the amplified products.

DNA purification and sanger sequencing

The DNA obtained from the amplification of cDNA from selected samples was purified for subsequent Sanger sequencing to confirm the identity of the bands observed in agarose gels. Duplicate master mixes with a final volume of 50 µl, including 6 µl of template, were prepared. The bands visualized in the gels were excised and purified using the QIAquick® Spin Kit (QIAGEN), following the manufacturer's instructions for DNA extraction from gels.

Ten microliters of each purified product were submitted without prior confirmation of DNA concentration (required: 10 pg/µl). However, based on the intensity of the bands observed in the purification

TABLE 3. Initial master mix used for cDNA amplification. The volume of each component and its final concentration are shown

Component	Volume (µl)	Final concentration
Forward and reverse primers	1.25 µl	0.5 pmol
dNTPs	0.5 µl	0.2 mmol
10× Buffer	2.5 µl	1x
DNase-free water	18.25 µl	NA
DreamTaq DNA Polymerase	0.25 µl	0.05 U/µl
cDNA template	1 µl	NA
Total	25 µl	

Source: own elaboration.

confirmation gel, it was assumed that the concentration was adequate. In addition, 5 µl of both primers from the NP1 set, at a concentration of 5 pmol/µl, were also submitted.

In a second round, purified products obtained using the same methodology were analyzed at the Universidad Nacional de Colombia. DNA concentrations were measured with a Nanodrop Lite Plus spectrophotometer (Thermo Fisher Scientific), yielding average values of 10 ng/µl, which were considered optimal for sequencing. Consequently, 10 µl of each purified sample, together with 5 µl of primer NP1F at 5 pmol/µl, were submitted to the Institute of Genetics of the same university for sequencing.

Results and discussion

Two *Boa constrictor* individuals (BC1 and BC2) were initially selected empirically as positive controls, as they exhibited the highest number of clinical signs consistent with the disease. Both presented respiratory symptoms, flaccid paralysis, tremors, ataxia, partial loss of righting reflex, and severe immunosuppression, all associated with IBD. However, the initial PCR results under temperature gradient conditions did not show detectable bands in the agarose gel, which may suggest either a low concentration of viral RNA or a suboptimal reaction.

For this reason, PCR parameters were systematically adjusted to determine the combination that yielded the most reliable amplification. Magnesium concentration, template cDNA volume, and annealing temperature were evaluated. The optimal conditions were determined to be a magnesium concentration of 2.75 mmol, 2 µl of cDNA template, and an annealing temperature of 55.0 °C or 53.9 °C (figure 2).

Additionally, to confirm the absence of cross-reactivity between primers, a negative control (C–) consisting of a reaction without template cDNA was included (figure 2). In parallel, a non-target DNA control of Avian Infectious Laryngotracheitis (CILT) was incorporated to rule out nucleic acid degradation or the presence of reaction inhibitors (figure 2). Regarding primer performance, NP1 primers demonstrated the highest efficiency. This was evidenced by the appearance of amplicon bands matching the theoretical size of the expected products, whereas NP2 primers did not yield consistent bands (figure 2). Using NP1 primers, two recurrent bands were observed across all snake samples belonging to the subfamily Boinae: one of approximately 220–250 bp, corresponding to the theoretical size expected for *Reptarenavirus* amplification with NP1 (252 bp), and another of ~500 bp (figure 2). Based on these findings, the 220–250 bp bands were suspected to indicate the presence of *Reptarenavirus*, which prompted the extension of sampling to the remaining individuals in the population.

Due to the suspected presence of the virus based on the bands observed in snakes of the subfamily *Boinae*, the 220–250 bp and ~500 bp bands were purified for Sanger sequencing. Blood from *Boa constrictor* BC2 and a liver sample obtained at necropsy from the chocolate boa BCH1 were used. The chromatograms obtained showed mixed, low-intensity signals, suggesting either contamination or the presence of multiple DNA sequences within the same band. Ultimately, BLAST analysis did not yield significant alignments with existing databases.

Subsequently, to improve band separation and rule out the possibility of co-migrating amplicons of similar size,

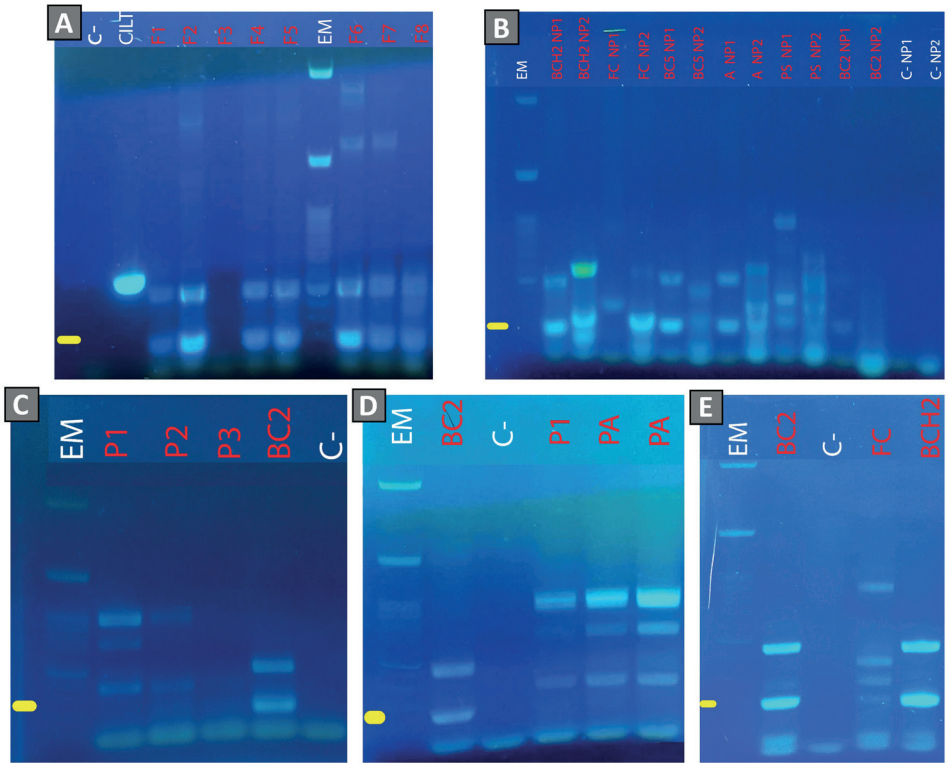

FIGURE 2. Agarose gels of PCR-amplified cDNA. In all gels, a molecular weight marker (EM) is shown. The yellow dash indicates the theoretical size of 250 bp expected for the presence of *Reptarenavirus.* (A) PCR results of samples from the experimental group designated F, performed with 2.75 mM $MgCl_2$ and an annealing temperature of 53.9 °C. This group included samples obtained from the necropsy of the chocolate boa (BCH1) and blood from Boa constrictor individuals (BC), labeled as follows: 1–Heart, 2–Liver, 3–Cyst, 4–Muscle, 5–Brain, 6–Blood BC2, 7–Blood BC3, 8–Blood BC4. A negative control (C–) and a non-target cDNA control of Avian Infectious Laryngotracheitis (CILT) were also included. (B) PCR results of blood samples from the chocolate boa BCH2, Boa constrictor BC5 and BC2, anaconda (A), Sumatran python (PS), and false coral snake (FC). In this experimental group, both NP1 and NP2 primers were tested, each with their respective negative controls (C–). (C, D, E) PCR results of samples from Indian pythons P1, P2, and P3, Boa constrictor BC2, chocolate boa BCH2, albino python (PA), and false coral snake (FC), all amplified with NP1 primers using 2.75 mM $MgCl_2$ and an annealing temperature of 53.9 °C.

Source: own elaboration.

electrophoresis parameters were optimized by using 2% agarose, which provided better resolution of the observed bands. At this stage, dimethyl sulfoxide (DMSO) was also tested, as it is known to enhance PCR specificity and stability. The addition of 1 µl increased band intensity without altering the reactions. Based on these results, new PCR assays were performed with blood samples from BC2, BCH2, and A1, as well as liver tissue from BCH1, under the following conditions: addition

of DMSO, 3 mM magnesium, annealing temperature of 55 °C, 2% agarose, and electrophoresis at 100 V for 75 min. Bands of 220–250 bp and ~500 bp were again detected, along with additional bands, with similarities noted among boas and differences compared to pythons (figure 3). The ~500 bp band proved difficult to purify (figure 3), restricting extraction to the 220–250 bp bands and a 300 bp band from A1. Following gel confirmation (figure 3), these products were submitted for Sanger sequencing using NP1F primers. The sequencing results produced chromatograms of higher purity and stronger signal compared to those obtained previously (figure 4).

Based on the sequencing results, the 220–250 bp bands obtained from all samples were analyzed. Visual inspection of the chromatograms indicated high similarity among these fragments, and subsequent analysis confirmed that they shared more than 92% identity. This suggests that the 220–250 bp bands represent the same DNA sequence across different individuals. However, BLAST analysis did not reveal significant alignments with available databases. In contrast, the 300 bp sequence obtained from the anaconda showed partial alignment with messenger RNA from Boinae species (70–88% identity). These findings led us to hypothesize that the consistently observed 220–250

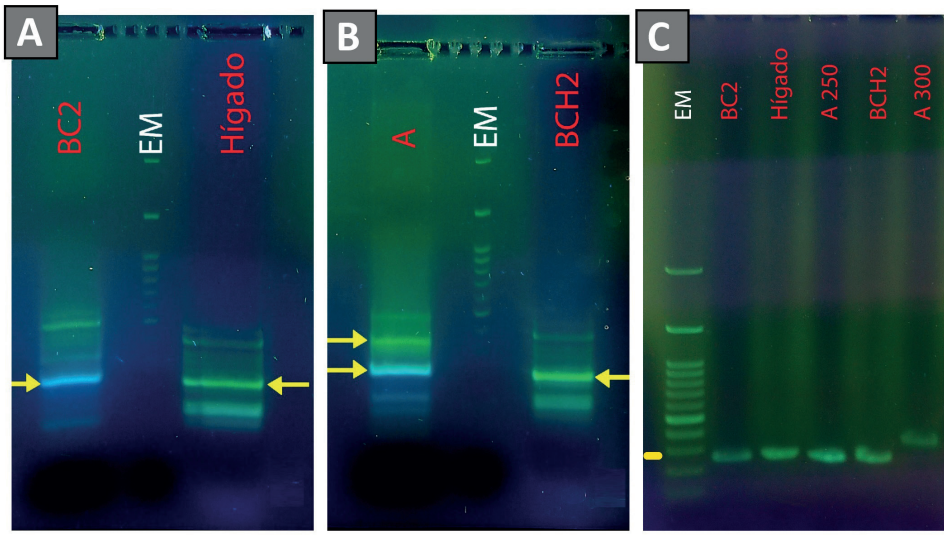

FIGURE 3. A molecular ladder (EM) is included in all gels. The yellow marker indicates the theoretical size of 250 bp, corresponding to the expected amplicon of *Reptarenavirus*. (A, B) Agarose gels showing extraction and purification of cDNA, with the purified bands marked by arrows. PCR amplicons were obtained from cDNA of blood samples from Boa constrictor BC2, anaconda A, chocolate boa BCH2, and liver tissue from the necropsy of chocolate boa BCH1. (C) Agarose gel confirming DNA purification, where distinct and well-defined bands are observed in each lane. In the case of the anaconda A sample, both the 250 bp and 300 bp bands are indicated.

Source: own elaboration.

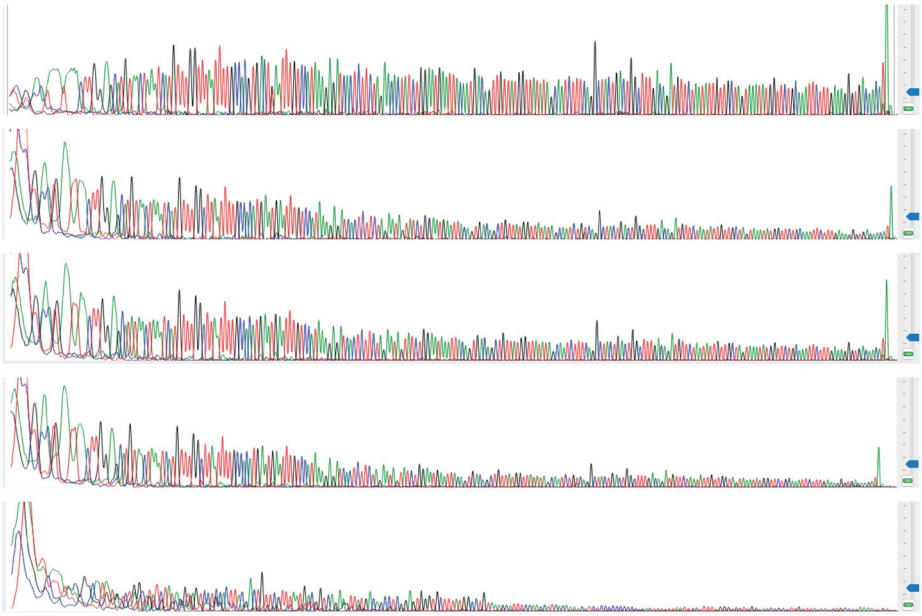

FIGURE 4. Chromatograms from Sanger sequencing reactions. From top to bottom: 220–250 bp band from the Boa constrictor BC2 sample, 220–250 bp band from the anaconda sample, 220–250 bp band from the chocolate boa BCH2 sample, 220–250 bp band from the liver sample obtained at necropsy of the chocolate boa BCH1, and 300 bp band from the anaconda sample.

Source: own elaboration.

bp bands may correspond to amplified mRNA sequences of snake origin that are not yet reported in current databases. Supporting this hypothesis, all individuals from the Boinae subfamily exhibited the same two bands (~220–250 bp and ~500 bp), which were absent in snakes outside this group. Conversely, all Pythonidae individuals consistently displayed highly similar bands—most prominently at 300, 700, and 1000 bp—that were not observed in Boinae snakes. In the false coral snake, the detected bands did not show similarities to those observed in either the Boinae or Pythonidae groups.

The specificity of the primers used in this study can therefore be questioned, as the preliminary results obtained were inconclusive. With regard to their design, the primers were deemed appropriate because they were developed from sequences within the S segment, which encodes the GP and NP genes and represents the most suitable target for Reptarenavirus detection (Argenta *et al.*, 2020; Hetzel *et al.*, 2021). However, although primers NP1 and NP2 showed alignment in PRIMER-BLAST primarily with the NP gene of species such as *R. giessenae* (University of Giessen Virus: UGV 1, 2, and 3) and *R. rotterdamense* (ROUT Virus: Boa Arenavirus NL), they did not align across the full diversity of *Reptarenaviruses* reported in the literature and databases, which is considerable (Hetzel *et al.*, 2021; Stenglein *et al.*, 2015). Given that the

assay did not yield conclusive evidence of *Reptarenavirus* detection, we conclude that the RT-PCR methodology employed here is not currently reliable, particularly in the absence of prior knowledge of the specific viral type or species circulating in snakes from the wildlife park. Although the observed bands corresponded to the expected amplicon size, these results should be regarded as false positives. For this reason, in the absence of a positive control, we recommend verifying band identity using an alternative method such as Sanger sequencing, as applied in this study. Even when positive controls are available, sequencing remains advisable due to the extensive genetic diversity of *Reptarenaviruses* and the experimental (rather than diagnostic) status of current PCR assays, with several "positive" results later disproven by sequencing (Hyndman *et al.*, 2019). To address the challenge posed by this high diversity, some studies have first conducted *de novo* sequencing of random samples from a population, followed by viral isolation and the development of RT-PCR assays specifically tailored to the *Reptarenavirus* types and species circulating in that population. These targeted assays can then be applied more reliably for large-scale screening (Argenta *et al.*, 2020; Baggio *et al.*, 2023; Thiele *et al.*, 2023).

Considering the above, several hypotheses can be proposed regarding the problem observed in the snakes from the RNBW. One possibility is that the snakes no longer harbor the virus but may have carried it in the past, as recent reports suggest that some *Boa constrictor* individuals can experience IBD subclinically and subsequently clear the infection (Dervas, 2024; Hetzel *et al.*, 2021; Thiele *et al.*, 2023). Another possibility is that the snakes are infected with a *Reptarenavirus* variant not detected by the RT-PCR assay used, given the extensive genetic variability of these viruses (Stenglein *et al.*, 2015) and the limited alignment of the primers with the full viral diversity currently available in databases. A further explanation is that blood samples may not represent the most suitable material for viral detection. In agreement with previous studies, higher viral loads are typically found in organs such as the liver, pancreas, and nervous tissue, while viremia is not consistently detectable in infected individuals (Baggio *et al.*, 2023; Moreira, 2010). This suggests that molecular testing should ideally be performed during peaks of clinical symptomatology. Finally, it is also possible that the snakes are affected by another virus, such as a member of the *Paramyxoviridae* family—particularly Ferlaviruses—which can produce clinical signs similar to IBD and are also associated with cytoplasmic inclusion bodies (Divers & Stahl, 2019; O'Rourke & Lertpiriyapong, 2015).

These results highlight the need for more precise and diversified methodologies for the detection of different *Reptarenaviruses*. This includes the use of a greater number of primers or assays such as multiplex PCR to simultaneously detect multiple types and species of *Reptarenaviruses* (Baggio *et al.*, 2023). Furthermore, the incorporation of positive controls is crucial, as they allow faster and simpler evaluation and standardization of newly designed PCR assays, although such controls were not available at the time of this study. Additional tools, such as real-time PCR, would enable in vivo quantification of viral genetic material in each sample. Finally, de novo sequencing and metatranscriptomic approaches could prove more effective in addressing and detecting the extensive viral diversity found in reptiles such as snakes.

As a final recommendation, whenever individuals in a snake population present neurological signs and evidence of cytoplasmic inclusion bodies in blood samples or biopsies, they should be maintained under indefinite quarantine or considered for euthanasia to protect the rest of the population. Both *Reptarenavirus* and Paramyxovirus (Ferlavirus) infections should be considered potential etiological agents, and efforts should be made to confirm the diagnosis using a robust molecular technique such as sequencing. Moreover, since the reptile mite (*Ophionyssus natricis*) has been proposed as a potential vector of the disease, strict hygiene and deparasitization protocols in snake enclosures are strongly recommended. In cases of mite infestation, a single oral administration of afoxolaner at a dose of 2.5 mg/kg has been demonstrated to be effective for mite elimination while remaining safe for snakes (Mendoza-Roldan *et al.*, 2023).

Conclusions

The RT-PCR assay designed in this study did not detect Reptarenavirus in the analyzed samples, and uncertainties remain regarding the identity of the bands observed. Therefore, this assay is not suitable for routine use in detecting Reptarenaviruses. For future studies, the inclusion of a positive control is recommended whenever available, as it facilitates the evaluation of the assay and its subsequent application. Nonetheless, band identity should always be confirmed by sequencing to avoid false positives.

With respect to primer design, it is concluded that the BLAST tool should be used as a guide to evaluate primer specificity; however, it must be considered that BLAST comparisons are limited to sequences reported in databases, which may not represent the full range of sequences present in a sample (e.g., host messenger RNA or DNA sequences not yet reported).

Finally, the health issues affecting snakes at the RNBW could be explained by several possible scenarios; therefore, further studies are required. These should incorporate a broader panel of primers and positive controls, or, when available, more robust diagnostic methods (e.g., de novo sequencing, multiplex PCR, qPCR, viral isolation, and direct observation). In addition, a greater diversity of sample types (biopsies, hemoglobin-free serum, urates, feces) should be included, while also considering alternative etiological agents such as Paramyxoviruses (Ferlaviruses).

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Conflict of interest

The authors declare no conflicts of interest.

Use of artificial intelligence

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