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> Ogata N. (2003a).
> Ogata N. (2003b).

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Comparison of a capture ELISA and Intradermal test for diagnosis of Bovine Tuberculosis in Mexico

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ABSTRACT

Objective: A comparative study was conducted to evaluate two immunodiagnostic techniques capture ELISA and the intradermal test for detecting bovine tuberculosis, aiming to establish the optimal test for early diagnosis in cattle.

Design/Methodology/Scope: Initially, the intradermal test was used as a screening tool in cattle, after which reactor animals underwent the comparative cervical test. Reactor animals (positive to the intradermal test) were then evaluated using a capture ELISA for bovine IFNγ. Intradermal testing is widely used in Mexico; however, its application presents several limitations. Identifying a faster and more accessible diagnostic test would significantly enhance disease control efficiency.

Results: The prevalence of bovine tuberculosis detected by the intradermal test was 4.9%, whereas the capture ELISA test identified 8.95%, showing a significant difference in the identification of positive cases between the two methods. The likelihood ratio met the standard parameters (LR+ >10 and LR− <0.5), confirming that the capture ELISA test for bIFNγis a valuable diagnostic tool. Additionally, the capture immunoassay detected a higher number of infected cattle compared to the intradermal test.

Conclusions/Limitations: The capture ELISA technique for bovine IFNγ proves to be a more effective diagnostic test. However, further studies are required, including a larger study population and expanded geographical coverage within the country.

Keywords: Bovine tuberculosis, Interferon gamma, Capture ELISA, Tuberculin skin test, sensitivity, specificity.

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INTRODUCTION

Bovine tuberculosis is a chronic infectious disease caused by *Mycobacterium bovis*, an acid-fast bacterium capable of infecting a wide range of hosts, including humans, making it a significant public health concern. In cattle, the disease leads to reduced body condition, decreased milk production, confiscation of affected organs, and quarantine

of infected animals, ultimately limiting livestock trade. These factors contribute to global economic losses exceeding $3 billion annually (Pérez-Morote, 2020). Bovine tuberculosis is present worldwide; however, its prevalence varies significantly between developed and developing countries. One of the primary factors influencing disease persistence is the ability to achieve timely diagnosis (Gormley, 2018; Lombard, 2021; Malama, 2013). Among the classical diagnostic methods, the intradermal tuberculin skin test is widely used. This test is based on detecting a type IV hypersensitivity immune response, requiring the inoculation of an antigen that stimulates memory lymphocytes, triggering a localized inflammatory reaction at the injection site, which becomes visible 72 hours post-inoculation (Carrisoza-Urbina J, 2019; Wangoo A, 2005). Currently, the most commonly used antigen for tuberculin testing is the purified protein derivative (PPD), obtained from secretion products of *M. bovis* strain AN5. The tuberculin testing protocol begins with a simple caudal fold test, in which only bovine PPD is administered. If the test is positive, a comparative cervical test must be performed to differentiate between reactions caused by *Mycobacterium avium* (NOM-031-ZOO-1995). There are also immunoassay-based diagnostic tests that detect key molecules involved in the immune response against *M. bovis*. These assays use blood samples and mycobacteria-derived antigens (such as PPD) to assess memory immune responses, primarily mediated by Th1 lymphocytes, which can be quantified through INF-γ detection (NOM-031-ZOO-1995; Ryan TJ, 2000). The first commercially available IFN-γ detection test for bovine tuberculosis was BOVIGAM® (Thermo Fisher, USA), which has been widely used in developed countries (Dean GS, 2005; Lahuerta-Marin A, 2015; Pollock JM, 2006; Praud A, 2015). However, high costs and lengthy import processes have driven the development of more affordable and accessible alternatives, particularly for developing countries, including various immunoassays that detect bovine INF-γ production, such as the one proposed in this study. Intradermal tests are widely used in Mexico; however, they present several practical limitations. These include the need for trained and certified personnel, as well as a minimum of 72 hours to obtain results. Consequently, the implementation of immunoassays could provide a faster and more accessible diagnostic approach for tuberculosis. In this study, the tuberculin test was compared against a specific capture ELISA for IFN-γ to evaluate the diagnostic potential of both methods in detecting bovine tuberculosis in cattle from various regions of Mexico.

MATERIAL AND METHODS

Ethical statement

This study was approved by the Institutional Committee for the Care and Use of Experimental Animals of the Universidad Nacional Autónoma de México, FES Cuautitlán (SICUAE-DC-2020/4-1), in compliance with the NOM-062-ZOO-1999 regulations.

Study animals

A random selection was conducted based on inclusion criteria, considering dairy, beef, and dual-purpose herds. The production system was batch-based and optimized according to the productive stage. Cattle were fed under a mixed system, consisting of

grains and coarse forages, with minimal concentrate supplementation and free access to water. A total of 384 cattle, aged 1 to 12 years, were included in the study, with their general characteristics detailed in Table 1. During the testing period and until the final determination of infection status, all cattle were kept in isolation. Animals confirmed as infected by any of the diagnostic tests were culled.

Intradermal test (Tuberculin skin test)

The two intradermal tests used in this study were performed by a licensed veterinarian. All animals included were pre-evaluated based on their clinical history. Cattle with records of vaccination, deworming, or tattooing within 30 days prior to the study were excluded.

Simple flow test

On day 0 of the experiment, the test was used as a general screening tool for the population. Proper livestock handling was ensured, and the skin fold thickness was measured at the center of the shaved area using a cutimeter. Subsequently, 0.1 mL of bovine PPD was intradermally inoculated into the caudal fold, with results interpreted 72 hours post-inoculation. Animals displaying swelling and/or other significant reactions were classified as reactor animals and were immediately subjected to the comparative cervical test.

Comparative Cervical Test

The comparative cervical test was performed 60 days after the caudal fold test. A total of 0.1 mL of avian PPD was inoculated into the upper middle third of the neck, while 0.1 mL of bovine PPD was administered in the lower neck area.

Bovine blood samples

Blood samples were collected in sodium heparin tubes (Heparin, BD®) via coccygeal and/or jugular venipuncture. Samples were transported at room temperature, protected from light exposure. A total of 250 μL of whole blood was placed in 96-well plates (Costar,

Table 1. General characteristics of the evaluated bovine study population.

Region	Number of bovines	*Bos indicus*	*Bos taurus*	Sex	
				Female	Male
State of Mexico (San Sebastián, Xhala, Cuautitlán Izcalli)	164	2	315	65	55
State of Mexico (San Sebastián, Teoloyucan)	62	8	13	25	30
Tabasco (Emiliano Zapata)	14	1	26	50	70
Querétaro (Colón, Querétaro Arteaga, Mexico)	144	2	13	69	20
Total	384	15	369	209	175

The different geographical regions of the animals were considered.

Corning). Cell stimulation was performed using 40 μg/well of bovine PPD (Pronabive, Mexico) for 24 hours at 37 °C. Plasma was then recovered by centrifugation at 3,000 rpm for 20 minutes, transferred into sterile tubes, and immediately evaluated.

Production of recombinant antigen (IFNγ)

The IFNγ gene was reamplified and cloned into the pET11a protein expression system (Novagen, Darmstadt, Germany) at the NdE1 and BamH1 sites, incorporating a 6x histidine tag at the carboxy terminus. Protein purification was carried out under native conditions using a Ni^{2+}-NTA affinity column (Qiagen, Chatsworth, CA, USA), following the manufacturer's instructions.

Obtaining monospecific polyclonal antibodies used in the capture ELISA technique

Specific antibodies against bIFNγ were generated in two species for use as primary and/or detection antibodies (anti-rabbit) and as secondary antibodies (anti-rabbit) for detection and quantification. The recombinant bovine IFNγ protein was utilized in the capture ELISA assay to measure bIFNγ production from bovine plasma.

Standardization of the capture ELISA technique for bovine IFNγ

The detection and quantification of anti-bovine IFNγ antibodies were performed using the IFNγ capture assay on serum samples from this study. The standardization of this technique and the production of the recombinant bovine IFNγ antigen were donated by the Laboratory of Molecular Genetics, UNAM, FMVZ.

Statistic analysis

The analysis included categorical data based on the positive or negative responses to both the intradermal test and the capture ELISA. An analysis of variance (ANOVA) with log-transformed data was performed to evaluate observed responses and conduct a comparative analysis between tests.

Statistical analysis was conducted using IBM® SPSS Statistics® Version 25 (IBM Corp.). Significant differences between diagnostic tests were determined using the McNemar test. The level of agreement between tests was assessed using Cohen's Kappa coefficient, while the likelihood ratio was also evaluated. Additionally, a comparative analysis of diagnostic tests was performed, measuring sensitivity (SE), specificity (SP), and positive predictive value (PPV).

RESULTS AND DISCUSSION

Intradermal test

All animals were evaluated using both intradermal test variants (CS and CC), with interpretation conducted by the same veterinarian. A total of 71 animals tested positive in the comparative cervical test, while 33 samples showed increased bovine IFNγ (bIFNγ) production in the capture ELISA test, a value compared against the test's negative controls. Approximately 3.1% of the animals were identified as positive in the comparative cervical

test, a finding consistent with previous reports by other authors (Perea-Razo, 2018; SENASICA, 2024).

Preliminary test to determine the sensitivity of the final bIFNγassay

Rabbit IgGs, at optimal dilutions of 4 μg and 8 μg, enabled clear discrimination between positive and negative samples. The minimum concentration of recombinant bIFNγ was 12 ng, while the maximum reached 200 ng. Serial double dilutions of bIFNγ were applied, as illustrated in Figure 1, showing IgG reactivity towards bIFNγ. These results confirm the proper functionality of the system. This assay allowed for the estimation of sensitivity values while also serving as a control curve within the system, providing both qualitative and quantitative data on bIFNγ detection in tuberculin-positive and negative animals.

Test with problem sera

Under the conditions established for the capture ELISA assays using a defined amount of rabbit IgG (4 μg) and guinea pig IgG (8 μg) bIFNγ was detectable within a range of 200 ng to 12 ng. The entire cattle population was evaluated, from which 22 serum samples were selected (12 positive and 10 negative for TB) based on reactivity to the tuberculin skin test (CC variant). Additionally, a positive control with varying concentrations of recombinant bIFNγ and a negative control (sample without IFNγ) were included, with each serum sample tested in duplicate. The assay results are presented in Figure 2, Population Evaluated Using Different Diagnostic Tests.

The commercial BOVIGAM test detects bIFNγ at a sensitivity of 80 pg/mL, which directly impacts the test's sensitivity when compared to the SE of this study (WOAH, Procedure for Registration of Diagnostic Kits, 2018).

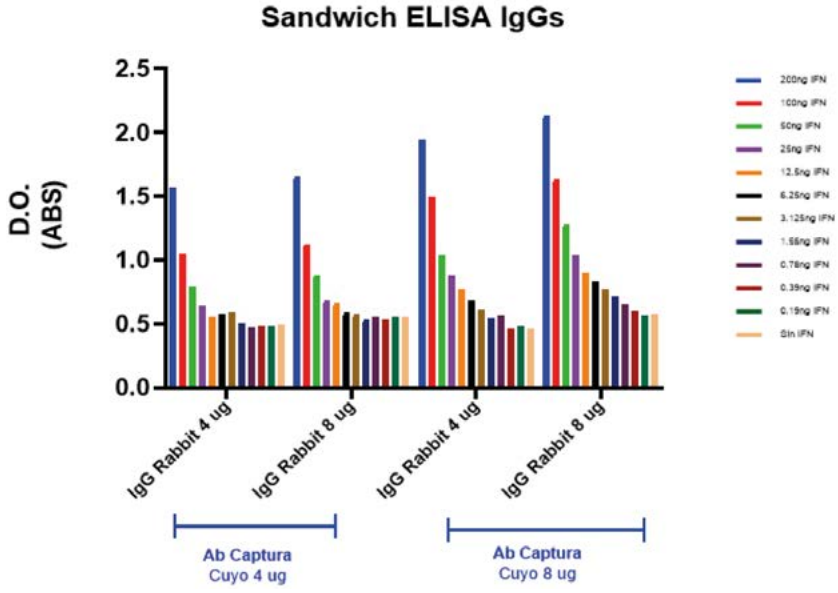

Figure 1. Reactivity of IgGs towards bovine gamma IFN. Sensitization of a rabbit and guinea pigs IgGs towards bovine IFNg, using monospecific polyclonal antibodies.

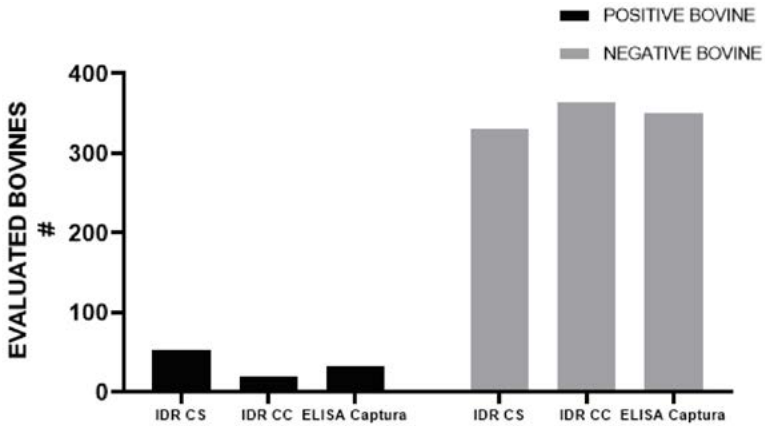

Figure 2. Total population evaluated towards different tests for the Diagnosis of Bovine Tuberculosis. Reactor animals ■ and animals negative ■ for 2 variants of tuberculinization are shown: simple caudal intradermorreaction (IDR-CS), cervical comparative reaction (DR-CC) and the capture ELISA test.

Capture ELISA Assay Standardization

The standardization of this assay enabled the evaluation of the capture ELISA test in comparison with the tuberculin skin test, identifying concordant elements between both methods and correlating the increase in bIFNγ levels in serum samples from bovines reacting to the comparative cervical test (Figure 3, Capture ELISA for the Quantification of bIFNγ). The rise in this cytokine is indicative of an active *Mycobacterium bovis* infection. Experimental and clinical data suggest that IFN-γ plays a crucial role in host defense against *Mycobacterium tuberculosis*. Deficiencies in IFN-γ production are considered a significant risk factor for *M. tuberculosis* infection and disease progression in humans (Dean GS, 2005).

Currently, global diagnostic strategies aimed at eradicating bovine tuberculosis have demonstrated that tests assessing cellular immunity such as this assay significantly reduce

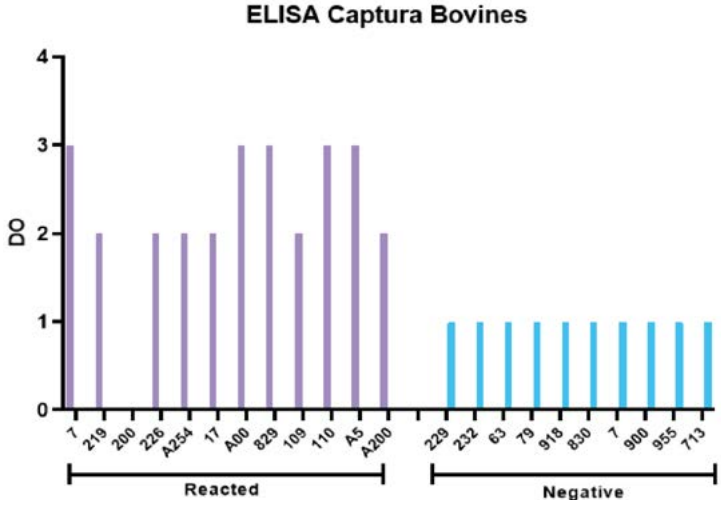

Figure 3. Capture ELISA based on the detection and quantification of bovine gamma IFN. The evaluation was carried out based on the selection of plasma from bovines that reacted to comparative cervical tuberculinization and from negative animals (n=12); The total number of samples is not included (n=384).

the presence of infected animals on farms (Alcaraz-López OA, 2016; Clegg TA, 2011; Clegg TA, 2017; Müller B, 2015).

Of the 384 cattle evaluated, 52 were identified as reactors to the intradermal screening test using the single caudal fold test. Among these, 19 animals tested positive in both the intradermal test and the capture ELISA assay. When comparing the effectiveness of both diagnostic methods in detecting positive animals, the intradermal test identified 4.94% of cases, whereas the capture ELISA for bIFNγ detected 8.95%. The discrepancy between the two tests may be attributed to factors such as sample handling during shipment, the time elapsed between collection and processing, and the low concentration of viable IFNγ in serum samples. According to WOAH (2018), the global prevalence of bovine tuberculosis varies across different geographic regions. In Mexico, data from SENASICA indicate that 86.27% of the national territory has reached the eradication phase, with a prevalence of less than 0.5% (SENASICA, 2024). However, in dairy cattle, an increase in prevalence of up to 16% has been reported (Perea-Razo, 2018).

The comparative analysis of diagnostic tests revealed that the capture ELISA for bIFNγ demonstrated 68% sensitivity, 95% specificity, a positive predictive value of 42%, a negative predictive value of 98%, and an overall prevalence of 0.05%. In contrast, commercial capture ELISA assays utilizing monoclonal antibodies, such as BOVIGAM, have reported a sensitivity range of 84-93% and specificity between 94-100% (Wood, 2001). The lower sensitivity observed in this study may be associated with the use of polyclonal antibodies in the ELISA assay; however, the test maintains high specificity. Additionally, the BOVIGAM test detects lower levels of bIFNγ compared to the ELISA proposed in this study. As noted by previous authors, tuberculosis diagnosis is influenced by several factors, including: (1) inadequate sample quantity and volume, (2) utilization of the same sample for multiple diagnostic tests, which may lead to poor microorganism distribution, (3) sample location, and (4) inefficiencies in sample processing techniques worldwide (Carrizosa-Urbina, 2015 & 2019). The Kappa coefficient (κ) analysis yielded a value of 0.49, with a 95% confidence interval (0.39-0.58), indicating a moderate level of agreement between the two diagnostic tests. Statistical analysis was performed using a non-parametric test for paired nominal variables with a binomial distribution, assessed through McNemar's Chi-square test ($\chi^2=6$, $p=0.0143$, $p<0.05$). This result provides evidence of a statistically significant difference in the identification of positive cases between the two methods. According to Gilchrist, as cited by Carrizosa-Urbina (2015), a Kappa value between 0.40 and 0.75 suggests moderate agreement; therefore, the obtained value of 0.49 supports an acceptable level of concordance between the tests (Gilchrist, 2009; Carrizosa-Urbina, 2015).

The evaluation of the likelihood ratio (LR) revealed an LR+ of 13.87 and an LR− of 0.33, placing the tests within the established parameters (LR+ >10 and LR− <0.5). This indicates that the capture ELISA test for bIFNγ is a useful diagnostic tool, as an individual infected with *Mycobacterium bovis* is 13 times more likely to test positive through the capture ELISA than a non-infected individual (Figure 4, Fagan Nomogram for the Likelihood Ratio of the Evaluated Tests).

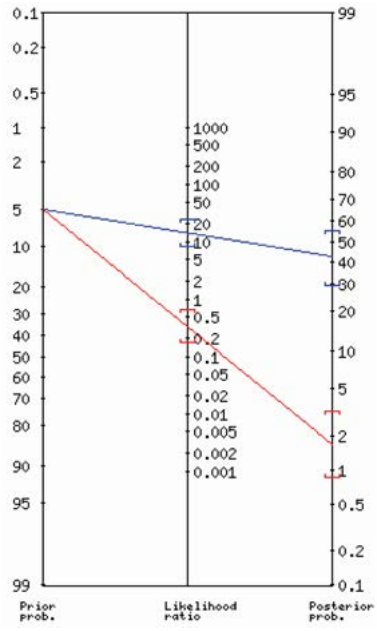

Figure 4. Fagan Nomogram for the Likelihood Ratio of the Evaluated Tests. This analysis aims to determine the post-test probability of positive results in both the intradermal test and the capture ELISA, thereby validating the plausibility of the obtained results within the evaluated cattle population (n=384).

CONCLUSIONS

This study represents the first strategic approach to implementing a capture ELISA test for determining bIFNγ levels in bovine serum samples. The use of specific polyclonal antibodies targeting bIFNγ rather than commercial monoclonal antibodies was successfully validated within the capture ELISA assay. This approach leveraged recombinant antigen technology and hyperimmune sera as a diagnostic alternative for bovine tuberculosis. Biological samples were obtained from beef cattle, dairy cattle, and dual-purpose herds originating from at least three different geographic regions of Mexico. Once validated across multiple samples, the bIFNγ capture ELISA assay could be effectively applied to the control and eradication of bovine tuberculosis in Mexico. This study demonstrated that the concordance between the intradermal test and the capture ELISA immunoassay for diagnosing bovine tuberculosis at the national level is satisfactory. Furthermore, our results align with the current prevalence rates reported by national zoosanitary authorities regarding infection in Mexican herds. The development of domestically produced diagnostic tests could significantly reduce import costs and improve accessibility.

DATA AVAILABILITY

The extensive data presented in this study are available upon request from the corresponding author.

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CONFLICT OF INTERESTS

None of the authors declare any conflict of interest regarding this publication.

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Caseous lymphadenitis in small ruminants. A review

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ABSTRACT
Objective: The main articles published on *Caseous lymphadenitis* (CL) in small ruminants were analyzed, consulting Google Academic, Scopus, and PubMED web pages available on the Internet.
Results: CL infection is an endemic disease, mainly in the herds of Mexico. There are strategies to prevent CL, although vaccines are not widely efficient. The clinical signs are evident, and CL can be prevented.
Study Limitations/Implications: Few studies in Latin America have described the etiology, diagnosis, and prevention of CL.
Findings/Conclusions: CL is a common disease in sheep and goat flocks. The disease affects the productivity of animals. However, there are good strategies for diagnosis, treatment and prevention.

Keywords: *Caseous lymphadenitis*; small ruminants; diagnosis; control; prevention.

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INTRODUCTION

Caseous lymphadenitis (CL) is a chronic infectious disease characterized by abscess formation and two clinical manifestations: visceral (subclinical) and cutaneous (clinical). The etiological agent, *Corynebacterium pseudotuberculosis*, is widely distributed in sheep and goat production areas, leading to significant economic losses (Szwako & Ortíz, 2014). In Canada, 36% of goat carcasses were found to have abscesses caused by *C. pseudotuberculosis*, which was associated with a substantial increase in carcass trimming and confiscation (Arsenault *et al.*, 2003). CL negatively impacts animal health and productivity, leading to systemic deterioration, reproductive disorders, mastitis, skin lesions, reduced carcass quality, and mortality (Alves *et al.*, 2018; Araujo *et al.*, 2020). The incidence of CL increases with age, with morbidity rates reaching up to 40% of the herd and mortality rates up to 4% in goat populations (Debien *et al.*, 2013). CL is characterized by the formation of abscesses

within the subcutaneous lymphatic system and the abdominal and thoracic cavities. The causative bacterium is a facultative intracellular pathogen that induces granulomatous hypersensitivity reactions (Delgado *et al.*, 2015). CL presents as a chronic pyogenic infectious disease with two forms: clinical, characterized by hypertrophy and abscesses of superficial lymph nodes, and subclinical, affecting internal organs and/or lymph nodes within the abdominal and thoracic cavities (Barnabé *et al.*, 2019). External CL lesions initially manifest as abscesses that later develop into pyogranulomas, predominantly within the superficial lymph nodes. Hair loss over affected areas occurs due to the dermonecrotic action of *C. pseudotuberculosis* exotoxins, while atrophy results from pressure exerted by the overlying skin. Visceral CL lesions are not clinically detectable but contribute to progressive weight loss, respiratory disorders, and ruminal tympany (Oreiby, 2014). The presence of one or more superficial swellings anatomically linked to lymph nodes strongly suggests CL and requires laboratory confirmation. Identification of *C. pseudotuberculosis* is achieved through bacterial culture of isolates obtained from lesions (Bastos *et al.*, 2011). For bacteriological diagnosis, lesion contents must be aseptically collected after thorough disinfection of the granuloma with an antiseptic solution. Proper disinfection prior to sampling is critical, as contamination with saprophytic bacteria may obscure *C. pseudotuberculosis* growth in culture media (Oreiby, 2014; Harwood & Mueller, 2018). The eradication of CL remains challenging due to the rapid transmission of the disease once introduced into a herd (Oreiby *et al.*, 2013). This review aims to provide an updated overview of *Corynebacterium pseudotuberculosis*, including its pathogenesis, diagnosis, control, prevention, and treatment in sheep and goats. To achieve this, an extensive literature search was conducted to compile recent findings on *Caseous lymphadenitis* in small ruminants.

Etiology

Corynebacterium belongs to the suborder *Corynebacterineae*, which includes the families *Corynebacteriaceae*, *Mycobacteriaceae*, and *Nocardiaceae*. This group shares common characteristics, including a cell wall primarily composed of peptidoglycan, arabinogalactan, and mycolic acids, as well as a high guanine-cytosine (G+C) content in the genome (Bastos *et al.*, 2012; Parise *et al.*, 2021). *Corynebacterium pseudotuberculosis* is a pleomorphic, Gram-positive bacterium that is facultative anaerobic and intracellular, with a size ranging from 0.5 to 0.6 μm (Díaz *et al.*, 2014; De Oliveira *et al.*, 2018). This bacterium infects both animals and humans, causing chronic diseases such as mastitis, *caseous lymphadenitis* in small ruminants, ulcerative lymphangitis in horses, and necrotizing lymphangitis in humans (Shi *et al.*, 2019). *C. pseudotuberculosis* strains are classified into two biovars. Strains capable of nitrate reduction belong to biovar equi, predominantly isolated from horses and cattle, while strains that do not perform nitrate reduction are classified as biovar ovis, frequently found in sheep and goats (Araujo *et al.*, 2016; Auad *et al.*, 2017). The genetic determinants of *C. pseudotuberculosis* virulence remain poorly characterized. A total of 19 identified proteins and approximately 1,230 genomic sequences have been studied, most of which are associated with virulence factors or act as positive modulators of genes encoding pathogenic virulence factors (D'Afonseca *et al.*, 2008). Among the most significant genes is PLD, which encodes an exotoxin likely involved in bacterial dissemination. The *FagA*,

FagB, *FagC*, and *FagD* genes facilitate iron acquisition, a critical factor for bacterial survival within the host. Heat shock proteins (HSPs) are known to elicit humoral and cellular immune responses, granting them immunogenic properties. The RecA protein plays a role in homologous recombination and DNA repair, while the rpoB gene encodes the β subunit of DNA-dependent RNA polymerase. Notably, evidence suggests that rpoB is also associated with rifampin resistance (D'Afonseca *et al.*, 2008; Galvão *et al.*, 2017; Marques da Silva *et al.*, 2021; Meng *et al.*, 2023).

Epidemiology

Corynebacterium pseudotuberculosis is a microorganism that is difficult to control due to its poor response to treatment, persistence in the environment, and the limited availability of diagnostic tests to detect subclinically infected animals (Abebe & Sisay, 2015). The incubation period ranges from 2 to 8 months, with detection typically occurring only when abscesses become visible in the superficial lymph nodes (Firdaus *et al.*, 2017; Harwood & Mueller, 2018). However, the epidemiology of CL varies across different animal production systems, with higher incidence rates reported in regions practicing intensive farming (Abebe & Sisay, 2015). In Brazil, prevalence rates of 93.88% have been reported in sheep, while goats showed a prevalence of 87.8% (De Farias *et al.*, 2019; Alves *et al.*, 2020b). In Egypt, prevalence in sheep was recorded at 83.65% (Selim *et al.*, 2021). In goats in Italy, a prevalence of 49.34% was reported (Bettini *et al.*, 2022). In Mexico, a prevalence of 33% was found in sheep and goats in Jalisco (Hernández *et al.*, 2019). Several risk factors contribute to high disease prevalence, including purebred status, lack of segregation by sex or age, failure to treat abscesses before spontaneous rupture, and delayed culling of seropositive animals (De Farias *et al.*, 2019; Alves *et al.*, 2020a; Alves *et al.*, 2020b). Surface lipids, known as mycolic acids, confer resistance to the bacterium by preventing drug penetration (Sá *et al.*, 2018). This microorganism can survive in the environment for extended periods: up to 8 months in soil within pus, 4 months in contaminated pens, and 2 months in straw, hay, and fomites. Low temperatures and high humidity further prolong its survival (Zeru & Kahsay, 2014; Constable *et al.*, 2017).

CL presents in two distinct forms: (A) The superficial form, characterized by abscesses in the superficial lymph nodes and subcutaneous tissue, with the mandibular (36.8%) and parotid (31.5%) lymph nodes being the most affected (Chikhaoui *et al.*, 2014). (B) The visceral form, involving abscesses in internal organs, particularly the lungs, liver, kidneys, mediastinal lymph nodes, and other lymphatic tissues (Figure 1) (Izgür *et al.*, 2010; Singh *et al.*, 2016).

Lesions caused by CL contribute to chronic weakness in affected animals (Gururaj *et al.*, 2018). The visceral form is less common in goats than in sheep (Oreiby *et al.*, 2013), and goats with internal abscesses do not always exhibit enlarged peripheral lymph nodes (Habuš *et al.*, 2015; Matthews, 2016).

Transmission

Infection can occur through direct contact with pus, consumption of contaminated water or food, and inhalation of aerosols or contaminated materials (Constable *et al.*,

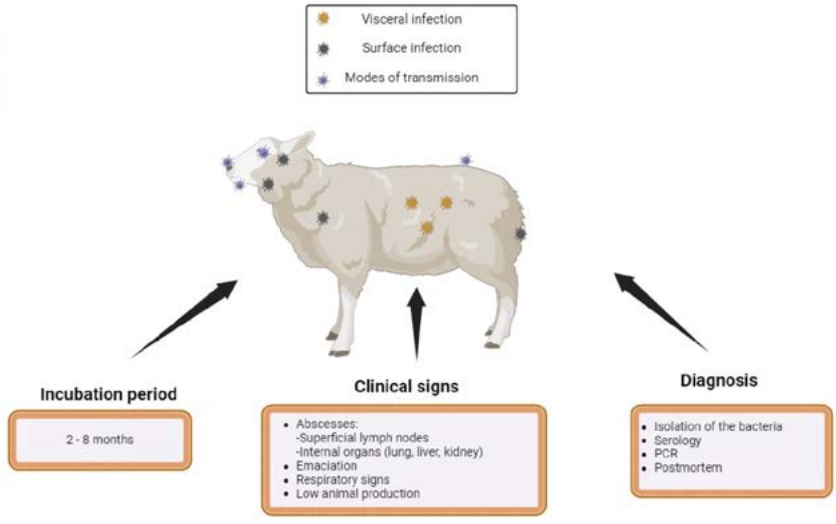

Figure 1. Most affected anatomic regions by *caseous lymphadenitis* in small ruminants.

2017; Barnabé *et al.*, 2019; De Oliveira *et al.*, 2021). Transmission between animals typically results from the contamination of superficial wounds caused by shearing, castration, vaccination, and tethering (Habuš *et al.*, 2015; Quiroga *et al.*, 2019; De Oliveira *et al.*, 2021). Vectors such as *Musca domestica* and *Hippobosca equina* have been implicated in the transmission of *C. pseudotuberculosis*, as documented in the United States, Israel, and Egypt (Costa *et al.*, 2013; Viana *et al.*, 2017). Another possible route of transmission is through mucosal contact (Harwood & Mueller, 2018). The bacteria can colonize the tonsils following ingestion, with young animals becoming infected by consuming contaminated milk directly from the mother's udder (Matthews, 2016). Spontaneous rupture and drainage of abscesses facilitate the environmental dissemination of *C. pseudotuberculosis*, increasing the risk of transmission (Rezende *et al.*, 2016). Once discharged from an abscess, the bacteria can persist in the environment, as well as in hay, soil, and manure (Matthews, 2016; Schlicher *et al.*, 2021). The spread of the pathogen between farms commonly occurs through the introduction of infected animals, contaminated equipment, and shared livestock facilities (Matthews, 2016).

Pathogeny and Immune Response

All *Corynebacterium pseudotuberculosis* strains produce an exotoxin known as phospholipase D (PLD), which is a key virulence factor responsible for pathogen dissemination from the initial infection site within the host (Jeber *et al.*, 2016; De Oliveira *et al.*, 2018). This toxin hydrolyzes phosphatidylcholine and sphingomyelin in mammalian cell membranes, facilitating bacterial spread and causing endothelial cell necrosis. It promotes bacterial translocation from the dermis into the bloodstream and lymphatic vessels (Baird & Malone, 2010). Additionally, PLD activates the complement system, leading to necrosis and thrombosis of the lymphatic vessels (Bastos *et al.*, 2012; Barros *et al.*, 2021). The disease begins with a primary infection at a lymphatic wound, which then spreads hematogenously. Following infection, neutrophils and macrophages are rapidly

activated, initiating signaling pathways that trigger an adaptive immune response against the pathogen (Sá *et al.*, 2018). The cellular adaptive response involves CD4+ T cells, which produce Th1-type cytokines, including interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), to control infection. Proinflammatory cytokines enhance macrophage bactericidal activity and activate CD8+ T lymphocytes, which attempt to eliminate *C. pseudotuberculosis* (De Souza *et al.*, 2014; Fu *et al.*, 2020). Macrophage activation follows two pathways: classical activation (M1 macrophages), mediated by MHC class II, Toll-like receptors (TLRs), and IFN-γ, which promotes the Th1 response and enhances phagocytosis; and alternative activation (M2 macrophages), induced by IL-4, IL-13, and IL-1, which secrete anti-inflammatory molecules such as TGF-β, thereby promoting a Th2 immune response (Sá *et al.*, 2018). To evade the immune system, *C. pseudotuberculosis* can be phagocytosed by neutrophils and macrophages, forming a phagolysosome. However, PLD interferes with opsonization, allowing the bacteria to escape neutrophil and macrophage activity. It also forms a lipid layer, shielding it from proteolytic enzymes within phagolysosomes, while inhibiting macrophage nitric oxide production. These mechanisms enable the bacterium to persist intracellularly, evade immune destruction, and disseminate throughout the host (Bastos *et al.*, 2012; Jeber *et al.*, 2016; De Oliveira *et al.*, 2018; Rebouças *et al.*, 2020; Umer *et al.*, 2020; Barros *et al.*, 2021; Marques da Silva *et al.*, 2021). The humoral immune response occurs 6 to 11 days post-infection, during which antibody production helps prevent bacterial dissemination. Cytokines such as TNF-α, IL-1β, and IL-6 are primarily produced at the inoculation site by macrophages, while IL-2, IL-4, and IFN-γ are produced in the lymph nodes, contributing to T-cell activation (Guimarães *et al.*, 2011a; Bastos *et al.*, 2012; Barral *et al.*, 2022a). Uncontrolled bacterial growth within macrophages induces inflammatory cell death, leading to the formation of pyogranulomas with a caseous necrotic center one of the hallmarks of the disease. Prolonged stimulation of TLRs promotes the formation of multinucleated giant cells, which respond to cellular damage. Simultaneously, M2 macrophages attract fibroblasts, stimulating collagen deposition and forming a capsule around the lesion. As a result, these granulomatous lesions consist of a core of infected macrophages and giant cells, surrounded by T cells, B cells, neutrophils, epithelioid macrophages, and fibrous tissue. However, some bacteria remain viable within abscesses for years, leading to latent infections (Bastos *et al.*, 2012; Tizard, 2018). When an internal abscess ruptures, pus containing millions of bacteria is released, facilitating hematogenous spread to multiple organs (Barnabé *et al.*, 2019).

Granuloma formation acts as a host defense mechanism, restricting bacterial dissemination to vital organs while promoting an effective immune response (Al-Gaabary *et al.*, 2010; Bastos *et al.*, 2012). However, abscess formation provides *C. pseudotuberculosis* with immune protection, limiting antimicrobial effectiveness and enabling immune evasion (Habuš *et al.*, 2015). The pyogranuloma undergoes continuous transformation through cytokine secretion by immune cells. A chronic inflammatory reaction is sustained at the lesion center, while tissue repair processes are initiated at the periphery. This response is driven by cytokines such as IFN-γ, TNF-α, IL-4, IL-1, and IL-6 (Lefevre *et al.*, 2010; Bastos *et al.*, 2012).

Clinical Signs

This disease is characterized by the formation of abscesses in the lymph nodes and internal organs of small ruminants. However, in sheep and goats, CL does not typically cause obvious clinical signs unless the lesions are progressive, excessively large, or numerous enough to be detected clinically, or if they impair the function of a vital organ (Jeber *et al.*, 2016). The most common clinical manifestation is the presence of abscesses in the superficial lymph nodes, with the parotid, submandibular, prescapular, femoral, popliteal, and supramammary nodes being the most frequently affected (Firdaus *et al.*, 2017). Goats and sheep with internal abscesses may exhibit signs of emaciation, while involvement of thoracic lymph nodes can lead to respiratory symptoms (Matthews, 2016; Firdaus *et al.*, 2017). The incidence of CL has been shown to increase with age, with animals between 2 and 3 years being the most affected (Selim *et al.*, 2021; Bettini *et al.*, 2022).

Differential Diagnoses

When evaluating lymph node abscesses, other potential etiologies should be considered, including tuberculosis infections and bacterial abscesses caused by *Actinomyces pyogenes* or *Trueperella pyogenes* (Abebe & Sisay, 2015; Harwood & Mueller, 2018; Gascoigne *et al.*, 2020). *Staphylococcus aureus* subspecies *anaerobius* produces abscesses similar to those seen in CL, a condition known as Morel's disease, which primarily affects young animals. This disease has an incubation period of approximately three weeks, and its lesions are not always located near lymph nodes (Gascoigne *et al.*, 2020). *Actinobacillosis lignieresii* is a commensal organism in the oral cavity of ruminants and is occasionally isolated from abscesses around the face and neck in goats. Additionally, infections caused by *Yersinia pseudotuberculosis* and various Mycobacterium species can occasionally produce lesions resembling those of *C. pseudotuberculosis* (Matthews, 2016). The superficial form of CL must be distinguished from conditions such as submandibular edema caused by *Fasciola hepatica* and *Haemonchus* spp., salivary cysts, lymphosarcoma, and abscesses resulting from vaccine inoculation (Guimarães *et al.*, 2011a). The visceral form may present clinical similarities to chronic parasitism, alveolar periodontitis, malnutrition, chronic systemic diseases, pulmonary adenomatosis, neoplasms, small ruminant lentivirus infections, paratuberculosis, and scrapie (Lefevre *et al.*, 2010; Guimarães *et al.*, 2011b). In sheep, orchitis and epididymitis caused by *C. pseudotuberculosis* must be differentiated from lesions induced by *Brucella ovis*, *Actinobacillus seminis*, *Histophilus somni*, and *Pasteurella* spp. (Guimarães *et al.*, 2011b).

Sampling and Diagnosis

The clinical diagnosis through the detection of lymphadenomegaly is the method routinely performed by veterinarians, but it is nonspecific, and the standard diagnostic test is bacterial culture, followed by the identification of *Corynebacterium pseudotuberculosis* using biochemical tests (Nicoletti *et al.*, 2022). Bacterial isolation is performed by puncturing the abscess with 18G needles and sterile 10 mL syringes, ensuring prior trichotomy of the area. The sample is refrigerated, labeled, and sent to the laboratory. For lesions with superficial or ruptured abscesses, a deep smear can be obtained using sterile cotton swabs; samples are preserved in Stuart culture medium and refrigerated. The material for bacterial

culture is collected from the abscess and inoculated into brain heart infusion (BHI) agar with 5% sheep blood (Seyffert *et al.*, 2010; Oreiby *et al.*, 2013; Díaz *et al.*, 2015). This bacterium is a facultative anaerobe that grows optimally at 37 °C, with a pH of 7.0-7.2. Initial growth on agar surfaces appears sparse but later forms clumps or palisades, with a cream to orange coloration. Colonies are dry, opaque, and concentrically ringed. Growth in liquid media develops as a granular deposit with a surface pellicle. Hemolysis on blood agar is variable, but large hemolysis zones appear in the presence of *Rhodococcus equi*. *C. pseudotuberculosis* toxin also inhibits the action of staphylococcal β-lysin (Dorella *et al.*, 2016). Serological diagnosis includes indirect hemagglutination test, microagglutination assay, double immunodiffusion test, hemolysis inhibition test, interferon-gamma (IFN-γ) detection by ELISA, and the enzyme-linked immunosorbent assay (ELISA) using bacterial cells, toxins, culture supernatants, and secreted proteins (PLD) (Bastos *et al.*, 2012; Rezende *et al.*, 2016; Silva *et al.*, 2019). The ELISA test is highly specific but has low sensitivity, which may result in false negatives. However, repeating the test one month later in negative animals confirms the diagnosis (Matthews, 2016). Nevertheless, ELISA formats using recombinant proteins are promising due to their high specificity and significant sensitivity, as they use purified antigens (Silva *et al.*, 2019). The synergistic hemolysis inhibition (SHI) test measures antibodies against PLD and has a 98% sensitivity in goats, but its low specificity makes it an unreliable predictor of clinical disease in infected herds (Washburn *et al.*, 2013; Matthews, 2016). The quantification of IFN-γ produced by peripheral leukocytes following antigenic stimulation is an accurate diagnostic method but has low sensitivity and is expensive (Bastos *et al.*, 2012). The Multiplex Polymerase Chain Reaction (mPCR) is useful for diagnosing CL in small ruminants. This method is highly sensitive and specific, does not require primary isolation, and is employed in epidemiological surveillance and experimental studies (Quiroga *et al.*, 2019). Detection of the PLD gene serves as a diagnostic tool for CL. Recently, partial sequence analysis of the RNA polymerase (rpoB) β subunit gene has been used for identifying *Corynebacterium* species (Nabih *et al.*, 2018). Over the past decades, various molecular techniques such as conventional, real-time, and multiplex PCR, DNA-DNA hybridization, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) have been applied to complement traditional diagnostic methods. These techniques also aid in studying the virulence profile, genetic diversity, and geographical distribution of *C. pseudotuberculosis* strains worldwide (Dorneles *et al.*, 2014; Parise *et al.*, 2018; Zamprogna *et al.*, 2021; Park *et al.*, 2022). Most visceral CL cases are diagnosed postmortem, revealing internal abscesses during slaughterhouse inspection (Abebe & Sisay, 2015). In goats, abscesses are typically creamy white or yellow, whereas in sheep, they are often green. Calcification is rare, and the characteristic "onion ring" appearance is observed in sheep but not in goats. The thick, sticky pus found in goats is attributed to enzymatic activity from phagocytic cells (Valdivia, 2015; Matthews, 2016).

Treatment

The animal scheduled for abscess debridement should be removed from the pen and relocated to a sun-exposed area. Preferably, the procedure should be performed in a

location with a cement floor, which facilitates washing and disinfection (Díaz *et al*., 2015). Surgical treatment is described in Figure 2.

Corynebacterium pseudotuberculosis is susceptible to various antibiotics in vitro; however, it is generally refractory to antibiotic therapy (Baird & Malone, 2010). This resistance is attributed to the intracellular nature of the organism during much of its pathogenesis and the presence of a thick fibrous abscess wall. Once an abscess has formed, treatment with commonly used antibiotics is ineffective (Izgür *et al*., 2010; Matthews, 2016; Bezerra *et al*., 2021).

Control and Prevention

Animals exhibiting clinical signs of CL and enlarged lymph nodes should be isolated from the herd to prevent abscess rupture and pus release (Harwood & Mueller, 2018). Rubefacient ointments, hot compresses, and poultices are therapeutic measures that promote abscess maturation for manual drainage (White, 2006; Tuemmers & Saldivia, 2015). The most effective strategy for CL control is identifying infected animals through clinical examination or serological testing. Culling infected animals has been successful in disease prevention (Chikhaoui *et al*., 2014; Harwood & Mueller, 2018). Animals should be examined every three months to detect visible abscesses. Serodiagnostic tests for *C. pseudotuberculosis* antibodies have been successfully implemented to identify asymptomatic infections. ELISA has been widely used in CL control and eradication programs due to

Figure 2. Procedure to debride an abscess. A) Incision in the distal region of the abscess with a sterile scalpel blade, previously disinfection of the area with 1% iodine solution or 70% ethanol must be carried out, B) Drain the purulent content into a plastic bag, C) The cavity is washed with hydrogen peroxide, 10% iodine or chlorhexidine, D) Moisten the gauze with a disinfectant solution and introduce the gauze into the cavity, scraping off the infected tissue, E) The cavity is washed again, F) Collect the contaminated material and discard.

its cost-effectiveness, ease of use, and suitability for routine clinical application (Silva *et al.*, 2019). Bacteriological and serological tests for *C. pseudotuberculosis* are highly specific, and any positive animal must be separated from seronegative groups. If an animal yields an uncertain result, it should be retested after one month and kept isolated until confirmation (Matthews, 2016).

In countries where vaccination is available, herd control measures significantly reduce disease prevalence, although complete eradication remains unattainable (Baird & Malone, 2010; Sobrinho *et al.*, 2018). The ineffectiveness of antibiotics against *C. pseudotuberculosis* has led to the development of various vaccines, including attenuated, inactivated, cell membrane fraction, and DNA-based vaccines (Guerrero *et al.*, 2018). Commercial vaccines against CL are currently available; however, their efficacy varies, and vaccination is recommended only for abscess-free animals (Barral *et al.*, 2022b). Most commercial vaccines for *C. pseudotuberculosis* are combined with *Clostridium* vaccines targeting *C. tetani*, *C. perfringens*, *C. septicum*, *C. novyi*, and *C. chauvoei*. These vaccines use inactivated phospholipase D (PLD) and are referred to as toxoid vaccines (Dorella *et al.*, 2006). PLD and CP40 proteins have been identified as potential vaccine targets, with PLD inducing a strong antibody response and CP40 activating cellular immunity (Barral *et al.*, 2022b). Vaccination of goats with a formalin-inactivated PLD exotoxin has been shown to prevent bacterial dissemination following experimental challenge, while antitoxin administration further inhibits bacterial spread within the host (Dorella *et al.*, 2009). Toxoid vaccines have demonstrated efficacy in reducing the number and size of CL lung abscesses and limiting bacterial spread. In Brazil, a live attenuated *C. pseudotuberculosis* strain (strain 1002) was approved for use, conferring 83% protection against CL in goats (Dorella *et al.*, 2006). Additionally, experimental immunogens are being tested to enhance safety and protection levels (Barral *et al.*, 2022b). Virulence genes of *C. pseudotuberculosis* are considered promising targets for vaccine development (D'Afonseca *et al.*, 2008). Most vaccine development studies focus on bacterial genes (DNA vaccines) or bacterial proteins (subunit vaccines). Another approach involves gene knockout-attenuated strains. A recent study developed an attenuated *C. pseudotuberculosis* strain with a *ciuA* gene knockout (a gene involved in iron citrate transport for bacterial metabolism). This vaccine successfully induced both cellular and humoral immune responses after challenge with the wild-type strain (Galvão *et al.*, 2017). Additionally, sheep vaccinated with a DNA vaccine expressing bovine CTLA-4 fused with HIg and genetically detoxified phospholipase D exhibited extracellular immune dominance. However, this genetically attenuated vaccine was only partially effective in experimental trials (Dorella *et al.*, 2006).

Zoonosis

As in small ruminants, *Corynebacterium pseudotuberculosis* can infect humans, causing necrotizing granulomatous lymphadenitis (Bastos *et al.*, 2012; Heggelund *et al.*, 2015; Tan *et al.*, 2021). CL has also been associated with pneumonia, particularly in individuals who have had contact with infected animals. It is considered an occupational zoonosis; however, human infections are rare. Most cases of human lymphadenitis have been reported in Australia, primarily affecting workers with regular exposure to infected sheep (Schlicher *et*

al., 2021). Transmission typically occurs through direct contact with the skin or wounds, although consumption of contaminated raw goat's milk and raw goat's cheese may also serve as potential routes of infection (Heggelund *et al*., 2015; Sigirci *et al*., 2019).

CONCLUSION

Caseous lymphadenitis is widely distributed worldwide, impacting small ruminant production and causing economic losses due to decreased productivity in these species. Given the significant effect of this disease on livestock production, we emphasize the importance of surgical debridement of abscesses to prevent environmental contamination and reduce the persistence of *Corynebacterium pseudotuberculosis* within the herd. However, based on the information compiled in this review, we highlight that preventive measures remain the most critical strategy for effective caseous lymphadenitis management.

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Colegio de Postgraduados

Nanotechnology is an innovative tool in the treatment of diseases that affect animals

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ABSTRACT
Nanotechnology is defined as the art of manipulating matter atom by atom. Its materials have a size between 1 and 100 nanometers. In the veterinary field, nanometric-scale devices and systems have been applied for the diagnosis, treatment, monitoring and traceability of agricultural inputs. Some notable applications include sensors used to detect specific substances in biological samples, dispensers that allow the controlled release of medications or nutrients, immunogens that help develop immune responses, and chemotherapy drugs that are used to treat diseases, among other applications. This work describes the application of nanotechnology in different areas of veterinary medicine.

Key words: nanotecnology, essential oils, antiviral, cell morphology, additives.

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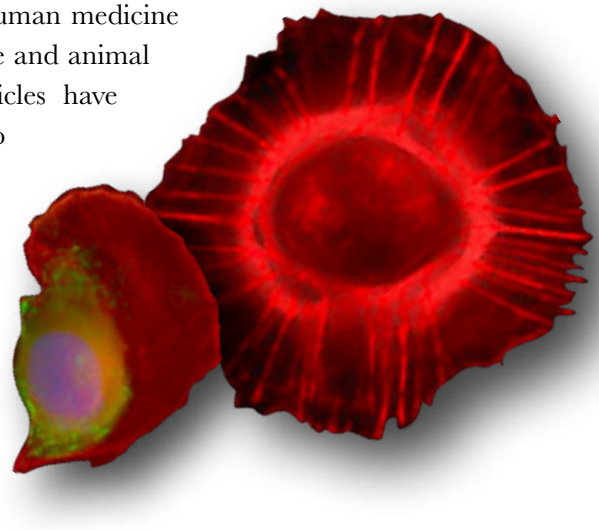
INTRODUCTION

Nanotechnology in veterinary medicine is an emerging field that offers innovative solutions for the diagnosis, treatment, and prevention of animal diseases. It uses nanoscale materials (between 1 and 100 nanometers) to create devices, substances, and systems with unique properties (Arjamend *et al.*, 2023; Coppo, 2009). These may include sensors, dispensers, immunogens, and chemotherapeutics that work at the molecular level to improve the effectiveness and reduce side effects of treatments.

Nanotechnology has proven to be an effective tool contributing to the area of diagnosis and therapy in human medicine and recently its use in veterinary medicine and animal production (Ali *et al.*, 2021). Nanoparticles have been shown to have great potential to revolutionize the veterinary sector in terms of drug and vaccine delivery, diagnostics, animal husbandry and reproduction, and even in the field of animal nutrition.



Natural alternatives are currently being investigated as adjuvants in

animal treatments. Nanotechnology allows the creation of controlled release systems that can improve the delivery of natural compounds, such as essential oils and plant extracts, that have antimicrobial and therapeutic properties. These systems can ensure that the active ingredients reach the site of action more effectively, increasing their efficiency and reducing the amount needed, which is beneficial for both animal health and the environment.

Use of Nanotechnology and natural products in the treatment of bovine mastitis

One of the great challenges in the pharmaceutical and food industry is to be able to use the benefits of natural components, avoiding the evaporation of some essential oils and directing the active components more efficiently to the site of action, which is why nanotechnology is a current tool that is managing to innovate the way drugs are delivered (Corona-Gómez *et al.*, 2022). They have conducted significant studies in this field, particularly about bovine mastitis, defined as mammary gland inflammation commonly caused by bacterial infections (Corona-Gómez *et al.*, 2022). One of their studies analyzed the in vitro microbicidal activity of tea tree essential oil, thymol, and carvacrol (compounds of oregano and thyme essential oils) on microorganisms isolated from cases of bovine clinical mastitis. The results showed that these natural substances have potential as alternatives to traditional antibiotics, which is crucial at a time when antibiotic resistance is a growing concern.

Ionic gelation is a method used to manufacture chitosan nanoparticles; a natural polysaccharide derived from chitin. This process involves the interaction of charges between chitosan, which is positively charged, and a negatively charged gelling agent, such as tripolyphosphate (TPP) or sodium alginate (Herrera Barros *et al.*, 2016).

Chitosan nanoparticles manufactured by ionic gelation (Figure 1) have several applications in veterinary medicine, such as the controlled release of drugs, and improving the bioavailability and stability of active compounds (Herrera Barros *et al.*,

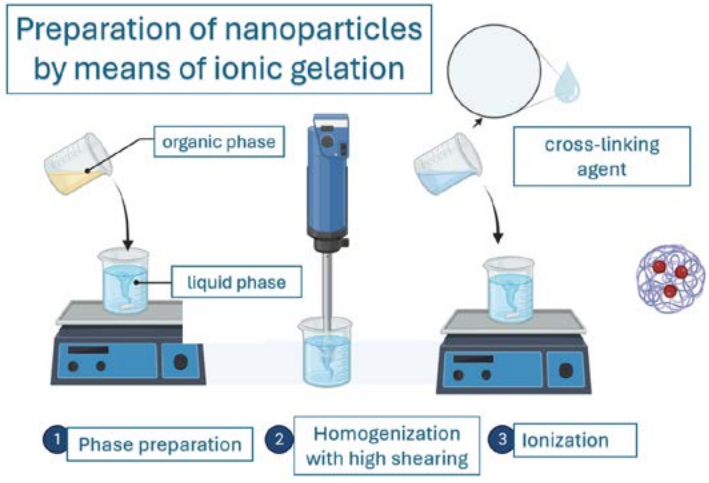


Figure 1. Ionic gelation process to prepare nanoparticles (image designed by Corona-Gómez Lysett with BioRender).

2016). Additionally, they can help mask undesirable odors and flavors and offer better physicochemical stability during processing and storage (Ramírez *et al.*, 2021).

This method is particularly useful for the encapsulation of essential oils due to its ability to protect sensitive compounds and allow a targeted and controlled release at the site of action, which may be beneficial for treating infections or inflammations in animals.

Nanoparticles containing glycyrrhizic acid and the evaluation of their antiviral activity against PRRS virus

Another interesting approach of nanomedicine in the veterinary field is the preparation of nanoparticles with the antiviral agent glycyrrhizinic acid (GA), the most important component from licorice extract (Obolentseva *et al.*, 1999). Urbán-Morlán *et al.* (2018) proposed to study GA due to its different therapeutic properties including its antiviral activity against human and animal viruses (Urbán-Morlán *et al.*, 2018).

In this research, they tested GA water solutions and solid lipid nanoparticles (SLN) containing GA on PRRS-infected cell culture. The Porcine Reproductive and Respiratory Syndrome (PRRS) is caused by a virus and elicits enormous economic losses in hog-producing countries worldwide (Darwich *et al.*, 2010) because currently there is no efficient treatment against this virus. The clinical presentation of the syndrome involves severe reproductive failure mainly in sows and respiratory signs in pigs of all ages; specifically, there is an increase in the number of stillbirths, weak-born piglets, mummified, and an increase in cases of severe pneumonia; sometimes the disease is presented with dyspnea, cyanosis of ears and extremities, pyrexia, lethargy, and anorexia at first stages (Corzo *et al.*, 2010; Huang & Meng, 2010).

The first step of the research was to evaluate the effect of GA water solutions on uninfected and PRRS virus-infected MARC-145 cell cultures. Cytotoxicity and antiviral activity were calculated using the trypan blue dye exclusion test, MTT, and virus titer reduction.

In the second stage, the authors developed SLN with GA. Since the '90s, SLN has been used as carriers of a wide variety of different active molecules because of their advantages, such as high drug payload, low toxicity, increased bioavailability, and drug targeting (Scioli Montoto *et al.*, 2020; Nguyen & Duong, 2022). These colloidal carriers are composed of a lipid matrix stabilized by a surfactant with a size under 1 μm. SLN containing GA (0.54 mg/ml) was obtained by the microemulsion method and tested on cells previously infected with PRRS. After exposure to the SLN, MTT assay, and trypan blue staining were performed.

The results showed that the cytotoxic concentration of GA that reduced cell viability to 50% (CC50) was 4.2 mg/ml and the effective concentration of GA required to inhibit the cytopathic effect to 50% (EC50) was 0.48 mg/ml. Virus titer decreased two logarithms compared to the final titer of a control assay without GA treatment (Figure 2). At this point, these results represent a potential alternative to treat PRRS infection.

Moreover, the effect of GA-loaded SLN on cell culture demonstrated that cell viability by MTT assay was comparable to that exerted by the virus-infected control cells, and the formation of needle-like structures was observed at 48 h, probably due to the presence

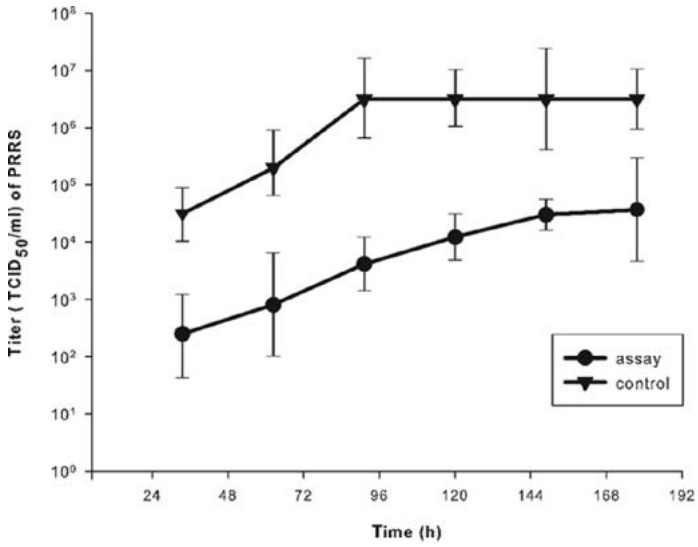


Figure 2. Virus titer was calculated by the Reed and Muench method, where increased concentrations of GA were added after 1 h of incubation of the cells infected with PRRs. n=3. (figure from Urbán-Morlán *et al.* 2018).

of the lipid forming the nanocarrier. The authors concluded that cell culture exposed to SLN interferes with the reliability of MTT assay and it is necessary to combine methods to assess viability. Figure 3 shows the SLN obtained by the microemulsion method.

Drug transporting nanoparticles and their effect on cell morphology

Nanotechnology allows for the development of new drug delivery methods using carriers to transport active ingredients. Nanoparticles can traverse biological barriers and target specific cells, potentially reducing side effects and improving treatment effectiveness. New systems need to be developed with high safety standards, allowing cells to monitor the environment and respond to external signals to survive (Ramos *et al.*, 2018; Kou *et al.*, 2018; Irache, 2008; Jabr-Milane *et al.*, 2008; Tsuji *et al.*, 2006).

The safety of nanosystems can be evaluated by creating cellular models to assess their impact on structure and internal cell function.

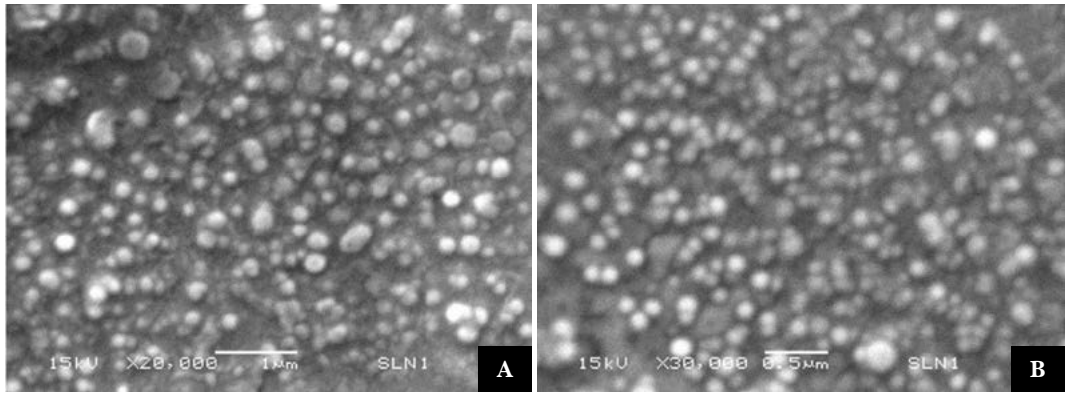


Figure 3. SEM images of SLN were obtained by the microemulsion method. SLN at t=0 (A), and at t=15 days (B). Bars represent 1 μm or 0.5 μm, as specified (figure from Urbán-Morlán *et al.* 2018).

The assessment of cell structure involves studying the rearrangements of the cytoskeleton, a dynamic network composed of different proteins. The most predominant protein is actin, which is responsible for maintaining the cells architecture and structural integrity. These functions are primarily associated with changes in shape and movement through filament rearrangements (Liu *et al.*, 2013).

The actin cytoskeleton induces changes in cell shape and migration in response to various extracellular signals, which can take on multiple arrangements such as bundles (parallel and contractile) and networks (mesh-like and dendritic). Parallel bundles consist of closely associated actin filaments that support structures such as filopodia and microvilli (Liu *et al.*, 2013).

The internal cellular activity of cell lines exposed to nanovehicles is assessed using cytotoxicity tests, which evaluate mitochondrial and lysosomal activity and plasma membrane integrity. Additionally, drug nanocarrier systems can be equipped with fluorophores, which enable their observation using fluorescence microscopy and confocal microscopy to determine the internalization and uptake potential of the nanovehicles.

Effects of additives in nanometric size in veterinary

For a long time, nanotechnology has focused only on humans in areas of health, food, and medicine, among others. Recently, nanotechnology has been applied in veterinary medicine, where several types of nanostructures and nanoparticles have been developed to revolutionize animal science. This is to study disease diagnosis and treatment, nano-vaccines and nano-adjuvants, animal health and nutrition, animal reproduction, and pet care (El-Sayed & Kamel, 2020).

Applications of drug delivery can be applicable in animal nutrition. Drug delivery refers to transporting a pharmaceutical substance to specific areas of the body, such as organs and cellular and subcellular levels of specific tissue, to achieve the desired therapeutic effect (Tewabe *et al.*, 2021). The same strategy used in nanomedicine to reach a specific body area is also applicable in veterinary nutrition.

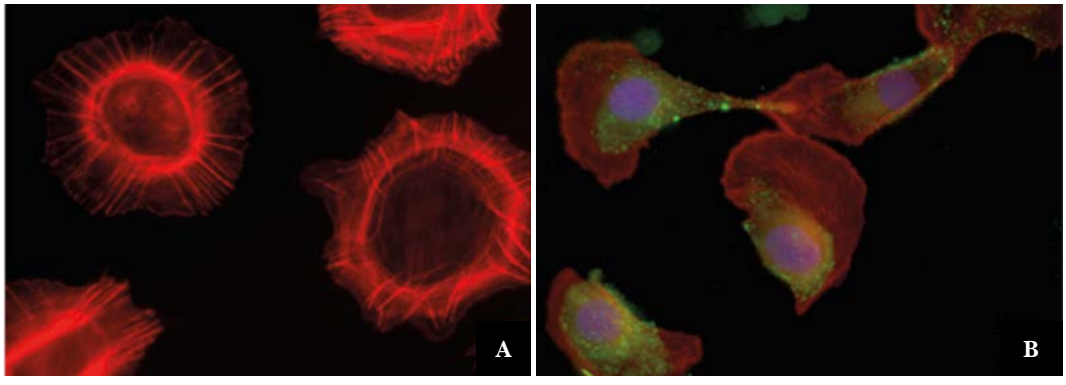


Figure 4. Morphological evaluation of nanoparticle interaction in cell culture. [A] Actin cytoskeleton in MARC-145 cells Direct fluorescence assay, actin filaments stained with TRITC-labeled phalloidin. [B] Internalization of nanoparticles into the cells at 4 h of exposure based on coumarin C6 staining fluorescence microscopy at x40 magnification.

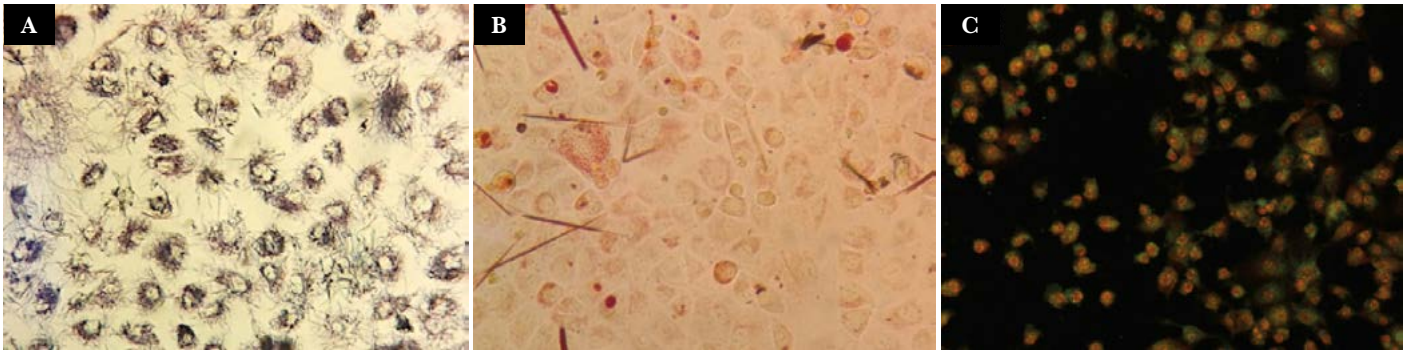


Figure 5. Cytotoxicity after exposure to nanovehicles evaluated by internal cellular activity. [A] Measurement of mitochondrial activity with the MTT assay; [B] determination of lysosomal activity with the neutral red assay; [C] acridine orange staining to evaluate the effect on nuclear integrity.

Administering nutrients to livestock presents inherent challenges in designing carrier nanoparticles, as each compartment of the gastrointestinal tract has its physiological characteristics, such as enzymes and pH level (Hill & Li, 2017). Therefore, nanoparticles must be able to cross many barriers to the delivery of nutritional cargo, particularly to the small intestine.

The delivery of nutrients in the form of carrier nanoparticles has a higher bioavailability than their conventional counterparts. Nanoencapsulation of vitamins allows them to pass through the gastrointestinal tract, deliver the vitamins into the bloodstream, and increase their bioavailability (Thulasi & Sellappan, 2013).

Eggs are consumed for their low price and high nutritional value. To enrich food at the farm level, micro and macro elements are added. Using nano-selenium in laying hens' nutrition decreases total lipids and total cholesterol levels. Additionally, chromium picolinate nanoparticles improve egg yield and increase the accumulation of chromium, calcium, and phosphorous (Konkol D. & Wojnarowsk K., 2018). Similarly, chickens are the most consumed meat in the world due to their easy care and growth. To improve their production, silver nanoparticles were administered to broiler chickens, which improved body weight gain compared to the control group (Reddy *et al.*, 2022).

A study was conducted to evaluate the effects of nanoparticles on growth, carcass characteristics, pork quality, and lipid metabolism in finishing pigs. Nanoparticles of chromium-loaded chitosan were added to the diet of finished pigs destined for market. The results indicated that dietary supplementation with chromium improves growth, carcass characteristics, and pork quality. The chromium-loaded chitosan nanoparticles elevated the activity of hormone-sensitive lipase in adipose tissue while decreasing fatty acid synthase activity (Wang *et al.*, 2014). Additionally, silver and Cu- montmorillonite nanoparticles were used as feed additives to increase the weight gain of pigs. Selenium is an essential element for animal health. Administration of nano-Se and selenomethionate as feed additives for crucian carp showed improvements in final weight in this aquaculture species (Zhou *et al.*, 2009).

Nanotechnology may represent a new and specific product for animal nutrition. Minerals, supplements, and vitamins are important. Through food delivery, many essential

nutrients that are not appropriately assimilated could reach a specific body area in the shape of carrier nutrients. Nevertheless, there are several limitations that require solutions. For example, design nanoparticles that must be resilient against enzymatic in multiple environments, and these nanoparticles must be able to either degradation or excretion of the body (Ali *et al.*, 2021).

The inclusion of nanoparticle supplements in livestock, poultry, and aquaculture will be beneficial in increasing the quality of meat for consumers; another advantage is that they are cheaper and needed in lower concentrations.

Nanosensors in Disease Diagnosis

Nanosensors represent an advance in the diagnosis of infectious diseases, particularly those with zoonotic risk. Different strategies using nanoparticles have been examined for both the diagnosis and treatment of these diseases, as well as for prevention (Dhakal, 2023).

Nanoparticles in Vaccine Development

The use of nanoparticles for the development of veterinary vaccines has had a great boom since they have demonstrated greater benefits and are safer than conventional formulations. Self-assembling nanoparticle vaccines (SAPNs) can produce robust cellular and humoral immune responses and have been shown to protect against various animal infectious diseases (Sun *et al.*, 2024).

Improvement of digestive efficiency and quality of products of animal origin

Feeding with nanoparticles has been shown to improve digestive efficiency, immunity, and the quality of milk, meat, and eggs. Nano-minerals offer low-dose use and improved bioavailability, making them an effective alternative to antibiotics, and can also be incorporated into natural feed ingredients (Gelaye, 2024).

CONCLUSIONS

Nanotechnology offers significant potential to improve animal health and welfare, and its continued research will continue to transform veterinary practice. It is essential that scientists, veterinarians and industry work together to make the most of these innovations and ensure their safety and effectiveness in clinical care.

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Colegio de Postgraduados

Detection of *Parvoviridae* and *Circoviridae* family species from blood of gilts in central Mexico backyard farms

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ABSTRACT

Objective: To determine the DNA prevalence of Parvoviridae family species (PPV1, PPV2, PPV5, and PPV6) and Circoviridae family species (PCV2 and PCV3) in PCV2-unvaccinated backyard farms in central Mexico through gilt blood samples.

Design/Methods/Approach: Blood samples were collected from 60 gilts raised in backyard farms located in the State of Mexico, Hidalgo, and Querétaro. Genomic DNA was extracted using a commercial kit. Nested PCR was performed on each sample using previously reported primers and amplification conditions for PPV1, PPV2, PPV5, and PPV6, PCV2, and PCV3.

Results: Positive cases were detected for each viral species tested: 5.2% (3/58) for PPV1, 89.6% (52/58) for PPV2, 67.2% (39/58) for PPV5, 25.9% (15/58) for PPV6, 50% (29/58) for PCV2, and 69% (40/58) for PCV3. The most prevalent species was PPV2, followed by PCV3 and PPV5, while PPV1 showed the lowest prevalence. Overall, 96.5% of the samples exhibited co-infection with at least one other species, with triple assortments being the most frequent, particularly in backyard farms from the State of Mexico. However, double assortments were predominant in Querétaro. The most common viral combinations were PPV2/PCV3 and PPV2/PPV5. The former was dominant in Querétaro, while the latter was widespread in Hidalgo and the State of Mexico. Additionally, PCV2 was significantly associated with co-infections in Querétaro, whereas both PCV2 and PCV3 were predominant in the State of Mexico and Hidalgo.

Study Limitations/Implications: Backyard farms have a low number of sows and limited accessibility for sampling. Increasing the sample size would provide a more comprehensive understanding of the interactions between these viral species in non-industrial farms.

Findings/Conclusions: All viral species analyzed were detected in the blood of backyard sows. Prevalence and co-infection patterns varied by state and farm. Notably, PPV5 was the predominant virus in Hidalgo and the State of Mexico, often co-occurring with PPV2, PCV2, and PCV3, in that order. In contrast, PPV2 and PPV5 were the most frequent double co-infections in Querétaro. This is the first report documenting the DNA prevalence of PPV2, PPV5, PPV6, and PCV3 in backyard farms. Given the high co-infection rates observed in reproductive sows, further studies are necessary to assess their impact on swine health and productivity.

Key words: *Parvoviridae*, *Circoviridae*, backyard farms, sows, PCR, co-infection.

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INTRODUCTION

In Mexico, porcine production is a highly sustainable economic sector that appeals to large corporations while also playing a crucial role in household economies. Nationwide, backyard farms account for approximately 30% of the swine trade (SENASICA, 2020). These farms are considered high-risk areas due to the lack of rigorous husbandry, biosecurity, and immunization protocols (Carrero, 2005), creating an environment where controlled diseases can spread within the porcine population. Reproductive issues in the swine industry lead to significant economic losses, ultimately affecting consumer prices. Viral agents are among the primary causes of reproductive failure (RF) in sows, as they can induce pregnancy miscarriages, with maternal viremia being essential for transplacental transmission (Zimmerman *et al.*, 2019). RF in sows encompasses a broad spectrum of clinical manifestations, with *Protoparvovirus ungulate* 1 commonly known as porcine parvovirus 1 (PPV1) being one of the main pathogens involved (Chen *et al.*, 2023). However, due to widespread vaccination over the past 40 years, the global incidence of PPV1 has remained low. PPV1 outbreaks are primarily linked to vaccination deficiencies, suggesting that infections may be more frequent in backyard farms where immunization practices are infrequent. Similarly, Porcine circovirus 2 (PCV2) is a well-documented pathogen associated with sow RF (Chen *et al.*, 2023). Although PCV2 vaccination has been available since 2008, its implementation has been largely restricted to commercial farms, with backyard farms exhibiting a low immunization rate. Meanwhile, Porcine circovirus 3 (PCV3), a recently identified agent, has been reported in Mexico. Although global studies have suggested its involvement in piglet diseases and RF outbreaks, its precise role in disease progression remains unclear. Furthermore, data on PCV3 prevalence in Mexico are scarce, with no available reports on backyard farms. Recently, seven emerging porcine parvovirus species (PPV2-8) have been identified, all belonging to the same family as PPV1 (Walker *et al.*, 2022). Numerous studies have investigated their potential role in disease, either as primary pathogens or in co-infections with other agents (Li *et al.*, 2021). In Mexico, a retrospective study analyzing PCV2-unvaccinated commercial farms from 2001 to 2015 reported a high prevalence of PPV2 to PPV6, markedly differing from global prevalence trends (García-Camacho *et al.*, 2020). However, no studies have assessed the prevalence of these viral agents in backyard farms, particularly those associated with sow RF. Therefore, the aim of this study is to determine the DNA prevalence of PPV1-6, PCV2, and PCV3 in backyard swine farms in central Mexico using blood samples from gilts.

MATERIAL AND METHODS

Sampling: Fifty-eight blood samples were collected from gilts in 2023 from PCV2-unvaccinated backyard farms located in three Mexican states: Hidalgo (Tepatepec, 16 samples), State of Mexico (Teotihuacán de Arista, 10 samples; Melchor Ocampo, 4 samples; Teoloyucan, 7 samples), and Querétaro (San Juan del Río, 21 samples). Venipuncture was performed via the jugular vein using EDTA-coated tubes (Vacutainer™).

DNA extraction: DNA extraction from each sample was performed using a commercial kit (QuickGene DNA Whole Blood, Fujifilm, United Kingdom) following the manufacturer's

instructions. The total DNA concentration was measured using a spectrophotometer (NanoDrop Lite, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Nested PCR: Each genomic DNA sample was used as a template in 25 μL reactions containing 1× master mix (RealQ Plus 2× Master Mix Green, Ampliqon, Denmark). Nested PCR was carried out in a Master Cycler Gradient (Eppendorf, Hamburg, Germany) using previously reported primers and amplification conditions: García-Camacho *et al.* (2020) for PPV1, PPV2, PPV5, and PPV6; Kim *et al.* (2001) for PCV2; and Agatón-Flores (2024) for PCV3. Amplicons were visualized by electrophoresis on 1.5% agarose gels.

RESULTS

Positive cases were detected for each viral agent tested in this study, as summarized in Table 1. The overall prevalence for PPV1, PPV2, PPV5, PPV6, PCV2, and PCV3 was 5.2%, 89.6%, 67.2%, 25.9%, 50%, and 69%, respectively. In general, viral frequencies were similar across states, though some variations were observed. PPV2 was the most prevalent, with a lower proportion in Hidalgo (56.2%), while PPV1 was the least prevalent, ranging from 0% to 10%. Notably, PPV5 was less frequent in Querétaro (9.5%) than in Hidalgo and the State of Mexico. Similarly, PPV6 prevalence was lower in Querétaro (9.5%) compared to Hidalgo (37.5%) and the State of Mexico (33.3%, on average). Fluctuations were also observed in PCV2 prevalence, with the highest frequency in the State of Mexico (71.4%, on average), followed by Querétaro and Hidalgo. Conversely, PCV3 showed the highest prevalence in Querétaro (100%), surpassing levels found in the State of Mexico and Hidalgo.

Regarding co-infections, 96.5% of the samples exhibited at least one type of co-infection (Figure 1). The most frequent were triple co-infections (50%), followed by double co-infections (27.6%) and quadruple co-infections (13.8%).

Quintuple co-infections accounted for 8.6% of cases, while no sextuple co-infections were identified. In the State of Mexico, co-infections involving three or more viruses were predominant, whereas double and triple co-infections were more common in Querétaro

Table 1. Frequency of viral species detected by nested PCR in the study population.

Farm	Samples	PPV1		PPV2		PPV5		PPV6		PCV2		PCV3	
		#	%	#	%	#	%	#	%	#	%	#	%
Hidalgo													
Tepatepec	16	1/16	6.2	9/16	56.2	15/16	93.7	6/16	37.5	5/16	31.2	6/16	37.5
State of Mexico													
Teotihuacan	10	1/10	10	9/10	90.0	10/10	100	3/10	30.0	8/10	80.0	7/10	70.0
Melchor Ocampo	4	0/4	0	4/4	100	4/4	100	3/4	75.0	1/4	25.0	1/4	25.0
Teoloyucan	7	0/7	0	7/7	100	7/7	100	1/7	14.2	6/7	85.7	3/7	42.8
Average	21	1/21	4.8	20/21	95.2	21/21	100	7/21	33.3	15/21	71.4	11/21	52.4
Querétaro													
San Juan del Río	21	1/21	4.8	21/21	100	2/21	9.5	2/21	9.5	8/21	38.0	21/21	100
Overall	58	3/58	5.2	52/58	89.6	39/58	67.2	15/58	25.9	29/58	50.0	40/58	69.0

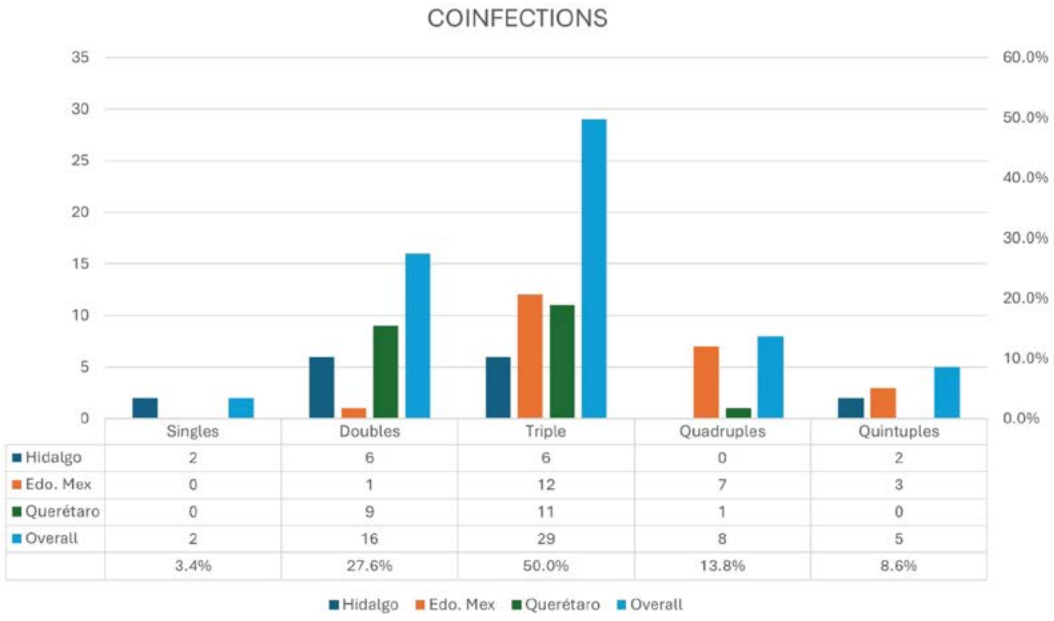

Figure 1. Frequency of co-infections by state.

and Hidalgo. Notably, Hidalgo was the only location where single infections were observed (two cases). The distribution of viral assortments in co-infections is illustrated in Figure 2. Among triple co-infections, the most frequently identified combinations were PPV2/PCV2/PCV3 (28.57%), PPV2/PPV5/PPV6 (17.2%), PPV2/PPV5/PCV2 (13.8%), and PPV2/PPV5/PCV3 (6.9%). In double co-infections, the most prevalent combination was PPV2/PCV3 (56.2%), primarily observed in Querétaro, followed by PPV2/PPV5 (37.5%). The overall frequency of PPV5 co-occurring with either PPV6 or PCV3 was 6.25%. Regarding quadruple co-infections, two viral assortments were identified: PPV2/PPV5/PCV2/PCV3 (87.5%) and PPV2/PPV6/PCV2/PCV3 (12.5%). A single combination was detected in quintuple co-infections, consisting of PPV2/PPV5/PPV6/PCV2/PCV3.

Regarding the distribution of viral combinations by state, in Hidalgo, PPV5 was detected in all cases except one. Two single infections were observed, while PPV5 showed a strong association with PPV2. The next most frequently linked viruses in co-infections were PCV3 and PCV2, in that order. A similar pattern was observed in backyard farms in the State of Mexico, where PPV5 and PPV2 were closely associated. However, a greater

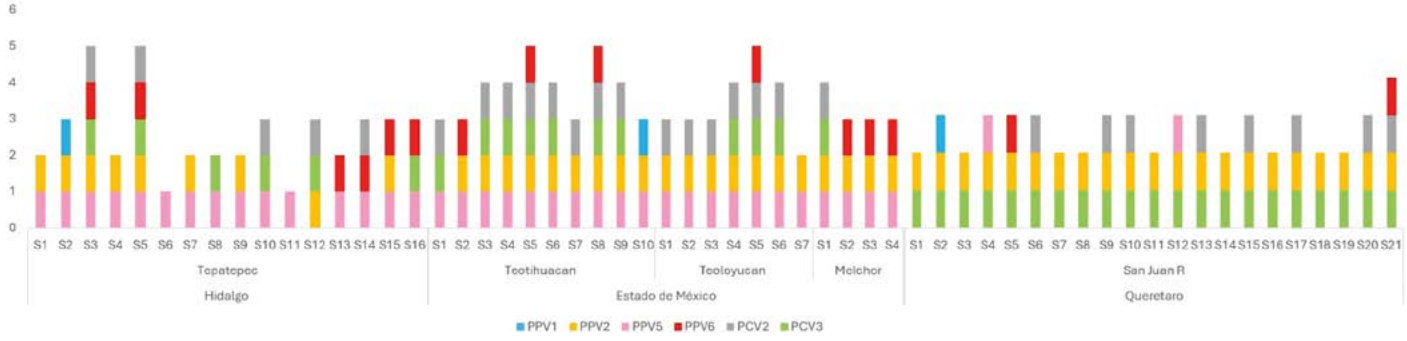

Figure 2. Assortments of viruses in co-infections by state. S=sample.

number of viral species were involved in this region, with a high prevalence of PCV2 and PCV3. In contrast, the most frequent viral combination in Querétaro was PPV2/PCV3, with PCV2 playing a significant role in triple co-infections.

DISCUSSION

In porcine backyard farms, basic diagnostic practices are rarely implemented. As a result, the prevalence of infectious agents and diseases remains largely unknown, potentially creating conditions favorable for disease outbreaks (Alonso & Maqueda, 2020). In this study, PPV2, PCV3, and PPV5 were the most prevalent viruses detected, exhibiting distinct distributions and assortments across different states and farms. Globally, PPV2 has consistently been reported as the most prevalent Parvoviridae species (Streck *et al.*, 2013; Saekhow & Ikeda, 2015; Saekhow *et al.*, 2014; García-Camacho *et al.*, 2020), a finding that aligns with the results of this study. The potential association of PPV2 with disease has not yet been fully elucidated. Regarding PPV5, its reported seroprevalence in China, South Korea, and Poland ranges from 20.0% (Li *et al.*, 2021; Kim *et al.*, 2022; Milek *et al.*, 2020) to 41.3% in oral fluid samples (Milek *et al.*, 2019), which is lower than the overall PPV5 prevalence (67.2%) observed in this study. In Mexico, a retrospective study using PCV2-affected and PCV2-unaffected paraffin-embedded tissues reported an overall PPV5 prevalence of 32.4% and a prevalence of 28.0% in reproductive failure (RF) cases. That study found a significant association between PPV5 and Porcine circovirus-associated disease (PCVAD), as well as with RF cases in PCV2-affected farms. Although PPV2 was the most prevalent Parvoviridae species in that case series, no direct association with PCVAD or RF was established (García-Camacho *et al.*, 2020). In this study, PCV3 exhibited a high overall prevalence (69.0%), followed by PCV2 (50.0%). While PCV2 is widely recognized as a pathogenic virus causing PCVAD, PCV3 has been detected in pigs with clinical conditions such as Porcine Dermatitis and Nephropathy Syndrome (PDNS), RF, and multisystemic inflammation (Palinski *et al.*, 2017; Phan *et al.*, 2016), suggesting its potential involvement in disease. However, because the gilts sampled in this study were clinically healthy, no direct correlation with disease can be made. Nonetheless, co-infections among different viral species are increasingly common in animal populations, and these viral interactions may contribute to disease severity. The impact of specific viral co-infections on animal health remains unclear (Vargas-Bermúdez *et al.*, 2023). The distinct viral distribution patterns identified in this study could play a role in disease outbreaks, as the detected viruses have either been directly associated with disease or reported in clinical cases. Hidalgo and the State of Mexico displayed particularly high PPV5 prevalence (93.7% and 100.0%, respectively). In Hidalgo, PPV5 appeared to be a key viral agent, detected in single infections and primarily co-infecting with PPV2, followed by PCV3. Notably, the only sample negative for PPV5 exhibited a viral assortment of PPV2/PCV3/PCV2, emphasizing the relevance of these four viruses in the local farm population. Similarly, in the State of Mexico, the PPV5/PPV2 combination was dominant (20 out of 21 samples). Triple and quadruple co-infections involving three or more viruses were more frequent in this region than in the other states, including quintuple co-infections. In this setting,

PCV2 and PCV3 were detected in 71.4% (15/21) and 52.4% (11/21) of the samples, respectively. In contrast, PPV5 prevalence was significantly lower in Querétaro (9.5%). Interestingly, co-infection of PPV2 with PCV3 was observed in all samples from this location, primarily as double co-infections, with a considerable presence of PCV2 in triple co-infections. This finding contradicts previous studies, where PCV3 was detected at lower rates (30.2%-40.9%) in RF cases (Saporiti *et al.*, 2021; Wang *et al.*, 2019; Chang *et al.*, 2020; Xu *et al.*, 2021). It is well established that co-infections involving multiple viruses can exacerbate disease severity. For example, PCV2 co-infection with other pathogens, such as PPV1 and Porcine Reproductive and Respiratory Syndrome Virus (PRRSv), has been linked to increased pathogenicity (Cui *et al.*, 2023). Additionally, viral co-infections may enhance the likelihood of recombination events, potentially leading to increased virulence or the emergence of new genotypes (Kwon *et al.*, 2017). These findings suggest that the observed viral assortments may contribute to disease outbreaks. PPV5 has already been associated with PCVAD in Mexico (García-Camacho *et al.*, 2020), a possible link between PPV2 and PCV2 has been proposed (Novosel *et al.*, 2018), and ongoing research continues to investigate PCV3's role in disease (Phan *et al.*, 2016). Additionally, PCV3 prevalence has shown an increasing trend in both the U.S. and Taiwan (Wang *et al.*, 2019; Chang *et al.*, 2020). Conversely, PPV6 and PPV1 were the least prevalent species in backyard farms, consistent with previously reported data indicating prevalence rates of 19.4%-25.8% for PPV6 (Milek *et al.*, 2020; Kim *et al.*, 2022; Faustini *et al.*, 2024) and 0.0%-3.5% for PPV1 (Cui *et al.*, 2017; Milek *et al.*, 2019; Kim *et al.*, 2022). A potential role of PPV6 in RF has been suggested, as its prevalence in fetal heart tissue from aborted fetuses was high (57.0%), particularly in PCV2-affected farms (García-Camacho *et al.*, 2020). However, in this study, the PPV6/PCV2 co-infection rate was relatively low (12.5%). Since the gilts were clinically healthy with no reproductive history, no assumption can be made about PPV6's role in RF in backyard farms, though its involvement seems unlikely based on these findings. As for PPV1, its low prevalence was expected, as it has been successfully controlled through vaccination. In fact, in the backyard farms sampled in this study, the pig population is vaccinated against PPV1, similar to high-density commercial farms. Further follow-up studies in these farms could provide valuable insights into the role of each viral agent and the potential impact of viral co-infections on disease development.

CONCLUSIONS

In this study, all assessed viral species (PPV1, PPV2, PPV5, PPV6, PCV2, and PCV3) were detected in gilts from backyard farms. This represents the first report on the prevalence of PPV2, PPV5, PPV6, and PCV3 in such settings. Among the experimental population, PPV2 was the most prevalent species, followed by PCV3, a recently described virus in Mexico. The co-existence of multiple viral species within gilts exhibited distinctive patterns that warrant further investigation to determine their potential contribution to disease.

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Solid lipid nanoparticles loaded with Jackfruit (*Artocarpus heterophyllus* Lam) seed-derived bioactive peptides: Characterization and antioxidant activity

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ABSTRACT

Objective: Characterize and evaluate the antioxidant activity of solid lipid nanoparticles loaded with bioactive peptides derived from the cotyledon of jackfruit.

Design/methodology/approach: The peptides were obtained from seed cotyledon by ultrasound-assited sequencial enzymatic hydrolysis. Four peptide fractions (PF) of ≥ 10 kDa, ≤ 10 kDa, ≥5 kDa, and ≤5 kDa, and their antioxidant activities were evaluated by using the DPPH radical scavenging method. A double emulsion method encapsulated PF≥ 5 kDa and ≤ 5 kDa into solid lipid nanoparticles (SLN).

Results: The UA-AF 2 treatment resulted in higher HD (40.39±2.82%). Besides, the effect of ultrasound improved the antioxidant activity on the DPPH. The SLN had a particle size (Ps) mean of 235 nm, polydispersity index < of 0.4, and negative zeta potential (ζ) > −30 mV and the SLNs were considered stables. The antioxidant activities of the peptide fractions in SLN decreased by 67.65±3.19% and 52.21±1.15%, associated with higher encapsulation efficiencies of peptide fraction in SLNs, ≥ 5 kDa (84.43±7.44 %) and ≤ 5 kDa (81.68±8.31%).

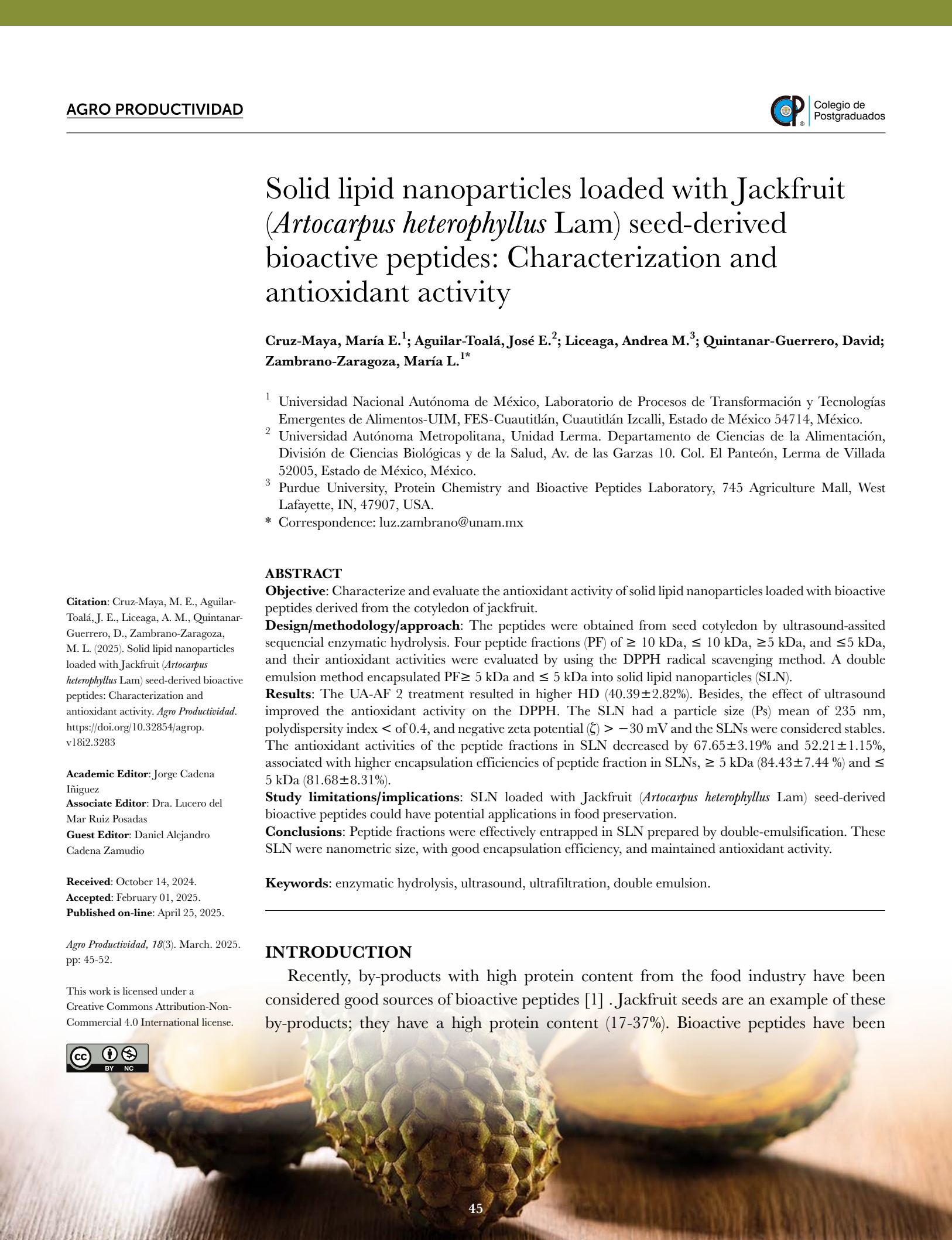
Study limitations/implications: SLN loaded with Jackfruit (*Artocarpus heterophyllus* Lam) seed-derived bioactive peptides could have potential applications in food preservation.

Conclusions: Peptide fractions were effectively entrapped in SLN prepared by double-emulsification. These SLN were nanometric size, with good encapsulation efficiency, and maintained antioxidant activity.

Keywords: enzymatic hydrolysis, ultrasound, ultrafiltration, double emulsion.

INTRODUCTION

Recently, by-products with high protein content from the food industry have been considered good sources of bioactive peptides [1] . Jackfruit seeds are an example of these by-products; they have a high protein content (17-37%). Bioactive peptides have been



identified from jackfruit seeds, including the antioxidant peptides JFS-2 and glutathione, the hormonal peptide leptin, and the antiviral α-peptide from jacalin [2,3].

Sequential hydrolysis has been used to extract bioactive peptides, resulting in a higher degree of hydrolysis and yielding bioactive peptides, thereby enhancing the peptide profile bioactivity. Additionally, ultrasound-assisted hydrolysis has been considered a promising method for improving hydrolysis and enhancing peptide bioactivity, owing to its positive effects on enzyme activity, protein structure, and enzyme-substrate interactions [4].

Encapsulation ensures the peptides' functionality by delaying their degradation, preventing undesired interactions with components of the food matrix, and increasing consumer acceptance by masking unpleasant tastes [5]. In this context, solid lipid nanoparticles (SLN) represent one of the main strategies used to encapsulate hydrophilic or lipophilic peptides, offering significant advantages [6]. Thus, the aim of this study was to characterize and evaluate the antioxidant activity of solid lipid nanoparticles loaded with bioactive peptides derived from the cotyledon of jackfruit.

MATERIALS AND METHODS

Materials

The ripened Jackfruits (*Artocarpus heterophyllus* Lam.) were collected in El Llano, San Blas, Nayarit, Mexico. The peptidases Alcalase® from *Bacillus licheniformis*, Subtilis A (specific activity ≥2.4 U/g), Flavourzyme® from *Aspergillus oryzae* (specific activity ≥500 U/g), DPPH (1,1-diphenyl-2-picrylhydrazyl) radical and Folin-Ciocalteu were purchased from Sigma-Aldrich Chemical® S. A. de C. V. (State of Mexico, Mexico). The surfactants Tween® 80, soy lecithin, cacao butter, and beeswax were provided by Drogueria Cosmopolita S. A. de C. V. (CDMX, Mexico).

Obtention and hydrolysis of jackfruit cotyledon

Jackfruit seed parts were manually separated, and the cotyledons were washed and dipped in 0.05% (w/v) sodium metabisulfite solution for 24 h. The cotyledons were ground (Nutribullet® food processor), dried (Sunix® dehydrator), and milled (KRUPS® mill) into a powder. The hydrolysis of jackfruit cotyledons (5% w/v) in two sequential stages of enzymatic hydrolysis at 55 °C/90 min by enzyme [7]. Initially, Alcalase® (8%) was used, followed by Flavourzyme® (6%) considering enzyme/protein relation. The enzymes were then inactivated at 80-85 °C/10 min.

Two treatments with ultrasound were used to assist the hydrolysis process. An ultrasound equipment UP200HT with a sonotrode of 14 mm diameter at 50 W and 50 kHz (Helshier, Warthestrasse, Germany) was used. The treatment conditions were UA-AF1 (3 pulses/60s-on/60 s-off) and UA-AF2 (5 pulses/40s-on/20s-off). After hydrolysis (65-and 125 min), each treatment's aqueous extract was recovered to determine the protein content and hydrolysis degree (HD).

Hydrolysis Degree (HD)

The hydrolysate samples were centrifuged at 3,000 g/10 min and separated from the supernatant. The protein content was measured using the modified Lowry assay, and

the HD was calculated by dividing the protein content by the protein content of seed flour [1,8].

Obtention of peptide fractions from hydrolysates

The hydrolysates from ultrasound and control treatments were sequentially separated and filtered through 10 kDa and 5 kDa molecular weight cut-off (MWCO) ultrafiltration membranes using two centrifugal filters (Visvaspin® 2, Sigma Aldrich, UK) under centrifugation at 2408 RCF/10 min (centrifuge Frontier™ 5707, Ohaus, Germany). The resulting peptide fractions were named PF ≥10 kDa, ≤ 10 kDa, ≥ 5 kDa, and ≤ 5 kDa.

DPPH Radical scavenging

The DPPH radical scavenging activities of the peptide fractions and SLNs were determined [8]. The reaction mixture's absorbance was measured at 515 nm against a standard curve of Trolox using a UV-Vis spectrophotometer. The DPPH radical scavenging activity was expressed in μM Trolox/L.

Preparation of solid lipid nanoparticles (SNL)

The SNL loaded with PF > 5 kDa and < 5 kDa were prepared using the double emulsification method assisted with ultrasound, as illustrated in Figure 1.

Characterization and encapsulation efficiency (EE)

The particle size (Ps) and polydispersity index (PDI) were determined by dynamic light scattering, and the zeta potential (ζ) by electrophoretic motion, using a Zetasizer Nano® ZS90 instrument (Malvern Instruments Ltd, Worcestershire, U.K.). The EE was determined by the indirect method after centrifugation at 16278 RCF/10 min

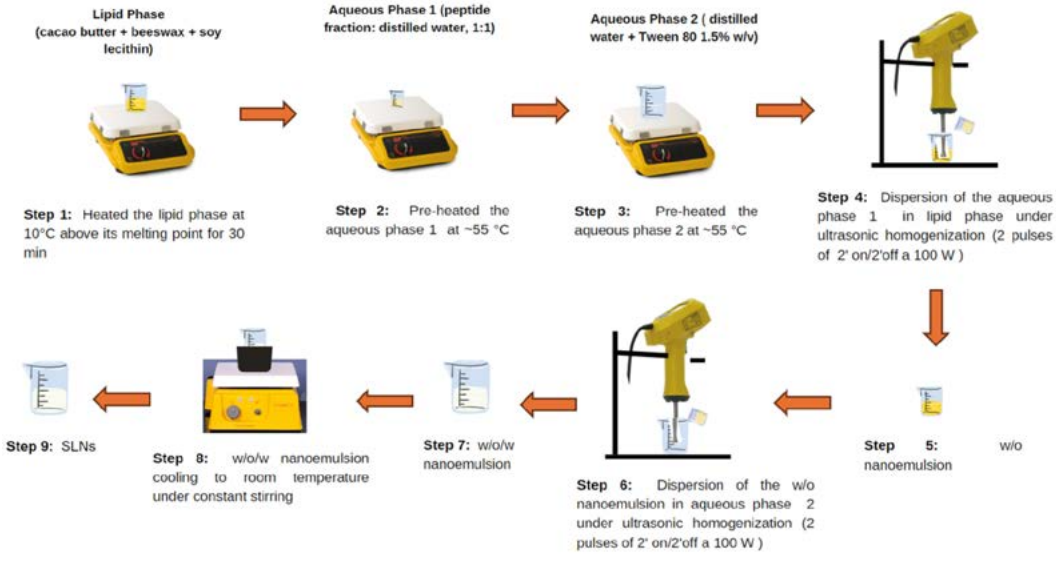


Figure 1. Double emulsification-ultrasound assisted in preparing SLN encapsulating bioactive peptides from jackfruit cotyledon.

(HERMLE Z323K, Germany). Protein content was determined using the Lowry assay, and the difference between protein content in the supernatant and peptides aggregated was reported as EE (%).

SLN stability

The backscattering profile was obtained using diffuse reflectance in a Turbiscan MA200 (Formulation, Toulouse, France), employing a detection wavelength of 55 nm throughout 24 h at 25 °C.

Statistical analysis

Statistical analysis was performed using Minitab® 19 Statistical Software, ANOVA, and Tukey test to mean differentiation, with significance set at $P<0.05$.

RESULTS AND DISCUSSION

Hydrolysis Degree (HD)

Table 1 shows the final HD; the most significant increase in final HD was observed with UA-AF2. The longer the ultrasound exposure time, the higher the HD [9]. However, this holds only if the ultrasound time is not excessive.

DPPH radical scavenging of the peptide fractions

Figure 2 shows the DPPH radical scavenging activities. The results demonstrated that ultrasound increased the DPPH activities of protein extract (66.34±14.47 %) and PF ≥ 5 kDa (5.31 ± 1.68 %) compared to the control. The higher DPPH radical scavenging activities of PF ≤ 10 kDa and PF ≥ 10 kDa observed can be attributed solely to enzyme specificity. In this case, Alcalase®, primarily an endopeptidase, exhibits broad specificity cleaving at Glu, Met, Leu, Tyr, Lys, Trp, and Gln. In contrast, Flavourzyme®, an exopeptidase, cleaves only the peptide bond between Leu-Pro and Pro-Pro [10]. This enhancement is attributed to the sonication effects, which promote hydrolysis, releasing peptides with diverse amino acid compositions that enhance antioxidant activity [11].

SLN characterization

Table 2 presents Ps, PDI, and ζ of SLN, both empty and loaded with PF with molecular weights ≥ 5 kDa and ≤ 5 kDa. These SLNs exhibited Ps ranging between 200-600 nm and PDI values ≤ 0.4. So, a PDI ≤ 0.5 indicated homogeneity in the Ps distribution, while a PDI ≥ 0.7 suggests particle aggregation [12]. The ultrasound conditions employed

Table 1. Effects of Ultrasound Treatment in the HD.

	UA-AF 1	UA-AF 2	Control
Total ultrasound time (s)	720	800	0
H.D. (%)	36.32±1.64 [a]	40.39±2.82 [a]	30.77±0.70 [b]
Increment (%)	18.12±7.20	31.18±6.46	---

Mean values with different letters in each row are statistically different ($P<0.05$).

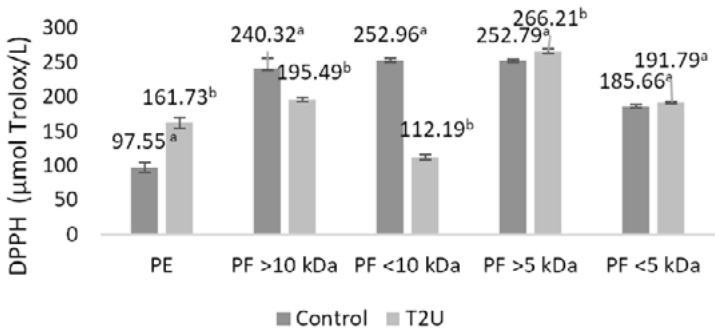


Figure 2. PF's antioxidant activity from DPPH assay. the mean values with different letters were statistically different (P<0.05).

Table 2. Particle sizes and polydispersity indexes of SLNS.

Parameter	NLS empty	NLS PF > 5kD	NLS PF < 5kD
Ps (nm)	218.7±4.980 [a]	228.8±2.501 [b]	235.2±3.993 [b]
PDI	0.258±0.037 [a]	0.336±0.055 [a]	0.412±0.094 [a]
ζ(mV)	−30.7±0.458 [a]	−34.8±0.100 [b]	−36.0±0.651 [c]

Mean values with different letters in each row are statistically different (P<0.05).

during SLN preparation with loaded PF contributed to the production of nanoparticles with smaller Ps and narrow distribution. The shear stress and shock waves generated by acoustic cavitation during the ultrasound reduced Ps [13].

The zeta potential (ζ) of empty SLN and those loaded with PF was around −30 mV, indicating electrostatic stables. However, there was a significant difference in the ζ between SLN loaded with PF ≥ 5kDa and those loaded with PF ≤ 5kDa. The differences are attributed to specific peptides loaded with exposed negatively charged amino acid residues on the nanoparticle surface, thereby increasing the negative potential of the SLN [14]. Establishing that the double nanoemulsion approach enables the production of a system for encapsulating and protecting bioactive peptides at the nanometric size.

Encapsulation efficiency (EE)

The SLN loaded with PF ≥ 5 kDa exhibited a higher EE (84.43±7.44%) compared to SLN ≤ 5 kDa (81.68±8.31) and demonstrated a statistically significant difference (P<0.05). The double emulsion facilitated the entrapment of hydrophilic peptides by incorporating the peptides into the inner aqueous phase of the W/O/W structure [14]. The combination of cacao butter (4.5%) and beeswax (0.5%) results in a less ordered lipid crystal structure with ample space for peptide embedding in the lipid matrix due to their different lipid composition and properties [15].

Antioxidant activity of the peptides in the SLN

Figure 3 illustrates the DPPH radical scavenging activities. The DPPH radical scavenging activities were lower in SLN with PF ≥ 5kDa (67.65±3.19%) compared with

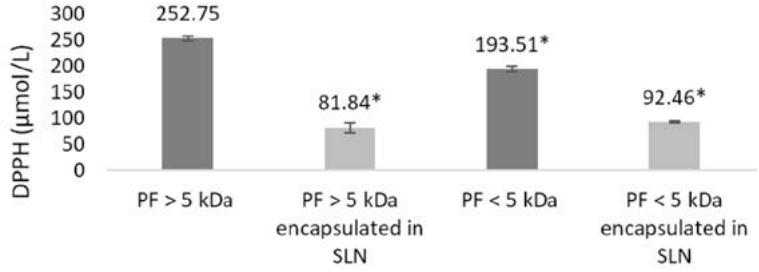


Figure 3. Antioxidant activity of the P.F. > 5 kDa and P.F. < 5 kDa with and without encapsulation in the SLN.

PF ≤ 5 kDa (52.21±1.15%). This behavior is attributed to a significant portion of the PF being entrapped within the SLN matrix, reducing the number of amino acid residues available to donate a proton to the DPPH radical, consequently decreasing the DPPH radical scavenging activities. Thus, encapsulating peptides from the cotyledon of jackfruit seeds in the SLN contributed to preserving their antioxidant activity.

SLN stability

Figure 4 (a and b) shows the backscattering (B.S.) profiles of the loaded SLNs. The figure depicts a slight increase in the B.S. intensity in the middle of the cell. However, the stability of SLN remained unaffected by this instability phenomenon, as indicated by the B.S. intensity variation in the middle (18-25 mm length), being less than 10% [16]. Following these results, SLNs loaded with PF ≥ 5 kDa and ≤ 5 kDa were deemed stable since they showed no significant variation in B.S. intensity. The stability of the SLNs can be attributed to higher ζ (>±30 mV), smaller Ps, and low PDI. Increased ζ results in higher electrostatic repulsion between nanoparticles, thus reducing instability phenomena [17]. Meanwhile, the hydrophilic surfactant Tween 80® stabilized the O/W_2 nanoemulsion by absorbing in the interface oil-water with the hydrophobic tail (carbon chain of oleic acid) directed towards the lipidic phase and the hydrophilic groups (hydroxyl moiety groups) oriented towards the aqueous phase [18].

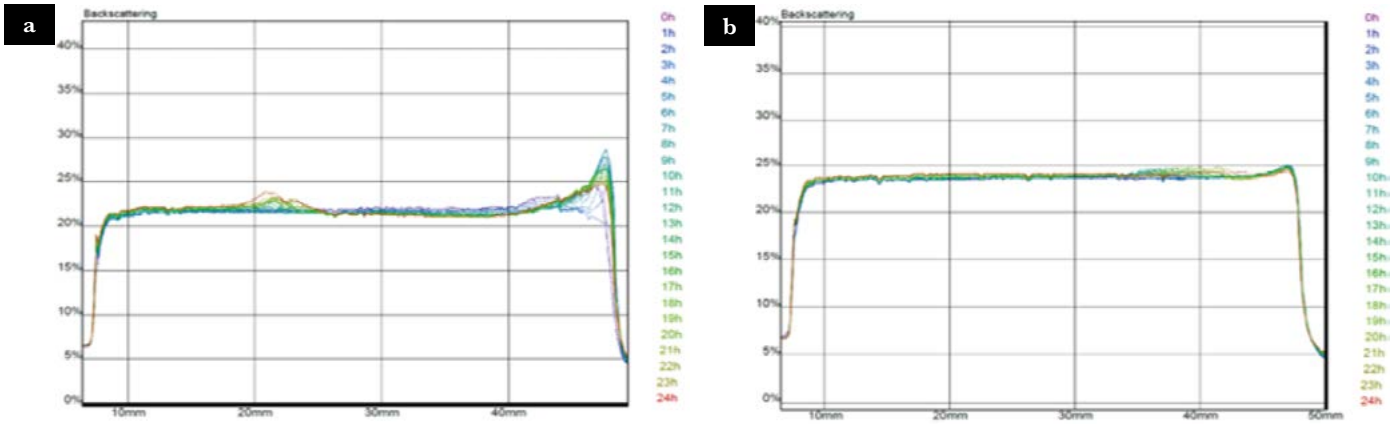


Figure 4. Backscattering profiles of the SLNs loaded with P.F. > 5 kDa (a) and P.F. < 5kDa (b).

CONCLUSIONS

Sequential enzymatic hydrolysis using Alcalase® and Flavourzyme® increased the degree of hydrolysis, thereby enhancing the antioxidant activity of the protein extract and protein fractions. The combination of the double emulsion method with ultrasound assistance, along with the selective use of lipids (beeswax and cacao butter) and surfactants (soy lecithin and Tween 80®), facilitated the encapsulation of antioxidant peptides into SLNs with higher encapsulation efficiency and good stability, as evidenced by low backscattering.

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Colegio de Postgraduados

Artemisia cina ethanolic extract reduces the infection of *Haemonchus contortus* in lambs

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ABSTRACT

Objective: This study aimed to evaluate the anthelmintic efficacy of an ethanolic extract (Ac eth/ext) derived from leaves and stems of *Artemisia cina* in lambs artificially infected with *Haemonchus contortus*.

Design/Methodology/Approach: Three experimental groups of *H. contortus*-infected lambs (n=5) were assigned to the following treatments: (1) Ethanolic extract of *A. cina* (Ac eth/ext) at 4 mg/kg body weight (BW); (2) Albendazole at 5 mg/kg BW and (3) control group (5 mL of water). Treatments were administered orally as a single dose, and fecal egg count (FEC) along with ocular mucosa color were monitored for seven days.

Limitations/Implications: At the end of the study,all lambs were sacrificed, and the abomasum was extracted to recover adult parasites, which were subsequently counted and sexed.

Results: The Ac eth/ext group exhibited a significant 47% reduction in FEC compared to the control group ($p<0.05$). In contrast, albendazole treatment resulted in a 100% reduction in FEC values. The ethanolic extract also reduced the parasite burden by 30%, whereas albendazole led to a 5.31% reduction.

Findings/Conclusions: The ethanolic extract of *A. cina* (Ac eth/ext) could serve as a valuable complementary tool for the control of ovine haemonchosis. However, further research is necessary to identify the bioactive molecules responsible for its anthelmintic properties. Additionally, reducing the parasite burden in female worms may have a positive impact by lowering FEC values and subsequently decreasing pasture contamination.

Keywords: *Artemisia cina*, anthelmintic, *Haemonchus contortus*, ethanolic extract, fecal egg count.

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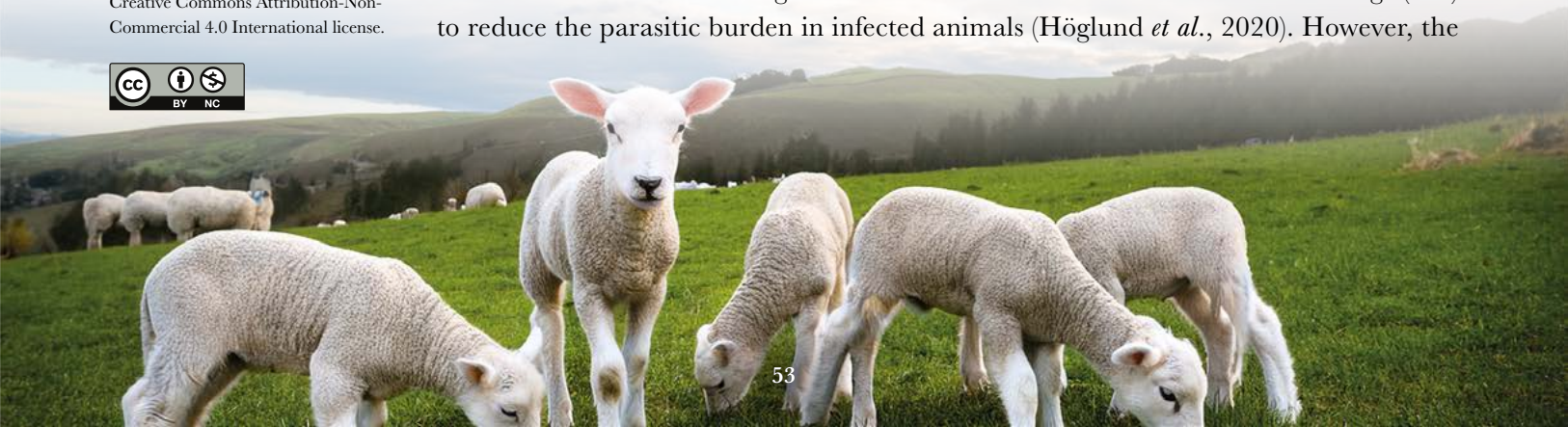
INTRODUCTION

Haemonchus contortus is the most significant parasite affecting small ruminants raised under grazing systems (Lalramhluna *et al.*, 2020). This parasite induces severe clinical symptoms, including anemia, submandibular edema, and, in some cases, fatalities in young animals (Lambacher *et al.*, 2019). The primary control strategy for this nematodiasis relies on the regular administration of chemical anthelmintic drugs (AH) to reduce the parasitic burden in infected animals (Höglund *et al.*, 2020). However, the



widespread and continuous use of these drugs has led to the inevitable development of resistance in *H. contortus* populations (Fávero *et al.*, 2020). In response to this challenge, alternative control methods for haemonchosis and other parasitic nematodiases are being investigated as sustainable strategies distinct from conventional AH drug use. Among these, the application of medicinal plants has emerged as a promising approach, offering herbal-based therapeutic solutions with encouraging results (Szulc *et al.*, 2020). *Artemisia* is a plant genus within the Asteraceae family, widely recognized for its medicinal properties. Comprising 474 species, this genus is distributed globally, spanning polar regions to the tropics (Rustaiyan *et al.*, 2021). *Artemisia cina* Berg ex Poljakov, a shrub native to the eastern Caspian Sea region in Afghanistan, emits a distinctive aromatic odor, grows between 40 and 60 cm in height, and possesses erect, cottony stems. The leaves are small, with short petioles, and the upper leaves are sessile. This species thrives in semi-desert environments with extreme temperatures, favoring saline soils. Commonly referred to as cina or marine wormwood, *A. cina* has been traditionally employed as an antiparasitic remedy (Woerdenbag, 1997). Between the 1950s and 1970s, plant-based formulations derived from *A. cina* were used for deworming children in schools and childcare facilities. The active compound responsible for this effect, santonin, was initially formulated as tablets and later as flavored sweets, targeting the treatment of ascariasis, a highly prevalent parasitic infection (Zhang *et al.*, 2019). However, its clinical application was discontinued due to accumulated toxicity, with adverse effects observed at 60 mg in children and 200 mg in adults. Beyond its historical use, *A. cina* harbors bioactive compounds with diverse pharmacological properties. For instance, artemisinin, a molecule present in *A. cina* leaves, has been extensively recognized for its antimalarial activity and has been utilized for decades as an alternative treatment for malaria (Saitbaeva & Sidyakin, 1971). Additionally, *A. cina* exhibits significant anthelmintic potential. Ethanolic extracts of this plant have demonstrated notable *in vitro* anthelmintic activity, achieving up to an 80% efficacy against *H. contortus* infective larvae (L3) (Van Agtmael *et al.*, 1999). Interestingly, the anthelmintic properties of *A. cina* (commonly referred to as "worm killer") are attributed to phytoconstituents located in its aerial parts. Several bioactive compounds identified in these plant structures have exhibited efficacy against roundworms, pinworms, and amoebal infections in pigs and companion animals (Lans *et al.*, 2007). Beyond its *in vitro* effectiveness against *H. contortus*, *A. cina* has also demonstrated cestodicidal activity against *Moniezia expansa*, a tapeworm species infecting small ruminants (Bashtar *et al.*, 2011). The aim of this study was to evaluate the effects of orally administered ethanolic extracts of *A. cina* leaves on fecal egg count (FEC) and parasitic burden in lambs experimentally infected with *H. contortus* under controlled conditions.

MATERIALS AND METHODS

Location

This study was conducted at Laboratory 3 of the Multidisciplinary Research Unit, Faculty of Advanced Studies Cuautitlán – National Autonomous University of Mexico (UNAM). The animals were kept in paddocks at the Postgraduate Area of the same Faculty.

Plant material

Ten kilograms of *A. cina* Berg ex Poljakov leaf material at the pre-flowering stage were commercially obtained from Millennium Laboratories in Mexico City. The plants in this facility are cultivated in a greenhouse under controlled environmental conditions, including humidity (24.6%), temperature (24 °C), pH (8.7), and salinity (1.6%).

Obtaining plant extract

The plant material was dried at 60 °C for one week. The extract was obtained through organic extraction using ethanol for 24 hours. The resulting plant extract was then concentrated using a rotary evaporator (Heidolph Laborota 4000, Heidolph Instruments, Schwabach, Germany) under reduced pressure at 40 °C (Iqbal *et al.*, 2004). Finally, the extract was lyophilized using a Labconco FreeZone™ Freeze-Dry System (4.5 L).

Experimental animals

Fifteen three-month-old Hampshire male lambs, born nematode-free, were identified with metallic earrings and kept under full confinement conditions throughout the experiment. The animals were fed alfalfa (*Medicago sativa* L.) and had *ad libitum* access to water.

Artificial *H. contortus* infection

Lambs were randomly assigned to four groups and monitored for 14 days to ensure the absence of gastrointestinal nematode (GIN) eggs in their feces, thereby confirming that the experimental flock was free of parasitic infections. Each lamb was then intraruminally inoculated with 5,000 L3 larvae of H. contortus FESC strain. The animals were monitored weekly to determine their fecal egg count (FEC) starting from the third week post-infection. The lambs were included in the study once the flock reached a mean FEC of 5,000 eggs per gram.

Experimental procedure

Animals were randomly distributed into three groups of five animals each as follows: Group 1, *A. cina* ethanolic extract (Ac eth/ext), orally administered at a single dose of 4 mg/kg body weight (BW); Group 2, a single dose of albendazole (Albendaphorte 2.5% Co, Health and Animal Welfare Lab), orally administered at a dose of 5 mg/kg BW; Group 3, control, untreated. Fecal samples were collected daily directly from the rectum of each animal for seven days post-treatment to conduct the McMaster technique (Coles *et al.*, 1992). Additionally, a record of the FAMACHA© index was measured daily during the same period (Torres-Chable *et al.*, 2020).

Effect of *A. cina* ethanolic extract on the *H. contortus* faecal egg count reduction

Means of the faecal egg counts per group were estimated and compared with the control group. The results of this evaluation were expressed as the FEC reduction percentage, based on the following formula:

$$\%\ Reduction = 100 \times \frac{C - T}{C}$$

where C=FEC arithmetic mean in the control group and T=FEC arithmetic mean in the treated group.

Effect of *A. cina* ethanolic extract on reducing the *H. contortus* adult parasitic population in lambs at necropsy

The three experimental groups of lambs were slaughtered on day seven post-treatment under humane conditions, following the Norma Oficial Mexicana NOM-033-ZOO-1995, which regulates the humane slaughter of domestic and wild animals. The procedure for evaluating anthelmintic efficacy in ruminants, established by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Wood *et al.*, 1995), was followed.

The abomasum was removed, placed in individual plastic containers, and washed three times with preheated phosphate-buffered saline (PBS) at 30 °C. The contents were filtered through sieves with 1.25 mm and 0.25 mm diameters. The final filtrate was diluted with water to a total volume of 2000 mL and fixed with 10% formaldehyde. Next, 10 mL of this suspension was transferred onto a 60×15 mm plastic Petri dish to observe and recover adult parasites, which were then counted and sexed into two groups: males and females. This process was repeated until the entire initial volume was analyzed. The efficacy of the treatments was determined by comparing the mean number of *H. contortus* adult specimens recovered from each group with the control group, and the percentage reduction was calculated following the previously described formula (González-Garduño *et al.*, 2011).

Statistical analysis

The FEC and genus (male/female) data obtained from the three experimental groups were analyzed using the variance-ANOVA and the complementary Tukey tests to identify possible differences among groups using the Statgraphics program (Centurion XV®).

Ethical note

The lambs were managed by the guidelines of the Institutional Committee for the Care and Use of Experimental Animals of the FESC, University National Autonomous of Mexico (CICUAE-FESC-UNAM), protocol No. DC—2014-14.

RESULTS

Fecal egg count reduction

The results regarding the dynamics of fecal egg count (FEC) excretion in the three experimental groups are presented in Figure 1. Since the day with *A. cina* treatment, an increase in FEC values was observed for the three lamb-groups. Although, groups treated with albendazole and Ac eth/ext, this increase was significantly lower compared to the untreated control group.

Notably, from day 1 to day 6, the Ac eth/ext-treated group exhibited a continuous decline in FEC values. In contrast, the albendazole-treated group showed a significant reduction

in FEC values from day 1, reaching zero on days 4, 5, and 6 of the experiment. The percentage reductions attributed to the anthelmintic effect of each treatment throughout the experiment are summarized in Figure 1. The mean FEC reduction percentages recorded during the experiment were 46.97% for the Ac eth/ext-treated group and 75.45% for the albendazole-treated group ($p<0.05$).

FAMACHA® values

The FAMACHA® values recorded throughout the experiment for the three experimental groups of lambs are presented in Table 1. Famacha score was observed since the day of treatment. Results showed that the untreated control group was consistently scored three in the FAMACHA® record, maintaining this value until the end of the experiment. In contrast, albendazole-treated group exhibited an improvement, with the FAMACHA® score decreasing to two, where it remained stable until the conclusion of the study. Similarly, in the Ac eth/ext-treated group, the FAMACHA® score remained at three until day four, after which it decreased to two on days five and six.

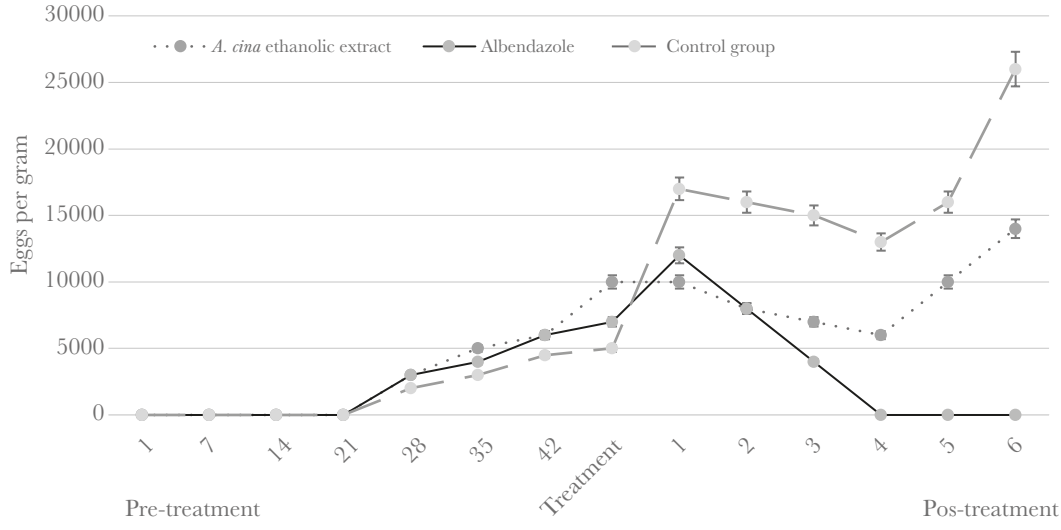


Figure 1. Records of the *Haemonchus contortus* EPG excreted by three groups of artificially infected lambs to determine the anthelmintic efficacy of *Artemisia cina* ethanolic extract.

Table 1. Ocular mucosa color (FAMACHA index) in artificially infected lambs with *Haemonchus contortus* treated with *Artemisia cina* ethanolic extract.

	FAMACHA index		
	Control group	***Artemisia cina* ethanolic extract**	**Albendazole**
Day 42	3 ± 0.5^{a}	3 ± 1^{a}	3 ± 1^{a}
Treatment	3 ± 1^{a}	3 ± 0.5^{a}	3 ± 1^{a}
Day 7 Post-treatment	3 ± 0.5^{a}	2 ± 1^{b}	2 ± 1^{b}

Superscripts show significant difference ($p<0.05$).

Anthelmintic effect of *A. cina* in lambs

Table 2 summarizes the data on the mean numbers of *H. contortus* males and females recovered at necropsy, along with the anthelmintic efficacies of Ac eth/ext and albendazole. The Ac eth/ext treatment resulted in a 30% reduction in total parasitic burden, whereas albendazole demonstrated only a 5.31% reduction. The efficacy of Ac eth/ext against male and female parasites did not show any statistically significant differences between them.

DISCUSSION

Reduction of FEC values attributed to the effect of *A. cina* ethanolic extract

The results obtained in the present study provide clear evidence of the anthelmintic effect of Ac eth/ext, which was observed from the first record (day 1) and persisted until the last record (day 6), with efficacy ranging from 37.5% to 53.8% compared to the control group. In this regard, it is noteworthy that, to date, no previous records exist regarding the anthelmintic effect of *A. cina* organic extracts in reducing *H. contortus* FEC excretion in lambs. Therefore, this study could be considered the first report documenting the anthelmintic properties of this plant against *H. contortus* in lambs. On the other hand, a study conducted in Cairo, Egypt, evaluated the anthelmintic effect of an *A. cina* hydroalcoholic extract under *in vitro* conditions, demonstrating that the extract induced structural damage to the scolex and other tissues of the cestode *Moniezia expansa* as observed through electron microscopy. Additionally, the authors reported that oral administration of 2 g of *A. cina* crude extract per sheep eliminated *M. expansa* fecal egg counts by day 9 post-treatment (Bashtar *et al.*, 2011). It is also noteworthy that, during the first three days post-treatment, the FEC reduction effect of albendazole was lower than expected, considering that some studies report that albendazole reaches its peak plasma concentration (approximately 1.2 μg/mL) around eight hours post-administration (Evrard *et al.*, 2002). However, the subsequent total reduction in FEC excretion observed in the following days confirms that albendazole remains a highly effective compound in controlling *H. contortus* infections in lambs. Nevertheless, it is crucial to acknowledge that the recurrent use of any anthelmintic drug in farm animals inevitably leads to the development of anthelmintic resistance, which in turn rapidly diminishes their efficacy (Kellerová *et al.*, 2020).

Table 2. Number, prolificity, length, male:female ratio of *Haemonchus contortus* females recovered from artificially infected lambs with *H. contortus* treated with *Artemisia cina* ethanolic extract.

Treatment	Eggs eliminated by adults of *H. contortus*	Number of *H. contortus* adult females*	Female length (mm) of *Haemonchus contortus*	No. of male adults of *H. contortus**	*H. contortus* lenght of adult males (mm)	Male: female ratio
Ac eth/ ext	$51{,}134 \pm 544^{b}$	365 ± 12^{a}	2.09 ± 0.2^{a}	306.25 ± 23^{a}	1.4 ± 0.2^{a}	$0.83{:}1^{a}$
Control	$37{,}319 \pm 251^{c}$	496.25 ± 15^{b}	1.9 ± 0.3^{b}	463 ± 27^{b}	1.47 ± 0.2^{a}	$0.93{:}1^{a}$
Albendazole	531 ± 31^{d}	276 ± 15^{c}	2.07 ± 0.2^{a}	632.5 ± 17^{c}	1.48 ± 0.2^{a}	$2.2{:}1^{c}$

**Haemonchus contortus* adult stages were collected from the abomasum of infected lambs and kept in incubation with RPMI medium to be counted and measured. Ac eth/ext=*Artemisia cina* ethanolic extract; superscripts show a significant difference ($p<0.05$).

Effect of *A. cina* ethanolic extract on the FAMACHA records

The FAMACHA® records in animals treated with Ac eth/ext showed an improvement on days 3, 5, and 6 of the experiment compared to the control group. This suggests that the Ac eth/ext-treated group likely had a lower parasitic burden, leading to reduced blood loss and, consequently, the maintenance of packed cell volume (PCV) values. This observation was further supported by the post-mortem reduction in parasitic burden in the Ac eth/ext-treated lambs compared to the control group. In the case of albendazole, a substantial reduction in FAMACHA® scores was observed from day 2, and this reduction persisted throughout the experiment until its conclusion.

These findings support the strategic deworming approach, reinforcing the principle that not all animals in a flock should be dewormed, but only those that require it. Furthermore, the results provide compelling evidence that Ac eth/ext represents a valuable complementary tool for minimizing the unnecessary use of anthelmintics, thereby indirectly contributing to reducing the spread of anthelmintic resistance.

Effect of administration of *A. cina* ethanolic extract on reducing the parasitic burden at necropsy in lambs

The reduction in the number of adult parasites recovered at necropsy in the Ac eth/ext-treated group, compared to the nematodes recovered from the untreated control group, indicates that although the reduction was moderate (30%), it can still be considered significant. This is particularly relevant when considering that albendazole demonstrated an efficacy closer to 100% (5.3%). The high efficacy of albendazole observed in this study suggests that the *H. contortus* strain used in the experiment may have developed anthelmintic resistance. However, to confirm this assumption, additional parameters such as body condition, packed cell volume (PCV), and the FAMACHA® index should be analyzed in conjunction with the Fecal Egg Count Reduction Test (FECRT) (Wood *et al.*, 2002; Höglund *et al.*, 2020). Similarly, albendazole has been widely reported in Mexico as a reference anthelmintic; however, its continuous use has led to documented cases of resistance, as reported by various authors listed in Table 3. The frequent and prolonged use of anthelmintic drugs has similarly contributed to resistance in sheep and goats. Evidence presented in Table 3 suggests that anthelmintic resistance is a growing concern in Latin America (Torres-Acosta *et al.*, 2012).

Regarding the anthelmintic efficacy of albendazole against gastrointestinal nematodes (GIN), several reports highlight a worrisome increase in resistance to this and most commercially available anthelmintic drugs across multiple countries (Table 3).

Due to the limited information regarding the use of *A. cina* extracts as potential anthelmintics in ruminants, further studies are necessary to enhance the anthelmintic efficacy observed with Ac eth/ext. For instance, research should explore the effects of increasing extract concentrations or administering higher doses. Additionally, it is crucial to design bio-guided experiments to identify the specific compounds responsible for the anthelmintic activity, ultimately paving the way for the development of a novel, alternative, and sustainable method for controlling sheep haemonchosis (Delgado-Nuñez *et al.*, 2020). Since ancient times, plants and natural products have been regarded as valuable resources

Table 3. Recent records about albendazole anthelmintic resistance in ruminant parasitic nematodes in different countries.

Country	Resistance (%)	Parasitic genera	Reference
Bangladesh	25-47	*H, O, Tr*	Dey *et al.*, 2020
Sudan	73.5-90.2	*Hc, Tr, C*	Mohammedsalih *et al.*, 2020
Brazil	100	*C, Hc, Tr*	Ramos *et al.* 2020
Iran	71	Natural infection	Ebrahimi *et al.*, 2020
Egypt	13.16	*Hc*	Aboelhadid *et al.*, 2020
France and Italy	0-5	Natural infection	Chartier *et al.*, 2020
Indonesia	16.19	*Tr, As*	Kholik *et al.*, 2019
Sudan	18	*Hc*	Mohammedsalih *et al.*, 2019
Mexico	83	*C, Tr*	Mondragón-Ancelmo *et al.*, 2019
Malang Regency	7.77	Natural infection	Sasangko *et al.*, 2019
Brazil	34	Natural infection	Nagata *et al.*, 2019
Mozambique	52.9	*Hc*	Atanásio-Nhacumbe *et al.*, 2019
Pakistan	54	Natural infection	Muhammad *et al.*, 2019
Brazil	1-38	Natural infection	De Fátima Lela Pererira *et al.*, 2019
Jordan	60	Natural infection	Hayajneh *et al.*, 2019
Brazil	100	Natural infection	Costa *et al.*, 2019
Brazil	100	*Co, Hc, Oes, Tr*	Ramos *et al.*, 2018
Brazil	95	*Hc, Tr, Oe, Tri*	Flávida da Silva *et al.*, 2018
Australia	100	*Hc, Tr, Os, C*	Rashid *et al.*, 2018
Madhya Pradesh	28	Natural infection	Shakya *et al.*, 2018
Iraq	100	*Tr, Mar, Tri, Ne*	Dyary, 2018
India	33	*Hc*	Sahoo *et al.*, 2018
Slovakia	13.2-30.8	Natural infection	Babják *et al.*, 2018

Hc: *Haemonchus contortus*, *Tr*: *Trichostrongylus axei*, *Oe*: *Oesophagostomum colombianum*, *Tri*: *Trichuris ovis*, *Os*: *Ostertagia ostertagi*, *Mar*: *Marshallagia* spp., *Ne*: *Nematodirus* spp.

for human and veterinary medicine. This traditional knowledge is essential for exploring phytochemical and pharmacological properties, providing scientific foundations for their sustainable use (Dassou *et al.*, 2020). The anthelmintic properties of *A. cina* against intestinal parasites in children, particularly Ascaris lumbricoides, were first documented in 1950 in China. The active compound santonin, a sesquiterpene, was identified as the primary agent responsible for its antiparasitic activity. For many years, santonin was one of the most widely used agents for the control of intestinal parasitic diseases in China (Zhang *et al.*, 2019). The anthelmintic efficacy of various *Artemisia* species against *H. contortus* has been demonstrated in several studies (Table 4).

High variability in *Artemisia* spp. anthelmintic activity against *H. contortus*

One of the most practical approaches to integrating plant-based anthelmintic treatments into flock management is by incorporating plant material into the diet as a nutritional supplement. For instance, the addition of *Artemisia absinthium* L. leaves to the

Table 4. Recent reports concerning the anthelmintic effect of *Artemisia* spp. against *Haemonchus contortus in vitro* and *in vivo.*

Artemisia spp.	Part of the plant	Extract	Experimental condition	Anthelmintic efficacy*	Author
A. absinthium	Stems	Dietary supplement	*In vivo*	45%	Váradyová *et al.*, 2017
A. absinthium	Leaves	Ethanolic Aqueous	*In vivo*	100% 80%	Alam *et al.*, 2016
A. absinthium	Leaves	Dietary supplement	*In vivo*	100%	Valderrábano *et al.*, 2010
A. absinthium	Leaves and stems	Aqueous	*In vivo*	No differences	Worku *et al.*, 2009
A. absinthium	Leaves	Aqueous Ethanolic	*In vitro*	80.49% 90.46%	Tariq *et al.*, 2009
A. absinthium	Leaves	Dietary supplement	*In vitro* *In vivo*	99% No differences	Mravcakova *et al.*, 2020
A. absinthium	Stems	Methanolic Aqueous	*In vitro*	100%	Váradyová *et al.*, 2018
A. annua *A. absinthium*	Leaves	Aqueous Ethanolic Essential oil	*In vivo*	No significant effects	Squires *et al.*, 2011
A. annua	Leaves	Hydroalcoholic	*In vitro*	93.22%	Sprenger *et al.*, 2016
A. annua	Leaves	Aqueous	*In vivo*	No differences	Cala *et al.*, 2014
A. brevifolia	Whole plant	Aqueous Methanol	*In vivo*	67.2%	Iqbal *et al.*, 2004
A. campestris	Leaves	Aqueous Ethanolic	*In vitro*	50%	Akkari *et al.*, 2014
A. campestris	Aerial parts	Essential oil	*In vitro*	100%	Abidi *et al.*, 2018
A. campestris L.	Leaves	Aqueous	*In vitro*	<50%	Boyko *et al.*, 2019
A. cina	Leaves	Homeopathic	*In vivo*	69%	Higuera-Piedrahita *et al.*, 2020
A. cina	Leaves and stems	Ethanolic	*In vivo*	47%	Present study
Artemisia afra	Leaves	Aqueous	*In vitro*	100%	Molefe *et al.*, 2012
A. herba-alba	Leaves	Methanolic	*In vitro*-IEH	67%	Ahmed *et al.*, 2020
A. herba-alba	Leaves	Dietary supplement	*In vivo*	100%	Idris *et al.*, 1982
A. lancea	Leaves	Essential oil	*In vitro*	99%	Zhu *et al.*, 2013
A. parviflora *A. sieversiana*	Leaves	Methanolic	*In vitro*	100% 90%	Irum *et al.*, 2017
A. vestita *A. maritima*	Leaves	Aqueous	*In vivo*	87.2% 84.5%	Irum *et al.*, 2015
A. vulgaris L.	Leaves	Essential oil	*In vitro*	No differences	Malik *et al.*, 2019
A. tridentata	Leaves	Aqueous	*In vitro*	96.30%	Luck-Montero *et al.*, 2018
A. vulgaris	Aerial parts	Aqueous Ethanolic	*In vitro*	100% 100%	Karim *et al.*, 2019

***** The anthelmintic efficiency value described in the table was taken as the highest reported in each paper.

diet of a Rasa Aragonesa lamb flock in Zaragoza, Spain, resulted in a 100% reduction in *H. contortus* fecal egg count (FEC) (Valderrábano *et al.*, 2010). In a similar study, Váradyová *et al.* (2018) reported a 45% FEC reduction in a Vallachian lamb flock in the Slovak Republic following the voluntary consumption of *A. absinthium* pellets. However, another study conducted in the Slovak Republic, in which *A. absinthium* was administered as part

of a mixed plant diet, did not demonstrate a significant FEC reduction (Mravčáková *et al.*, 2020). The high variability observed in the anthelmintic response across different studies conducted in various agroecological zones may be explained by the biological activity of plants, which is influenced by numerous biotic and abiotic factors. These factors, primarily associated with the environment where the plants grow, subject plants to various stresses that may modify their morphological, physiological, and biochemical activities (Sardharal & Mehta, 2018).

Recent studies have identified several key factors regulating plant biosynthesis, including light exposure, plant hormones, temperature, and saline and drought stress. Notably, a study demonstrated that abiotic stressors, such as ultraviolet B irradiation and the presence of phytohormones, stimulated artemisinin accumulation in *Artemisia annua* (Ma *et al.*, 2020). Regarding the anthelmintic activity of Artemisia species extracted using different organic solvents under in vitro conditions, researchers have reported high variability in efficacy, ranging from 50% (*e.g.*, *Artemisia campestris* L.) to close to 100% (*e.g.*, *A. absinthium*, *A. annua*, and *A. campestris*) (Sprenger *et al.*, 2016; Váradyová *et al.*, 2018; Abidi *et al.*, 2018; Mravčáková *et al.*, 2020). Furthermore, the inclusion of Artemisia leaves in the diet as a nutritional supplement for small ruminants has been associated with FEC reductions ranging from 45% to 100% (Table 4). Similarly, when organic extracts of Artemisia species were administered orally, reported FEC reductions ranged from 67.2% (*e.g.*, *A. brevifolia*) to 100% (*e.g.*, *A. absinthium*) (Table 4). It is important to consider that secondary metabolites responsible for anthelmintic activity, such as flavones, flavonoids, alkaloids, coumarins, sesquiterpenes, and santonins, are typically extracted using organic solvents, due to their specific polarity (Muhammad *et al.*, 2021). Consequently, aqueous extractions are not generally expected to exhibit strong anthelmintic properties. However, a study by Alam *et al.* (2016) reported an 80% FEC reduction in *H. contortus*-infected calves treated with an aqueous extract of *A. absinthium*. Additionally, essential oils extracted from various Artemisia species have been found to contain monoterpenoids, including camphor, 1,8-cineole, camphene, and α-pinene, which are associated with antioxidant and antimicrobial properties (Sharopov *et al.*, 2020). Notably, monoterpenes, such as thymol (reagent grade), have also been linked to antiparasitic activity against other helminth species of veterinary importance, demonstrating high efficacy in rodent models (Mirza *et al.*, 2020).

CONCLUSIONS

Artemisia cina ethanolic extract demonstrated a 47% reduction in fecal egg count (FEC) in *Haemonchus contortus*-infected lambs under controlled conditions. This treatment also resulted in a 30% decrease in parasitic burden. *Artemisia cina* ethanolic extract represents a valuable tool for the control of haemonchosis in lambs, and further studies are necessary to assess its efficacy within an integrated control system, combining alternative control strategies to enhance flock health from a sustainable perspective. Additionally, the identification of bioactive metabolites responsible for the anthelmintic activity should be pursued to evaluate their potential application in the control of gastrointestinal parasitic nematodes.

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UV-C light irradiation and ultrasonic infiltration of rosemary (*Rosmarinus officinalis* L.) essential oil nanocapsules into refrigerated chicken fajitas

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ABSTRACT

Objective: To evaluate the effect of the application of ultraviolet light (UV-C) treands and/or ultrasonic infiltration of a marinade with and without rosemary (*Rosmarinus officinalis* L.) essential oil nanocapsules on the physical, physicochemical, and textural properties of vacuum-packed chicken fajitas stored at 2 °C for 11 days.

Design/Methodology/Approach: Water holding capacity (WHC), lactic acid, electrical conductivity, shear force, pH, and color changes (ΔE) were evaluated. The treatments applied were fajitas without any treatment (C), marinated fajitas (M), UV-C irradiated, marinated with ultrasound (US), marinated with nanoencapsulated rosemary into zein nanocapsules (NCS), UV-C/US, NCS/UV-C, NCS/US, and NCS/UV-C/US. Each was marinated with vinegar and salt, except the control lot. To analyze the correlation between variables, the Pearson evaluation is used in the Minitab®19 program, with $\alpha=0.05$.

Results: The NCS with US, UV-C, or US/UV-C, presented the highest WHC values (52.49±1.43%), and lactic acid concentration at the end of storage (increase 18.52±0.44%). NCS treatment in combination with US and UV-C presented the highest EC values (7.31±0.37 and 6.25±0.33 mS/cm respectively). Maintaining pH around 5.5 during 11 days of storage and presenting 30% of stability in ΔE.

Limitations on study/implications: The use of UV-C requires additional training of operators for its correct operation, in addition, the use of ultrasound as a food treatment technology may present inconveniences for its scaling and application in an industrial production line.

Conclusions: The application of marinade in combination with emerging technologies and nanocapsules with rosemary essential oil favors their infiltration and chicken fajitas preservation during 11 days of refrigerated storage (2 °C).

Keywords: Emerging technologies, zein, meat, preservation, marination.

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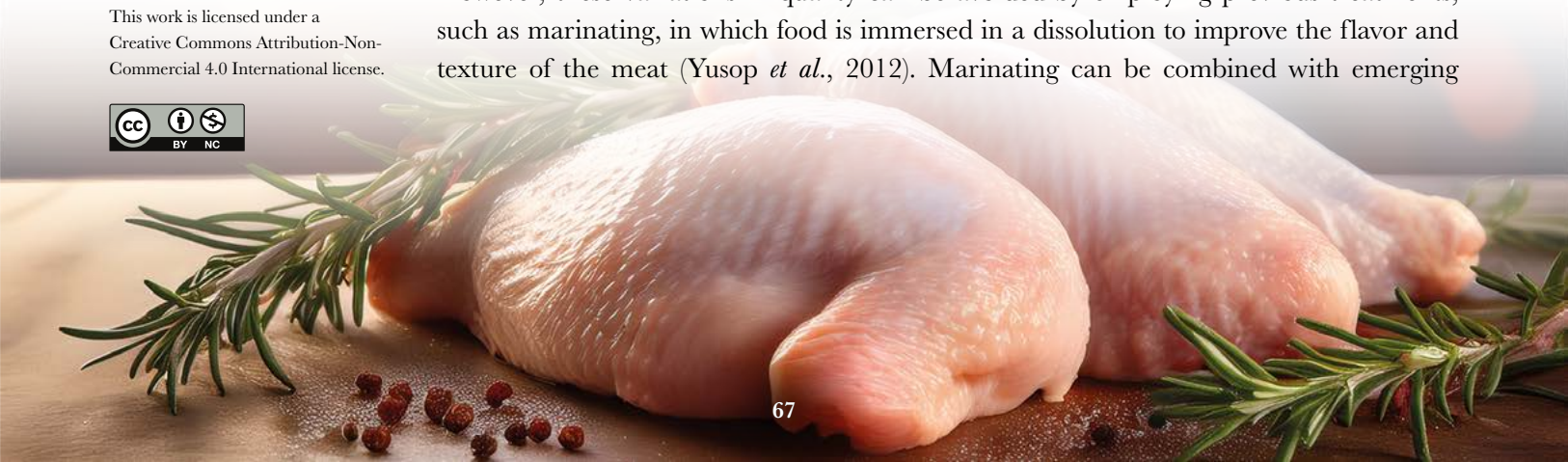
INTRODUCTION

Fresh chicken breast is susceptible to surface contamination, affecting quality parameters during their storage and decreasing consumer acceptability (Rouger *et al.*, 2017). However, these variations in quality can be avoided by employing previous treatments, such as marinating, in which food is immersed in a dissolution to improve the flavor and texture of the meat (Yusop *et al.*, 2012). Marinating can be combined with emerging



technologies such as ultraviolet light (UV-C), nanotechnology, and ultrasound to promote the infiltration of marinade ingredients into the chicken. The emerging technologies are an alternative for food preservation and processing, decreasing food matrix degradation, maintaining the nutritional composition, as well as the organoleptic and physicochemical properties. Nanocapsules act as a reservoir, that can modify the release profile of bioactive substances, such as rosemary (*Rosmarinus officinalis* L.) essential oil, which has antibiotic, antimicrobial and antioxidant properties and is considered safe for human consumption, due its low toxicity level (Granata *et al.*, 2018). However, lipophilic nature compounds incorporation into mainly water matrix, such as food, is complicated. Nanoencapsulation of these components represents an alternative to their incorporation into food, enhancing its solubility (Bazzano *et al.*, 2016). For this reason, emerging technologies in conjunction with refrigeration can maximize the efficiency of food preservation and increase its shelf life by providing the necessary conditions to maintain the final quality of the product, *i.e.*, its physical and physicochemical properties during storage. This work aimed to evaluate the effect of the application of ultraviolet light treatments (UV-C) and ultrasonic infiltration (US) of a marinade (M) with nanoencapsulated rosemary (*Rosmarinus officinalis* L.) essential oil (NCS) on the physical, physicochemical, and textural properties of chicken fajitas, vacuum packed and refrigerated storage at 2 °C for 11 days.

METHODOLOGY

A chicken breast was processed to obtain fajitas (7×1.5×0.8 cm) that were pretreated under different conditions before their refrigerated storage (2±1 °C) for 11 days, evaluating the variation in quality parameters every third day to determine the effect of the application of individual and combined technologies on chicken fajitas for their preservation. The different studied treatments were fajitas without any treatment (C), marinated fajitas (M), UV-C irradiated, marinated with ultrasound (US), marinated with nanoencapsulated rosemary into zein nanocapsules (NCS), UV-C/US, NCS/UV-C, NCS/US, and NCS/UV-C/US. Subsequently, pretreated fajitas (20 g) were packed in a high-density polyethylene bag (10×15 cm) under vacuum conditions (50 bar). US treatment application required a Hielscher® 200Ht equipment, with a 40 mm sonotrode (S26d40), at 26kH frequency, 80% amplitude, and 50% duty cycles, during 15 s. A UVP XX-15S lamp (254 nm, 15 W) was used for the UV-C irradiated samples for 2 min. The marinated samples were immersed for 5 min at room temperature on an aqueous dissolution of apple cider vinegar (100 mL/L) and NaCl (75 g/L). The marinade samples with NCS incorporate rosemary essential oil (1 g/L) zein nanocapsules (150±18 nm). Changes in the chicken fajitas were monitored during storage. The color variation (ΔE) of the surface was carried out with a Konica Minolta® colorimeter (CM-600d), according to (Domínguez *et al.*, 2022).

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \qquad \text{(Equation 1)}$$

The color parameter is expressed on the CIELAB scale (L*, a*, b*). L* represents Luminosity, a* green-red scale, and b* yellow-blue scale. The Brookfield® CT3 texturometer

equipped with a 25 kg load cell was used for shear resistance evaluation, employing a Warner-Braztler cell (0.07 N, 1 mm/s). It also was used for the water holding capacity (WHC) determination, using a compression method as described by (Barbut, 2024) and (Jankowiak *et al.*, 2021). Samples of meat were put between two layers of filter paper (5 cm diameter) and a constant compression force (10 N, 20 s), using an acrylic cylinder (TA 25/1000) and an activation force of 0.07 N. The WHC was calculated based on

$$WHC\left[\frac{g_{H_2O}}{100\,g_{H_2O}}\right]=\frac{(m_1*H)-(m_2-m_3)}{(m_1*H)}*100 \qquad \text{(Equation 2)}$$

and the results were reported as g of water per 100 g of water in the sample. Where m_1 is the sample mass, m_2 is the initial mass of the filter paper, m_3 is the mass of the filter paper after the test, and H is the theoretical moisture of the chicken meat. The pH determination was performed with a pH-meter with a knife blade electrode, model HI99163, from Hanna Instruments (Woonsocket, USA) as reported by (Jankowiak *et al.*, 2021). Lactic acid was measured by titration, employing a 0.1 N NaOH solution (Terefe, 2017). The obtained result is reported as the percentage of acidity, calculated according to:

$$Lactic\ acid(\%)=\frac{m_{eq}*V*N*V}{W} \qquad \text{(Equation 3)}$$

Where m_{eq} is the milliequivalents for the quantified acid (lactic acid), V is the volume of NaOH solution spent, N is normality, V is the volume of water used in the dilution and W is the weight of the sample. The electrical conductivity was measured as an indicator of membrane integrity in meat according to (Saelin *et al.*, 2017), 5 g of sample were homogenized with 50 mL of distilled water, and electrical conductivity was evaluated using a conductivity meter, model ST3100C from Ohaus.

RESULTS

Water-holding capacity (WHC)

The WHC presented a statistically significant difference ($p \leq 0.05$) during the storage time. Figure 1 shows a decrease for all treatments. The increase in water loss during meat storage is related to protein degradation and structure modification due to oxidation, and protein denaturation leaving the hydrophobic groups exposed (Saelin *et al.*, 2017).

WHC results for the first day do not present significant differences ($p \geq 0.05$) between treatments except for the C, M, US, and the NCS/UV/US. The samples treated with UV-C individually or combined, were the ones that presented the highest WHC values at the beginning ($52.33 \pm 1.5\%$) and the end of the storage ($26.67 \pm 0.83\%$). UV-C irradiation can stimulate reactive oxygen species (ROS) formation, leading to an increased protein charge state by increasing ionic strength and the subsequent exposure of more (Monteiro *et al.*, 2019). The samples with infiltrated nanocapsules (NCS) did not demonstrate significant

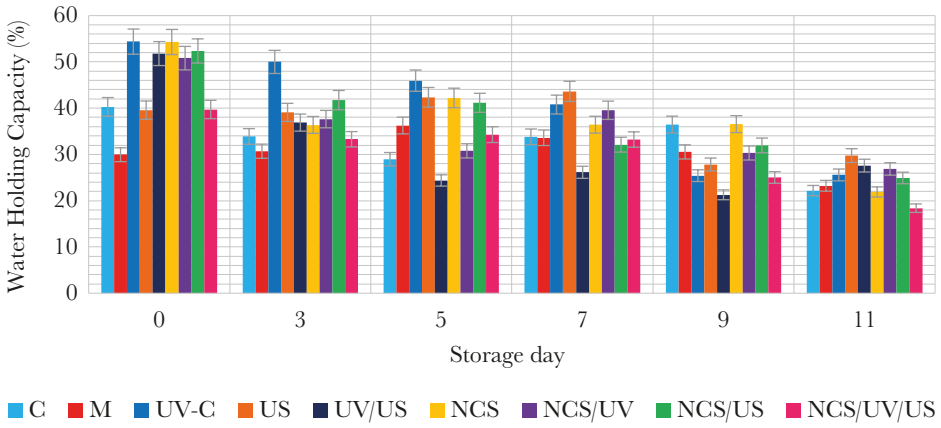


Figure 1. Water-holding capacity (WHC) variation during storage.

differences with UV-C samples, presenting high WHC values (52.49±1.43%) related to the zein polymeric structure of the NCS and the lipophilic nature of rosemary essential oil. Some researchers have demonstrated that the protein oxidation with ROS like hydrogen peroxide (H_2O_2) can bring about formation of hydroxyl and carbonyl groups even on hydrophobic amino acid residues with aliphatic side chains such as leucine, isoleucine, valine, and alanine, and oxidize proline to hydroxyproline and pyroglutamic acid. These amino acids are predominant in zein (Taylor *et al.*, 2016), enhancing the WHC even in zein that contains more than 50% hydrophobic amino acids and low water solubility (soluble at 55-90% aqueous ethanol solutions) (Chen *et al.*, 2024). Also, the addition of NCS from hydrophobic compounds can enhance the WHC by acting like a polymeric (Ranjan *et al.*, 2016). Despite US and UV-C treatments suggesting higher cell damage, the application in combination with NCS may generate a preservation system with the formation of ROS that could enhance WHC by water bid points formation in protein structure, without incrementing the tissue deterioration due to the rosemary essential oil antioxidant effect. Usually, the US is used as a tenderization process for meat, due to its effect on myofibril protein fragmentation (Yoon *et al.*, 2024). The lower WHC presented by M samples implies that meat cannot bind successfully aggregated water without the implementation of an external technology such as UV-C, US, or NCS. In contrast, the marinated samples were the samples that presented the lower decrease of WHC during the storage time (22.42±0.62%) due to the lack of external energy addition during the process that promotes the formation of ROS, indicating more stable WHC values during the storage, only exhibiting significant differences until the fifth storage day. WHC presented a significant correlation ($p \leq 0.05$) with electric conductivity, lactic acid, due to the leakage of hydrophilic compounds from cell structure in samples with low WHC, and with shear force, related to the amount of water trapped in the meat tissues.

Lactic acid

Figure 2 shows the inverse correlation between lactic acid production and WHC variation during the storage time for the different treatments. The samples with a higher

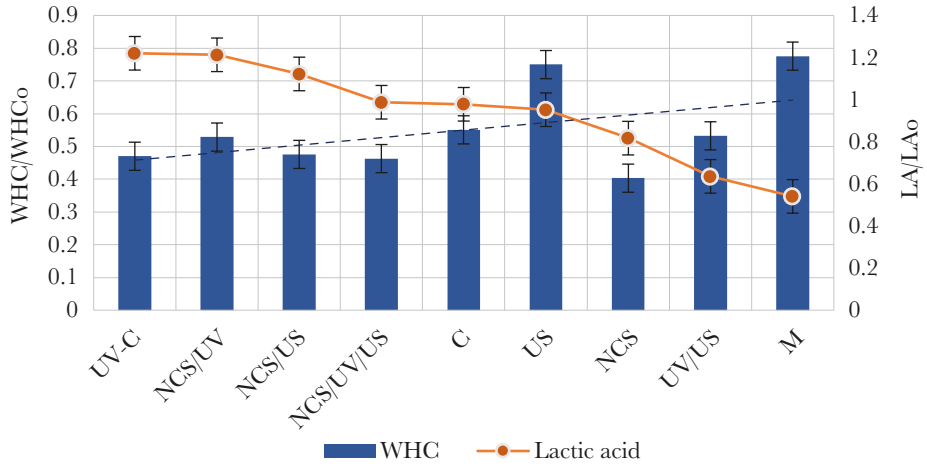


Figure 2. WHC and lactic acid correlation.

difference between initial and final WHC presented higher lactic acid concentrations. The lactic acid concentration of US, NCS/UV-C/US, and C samples did not present a significant difference ($p \geq 0.05$) during the storage time. The irradiated samples with UV-C and infiltrated with NCS in combination with UV-C, and US, presented an increase in the concentration of lactic acid at the end of storage, not presenting a significant difference between treatments (18.52±0.44%). The increase of the concentration of lactic acid in the tissues favors protein denaturation and chemical changes in the meat structure, decreasing the WHC during the storage. However, since these samples are the ones with the highest initial WHC, they continue exhibiting the highest net values at the end of storage.

Electrical conductivity

Figure 3a shows the effect of WHC during the last day of storage on the evaluated parameters, electrical conductivity (EC), and shear force (SF). A significant direct correlation existed between the lower WHC value on the last day of storage and a lower EC, due to the release of electrolytes and water-soluble minerals. NCS/ UV-C/US samples presented the lowest EC (2.02±0.16 mS/cm) and the lowest WHC (18.38±0.93%), due to greater myofibrillar structural damage, originated by the use of all treatments at the time, promoting protein oxidation and cell injury (Mancini, 2013).

Despite US samples presenting the highest WHC value (29.75±0.18%) at the eleventh storage day, they exhibited an EC with no significant differences ($p \geq 0.05$) with the NCS/ UV-C/US treatment (1.99±0.14 mS/cm). This can be explained by the "sponge effect" or mechanical stress, successive compressions and expansions of the food material like a sponge because of acoustic vibrations during US treatment (Astráin-Redín *et al.*, 2021). This phenomenon promotes the release of salts due to cellular and tissue deterioration, not affecting the WHC because the change in the structure of the proteins and ROS formation can originate cross-linking and a gel-like structure capable of retaining water (Domínguez *et al.*, 2022). NCS treatment in combination with US and UV-C presented the highest EC values (7.31±0.37 and 6.25±0.33 mS/cm respectively). Figure 3 shows despite not finding

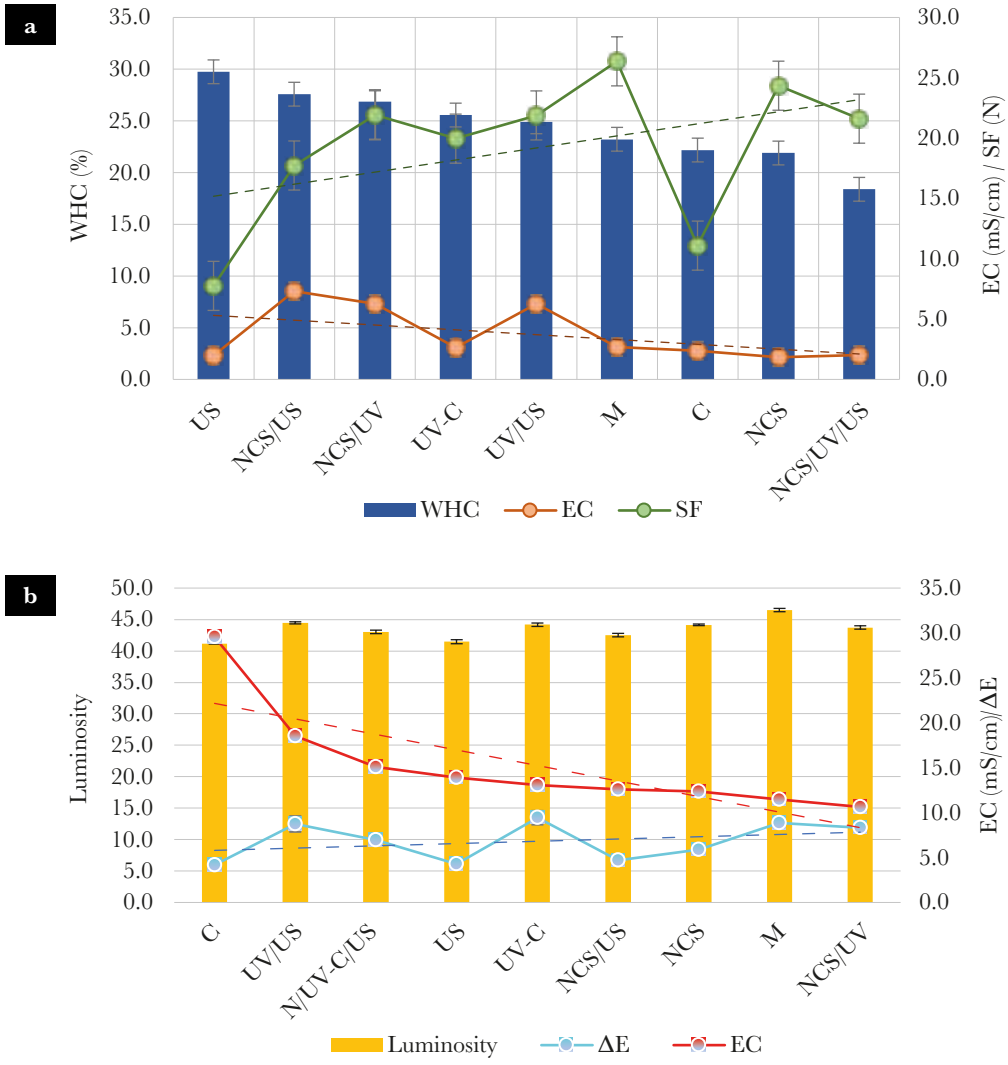


Figure 3. (a) WHC, EC, and SF correlation (b) Luminosity, EC, and ΔE correlation.

a significant correlation ($p \geq 0.05$) between WHC and ΔE, the variation in electrical conductivity could exhibit an inverse relation in both parameter behavior during meat storage. In Figure 3a a direct relation between the decrease in electrical conductivity and WHC is associated with a leakage of salts and inorganic compounds due to cell disruption (Mancini, 2013). Figure 3b exhibits an inverse relation between average ΔE and average electrical conductivity during the storage time. A higher ΔE is related to myoglobin oxidation from oxymyoglobin (Fe^{2+}) to metmyoglobin (Fe^{3+}) and myoglobin release due to its hydrophilic nature (Mancini, 2013), favored by marination, enhancing pigment loss.

Shear Force

As Figure 3 demonstrated, the shear force (SF) presented a significant inverse correlation ($p \leq 0.05$) with the WHC. The less water retained in the meat tissues increases the required SF, developing hardness and loss of tenderness in the meat. The control sample is the

only did not found within this behavior, presenting a lower SF (8.7±0.37 N) at a low WHC (22.18±1.08%). In the absence of an external treatment that would increase the presence of external ROS and provide energy, the oxidation of lipids and proteins resulted in polymeric fragmentation and a decrease in hardness as the meat structure lost its integrity.

pH

In the NCS, NCS/US, NCS/UV and NCS/UV/US treatments, pH values were lower and remained stable, with no statistically significant differences ($p \geq 0.05$) during the 11 days of storage (Figure 4). The quality of the final meat is a result of the evolution of temperature and pH during the post-mortem period (Hamoen *et al.*, 2013). It has been reported that if the pH is above the isoelectric point (5.0 to 5.1) for myofibrillar proteins, the filaments open, and more space is left for water molecules (Andújar *et al.*, 2003). In the same way, the excess of positive charges at low pH values causes repulsion and increases the volume of the myofilaments, which can favor the infiltration of the nanocapsules. In addition, the incorporation of the nanocapsules generated an effect on the surface luminosity of the chicken fajitas during storage at 2 °C due to the modification of the pH of the product. The ingredients used for marinating, in this case apple cider vinegar present a dark color, similar to coffee color, for this reason, luminosity in all treatments is low. However, the application of ultrasound has a direct relationship with the changes in luminosity because it favors the infiltration of the marinade into the fajitas, decreasing the luminosity and influencing the penetration of the color from the marinade ingredients (Chemat *et al.*, 2011). In addition, the nanocapsules, due to their nanometric size, favored the infiltration of the rosemary oil and provided stability to the system. It has been shown that there is a relationship between breast pH and luminosity (Chun *et al.*, 2010). When marinating chicken fajitas, the luminosity increases and is maintained during the conservation of the food due to the stabilization of the surface acidity caused by the incorporation of vinegar. The treatment (C) had a decreasing trend in luminosity, due to the physicochemical changes in the chicken breast caused by the deterioration of the breast causing a darkening of the surface of the product.

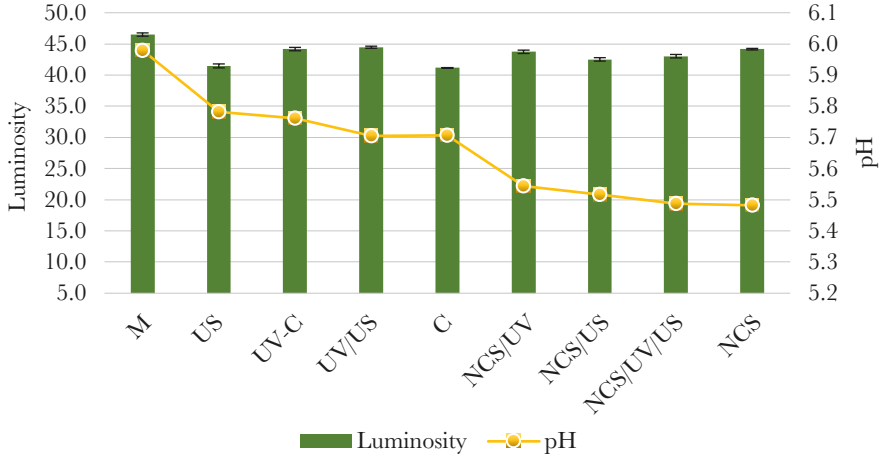


Figure 4. Luminosity and pH correlation.

Changes in ΔE

Color changes are very important, as it is the most important sensory characteristic for consumers at the point of sale. The values of ΔE obtained are between (3.0-6.0) (Inguglia *et al.*, 2021) and considered very visible, where treatments C, US, NCS, NCS/US are found, however, they present a great variation of color during the 11 days of storage (Figure 5). In the remaining treatments, the ΔE is between (6.0 and values above 12.0) and is highly visible (Inguglia *et al.*, 2021). With the above, the treatments that showed less color variation during the 11 days of storage are mainly preferred, being treatment C with 33%, NCS/UV and NCS/UV/US with 30%, where again the infiltration of the NCS in combination with UV and UV/US, presented greater color stability, due to their infiltration into the tissue.

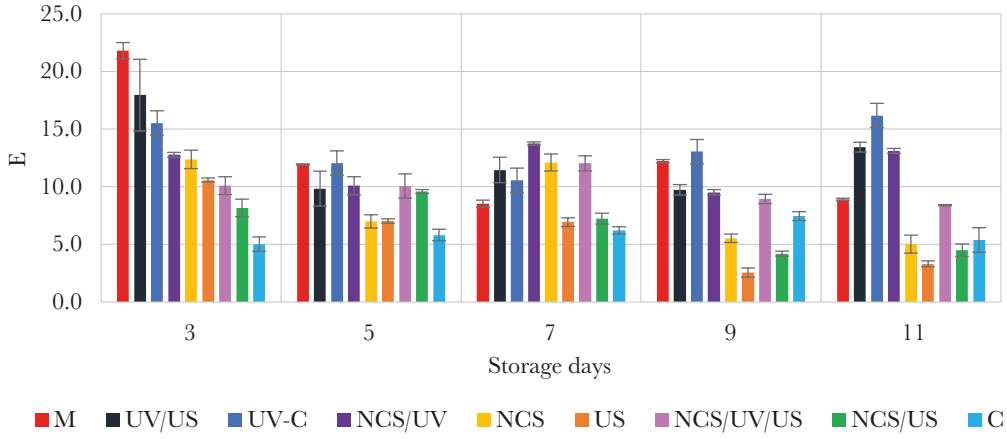


Figure 5. Changes in ΔE.

CONCLUSION

In this research, the combined use of emerging technologies for meat pre-treatment before refrigerated storage has demonstrated an inhibition of meat quality parameters variation. The marinate exhibits its importance in preserving the WHC during storage. Nevertheless, the incorporation of zein NCS in combination with other technologies presented higher WHC on the first and final storage day, affecting the electrical conductivity and shear force on the final storage day. These treatments presented a lower shear force compared to marinated samples (25.86%) and an electrical conductivity increase versus marinated (42.8%) and control samples (38.17%), suggesting less generated cell damage due to the treatment and storage. Despite these advantages, the marinated samples single and combined with other technologies presented higher ΔE due apple cider vinegar effect, meaning the control sample presented a lower variation. However, the NCS combined with US, UV-C, and US/UV-C also exhibits color stability.

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Colegio de Postgraduados

MitoTEMPO® is an antioxidant that protects the bovine sperm acrosome during cryopreservation

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ABSTRACT

Objective: To evaluate the addition of different concentrations of the mitochondrial-targeted antioxidant MitoTEMPO® in a commercial egg yolk-free diluent.

Design/Methods/Approach: Reactive oxygen species (ROS) and ATP production were measured post-thawing by fluorescence, while sperm viability, as well as membrane and acrosome integrity, were assessed using eosin-nigrosin and hypoosmotic Coomassie brilliant blue (HOST/Coomassie) staining of cryopreserved bovine spermatozoa in the commercial medium AndroMed®. Three healthy, fertile 3-year-old Brangus bulls were used, with three ejaculates collected from each bull using an artificial vagina. Ejaculates were divided into three groups and frozen: Group 1 (control), Group 2 (25 μM MitoTEMPO®), and Group 3 (50 μM MitoTEMPO®), with the antioxidant added at the time of strawing. One-way ANOVA (Tukey's multiple comparison test) was used to compare the means of motility, live vs. dead sperm, membrane integrity, and acrosome integrity between treatments, with statistical analysis performed using GraphPad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA) at a significance level of $p<0.05$.

Results: The study demonstrated that the 50 μM concentration improved sperm motility compared to the 25 μM concentration (81.6% *vs.* 76.6%). Coomassie blue staining revealed that the 25 μM group had a higher percentage of sperm with intact acrosomes compared to both the control and the 50 μM groups (58.88% *vs.* 36.21% and 36.34%, respectively). No statistically significant differences were observed in eosin-nigrosin staining, the HOST test, ROS production, or ATP levels between the groups.

Findings/Conclusions: It is concluded that the 25 μM concentration of MitoTEMPO® helps preserve the acrosomal integrity of spermatozoa after the freeze-thaw process.

Keywords: Acrosome, antioxidant, ROS, MitoTEMPO, bovine spermatozoa.

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INTRODUCTION

During sperm cryopreservation, damage often occurs due to ice crystal formation, osmotic shock, and oxidative stress, leading to decreased sperm motility, impaired viability, and poor membrane stability (Gómez-Torres *et al.*, 2017; O'Connell *et al.*, 2002). Oxidative stress reduces sperm quality by generating reactive oxygen species (ROS) and promoting lipid peroxidation during cryopreservation (O'Connell *et al.*, 2002). Antioxidant supplementation during semen processing has been suggested to improve the quality of frozen-thawed sperm (Banihani *et al.*, 2014; Fontoura *et al.*, 2017; Ghorbani *et al.*, 2016; Kalthur *et al.*, 2011; Karimfar *et al.*, 2015), yet the need for an effective antioxidant

remains. MitoTEMPO® (MT) is a novel, cell-permeable ROS scavenger composed of the piperidine nitroxide unit TEMPOL and the lipophilic triphenylphosphonium (TPP) moiety. TPP allows MitoTEMPOL to cross lipid bilayer membranes and accumulate in energized mitochondria, the primary site of ROS generation (Jiang *et al.*, 2015). TEMPOL serves as an effective intracellular ROS scavenger, reducing stress-induced apoptosis and necrosis (Hu & Li, 2016; Trnka *et al.*, 2009). Freezing and thawing cause significant cellular damage, and various studies have sought to analyze these effects (Nieves-Osorno, 2012). While some researchers have proposed mitochondria, lysosomes, and other organelles as primary damage sites, most studies indicate that the plasma membrane is the most affected structure (Barrientos, 2008). During cryopreservation, spermatozoa experience osmotic fluctuations, dehydration, and ice formation, followed by further stress during thawing.

Factors such as cryoprotectant concentration, cooling rate, and thawing temperature contribute to increased cellular stress (Córdoba *et al.*, 2002; Martínez *et al.*, 2006), resulting in morphological alterations in the plasma membrane, acrosome, mitochondria, cytoskeleton, and nucleus (Petrunkina *et al.*, 2004; Petrunkina *et al.*, 2005). Evaluating the impact of these alterations on sperm function requires physiological assays that accurately assess the extent of damage (Petrunkina *et al.*, 2007). To minimize cryopreservation-induced damage, modifications to freezing protocols have been explored, including adjustments in cooling rates, thawing temperatures, and improvements in freezing diluents, with the latter showing the most significant advancements (Flores-González, 2005). However, few studies have investigated the effects of supplemental antioxidants such as MitoTEMPO® on bovine semen cryopreservation. Therefore, the aim of this study was to evaluate the addition of different concentrations of the mitochondrial-targeted antioxidant MitoTEMPO® in the commercial medium AndroMed®, assessing its potential to improve sperm integrity, ATP production, and ROS levels in cryopreserved bovine sperm.

MATERIALS Y METHODS

Semen Collection and Preparation: Using the artificial vagina method, three ejaculates were collected from each of three healthy, fertile 3-year-old Brangus bulls from the ranch "La Sábila" in Moyotepec, municipality of Plan de Ayala, State of Morelos. Samples were diluted in AndroMed® (80%) and maintained in a water bath at 35 °C for evaluation and eventual strawing. Only ejaculates containing at least 80% live motile spermatozoa were used. Semen evaluation included assessments of progressive motility and sperm concentration. Ejaculates with less than 80% progressive motility or more than 20% morphological abnormalities were discarded. Sperm concentration was determined using a hemocytometer (Neubauer chamber) and calculated with the following formula:

$$\textit{No. of sperm} \times 20 \times 10{,}000 \times 5 = \textit{sperm concentration per } \text{mL}$$

where 20 is the dilution factor (25 μL of semen diluted in 500 μL of 0.1% Triton X-100 in PBS), 10,000 corresponds to the chamber volume, and 5 represents the number of frames counted. The number of abnormal spermatozoa was recorded during the counting process.

Once these parameters were evaluated, semen was frozen. Samples were divided into three groups: Group 1 (control, without antioxidant), Group 2 (25 μM MitoTEMPO®), and Group 3 (50 μM MitoTEMPO®), with the antioxidant added immediately before strawing. The freezing protocol followed the guidelines for AndroMed®, an egg yolk-free medium prepared with Milli-Q water, containing phospholipids, TRIS, citric acid, sugars, antioxidants, buffers, glycerin, high-purity water, and antibiotics (Tylosin, Gentamicin, Spectinomycin, Lincomycin) (Nabiev *et al.*, 2003). Sample strawing was performed using 0.25 mL straws at a concentration of 20×10 spermatozoa/mL, equilibrated for 4 h at 4 °C. The samples were then fast-frozen by exposing the straws to nitrogen vapors for 10 minutes before being immersed in liquid nitrogen at −196 °C. The frozen samples were stored in goblets and placed in labeled baskets within liquid nitrogen tanks for at least one month before use.

Thawing and post-thaw sperm evaluation: Thawing was performed by removing the straws, exposing them to room temperature for 10 s, and then placing them in a water bath at 37 °C for 40 s. After thawing, sperm viability was evaluated, and acrosomal integrity was analyzed using non-fluorescent eosin-nigrosin staining and the hypo-osmotic test (HOST) in combination with Coomassie brilliant blue (Mejía *et al.*, 2021). Mitochondrial activity was assessed by measuring ATP production and ROS levels spectrophotometrically. To evaluate post-thaw sperm motility, a 20 μL drop of diluted semen was placed on a pre-warmed slide (37 °C), covered with a coverslip, and examined at 400x magnification using a microscope equipped with a heated stage. The percentage of spermatozoa exhibiting coordinated forward movement was estimated. Three ejaculates were evaluated per bull, with all measurements performed in triplicate.

Measurement of reactive oxygen species (ROS): Reactive oxygen species were measured in spermatozoa upon thawing using Amplex Red (Invitrogen, Molecular Probes) following the protocol described by Guerrero-Castillo *et al.* (2012). Thawed samples were incubated in NaCl at 37 °C, after which × cells were placed into a 96-well microplate containing 20 μL of working solution (10 μM Amplex Red, 0.2 units/mL horseradish peroxidase, and 0.2 units superoxide dismutase/mL in 250 mM sodium phosphate, pH 7.4), with a final volume of 100 μL. Fluorescence was measured after 30 minutes using a POLARstar Omega detector (BGM LABTECH, Offenburg, Germany) set at excitation and emission wavelengths of 571 and 585 nm, respectively. Results were interpolated against a calibration curve.

Measurement of intracellular ATP: ATP concentration was determined by bioluminescence using the ATP-dependent luciferase-catalyzed oxidation of luciferin, which enables the detection of extremely low ATP concentrations (Deluca & McElroy, 1978). Intracellular ATP levels were measured using the ATP Bioluminescence Assay Kit CLS II (Roche), optimized for luminometer-based detection and designed to produce a sustained light signal. To quantify intracellular ATP, a fresh ATP calibration curve was prepared daily following the manufacturer's instructions, using lyophilized luciferase reagent. The procedure was as follows: 8×10^6 cells/mL were resuspended in 100 mM Tris-HCl, 4 mM EDTA, pH 7.8. The samples were then immersed in boiling water for 2 min, followed by centrifugation at 15,000x g in a BenchMark MC-12 centrifuge to remove

cell debris. The resulting supernatants were collected and used to quantify endogenous ATP concentrations. Supernatants were pipetted into a microplate, and luciferase reagent was added to each well (Mendoza-Hoffmann *et al.*, 2018). Fluorescence for both ROS and ATP was measured using a POLARstar Omega detector (BGM LABTECH, Offenburg, Germany) at excitation/emission wavelengths of 571-585 nm. Fluorescence signals were interpolated against a calibration curve, and results were normalized to their non-effector control, which was set at 100% (Mendoza *et al.*, 2018).

Evaluation of sperm viability and acrosomal integrity: Sperm viability and acrosomal integrity were assessed upon thawing using the hypo-osmotic test (HOST) in combination with Coomassie brilliant blue staining. Samples were adjusted to a concentration of 35×10^6 spermatozoa/mL and washed by centrifugation with SSF at 2,500 rpm for 3 min to remove the diluent. A control smear was prepared by mixing 100 μL of semen with 100 μL of 4% paraformaldehyde, incubating for 10 minutes at room temperature, washing twice with PBS at 2,500 rpm for 3 min, reconstituting in 100 μL of ammonium chloride, and then smearing and air-drying the sample. For the test smear, 100 μL of semen was mixed with 100 μL of hypo-osmotic solution (50 μL of 1.46% sodium citrate and 50 μL of 2.7% fructose) and incubated for 60 minutes at 37 °C. Subsequently, 200 μL of 4% paraformaldehyde was added for 10 minutes, followed by two washes with PBS and reconstitution in 100 μL of ammonium chloride. A 20 μL aliquot was then smeared onto a slide and air-dried. Both smears were stained by immersion in Coomassie brilliant blue for 10 to 15 min at room temperature, then rinsed in a Coplin jar with distilled water to remove excess dye. Once dried, the slides were examined under a microscope.

Hypo-Osmotic Swelling (HOST) and coomassie blue staining: The HOST test is used to evaluate sperm membrane functionality. A positive result, indicated by bent or curled flagella, signifies an intact and functional membrane, whereas a negative result, characterized by straight flagella, suggests membrane damage. Coomassie blue staining is employed to assess the presence of the acrosome in spermatozoa. A well-defined, deep blue-stained acrosome indicates acrosomal integrity, whereas a light blue or poorly defined staining pattern suggests acrosome loss or damage (Mejía *et al.*, 2021).

Eosin-Nigrosin staining for sperm viability: After thawing the semen straws, 5 μL of semen was mixed with 2.5 μL of dye and incubated for 5 min at 37 °C. Smears were then prepared on defatted slides, air-dried, and mounted with resin before placing the coverslip. Finally, samples were examined under a microscope. Live spermatozoa, having intact membranes, remained unstained, while dead spermatozoa exhibited staining due to plasma membrane damage (Mejía *et al.*, 2021).

Statistical analysis: Two-way ANOVA (Tukey's multiple comparison test), was employed to compare the means of ROS and ATP production between treatments with two concentrations MitoTempo (MT), the Tx 2 (25 μM) and Tx 3 (50 μM) as well as evaluation with eosin-nigrosine and Coomassie staining in combination with the HOST test, using GraphPad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA) with a value of $P\leq0.05$.

RESULTS AND DISCUSSION

Production of Reactive Oxygen Reactive Species (ROS)

No significant differences were observed in ROS concentrations among the groups (Figure 1A). The mean ±SE values were as follows: control group (184.6 nM ±53.70), 25 μM MitoTEMPO® group (157.6 nM ±44.17), and 50 μM MitoTEMPO® group (187.3 nM ±59.36). The results of this study indicate no significant variation in ROS production between the bovine groups. However, a previous study in buffalo reported by Kumar *et al.* (2022) found a reduction in ROS concentration when MitoTEMPO® was added at a specific concentration. This discrepancy may be attributed to the unique characteristics of buffalo semen, which contains higher levels of polyunsaturated fatty acids and lower antioxidant concentrations compared to bull semen (Kumar *et al.*, 2022).

ATP production after thawing

No significant differences were observed in ATP production among the three groups (Mean ±SE): control group (27.62 nM ±7.30), 25 μM MitoTEMPO® group (29.95 nM ±2.50), and 50 μM MitoTEMPO® group (28.44 nM ±7.55) (Figure 1B). Despite advancements in cattle sperm preservation and continuous efforts to improve sperm quality, oxidative stress remains an unavoidable consequence of the freeze-thaw process. Recently, researchers have focused on mitochondria-targeted antioxidants due to their broad applications, high efficiency, and low toxicity (Zhang *et al.*, 2019). Studies on ram spermatozoa, as reported by Zarei *et al.* (2020), have demonstrated that MitoTEMPO® at concentrations of 5 and 50 μM reduces oxidative stress and mitochondrial damage during temperature fluctuations, thereby enhancing sperm preservation. In this study, no significant differences in ATP production were observed between the groups. Sánchez (2023) investigated whether ATP production could serve as an additional parameter alongside motility and viability to assess bull sperm quality, finding that ATP levels were influenced by the preservation medium, partly independent of motility and viability.

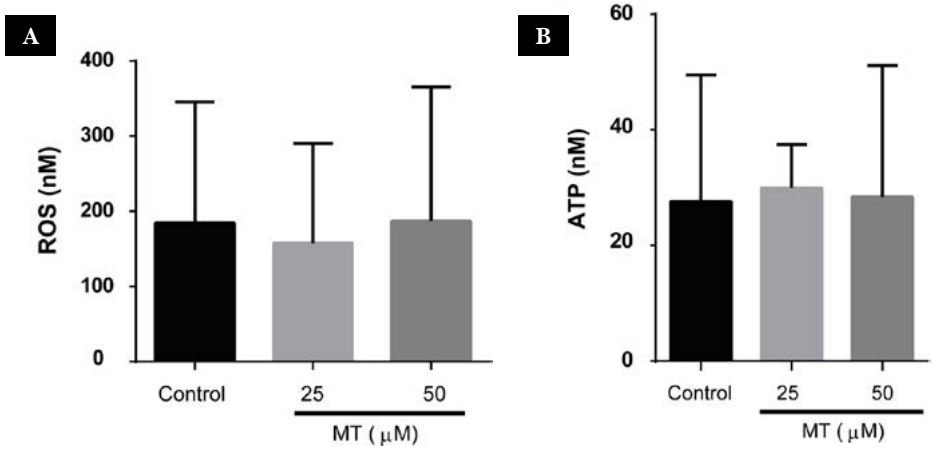

Figure 1. (A) ROS concentration (nM) in bovine spermatozoa; (B) ATP production (nM) in bovine spermatozoa after cryopreservation. Different concentrations of MitoTEMPO® (MT) are indicated. Data represent the average of three biological replicates ± standard deviation ($p \leq 0.05$).

Similarly, Barbosa *et al.* (2011) found no direct correlation between sperm motility and mitochondrial activity.

Sperm viability and acrosomal integrity. Hypo-osmotic test in combination with Coomassie brilliant blue

The effects of MitoTEMPO® supplementation on sperm viability and acrosomal integrity were analyzed, revealing that the percentage of spermatozoa with a positive response to the HOST test was significantly higher in Group 2 (25 μM MT) compared to both the control group and the group supplemented with 50 μM MT (Table 1, Figure 2).

Coomassie brilliant blue staining

The presence of the acrosome in spermatozoa was evaluated using Coomassie brilliant blue staining. The results are presented in Table 2, showing that the group supplemented with 25 μM MitoTEMPO® had a significantly higher percentage of spermatozoa with intact acrosomes compared to the other groups (Figure 3).

Eosine-nigrosine staining

Membrane integrity was evaluated using eosin-nigrosin staining. Although no statistical differences were observed between the groups, semen treated with 25 µM MitoTEMPO® showed a higher percentage of spermatozoa with intact membranes (Table 3, Figure 4).

Table 1. Effect of MitoTEMPO® supplementation at different concentrations on sperm response to the HOST test.

Host	Group 1: without MT	Group 2: 25 μM MT	Group 3: 50 μM MT
Positive to Host	94.33±0.92[a]	95.92±0.92[a]	92.71±0.92[a]
Negative to Host	5.664±0.92[a]	4.080±0.92[a]	7.29±0.92[a]

HOST test results from 3 treatments (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTempo®, Group 3: 50 μM MitoTempo®), showing the proportion of live/dead sperm. Data are shown as average of three biological replicates ± standard deviation, there was no statistical difference between treatments ($p \leq 0.05$).

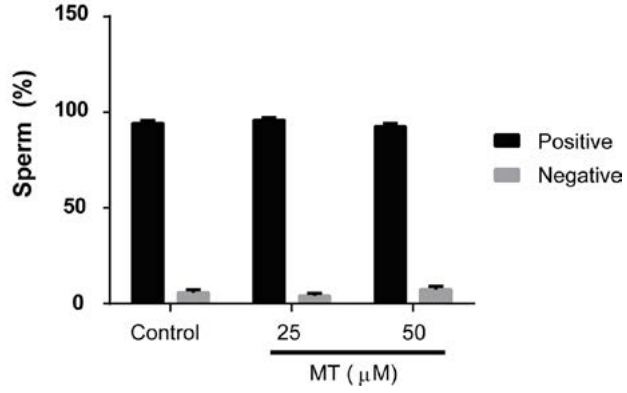

Figure 2. HOST test results for the three treatment groups (Group 1: Control without MitoTEMPO®, Group 2: 25 µM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®), showing the positive/negative sperm ratio. Data represent the average of three biological replicates ± standard deviation ($p \leq 0.05$).

Table 2. Effect of MitoTEMPO® supplementation on acrosomal integrity.

Coomassie stain	Group 1 without MT	Group 2 25 μM MT	Group 3 50 μM MT
Sperm with acrosome	36.21±7.53[a]	58.88±7.53[b]	36.34±7.53[a]
Sperm without acrosome	58.42±6.71[a]	37.77±6.71[b]	37.33±6.71[a]

Coomassie stain results for the three treatment groups (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®), showing the ratio of sperm with an intact acrosome to sperm without an acrosome. Data represent the average of three biological replicates ± standard deviation. Values with different superscripts indicate significant differences ($p \leq 0.05$).

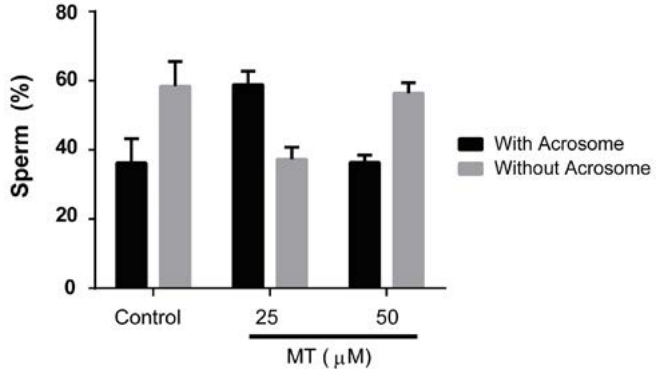

Figure 3. Coomassie staining results showing the ratio of spermatozoa with and without an acrosome after each of the three treatments (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®). Data represent the average of three biological replicates ± standard deviation ($p \leq 0.05$).

Table 3. Effect of MitoTEMPO® supplementation on sperm membrane integrity.

Eosine-nigrosine stain	Group 1 without MT	Group 2 25 μM MT	Group 3 50 μM MT
Alive sperm	56.46±1.21[a]	60.53±1.21[a]	59.47±1.21[a]
Dead sperm	43.53±1.21[a]	39.48±1.21[a]	40.47±1.21[a]

Eosin-nigrosin staining results for the three treatment groups (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®), showing the ratio of live to dead sperm. Data represent the average of three biological replicates ± standard deviation. No statistical differences were observed between treatments ($p \leq 0.05$).

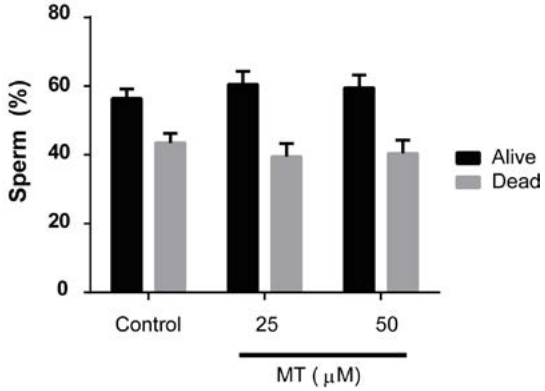

Figure 4. Average eosin-nigrosin staining ratio of live to dead sperm after cryopreservation in the three treatment groups (Group 1: Control without MitoTEMPO®, Group 2: 25 μM MitoTEMPO®, Group 3: 50 μM MitoTEMPO®). Data represent the mean of three biological replicates ± standard deviation ($p \leq 0.05$).

For the evaluation of sperm viability and acrosomal integrity, the HOST test was used. The results showed that spermatozoa treated with 25 μM MitoTEMPO® were better able to compensate for the osmotic imbalance induced by the hypo-osmotic medium compared to both the control group and the 50 μM MitoTEMPO® group. This was evidenced by morphological changes in the flagella, such as dilation and coiling. By combining this test with Coomassie blue staining, the acrosomal state of the spermatozoa was also assessed, revealing that Group 2 (25 μM) had a higher number of spermatozoa with intact acrosomes. These findings represent the first evidence in bovine spermatozoa demonstrating an improvement in acrosomal integrity after the freeze-thawing process. Atuesta *et al.* (2012) reported that cryopreservation negatively affects sperm viability. The acrosome, located in the apical region of the spermatozoon, is essential for fertilization (Pérez *et al.*, 2020), as only spermatozoa capable of undergoing the acrosomal reaction in synchronization with oocyte penetration can successfully complete fertilization (Cox *et al.*, 1998). During the freeze-thaw process, oxidative stress leads to excessive ROS production, triggering lipid peroxidation and causing structural and functional damage to the sperm plasma membrane (Kumar *et al.*, 2021). Zarei *et al.* (2020) explained that MitoTEMPO® not only reduces ROS production but also decreases lipid peroxidation, thereby protecting the sperm plasma membrane. The results of this study differ from those reported by Kumar *et al.* (2021), who found that viability, acrosomal integrity, and the HOST response were improved in buffalo semen with 50 μM MitoTEMPO®. The discrepancy in optimal antioxidant concentration between studies may be attributed to species-specific differences, as buffalo semen has distinct biochemical and physiological properties compared to bovine semen.

Motility

A significant difference was observed in sperm motility between the control group (67.22±1.69) and the groups treated with 25 μM (74.44±1.30) and 50 μM (78.33±1.44) MitoTEMPO®, indicating that the addition of the antioxidant improved motility (Figure 5).

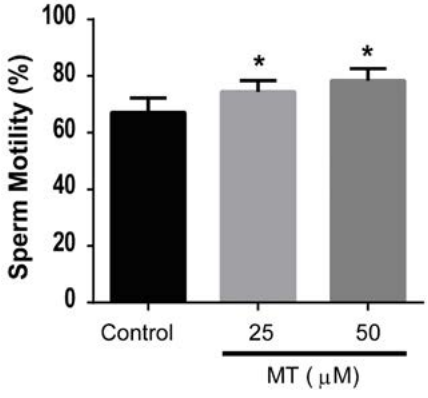

Figure 5. The percentage of sperm motility shows that Group 2 (25 μM MitoTEMPO®) and Group 3 (50 μM MitoTEMPO®) exhibited higher motility after thawing compared to the control group. Values marked with * indicate a statistically significant difference. Data represent the mean of three biological replicates ± standard deviation ($p \leq 0.05$).

The results show a significant improvement ($p \leq 0.05$) in sperm motility in the groups supplemented with MitoTEMPO® (25 and 50 μM) compared to the control group. These findings align with those reported by Valdez (2017), who observed enhanced motility following the addition of natural antioxidants. Similarly, Kumar *et al.* (2021) reported improved progressive motility in buffalo semen treated with 50 μM MitoTEMPO®. However, Foote (2002) found no improvement in motility when Tempo and Tempol were added to bull semen.

CONCLUSIONS

The addition of mitochondria-targeted antioxidants can effectively prevent membrane damage; however, the protection was partial, and only one MitoTEMPO® concentration proved effective. The group supplemented with 25 μM of the antioxidant exhibited a significantly higher percentage of spermatozoa with intact acrosomes compared to both the control and 50 μM groups after the freeze-thaw process. These findings provide valuable insights and highlight the need for further research.

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Colegio de Postgraduados

Synchrotron radiation techniques for soil analysis: a review of basics and methods

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ABSTRACT

Objective: The objective of this work is to present the basics of synchrotron radiation and the fundamentals of some synchrotron-based analytical techniques useful in soil research.

Approach: Synchrotron radiation has revolutionized all areas of human activities. The unique opportunities that synchrotron-based analytical tools offer to vast research areas in the natural sciences is relatively unknown in Mexico and Latin America, with the notable exception of Brazil. This review offers a brief introduction to the principle of operation of a synchrotron radiation facility, its main components and some spectroscopic tools which can be applied to research on soils. We aim to motivate the reader and the researcher on soils to consider the inclusion of synchrotron-based techniques in his/her research activities.

Limitations on study/implications: Synchrotron-based analytical tools offer the possibility of performing analysis at the nanometric scale with elemental sensibility, chemical state (though charge) and orbital selectivity. Other key points of these techniques are the low volume of sample required and the nondestructive nature of the probes, which allow to correlate chemical composition, structure, and physicochemical properties of soils at a very detailed and fundamental level.

Conclusions: Soils are fundamental for the sustainable development of humanity. Regardless of the specific area of soil research in question (composition, fertility, productivity, pollution, remediation), it is possible and desirable to consider the application of synchrotron based spectro-microscopic analytical tools to deepen and broaden our current understanding on any specific topic related to soil science.

Keywords: Synchrotron, X-ray Absorption, Fluorescence, Soils.

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INTRODUCTION

Soil is the mineral and organic material that has been generated on the Earth's surface formed by environmental and biological factors, and that allows the growth of plants. It is a fundamental component for supporting life on Earth (Dumanski and Peiretti 2013). The integrity of terrestrial ecosystems depends on the relationships between all the processes that occur in the soil. A deep understanding of the soil requires research, for example, on the chemistry, uptake and distribution of contaminants, amount of dissolved organic matter, among others. However, spectroscopic and analytical methods conventionally used to investigate such phenomena lack the sensitivity necessary for investigation under relevant conditions (Bertsch and Hunter 2001).On the other hand, synchrotron-based techniques are currently established tools sometimes tailored to obtain specific information

about several of the processes that occur in the soil, surpassing conventional methods by multiple orders of magnitude (Lehmann and Solomon 2010). In this sense, techniques that use synchrotron radiation have begun to open various opportunities for the study of the phenomena that occur on the soil. This has been possible due to the significant improvements in the spectral and spatial resolution of synchrotron-based instruments in hand with the inherent advantages of synchrotron radiation (SR). Techniques like SR-FTIR (Fourier Transform Infrared Spectroscopy), SR-XRD (X-ray Diffraction), different modalities of X-ray absorption (XAS), emission spectroscopy (XES), and tomography are almost a requirement for the development of a complete understanding of the behavior of elements in soil. Furthermore, this information is necessary to more optimally predict the processes that occur in the soil, develop strategies for soil remediation, in the case of contaminants, and provide strategies to accurately assess risks (Bertsch and Hunter 2001). In this review we present the main components and operation principle of a synchrotron radiation facility. We then discuss the key characteristics of SR and how it is produced. Then we present the fundamentals of several SR-based techniques and a few examples where these techniques found application in various fields of study related to soil science.

Synchrotron radiation basics

Synchrotron radiation (SR) is electromagnetic radiation emitted when electrons, moving at relativistic velocities (close to the speed of light) change their direction under an applied magnetic field. Its virtues are: high intensity and brightness (synchrotron light is hundreds of thousands of times more intense than that from conventional X-ray tubes), beam highly collimated (narrow cone of emission), continuous and tunable in a wide range of photon energies spanning from the infra-red (IR) to the hard X-rays regime (thousands of eV), allows control over its polarization (linear, circular, elliptical) and has time structure due to the time separation of electron bunches in the ring (ns pulse separation) and their width (ps pulse duration) (Margaritondo, 2002) (see Figure 2). It was first observed in 1947 at the General Electric research laboratories in Schenectady, New York. A theoretical description of the phenomena was made by Schwinger (Schwinger, 1949), Sokolov and Ternov (Sokolov and Ternov, 1967). At the beginning, spectroscopists used this radiation in a parasitic manner. In mid-1980s, insertion devices (IDs, wigglers (W) and undulators (U)) using permanent magnets were developed and were implemented in the straight sections of the 2nd generation machines (see Figures 1 and 2). The 3rd generation sources are characterized by being built for the optimal use of undulators. In 1994, the European Synchrotron Radiation Facility (ESRF) in Grenoble opened the era of the 3rd generation light sources. The first batch of machines followed immediately in USA and Asia in the soft (100 eV - 3 keV) and in the hard X-ray regimes (up to 50 keV). The 'Lorentz factor' (γ) is a dimensionless parameter that expresses the ratio of the electron energy Ee to the rest mass energy of the electrons $m_e c^2$ = 511 keV ($m_e = 9.109 \times 10^{-31}$ kg is the electron rest mass and $c = 2.9979 \times 10^8$ m/s is the speed of light). More conveniently can be expressed as $\gamma = 1957\, Ee$ (GeV) and it helps to characterize different synchrotron facilities. Hence, for a soft X-ray optimized synchrotron like the ALS in Berkeley with $Ee = 1.9$ GeV, $\gamma = 3718$, while for a synchrotron radiation facility

like the ESRF, optimized for hard X-rays, with $Ee = 6$ GeV, $\gamma = 11800$. Other parameters like circumference, bunch length, energy spread, filling pattern, etcetera, are used to characterize different facilities (Atwood, 1999). Today, there are about 70 SR facilities in all the continents of the world except mainland Africa (http://www.lightsources.org).

Main components and operation of a synchrotron facility

The main components of a SR facility are presented in panel a) of Figure 1. Synchrotrons operate typically with electrons produced under vacuum conditions at the **electron gun** by thermionic emission from a heated tungsten cathode. The emitted electrons packed into bunches nanoseconds apart from each other (Figure 2) are then accelerated to energy in the order of tens of keV by a potential difference applied across the gun. Then they are moved into the linear accelerator. **Vacuum** is crucial in a synchrotron facility because if the electrons were to travel through air inside the storage ring, they would scatter off air molecules and be lost from the electron beam, resulting soon in no emission of SR. Therefore, the electrons circulate around the ring in a vacuum chamber with pressure in the order of 10^{-10} torr. The electrons fired from the electron gun travel through vacuum chambers and reach the **linear accelerator** or **LINAC**. This device accelerates the electron beam in the form of electron bunches to an energy of few hundred MeV by means of a series of **radio frequency** (**RF**) cavities placed over a distance of about 10 meters. At the ESRF for example they reach 200 MeV. After the first meter of acceleration in the LINAC, the electrons are already travelling at more than 99.99% of the speed of light. From the LINAC the electron bunches are transferred into the **booster ring**, which boosts the energy of the electrons from approximately 200 MeV to approximately 1.9 GeV (ALS) to 6 GeV (ESRF), depending on the facility. The booster ring increases the

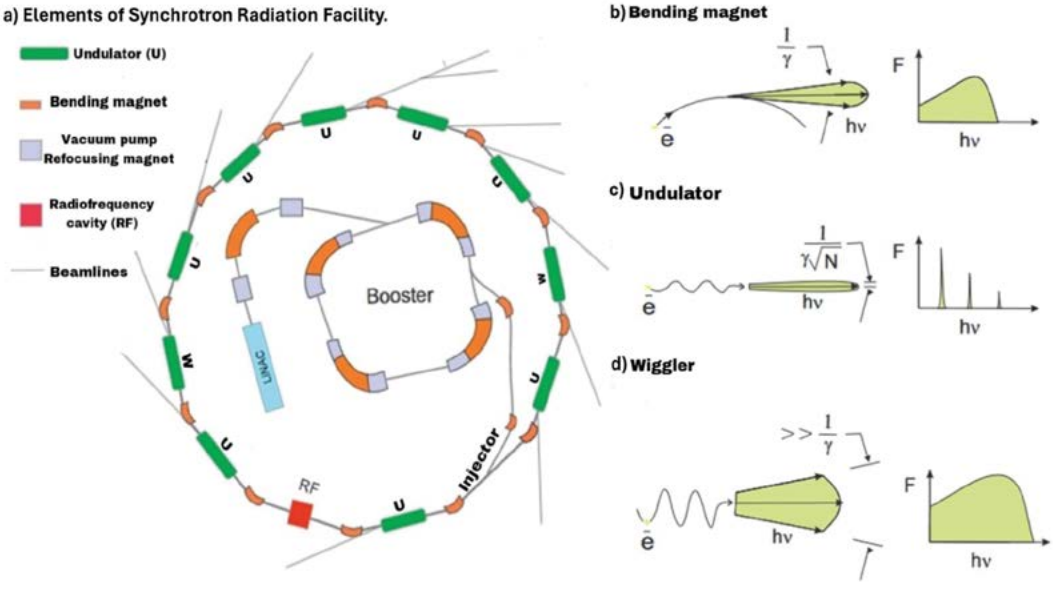

Figure 1. a) Main components of a synchrotron radiation (SR) facility. b), c) and d)are the angular aperture and energy distribution profile of emitted SR from a bending magnet, an undulator and a wiggler, respectively. (see text for details).

speed of the electrons even closer to the speed of light (approximately 99.9999985% of the speed of light) on each turn using a RF voltage source. As the electrons travel around a booster ring, they are kept into a near-circular path by dipolar bending magnets and by quadrupolar focusing magnets known as the lattice. The electrons change their trajectory because of the bending magnets and emit SR, which results in the electrons losing energy and momentum. The RF system provides a synchronous boost to the electrons every time they complete a turn within the storage ring, compensating the energy and the momentum lost through SR emission. At the ESRF, the booster synchrotron has a circumference of 300 meters and a 10 Hz cycling frequency. Once the electrons have reached the nominal energy (1.9 GeV for ALS, 6 GeV for ESRF) they are transferred into the **storage ring** by an **injection system**. Each storage ring can hold few hundred mA circulating inside. For example, the ALS typically operates with 500 mA while the ESRF with 200 mA. Depending on the facility the electrons keep circulating for few hours or even for days in the so called top-off (continuous) mode. Each time electrons pass through magnets which alter their trajectory the electrons lose energy by emitting SR. Each of these magnetic arrays (bending magnets, wigglers and undulators) display a characteristic electromagnetic emission pattern (in terms of energy, brilliance, polarization, and angular aperture, see Figures 1-2) which is directed towards the beamlines. Insertion devices (IDs) as undulators and wigglers are magnetic structures made up of a series of small magnets with alternating polarity that make electron beam to move onto a sinusoidal trajectory (See Figures 1-2). The beams of X-rays they produce are a million times more intense than those generated by the bending magnets and, particularly the undulators have the smallest angular aperture (see Figures 1c-2), the highest brightness and coherence properties that are close to lasers. In an undulator, the electron motion in the transverse direction is set to be on the order of the opening angle. In a wiggler, however, the motion is made to be larger than the opening angle and therefore a wider beam results (See Figure 1 c-d). In an undulator, radiation from the various periods interfere coherently. Sharp peaks are produced at odd harmonics of the resonant frequency (Figure 1c), which depends on the electron energy, the undulator period (λ_u), the field strength of the magnets, and the observation position (Atwood 1999) (see Figures 1-2). Again, to compensate the energy lost by emission of SR, synchronized RF cavities restore and maintain the energy of the electron bunches close to the nominal storage ring energy. After each ID or bending magnet, there is a photon port to allow the tangential extraction of SR light towards the **beamlines** and their research **endstations**. The extraction of SR is achieved by optical devices (slits, mirrors, gratings). The components of each endstation are experiment specific. The 6 GeV storage ring at ESRF has a circumference of 844 m and delivers SR to 44 highly specific beamlines. A complete set of parameters for each SR facility can be found in the machine/parameters section of each facility website.

METHODS

X-ray Absorption Spectroscopy (XAS)

Atoms absorb X-rays sharply at certain energies called absorption edges that are characteristic of that atomic species. Each of these absorption edges have different

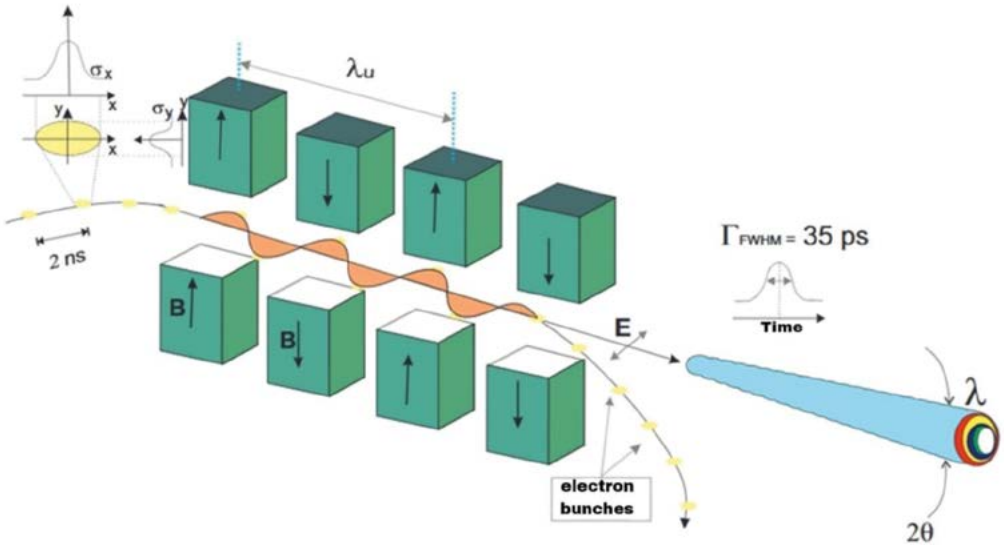

Figure 2. An undulator with its alternating periodic poles (λ_u) induces electron bunches (separated nanoseconds apart from each other) to move in a sinusoidal trajectory between the magnetic arrays, resulting in a narrow cone of SR emission.

electron occupation numbers with characteristic binding energies (see Figure 3 a-d). **XAS** can have different names depending on the energy range used. It is an element and orbital specific technique governed by dipolar radiation selection rules (Atwood, 1999; Groot and Kotani, 2008). Today, **Near Edge X-Ray Absorption Fine Structure (NEXAFS)** is typically used for soft X-ray absorption spectra (low Z elements) (Figure 3a) and **X-ray Absorption Near Edge Spectroscopy (XANES)** for hard X-rays (heavy elements, metal atoms). With more detail, XANES refers to the absorption of X-rays about 10 eV below and 20 eV above the absorption edge. This region usually shows the largest variations in the X-ray absorption coefficient and is often dominated by intense, narrow resonances (see Figures 3b and 3f). Either as NEXAFS or XANES, XAS provides information on the electronic structure of the unoccupied levels or conduction band (CB) (see Figures 3a-b) and the geometry surrounding the absorbing element. Different oxidation states of transition metals are observed in this region by relative shifts in the absorption onsets: the more oxidized the species the more it shifts towards higher photon energies. The pre-edge structures at the onset of the XANES spectrum (Figure 3f) typically corresponds to quadrupolar transitions (for example 1s to 3d orbitals) in transition metal elements (Groot and Kotani, 2008). The higher energy region in XANES spectra is typically referred to as the **Extended X-ray Absorption Fine Structure (EXAFS)**, typically starts ~50 eV above the absorption edge. The so-called EXAFS oscillations originate from the scattering of photoelectrons as waves ejected from the absorbing atom and its neighbors interfering constructively and destructively among them (Figure 3g). Therefore, EXAFS is typically used to determine the local structure of the studied element and its surrounding atoms (Groot and Kotani, 2008).

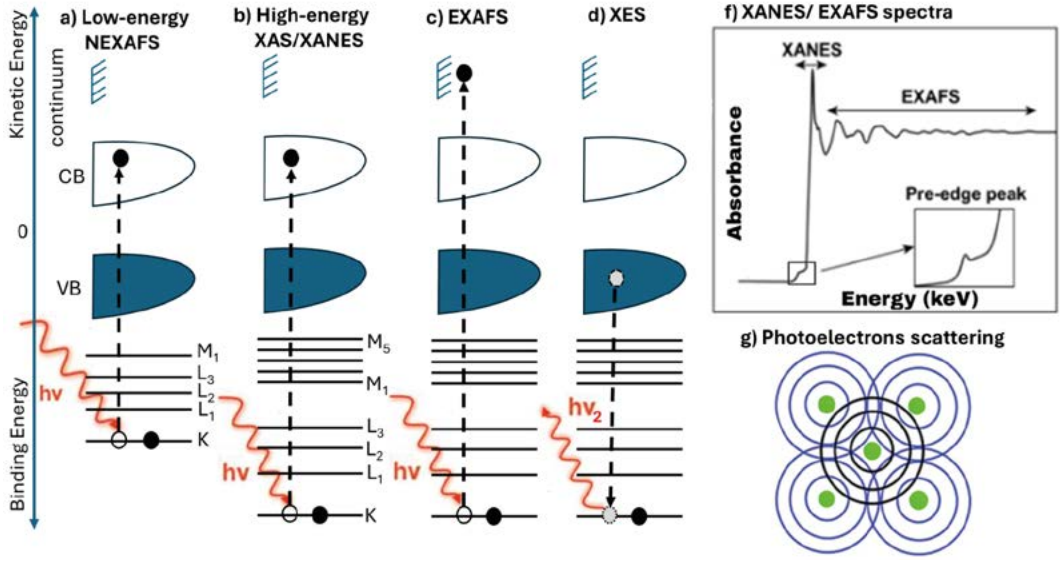

Figure 3. Basic processes in X-ray absorption spectroscopy (XAS) in its different varieties: a) NEXAFS, b) XANES, c) EXAFS, f) XANES/EXAFS spectrum and the scattering process of photoelectrons which produces EXAFS oscillations. d) X-ray emission process in XES spectroscopy. K-M are characteristic absorption edges of atoms in a solid with its valence band (VB) and conduction band (CB) $h\nu/h\nu_2$ are the incoming/outgoing X-rays. See text for details.

X-ray emission spectroscopy (XES)

XES is also known as **X-ray Fluorescence (XRF)** in the literature (Groot and Kotani, 2008). X-ray emission occurs as a core hole is relaxed after photoionization (ejection of an electron after X-ray irradiation) from a core level. The photon generated during the relaxation of the core hole has the energy corresponding to the energy difference of the atomic levels involved, in Figure 3d $h\nu_2 = E(1s) - E(VB)$. A XES spectra provides information on the elemental/orbital composition of the valence band (VB). The relaxation of the core hole can also occur through the Auger process, where instead of emitting X-rays the excited atom emits electrons with well-defined kinetic energies. Both processes compete and are Z and absorption-edge dependent: core holes in lighter atoms predominantly decay via Auger processes, while heavier elements do it via emission of X-rays (Atwood 1999). At this point is important to emphasize that both techniques XAS and XES require a SR facility. This is so because to register the XAS/XES spectra we need to scan the incoming photon energy and tune it to the absorption edges of the various elements in our sample(s). In addition, to enhance the X-ray emission signal, we need to create the largest possible number of core holes in our atom, and thus we need an undulator beamline with the highest photon flux possible. At this point, the potential that these techniques offer for agriculture research should be evident, see for examples the work of Castillo *et al.* on plants and engineered nano materials (Castillo-Michel, Larue *et al.*, 2017) and Lanziroti *et al.* on soils (Lanzirotti, Tappero *et al.*, 2010). Having reviewed the basic atomic processes involved in X-ray spectroscopies, we will proceed to discuss some other SR techniques bassed in these processes and their application to soil research.

X-ray computed μ-tomography (SR-μCT)

X-ray tomography is the construction of a three dimensional image from two dimensional projections (slices) taken at different orientations. According to Lombi and Susuni (Lombi and Susini, 2009), X-ray computed μ-tomography (SR-μCT) is a technique that provides high contrast and high resolution 3D images by combining μCT and SR that leads to detailed intern structure analysis for materials inner structure, without the need of dissecting. Therefore, X-ray computed μ-tomography (SR-μCT) is a non-destructive technique. There are three modes of contrast for imaging with X-rays: X-ray absorption, fluorescence and phase contrast. The X-ray beam used permits the imaging of non-geometrical shapes in the sample. Many applications in soils and vegetable tissue are described for this technique. Peth (Peth, 2010) highlights the use of SR-μCT for studying the biophysical and physicochemical properties of soil aggregates. In this case, the technique allowed to image natural undisturbed soil samples, showing the patterns of internal pore structures. These spaces are important because it is habitat for soil microbiome, and the physical properties of the pore may be involved in regulation of microecological relations. SR-μCT also was used for comparing topology and morphology of pores in different tilled soils.

Absorption SR-μCT

In X-ray absorption computed μ-tomography (SR-μCT), the contrast is achieved by registering the response of the sample at energies below and above the energy of the element of interest, it works better for heavy elements that strongly absorb X-rays. The use of a monochromator to select the photon energy is mandatory. This technique requires less sample to work with but needs a long distance between the SR source and the tomography facility. First, the X-rays beam needs to be attenuated with a scintillator that converts it into visible light. Then, the absorption projection is captured by a detector (usually a CCD camera). The sample is slightly rotated to receive another beam and generate the next image. The process is repeated and finishes when the sample completes an arc between 0° and 180°. The images generated are reconstructed to allow a 3D visualization. As mentioned above, it is possible to obtain a set of images above and below an elemental absorption edge and get a 3D distribution of it in the sample. The exposure of the sample to SR is only a few seconds per image with a micrometric resolution. Scheckel *et al.* (2007) used this technique to enlighten how *Iberis intermedia* absorbs and bioaccumulates thallium in a differential way within the plant structures. This study shows a promising area for phyto-mining and soil phytoremediation because thallium is a precious metal.

SR-XRF-μCT

This technique combines the quantitative approach of XRF and μCT for 3D imaging and composition information of the analyzed sample yielding the spatial distributions of the elements. Xie *et al.* (Xie, Deng *et al.*, 2020) describe XRF computed tomography (XFCT) used in the Shanghai SR facility. A beam of SR X-rays is used to create core holes in the atoms of the elements of interest within the sample. This generates a fluorescent

X-ray whose energy is unique for each element. The sample is then rotated to acquire various images that will be processed to create a 3D representation. For this technique raster scanning is needeed, therefore this technique is slow in comparison with phase contrast and X-ray absorption modes, and hence radiation damage maybe an issue for high resolution measurements. Other drawbacks to consider are 1) the energy at which the analysis is done impacts the elements to be detected and 2) low (Z) energy can only be analyzed in thin samples (remember low XES probabilty vs Auger for low Z), else XRF form within the sample does not reach the detector. This robust technique has been widely used for studying the interactions of pollutant removing plants and soil, showing that chemical speciation of contaminants varies in different plant structures (Lombi and Susini, 2009).

Phase contrast SR-μCT

This technique is based on the fact that SR is affected by both the absorption of the materials and their phase.The contrast between phases is more remarkable at the absorption edge, especially if materials at the interface have different refractive index. While producing μ-CT images, SR through the sample lowers its energy, so the attenuation is registered and used to build the 3D image. Due the relation between refractive index an electron density, the images generated show a 3D map for electron density at micrometric scale (Lombi and Susini, 2009). The procedure is similar to absorption SR-μCT (Indore, Karunakaran *et al.*, 2022) but differs in the distance from the detector. This method is more sensitive to small differences in refractive index, so it increases the contrast of edges in the sample's borders allowing to detect soft materials in it. Experimentally, this technique is fast and, as mentioned it is very sensitive. It is typical to use high energy X-rays (energies greater than 20 keV) directly form the source as white or pink beam, and hence no monochromator is needed. This technique has been used by Ma *et al.* (Ma, Cai *et al.*, 2015) for studying changes in the pore inner structure in two different soils during various cycles of wetting and drying. It showed the presence of a very complex pore network due the clay composition in soil while wetting.

Synchrotron Radiation X-Ray Diffraction (SR-XRD)

The conventional X-ray diffraction system is limited to crystalline materials and has the drawbacks of requiring large sample quantities, long time for analysis, low resolution, beam hardening, low photon energy, low photon flux, and fixed wavelength. SR-XRD provides outstanding resolution and sensitivity, even with very small samples. This enables the identification and quantification of trace phases, something not achievable with conventional X-ray sources (Surabhi, 2021). Compared with lab-based sources, SR based X-rays have a much shorter, providing much greater spatial resolution and penetration capability. They interact with the atoms of the sample, producing characteristic diffraction patterns that can be detected (Manceau, Marcus *et al.*, 2002). SR-XRD enables the non-invasive study of trace matters, amorphous minerals, hydrated and oxygen samples, thin films and solution phase. The study of areas as small as 1 mm^2 is rutinary (Surabhi, 2021) although it is also possible t perform SR-XRD-μCT.

SR-Fourier Transform Infrared Spectro-microscopy (SR-FTIR)

In general, infrared radiation interacts with the sample and is absorbed to varying degrees depending on the molecular vibrations present in the sample. The absorption of energy results in absorption spectra containing information about the characteristic vibrational bands of the chemical bonds present in the sample. SR-FTIR spectro-microscopy integrates three methodologies: microscopy, mid-infrared spectroscopy, and SR. Due to its superior spatial resolution (down to micrometers) and enhanced signal-to-noise ratio (SNR) (given the high photon flux from SR facilities in comparison to traditional FTIR methods), this approach enables a quantitative analysis, composition, structure, and distribution of chemical constituents within the sample. SR-FTIR spectro-microscopy allows for the extraction of more intricate structural details, characterization of soil mineral components, including mineral identification, structural assessment, and in situ monitoring of mineral formation (Margenot, Calderón *et al.*, 2017).

Scanning Transmission X-ray Microscopy (STXM)

STXM is a transmission microscopy technique able to perform XAS with spatial resolution of about 20-nm. STXM provides two-dimensional (2D) quantitative information about the distribution of chemical components and interactions in the soil (Obst and Schmid, 2014). The technique can be adapted for the use of hard and soft X-rays. Each regime has its own optics and instrumentation and offers different advantages. Hard X-rays, have higher energies, can penetrate deeper into samples (which is useful for studying internal structures and buried components of the soil). Therefore, they are used for the detection of heavy elements and to obtain structural information at greater depths. Typical applications include the characterization of heavy minerals, detection of metallic contaminants, and evaluation of soil microstructure. Soft X-rays, on the other hand have lower energies (typically between 100 and 2200 eV), are more sensitive to light elements and organic compounds, providing detailed information about the surface chemistry and the upper layers of the soil. Therefore are used to study organic composition, chemical interactions, investigation on nutrient cycles, and the speciation of light elements in the surface of the soil (Obst and Schmid, 2014). STXM in both X-ray regimes allows for a comprehensive and multidimensional characterization of soil, encompassing both organic and inorganic components, and providing a more integral understanding of soil chemistry and structure.

Limitations for Mexican and Latin American Researchers

Probably the four most important limitations for employing SR in soil studies for Mexican researchers are: 1) the lack of a SR facility in Mexico, 2) the necessary funding for even a small team (2 people) traveling abroad to perform experiments, even to the SR facilities in America (USA, Canada and Brazil), 3) the lack of previous experimental experience and 4) the very competitive process of beamtime allocation (which often requires previous SR experience). Fortunately, it is possible to reach for experienced users aiming to collaborate. It is also advisable to contact the beamline scientists in charge of the targeted instrument in advance to discuss the viability of our research project, and who sometimes even help

with preliminary measurements to support our research proposals. It is necessary to create options, several alternatives are possible: like developing collaborative schemes at the University-SR facility or even at binational level to favor the training of graduate students. For example, the Brazilian SR facility partially supports the trip of Latin American students provided beamtime has been awarded through the regular proposal mechanism. Despite all these limitations, we must keep trying to incorporate SR techniques into our research, we must continue submitting research proposals and continue to train the next generation of Mexican researchers well versed in SR techniques, especially if we ever aspire to host a SR facility.

CONCLUSIONS

In this brief review we presented the most important SR based techniques applied to soil science. We discussed the basic operation of a SR facility as well as the main characteristics of the SR. The properties of synchrotron radiation allow for the development of unique tools with extraordinary analytic capabilities with spatial resolution at the nanometric level, with molecular, elemental, charge, and orbital resolution. The aim of this review is to promote the incorporation of SR based techniques among Mexican and Latin American researchers performing research in soils.

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Serological response of lambs vaccinated with a biological bacterin-toxoid type against *caseous lymphadenitis*

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ABSTRACT

Objective: To evaluate the serological response to somatic, secretory, and soluble antigens in sheep vaccinated and revaccinated with an experimental bacterin-toxoid biological against ovine caseous lymphadenitis.

Design/Methodology/Approach: The serological response to different antigens was assessed in sheep vaccinated with an experimental bacterin-toxoid biological against *caseous lymphadenitis*. The animals' sera were tested using indirect enzyme-linked immunosorbent assay (ELISA) with different antigens. The antigens were obtained from a wild strain isolated from an ovine abscess.

Results: The antigens with the strongest immunogenic response were those obtained by sonication and the supernatant rich in phospholipase D.

Study Limitations/Implications: In Mexico, no commercial vaccines are currently available for the prevention of *caseous lymphadenitis*. Therefore, conducting research to develop effective control strategies for this disease is of great importance.

Findings/Conclusions: The most effective antigens were those obtained by sonication and the supernatant rich in phospholipase D, which is significant since phospholipase D is the primary pathogenic factor of *Corynebacterium pseudotuberculosis*. Vaccination against caseous lymphadenitis using a bacterin-toxoid biological could serve as a promising alternative for disease prevention, given the strong antigenic response observed in this study.

Keywords: *caseous lymphadenitis*, bacterin-toxoid, sheep, antibodies.

INTRODUCTION

Caseous lymphadenitis (CL) is a chronic infectious disease that affects sheep and goats. It is caused by the Gram-positive bacterium *Corynebacterium pseudotuberculosis* and is characterized

by suppurative and necrotizing lesions in the lymph nodes, lungs, and other visceral organs, leading to economic losses due to its impact on carcass and wool quality (Sánchez *et al.*, 2021; Ruíz *et al.*, 2020; Windsor *et al.*, 2011). *Corynebacterium pseudotuberculosis* is a facultative intracellular pathogen capable of replicating and surviving within phagocytes. Its virulence is primarily attributed to cell wall lipids and the production of phospholipase D (PLD) (Guimarães *et al.*, 2011). PLD is a 31 kDa protein that hydrolyzes sphingomyelin in cell membranes, releasing choline and facilitating bacterial dissemination (permeability factor). This toxin exhibits cytotoxic activity, particularly affecting endothelial cells and causing erythrocyte lysis in sheep and cattle. Additionally, it exerts a cytotoxic effect on goat macrophages (Sánchez *et al.*, 2021; Ruíz *et al.*, 2020; Guimarães *et al.*, 2011; Delgado *et al.*, 2016). The lipid layer of *C. pseudotuberculosis* functions as a chemotactic agent for phagocytes, while also exhibiting leukotoxic properties, leading to cell degeneration and lysis. Furthermore, it contributes to abscess formation and enhances resistance to lysosomal lytic enzymes (Rodríguez *et al.*, 2022; De La Fuente, 2012). The primary mode of transmission occurs through skin lesions via direct contact with contaminated shearing equipment, animal handling tools, or environmental surfaces. Additional transmission routes include inhalation and ingestion (Rodríguez *et al.*, 2022). Following infection, *C. pseudotuberculosis* multiplies locally, forming microabscesses. The bacterium is subsequently phagocytosed by macrophages and neutrophils, yet remains viable, allowing it to be transported to regional lymph nodes, where it induces caseous abscess formation and necrosis. PLD inhibits chemotaxis, prevents phagocytic cell degranulation, and activates the complement system via the alternative pathway, resulting in lymph node necrosis and thrombosis. Additionally, the bacterium utilizes extracellular iron through its serine- and protease-related proteins, facilitating lesion development (Rodríguez *et al.*, 2022). Cellular immunity is considered the primary immune response involved in CL pathogenesis; however, *C. pseudotuberculosis* is a complex pathogen, and both humoral and cellular immune mechanisms play significant roles in disease progression (Miranda *et al.*, 2010). The control of *caseous lymphadenitis* is challenging, necessitating serological diagnostic tests to identify and eliminate infected animals. Additionally, hygiene measures, including disinfection of surgical instruments, shearing equipment, and animal facilities, are crucial for disease prevention (Sánchez *et al.*, 2021). In countries where CL is endemic, vaccination is recommended, and several biological formulations have been developed for disease prevention (Braga *et al.*, 2007). In Mexico, no commercial vaccines are available for CL prevention. Therefore, conducting research to develop effective control strategies is of paramount importance. The aim of this study was to evaluate the serological response to somatic, secretory, and soluble antigens in sheep vaccinated and revaccinated with an experimental bacterin-toxoid biological against ovine *caseous lymphadenitis*.

MATERIAL AND METHODS

Thirty-three lambs between three and six months of age, from sheep production units with no clinical history of *caseous lymphadenitis*, were used. The animals were divided into four groups. Groups 1 and 2 consisted of ten lambs each, which were vaccinated and revaccinated 15 days later with 2 mL of an experimental bacterin-toxoid vaccine against

Corynebacterium pseudotuberculosis-induced *caseous lymphadenitis*. This biological preparation contained a concentration of 1×10^9 CFU/mL of bacteria inactivated with 0.4% formalin and enriched with a 48-hour culture supernatant incubated at 37 °C. Group 3, serving as control group 1, consisted of ten lambs that received only sterile physiological saline solution administered subcutaneously. Group 4, control group 2, comprised three lambs that did not receive any treatment.

Blood samples were collected weekly before and after vaccination for ten weeks to obtain serum samples. In week 7, the vaccinated animals and control group 1 were challenged with a live strain of *C. pseudotuberculosis* to assess the protective capacity of the biological; however, these results are not included in this publication. Serum samples were analyzed using indirect ELISA to detect antibodies against somatic, secretory, and soluble antigens. The antigens used included a crude supernatant from a liquid culture of *C. pseudotuberculosis* rich in phospholipase D (PLD), a protein extract from the bacterial cell wall obtained through chemical denaturation and heat (DCPE), a protein extract obtained solely through heat treatment (BCPE), and an antigen extracted using fluent steam.

All antigens were obtained from a wild *C. pseudotuberculosis* strain isolated from an abscess in a three-year-old ram. This strain was biochemically characterized and identified by polymerase chain reaction (PCR) and designated as the "ROBUS" strain. The BCPE antigen was obtained by culturing *C. pseudotuberculosis* on sheep blood agar at 37 °C under aerobic conditions for 48 hours, harvesting the colonies, suspending them in phosphate-buffered saline (PBS), and centrifuging at 3000 × g, repeating the process three times. The pellet was resuspended in PBS, heated at 100 °C for five minutes, centrifuged, and the resulting supernatant was used as the cell wall protein antigen. The DCPE antigen followed the same initial steps as BCPE but was resuspended in an antigen preparation buffer containing 0.5 M Tris pH 6.8, 5.2% SDS, and 8.7% 2-mercaptoethanol, boiled at 100 °C for five minutes, centrifuged at 3000 × g, and the supernatant was used as the antigen.

The fluent steam antigen was prepared by culturing *C. pseudotuberculosis* under the same conditions, harvesting the colonies, suspending them in physiological saline solution (SSF), and centrifuging at 3000 × g for 30 minutes. The supernatant was removed, the cells were washed, recentrifuged, and the final suspension was autoclaved in fluent steam (without pressure) for 30 minutes. After cooling for 30 minutes, it was centrifuged again for 30 minutes at 3000 × g, dialyzed with two changes of distilled water, centrifuged at 3000 × g for 50 minutes, repeated twice, and left to stand for 15 minutes. The final supernatant was filtered through 0.45 μm membranes.

The sonication antigen was obtained from *C. pseudotuberculosis* colonies cultured under the same conditions, suspended in 10 mL of SSF, and inactivated in a water bath at 90 °C for 15 minutes. The suspension was sonicated for ten cycles of 30 seconds, followed by 30 seconds of rest. After sonication, it was centrifuged at 3000 × g for 10 minutes, followed by a second centrifugation at 6000 × g for six minutes. The final supernatant, containing all cell fractions, was designated as the somatic antigen for ELISA testing.

All sera were analyzed using the indirect ELISA technique. The standardization method known as "Chessboard Titrations" (CBT) or the "Checkerboard Method" was

used to determine the optimal antigen and serum concentrations. For ELISA, the plates were sensitized with the respective antigens, incubated for 24 hours at 4 °C, and washed four times with PBS-Tween 20. The plates were blocked with 2.0% skim milk, incubated for one hour at 37 °C, washed again, and dried. The primary antibody (test serum) was added and incubated for one hour at 37 °C, followed by four washes with PBS-Tween 20. The secondary antibody (anti-ovine IgG conjugated with peroxidase) was added at a 1:1000 dilution, incubated for one hour at 37 °C, and washed again. The substrate 2,2'-Azino diethylbenzothiazoline sulfonic acid (ABTS) was added and left to react for 15 minutes. The optical density was measured at a wavelength of 450 nm. Statistical analysis was performed using an analysis of variance (ANOVA) test, and differences between means were established using the Tukey test.

RESULTS

The animals immunized with the experimental bacterin-toxoid biological for the prevention of *caseous lymphadenitis* exhibited a significant increase ($p<0.05$) in their serological response to the different antigens used in the ELISA test. This difference was observed only in the immunized animals, compared to the non-immunized group, while no significant differences were detected in the serological response among the different evaluated antigens (BCPE, DCPE, somatic, and PLD). Figure 1 illustrates the serological response to somatic antigens (sonicated antigen) of *C. pseudotuberculosis*. As observed, from the second week, antibody levels began to increase in the two immunized groups. Following the fourth week, corresponding to revaccination, a pronounced rise in antibody levels was detected in the vaccinated groups, markedly distinguishing them from the control groups.

After the seventh week, when both vaccinated groups and control group 1 were challenged, a similar increase in antibody levels was observed among these three groups, with a significant difference compared to control group 2. The Figure 2 presents the

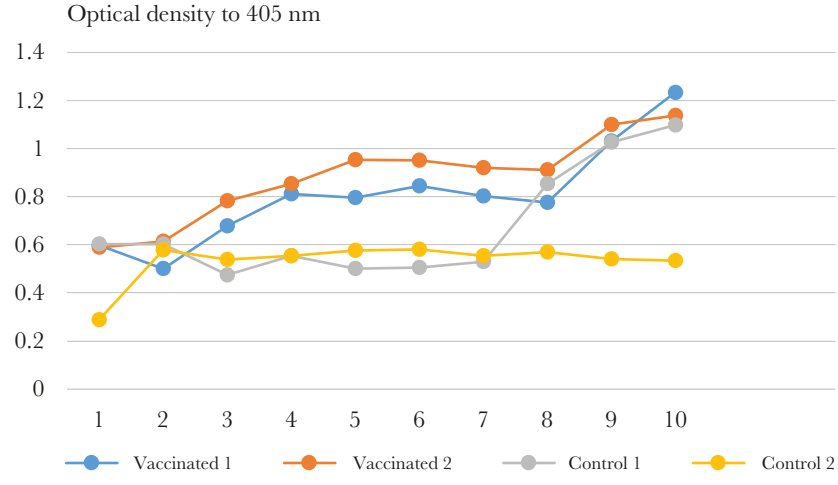

Figure 1. Serological response to somatic antigens of *Corynebacterium pseudotuberculosis*. Vaccinated groups 1 and 2 exhibited a notable increase in antibody levels following vaccination against somatic antigens, with this response becoming more pronounced after the challenge. Meanwhile, control group 1 showed a gradual increase in antibody levels only until the challenge.

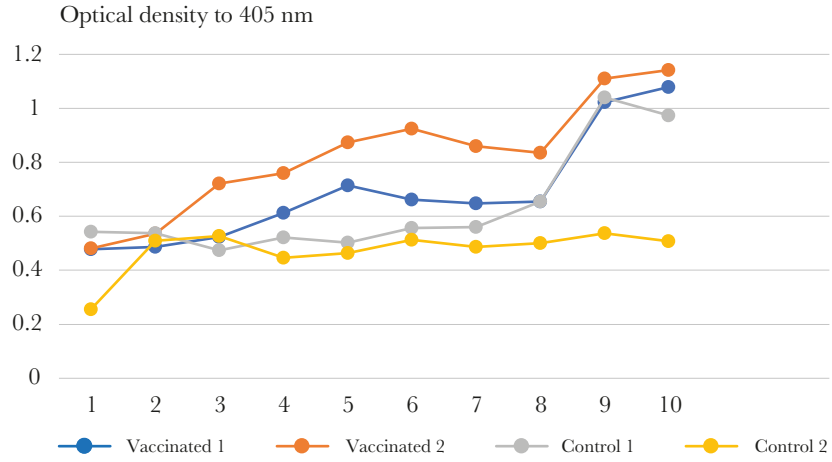

Figure 2. Serological response to heat-derived antigens (BCPE) of *C. pseudotuberculosis*. Vaccinated groups 1 and 2 showed increased antibody levels after vaccination against these antigens, this increase being more evident after challenge. Control group 1 increased its values until the challenge.

serological response to surface antigens of *Corynebacterium pseudotuberculosis* (BCPE). By the third week, antibody levels increased in vaccinated group 2, whereas vaccinated group 1 showed an increase in antibody levels by the fourth week. By week 7, the two immunized groups and control group 1 exhibited comparable antibody levels, whereas control group 2 maintained consistently low levels.

In the Figure 3 illustrates the antibody response to antigens obtained through heat and chemical denaturation. Antibody levels showed a gradual increase over time; however, by week 8, a notable rise was observed in both vaccinated groups compared to control group 1.

Figure 4 illustrates the serological response to *Corynebacterium pseudotuberculosis* toxin. Following immunization, a rise in antibody levels was observed in the vaccinated group,

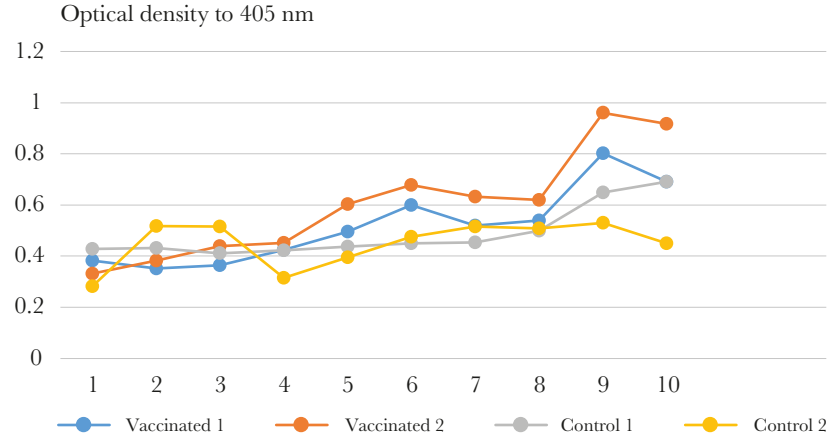

Figure 3. Serological response to *C. pseudotuberculosis* antigens obtained by heat and chemical denaturation (DCPE). Vaccinated groups 1 and 2 showed slightly increased antibody levels after vaccination against these antigens, this increase being more evident after challenge. Control group 1 increased its values until the challenge.

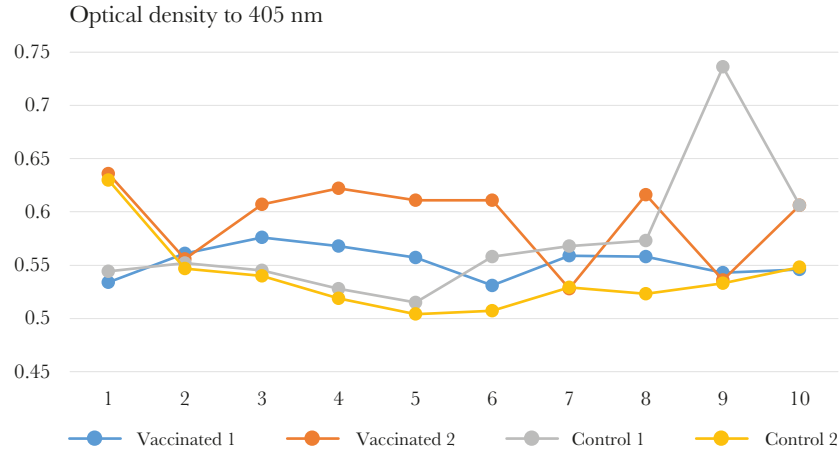

Figure 4. Serological response to *C. pseudotuberculosis* phospholipase D. Vaccinated groups 1 and 2 showed increased antibody levels after vaccination against this antigen. Control group 1 considerably increased its values up to the challenge.

followed by a subsequent decline. However, after the challenge, control group 1 exhibited a considerable increase in antibody levels.

Finally, Figure 5 shows the behaviour of the serological response of vaccinated and unvaccinated sheep against the antigens obtained by fluent vapour. Vaccinated group 2 showed a significant increase in antibody levels compared to the other groups, both at vaccination, revaccination, and challenge.

Figure 5. Serological response to antigens obtained by fluent vapour of *C. pseudotuberculosis*. Only vaccinated group 2 showed increased antibody levels after vaccination against this antigen. The increase was more evident after the challenge.

DISCUSSION

This study evaluated the serological response induced by an experimental bacterin-toxoid vaccine against *Corynebacterium pseudotuberculosis*, focusing on different antigenic preparations. The findings demonstrated that antigens obtained through sonication and heat extraction (BCPE) elicited the strongest immune responses, making them valuable

tools for assessing immunogenicity. Similar results were reported in the United Kingdom, where the use of sonicated antigens in ELISA tests on sera from clinically affected sheep yielded a sensitivity of 83% and a specificity of 71%, confirming the reliability of this approach (Corona *et al.*, 2011; Farias *et al.*, 2020). Consistently, the present study revealed that vaccinated animals developed a robust antibody response against somatic and surface antigens, reinforcing the potential of these antigens for immunodiagnostic applications. A detailed analysis of heat-extracted antigens revealed that BCPE antigens were more effective in detecting surface antigens than those obtained through chemical denaturation plus heat (DCPE). This finding contrasts with a previous study (Corona *et al.*, 2011), which found DCPE antigens to be more effective in diagnosing *caseous lymphadenitis*. However, an important distinction between these studies lies in the immune status of the evaluated animals. While the earlier research focused on naturally infected animals, this study analyzed the immune response following vaccination. Given that *C. pseudotuberculosis* is a facultative intracellular pathogen, the immune response elicited by active infection differs significantly from that triggered by vaccination, which could explain the discrepancy in antigen performance (Corona *et al.*, 2011; Abbas *et al.*, 2022). Additionally, alternative heat extraction techniques have been explored in other studies, but results have been inconsistent, likely due to variations in temperature protocols and antigen stability (De La Fuente *et al.*, 2012). Beyond surface and somatic antigens, this study also assessed the immune response to secretory antigens, particularly phospholipase D (PLD), which is the primary virulence factor of *C. pseudotuberculosis*. A strong serological response to PLD was observed in vaccinated animals, aligning with previous findings that identified neutralizing anti-PLD antibodies as key indicators of protective immunity (Tachedjian *et al.*, 1995). This is particularly relevant given that Hoelzle *et al.* (2013) demonstrated that PLD-based ELISA detected 100% of infected goats but only 70% of infected sheep, emphasizing the need for multiple antigenic targets to improve diagnostic sensitivity. In fact, these authors suggested that combining immunodominant bacterial proteins (150, 74, 48, and 30 kDa) with PLD enhances diagnostic accuracy, a strategy that could further optimize serological tests for *caseous lymphadenitis*. Another antigenic preparation that demonstrated strong immunogenicity was the fluent steam-extracted antigen. This response may be attributed to the continuous heat and moderate pressure applied during extraction, which likely preserved a broader spectrum of bacterial antigens. The development of ELISA assays for *C. pseudotuberculosis* detection has been a global priority, yet many of these tests remain commercially unavailable. For instance, Dercksen *et al.* (2000) developed four ELISA protocols, with Double-Sandwich ELISA using purified PLD antigen and hyperimmune sera demonstrating a sensitivity of 79% and specificity of 99%, making it particularly suitable for disease eradication programs. In addition to diagnostics, ELISA has been widely employed for monitoring vaccine-induced serological responses. De Oliveira *et al.* (2022) followed up on sheep vaccinated with either a PLD-rich bacterin or a recombinant PLD vaccine, demonstrating that ELISA using sonicated and toxoid antigens effectively measured humoral immunity against PLD. Their findings showed that the formalin-inactivated PLD vaccine conferred 95% protection, whereas the recombinant PLD vaccine provided only 44% protection

(Farias *et al.*, 2020). These results reinforce the protective potential of PLD, which led to the development of the Glanvac22-24 toxoid vaccine in Australia (1984). This vaccine has demonstrated consistent efficacy in preventing *C. pseudotuberculosis* infections in multiple challenge studies (De Oliveira *et al.*, 2022). Further supporting the role of PLD in protective immunity, a study conducted in the United Kingdom evaluated different vaccination strategies, including a recombinant PLD derivative, a formalin-inactivated bacterin, and a bacterin containing recombinant PLD. ELISA-based serological follow-ups confirmed that PLD-containing vaccines provided statistically significant protection, prevented disease dissemination, and reduced the incidence of visceral forms of the disease (Fontaine *et al.*, 2006). In Mexico, Ibarra *et al.* (2016) tested an aroA mutant strain of *C. pseudotuberculosis* in cellular and murine models to evaluate attenuation and immunogenicity. Their study confirmed that the aroA mutant strain was significantly attenuated, yet failed to provide at least 80% protection, a threshold considered necessary for an effective immunogen. This highlights the challenges of developing attenuated vaccines and underscores the need to explore alternative antigenic targets (Ibarra *et al.*, 2016). The selection of bacterial strains for vaccine development is another critical factor. Parise *et al.* (2018) conducted phylogenomic analyses of *C. pseudotuberculosis* isolates from different hosts, revealing that genetic diversity in ovine strains is influenced more by animal transportation than host specificity. They also identified nrdF2, a gene previously described as a potential vaccine target, which could aid in developing novel diagnostics, vaccines, and therapeutics (Parise *et al.*, 2018). As highlighted in multiple studies, ELISA remains an essential tool for epidemiological surveillance and evaluation of vaccine efficacy. However, in Mexico and other countries, the lack of standardized ELISA tests for *caseous lymphadenitis* hinders diagnostic efforts. This has led researchers to explore alternative antigen extraction methods to enhance diagnostic accuracy and serological monitoring in vaccinated animals. Continued advancements in antigen selection and vaccine formulation will be crucial for improving disease control strategies and preventing the spread of *C. pseudotuberculosis* in sheep and goat populations.

CONCLUSIONS

Finally, this study successfully obtained *Corynebacterium pseudotuberculosis* antigens through various extraction methods, allowing for the evaluation of immune responses in vaccinated animals. Among the antigens tested, those obtained by sonication and the PLD-rich supernatant elicited the strongest immune responses in the vaccinated groups. Additionally, antigens derived from the native bacterial strain demonstrated acceptable immunoreactivity, indicating their potential for use in serological assessments. Given the robust antigenic response observed in this study, vaccination against *caseous lymphadenitis* using a bacterin-toxoid biological represents a promising alternative for the prevention and control of this disease.

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The structure and geographic distribution of the live-sheep market in Mexico, 2007-2021

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ABSTRACT

Objective: This study aimed to analyze the structure and geographic distribution of the sheep market mobilized for slaughter, breeding, rearing, fairs, and fattening in Mexico from 2017 to 2021.

Design/methodology/approach: The data on sheep mobilizations between source and destination municipalities were sourced from Mexico's Sistema Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA). Via social network analysis theory, national cohesion and centrality measures were calculated per purpose of sheep mobilization.

Results: Of all sheep mobilized, 98.2% moved between states, with 97.0% of them destined for slaughter. The national livestock mobilization network had 54.0% of possible connections, an average out-degree of 17.3, and an average in-degree of 16.7. The highest out-degree centrality was found in the state of Jalisco, while the highest in-degree centrality was found in the state of Puebla.

Limitations on study/implications: The primary constraint of the study was the failure to consider the mobilizations of sheep not passing through livestock control centers, despite assuming a minimal level.

Findings/conclusions: The sheep mobilization structure in Mexico is determined by the market of sheep destined for slaughter and an interstate mobilization pattern.

Keywords: Network analysis, sheep mobilization, market connectivity, distribution networks.

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INTRODUCTION

Over the past decade, sheep production systems in Mexico have accounted for 12.5% of the national red meat inventory (SIAP, 2023). Although these systems are distributed nationwide, a significant gap in data persists regarding the relationship between production and consumption centers, creating challenges for national planning. In contrast, cattle production systems are found throughout the country but exhibit a highly concentrated mobilization pattern, primarily shaped by slaughter and fattening markets. This structure is characterized by high-density networks, low centrality, significant intra-state movement,

and a low degree of market specialization (Callejas and Salas, 2023). Network Analysis, traditionally employed in epidemiological research, provides a theoretical framework for studying relationship structures involving the exchange of information, products, and services within markets. The role of livestock mobilization in disease transmission has been well documented. In the United Kingdom, sheep movements contributed to the rapid spread of foot-and-mouth disease in 2001 (Kiss *et al.*, 2006), while in Scotland, researchers identified sheep mobilization patterns as a key factor in disease dissemination (Volkova *et al.*, 2010). Similarly, studies conducted in Paraguay demonstrated that cattle movement networks facilitate the spread of foot-and-mouth disease and brucellosis (Avalos *et al.*, 2022). When applied to market dynamics, social network analysis enables the examination of attributes within dyadic relationships as well as structural patterns across entire networks (Borgatti and Everett, 1997). Given the limited availability of information on livestock mobilization in Mexico, this study aimed to analyze the structure and geographic distribution of the live-sheep market mobilization network, specifically for slaughter, breeding, rearing, fairs, and fattening from 2017 to 2021.

MATERIALS Y METHODS

The analysis considered the daily number of sheep mobilized (X_{ij}) from all source municipalities (x_i) to all destination municipalities (x_j) across Mexico between 2017 and 2021. The dataset was derived from official records of sheep mobilization for slaughter (Y_1), breeding (Y_2), rearing (Y_3), fairs (Y_4), and fattening (Y_5), obtained from the Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA).

Given that Mexico comprises 2,476 municipalities across 32 states, state- and municipal-level samples were constructed for analysis. Each sheep mobilization purpose was examined as both a source and destination market (Table 1).

Nationwide mobilization matrices (N_{ij}) were constructed using the daily number of sheep mobilized X_{ij} for each mobilization purpose at both municipal and state levels:

Table 1. Municipal and state samples per source and destination sheep markets, 2017-2021.

Purpose/ source	Percent	Purpose/ destination	Percent
Municipal level			
Slaughter	12.0	Slaughter	15.0
Breeding	5.6	Breeding	15.6
Rearing	4.4	Rearing	9.4
Fairs	4.1	Fairs	3.5
Fattening	0.9	Fattening	1.2
State level			
Slaughter	91.3	Slaughter	93.1
Breeding	87.5	Breeding	122.0
Rearing	80.0	Rearing	92.5
Fairs	67.5	Fairs	73.1
Fattening	35.6	Fattening	39.4

slaughter (Y_{1ij}), breeding (Y_{2ij}), rearing (Y_{3ij}), fairs (Y_{4ij}), and fattening (Y_{5ij}). In each matrix, rows represented the source states of the mobilized sheep, while columns represented the destination states.

Sheep mobilization patterns were analyzed at the municipal level, distinguishing between inter-municipal (M_{ij}) and intra-municipal (M_{ii}) shipments. The former represents shipments from x_i to x_j, while the latter accounts for shipments within the same municipality x_j to x_i. Similarly, the analysis was extended to the state level to evaluate broader movement trends.

To examine the structure and geographic distribution of the sheep market in Mexico, the study applied social network analysis theory (Borgatti *et al.*, 2018). According to these authors, cohesion and centrality measures are particularly effective for assessing network structure, providing insights into connectivity patterns, market integration, and dominant mobilization hubs.

The market network density (ND) measures the proportion of connections between source and destination municipalities or states, indicating the extent of direct and indirect linkages within the network. A fully connected network is considered complete, meaning all nodes are directly or indirectly linked. Higher network density corresponds to a greater number of links, reflecting a more interconnected market.

While ND is a fundamental measure for understanding overall network structure, additional metrics provide deeper insights. Among these, outbound centrality (OC) and inbound centrality (IC) assess the dominance of specific nodes in sheep mobilization. The average degree (AD) measures the mean number of connections per node, while connectivity (CO) evaluates network cohesion. Mutual nodes (MD) identify the reciprocal exchange of sheep between locations, and dyadic reciprocity (DR) quantifies bidirectional transactions within the network. Each node was analyzed using density and centrality measures to determine its role in the national mobilization structure.

To achieve a comprehensive understanding of sheep mobilization networks in Mexico, the study incorporated the theory of spatial location of production systems, particularly focusing on the motivations for mobilization. Following the framework of Lira and Quiroga (2009), the economic structure of each state and the purposes for mobilization were examined using two measures of relative regional specialization.

The localization quotient (R_E) quantifies a state's participation in a specific production system, comparing it to the national level. Here, F_{ij} represents the flow of sheep for purpose i in state j. An $R_E > 1$ indicates relative or interregional specialization, signifying that the state plays a disproportionately large role in that particular aspect of sheep production or trade.

$$R_E = \frac{\frac{F_{ij}}{\sum_i F_{ij}}}{\frac{\sum_j F_{ij}}{\sum_{ij} F_{ij}}}$$

The specialization coefficient (R_M) assesses the degree of similarity between a region's economic structure and the national structure. An $R_E \sim 1$ indicates a specialized production system, meaning the region is highly focused on a particular aspect of sheep production or trade. Conversely, an $R_M \sim 0$ suggests a diversified production system, where economic activities are more evenly distributed across multiple production purposes.

$$R_M = 0.5 \sum_i \left| \frac{F_{ij}}{\sum_i F_{ij}} / \frac{\sum_j F_{ij}}{\sum_{ij} F_{ij}} \right|$$

RESULTS AND DISCUSSION

The findings are presented for both the national market (N_{ij}) and per product (Y_{ij}). During 2017-2021, an annual average of 1,114,147 livestock heads were mobilized in Mexico. Five types of markets comprised the sheep market: slaughter, breeding, rearing, fairs, and fattening. All markets showed significant linear correlations ($p<0.05$), except for fattening with other types and slaughter with fairs.

Of all mobilized sheep in Mexico's markets, 97.0% were destined for slaughter, 1.3% for breeding, 0.9% for rearing, 0.6% for fairs, and 0.2% for fattening. The domestic market grew at an average annual growth rate (AAGR) of 1.5%, increasing from 1.08 million head in 2017 to 1.1 million head in 2021. The slaughter and fattening markets grew at AAGRs of 1.9% and 52.7%, respectively, while the breeding, rearing, and fair markets recorded negative AAGRs of 10.3%, 13.8%, and 9.4%, respectively.

Sheep supply and demand in Mexico were highly concentrated at the state level, with the top quartile of states accounting for 79.6% of the national market. These states mobilized 80.7% of sheep for slaughter, 78.6% for breeding, 64.6% for rearing, 72.3% for fairs, and 74.5% for fattening. Meanwhile, the top quartile of states with the highest demand concentrated 94.1% of the national sheep market, including 95.5% for slaughter, 56.6% for breeding, 59.5% for rearing, 74.6% for fairs, and 73.6% for fattening.

The leading states in national sheep supply were Jalisco (32.6%), San Luis Potosí (10.9%), and Campeche (10.0%). Other significant contributors included Chihuahua, Coahuila, Guanajuato, Zacatecas, and Querétaro. The supply of sheep for slaughter determined the national sheep supply, reflecting the same states and market shares as the national market. The top states for breeding were Hidalgo (25.2%) and Jalisco (21.2%); for slaughter, the State of Mexico (14.4%) and Puebla (11.6%); for fairs, Guanajuato (25.1%) and Jalisco (19.3%); and for fattening, Campeche (25.5%), Colima (20.4%), and Nayarit (10.0%).

During the analyzed period, only 34.4% of the markets exhibited positive AAGRs, with an average growth rate of 1.5%. The most critical period occurred between 2018 and 2019, when the market experienced a 6.3% decline.

The leading sheep market, located in Jalisco, demonstrated a notable average annual growth rate of 9.3%, while the second-largest market recorded a 7.3% decrease. Significant variations were observed in the supply of certain markets with outstanding AAGRs. The market in Coahuila grew at an average rate of 27.0%, Tabasco at 26.2%, and Sinaloa

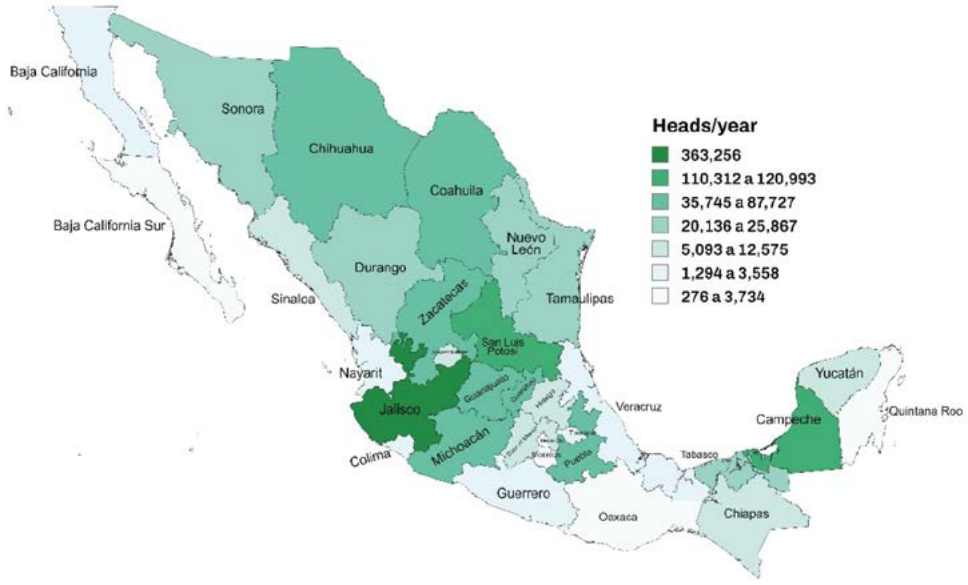

Figure 1. Average Spatial Distribution of Cattle Supply in Mexico.

at 412.8%. Despite this growth, Tabasco and Sinaloa maintained low market shares, accounting for only 1.4% and 0.2%, respectively.

Regarding national sheep demand, the leading states were the State of Mexico (45.0%), Hidalgo (22.6%), and Puebla (11.7%) in terms of mobilized livestock, followed by Guanajuato, Morelos, Querétaro, Jalisco, and Nuevo León. Similar to sheep supply, the slaughter market dictated national demand, involving the same states but in different proportions. The most significant states in the slaughter market were the State of Mexico (46.2% of all mobilizations), Hidalgo (23.1%), and Puebla (11.8%). In the breeding market, the key states were Querétaro (8.6%), Guanajuato (8.0%), and Veracruz (7.6%). For the rearing market,

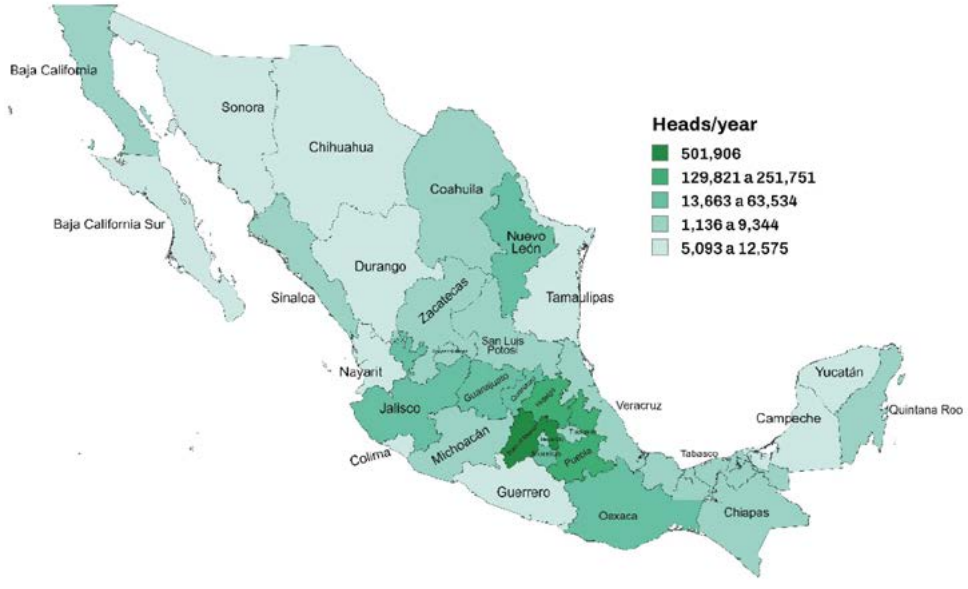

Figure 2. Average spatial distribution of cattle demand in Mexico.

the most important states were Hidalgo (10.8%), Puebla (8.9%), and Veracruz (8.3%). In the fairs market, Guanajuato (31.6%), Querétaro (14.3%), and Chiapas (8.9%) stood out. Lastly, for the fattening market, the leading states were Jalisco (30.9%), Querétaro (8.8%), and Puebla (7.6%). Sheep demand exhibited positive AAGRs in only 25.0% of its markets. A notable trend was observed in the three main destination markets: the State of Mexico grew at an AAGR of 2.0%, while Hidalgo and Puebla experienced declines of 1.6% and 3.7%, respectively. Other states with smaller market shares recorded significant growth rates, such as Guanajuato (41.0%), Nuevo León (48.5%), and Sinaloa (60.1%).

Networks

At the national level, the sheep mobilization network consisted of 31.2±0.4 source markets (red circles) and 32 destination markets (blue squares), with the exception of Quintana Roo (2017-2018) and Oaxaca (2020-2021).

The sheep market was predominantly driven by inter-state trade, as only 1.8% of sheep remained within their state of origin. Additionally, 51.4% of all potential links were present. The analysis revealed that Baja California Sur (destination) and Baja California Norte (source) were distant from major markets such as Jalisco and the State of Mexico, which functioned as key source and destination states, respectively. The low out-degree centrality (45.2%) and even lower in-degree centrality (38.5%) highlighted a significant imbalance in the states' roles as primary suppliers and consumers of sheep (Figure 3).

For all purposes of sheep mobilizations, the measures of cohesion were lower than for the whole network. A significant direct linear correlation ($p<0.05$) was found between the number of animals mobilized and the measures of cohesion.

For all sheep mobilization purposes, the cohesion measures were lower than those observed for the entire network. A significant direct linear correlation ($p<0.05$) was found between the number of animals mobilized and the cohesion measures.

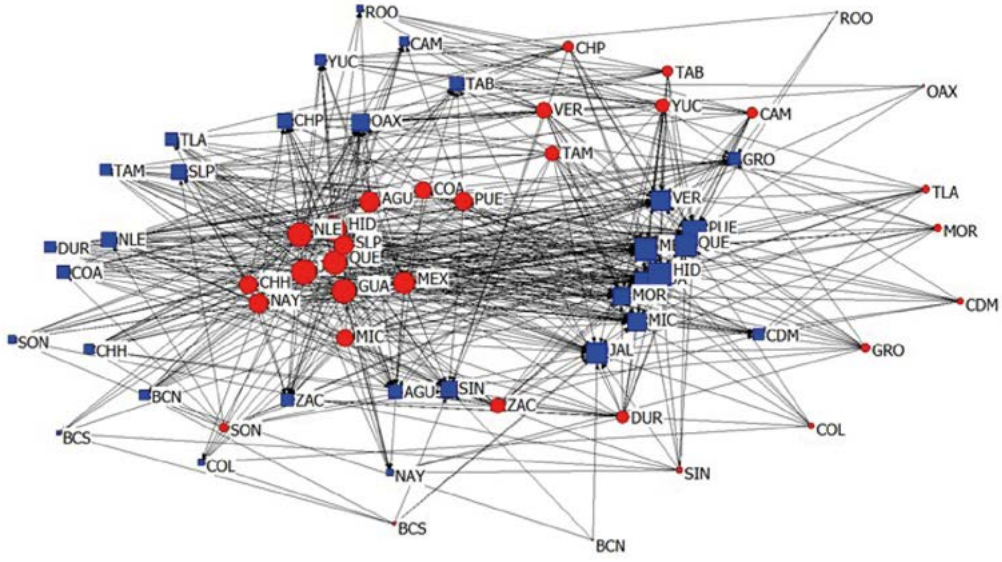

Figure 3. Network of Mexico's national sheep market (2017-2021).

Jalisco was identified as the most central source market, accounting for 96.9% of all sheep mobilizations to destination markets. The State of Mexico was the main destination market, receiving 90.6% of all links. In contrast, Baja California Norte received 9.4% of all shipments, while Baja California Sur sent 18.8% of all shipments.

Analysis

The first analysis of Mexico's sheep market network revealed its large size, driven by significant distances between markets and the country's numerous municipalities. Additionally, intrastate markets were found to be substantial. Mexico's diverse human, monetary, and natural resources sustain five distinct production systems and their respective markets. However, social network analysis indicated a centralization of these production systems and markets. Network centrality was used to identify key elements within the network, revealing that the national network's centrality was lower compared to the per-product networks. Four markets contributed to increased national centrality: Jalisco (supply) and Estado de México, Hidalgo, and Puebla (demand). For slaughter, Jalisco and San Luis Potosí led in supply, while Estado de México and Puebla dominated demand. In breeding, Hidalgo and Jalisco were major suppliers, but no significant demand markets were identified. For fattening, Campeche and Colima served as key suppliers, with Jalisco as the main demand market. No important markets were found for rearing, while in fairs, Guanajuato and Jalisco were key suppliers, and Guanajuato and Querétaro were leading demand markets.

Market cohesion measures belonging and access to information for all source and destination markets within a network. In the national sheep mobilization network, every source and destination market, including the smallest (fattening), exhibited high connectivity. However, the overall network density was low, and even lower in the per-product networks. The states with the highest centrality and market density within the national network were Jalisco, Estado de México, San Luis Potosí, Campeche, Puebla, Colima, and Querétaro. The centrality and cohesion observed in the sheep market network

Table 2. National network and product cohesion metrics (2017-2021).

	Y	Y1	Y2	Y3	Y4	Y5
Possible links	1024	992	1024	992	812	456
Existing links	553	372	377	288	183	82
Avg degree	17.30	10.97	11.13	8.13	5.89	2.88
Deg centrality	0.34	0.48	0.47	0.47	0.45	0.33
Out-degree centrality	0.45	0.53	0.63	0.62	0.43	0.23
In-degree centrality	0.39	0.47	0.26	0.20	0.43	0.19
Density	0.53	0.35	0.36	0.27	0.22	0.13
Transitivity/Closure	0.71	0.65	0.62	0.49	0.44	0.27
Mutual	0.38	0.22	0.19	0.11	0.08	0.03
Null	0.32	0.52	0.47	0.57	0.64	0.78
Arc Reciprocity	0.72	0.63	0.53	0.41	0.36	0.26
Dyad Reciprocity	0.57	0.46	0.36	0.26	0.22	0.15

were attributed to the economic structure of the production systems. At the national level, economic specialization was low (45.0%), primarily due to minimal specialization in slaughter (1.5%). Other systems exhibited higher specialization levels: 64.5% in fattening, 58.3% in rearing, 51.8% in fairs, and 49.3% in breeding. Additionally, 51.3% of sheep-producing states considered themselves specialized overall. Specifically, 40.6% specialized in sheep for slaughter, 65.6% for breeding, 62.5% for rearing, 43.8% for fairs, and 43.8% for fattening.

CONCLUSIONS

The live-sheep mobilization network in Mexico is primarily driven by the slaughter market, while production systems for breeding, fattening, and fairs are nearly non-existent. These production systems are characterized by interstate markets, which are heavily concentrated in the central region of the country. The national state network exhibited high cohesion among states, with strong centrality for both entry and exit. However, the densities for specific mobilization purposes remained low. The national municipal network displayed even lower density and centrality. Jalisco (as an origin) and Puebla (as a destination) were identified as the two best options for disseminating information within the sheep mobilization network. Additionally, sheep production systems in Mexico should be regarded as diversified.

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The dynamics of the pork-market structure in the State of Mexico, 2017-2021

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ABSTRACT

Objective: Identify the main entities of origin and/or destination in the transfer flows of live pigs across slaughterhouses, fattening farms, breeding facilities, and fair markets in the State of Mexico. Additionally, detect potential information asymmetries to design public policy strategies that enhance efficiency and equity in pig mobilization.

Design/methodology/approach: This study employs the Social Network Analysis (SNA) approach, analyzing data from 2017 to 2021 across four market types (*i.e.*, slaughter, fattening, breeding, and fairs). The study identifies 24 source entities and 29 destination entities, some of which function as both origins and destinations.

Results: Pig mobilization in the State of Mexico reveals a network of interactions among different market types, influenced by geographical, productive, and economic factors, as well as consumer preferences. The network exhibits significant centralization in certain markets, particularly slaughterhouses, where Puebla, Jalisco, and Veracruz emerge as the main contributors to pig livestock mobilization. Challenges related to reciprocity in trade relationships suggest imbalances in the distribution of pork commerce within the State of Mexico.

Limitations/implications: The lack of participation by some states in government-sponsored fairs, coupled with restrictive pig-breeding regulations in certain regions, highlights the need for targeted strategies to address the challenges within each market type.

Findings/conclusions: Public policies should foster transparency, competition, and equitable access to resources and markets. Additionally, addressing information asymmetries is crucial to ensuring food security and promoting animal welfare in the pork industry of the State of Mexico.

Keywords: Network analysis, pork industry structure, market connectivity, distribution networks.

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INTRODUCTION

Pig farming is intrinsically linked to the agri-food and agro-industrial production of nations, playing a pivotal role in their economies due to its strong connections with input

supply chains and processing industries (Sosa *et al.*, 2017; Iglesias *et al.*, 2017; Peña, 2011). In Mexico, it stands as one of the most dynamic livestock-related activities, distinguished by its significant multiplier effect, with a product factor of 3.40 (Sosa *et al.*, 2017). It is regarded as a key driver of economic growth, given its extensive ripple effect across multiple industrial sectors. Mexico ranks among Latin America's leading producers and consumers of pork. In 2021 alone, the country produced 1,693,007 tons, with the primary pork-producing states being Jalisco (22% of total production), Sonora (18%), Puebla (11%), Yucatán (9%), and Veracruz (9%), collectively accounting for 69% of the nation's total output (SIAP, 2022a). From a zootechnical perspective, this production is categorized into six segments: 'breeding stock' (106,068), 'cull pigs' (188,835), 'replacement pigs' (228,683), 'sows' (1,246,276), 'piglets' (3,987,715), and 'fattening pigs' (10,263,741) (INEGI, 2022). The main distribution destinations were the State of Mexico (with a quarterly expenditure of MXP 1,950 million), Mexico City (MXP 924 million), Veracruz (MXP 870 million), Puebla (MXP 859 million), and Jalisco (MXP 747 million), driven by factors such as population density, purchasing power, and production capacity. With a population of 16.9 million inhabitants, the State of Mexico plays a crucial role in pork production and consumption. In 2021, it ranked as the 12th most significant pork-producing state, with an average pig weight of 74.86 kg per head and a market price of MXP 50.70 per kg of pork (SIAP, 2022b). However, while these figures reflect the final consumer stage of the cold carcass market, they fail to account for the primary production stage, the origins and destinations of live pigs, or the specific type of market (*i.e.*, slaughter, fattening, breeding, or fairs) toward which the supply and demand are directed (Callejas *et al.*, 2020). Given this context, pig trade and mobilization across various regions and market types constitute a fundamental component of the value chain. Consequently, this study proposes analyzing the pig-farming system in the State of Mexico as a network structure, enabling small producers to strategically plan, enhance productivity, and access regional markets (Gelabert *et al.*, 2017). Moreover, such an approach fosters the development of innovative ecological management systems for agricultural lands (Staver *et al.*, 2004), strengthens mechanisms for disease detection and control, and facilitates a deeper understanding of the social complexity underlying interactions among pig farmers and other key stakeholders in the information-exchange process (Andico *et al.*, 2021). These measures, in turn, would enable the formulation of public policy strategies aimed at optimizing pig mobilization from source to destination, thereby mitigating delays, streamlining logistics, reducing costs, and ensuring stringent monitoring to prevent disease transmission along the production chain. Given the imperative to further develop the pork industry in the State of Mexico, this research aims to analyze the mobilization of live pigs by market type (slaughter, fattening, breeding, and fairs), identifying key actors, intermediation hubs, and information asymmetries. This will be achieved using Social Network Analysis (SNA) to evaluate transfer flows and network centralization. This methodological approach will facilitate the identification of hierarchies and roles within the pork market, shedding light on the influence of information asymmetry and guiding the design of public policies to enhance transparency and efficiency within the sector.

MATERIALS AND METHODS

The study examined the interstate pig-mobilization system in the State of Mexico, which comprises 125 municipalities, a population of 16.9 million inhabitants (representing 13.5% of Mexico's total population), and holds significant importance in the country's agriculture and livestock sector (INEGI, 2020). The data were obtained from the daily records database for the period 2017-2021, with the explicit authorization of SENASICA, considering the available information from the states that supply pigs to the State of Mexico and receive them from this entity for slaughter, fattening, breeding, and fairs. The analysis encompassed 24 source entities (xi) and 29 destination entities (xj).

Data analysis: Social-network analysis (SNA)

Social Network Analysis (SNA) was employed to examine topological trends in the pork market and identify social hierarchies and roles within the analyzed structure. The study utilized UCINET (version 6.27) and VISONE (version 2.2) software for network visualization and analysis. To determine social density, mathematical centrality and clustering equations were applied (Velázquez *et al.*, 2005; Núñez *et al.*, 2022), while degree centrality was assessed following the definition of Proctor and Loomis (1951). The total number of heads per source-node and destination-node was calculated using in-degree data (Wasserman *et al.*, 2013) and out-degree data (Pérez, 2021). Furthermore, each source and destination state's influence and power were analyzed within the mobilization-network structure, following the asymmetric-information theory. Information asymmetry arises when market entities do not have equal access to information, leading to market failures (Ayat *et al.*, 2020). Since such asymmetry allows for an in-depth examination of how unequal access to information affects decision-making and economic outcomes across different sociopolitical and economic contexts, its implications were assessed through various dimensions, including competition promotion, supply-chain diversification, transparency in business transactions, information-system development, access to communication technologies, direct market-initiative subsidies, regulatory oversight, and livestock traceability and quality control. The degree of information asymmetry was quantified using mathematical equations to determine network intermediation (Freeman, 1977) and cliques (Brandes, 2005; Núñez-Espinoza *et al.*, 2022).

RESULTS AND DISCUSSION

The State of Mexico maintains a negative trade balance in the pig market across all categories: slaughter, fattening, breeding, and fairs. The analysis revealed that while 48.56 million heads were mobilized into the State of Mexico, only 122,636 heads were mobilized out. A significant 91.7% of incoming shipments originated from Puebla (31.2%), Jalisco (25.8%), Veracruz (21.4%), and Guanajuato (10.1%), while the primary destination states were Hidalgo (23.85%), Tlaxcala (5.77%), and Puebla (4.09%). The distribution by market type showed that 99.4% of pigs mobilized to the State of Mexico were intended for slaughter, followed by those for the fattening market (0.57%), breeding (0.04%), and fairs (less than 0.01%). Regarding the origins of pig shipments by purpose, the pigs destined for slaughter primarily came from Puebla (34.31%), Jalisco (25.88%), and Veracruz (21.48%),

while those intended for fattening originated mainly from Puebla (17.89%), Morelos (17.19%), and Jalisco (16.44%). In terms of breeding, the largest contributions were from Jalisco (40.65%), Zacatecas (7.89%), and Guanajuato (6.83%), whereas the pigs destined for fairs predominantly came from Jalisco (64.33%), Guanajuato (23.94%), and Sonora (11.70%). On the other hand, the mobilization of pigs from the State of Mexico to other states followed a distinct pattern. The majority of shipments for slaughter were directed to Hidalgo (30.08%), Querétaro (5.99%), and Guerrero (2.75%), while those for fattening were sent to Tlaxcala (14.93%), Hidalgo (13.30%), and Puebla (6.50%). The breeding market primarily received pigs in Morelos (8.68%), Hidalgo (18.31%), and Chiapas (9.39%), whereas the vast majority of pigs mobilized for fairs were destined for Mexico City (99.47%) and Baja California Sur (0.53%). In total, 264,273 pig mobilizations were recorded into the State of Mexico, with 33.6% originating from Puebla, 26.6% from Jalisco, 20.7% from Veracruz, and the remainder from other states. Additionally, 42.9% of all pig mobilizations within the State of Mexico were intrastate, while 21.0% were destined for Hidalgo, with the rest distributed across 28 other states with which the State of Mexico maintains trade links (Figure 1). These data underscore the complexity and

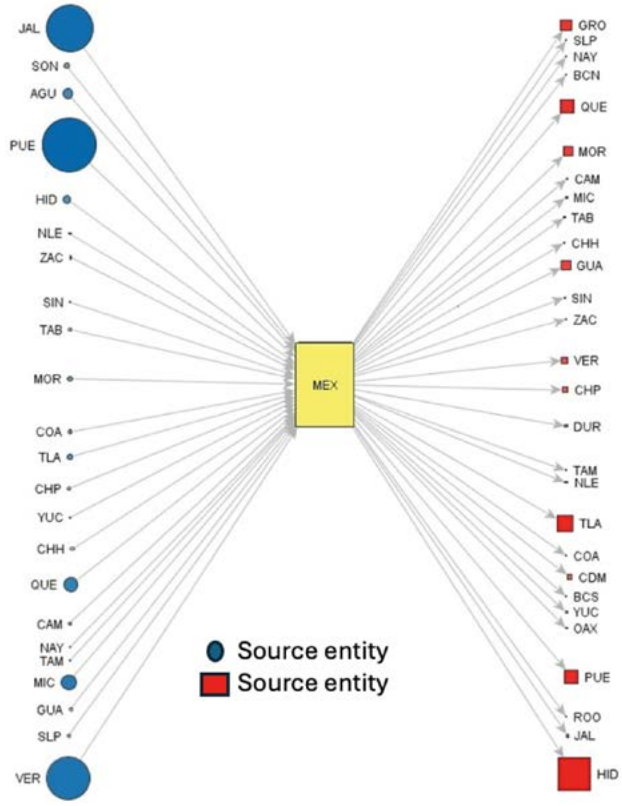

Figure 1. Pork-market dynamics in the State of Mexico by type of mobilization, 2017-2021.
BCN=Baja California Norte; BCS=BAJA California Sur; CDM=Mexico City; COL=Colima; DUR=Durango; GRO=Guerrero; ROO=Quintana Roo; YUC=Yucatán; CAM=Campeche; CHP=Chiapas; NAY=Nayarit; OAX=Oaxaca; TAB=Tabasco; CHH=Chihuahua; PUEB=Puebla; MIC=Michoacán; VER=Veracruz; SIN=Sinaloa; SON=Sonora; MEX=State of Mexico: ZAC=Zacatecas; GUA=Guanajuato; JAL=Jalisco; SLP=San Luis Potosi; AGU=Aguascalientes; TLA=Tlaxcala; QUE=Querétaro; NLE=Nuevo León; COA=Coahuila; MOR=Morelos; TAM=Tampico; HID=Hidalgo.

concentration of the pig trade network, revealing both strong regional dependencies and market asymmetries in the mobilization dynamics of the sector.

In addition to the shortage of pigs, the decline in pig transfers between 2019 and 2021 is closely linked to a global disruption in the supply chain of agri-food products (Kerr, 2020). One of the most significant factors was the impact of African Swine Fever (ASF) on the pork industry worldwide, which may have begun to affect pig mobilization in Mexico as early as 2019. However, the most pronounced decrease occurred in 2020, coinciding with the onset of the COVID-19 pandemic, which severely disrupted supply chains, hindered transportation and processing, and led to temporary market closures. The pandemic's effects persisted into 2021, with residual restrictions and a gradual recovery in the livestock sector continuing to influence the transfer of live pigs (Table 1).

However, the State of Mexico exhibited a deficit in the supply of pigs for slaughter, fattening, reproduction, and fairs, which complicated its commercial interactions with other states. Between 2017 and 2021, the main pig-supplying states to the State of Mexico were Puebla, Jalisco, Veracruz, and Guanajuato. Puebla, located approximately 130 km east of the capital of the State of Mexico, stood out as the largest supplier, accounting for 31.2% of the pigs mobilized. Jalisco, situated about 350 km to the west, contributed 25.8%, while Veracruz, approximately 450 km to the east, supplied 21.4%, and Guanajuato, around 300 km to the northwest, accounted for 10.1% of the total supply. In terms of demand, the State of Mexico maintained significant trade interactions with other entities, particularly Hidalgo, Tlaxcala, and Puebla. Hidalgo, located 250 km to the north, was one of the primary destinations, receiving 23.85% of pigs mobilized from the State of Mexico. Tlaxcala, about 200 km to the east, and Puebla, 130 km away, were also key recipients of pig livestock from this region. Mobilization data from 2017 to 2021 revealed a centralized distribution network, with a few states functioning as major nodes in pig mobilization. This centralization resulted in a star-shaped structure, where states such as Puebla and Jalisco held dominant positions due to their high concentration of connections. The asymmetry of information between origin and destination states can significantly impact market efficiency and equity. Public policies must address these imbalances, ensuring greater transparency in transactions. According to Callejas *et al.* (2020), it is crucial to analyze the first link in the supply chain —the producer— alongside the origin, destination, and type of market (slaughter, fattening, reproduction, or fairs) to which the supply and demand of pigs are directed. However, previous research has primarily focused on the cold-channel final

Table 1. Pig movement by market type in State of Mexico (2017-2021).

Year	Slaughter	Fattening	Breeding	Fairs	Total (year)
2017	10,700,053	55,526	1,875	36	10,757,490
2018	10,624,564	75,426	3,397	129	10,703,516
2019	10,062,205	38,760	2,653	0	10,103,618
2020	8,685,878	45,182	1,608	6	8,732,674
2021	8,282,506	45,882	2,428	0	8,330,816
Total (market)	48,355,206	260,776	11,961	171	48,628,114

Source: Own elaboration with SENASICA (2017-2021).

consumer value chain, often overlooking these critical aspects. The overconcentration of power in the hands of a few key actors can exacerbate information asymmetry, limit choices for producers and consumers, and create an imbalanced competitive landscape. Public policies should promote competition in the pork market, encourage the entry of new players, and diversify both distribution and sales channels. A highly concentrated market structure can generate distorted price signals, ultimately influencing producers' economic and financial decision-making in future market conditions (Martínez-Castañeda *et al.*, 2024).

Interstate network of pig mobilizations for purposes of slaughter

The State of Mexico's trail network exhibited a network centralization of 3.7% for outbound links and 11.8% for inbound links (Figure 2A). The distribution of pig mobilization was found to be highly polarized, as a small number of entities maintained a disproportionately high number of connections. This pattern reflects a star-shaped network structure, in which certain states function as major hubs, concentrating trade flows and influencing market dynamics. Several source entities, such as Mexico City, Guerrero, and Oaxaca, as well as peripheral destination entities, including Coahuila, Nayarit, Nuevo León, Sinaloa, Sonora, Tamaulipas, Yucatán, and Zacatecas, were identified as being at a disadvantage in terms of negotiating power and access to information. This asymmetry in market participation underscores the need for public policies that enhance transparency in the pig-farming sector. Ensuring clarity in business contracts and agreements is essential to leveling the playing field, allowing both producer and consumer states to access comprehensive and reliable information regarding transaction terms and market prices.

Interstate network of pig mobilizations for purposes of fattening

We recorded 1,327 entry mobilizations, with the State of Puebla leading in connectivity, registering 265 connections, followed by Morelos with 206 connections and Michoacán with 197 connections. Conversely, as a source state, the State of Mexico maintained only 182 business links, with its primary purchasers being Hidalgo (45 links) and Tlaxcala (44 links). The mean number of entry and exit connections was 79.42, with the State of Mexico and Puebla emerging as the most highly connected states, highlighting their strategic importance within the network. Network centralization was 4.10% for exit links and 60% for entry links, indicating a decentralized trading structure for source states, whereas destination-state trading relationships were more centralized, with the State of Mexico serving as the central node in the network (Figure 2B). According to Santos-Barrios *et al.* (2021), despite the State of Mexico's strong involvement in pig farming, the number of pigs purchased for fattening exceeds those sold, creating information asymmetry for farmers and fostering a prevalence of informal production, particularly in rural areas and small-scale operations. Additionally, pig farmers face competitive disadvantages, as many are unfamiliar with the registration and compliance requirements established by SENASICA. The lack of access to reliable information, geographic challenges in reaching pig-farming zones, limited technical support, and financial constraints further hinder compliance with official regulations. Consequently, inspection and certification services remain scarce,

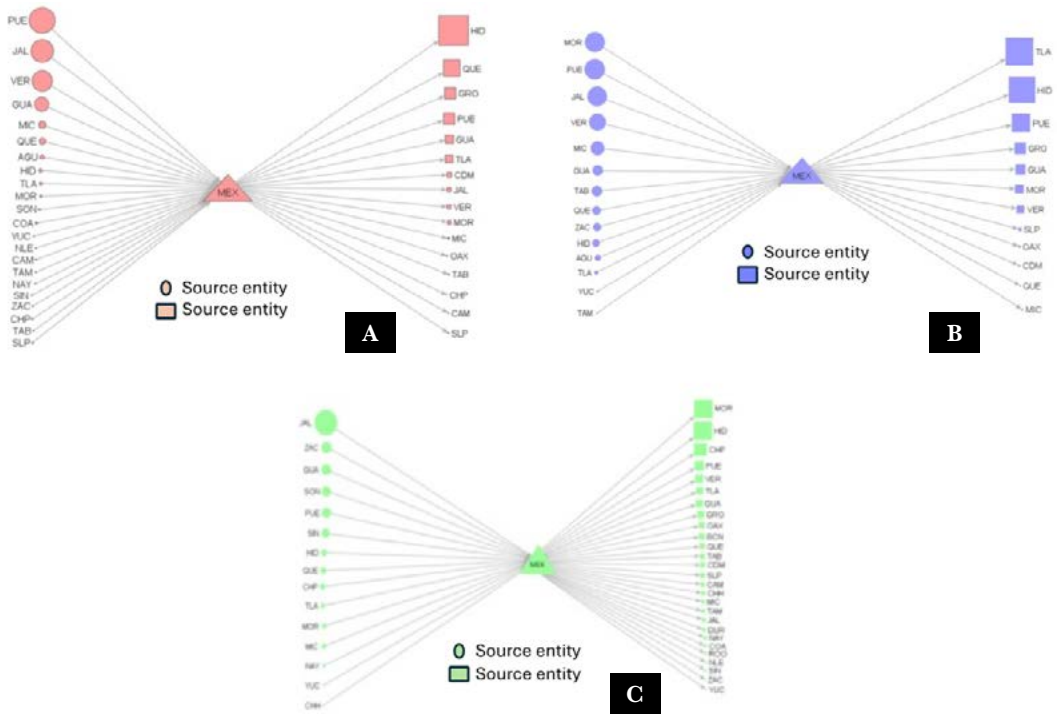

Figure 2. Pig-mobilization dynamics in the State of Mexico, 2017-2021.
A=slaughter; B=fattening; C=breeding.
BCN=Baja California Norte; BCS=BAJA California Sur; CDM=Mexico City; COL=Colima; DUR=Durango; GRO=Guerrero; ROO=Quintana Roo; YUC=Yucatán; CAM=Campeche; CHP=Chiapas; NAY=Nayarit; OAX=Oaxaca; TAB=Tabasco; CHH=Chihuahua; PUEB=Puebla; MIC=Michoacán; VER=Veracruz; SIN=Sinaloa; SON=Sonora; MEX=State of Mexico: ZAC=Zacatecas; GUA=Guanajuato; JAL=Jalisco; SLP=San Luis Potosi; AGU=Aguascalientes; TLA=Tlaxcala; QUE=Querétaro; NLE=Nuevo León; COA=Coahuila; MOR=Morelos; TAM=Tampico; HID=Hidalgo.

exacerbated by the perception that the pig-farming sector does not receive sufficient tangible benefits or incentives (Cuevas *et al.*, 2012).

Interstate network of pig mobilizations for purposes of breeding

The mean number of entry and exit trading connections in the State of Mexico was 41, with a total of 1,189 trading relationships, indicating a relatively balanced distribution within the network. The minimum and maximum numbers of relationships between source states were 0 and 563, respectively, while the minimum and maximum numbers of relationships between destination states were 0 and 626, respectively.

These results indicate that the degree of centralization of exit trading relationships was 7.02%, reflecting a moderate concentration within the network, while the degree of centralization of entrance trading relationships was similar, at 7.86%. The breeding-market network exhibited the highest number of trading relationships, with the State of Mexico participating in 563 trading relationships as a source state and 626 as a destination state. Jalisco also played a significant role, showing a strong source-state trend with 275 exit relationships, but a limited number of only 9 entrance connections. Meanwhile, Hidalgo had just 9 exit connections, yet was an active receiver of mobilizations, with 116 trading

relationships. Similarly, Morelos emerged as a major destination state, registering 65 destination relationships and only 5 source connections (Figure 2C). These findings are highly relevant, as they pertain to the commercial exchange of breeding stock and future breeders, which will eventually supply pork to the regions where this genetic material is distributed. In contrast, Mexico City and Veracruz, despite being geographically close, did not record any business transactions as source entities. However, both functioned as destination entities, with Mexico City registering 26 trading relationships and Veracruz 55. In the case of Mexico City, public policies discourage pig breeding due to its high population density, where municipal regulations either restrict or prohibit livestock production to mitigate issues related to noise, odors, public health, and animal welfare. Indeed, public-health and food-security policies should impose strict regulatory requirements on pig farming to ensure that pork products meet safety standards. Such regulations may include hygiene protocols, waste management systems, disease control measures, and sustainable production practices, which can be difficult to implement in an urban environment like Mexico City (Losada *et al*., 1982; Rivera *et al*., 2007). Similarly, in Veracruz, public policies also discourage pig breeding, though for environmental, sanitary, and economic reasons, as well as competition with other livestock industries and concerns related to tourism. Veracruz, characterized by significant environmental and ecological diversity, has policies designed to protect natural resources and fragile ecosystems. Consequently, environmental regulations may impose restrictions on pig farming to prevent water contamination, deforestation, and other ecological impacts associated with intensive production systems. Furthermore, Veracruz's economic and agricultural landscape prioritizes certain livestock activities over others for financial, environmental, and social considerations. The state is widely recognized for its diverse production systems, which include cattle and poultry farming, among others. Additionally, competition for essential resources, such as land, water, and feed, may influence producers to favor alternative livestock species over pig breeding. Lastly, Veracruz's status as a financial hub and a major tourist destination likely contributes to policy restrictions on activities that could negatively impact the environment and the region's appeal to visitors. Given the potential environmental and public-health concerns associated with intensive pig farming, this activity may not align with the broader public-policy objectives of the state (Gutiérrez *et al*., 2015).

Interstate network of pig mobilizations for purposes of fairs

The only available records for fairs pertain to events authorized and organized by government institutions. The average number of business relationships in this market was four per source state and destination state, with a total of 24 entry and exit links within the six-state network. Guanajuato was the most active source state, with 16 business relationships, yet it lacked entry connections, functioning primarily as a remitter rather than a receiver within the network. Meanwhile, the State of Mexico was the main destination entity, holding 18 entry business links, positioning itself as a central node in the fair-market network and establishing entry and exit links with other states involved in pig mobilization. The limited participation of State of Mexico producers in government-sponsored fairs is largely attributed to the fact that most are small-scale farmers who perceive the registration

process as overly bureaucratic and feel that they lack the necessary documentation to take part in such events. Some fairs require sanitary certification to verify that animals are free from contagious diseases and compliant with animal health regulations. Additional requirements may include vaccination records, veterinary treatment documentation, and official identification methods such as tags, microchips, or identification numbers. Moreover, certain fairs mandate enrollment fees or payments to cover event organization costs, while participants must ensure that their pigs meet optimal exhibition standards. Some events also require third-party liability insurance to cover any contingencies, along with strict registration deadlines and compliance with organizer-imposed conditions (SENASICA, 2017). Given these barriers, pork producers in the State of Mexico remain underrepresented, placing them at a competitive disadvantage compared to other states in the design and implementation of public policies. Consequently, their needs and interests are often overlooked when organizing fairs that facilitate commercial interactions. According to Rebollar *et al.* (2016), economic growth in the midwestern region, which includes the State of Mexico, has been slow due to a lack of support policies, inefficient resource utilization, and low productivity levels. Therefore, state-level public policies should integrate targeted strategies to mitigate information asymmetry between pig-producing states, government institutions, and pig-purchasing entities. These structural deficiencies lead to inadequate, outdated, inefficient, or overly restrictive regulations, hindering state participation in fairs and failing to support the development of the pork industry. Bureaucratic hurdles, limited access to resources and financial aid programs, and inadequate infrastructure —such as exhibition facilities, animal transportation means, and logistical support— further restrict the effectiveness and reach of these events (Puente, 2013).

Additionally, pig fairs pose a biosecurity risk, as they can facilitate the spread of animal diseases unless proper preventive policies are in place. Public policies regarding the market should also address broader concerns, including environmental and social sustainability, waste management, responsible resource usage, and labor rights within the pork industry. Despite its potential as a strategic platform for the State of Mexico to showcase and market its pigs to other states, the absence of comprehensive market-development policies and structured growth strategies significantly limits its economic impact and income-generation potential.

Key factors of the mobilization of live pigs in the State of Mexico

The mobilization of pigs in the State of Mexico revealed a network shaped by multiple factors that influence the dynamics of live animal transfers. The debate over whether these mobilizations are primarily driven by geographical proximity, economic factors, or consumer preferences must take into account several interrelated aspects. Geographic proximity plays a crucial role in pig mobilization, as evidenced by the fact that states closest to the State of Mexico, such as Puebla (130 km away) and Morelos (approximately 100 km away), are the main suppliers of pigs for slaughterhouses and fattening, respectively. The reduction in transportation costs and the mitigation of risks associated with long-distance movement, such as mortality and stress in animals, provide a competitive advantage to nearby states. This proximity enhances logistical efficiency, reduces operating costs, and

optimizes the delivery process, making short-distance suppliers more competitive in the market. Beyond geographic factors, the economic and productive capacity of each state significantly influences pig mobilization dynamics. For example, Jalisco, which accounts for 22% of national production, and Puebla, which contributes 11%, possess highly developed livestock infrastructure and substantial production capacity. This allows them to not only supply large volumes of pigs but also compete in broader markets, including the State of Mexico. Their financial strength and investment in livestock infrastructure enable them to offer competitive prices and secure long-term supply contracts, particularly with the State of Mexico, which, despite being a major consumer, faces a local production deficit. Consequently, it must rely on external suppliers to meet demand, reinforcing the importance of supplier states with strong production capacity that can absorb additional costs and market fluctuations.

Consumer preferences also play a role in pig mobilization, as residents of the State of Mexico may have specific preferences regarding meat type, quality, and origin, influencing supplier selection. However, given that 99.4% of pigs mobilized to the State of Mexico are destined for slaughter, consumer preferences likely hold less weight compared to the overriding need to meet processing and distribution demands. Additionally, a lack of information regarding pig prices, quality, and availability can result in inefficient decision-making and an overreliance on specific supplier states. To address this information asymmetry, public policies should promote transparency, enhance access to market data, and introduce incentives for a more balanced distribution of production and supply.

CONCLUSIONS

The State of Mexico maintains a negative trade balance across all four pig markets slaughter, fattening, breeding, and fairs with a high degree of centralization in the slaughter market, which has significant implications for competition and access to information.

The mobilization of pigs in the State of Mexico is shaped by a combination of geographic proximity, economic conditions, and, to a lesser extent, consumer preferences. Proximity plays a crucial role by reducing transportation costs and risks, while the economic capacity of supplier states ensures a reliable and competitive supply chain. However, several challenges persist, particularly the lack of reciprocity in certain trading relationships between the State of Mexico and other federal entities. The asymmetry in the number of mobilized pigs highlights the urgent need for public policies that promote the timely generation of and access to comprehensive information across the entire value chain.

The information asymmetry theory underscores the importance of ensuring equitable access to market information within the pig farming sector of the State of Mexico. Addressing information gaps is essential to ensuring food security, protecting public health, and fostering animal welfare. In this regard, public policies play a crucial role by enhancing transparency in the supply chain, establishing clear and effective regulations, and implementing robust oversight mechanisms. These policies should ensure that producer states comply with quality and animal-welfare standards through incentives that encourage good practices and sanctions for regulatory non-compliance, thereby aligning the interests of producer states within the pig markets. Moreover, continuous

training and education on best production practices, animal management, and animal health are fundamental to improving pork production quality and sustainability. The findings of this study emphasize the necessity of conducting targeted research on the four pork-production systems, thereby mitigating risks to the long-term sustainability of this vital protein source in the State of Mexico.

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Colegio de Postgraduados

Nopal mucilage and its synergistic-humectant effect in combination with different polyalcohols

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ABSTRACT

Objective: This study aimed to evaluate the *in vitro* wetting capacity of cactus mucilage (*Opuntia ficus-indica*) in combination with three wetting agents through permeability and water retention capacity tests, as well as its *in vivo* effects by measuring transepidermal water loss (TEWL) and hydration levels in volunteers. The goal was to determine the efficacy of this agent and its potential synergistic effects.

Design/Methodology/Scope: Nopal mucilage films (1.5% and 3%) were formulated using glycerin, sorbitol, and propylene glycol (1.5% and 3%). To assess their performance, water retention capacity tests were conducted at relative humidities of 40%, 60%, 80%, and 90%. TEWL was measured using a Tewameter® TM210 on a 4×4 cm area of each participant's forearm. Additionally, film characterization was performed using Scanning Electron Microscopy, and water vapor permeability was determined by calculating the permeability constant.

Results: The water vapor permeability test indicated that 3% of mucilage and 3% of sorbitol combination exhibited the lowest permeability constant, making it the most effective formulation. Furthermore, TEWL and stratum corneum hydration assessments confirmed that this combination demonstrated the highest moisturizing properties, as it showed the greatest hydration value and the lowest TEWL compared to the other formulations.

Conclusions/Limitations: The application of cactus mucilage emerges as a viable strategy to enhance the moisturizing effect in cosmetic product formulations.

Keywords: mucilage, nopal, humectant.

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INTRODUCTION

The skin is the largest organ of the human body, with a surface area of approximately 1.8 m^2, accounting for 16% of total body weight (Chambers & Vukmanovic-Stejic, 2020). It is composed of three primary layers: the epidermis, dermis, and subcutaneous tissue. The epidermis, the outermost layer, is responsible for maintaining skin moisture and consists of five sublayers: the *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum*, and *stratum corneum*. Among these, the stratum corneum plays a crucial role in the skin's water barrier function. This layer, measuring approximately 10-20 μm in thickness, is primarily composed of corneocytes (dead keratinocytes devoid of nuclei) and intercellular lipids, which are organized in a "brick-and-mortar" structure that regulates and minimizes

transepidermal water loss (TEWL) (Bouwstra *et al.*, 2021). The water content in the *stratum corneum*, commonly referred to as skin hydration, must remain above 10% for healthy skin, with an upper limit of 30% to prevent overhydration (Chambers & Vukmanovic-Stejic, 2020). One approach to maintaining optimal moisture levels in the epidermis is the use of cosmetic formulations containing moisturizing agents, which ensure an adequate balance between water and lipid content. These agents, known for their hygroscopic properties, enhance the water-retention capacity of *stratum corneum* cells (Pavlou *et al.*, 2021). Humectants are classified into three categories based on their composition: inorganic, metal-organic, and organic. Among these, organic humectants exhibit the highest moisturizing efficacy and are therefore the most widely used in cosmetic formulations. Examples include glycerol, sorbitol, and propylene glycol. Additionally, mucilages naturally occurring in various plants, seeds, and husks are known for their exceptional water-binding capacity due to their high concentration of hydroxyl groups in the polysaccharide chain (Tosif *et al.*, 2021; Prajapati *et al.*, 2013).

Nopal (*Opuntia ficus-indica*), a cactus species native to semi-arid and arid regions of Mexico, is a rich source of mucilage. The annual production of nopal reaches approximately 812 tons, with 70% consumed fresh. Nopal cladodes contain significant amounts of polysaccharides, including mucilage, cellulose, and hemicellulose, among others (García-Barradas *et al.*, 2023). This study aims to evaluate the in vitro humectant capacity of *Opuntia ficus-indica* mucilage in combination with organic humectant agents through permeability and water retention capacity tests, as well as its in vivo effects by assessing TEWL and skin hydration in volunteers. The objective is to determine the efficacy and potential synergistic effects of the formulation.

MATERIAL AND METHODS

Glycerol, sorbitol, and propylene glycol were purchased from Droguería Cosmopolita, Mexico. *Opuntia ficus-indica* mucilage was freeze-dried at the Facultad de Estudios Superiores Cuautitlán and donated by Dr. Elsa Gutiérrez Aburto. NaOH was obtained from Sigma-Aldrich Corporation, St. Louis, MO, USA.

The evaluated formulations are presented in Table 1.

Water retention assay

This assay assesses the percentage of weight gained by the formulations when exposed to different relative humidity (RH) levels. Four desiccators were conditioned at 40%, 60%, 80%, and 90% RH using glycerol and NaCl solutions. A total of 10 mL of each formulation

Table 1. Evaluated Formulations of Mucilage-Organic Humectants.

Mucilage	Humectants								
	Sorbitol			Glycerol			Propylene Glycol		
	0%	1.5%	3%	0%	1.5%	3%	0%	1.5%	3%
0%		x	x		X	x		X	x
1.5%	x	x	x		X	x		X	x
3%	x	x	x		x	x		X	x

(Table 1) was placed in a 15 mL beaker. The weight gain of each sample was recorded at 2, 4, 6, and 8 hours from the start of the test, followed by measurements every 24 hours until a constant weight was achieved. Each test was conducted four times.

Transepidermal water loss assay (TEWL)

The effects on TEWL were measured using a TEWAmeter™ TM 210 (Courage + Khazaka Electronic GmbH, Cologne, Germany). Ten volunteers aged between 20 and 50 years, with healthy skin and no application of cosmetic treatments on the tested area for one week, were selected for this evaluation. The results were expressed in $g/h/mm^2$. Two 4×4 cm areas were marked on each volunteer's arm. In quadrant 1, 5 mL of each formulation (Table 1) was applied and left to rest for 30 minutes, while quadrant 2 served as a reference for basal TEWL measurements. After the resting period, excess formulation was removed. Each test was performed in triplicate.

Skin hydration

Skin hydration measurements were conducted using a Corneometer® CM 825 (Courage + Khazaka Electronic GmbH, Cologne, Germany) mounted on a Derma Unit SSC3 (Courage + Khazaka Electronic GmbH, Cologne, Germany). This device measures the electrical capacitance of the skin surface, thereby indirectly assessing skin hydration, as electrical capacitance is dependent on water content (Vater *et al.*, 2021). All experiments were performed in triplicate.

Scanning Electron Microscopy (SEM)

The morphological analysis of the films formed by mucilage-organic humectant formulations was conducted using a JEOL scanning electron microscope (Model JSU-5600) at a resolution of 5 nm, with accelerating voltages of 10 and 15 kV, and a chamber pressure of 12-20 Pa (García-Betanzos *et al.*, 2016).

Water Vapor Permeability (WVP)

Water vapor permeability was determined at 25 ± 2 °C following the ASTM E96/E96M-05 method (ASTM, 2005a, b) with slight modifications. Glass vials were filled with 8 mL of deionized water (100% RH), covered with the conditioned films, sealed, and placed in a chamber with water (0% RH). Weight changes in the vials were recorded three times until the samples lost 10% of their initial weight. Determinations were performed in triplicate for each batch of film.

RESULTS AND DISCUSSION

Water retention assay

The water retention capacity test, shown in figure 1a, indicates that at the lowest relative humidity levels, mucilage at 1.5% and 3% behaves similarly to glycerin (1.5% and 3%) and sorbitol (1.5%). This graph also reveals that propylene glycol exhibits the lowest wetting capacity under low relative humidity conditions. This phenomenon occurs because, in low-humidity environments, moisturizing agents can absorb water from the epidermis

and dermis, leading to increased skin dryness (Wolff, 2003). At 60% RH (Figure 1b), mucilage, propylene glycol, sorbitol, and glycerin at 3% exhibit a comparable effect, with no significant differences observed, indicating that all three wetting agents and mucilage have a similar water retention capacity. However, at this relative humidity, glycerin at 1.5% demonstrates the highest water retention compared to the other humectants. In Figure 1c (80% RH), mucilage at 3% displays the lowest water retention, whereas glycerin, at both tested concentrations, exhibits the highest retention capacity. The remaining substances show no significant variations. Finally, at 90% RH (Figure 1d), glycerin at 1.5% continues to demonstrate the highest humectant capacity, while mucilage and propylene glycol at 1.5% exhibit lower water retention.

Nopal mucilage has greater moisturizing capacity at low relative humidity, explained since mucilage is a component whose main function is water retention in nopal.

Transepidermal water loss assay (TEWL)

TEWL measurement is a fundamental parameter for assessing the integrity of the stratum corneum and is considered a powerful non-invasive method to evaluate the effects of chemical compounds on the epidermal barrier function (Sotoodian & Maibach, 2012). As shown in Figure 2, the mean TEWL values for the mucilage 3% sorbitol 3% and mucilage 3% propylene glycol 3% systems exhibit statistically significant differences compared to the other formulations. This finding supports previous observations, as the mucilage 3%-propylene glycol 3% system presented the highest TEWL value, likely due to the drying effect of propylene glycol. Conversely, the mucilage 3% sorbitol 3% system demonstrated the greatest humectant effect, as evidenced by the lowest TEWL value.

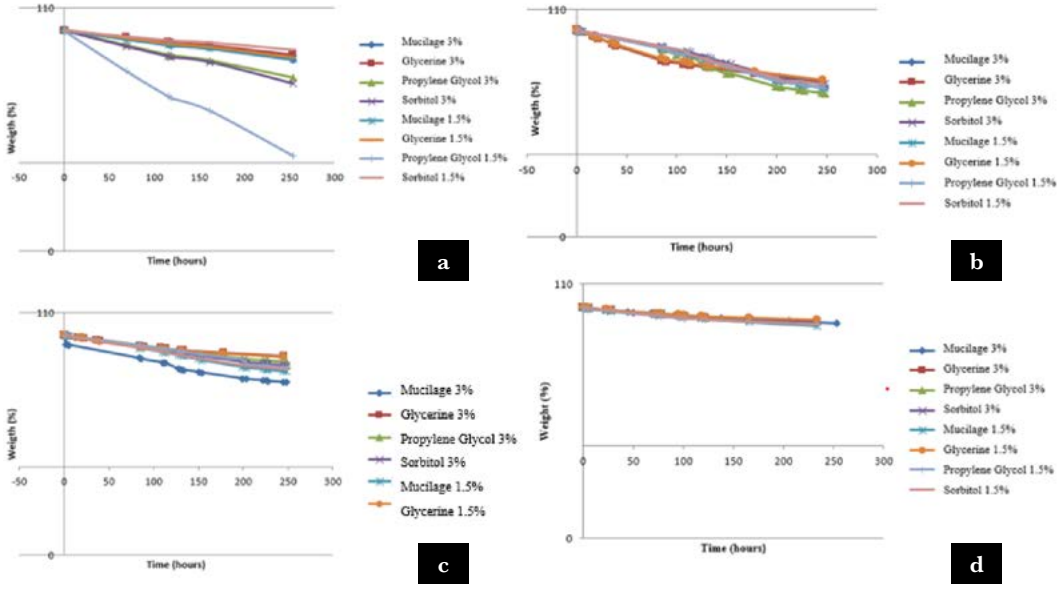

Figure 1. Water retention capacity over time for mucilage, sorbitol, glycerin, and propylene glycol at 1.5% and 3% under different relative humidity conditions: (a) 40% RH, (b) 60% RH, (c) 80% RH, and (d) 90% RH.

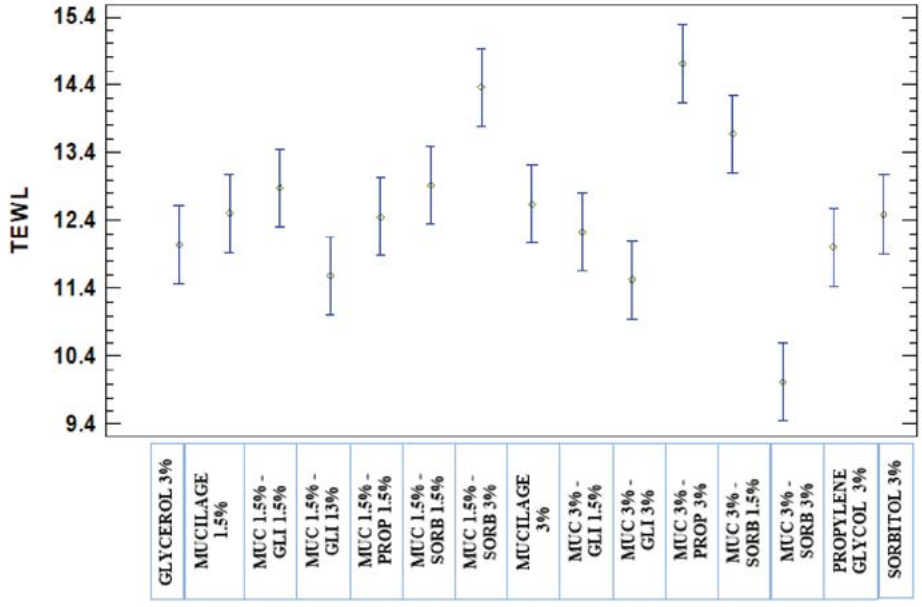

Figure 2. Least Significant Difference (LSD) plot of mucilage humectant systems for TEWL.

Skin hydration

As shown in Figure 3, the mean hydration values for the mucilage 3% sorbitol 3%, sorbitol 1.5%, and sorbitol 3% systems exhibit statistically significant differences compared to the other formulations. The mucilage 3% propylene glycol 3% and mucilage 3% propylene glycol 1.5% systems displayed the lowest hydration values, likely due to the drying effect of propylene glycol. Conversely, the mucilage 3% sorbitol 3% system demonstrated the highest hydration value, confirming its superior humectant effect. Regarding the water content in the *stratum corneum*, the mucilage 3% sorbitol 3% (Figure 3) and mucilage 3% glycerin 1.5%

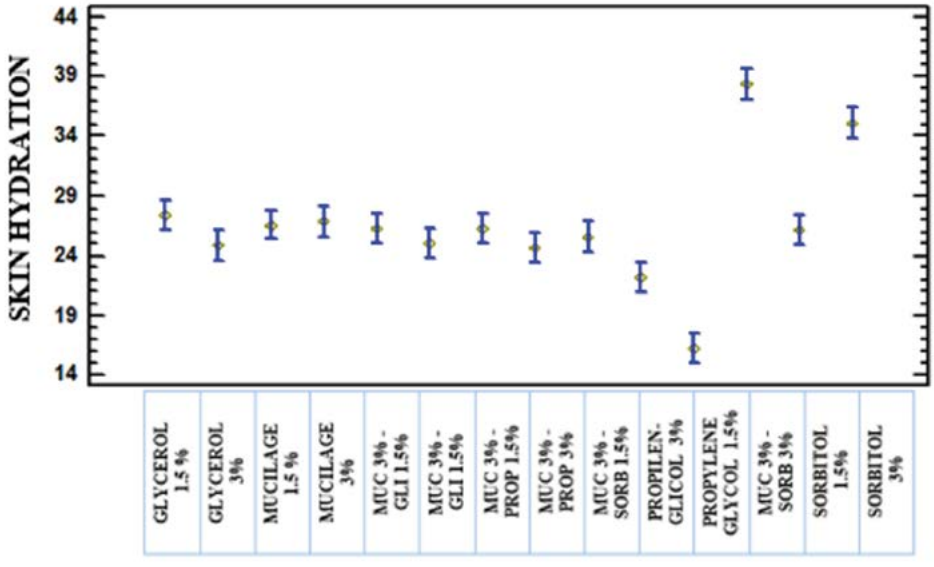

Figure 3. Least Significant Difference (LSD) graph of mucilage-humectant systems for hydration.

mixtures were the most effective, maintaining a level of hydration twice as high as that achieved by the humectants individually. The combination of nopal mucilage with sorbitol and glycerin forms an occlusive substance, leading to the formation of a film on the skin's surface that reduces transepidermal water loss and enhances *stratum corneum* hydration.

In this manner, the water retention capacity of the stratum corneum is enhanced. Additionally, it is well established that glycerol, sorbitol, mannitol, sucrose, propylene glycol, and polyethylene glycol act as plasticizers, facilitating the formation of the mucilage film in combination with the wetting agent (Olawuyi, 2021).

Scanning Electron Microscopy (SEM)

The films containing sorbitol at both concentrations (1.5% and 3%) exhibited a continuous structure, as did the film obtained with 1.5% glycerin. These films were flexible to the touch, in contrast to the one formed with 3% propylene glycol, which developed cracks and exhibited slight brittleness. Notably, the film produced with 3% glycerin was extremely thin and highly adhesive, making it difficult to handle.

Figure 4 presents micrographs of representative fields of the films formed by the mucilage-humectant mixtures. Polysaccharides, such as cactus mucilage, are effective film formers; however, they tend to be fragile and brittle unless a plasticizer is incorporated (Ghanbarzadeh, 2007). The humectants propylene glycol, sorbitol, and glycerin serve this function, as they are low-molecular-weight organic compounds that reduce intermolecular forces between the polymer chains of the mucilage. This interaction decreases tensile strength while enhancing the flexibility of the films (Pastor, 2010).

The film with the best characteristics was the 3% mucilage–3% sorbitol formulation (Figure 4d), as it exhibited the most uniform and continuous structure, followed by

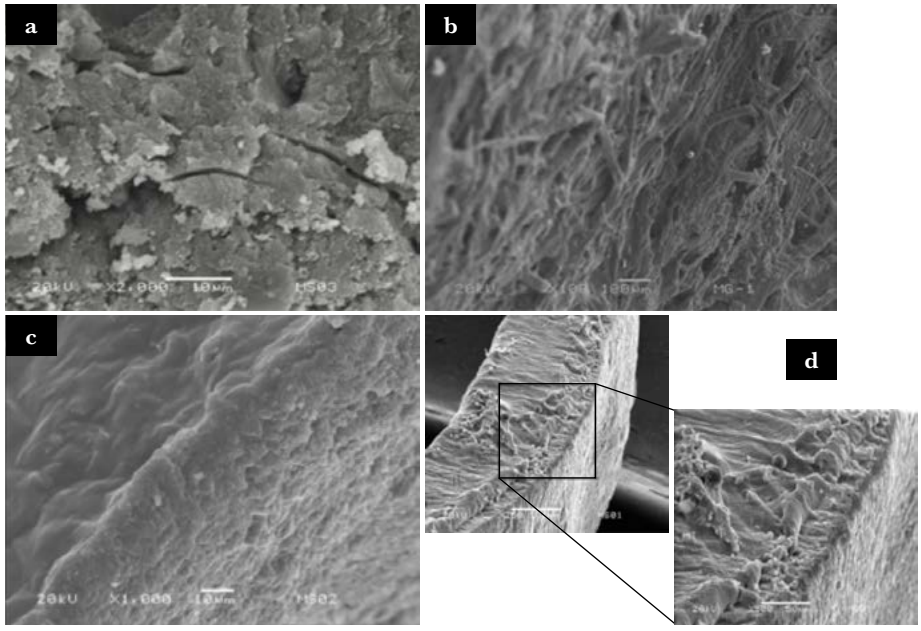

Figure 4. Micrographs of the films obtained: (a) 3% mucilage 3% propylene glycol, (b) 3% mucilage 1.5% glycerin, (c) 3% mucilage-1.5% sorbitol, (d) 3% mucilage 3% sorbitol.

the 3% mucilage-1.5% sorbitol and 3% mucilage-1.5% glycerin films (Figure 4). This evident continuity is attributed to the ability of plasticizer molecules to bind with water molecules, protecting the active centers along the polymer chains and thereby reducing intermolecular distances (Koc-Bilican, 2024). In contrast, the 3% mucilage-3% propylene glycol film presented a particular case, as its micrograph revealed structural gaps and surface particles, suggesting incomplete incorporation of the plasticizer (Ghanbarzadeh *et al*., 2006). Consequently, this formulation failed to produce a continuous film.

Water Vapor Permeability (WVP)

The table below presents the permeability constant values obtained for the mucilage systems formulated with different humectants.

Table 2. Permeability constants for mucilage-humectant systems.

System	Water Vapor Permeability ($*10^{-7}$) (mg mm/mm^2 h Pa)
mucilage 3% - sorbitol 3%	2.606
mucilage 3% - sorbitol 1.5%	3.351
mucilage 3% - glycerine 3%	4.730
mucilage 3% - glycerine 1.5%	3.424
mucilage 3% - Propylene glycol 3%	3.520
Control	5.791

Since the permeability constant represents water vapor permeability, a higher constant indicates greater water loss from the system to the external environment. According to Table 2, the 3% mucilage-3% sorbitol system exhibited the lowest permeability constant, making it the most effective film. This can be attributed to the molecular structure of sorbitol, which contains a higher number of hydroxyl groups compared to glycerol and propylene glycol. This increased hydroxyl content enhances interactions with the carboxyl groups of mucilage, reducing the mobility between mucilage chains (Mannai *et al*., 2023). As a result, a more uniform film is formed, which effectively minimizes water vapor permeability. When sorbitol is used at 1.5%, an increase in the permeability constant is observed, likely because this concentration is insufficient to produce a film with higher resistance. The 3% mucilage-1.5% glycerin and 3% mucilage-3% propylene glycol systems exhibit higher permeability constants, as these plasticizers are more hydrophilic than sorbitol, leading to greater water permeability (Koc-Bilican, 2024).

CONCLUSIONS

The water retention capacity of mucilage in combination with glycerin, sorbitol, and propylene glycol was evaluated, with the glycerin and sorbitol mixtures demonstrating the highest retention capacity. The water vapor permeability test confirmed that the 3% mucilage-3% sorbitol mixture, exhibiting the lowest permeability constant, was the most effective formulation. TEWL and stratum corneum hydration assessments further reinforced that the 3% mucilage-3% sorbitol system possesses the greatest moisturizing

properties, as it exhibited the highest hydration value and the lowest TEWL compared to the other formulations. The combination of cactus mucilage with conventional moisturizing agents presents a promising strategy for developing various cosmetic and dermatological formulations, including solutions, lotions, creams, suspensions, masks, and scrubs.

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Colegio de Postgraduados

In vitro anthelmintic effect of Malvaceae family plants in sheep and goat parasites: Review

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ABSTRACT

Objective: To analyze scientific database information on the *in vitro* anthelmintic effects of plant species from the Malvaceae family in sheep and goats.

Materials and Methods: A selection of articles was retrieved from the following databases: Biblat, Google Scholar, Reaxys, ScienceDirect, Scopus, and Springer. The inclusion criteria encompassed original studies published between 2002 and 2022 that investigated Malvaceae species, assessing their *in vitro* anthelmintic effects on parasites at any developmental stage in sheep and goats. Articles published in English, Spanish, and Portuguese were considered.

Results: A total of 4,020 results were identified. After abstract screening, 13 articles meeting the inclusion criteria were selected, highlighting the relevance of 10 plant species within the Malvaceae family, with particular emphasis on *Abutilon* spp. (shrubs) and *Guazuma ulmifolia* (tree). The most significant findings indicate that seven plant species exhibit effects on *Haemonchus contortus*, three on *Trichostrongylus* spp., two on *Oesophagostomum* spp., and one species each on *Moniezia expansa*, *Teladorsagia* spp., *Cooperia* spp., and *Nematodirus* spp.

Implications: Ovicidal activity has been reported with methanolic, aqueous, and hexanic extracts of *Abutilon theophrasti* against *H. contortus*, as well as with aqueous extracts of *Urena lobata* leaves, which demonstrated over 70% inhibition of *H. contortus* egg hatching.

Conclusions: Plant species within the Malvaceae family exhibit anthelmintic properties and could serve as a valuable tool in the integrated parasite control strategies for small ruminants.

Keywords: Plant extracts; animal helminthiasis; parasite management; secondary metabolites; alternative methods; small ruminants

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INTRODUCTION

The production of sheep and goats is one of the most significant agricultural activities in several Latin American countries, providing economic security that other sectors often cannot offer. Additionally, it contributes to the supply of meat and milk

from these species (Hernández-Valenzuela *et al.*, 2019). However, small ruminants face multiple challenges, particularly in nutrition, reproduction, and health. Among these, gastrointestinal parasites (PGI) represent the primary health concern, significantly reducing productivity in grazing systems (Cruz-Tamayo *et al.*, 2020). The major PGI classes affecting sheep and goats flukes, cestodes, nematodes, and protozoa are responsible for substantial economic losses (Kumsa & Hagos, 2020; Rodríguez-Vivas *et al.*, 2017; Starling *et al.*, 2019; Ilangopathy *et al.*, 2019). Numerous studies highlight the high prevalence and pathogenicity of many parasite species (Arsenopoulos *et al.*, 2021; Cobon & Osullivan, 1992; López *et al.*, 2013), particularly in tropical regions where favorable climatic conditions enhance their development. This includes countries such as Mexico (Rodríguez-Vivas *et al.*, 2017) and Brazil (Starling *et al.*, 2019), where both economic and health risks associated with parasitic infections have been documented. Among these parasites, *Haemonchus contortus* is the most economically significant due to its global distribution and high pathogenicity in small ruminants (Arsenopoulos *et al.*, 2021; Cobon & Osullivan, 1992; López *et al.*, 2013; Besier *et al.*, 2016). This hematophagous nematode colonizes the abomasum, causing severe blood loss, hemorrhagic gastritis, and anemia, often leading to high mortality rates, particularly among lambs (López *et al.*, 2013; Besier *et al.*, 2016). Other nematodes of veterinary importance include *Trichostrongylus colubriformis*, *Cooperia curticei*, *Oesophagostomum columbianum*, *Trichuris* spp., and *Strongyloides papillosus*, among others (López *et al.*, 2013). PGI control is traditionally managed through anthelmintic drugs; however, their long-term effectiveness is compromised by the increasing resistance of parasites, reducing treatment efficacy (Claerebout *et al.*, 2020; Hodgkinson *et al.*, 2019; Santiago-Figueroa *et al.*, 2019). Consequently, alternative strategies for parasite control have been developed, including plant-based bioactive compounds as a sustainable approach to reducing dependence on synthetic anthelmintics (García-Hernández *et al.*, 2017; Medina *et al.*, 2014). The Malvaceae family includes economically important plant species such as cocoa, cotton, and durian, widely utilized in the textile and food industries (Wang *et al.*, 2020). Furthermore, dietary inclusion of Malvaceae species in small ruminant feed has demonstrated positive effects on animal productivity (Mayren-Mendoza *et al.*, 2018; Valdivié & Martínez, 2022). The family comprises 243 genera and 4,225 species distributed worldwide (Vadivel *et al.*, 2016). Among its secondary metabolites with therapeutic potential are thylyroside, lespedin, rutin, myricetin, quercetin, and apigenin, recognized for their antioxidant properties, as well as taraxerol, known for its anti-inflammatory effects (Fernandes de Oliveira *et al.*, 2012; Khanra *et al.*, 2015). Additionally, Calixto *et al.* (2016) identified bioactive compounds such as chlorogenic acid, rutin, and luteolin in ethanolic extracts of *Guazuma ulmifolia* leaves, which have demonstrated antiparasitic activity in previous studies on other plant species (Liu *et al.*, 2020). A selection of the most representative species is presented in Table 1.

The aim of this study is to conduct a comprehensive review of the key findings from *in vitro* studies on plant extracts from the Malvaceae family, evaluating their potential for controlling helminths in small ruminants.

Table 1. Main species of Malvaceae family.

Generus	Scientific name
Abelmoschus	*Abelmoschus moschatus, A. esculentus, A. Manihot, A. esquirolii, A. mindanaensis*
Abroma	*Abroma augustum, A. alata, A. angulata, A. angulosa, A. augusta, A. communis, A. denticulata, A. elongata, A. fastuosa, A. javanica, A. mariae, A. mollis, A. nítida, A. obliqua, A. sinuosa, A. tomentosa, A. wheleri*
Abutilon	*Abutilon albescens, A. auritum, A. bedfordianum, A. berlandieri, A. californicum, A. darwinii, A. eremitopetalum, A. fruticosum, A. hirtum, A. hulseanum, A. hyoleucum, A. incanum, A. indicum, A. pakistanicum, A. grandiflorum, A. theophrasti, A. insigne, A. leonardi, A. mollicomum, A. mollissimum, A. niveum, A. malacum, A. megapotamicum, A. menziesii, A. ochsenii, A. palmeri, A. pannosum, A. parishii, A. parvulum, A. permolle, A. pictum, A. purpurascens, A. reventum, A. sachetianum, A. sandwicense, A. thurberi, A. thyrsodendron, A. trisulcatum, A. venosum, A. virginianum, A. vitifolium wrightii*
Bombax	*Bombax albidum, B. anceps, B. blancoanum, B. buonopozense, B. ceiba, B. costatum, B. insigne*
Duboscia	*Duboscia acuminata, D. brieyi, D. macrocarpa, D. polyantha, D. viridiflora*
Guazuma	*Guazuma crinita, G. invira, G. iuvira, G. longipedicellata, G. ulmifolia*
Hibiscus	*Hibiscus sabdariffa, H. cannabinus, H. rosa-sinensis, H. syriacus, H. trionum*
Helicteres	*Helicteres lhotzkyana, H. longepedunculata, H. macropetala, H. macrothrix, H. microcarpa, H. muscosa, H. nipensis, H. ovata, H. pentandra, H. pilgeri, H. pintonis, H. plebeja*
Herissantia	*Herissantia crispa, H. dressleri, H. nemoralis, H. tiubae, H. trichoda*
Lavatera	*Lavatera bryoniifolia, L. cachemiriana, L. cashemiriana, L. flava, L. oblongifolia, L. trimestris, L. thuringiaca, L. olbia, L. punctata, L. triloba*
Malva	*Malva alcea, M. pánica, M. borealis, M. parviflora, M. aegyptia, M. sylvestris* L. *crispa, M. coromandelianum, M. cretica, M. erecta his, M. iljini, M. ilindsayi, M. multiflora, M. neglecta, M. arborea, M. nicaeensis, M. cathayensis, M. occidentalis*
Pavonia	*Pavonia xanthogloea, P. multiflora, P. spinifex, P. arechavaletana, P. intermedia, P. hastata*
Sida	*Sida acuta, S. antillensis, S. cardiophylla, S. carpinifolia, S. ciliaris, S. cleisocalyx, S. clementii, S. cryphiopetala, S. cordifolia, S. rhombifolia, S. hermaphrodita, S. tuberculata, S. echinocarpa, S. fallax, S. hederifolia, S. intricata, S. kingii, S. hermaphrodita, S. nesogena, S. phaeotricha, S. physocalyx*
Theobroma	*Theobroma angustifolium, T. bicolor, T. cacao, T. glaucum, T. grandiflorum, T. leiocarpa, T. mammosum, T. mariae, T. martiana, T. microcarpus, T. obovatum, T. pentagona*
Urena	*Urena lobata, U. sinuata*

Modified from The World Flora Online, WFO (2024).

METHODOLOGY

Data source and search strategy. We conducted a search for scientific articles on the use of plant species from the Malvaceae family for parasite treatment in sheep and goats. The information was retrieved from the following digital databases: Biblat, Google Scholar, Reaxys, ScienceDirect, Scopus, and Springer, considering original articles published between 2002 and 2022. The search terms used in each database were: (a) Biblat: Malvaceae, sheep, and goats; (b) Google Scholar: Malvaceae, anthelmintic, sheep, or goat; (c) Reaxys: Malvaceae, anthelmintic; (d) ScienceDirect: Malvaceae, anthelmintic; (e) Scopus: Malvaceae, anthelmintic, sheep, or goat; and (f) Springer: Malvaceae, anthelmintic, sheep, or goat. The abstracts of the retrieved articles were analyzed, and

those meeting the inclusion criteria were selected for further evaluation. Additionally, a search was conducted for the 243 genera within the Malvaceae family.

Inclusion criteria. Only original papers published in English, Spanish, or Portuguese, focusing on plants from the Malvaceae family and related to the *in vitro* evaluation of bioactive metabolite extracts, were included. The studies considered involved internal parasites of sheep or goats, employing various techniques to assess the effectiveness of plant-derived products at different stages of parasite development. The article selection process is illustrated in Figure 1.

The abstracts of the retrieved articles were analyzed, and those meeting the inclusion criteria were selected for evaluation. Additionally, a search was conducted for the 243 genera within the Malvaceae family. The information from the selected articles was organized into Excel sheets, identifying the author, country, year of publication, and plant species evaluated. Furthermore, the parasite involved, life cycle stages, and the technique used for the evaluation were recorded. A total of 4,022 documents were obtained: 2,800 from Google Scholar, 1,089 from Biblat, 85 from Springer, 44 from Reaxys, 3 from Scopus, and 2 from ScienceDirect. After analyzing the abstracts, eleven articles met the inclusion criteria and were selected as the basis for this study.

RESULTS

Table 2 presents the reported in vitro evaluations of plant species from the Malvaceae family against gastrointestinal parasites of small ruminants, along with the techniques used for assessment.

Table 3. Presents the species of the Malvaceae family evaluated *in vitro* against gastroenteric nematodes of sheep and goats, the types of extracts developed, and the secondary metabolites reported.

DISCUSSION

India and Mexico have conducted the highest number of *in vitro* experimental trials on plants from the Malvaceae family, primarily between 2018 and 2022. These studies have focused mainly on the gastrointestinal nematodes *Haemonchus* spp., *Trichostrongylus* spp., and *Oesophagostomum* spp. Within this plant family, the most extensively studied species for

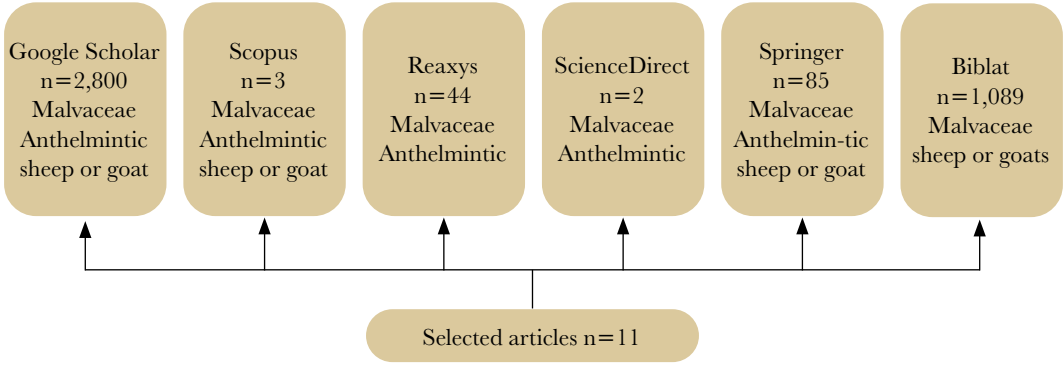


Figure 1. Characteristics of the selected scientific articles included in this analysis.

Table 2. *In vitro* evaluation reports on the effectiveness of Malvaceae species in controlling gastrointestinal nematodes in small ruminants.

Country	Animal	Parasite	Technique	Reference
Slovakia	Ovine	*Haemonchus contortus*	IEH - DL	Váradyová *et al.* (2018)
France	Goat	*Haemonchus contortus, Trichostrongylus colubriformis*	Larval sheath inhibition	Quijada *et al.* (2015)
India	Ovine	*Moniezia expansa*	Motility inhibition - ML	Thooyavan *et al.* (2018)
India	Ovine	*Haemonchus contortus*	IEH - ML	Hassan *et al.* (2019)
Indonesia	Ovine	*Haemonchus contortus*	IEH - DL - Adult motility	Suteky & Dwatmadji (2015)
Mexico	Ovine	*Haemonchus* sp. *Oesophagostonum* sp. *Trichostrongylus* sp. *Cooperia* sp. *Nematodirus* sp.	IEH	Antonio-Irineo *et al.* (2021)
Mexico	Ovine	*Haemonchus contortus*	ML	García-Arce (2019)
Mexico	Ovine	*Haemonchus contortus*	IEH - ML	Reséndiz-González *et al.* (2022)
Pakistan	Ovine	*Haemonchus contortus*	ML	Zia-Ul-Haq *et al.* (2012)
South Africa	Ovine	*Haemonchus* sp., *Oesophagostomum*, *Trichostrongylus Teladorsagia*	IEH - DL - ML	Molefe *et al.* (2013)
Ukraine	Goat	*Strongyloides papillosus*	ML	Boyko & Brygadyrenko (2021)

DL: larval development test, IEH: Egg hatching inhibition test, ML: Larval mortality test.

anthelmintic purposes in small ruminants over the past two decades are *Guazuma ulmifolia* and species of the genus *Abutilon* spp. (Table 3). Antonio-Irineo *et al.* (2021) conducted a preliminary study on the *in vitro* efficacy of aqueous extracts from the leaves of four plant species at three different concentrations (0.75, 1.0, and 1.25 mL), including *Guazuma ulmifolia*, a tree widely distributed in the Americas, particularly in Brazil and Mexico. Their findings indicated that the aqueous extract of *G. ulmifolia* exhibited egg hatching inhibition (IEH) of 62.4%, 59.8%, and 22% at 1.25, 1.0, and 0.75 mL, respectively. Various secondary compounds, such as tannins, flavonoids, saponins, mucilages, alkaloids, and terpenes, have been identified in the leaves and fruits of *G. ulmifolia* (Pereira *et al.*, 2019). Moreover, condensed and hydrolyzable tannins, along with certain phenolic acids, have been isolated from other plant families with known antiparasitic potential (Cortes-Morales *et al.*, 2022; Olmedo-Juárez *et al.*, 2017). A recent study by Reséndiz-González *et al.* (2022) demonstrated that a hydroalcoholic extract of *G. ulmifolia* possesses ovicidal properties against *Haemonchus contortus*. The authors identified key bioactive compounds, including kaempferol, ethyl ferulate, ethyl coumarate, flavonol, luteolin, ferulic acid, luteolin rhamnoside, apigenin rutinoside, coumaric acid derivatives, luteolin glycoside, and quercetin glycoside, which have been shown to disrupt the biological cycle of gastrointestinal nematodes. Regarding the genus *Abutilon* (*A. indicum* and *A. theophrasti*), the primary groups

Table 3. *In vitro* reports of Malvaceae on gastroenteric nematodes of sheep and goats.

Species	Common name	Extracrit	Concentration			Life Cycle Phase	Isolated Compounds	Reference
			*(mg/ml)	**(μg/mL)	***%			
Abutilon indicum	Indica Mallow	Methanolic	25, 50 and 100*			Adult	Alkaloids, Flavonoids, Tannins, Phenols, Terpenoids, Diterpenoids, Steroids and Cardiac Glycosides	Thooyavan G. *et al.* (2018)
Abutilon theophrasti	Velvet Sheet	Aqueous Hexanic Methanolic	500, 250, 125, 62.5, and 31.25*			Egg	-	Hassan *et al.* (2019)
Althaea officinalis L.	Marshmallow	Aqueous Methanolic		Methanolic	Aqueous	Egg, L 1 and L 3	Routine	Váradyová *et al.* (2018)
			IEH	1024, 256, 64, 16, 4, 1**	50, 25, 12.5, 6.25, 3.125*			
			DL	1365-0.66**	40-0.019			
Malva sylvestris L.	Common Mallow	Aqueous Methanolic		Methanolic	Aqueous	Egg, L 1 and L 3	Rutin, Gallic Acid, Quercetin, Kaempferol	Váradyová *et al.* (2018)
			IEH	1024, 256, 64, 16, 4, 1**	50, 25, 12.5, 6.25, 3.125*			
			DL	1365-0.66**	40-0.019			
Grewia asiatica	False	Methanolic	50, 25, 12.5, 6.25 and 3.12			L3	-	Zia-Ul-Haq *et al.* (2012)
Guazuma ulmifolia	Guácima	Aqueous	0.75, 1.00 and 1.25*			Egg	Phenols, Condensed Tannins and Total Tannins	Antonio-Irineo *et al.* (2021)
G. ulmifolia	Guácima	Hydroalcoholic	10, 5, 2.5 and 1.25			Egg & L3	Kaempferol, Ethylferulate, Ethylcoumarate, Flavonol, Luteolin, Ferulic Acid, Luteolin Rhamnoside, Apigenin Rutinoside, Coumaric Acid Derivatives, Luteolin Glycoside, and Quercetin	Reséndiz-González *et al.* (2022)
Hermannia depressa	-	Aqueous Acetonic	2.5, 5.0 and 7.5*			L1, L2 and L3	-	Molefe *et al.* (2013)
Thuringiac lavatera	Royal Mallow	Aqueous extract	3.0, 0.75 and 0.19***			L 1 and L 2	-	Boyko & Brygadyrenko (2021)
Theobroma Cacao	Cocoa	Acetone-water 70:30	600, 300, 150, 75 and 37.5**			L 3	Tannins	Quijada *et al.* (2015)
Urena lobata	Guaxima from Brazil	Aqueous	3.125, 6.25, 12.5, 25 and 50*			Egg, L 3	Alkaloids,Flavonoids, Tannins, Coumarins, Saponins, Triterpenoids,, Mangiferin, Quercetin	Suteky & Dwatmadji (2015)
American Waltheria	White Brush	Methanol, Hexanic Dichloromethane	50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78*			L3, L4	-	Garcia-Arce (2019)

IEH Inhibition of Egg Hatching, DL Larval Development Test, L1 first stage larvae, L2 second stage larvae, L3 third stage larvae.

of secondary metabolites identified include alkaloids, flavonoids, catechins, anthocyanidins, sterols, vitamins, sugars, tannins, phenols, terpenoids, diterpenoids, steroids, and cardiac glycosides (Thooyavan *et al.*, 2018; Hassan *et al.*, 2019). The methanolic extract of *Abutilon indicum* demonstrated a concentration-dependent effect on *Moniezia expansa*, influencing both the time of paralysis and mortality of the parasite. This effect may be attributed to the presence of phenolic compounds and alkaloids in the extract (Thooyavan *et al.*, 2018). Similarly, *Abutilon theophrasti* exhibited *in vitro* antiparasitic effects depending on the concentration of methanolic, aqueous, and hexane extracts in both IEH and larval mortality (ML) assays. Among these, the methanolic extract showed the highest efficacy at 500 mg/mL, achieving 74.39% IEH and 79.79% ML (Hassan *et al.*, 2019). Additionally, *Grewia asiatica* demonstrated antiparasitic potential, with its methanolic extract achieving an LC50 of 17.21 mg/mL in ML tests against *H. contortus* (Zia-Ul-Haq *et al.*, 2012).

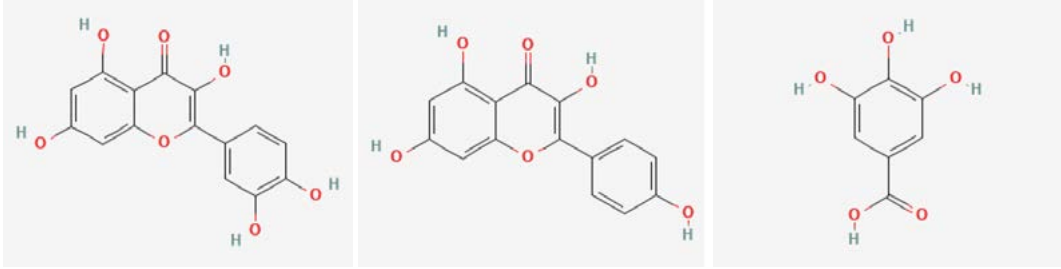
For *Hermannia depressa*, an aqueous extract at 7.5 mg/mL achieved 40.14% IEH, whereas the acetone extract resulted in only 7.4% inhibition. However, in larval development inhibition assays, the acetone extract demonstrated 100% efficacy at all tested concentrations, while the aqueous extract showed the highest inhibition at 7.5 mg/mL, reaching 66.69%. Regarding larval mortality, the aqueous extract at 7.5 mg/mL resulted in 100% mortality within 24 hours, with a clear concentration-dependent effect (Molefe *et al.*, 2013). For *Lavatera thuringiaca*, an aqueous leaf extract at a 3% concentration resulted in 97.4% ML of L1 and L2 larvae of *Strongyloides papillosus* (Boyko & Brygadyrenko, 2021). Additionally, phytochemicals such as alkaloids, cardiac glycosides, tannins, terpenoids, and saponins were identified in the leaves of *Urena lobata* (Suteky & Dwatmadji, 2015). The aqueous extract of *U. lobata* was tested for anthelmintic properties using IEH, motility, and larval development assays against *H. contortus*. The highest concentration (50 mg/mL) achieved 70.08% IEH and 57.8% ML (Suteky & Dwatmadji, 2015; Islam & Uddin, 2017). Váradyová *et al.* (2018) assessed the anthelmintic activity of 13 medicinal plants from Central Europe, including aqueous and methanolic extracts of *Althaea officinalis* roots and *Malva sylvestris* flowers, at concentrations ranging from 1 to 1,024 μg/mL against *H. contortus*. Aqueous extracts exhibited an IEH of 88.3% in *A. officinalis* and 40.4% in *M. sylvestris*. In ML assays, the methanolic extract of *M. sylvestris* had an LC50 of 53 μg/mL, while the aqueous extract had an LC50 of 90 μg/mL. For *A. officinalis*, the LC50 values were 157 μg/mL for the aqueous extract and 236 μg/mL for the methanolic extract. In Mexico, García-Arce (2019) evaluated extracts of varying polarity from the roots and leaves of *Waltheria americana* against *H. contortus* infective larvae. The highest ML percentages at 48 hours were observed with hexanic (root), methanolic (leaf), and dichloromethane (root) extracts, achieving 42.61%, 39.1%, and 30.25%, respectively. Additionally an acetone-water extract from *Theobroma cacao* seeds was tested against *H. contortus* and *Trichostrongylus colubriformis* infective larvae. This extract was fractionated to isolate condensed tannins, specifically prodelphinidins and procyanidins (Quijada *et al.*, 2015). The study demonstrated that prodelphinidins inhibited larval sheath development at lower concentrations compared to procyanidins. Based on the findings presented, it can be concluded that the anthelmintic efficacy of extracts from Malvaceae species varies depending on the extraction method, the concentration of secondary metabolites, and the specific bioactive compounds present.

Malvaceae Secondary metabolites. Secondary metabolites with proven anthelmintic effects in plants from other botanical families have been identified in species of the Malvaceae family (Castillo-Mitre *et al.*, 2017; von Son-de Fernex *et al.*, 2015; García-Hernández *et al.*, 2022). *Luehea paniculata* and *Guazuma ulmifolia* were evaluated using ethanolic extracts from leaves and bark, revealing the presence of flavonoids such as quercetin (Figure 2a), rutin, and kaempferol (Figure 2b), as well as gallic acid (Figure 2c), chlorogenic acid, and caffeic acid (Calixto *et al.*, 2016). Similarly, Tanaka *et al.* (2005) isolated the metabolite epicatechin from the stems and leaves of *L. divaricata*. In *Malva sylvestris* leaves, secondary metabolites such as gallic acid, rutin, quercetin, and kaempferol were identified, while *Althaea officinalis* leaves contained rutin (Váradyová *et al.*, 2018).

Similarly, the metabolite hydroxycinnamic acid was isolated from the aqueous extract of *Malva neglecta* leaves (Sharifi-Rad *et al.*, 2020), all of which have been reported to exhibit anthelmintic activity (Liu *et al.*, 2020). Research into the potential anthelmintic effects of plant species from the Malvaceae family could contribute to the development of alternative treatments, helping to reduce the prevalence of gastrointestinal parasites and their resistance to conventional drugs in small ruminants.

It is important to note that, out of the 4,225 plant species belonging to the Malvaceae family, only 10 species have been evaluated for their *in vitro* anthelmintic effects in small ruminants. Secondary metabolites are synthesized in limited amounts within plants and are often restricted to specific genera or botanical families, including certain plant species (Ávalos & Pérez-Urria, 2009; Hernández-Alvarado *et al.*, 2018). For this reason, the Malvaceae family presents new opportunities for research into the natural control of gastrointestinal nematodes in small ruminants.

Medicinal plants serve as an alternative source of bioactive compounds for the pharmaceutical industry. Ethnobotanical and ethnopharmacological knowledge provides an essential foundation for their identification and selection (Leitão *et al.*, 2014). Malvaceae species are widely used in traditional medicine due to their antibacterial, anti-inflammatory, antiviral, hepatoprotective, analgesic, expectorant, diuretic, and antioxidant properties, as well as their role in the treatment of urinary disorders, stomach ailments, and digestive pain (Ariharan & Revathi, 2021; Hamed *et al.*, 2014; Martínez & Jiménez-Escobar, 2017). However, the vast diversity of bioactive compounds within this botanical family presents significant research opportunities, as many species remain insufficiently studied regarding



Quercetin (A) Kaempferol (B) Gallic Acid (C)

Figure 2. Metabolites with anthelmintic effects isolated from species of the Malvaceae family. Adapted from PUBCHEM (National Center for Biotechnology, 2023).

their phytochemical composition, making them valuable targets for further investigation (Vadivel *et al.*, 2016).

Other Uses of Family Plants Malvaceae. Research has been conducted on the use of Malvaceae species in animal feed and their productive impact. Mayren-Mendoza *et al.* (2018) evaluated the effect of supplementing *Guazuma ulmifolia* foliage on the productive performance of Pelibuey sheep, reporting increased dry matter intake, weight gain (0.50 kg per animal, $p \geq 0.05$), and feed efficiency (0.02, $p \geq 0.05$) compared to animals that did not receive this supplementation. Similarly, Mata-Espinosa *et al.* (2006) assessed the supplementation of tulip tree flour (*Hibiscus rosa-sinensis*), mulberry (*Morus alba*), and cocoite (*Gliricidia sepium*) in pasture-fed lamb diets. Their findings showed higher daily supplement intake (167.2 *vs.* 149.7 *vs.* 97.7 g/day, $p < 0.05$) and greater consumption of star grass (*Cynodon nlemfuensis*) (941.8 *vs.* 848.6 *vs.* 796.1 g/day, $p < 0.05$) compared to the other evaluated forage trees. Additionally, daily weight gain (GDP) was statistically similar to that of lambs supplemented with concentrates (77.1 *vs.* 81.6 g/day, $p < 0.05$).

Ruiz-Sesma *et al.* (2006) investigated the inclusion of *Hibiscus rosa-sinensis* hay in diets for hair sheep, analyzing diet digestibility and GDP. The highest response was observed with a diet containing 60% hay from this forage shrub per kg of dry matter. Meanwhile, Le Bodo *et al.* (2020) evaluated the addition of 30% (dry basis) fresh foliage from *G. ulmifolia* in sheep diets over a 30-day period. Their study found no significant anthelmintic or anticoccidial effects, and the impact on GDP was inconsistent, likely due to the absence of an adaptation period and the short evaluation timeframe. In conclusion, certain plants from the Malvaceae family exhibit notable anthelmintic effects and can be considered as potential alternatives for inclusion in integrated parasite control strategies. However, further *in vitro* and *in vivo* studies are necessary, along with investigations into other genera within the same botanical family that have not yet been explored. This would open up new opportunities for evaluating the biological effects of Malvaceae species in controlling gastrointestinal nematodes.

Conflict of Interest

The authors declare no conflict of interest.

Thanks

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Colegio de Postgraduados

Resilience of the *milpa* (*ich kool*) to climate change

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ABSTRACT
Objective: To prove that the *milpa* agroecosystem is resilient to extreme weather events, as a result of indigenous agricultural practices and lore.
Design/Methodology/Approach: A literature review was carried out on Google Scholar to identify relevant publications. The SCOPUS database was used to estimate scientific publication metrics, addressing the resilience of the *milpa*, under the climate change context.
Results: The *milpa* has been in constant evolution and change, as a result of the practices that the *milperos* have been carrying out to reduce the risks posed by climate change and consequently to keep growing basic food for their families.
Study Limitations/Implications: Further research about the *milpa* practices should be carried out, in order to determine how they have managed to survive to the present day.
Conclusions: The *milpa* agroecosystem has evolved in order to achieve food security. People acknowledge its value and seek to reduce risks, guaranteeing food production.

Keywords: polycultures, agroecosystem, culture, agricultural practices, cosmovision.

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INTRODUCTION

Food production is threatened by radical changes in temperature and rain patterns, as a result of global climate change. This situation jeopardizes food safety, both at local and worldwide levels (Altieri, 2012). Global climate change is an unequivocal phenomenon that includes extreme weather events, which have an impact on humans, their properties, and their physical well-being, as well as on agricultural and forest production (Landa *et al.*, 2008). Resilience has been a frequent subject of study of different disciplines and different systems (Lorenz, 2013). It originated in the medical sciences (Pfeiffer, 1929); however, the foundation of its definition lays in ecology (Holling, 1996). Resilience can be understood as a resistance to endure impacts or alterations of a certain magnitude, that can destabilize the system. It is mostly considered as a positive feature of the systems (Béné *et al.*, 2014) and as one of their intrinsic properties (Folke, 2006). Resilience is usually mistaken for sustainability, because the link between them is strong (Brand, 2009). However, resilience plays a key role in the agroecosystems, because it allows

them to absorb disturbances and to keep functioning, as well as to renew and reorganize themselves (Balvanera *et al.*, 2017).

Recent researches have proven that, traditionally, many farmers have strived to adapt and prepare to face climate change within their agroecosystems. The actions carried out by farmers include: reduction of loses using drought-resistant local varieties, polyculture, agroforestry, and timely weeding (Altieri and Nicholls, 2009).

The *milpa* is one of these agroecosystems. *Milpas* date back to pre-Hispanic times and are known as *Ich kool* in the Mayan language. They are very important agroecosystems, because they provide staple foods to the Mesoamerican territories (Velasco-Murguía *et al.*, 2021). In the Yucatan peninsula, corn (*Zea mays* L.) —in association with lima bean (*Phaseolus lunatus* L.), bean (*Phaseolus vulgaris* L.), and squash (*Cucurbita pepo* L.), as well as other crops— is still grown, using the "brush, knock down, and burn" system (Cuanalo-de la Cerda, 1999). The *milpa* is the core of all the productive and reproductive strategies used for the exploitation and integral management of the jungle (Santos-Fita *et al.*, 2013).

Achieving resilience in the agricultural sector —particularly in traditional agroecosystems— must doubtlessly be the main objective of the Mexican agricultural sector, in face of the uncertainty caused by climate change and other associated phenomena. The hypothesis of this study was that the *milpa* is a traditional agroecosystem whose survival has depended on indigenous agricultural practices and lore, which have allowed it to adapt and endure the extreme weather events caused by climate change. Therefore, the objective of this study was to prove that the *milpa* agroecosystem is resilient to extreme weather events, as a result of the indigenous agricultural practices and lore.

MATERIALS AND METHODS

The SCOPUS database was used to identify publications about the resilience of the *milpa* in the face of climate change. The terms used in the search were: "*milpa*", "resilience and milpa", and "*milpa* and climate change." The information collected from SCOPUS was not limited to a specific period; however, it only included scientific publications written in Spanish and English. In addition, a literature review about resilience of the *milpa* in the face of climate change was carried out using Google Scholar. This literature review was used to prepare this study and led to an interesting discussion about this subject.

RESULT AND DISCUSSION

Using the abovementioned search words, the SCOPUS database provided the following results: 1,853 publications included the word "*milpa*", 19 publications included the words "resilience and *milpa*", and 80 publications included the words "*milpa* and climate change". In conclusion, the *milpa* in the face of climate change has been the subject of very few research works.

Resilience of the Ich kool to climate change

The Mayans from the Yucatan peninsula call the *milpero Ichkoolij maak* (or *milpa* man). The *milpero* is the person who observes, reflects, takes care of, improves, and sows the seeds during the appropriate seasons, in order to obtain a good harvest.

The *milpa* is a plot that is used for one to three years (maximum), to grow a wide variety of plants and vegetables. Specifically, the main crops of the *milpa* are corn, bean, and squash, always associated with other crops (Figure 1), which provide food for the animals and family members, as well as household tools.

Modern industrial agriculture is based on artificial monoculture areas, which are exploited in order to provide benefits to humans, while the *milpa* involves the management of the jungle, avoiding the intensive use of soils and enabling the regeneration of the area and the recovery of its fertility (Figure 2).

Figure 1. A milpero and the wide variety of crops sown in the milpa. A: bitter cassava (*Manihot esculenta* Crantz); B: water yam (*Dioscorea alata* L.); C: watermelon (*Citrullus lanatus* (Thumb.); and D: summer squash (*Cucurbita pepo* L.).

Figure 2. The plants that remain after the brush, knock down, and burn system is carried out. Afterwards, the sowing takes place in the milpas of the Yucatan peninsula.

Currently, the fallow or resting period have been reduced, as a result of ejido or land ownership issues. The *milpa* has its own resilience to climate change: *milperos* observe the plants in their *milpas* to determine the changes in their own areas. In addition, they possess considerable lore about the appropriate soil types for the *milpa* and they carry out the best practices to guarantee a good harvest, despite the adverse weather conditions.

As a result of the changes in the weather, *milperos* have adopted certain new practices; for example, they now delay the burning date, to match the sowing date with the start of the rainy season (Castillo López and Torres Carral, 2022). Rain is fundamental for *milperos*; however, they are aware that the rain patterns have become unstable. Consequently, they resown as many times as needed to guarantee a harvest. The burnings are usually perceived as a negative practice. Nevertheless, they are essential to the Yucatan peninsula, which lacks enough soil; consequently, the burnings provide the plants with the required nutrients. Estrada-Medina and Álvarez-Rivera (2021) pointed out that burnings release a nutrient flux to the atmosphere and the soil (the latter as a consequence of the settlement of ashes).

The Mayan *milperos* use a great number of varieties selected according to the climate and the scarce rainfall (Barrera *et al*., 1977). In fact, they still grow and select the seeds of their own communities (Boege, 2009), seeking to find the best phenotypic and genotypic characteristics. The *milpero* understands that crops have adapted and were produced under hard environmental pressures caused by climate change. Although they are temporal crops, they can grow in unfavorable soils.

Another important characteristic of the *milpa* is the diversity of crops grown in the same space. These crops are essential in the diet of Mayan families; nevertheless, the loss of one of these crops would not impact food security, given the diversity of products available. Méndez (2015) pointed out that, in order to obtain staggered harvests, the *milpero* grows two corn varieties during the same year: one long-term (4 months) variety, known in Mayan as *Xnuuk nal* and one short term (7-8 weeks) variety, known in Mayan as *xmejen nal*. Other species, such as pulses, help to fertilize the soil; these crops include bean, lima bean, and jícama (*Pachyrhizus erosus*). Squashes and *X-tóop*' (*Cucurbita argyrosperma* Sin. C. Mixta.) and sweet potato (*Ipomoea batatas* (L.) Poir.) prevent the wind and hydric erosion of the soil and their wide soil coverage helps to control weeds.

In addition, *milperos* have noticed that weeds can help to protect crops from sunlight; consequently, they allow weeds to develop next to the crops. However, they do not allow them to grow excessively. When weeds threaten the growth of the crops, *milperos* prune them, leaving the weeds only next to the crops or extended along the ground (Figure 3).

In addition, the *milpa* provides extra supplies for the families, including: firewood for the kitchens; wild animals hunted for family consumption; and the sale of the production surplus.

The *milpero* lore regarding the resilience to climate change

The *milpa* is directly linked to the cosmovision and practices carried out by *milperos* and their families. The *milperos* are constantly aware of the weather. An excellent example of this phenomenon is the weather forecasting practices known as cabañuelas or *Xook k'iin*,

Figure 3. Practices carried out by milperos. A: protecting corn or other crops with weeds. B: covering plants and soil with weeds that have been pruned or pulled out.

which take place at the beginning of every year. In addition, Méndez (2015) describe the *milpa* as a system that can completely adapt to the current climate conditions, as a result of the great environmental lore of Mayan farmers, which is crucial to the potential mitigation, adaptation, and reversion of climate change.

The dynamism of the *milpa* allows the *milperos* to maintain food production over the years; consequently, the milpa has managed to survive to the present day.

The small community *milpas* are the cornerstone of family diets, as a result of the wide diversity of products that can be harvested every year from a small plot. The cosmovision and the practices inherited from our ancestors still influence the new generations, allowing the culture and resilience of the *milpa* to successfully survive. In this regard, Núñez (2022) reaffirmed that sowing the *milpa* is a living memory because, through the circularity of its work, it reconsiders, strengthens, and reformulates the meaning of language itself, the division of work, the social organization, the belief systems, the narratives, and even the rituals.

In their cosmovision, the pre-Hispanic peoples were very close to nature (Balvanera *et al*., 2017). The current Mayan communities still understand that they are not the owners of the mountains and the jungle and, consequently, they are fully aware that they should not damage them; otherwise, the guardians of the forests or *K-Yuum K'aax* will punish their actions through the *milpa*. The respect and devotion that the Mayans have for this god has allowed dismantled areas to recover, because the jungle is understood as a living being and, therefore, what was taken should be returned to its rightful owner and allowed to rest for a while in order to recover its vitality.

Consequently, the interest for understanding how societies coevolve along with their environment and how the power relationships work in the environment-society interaction has arisen (Balvanera *et al*., 2017).

For thousands of years, the milpa has been a teaching/learning center of the Mayan language and, at the same time, it has been the source of the agricultural lore developed

by the ancestors (Castillo López and Torres Carral, 2022). Resilience is the result of the empirical lore of Mayan farmers that observe the results of their practice, striving to improve the resilience of the *milpa*, through the accumulation and sharing of learning.

Climate change has impacted agriculture as a whole and certain traditional agroecosystems may have a higher resilience than others, because they have responded to the changing weather conditions with strategies aimed to face droughts, floods, and hurricanes, with the same resilience shown by indigenous and famer populations (Altieri, 2013). Hofstede (2014) pointed out that the ancestral lore of indigenous communities can help to adapt and develop resilience to climate change, because these communities have an accumulated experience that can be transformed into lore and practices against climate change and variability. The products obtained from traditional agriculture are exclusively used for family consumption: most of the harvests meet food necessities (Salazar-Barrientos *et al*., 2016). Unlike conventional agriculture, the *milpa* has enabled the survival of thousands of families that depend on this type of production systems, because they include a wide variety of nutritious products, that guarantee food security without using external supplies. Therefore, the value of the *milpa* must be acknowledged, regardless of the adverse conditions of the different types of traditional agriculture. In addition, the milpa is a very important part of the life of indigenous families.

Finally, the *milpa* should be reconceptualized and seen as an indigenous and famer strategy, aimed to achieve food sovereignty, environmental communion, and social and identity reproduction, as well as to face extreme weather events. In other words, the *milpa* requires an interdisciplinary, comprehensive, and biocultural approach.

CONCLUSIONS

The *milpas* of the Yucatan peninsula have managed to survive to the present day, because the Mayan communities have an excellent handling of natural resources and have accumulated vast lore about this traditional agroecosystem. Therefore, the resilience of the *milpa* is always linked to the resilience and evolution of the *milpero's* ideas, enabling a natural equilibrium. The *milpa* and the *milpero* would seem to be two different things; however, the *milpa* would not exist without the *milpero* and vice-versa. The *milpa* and the *milpero* are one and the same and they change together to achieve resilience in the face of climate change and to prevent production losses, implementing practices, using handling methods, and adapting different varieties of plants to their own regions, in order to give priority to food production and the preservation of seeds for the following year.

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Drying and protein content in *Moringa oleifera* Lam. leaves

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ABSTRACT

Objective: To identify the appropriate climatic conditions for drying, as well as to validate the optimal procedure for solar dehydration and protein content in Moringa leaves from cultivars produced in the state of Veracruz.

Design/methodology/approach: The study period of this research was from October 2022 to September 2023. The plant material came from cultivars located in the Capital region and the Sotavento region, from the municipalities of Emiliano Zapata and Veracruz. The Dumas combustion method was applied. Moisture loss (ML) was determined using the gravimetric method, which involved measuring the change in weight of the sample before and after drying to calculate the moisture content. In the bromatological analysis (BA), the Acid Hydrolysis method was used, and it was quantified using the liquid chromatography (HPLC) technique to determine the nutritional composition of the foods.

Results: In the months of March, April and May, the relative humidity registered a low level, which is beneficial for the efficient operation of the dehydrator and accelerates the dehydration process. The results of the bromatological analysis reveal small differences in the contents of ash, protein, crude fiber and fat in the Moringa leaves of the municipalities studied.

Limitations on study/implications: The study period was less than one year.

Findings/conclusions: It was found that Moringa leaves from the municipality of Emiliano Zapata have higher protein, crude fiber and fat content than those from Veracruz. However, both are superior to other Moringa cultivars from the state of Veracruz.

Keywords: Bromatological content, indirect drying, proteins, relative humidity.

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INTRODUCTION

Moringa oleifera Lam. (Moringaceae) is a tree native to India and Pakistan, capable of adapting to hot climates and scarce rainfall (Olson and Alvarado-Cárdenas, 2016); therefore, it is considered a resilient species under the new conditions of climate change (Carrión-Delgado *et al.*, 2023). In addition, the various parts of the plant such as leaves, roots, bark, flowers and pods are consumed for therapeutic purposes to treat different diseases (Asensi *et al.*, 2017). These parts have a high nutritional value, such as vitamins, minerals and essential amino acids, which gives it an important role in preventing and combating malnutrition (Asensi *et al.*, 2017). Studies carried out by Iglesias Díaz *et al.* (2018) and Ruiz-Hernández *et al.* (2021) demonstrate the benefits of Moringa in human nutrition and health. Meanwhile, Salmerón Sánchez (2013) explains that growing this

species provides a series of advantages, such as positive impacts on food sovereignty and favoring an increase in the economic income of families; it also plays a fundamental role in the stability of ecosystems and is an essential part of cultural diversity. On the other hand, Meza-Carranco *et al.* (2016) mention that identifying the agroclimatic conditions of Moringa is essential to understand the factors that influence its cultivation and development, which can help increase its production and quality. Normally, conservation of the leaves is carried out through open-air solar dehydration. Dehydration involves removing moisture through the simultaneous transfer of heat via mass (Iglesias Díaz *et al.*, 2018a). However, protein can be denatured during the dehydration process due to temperature, which can affect its conservation (Quintanilla-Medina *et al.*, 2018). The artificial dehydration process affects its properties but facilitates its conservation, distribution and marketing (Sivipaucar *et al.*, 2010). In Veracruz, there are commercial crops of Moringa in areas with high agroecological potential for this crop (Carrión Delgado *et al.*, 2021), although producers lack an adequate dehydration method to guarantee the improvement of the leaf quality. Based on the above, the objective of this study was to identify the appropriate climatic conditions for drying, as well as to validate the optimal procedure for solar dehydration and protein content in Moringa leaves from cultivars in the state of Veracruz.

MATERIALS AND METHODS

The study was carried out from October 2022 to September 2023 on two cultivars in the state of Veracruz, in the municipalities of Emiliano Zapata (between parallels 19° 35' N and 19° 20' N, and meridians 96° 32' W and 96° 54' W) and Veracruz (between parallels 19° 06' N and 19° 16' N, and meridians 96° 07' W and 96° 21' W) because they belong to cooperating producers. A detailed characterization of the climatic conditions of these two municipalities was carried out, and open databases of the Global Energy Resources Project were consulted for this purpose (Nasa Langley Research Center, 2022), as well as historical climate records of average temperature, relative humidity, rainfall patterns, and other climatic factors that contributed to the determination of monthly relative humidity (MRH) and levels of monthly irradiance (E) in the municipalities in question. The data were analyzed through the NASA Power Web mapping application (NASA Prediction of Worldwide Energy Resources, 2021)

Sample selection

For sampling and collecting Moringa leaves, 10 plants were randomly selected, and the intermediate branches were cut until reaching a total of 1 kg. The compound leaves were separated from the stems, which were discarded, to obtain five 100 g samples (using an SF400 analytical scale), which were placed in transparent polyethylene bags with a hermetic seal. Each sample was labeled and placed inside a corrugated cardboard container in order to reduce cellular respiration during transfer to the dehydrator.

Solar dehydration and protein content

The dehydration process was carried out using a forced air solar dehydrator. The average external ambient temperature during the process was 23 °C (measured with a

Termio15 Datalogger thermometer). The internal temperature of the dehydrator was kept constant at 60 °C. The total dehydration time was 2 hours, following the recommendations by Iglesias Díaz *et al.* (2018b). At the end of this process, the samples were weighed again on an analytical scale (Electronic SF-400) to determine the moisture loss. After drying, the Dumas combustion method was used to determine the ash content (Muñoz-Huerta *et al.*, 2013; Miranda *et al.*, 2016), which consisted of charring 500 g of Moringa leaves to remove organic matter and obtain only inorganic ashes. The moisture loss (ML) was determined using the gravimetric method, which implied measuring the change in weight of the sample before and after drying. In the bromatological analysis (BA), the Acid Hydrolysis method was used, and the nutritional composition of the leaves was quantified using the liquid chromatography technique (HPLC). With this analysis, the percentages of macronutrients present in the sample were determined, as well as the content of crude fiber (CF), protein (Pro), fat (g), carbohydrates (CHO), and energy content (EC).

RESULTS AND DISCUSSION

Figure 1 presents the monthly relative humidity (RH) values by municipality. It shows that during the months of March, April and May, the RH registered a low level, which is beneficial for the efficient operation of the dehydrator and accelerates the dehydration process. However, in the other months, the high water vapor saturation makes the process difficult, resulting in longer dehydration time and increasing the risk of mold contamination. This situation can negatively affect the quality of the dehydrated product (Quintanilla-Medina *et al.*, 2018).

Throughout 2022, no differences were found in irradiance levels in the municipalities of Emiliano Zapata and Veracruz (Nasa Power, 2021). This is because they are located in the same geographical zone and have similar climatic conditions, as shown in Table 1. In both municipalities, the highest levels of irradiance are found in the months of March to August, which is beneficial for the generation of solar energy and contributes

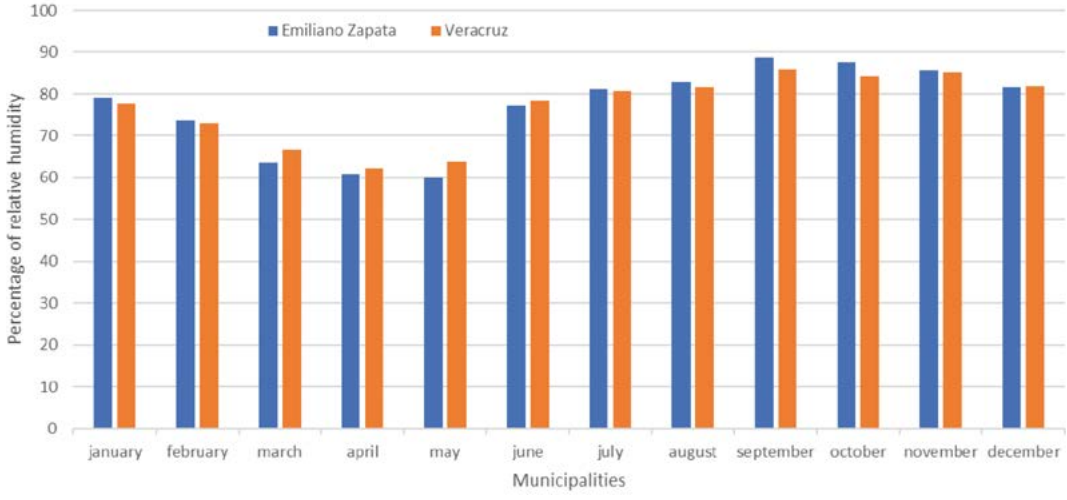

Figure 1. Average monthly relative humidity by municipality (2022).

significantly to the dehydration process of the raw material (Viveros Folleco and Mayorga Castellanos, 2017).

Table 2 presents the relevant data from the bromatological analysis of Moringa leaves, divided by municipality. It was observed that the ashes from the samples from Emiliano Zapata have a higher content, with 8.0%, compared to the samples from Veracruz (3.69%). It is important to highlight that the ashes provide us with information about the elements present in Moringa, such as copper, magnesium, potassium, phosphorus, calcium and zinc (Lezama *et al.*, 2017). In addition, when comparing the protein levels, it was seen that the samples from the crop in Emiliano Zapata have a percentage of 1.40% higher, compared to the samples from Veracruz. It is relevant to highlight that proteins are essential components for a balanced diet and play a crucial role in the body (Quintanilla-Medina *et al.*, 2018). Regarding crude fiber, the results indicate that the sample from Emiliano Zapata has 6.81% more than the sample from Veracruz. Crude fiber is the non-soluble combustible organic residue that represents the cellulose content, and its presence in food is important for good

Table 1. Average monthly irradiance by municipality (2022).

Month	Emiliano Zapata (W/m²)	Veracruz (W/m²)
January	3.565	3.565
February	4.310	4.310
March	5.426	5.426
April	5.967	5.967
May	6.334	6.334
June	5.595	5.595
July	5.925	5.925
August	6.095	6.095
Septiember	4.536	4.536
Octuber	4.300	4.300
November	3.924	3.924
December	3.539	3.539
Yearly E (W/m²)	4.965	4.965

E=irradiance; W/m^2=Watts by square meter.

Table 2. Bromatological content of dehydrated moringa leaves.

Parameter	Emiliano Zapata		Veracruz	
	Result	Unids	Result	Unids
Humidity	9.0	%	8.0	%
Ashes	8.0	%	3.7	%
Crude fiber	23.0	%	16.2	%
Proteins	25.0	%	23.6	%
Oil (ethereal extract)	6.0	%	2.3	%
Carbohydrates (Nitrogen-free extract)	29.0	%	18.0	%
Energy content	270.0	Kcal/100 g	263.0	Kcal/100 g

digestive health (Quintanilla-Medina *et al.*, 2018). Finally, in relation to fat levels, the data show that the samples from Emiliano Zapata contain 3.7% more lipids than those from Veracruz. The results of the bromatological analysis reveal small differences in the contents of ash, protein, crude fiber and fat in the Moringa leaves from the municipalities studied. However, these results, in terms of protein and ash, are superior to other plantations in the Sotavento region, which range between 17.7% and 19.9% protein and 10.6 and 12.3% ash (Ruiz-Hernández *et al.*, 2021). This is because the type of soil and its fertility affect the nutritional content of the leaves, so more studies are required with other cultivars from the state to have a better characterization (Cerdas-Ramírez, 2017).

CONCLUSIONS

The results show that the months of March, April and May are the most favourable for efficient operation of the dehydrator, due to the low relative humidity levels. This accelerates the dehydration process. However, in the remaining months, high water vapor saturation makes the process more difficult, resulting in longer dehydration time and a higher risk of mold contamination. These factors can negatively affect the quality of the dehydrated product. Therefore, it is important to take into account the variation in relative humidity throughout the year to ensure an efficient dehydration process and high quality of the final product.

The municipalities of Emiliano Zapata and Veracruz presented similar irradiance levels throughout 2022. These findings are relevant for the development of solar energy projects within these municipalities, as they indicate there is a consistent potential over the year to harness this renewable energy source.

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Growth of juveniles of (*Striostrea prismatica* G.) (Ostreida: Ostreidae) under two feeding regimes in a semi-closed circulation system

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ABSTRACT
Objective: The effect of two diets on the growth of juveniles of *Striostrea prismatica* was studied.
Design/Methodology/Approach: The experiment consisted of tasting two diets, each with two replicates of 70 organisms. The experiments were carried out over 45 days in seawater recirculation systems. These two diets consisted of 1) 50% *Chaetoceros muelleri* B. and 50% *Tetraselmis tetrathele*, both grown in the laboratory, and 2) Shellfish Diet1800® pasta.
Results: The results did not show differences between both treatments. The organisms' height of those fed with the pasta was 24.7±6.42mm, and that of those fed with the live food was 24.7±7.87mm.
Limitations on study/implications: L'ignorance of the species' biology can limit our understanding of growth and its impact on the crop. Since the duration of the experiment, a more significant increase has been observed in the left valve, which may be due to the absence of currents or waves as occurs in natural conditions.
Conclusions: Therefore, it is concluded that using both diets is indistinct to cultivate *S. prismatica* juvenile in semi-closed systems.

Keywords: Diets, Growth, *Striostrea prismatica*, juvenile.

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INTRODUCTION

The rocky oyster (*Striostrea prismatica* G.), a bivalve mollusk, is an essential species for fishing and the economy. It is heavily fished from Mexico's Gulf of California to Peru Mancora, which has put it under significant pressure (Arreguín-Sánchez & Arcos-Huitrón, 2011). To address this issue, domestication for cultivation is being explored. However, limited research on the species and its biology and development under laboratory conditions is poorly understood. Previous studies have focused on evaluating juvenile growth and gametogenic development in conditioning bioassays (Ríos-González *et al.*, 2018). Food plays a crucial role in the growth of organisms throughout their life cycle (Parker, 2011).

Therefore, it is essential to design diets that allow success in these early phases of development. It has been found that the growth of *Crassostrea corteziensis* and *Pinctada maxima* is influenced by the species composition and, consequently, the nutritional composition of each diet (Rivero-Rodríguez *et al.*, 2007; Haoujar *et al.*, 2022 and Yi *et al.*, 2023).

The larval and seed stages require high-quality nutrition (Marshall *et al.*, 2010), and microalgae species are commonly used to meet protein, lipid, and carbohydrate needs. Mixtures from production labs or commercial concentrates are preferred over monoalgal diets for optimal growth, and a varied diet leads to positive development outcomes (Helm, 2006).

Previous studies have tested concentrated foods and food substitutes like microparticulate food and industrial cheese whey in bivalves and invertebrates, but there is controversy surrounding their nutritional effectiveness (McCausland *et al.*, 1999; Ponis *et al.*, 2003; Enes & Borges, 2003; Espinosa & Allam, 2006; Duy *et al.*, 2016). (Duy *et al.*, 2015), evaluated the differences in the sea cucumber (*Holothuria scabra* J.) in the ingestion and digestion rate of various diets, including live food and commercial microalgae concentrate from the brand Shellfish Diet 1800© Red Mariculture, finding that both had similar results. In subsequent studies, Duy *et al.* 2016 reported that the monoalgal concentrates supplied have sufficient nutritional requirements to promote larval development, growth, and survival. Concentrated diets such as algae pastes have been used in larva and seed nurseries of marine organisms. McCausland *et al.* 1999 found that adding pastes to a natural phytoplankton diet improved growth rates in marine larvae. However, they did not observe growth differences between diets for the Pacific oyster (*Crassostrea gigas* T.). Feeding laboratory-formulated live microalgae diets did not show any difference in the growth of *Siriostrea prismatica* G. juveniles cultured in controlled systems compared to the development of juveniles fed commercial algae concentrates (pastes) in controlled systems. The present study evaluated the performance of pasta and live food feeding regimes for juvenile rock oysters in a semi-recirculating system.

MATERIALS AND METHOD

Experimental design

Each treatment was carried out in triplicate, giving six semi-closed recirculation systems. A pond individually integrated the semi-closed recirculation systems with a capacity of 300 L, connected to a 200 L filter with a section for sediments and sand (mechanical) and a biological filter section with perforated spheres (bioballs) that houses bacteria-denitrifying agents for the elimination of NH_3. The water was returned to the pond using an aquarium pump with a pumping capacity of 2000 L/h. Three replacements were made during the experiment period.

Collection and preparation of organisms

From "El Tizate," Nayarit, 420 juvenile oysters between 7 and 45 mm/length (average 22.33±6.24 mm) (attached to rocks) were collected and transported in 40 L seawater reservoirs to Laboratory Water Quality and Aquaculture from the Centro Universitario de la Costa (LACUIC), they were cleaned with a wire brush and fresh water to eliminate epibionts. In each replica, the rocks were placed with 70 oysters, and the total height was recorded (they were not detached from the rock since this caused severe damage to the organism).

The bioassay lasted 45 days, from December 19, 2015, to February 4, 2016. To determine the organism's growth in both feeding regimes, the total height was recorded at the beginning and end of the bioassay.

Feeding regimens

The diets for each treatment consisted of Diet 1) 50% (*Tetraselmis tetrathele* B.) and 50% (*Chaetoceros muelleri* L.) (cultivated in the LACUIC).

Microalgae cultivation

Tow strains of microalgae, *T. tetrathele* and *C. muelleri*, were cultivated. The Northeast Biological Research Center (CIBNOR) strains acquired the strains. They were maintained in standard batch cultures in 19l jugs and cultivated with the following procedure:

The jugs were sterilized with 2% sodium hypochlorite (NaClO) at 1 ml for every 10 ml of water. Then, the NaClO was neutralized with sodium thiosulfate (Na_2S2O_3). Finally, to avoid contamination, the jugs were covered until inoculation.

Seawater was treated using a sand and carbon filter and then placed in 40 L containers with 2% NaClO for disinfection. It was covered, left to rest for 12 hours, and neutralized with sodium thiosulfate, ensuring its safety and cleanliness. These cultures were scaled consecutively from 500 ml flasks to 2 L glass flasks to 19 L capacity glass jugs. The seawater was enriched with an F/2 culture medium PROLINE brand. In the case of diatoms, sodium metasilicate pentahydrate was added.

The jugs with the culture were kept in a light regime of 12 hours of light and 12 hours of darkness at an intensity of 1800 lux, with constant aeration to avoid self-shading. The cultivation temperature was 20 °C, and they were harvested in the exponential phase of their growth.

Diet 2) commercial Shellfish Diet1800® microalgae concentrate (Reed Mariculture®) containing *Tisochrysis lutea* (T-ISO) B & P., *Pavlova* sp., *Tetraselmis* sp., *Thalassiosira weissflogii* F., *Chaetoceros calcitrans* & H., *T. pseudonana* H & H. and *Chaetoceros calcitrans* T.

The food ration was calculated with a sample of 3 organisms per treatment. The total height, total length, total weight, and wet weight of the tissue were recorded. In each treatment, the food ration was calculated differently. In the case of Treatment 1, live food (AL), the method proposed by FAO 2006 (Helm, 2006) was considered, which consisted of 2% of the live weight of the organism following the formula (based on a mixture of 50% *T. tetrathele* and 50% *Ch. muelleri*.

$$V = (S * 0.2) / (7 * W * C)$$

Where V is the volume of microalgae (l) required for daily feeding, S is the weight of the live organisms, W is the weight of one million cells supplied (the weight of the cells was considered according to Helm *et al.*, 2006. C is the cell density of microalgae in the culture.

Treatment 2 utilized commercial microalgae paste (PCM) for seeding, following the company's recommended protocol of adding 5% of the product based on the organisms' live weight. The ratio had to be reduced to 4% due to microalgae remaining in the water after 12 hours.

The organisms were fed once a day after removing the feces and excess sedimented organic matter. The recirculation system was kept off for 12 hours to ensure the organisms were fed adequately. Aeration in ponds continued with two Elite model 802 aerators, and then the circulation system restarted. The feed portion was adjusted three times during the experiment. For adjustment, a sample of three organisms was taken from each pond, of which the total height and wet weight were recorded. With the latter, the portion was calculated using the formulas mentioned in the previous paragraphs.

The physicochemical variables of the water (temperature, salinity, dissolved oxygen, and pH) of each recirculation system were recorded once a day.

Statistical analysis

Data were tested for normality with a Kolgomorov-Smirnov test (Daniel, 2010). The average growth was calculated, and subsequently, an X^2 was performed to determine the existence of statistical differences between the treatments.

RESULTS AND DISCUSSION

The recirculation system model used in the experiment had the appropriate physicochemical conditions for maintaining the contained biomass since the variations of the monitored physicochemical parameters remained constant, with salinity and pH being the parameters that were controlled with the addition of Sodium Bicarbonate ($NaHCO_3$) and freshwater depending on the parameter to be controlled (Table 1.); nevertheless, McCausland *et al.*, 1999, suggests that variations between experiments may be a factor that influences the composition of diets and the response to growth.

The mortality rate was 4.76%, but growth was possible. This suggests that systems (RASs) are viable for breeding and conditioning bivalve mollusks to maximize production and maintain water quality. Previous studies by Frías & Segovia (2010) and Kamermans *et al.* (2016) have used RASs for seed conditioning and growth, demonstrating their ability to maintain the conditions for experiment success.

The average growth with PCM was 24.67±6.4mm, and with AL, 24.76±6.47mm. No significant statistical differences were recorded in oyster growth between feeding regimes ($X^2$0.8862, α=0.5, df=1) (Table 2). This is consistent with the findings of Yi *et al.*, 2023, who tested the effects on the growth of juvenile *Pinctada maxima* J., with live microalgae

Table 1. Average of the physicochemical parameters during the experimental period. PCM commercial microalgae paste, AL live food.

Diet	T (°C)	O.D. (mg L^{-1})	pH	S (UPS)
Commercial food PCM	22.1±1.8	5.6±0.4	7.4±0.10	35.4±2.3
Live cultivation al	22±1.6	5.7±0.4	7.4±0.4	35.4±2.7

Table 2. Average growth of juvenile rock oysters in semi-recirculating systems. PCM commercial microalgae paste, AL live food.

Organism size	Commercial food (PCM)	Live cultivation (AL)
Average starting size	22.33±6.24	22.33±6.24
Average final size	24.67±6.40	24.76±6.47

and spray-dried algae powder and didn't find significant differences in larval growth; however, they found differences in spat growth.

Oyster growth didn't show differences between the diets administered maybe because they were a species mix. Rivero-Rodríguez *et al.*, 2007 explain that the most successful diet for *Crassostrea corteziensis* H. spat was a monospecific dietary compound just for *Chaetoceros calcitrans* P., which gave sufficient nutrients to perform spat growth.

Microalgae production offers benefits and drawbacks when feeding bivalve mollusk larvae and seeds. Due to the food's immediate availability, the nutritional value of the product provided to the organisms during their production can be determined. However, because of the high cost of production and labor requirements for this activity, scientists and production farms have been employing food supplements or substitutes such as dehydrated microalgae, microalgae concentrate (pastes), grain flours, and other by-products like whey. There is a lot of debate about whether pasta is a nutritious food. Some scientists believe that when pasta is frozen or stored for a short period, it loses its nutritional value. Studies by Enes *et al.* (2003) and Espinoza *et al.* (2006) have explored this issue.

Espinosa *et al.* (2006) found that bivalve mollusks grow better when fed live microalgae than commercial products. Commercial food undergoes degradation during freezing, storage, and transportation, leading to lower nutritional quality. This highlights the importance of considering the food source for optimal growth (McCausland *et al.*, 1999).

Experiments with concentrated foods in laboratories show varying efficiencies in shelf life and concentration methods. These concentrates effectively feed larval and seed stages of Brown bivalve mollusks (Ponis *et al.*, 2003).

Duy *et al.* (2015) found that concentrates like Shellfish Diet 1800 are essential for the growth and survival of sea cucumber larvae. However, not all species of microalgae included in the concentrate may be fully digested within 24 hours, potentially leading to incomplete consumption by the larvae. This suggests the need for further research on optimal feeding strategies.

CONCLUSIONS

The effectiveness of pastes or microalgae for cultivation is inconclusive. Further research is needed to determine if culture systems impact growth rates.

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Evaluation of sargassum (*Sargassum* spp.) extracts as a method of organic fertilization of sugarcane

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ABSTRACT

Objective: To evaluate the effect of the application of commercial sargassum extract as foliar biofertilizer in sugarcane cultivation.

Design/methodology/approach: Five fertilization treatments were applied in an experimental plot cultivated with sugarcane: Conventional mineral fertilization (T1); Foliar fertilization with 100% sargassum extract (T2); Foliar fertilization with 50% sargassum extract (T3); Conventional mineral fertilization at 50% plus foliar fertilization with 50% sargassum extract (T4); and control without fertilization (T5), in a completely randomized experimental design.

Results: The greatest stem height was observed in the statistically equal treatments T1, T2 and T4, but higher than that of the control. The longest diameter was observed in the T4 treatment, significantly higher than that of the control, in which a statistically lower number of leaves was also observed. The highest field yield was observed in statistically identical treatments T1, T2 and T4, but higher than that found in the control treatment.

Limitations on study/implications: Further evaluation of commercial sargassum extracts applied as foliar fertilizers in crops is considered necessary to corroborate the results presented here. A study of the action mechanisms of the compounds found in sargassum extract on crop physiology, when applied as foliar fertilizers, is also considered necessary.

Findings/conclusions: Commercial sargassum extract is a sustainable alternative for sugarcane biofertilization, which does not pose a risk of chemical contamination like that of conventional mineral fertilization.

Keywords: Biofertilization, sustainability, sargassum, *Saccharum officinarum*, crop yield.

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INTRODUCTION

Sargassum (*Sargassum* spp.) is a holopelagic macroalgae that forms large floating masses in the oceans. In 2011, the first record of a massive arrival of sargassum in the waters of the Mexican Caribbean was made (Ortegón-Aznar and Ávila-Mosqueda, 2020); later, in 2018, some 47 thousand cubic meters of sargassum per kilometer of beach in Quintana Roo arrived on the Mexican coasts, with two types of algae predominating: *Sargassum fluitans* and *Sargassum natans* (Espinosa-Antón *et al.*, 2024). It is speculated that sargassum proliferation is linked to the increase in temperature in marine waters due to climate change and the influx of nutrients from rivers in Africa and Brazil (García, 2019).

Due to its massive arrival, sargassum began to be considered an environmental problem, since it obstructs the entry of the sun's rays to the seabed, preventing the photosynthesis process of species such as phytoplankton, so its population decreases and with it also the species that depend on it (Espinosa-Antón *et al.*, 2024). In order to try to reduce the amount of sargassum, options for its use have been sought; it has been discovered that, with prior treatment, it has the potential for consumption by some animal species (Casas-Valdez *et al.*, 2006); its use in the pharmaceutical area is being evaluated, and it has been used as a fertilizer in crops, mainly vegetables (Salazar-Salazar *et al.*, 2022).

The objective of the study was to evaluate the application of commercial sargassum extract as a foliar biofertilizer in sugarcane, an important crop in the state of Campeche. In 2018, nearly 16,000 ha were dedicated to growing it, an area that increases each year in the state (SADER, 2019). The use of sargassum extract as a foliar fertilizer would have a positive impact on the environment, and simultaneously the costs of fertilizing sugarcane could be reduced.

MATERIALS AND METHODS

Experimental site

The experiment was carried out on a sugarcane plot (variety Mex 69-290) belonging to a cooperating producer from the Villa de Guadalupe ejido, municipality of Champotón, Campeche, at the slashing stage 3. It was harvested in January 2023, and work began in February of the same year. The crop was managed under rainfed conditions. The climate is type AW1 according to the Koëppen classification modified by E. García (García, 1973), with summer rains. The soil of the experimental plot is clayey and deep, vertisol according to FAO, akalché according to the Mayan classification, quite homogeneous throughout the plot, with a slight slope and very slow internal drainage. It cracks when it dries.

Treatments, repetitions and experimental units

The following treatments were evaluated: T1.- Conventional fertilization according to the technological package of the region (160-80-100 NPK); T2.- Fertilization with commercial sargassum extract of the brand "Sarga extra" (Figure 1) applied on the leaves

Figure 1. Commercial sargassum extract evaluated in the experiment.

at a dose of 125 ml in 20 liters of water (manufacturer's recommendation); T3.- Fertilization with commercial sargassum extract applied on the leaves at 50% (62.5 ml in 20 liters of water); T4.- Combined fertilization with chemical fertilizers at a dose of 50%, plus the application of 50% sargassum extract (fertilization formula 80-40-50, mixed with T3); and T5.- Absolute control without fertilization. In T1 and T4, the fertilizers DAP (18-46-00), urea (46-00-00), and potassium chloride (00-00-60) were used, applying them only once at the beginning of the study, like the sugarcane producers in the area. Each treatment had four repetitions. The experimental units (EU) were five furrows, 1.5 m wide and 10 m long, separated by 2 m from each other to avoid the "edge effect". Four applications of the sargassum extract solution were made, on the dates: February 24, May 8, June 22, and August 6, in the morning, when the stomata of plants remain open.

Chemical composition of the sargassum extract

The container of the commercial sargassum extract does not indicate the elements it contains (Figure 1), so a chemical analysis was carried out in the soil, plant and water chemical analysis laboratory of the Tabasco Campus of Colegio de Postgraduados, the results of which are included in Table 1. It has high electrical conductivity and very high concentrations of sodium, potassium and magnesium. The last two can function as macro and microelements for crop nutrition, but sodium can eventually cause toxicity. It has very low concentrations of phosphorus and nitrogen in both its nitric and ammonia forms, so the only macroelement available is potassium. It is important to note that it is in high demand by the sugarcane, a fundamental element for the synthesis of sugar.

Experimental design

The experimental plot showed homogeneity in the soil type, so a completely randomized experimental design (CRD) was used.

Response variables

The height, diameter and number of leaves of the plants were evaluated in the six meters and three central rows of each EU. At the end of the study, when the sugarcane was 12 months old, the yield was evaluated by cutting and weighing the stems from each experimental unit.

Table 1. Chemical properties and elements present in the commercial sargassum extract evaluated in the study.

Chemical property		Element	Concentration ($mg\ L^{-1}$)	Element	Concentration ($mg\ L^{-1}$)
pH	3.4	$N\text{-}NO_3$	2.43	Na	2,283.00
CE	11.97 $dS\ m^{-1}$	$N\text{-}NH_4$	18.85	Fe	0.526
		P	3.17	Cu	0.033
		K	5,224.00	Zn	0.320
		Ca	240.30	Mn	0.391
		Mg	1,187.00	Cd	0.060

Rainfall

The incidental rainfall on one side of the sugarcane plot was quantified using a pluviometer. The crop was not irrigated, so the water stress to which it was subjected was very intense due to the absence of rainfall during a good part of the vegetative cycle, causing a considerable reduction in the size of the plants and in the final yield in all treatments.

Statistical analysis

The CRD analysis of variance was performed for the response variables ($p=0.05$), using the InfoStat statistical package (InfoStat, 2017). The Shapiro-Wilk normality test of the data was performed, as well as Tukey's multiple means comparison ($p=0.05$) in the variables with significant treatment effects (Tukey, 1991).

RESULTS AND DISCUSSION

Rainfall

Total precipitation was only 1,067.9 mm, insufficient to meet the water needs of sugarcane (between 1,487 and 2,122 mm; Garay Jácome *et al.*, 2022). From January to the end of June, there was practically no rain, a behavior that had not been recorded in recent years in Campeche.

Height

The average height of the plants was statistically the same in all treatments ($p>0.05$) for almost the entire cycle; only at the end of the study there were statistical differences observed, for the last three measurement dates. In these, the height in T1 was always higher, although with statistically similar values to those observed in T2, T3 and T4. For the last measurement date, T1, T2, T3 and T4 were statistically equal, although T3 was statistically equal to the control (Figure 2). The plants grew very little in the months without rain and only began to develop when the soil moisture favored growth, without

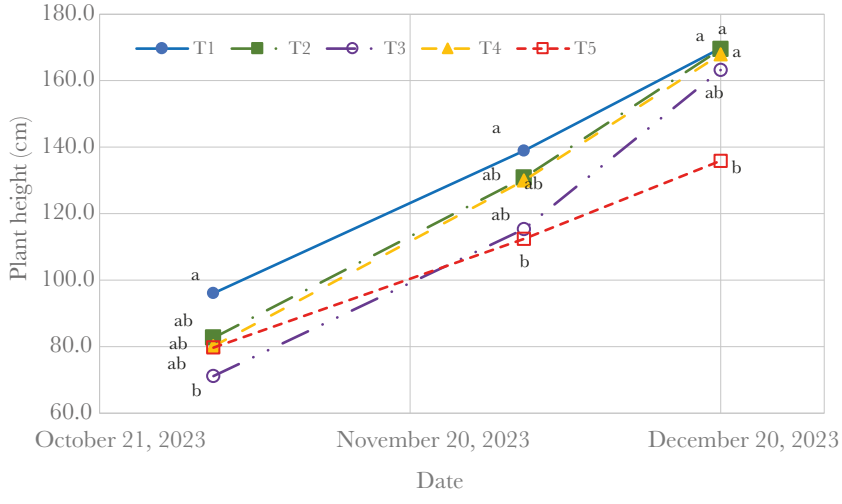

Figure 2. Temporal evolution of sugarcane height for the last three measurement dates in the five treatments. Means with different letters are statistically different (Tukey, $p\leq0.05$).

growing enough: the final average height barely exceeded 169 cm in the treatments with the greatest height, and in the control treatment they only grew 135.9 cm on average.

Stem diameter

No statistical differences were found during practically the entire study. Starting in the month of June, when the rainfall began, the highest values were observed in T1 or T4, although with statistically similar values in all treatments. In every case the diameter was small, barely exceeding two centimeters, because the plants did not have enough moisture to develop. Significant effects were found only on the last measurement date: the thickest stems were observed in T4, 2.44 cm on average, with the lowest value in the control.

Field performance

Statistically equal values and higher than the rest were observed in T1, T2 and T4 (Figure 3). A significantly lower value than the previous ones was observed in T3, and the statistically lowest value was recorded in T5, where the yield was very low due to drought.

No records of studies were found in which the application of sargassum extract had been evaluated in sugarcane, but it has been assessed in other crops and for other uses: Herrera-Monroy (2015) evaluated sargassum as a substrate for the production of jalapeño pepper and tomato seedlings; they concluded that it can be used as a substrate, but its high sodium concentrations limit its use in plants that are not very tolerant to the element. Sariñana-Aldaco *et al.* (2021) evaluated the effect of foliar application of 17 sargassum extracts on the growth of tomato seedlings. One of the extracts increased height, stem diameter, total fresh and dry matter of the seedlings, as well as the content of proteins, glutathione, phenols, flavonoids and antioxidant capacity of the leaves compared to the control. Salazar-Salazar *et al.* (2022) evaluated the effect of foliar application of fertilizers

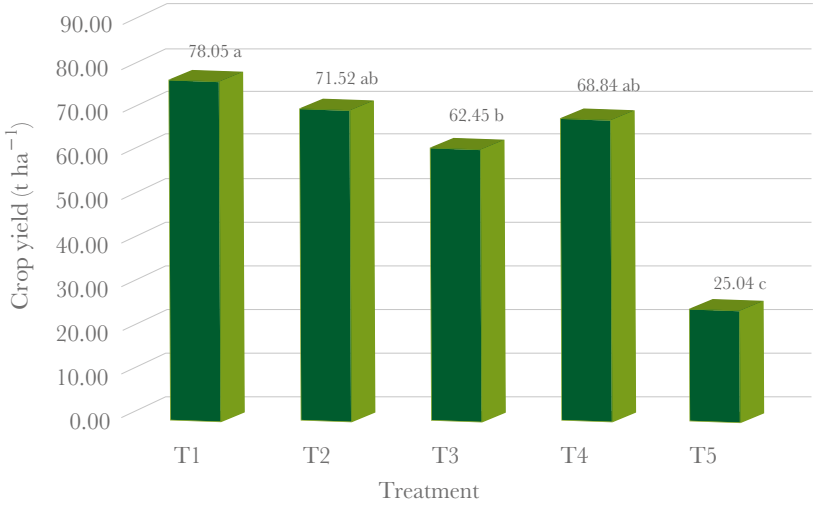

Figure 3. Field sugarcane yield in the evaluated treatments. Means with different letters are statistically different (Tukey, $p \leq 0.05$). T1: conventional fertilization; T2: application of 100% sargassum extract; T3: application of 50% sargassum extract; T4: 50% conventional fertilization plus application of 50% sargassum extract; T5: control treatment.

and sargassum extract on cucumber cv. Modan; with the application of the extract a significantly higher number of commercial and total fruits was obtained, as well as a significantly higher yield.

Although the application of sargassum extract did not significantly improve the growth or yield of the sugarcane obtained, in relation to chemical fertilization, it has the advantage of being of natural origin, so it does not generate the chemical contamination that can be caused by conventional mineral fertilizers. In addition, its use as a biofertilizer contributes to solving the problems of sargassum saturation that have taken place on the beaches of the Mexican Caribbean. Both aspects improve the sustainability of both the sugarcane agroecosystem and the coastal ecosystems. However, it is considered necessary to continue evaluating the effect of the application of commercial sargassum extracts as foliar biofertilizer in different crops, specifically in sugarcane, to corroborate the results presented in this study. Likewise, it is considered important to study the action mechanisms of the compounds present in the sargassum extract on the physiology of crops, when applied as foliar fertilizers.

CONCLUSIONS

The use of commercial sargassum extract as a biofertilizer in sugarcane allowed obtaining statistically equal values in the height and diameter of the stems to those found when conventional mineral fertilization was applied, as well as in the number of leaves in sugarcane, statistically higher than those observed in the control. Regarding the field yield of the cane, it was statistically equal because of the effect of the application of conventional mineral fertilization treatments and foliar sargassum extract (T1, T2 and T4), so the study concludes that commercial sargassum extract is a sustainable alternative for the biofertilization of sugarcane, which does not have the risk of chemical contamination that conventional mineral fertilization represents.

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Colegio de Postgraduados

Competitiveness indicators of Mexican bananas in the international market

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ABSTRACT

Objective: To analyze the variables and indicators of banana trade in the international market.

Design/Methodology/Approach: The revealed comparative advantage index of exports (RCAIE), the revealed comparative advantage index (RCAI), and the normalized revealed comparative advantage index (NRCAI) were used to analyze the competitiveness of Mexican bananas from 1994 to 2021.

Results: Mexico is one of the main banana exporters worldwide. Its main export destinations are the USA and Japan which consumed 84% and 15% of the Mexican production, respectively. The RCAI recorded positive values, showing that Mexican banana exports are competitive in the international market. This product is competitive in the American and Japanese markets and is more stable in the former. The >0 RCAIE confirmed that Mexico is a competitive country and one of the main banana exporters of the world.

Study Limitations/Implications: Competitiveness was analyzed based on a databases query from international organizations. These export databases have a delay of up to 2-3 years and part of the data showed significant differences.

Findings/Conclusions: Mexico is a competitive banana exporter worldwide. Based on the RCAI and NRCAI, the American and Japanese markets are the most competitive. However, Mexico exports a lower banana volume to Japan than to the USA.

Keywords: Exports, revealed comparative advantage index of exports, revealed comparative advantage index, normalized revealed comparative advantage index.

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INTRODUCTION

Bananas are grown in almost all the tropical regions of the world. More than 1,000 banana varieties are produced and consumed worldwide (Redagrícola, 2021). They are mainly produced in developing countries, where they are considered as a strategic crop for food security. They are the most exported and consumed fruit in the globalized world and are a source of employment and income in rural areas (Martínez and Rey, 2021).

Bananas are highly valued as a result of their pleasant flavor and high nutritional value. They are a source of potassium, iron, and vitamins A, B6, K, C, and D, benefiting human bones and muscles. In addition, bananas are available all year round (García *et al.*, 2013; Blasco and Gómez, 2014).

In 2021, the worldwide banana production reached 124,978,578 t. Nineteen-sixty-seven percent was exported, while the rest was used for self-consumption. India, China, Indonesia, Brazil, and Ecuador are the main banana producers and account for >50% of the worldwide production. Mexico is the twelfth banana producer worldwide and its production reached 2,405,891.33 t (FAOSTAT, 2024). Latin America and the Caribbean are the main regional exporters (15.9 million t) of the world (FAO, 2022). In 2021, Mexico was the tenth banana exporter (489,522.34 t), accounting for 1.99% of the total worldwide volume (FAOSTAT, 2024).

The competitiveness of a country, industry, or company is linked to the productivity increase, resulting from the technological development that allows them to reduce costs, to obtain higher profit, and to increase their share in both the domestic and international markets (Zamora and Ortiz, 2021). The implementation of technology, infrastructure, and sustained development should promote the economy of a country vis-à-vis other regions (Daza, 2014). In the agrifood chain, competitivity should be made clear by a strengthened production of assets and services aimed to constantly maintain, increase, and improve its share in the international market (Luquez *et al.*, 2022). In turn, it should specialize in the production and commercialization of the said asset, reducing production costs (Zavala *et al.*, 2023).

A statistical analysis about the banana commercialization should establish the advantages of the product in the international market. This analysis should include total exports, in order to determine the international share of the said country or product. Therefore, the objective of this study was to analyze the competitiveness of Mexican bananas from 1994 to 2021. After the North American Free Trade Agreement (NAFTA) came into force in 1994, Mexico became a platform for the exportation of several products to international markets. To establish the competitiveness performance of Mexican bananas in the main import markets, the revealed comparative advantage index (RCAI), the revealed comparative advantage index of exports (RCAIE), and the normalized revealed comparative advantage index (NRCAI) were determined.

MATERIALS AND METHODS

The statistical data used in this study for the 1994-2021 period were collected from the international databases of the Food and Agriculture Organization Statistics (FAOSTAT) and the Foreign Agricultural Service of the United States Department of Agriculture (FAS-USDA). The following variables about the commercialization of Mexican bananas were analyzed: value of the Mexican banana exports in the international markets; total agricultural exports from Mexico to the international markets; value of the Mexican banana exports to the USA; total agricultural exports from Mexico to the USA; value of the Mexican banana exports to Japan; and total agricultural exports from Mexico to Japan. Subsequently, the revealed comparative advantage index of exports (RCAIE), the revealed comparative advantage index (RCAI), and the normalized revealed comparative advantage index (NRCAI) of the Mexican bananas in the American and Japanese markets were calculated. The objective was to establish the competitiveness of Mexican bananas in the main import markets worldwide.

Calculation method

The revealed comparative advantage (RCA) indicates the advantages of the total exports of a given country and its share in the rest of the world. In addition, the value of the index shows the existence of a comparative or specialized advantage in the exports of a certain product (Ávila and González, 2012). The calculation method was:

$$RCAIE = \left(\left(X_a^i / X_n^i\right) / \left(X_a^r / X_n^r\right)\right)$$

Where: *RCAIE*=revealed comparative advantage index of exports of product *a* in the country; X_a^i =value of the exports of product *a* in the country; X_n^i =value of total exports without product *a* in the country; X_a^r = value of the exports of product *a* in the international market (without country *i*); X_n^r =value of the total exports (without the product) in the international markets (without country *i*). $A>1$ or positive *RCAIE* indicates a revealed comparative advantage.

The revealed comparative advantage index (RCAI) developed by Balassa analyzes the comparative advantages or disadvantages of a country, regarding the commercial exchange with its partners. It not only measures the importance of a given product as part of the exportations from one market to another, but it also indicates its competitiveness in the main import markets (Durán and Álvarez, 2008). The following formula is used to calculate RCAI:

$$RCAI_{ij}^k = \left(X_{ij}^k / XT_{ij}\right) / \left(X_{iw}^k / XT_{iw}\right)$$

Where: $RCAI_{ij}^k$ =revealed comparative advantage index of product *k* from country *i* to country *j*; X_{ij}^k =exports of product *k* from country *i* to country *j*; XT_{ij} =total exports from country *i* to country *j*; X_{iw}^k =exports of product *k* from country *i* to the world (*w*); XT_{iw} =total exports from country *i* to the world (*w*). $A>0$ *RCAI* indicates a comparative advantage of the country or product, showing that the country is competitive in international markets. $A<0$ indicates a comparative disadvantage of the country or the product, showing that the product is not competitive in international markets (Caamal *et al.*, 2017).

The normalized revealed comparative advantage index (NRCAI) improves the analysis of the RCAI. The index is normalized to a maximum of 1 and a minimum of −1, using the following formula:

$$NRCAI = RCAI - 1 / RCAI + 1 \quad \text{(Durán and Álvarez, 2008)}$$

Where: *NRCAI*=normalized revealed comparative advantage index and *RCAI*=revealed comparative advantage index. Results between +0.33 and +1 show a comparative advantage of the country and a favorable balance of trade regarding the analyzed country. Results

between −0.33 and −1 show a comparative disadvantage of the country. Values between −0.33 and +0.33 show a trend to exchange products within the same technological group (intra-industry trade).

RESULTS AND DISCUSSION

The value of Mexican banana exports during the 1994-2021 period showed a growing trend, increasing from US$80,418,000 in 1994 to US$242,792,000 in 2021. During the same period, the volume of exports increased by 135.42%. In 2021, exports accounted for 20.34% of the total domestic production, reaching a share of 1.9% in the international market (FAOSTAT, 2024).

The >0 RCAIE of Mexico during the 1994-2021 period indicated a revealed comparative advantage, showing the competitiveness of Mexico in the banana global market. Although the RCAI values recorded a slight decrease (from 0.2168 in 1994 to 0.1165 in 2021), they remained above 0 (Table 1). Consequently, the competitive advantage of Mexican banana exports was stable during the analyzed period.

The USA was the main destination of the Mexican exports (412,193.58 t) in 2021, followed by Japan (73,533.41 t) (FAOSTAT 2024). Mexico also exported bananas to the United Kingdom, the Netherlands, Canada, and Germany. Approximately 84% of the Mexican banana exports are sold to the USA. The production of this country (4,635,392.21 t in 2021) is not enough to supply its domestic market; consequently, the USA imports that volume of bananas from Mexico to meet its customer demand. Mexico exports 15% of its banana production to Japan and the remaining 1% is sold to the United Kingdom, the Netherlands, Canada, and Germany.

The USA and Japan markets are the main destinations of Mexican bananas and, consequently, they were analyzed using different total agricultural and banana exports data to determine RCAI and NRCAI (Table 1).

The USA is the main worldwide importer of bananas, which makes it an excellent market for the Mexican production. The RCAIE of Balassa for Mexican bananas sold in the American market during the 1994-2021 period recorded a constant performance,

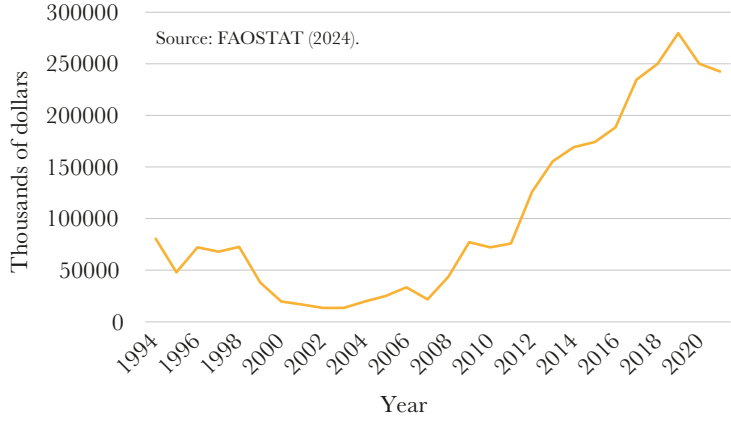

Figure 1. Performance of Mexican exports (1994-2021).

Table 1. Competitiveness indicators of bananas (Mexico to the world and Mexico and its main import markets - 1994-2021).

Year	VRE Mexico-World	IVCR Mexico-USA	IVCRN Mexico-USA	IVCR Mexico-Japan	IVCRN Mexico-Japan
1994	0.2168	0.9912	−0.0044	0.2413	−0.6112
1995	0.1804	0.7556	−0.1392	0.4270	−0.4015
1996	0.1699	0.8129	−0.1032	0.0000	−1.0000
1997	0.1528	0.5925	−0.2559	0.4939	−0.3388
1998	0.1251	0.4440	−0.3850	1.1743	0.0802
1999	0.0677	0.5054	−0.3286	0.0209	−0.9591
2000	0.0362	0.8127	−0.1033	0.0000	−1.0000
2001	0.0270	1.0632	0.0306	0.0000	−1.0000
2002	0.0211	1.1668	0.0770	1.8320	0.2938
2003	0.0198	1.0508	0.0247	2.8327	0.4782
2004	0.0256	0.7603	−0.1362	0.0307	−0.9404
2005	0.0268	0.5813	−0.2648	1.3978	0.1659
2006	0.0334	0.6879	−0.1849	1.7381	0.2696
2007	0.0210	1.0750	0.0361	3.5116	0.5567
2008	0.0372	1.1604	0.0743	1.7569	0.2746
2009	0.0650	0.8925	−0.0568	0.9075	−0.0485
2010	0.0642	1.0264	0.0130	0.5994	−0.2505
2011	0.0578	1.0957	0.0456	0.5127	−0.3221
2012	0.0992	1.0220	0.0109	0.2591	−0.5885
2013	0.1171	0.8289	−0.0935	0.2110	−0.6515
2014	0.1118	0.8638	−0.0731	0.2216	−0.6372
2015	0.1248	1.1481	0.0689	0.2854	−0.5559
2016	0.1235	0.8930	−0.0565	1.0468	0.0229
2017	0.1371	0.8466	−0.0831	2.2171	0.3783
2018	0.1396	0.9152	−0.0443	1.8692	0.3029
2019	0.1409	0.9250	−0.0390	3.9537	0.5963
2020	0.1405	0.8308	−0.0924	6.3661	0.7285
2021	0.1165	1.1029	0.0489	6.5519	0.7352

Source: FAOSTAT (2024); FAS-USDA (2024).

positive value, and few changes. The RCAI reached 0.9912 and 1.1029 in 1994 and 2021, respectively, showing a comparative advantage for Mexican banana exports to the USA. Therefore, this is the most stable market for this Mexican product. The highest RCAI values were recorded in 2002, 2008, and 2015, reaching 1.1668, 1.1604, and 1.1481, respectively (Table 1). The mean RCAI value during the analyzed period was 0.8875. Consequently, Mexican bananas are competitive and have a remarkable comparative advantage regarding the exports to the American market, reaching an export value of US$204,359,000 (FAOSTAT, 2024).

The NRCAI values of the Mexican bananas exported to the USA recorded −0.33 and +0.33. Therefore, this market showed an intra-industry trade or product exchange within the same technological group (Table 1).

The RCAI of Mexican bananas exported to Japan recorded variations throughout the studied period: 0.2413 in 1994 and 6.5519 in 2021 (Table 1). Mexican bananas have increased their share in this competitive market. Nevertheless, the export volume of this market is lower than the export volume of the USA. Nevertheless, Japan is one of the main destinations of Mexican banana exports and has comparative advantages, as a consequence of the increased exports from 2018 to 2021. Therefore, the Japanese market is increasingly attractive to Mexican banana exports. The NRCAI of bananas exported to the Japanese market recorded ups and downs throughout the studied period: it reached values ≈−1 in 1996, 2000, 2001, and 2004. Consequently, this market had a comparative disadvantage. In addition, it recorded values between −0.33 and +0.33 for most of the studied period, indicating an intra-industry trade (Durán and Álvarez, 2008). From 2019 to 2021, the NRCAI values increased from 0.59 to 0.73, suggesting a revealed comparative advantage for banana exports from 2019 (Table 1).

The data obtained showed that Mexico has the potential to be a competitive producer and exporters of bananas. However, Mexico is strongly dependent on the American market, because the USA is the main destination of its exports. Therefore, promoting the search for new market niches of this product is recommended.

CONCLUSIONS

The positive and growing values of the RCAIE and the RCAI of Mexican bananas showed that Mexico is competitive in the international market. Consequently, Mexico is one of the main worldwide exporters of bananas. Banana exports recorded a higher comparative advantage in both the American and Japanese markets. Furthermore, banana exports are competitive and increasing. The USA is a more stable market for the product, where Mexican banana exports are more competitive.

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Colegio de Postgraduados

Yield and nutritional quality of castilla bean (*Vigna unguiculata* L. Walp) under the application of liquid organic fertilizers

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ABSTRACT

Objective: To evaluate the effectiveness of liquid organic fertilizers on *Vigna unguiculata* under field conditions.

Design/methodology/approach: A randomized block design was used with the following treatments: bovine biol (5% and 10%), worm leachate (25% and 50%), and compost tea (20% and 40%), alongside a chemical foliar fertilizer control and a water control. Performance variables assessed included plant height (cm), days to flowering (days), grain yield (g/plant; kg/ha), weight of 100 seeds (g), plant dry matter (kg/ha), NPK content in grains and stems-leaves (%), and crude protein percentage of harvested beans. Most variables were measured at harvest.

Results: The results indicate that bovine biol (5% and 10%) and worm leachate (25% and 50%) were the most effective treatments for grain yield, achieving 640.30 kg/ha, 582.39 kg/ha, 1,519.68 kg/ha, and 509.73 kg/ha, respectively. These yields surpassed those of the chemical treatment (373.77 kg/ha) and the water control (352.49 kg/ha). Additionally, the 25% and 50% worm leachate treatments promoted greater NPK absorption compared to other treatments, resulting in higher crude protein content in the harvested biomass.

Limitations/implications: In the Chontalpa region of Tabasco, Mexico, small-scale farmers have largely abandoned *V. unguiculata* cultivation due to declining yields, primarily caused by poor agronomic management. Liquid organic fertilizers represent eco-friendly alternatives for foliar fertilization, with demonstrated efficacy in various crops, and can partially or fully replace chemical fertilizers.

Findings/conclusions: The findings suggest that applying bovine biol or worm leachate at the evaluated concentrations as foliar fertilizers can enhance both the yield and nutritional quality of *V. unguiculata* compared to chemical fertilization.

Keywords: Field yield, plant nutrition, fertilization, leachate, liquid organic fertilizers, agroecology.

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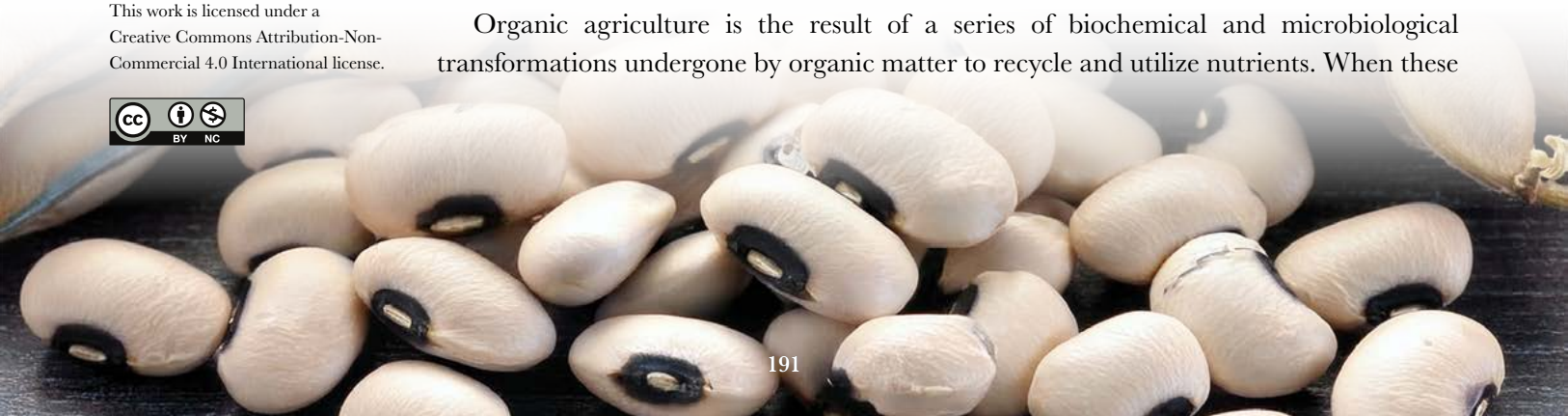
INTRODUCTION

Organic agriculture is the result of a series of biochemical and microbiological transformations undergone by organic matter to recycle and utilize nutrients. When these



processes are managed by humans, one of the byproducts of organic matter recycling is the production of liquid organic fertilizers, such as biols, worm leachates, and compost teas (Soto, 2003). The use of liquid organic materials (biols) is an alternative to meet the nutritional demands of crops due to their high total and ammoniacal nitrogen content and high chemical oxygen demand. Additionally, they can reduce production costs and dependency on mineral fertilizers (Jara-Samaniego *et al.*, 2021). Applying these effluents via foliar spray or soil incorporation increases crop yield and fruit quality while promoting plant health. This is attributed to the presence of beneficial microorganisms capable of suppressing certain crop diseases (Pant *et al.*, 2009). The Castilla bean (*Vigna unguiculata* L. Walp) remains essential to the economy of communities in the La Chontalpa region of Tabasco, Mexico. For the primary sector in this region, it serves as an important source of employment and income, as well as a key element of food security through self-consumption for low-income populations in both urban and rural areas, where it represents their main source of protein (Lagunes-Espinoza *et al.*, 2008; Estrada-Domínguez *et al.*, 2018). This crop provides significant amounts of protein, dietary fiber, carbohydrates, vitamins, and phytochemicals (Devi *et al.*, 2015). Almost the entire *V. unguiculata* plant is utilized: dry grains, green pods, growing tips, and the whole plant as forage. Its yield depends on various anatomical and morphological characteristics, such as the number of pods per branch, pods per plant, seeds per pod, and seed weight (Márquez-Quiroz *et al.*, 2015). The *V. unguiculata* bean is also a critical food source for rural populations in the states of Campeche, Chiapas, Guerrero, Jalisco, Oaxaca, Tabasco, Veracruz, Yucatán, and Tamaulipas (Lagunes-Espinoza *et al.*, 2008; Apáez-Barrios *et al.*, 2011). However, due to the higher commercial value of other bean varieties in the region, the primary use of *V. unguiculata* in La Chontalpa is for self-consumption (Estrada-Domínguez *et al.*, 2018). This study aimed to develop technology for the cultivation of Castilla beans by implementing new strategies for applying liquid organic foliar fertilizers to increase crop yields, maintain crop safety, and reduce the use of chemical inputs that can harm consumer health. Simultaneously, the research seeks to generate technologies for nutrient recycling by reusing raw materials available in the region.

MATERIALS AND METHODS

Description of the study area

The present study was conducted at the experimental field of the Tabasco Campus of the Colegio de Postgraduados, located at 18° 01' N and 93° 03' W, 21 kilometers from the city of Cárdenas along Federal Highway 180 toward Coatzacoalcos. The region has a humid tropical climate, classified as Am(g)"w" according to the Köppen system modified by García (1988), with abundant rainfall in summer and a prolonged dry season during March and April, accompanied by "nortes" (cold fronts) at the end of the year. The average annual temperature is 26 °C, with minimal variation. In the municipality of Cárdenas, the average annual rainfall is 2,324 mm. During the dry months (March and April), less than 50 mm of rain falls per month, while in the rainiest months (September and October), monthly precipitation reaches approximately 400 mm. The average annual evaporation is 1,400 mm (Rivera-Hernández *et al.*, 2016). The Figure 1 shows the

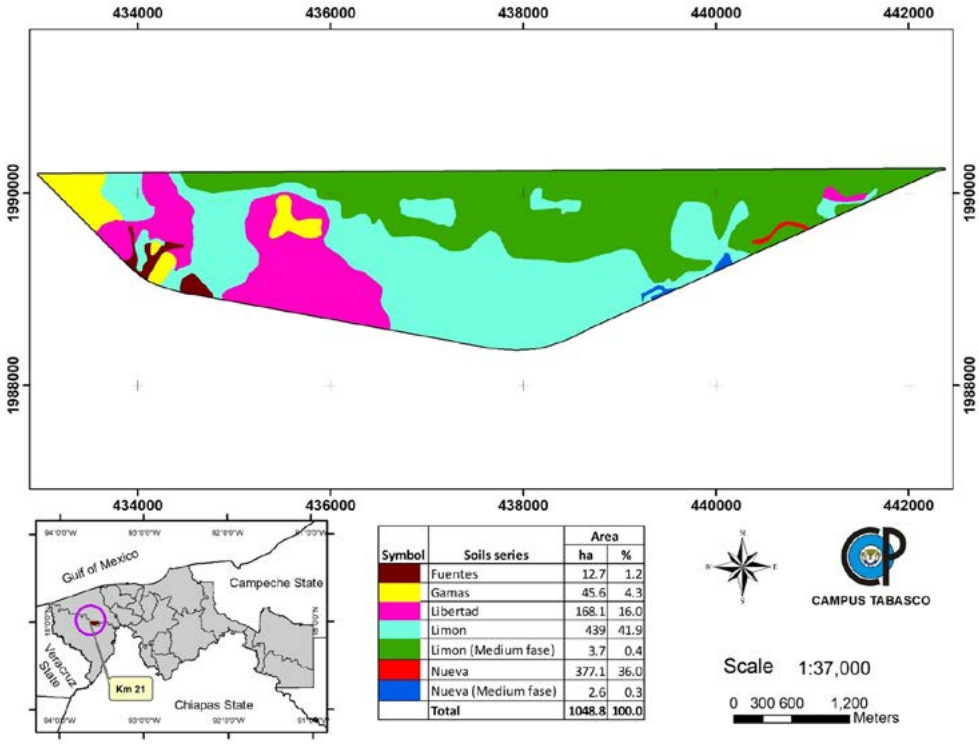


Figure 1. Representation of the soil series in the vicinity of the experimental field of the Colegio de Postgraduados Campus Tabasco. Source: Own elaboration.

soil series present in the experimental field. The study site corresponds to the Libertad soil series, classified as Cambisol Eutric in the World Reference Base for Soil Resources (Palma-López *et al.*, 2017).

The experiment was conducted on soil with a history of crop rotation, including cassava and legumes, used for green manure purposes. The soil was prepared using two passes of a heavy harrow in a cross pattern, followed by two passes of a light harrow and furrowing with 1.3 m spacing between rows. At the start of the experiment, a composite soil sample was collected using a Dutch auger at a depth of 0-30 cm. The composite sample consisted of 15 subsamples taken in a zig-zag pattern, covering the entire area designated for cultivation (Salgado-García *et al.*, 2013). The sample was mixed until homogeneous, dried in the shade on a plastic tray, manually ground, and sieved through a 2 mm (mesh 10) steel sieve. It was then stored in a plastic bag, ready for the following analyses: pH in water (1:2 ratio) using the potentiometric method, organic matter (OM) via the Walkley and Black AS-07 method, soil texture determined using the AS-09 method, cation exchange capacity (CEC) using the AS-12 method with ammonium acetate, electrical conductivity (EC) using the potentiometric method, total nitrogen (Nt) analyzed by the micro-Kjeldahl method (Bremner, 1965), phosphorus (P) by the Olsen method, and exchangeable potassium (K), calcium (Ca), magnesium (Mg), and sodium (Na) using the AS-12 method with ammonium acetate, measured by atomic absorption spectroscopy (Perkin Elmer 400). All methodologies followed the recommendations of NOM-021-RECNAT-2000 (DOF, 2002).

Study variables, experimental design and treatments

An experiment was established using a randomized block design with a total of eight treatments, each with four replications. The treatments included: biol at 5% (B5), biol at 10% (B10), worm leachate at 25% (WL25), worm leachate at 50% (WL50), compost tea at 25% (CT25), compost tea at 50% (CT50), chemical control (CC), and absolute control (AC). The chemical control consisted of a foliar application of Grow Feed® (20-30-10 NPK), while the absolute control involved a foliar application of water. Treatments were randomly distributed using R Studio 3.4.1 statistical software. Each experimental unit consisted of 25 *Vigna unguiculata* (Castilla bean) plants in a plot area of 6.25 m^2 (2.5 m×2.5 m), at a density of 40,000 plants/ha, equivalent to 50×50 cm spacing between plants. Treatments were applied foliarly every 15 days, for a total of six applications. To standardize experimental units, a basal fertilization of 00-14-14 (N-P-K) was applied 15 days after germination (DAG). Each experimental unit was composed of 25 plants, and border plants were used to avoid contamination from neighboring treatment applications. Although treatments were applied to all 25 plants, only the 9 central plants constituted the usable plot.

The comparative variables of the experiment were: plant height in cm (PH), days to flowering (DF), grain yield in g/plant and kg/ha (GY), weight of 100 seeds in g (SW), dry matter in kg/ha (DM), and nitrogen, phosphorus, and potassium content in grains and stems-leaves (%) (NPK%). These variables were measured at harvest. At the end of the Castilla bean crop cycle, 100 g of grain along with 3 plants from each replication of each treatment were collected to estimate nutrient extraction. The collected samples were stored in paper bags and dried in an oven at 65 °C for 72 hours. The dried samples were pulverized using a Wiley LABORATORY MILL, Model 4, equipped with a 1 mm sieve, to prepare them for nutrient quantification. For nitrogen and phosphorus, the Semi-micro Kjeldahl and Vanadium-Molybdate methods were used, respectively. Potassium was determined after digestion and analyzed using an Atomic Absorption Spectrophotometer (Perkin Elmer 400). The collected data were recorded in Microsoft Excel (Office 365, Microsoft Corporation, USA).

Statistical analysis

The data were organized to evaluate the assumptions of normality (Shapiro-Wilk) and homoscedasticity (Levene), which were met. The data were then subjected to an analysis of variance (ANOVA) using the general linear model procedure for the completely randomized block design. To determine the best treatments from a statistical perspective, the treatment means were subjected to Tukey's multiple mean comparison test ($\alpha=0.05$). All statistical analyses were performed using R Studio 3.4.1 (Posit, USA).

RESULTS AND DISCUSSION

The Table 1 shows the results of the physical and chemical analyses of the soil samples from the Castilla bean experiment. The soil was found to have a moderately acidic pH. Total nitrogen, organic matter, potassium, and cation exchange capacity were at medium levels, while phosphorus and calcium were at high levels, as per NOM-021-RECNAT-2000 (DOF, 2002). The soil texture was classified as loam, which is considered

Table 1. Results of soil analysis from the experimental area for the Castilla bean crop. Electrical conductivity (EC), bulk density (BD), organic matter (OM), cation exchange capacity (CEC).

Parameter	pH (H_2O)	EC	BD	P Olsen	K	Ca	Mg	Na	CIC	MO	N total	Clay	Silt	Sand	Classification
	rel. 1:2	dS m^{-1}	g cm^{-3}	mg kg^{-1}	Cmol(+) kg^{-1}					%					Texture
Experimental Field Km 21 Depth 0-30 cm	5.6	0.05	1.37	12.78	0.49	15.21	0.47	0.06	27.48	3.1	0.12	21	1	48	Loam

suitable for bean cultivation, and the bulk density indicated slight compaction issues (Salgado-García *et al.*, 2013).

Visually, the treatments with liquid organic fertilizers, compared to the chemical and water treatments, exhibited less stress due to changes in temperature and humidity. The plants remained more vigorous during high temperatures and rainy days, were more resistant to wind gusts, and showed faster recovery from damage caused by cultural practices and treatment applications. This aligns with Jara-Samaniego *et al.* (2021), who reported that the microorganisms produced during the preparation of biols may be responsible for chemical reactions that enable plants to withstand adverse environmental conditions. Figure 2 shows the growth in height of the Castilla bean plants over time.

The plants germinated seven days after sowing, and data were collected biweekly starting on day 15 after germination (DAG). During the first 30 days, growth was exponential across all treatments. At this stage, the plants reached their maximum vegetative growth

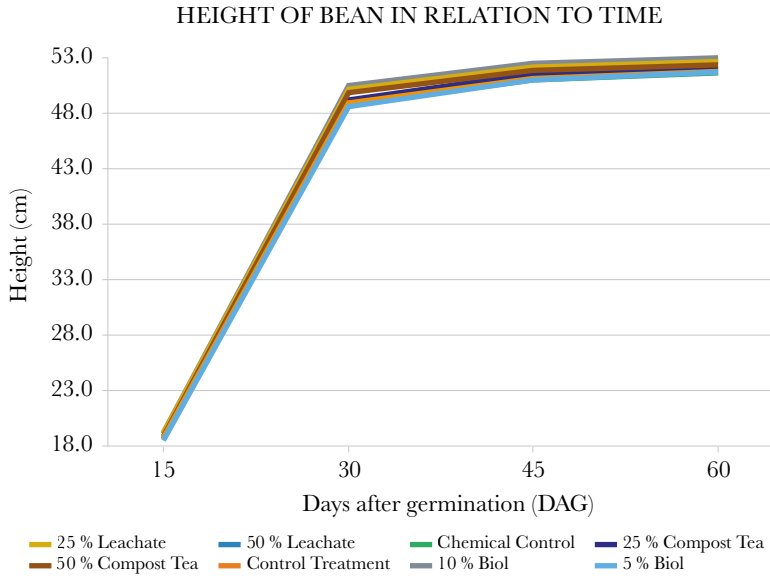


Figure 2. Height (cm) of Castilla bean plants in relation to treatments over time (DAG=Days After Germination). Source: Own elaboration.

and began their flowering process, consistent with the findings of Guillén-Molina *et al.* (2016), who reported that flowering in Castilla beans occurs between 44.16 and 53.16 days. At 60 days after germination, the maximum average plant height was 57.4 cm, while the plant vines reached lengths of up to 100 cm.

The population of pests and the incidence of pathogens varied across treatments, with the control and chemical treatments being the most affected. All treatments showed the presence of insects such as the fall armyworm (*Spodoptera albula*), cucumber beetle (*Cerotoma facialis*), green leafhopper (*Empoasca kraemeri*), black aphid of legumes (*Aphis craccivora*), and pod weevils (*Trichapion godmani* and *Trichapion auricalcium*). The application of bovine biol had a greater effect on the resistance of the beans to insect attacks, similar to the findings of Huallpa *et al.* (2016) and Cruz *et al.* (2021) for oats and forage grasses, respectively. Meanwhile, the application of compost tea appeared to enhance resistance to bacterial and fungal pathogens, as no diseases were observed in plants sprayed with this liquid fertilizer. This aligns with Ronga *et al.* (2021), who noted that compost tea has been used to prevent diseases both through foliar spraying and direct substrate application, resulting in vigorous and resilient crops.

The application of organic fertilizers such as biol, leachates, and compost tea had positive effects on inducing flowering between 30 and 45 days after germination (DAG). For this effect to be considered, at least 50% flowering in the treatments was required. The treatment with 5% biol induced flowering at 30 DAG across all four replications, followed by 10% biol, which initiated flowering at 34 DAG, both considered early flowering treatments. The water control treatment showed the latest flowering, occurring at 48 DAG, an 18-day delay compared to 5% biol. All treatments, except the water control, fell within the suggested range of 27-45 days for on-time flowering (Estrada-Domínguez *et al.*, 2018).

Table 2 shows the statistical differences for each yield variable used to evaluate the performance of Castilla bean under applications of liquid organic fertilizers. For variables such as the number of pods, grain yield (g/plant and kg/ha), and weight of 100 seeds, no significant statistical differences were found between treatments. However, the 5% biol treatment achieved the highest values, with 16.0 g/plant and 640.3 kg/ha, particularly when compared to the absolute control (water), which had a yield of 352.49 kg/ha, representing a difference of 287.81 kg/ha. Regarding biofortification of *V. unguiculata* with iron and zinc, Guillén-Molina *et al.* (2016) reported grain yield values per plant across all treatments ranging from 8.28 g/plant to 14.45 g/plant. These results were similar to those obtained with leachates, compost tea, and controls in this experiment, but lower than those achieved with biol treatments, highlighting that bovine manure biol may have a positive effect on grain yield. The weight of 100 seeds varies when compared to the ecotypes of *V. unguiculata* found in the region, such as: chickpea bean (19.12 g); "sin tiempo" bean (11.25 g and 13.67 g); "pelón criollo" bean (11.77 g); cowpea bean (12.41 g); Castilla bean with brown hilum (10.26 g); Castilla cow bean (18.11 g); and Castilla bean (17.56 g) (Lagunes-Espinoza *et al.*, 2008). In this study, the average weight of 100 seeds across all treatments ranged from 11.10 g to 12.83 g.

These results were intermediate compared to those reported in a collection for the registration of genetic diversity in beans from the Chontalpa region (Lagunés-Espinoza

Table 2. Grain yield and dry matter of Castilla bean (*V. unguiculata*) at the conclusion of the experiment. CV=Coefficient of Variation; Pr(>F)=Significance Value; NS=Not Significant; <.0001=Highly Significant, Tukey p≤0.05.

Treatments	No. of pods per plant	Grain yield (g plant^{-1})	Dry grain yield (kg ha^{-1})	Dry grain yield (kg ha^{-1})	Dry Vegetable Matter (g plant-1)	Aboveground biomass (kg ha-1)
5% Bovine Biol	13^{a}	16.0^{a}	640.30^{a}	11.57	6.68^{a}	267.00^{a}
10% Bovine Biol	15^{a}	14.56^{a}	582.39^{a}	12.05^{a}	6.80^{a}	271.66^{a}
25% Worm Leachate	17^{a}	12.99^{a}	519.68^{a}	12.60^{a}	6.90^{a}	276.11^{a}
50% Worm Leachate	11^{a}	12.74^{a}	509.73^{a}	12.68^{a}	6.14^{ab}	206.11^{bc}
25% Compost Tea	15^{a}	12.59^{a}	503.68^{a}	11.45^{a}	6.25^{ab}	250.11^{ab}
50% Compost Tea	12^{a}	11.46^{a}	458.66^{a}	12.08^{a}	5.64^{b}	225.67^{ab}
Chemical Foliar Fertilization (Grow Feed®)	18^{a}	9.34^{a}	373.77^{a}	12.83^{a}	6.14^{ab}	245.67^{ab}
Absolute Control	11^{a}	8.81^{a}	352.49^{a}	11.10^{a}	4.03^{c}	161.22^{c}
Mean	13.84	12.31	492.59	12.04	5.95	237.94
CV	47.02	40.90	40.90	9.54	9.25	9.25
Pr(>F)	0.674 NS	0.503 NS	0.503 NS	0.338 NS	<.0001	<.0001

et al., 2008; Apáez-Barrios *et al.*, 2011). Differences were observed in the dry matter variable, where the treatments with 10% biol, 5% biol, and 25% worm leachate showed the highest values, with 6.68 g/plant, 6.68 g/plant, and 6.90 g/plant, respectively. These values corresponded to yields of 271.66 kg/ha, 267.00 kg/ha, and 276.11 kg/ha, as shown in Table 2. The 50% worm leachate and absolute control treatments recorded the lowest values compared to the other treatments.

The nutritional quality of crops is a crucial parameter for human nutrition, as they provide proteins, vitamins, minerals, and essential compounds. Table 3 presents the macronutrient concentrations found in grains and aerial biomass (stems and leaves) of

Table 3. Nutrient content of N, P, and K expressed as a percentage in grains and aerial biomass of the Castilla bean crop. CV=Coefficient of Variation; Pr(>F)=Probability of F; NS=Not Significant; <0.01*=Highly Significant; (Tukey <0.05).

Treatments	Nutrient Concentration in Grains			Nutrient Concentration in Stem-Leaf Biomass		
	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)
5% Bovine Biol	3.19^{d}	0.48^{de}	1.19^{e}	2.46^{d}	0.33^{d}	1.08^{cd}
10% Bovine Biol	3.28^{cd}	0.54^{e}	1.22^{e}	2.62^{c}	0.36^{cd}	1.15^{bc}
25% Worm Leachate	3.39^{bc}	0.64^{bc}	1.45^{c}	2.71^{bc}	0.46^{b}	1.23^{b}
50% Worm Leachate	3.59^{a}	0.74^{a}	1.74^{a}	2.83^{a}	0.58^{a}	1.49^{a}
25% Compost Tea	3.30^{cd}	0.60^{c}	1.35^{d}	2.69^{c}	0.43^{bc}	1.22^{b}
50% Compost Tea	3.50^{ab}	0.67^{b}	1.57^{b}	2.78^{ab}	0.49^{b}	1.43^{a}
Grow Feed® (20-30-10 NPK)	3.00^{e}	0.45^{ef}	1.35^{d}	2.41^{d}	0.29^{de}	1.16^{bc}
Absolute Control (Water)	2.87^{f}	0.39^{f}	1.01^{f}	2.30^{e}	0.25^{e}	1.06^{d}
Mean	3.26	0.56	1.36	2.60	0.40	1.23
CV	1.50	4.50	2.67	1.46	7.54	2.99
Pr(>F)	0.001**	0.001**	0.001**	0.001**	<0.001**	0.001**

the Castilla bean crop. The treatment with 50% worm leachate stood out with the highest concentrations of NPK in grains: 3.59% for N, 0.74% for P, and 1.74% for K. This was followed by the 25% worm leachate and 50% compost tea treatments. The absolute control treatment recorded the lowest concentrations of N, P, and K, with 2.87%, 0.39%, and 1.01%, respectively.

In this study, the nitrogen content under liquid organic fertilizer applications ranged from 3.19% to 3.59%, equivalent to 17.38% and 19.56% crude protein, using the conversion factor of 5.45 proposed by Muranaka *et al.* (2016) for *V. unguiculata*. These values fall within the nitrogen content ranges reported for *V. unguiculata* grain by Muranaka *et al.* (2015) and Guillén-Molina *et al.* (2016), which ranged from 3,150 to 3,730 mg/100 g, equivalent to 3.15% to 3.373%, and 17.2% to 20.3% crude protein, respectively.

It has also been reported that protein content is negatively correlated with grain yield (Olunike, 2014), which was observed in this study. The 10% bovine biol and 5% bovine biol treatments had the highest grain yields but lower crude protein (nitrogen) content, as shown in Table 4. Crude protein (CP%) is calculated by multiplying the total nitrogen content of a food by the factor 5.45, as proteins contain, on average, 18.35% nitrogen. The factor 5.45 derives from the ratio 100/18.35. The CP value includes both true protein and other non-protein nitrogenous compounds, obtained via the Kjeldahl method (Muranaka *et al.*, 2016). Guillén-Molina *et al.* (2016) reported that phosphorus

Table 4. Nitrogen and crude protein percentage implemented with the conversion factor of 5.45 of Muranaka *et al.*, (2016) for *V. unguiculata*. Uneven letters in the columns indicate significant differences ($p<0.05$).

Treatments	Nutrient Concentration in Grains		
	Grain yield (kg ha^{-1})	Nitrogen (%)	Raw Protein (%)
5% Bovine Biol	640.30^{a}	3.19^{d}	17.35^{e}
10% Bovine Biol	582.39^{a}	3.28^{cd}	17.85^{de}
25% Worm Leachate	519.68^{a}	3.39^{bc}	18.47^{bc}
50% Worm Leachate	509.73^{a}	3.59^{a}	19.55^{a}
25% Compost Tea	503.68^{a}	3.30^{cd}	17.98^{cd}
50% Compost Tea	458.66^{a}	3.50^{ab}	19.06^{ab}
Grow Feed® (20-30-10 NPK)	373.77^{a}	3.00^{e}	16.34^{f}
Absolute Control (Water)	352.49^{a}	2.87^{f}	15.62^{g}
Treatments	**Nutrient Concentration in Stem-Leaf Biomass**		
	Stem-leaf biomass yield (kg ha^{-1})	Nitrogen (%)	Raw Protein (%)
5% Bovine Biol	267.00^{a}	2.46d	13.38^{e}
10% Bovine Biol	271.66^{a}	2.62^{c}	14.27^{d}
25% Worm Leachate	267.00^{a}	2.71^{bc}	14.76^{bc}
50% Worm Leachate	206.11^{bc}	2.83^{a}	15.40^{a}
25% Compost Tea	250.11^{ab}	2.69^{c}	14.64^{cd}
50% Compost Tea	225.67^{ab}	2.78^{ab}	15.18^{ab}
Grow Feed® (20-30-10 NPK)	245.67^{ab}	2.41^{d}	13.09^{e}
Absolute Control (Water)	161.22^{ab}	2.30^{e}	12.50^{f}

content in *V. unguiculata* grains ranged from 56 to 90 mg/100 g of grain, equivalent to 0.056% to 0.09%. These values are far below the averages of all treatments in this study, which ranged from 0.39% for the absolute control to 0.74% for the 50% worm leachate treatment (Table 3).

Potassium (K) levels in liquid organic fertilizer treatments ranged from 1.19% to 1.74% in Castilla bean grains, values that fall within the ranges reported for *V. unguiculata* by Márquez-Quiroz *et al.* (2015) and Guillén-Molina *et al.* (2016), which varied between 1,180 and 1,520 mg/100 g of grains, equivalent to 1.18% and 1.52% K. These same ranges have remained stable over 13 years (Frota *et al.*, 2008). For nutrient concentrations in aerial biomass (stems and leaves), the 50% worm leachate treatment showed the most significant differences, with 2.83% N, 0.58% P, and 1.49% K, followed by the 50% compost tea treatment. The 5% and 10% bovine biol treatments showed intermediate values compared to all treatments, while the absolute control treatment consistently showed the lowest nutrient percentages. Table 5 presents the nitrogen, phosphorus, and potassium composition (in kilograms) in the total aerial biomass of Castilla bean, including grains, stems, and leaves. Estimating nutrient extractions of nitrogen, phosphorus, and potassium is crucial to understanding the crop's requirements. For NPK in grains, no statistical differences were observed across treatments. However, the 5% biol treatment recorded the highest nitrogen value at 20.41 kg N/ha, while the absolute control showed the lowest value at 10.10 kg N/ha. For phosphorus in grains, the 50% worm leachate treatment had the highest value (3.78 kg P/ha), while the absolute control recorded the lowest (1.37 kg P/ha). For potassium, the 50% worm leachate treatment again showed the highest value, at

Table 5. Extraction of kg ha^{-1} of N, P, and K in grain and aerial biomass of castilla bean. CV=Coefficient of Variation; Pr(>F)=Probability of F; NS=Not Significant; <0.01**=Highly Significant; (Tukey <0.05).

Treatments	Nutrient Extraction in Grains (kg ha^{-1})				Nutrient Extraction in Stem-Leaf Biomass (kg ha^{-1})				Total Nutrient Extraction (kg ha^{-1})		
	Grain yield (kg ha^{-1})	N	P	K	Biomass yield (kg ha^{-1})	N	P	K	N	P	K
5% Bovine Biol	640.30^{a}	20.41^{a}	3.07^{a}	7.64^{a}	267.00^{a}	5.55^{ab}	0.74^{cd}	2.43^{a}	26.88^{a}	3.93^{ab}	10.47^{ab}
10% Bovine Biol	582.39^{a}	19.09^{a}	3.12^{a}	7.12^{a}	271.66^{a}	7.12^{ab}	0.98^{bcd}	3.13^{a}	26.28^{a}	4.06^{ab}	10.17^{ab}
25% Vermicompost Leachate	519.68^{a}	17.62^{a}	3.34^{a}	7.55^{a}	267.00^{a}	7.24^{a}	1.23^{a}	3.28^{a}	25.12^{a}	4.65^{a}	10.89^{a}
50% Vermicompost Leachate	509.73^{a}	18.30^{a}	3.78^{a}	8.87^{a}	206.11^{bc}	6.95^{b}	1.42^{ab}	3.65^{a}	24.14^{a}	4.97^{a}	11.89^{a}
25% Compost Tea	503.68^{a}	16.61^{a}	3.00^{a}	6.79^{a}	250.11^{ab}	5.54^{ab}	0.89^{abc}	2.52^{a}	23.34^{a}	4.06^{ab}	9.80^{ab}
50% Compost Tea	458.66^{a}	16.05^{a}	3.05^{a}	7.26^{a}	225.67^{ab}	6.96^{ab}	1.21^{abc}	3.56^{a}	22.27^{a}	4.15^{ab}	10.49^{ab}
Grow Feed® (20-30-10 NPK)	373.77^{a}	11.21^{a}	1.66^{a}	5.06^{a}	245.67^{ab}	6.64^{b}	0.80^{d}	3.20^{a}	17.12^{a}	2.37^{ab}	5.28^{c}
Absolute Control (Water)	352.49^{a}	10.10^{a}	1.37^{a}	3.55^{a}	161.22^{ab}	3.70^{c}	0.39^{e}	1.71^{b}	13.84^{a}	1.81^{b}	5.26^{c}
Mean	492.59	16.18	2.8012	6.69	237.94	6.19	0.94	2.91	22.37	3.75	9.60
CV	40.90	40.77	40.01	39.30	9.25	9.40	12.14	2.99	28.64	29.83	26.53
Pr(>F)	0.503 NS	NS	NS	NS	0.001**	0.001**	0.001**	0.001**	NS	0.0064**	0.0338 *

8.87 kg K/ha. For stem-leaf biomass, the 25% worm leachate treatment showed the most significant differences, with 7.24 kg N/ha and 1.23 kg P/ha, statistically different from the chemical and absolute control treatments. Regarding potassium in stem-leaf biomass, the absolute control treatment had the lowest yield at 1.71 kg K/ha, while all other treatments were statistically similar in potassium content for stem-leaf biomass.

Regarding the total nutrient concentration, no statistical differences were observed for nitrogen across treatments, but the bovine biol treatments at 5% and 10% showed the highest values with 26.88 kg N ha^{-1} and 26.28 kg N ha^{-1}, respectively, while the absolute control treatment had the lowest value with 13.84 kg N ha^{-1}. Phosphorus and potassium showed a similar trend, with the control treatment differing the most from the others. The vermicompost leachates at 25% and 50% had the highest phosphorus values, with 4.65 kg P ha^{-1} and 4.97 kg P ha^{-1}, respectively, while the vermicompost leachate at 50% and bovine biol at 5% had the highest potassium values, with 11.89 kg K ha^{-1} and 10.47 kg K ha^{-1}, respectively. This corresponds to the sum of the results for each NPK repetition in grains and NPK in stem-leaf biomass. Espinosa *et al.* (2000) conducted studies on the collection of legume diversity in the state of Tabasco with the aim of preserving culinary culture. However, since 2008, there have been no studies documenting local knowledge on the production and conservation of native grain legume varieties in the region (Lagunes-Espinoza *et al.*, 2008). Species of the genus *V. unguiculata* ranked second among legumes consumed in the region. The reported native varieties include "criollo negro," "Castilla," and "sin tiempo" (Lagunes-Espinoza *et al.*, 2008). Unfortunately, despite these native varieties achieving higher yields, small producers are abandoning them in favor of more commercial crops with lower yields for the region (Lagunes-Espinoza *et al.*, 2008; Delgado-Salinas *et al.*, 2021). The results of this study could help increase the use and production of this regional bean in La Chontalpa, Tabasco, Mexico; however, further studies focused on its nutritional qualities are needed to promote its consumption among local producers, as is done with common beans in other regions of Mexico (Florvil *et al.*, 2022).

CONCLUSIONS

Based on the grain yield and stem-leaf biomass, it is concluded that bovine manure biol treatments at 5% and 10% and vermicompost leachate at 25% and 50% are the best liquid organic nutrition alternatives for the Castilla bean crop. These treatments showed better field performance, growth, and nutritional quality compared to the chemical treatment and did not result in significant infections or damage from pest insects or diseases. The results suggest a positive impact on the yield of Castilla beans in La Chontalpa, Tabasco, Mexico, offering an agroecological alternative for managing this crop with lower impact on cultivated soils and reduced chemical fertilizer expenses.

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Colegio de Postgraduados

Association between *Anaplasma marginale* and semen quality in bulls

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ABSTRACT

Objective: to determine the association between the presence of *Anaplasma marginale* and sperm quality variables, including sperm concentration and percentage of individual motility.

Design/methodology/approach: A total of 92 animals from the Costa Chica region of Guerrero state, in Mexico, were evaluated. All animals were over 16 months of age, in good health and classified according to their origin and breed group. Semen samples were collected through an electroejaculation. Sperm concentration (SCo) and percentage of individual motility (%IM) were measured. Additionally, blood samples were collected via coccygeal vein puncture. The diagnosis of *A. margninale* was made by determination of antibodies. To analyze the association between *A. marginale* and other risk factors, a logistic model was used, with %IM and SCo as the dependent variables.

Results: More than 60% of the animals evaluated were from the municipality of Ometepec. Zebu-type cattle predominated, 75% of the sample was included in this category, followed by crossbred animals (Zebu×European). The incidence of *A. marginale* reached 50%. Logistic regression analysis indicated that origin and racial group are risk factors ($p<0.05$), whereas *A. marginale* is not a determinant ($p>0.05$) of seminal quality. However, its presence reduces the possibility of obtaining a sperm concentration and motility percentage suitable for reproductive function.

Limitations on study/implications: With Zebu×European crossbred animals, the possibility of males presenting better seminal characteristics increases.

Findings/conclusions: *A. marginale* is not a determining factor that modifies seminal characteristics in bulls.

Keywords: anaplasmosis, motility, sperm quality, ticks, tropics.

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INTRODUCTION

In the selection of breeding males, it is essential to evaluate semen characteristics alongside phenotypic traits such as live weight or breed type (Butler *et al.*, 2020). According

to Torrado *et al.* (2016), the reproductive fitness of sires should be assessed when they are acquired, before the mating season begins, or during the breeding period if there is suspicion of reproductive failure attributable to the sire. However, this practice is not widely adopted and is mainly applied to sires intended for semen collection and cryopreservation for straw production, as well as to bulls selected for sale as breeders (Páez-Barón & Corredor-Camargo, 2014). As a result, at least 20% of bulls in non-selected populations are classified as subfertile and are unable to provide acceptable service or exhibit poor semen quality (Torrado *et al.*, 2016).

Regardless of whether the sire is used for semen collection for cryopreservation or under a natural mating system, the evaluation must include three aspects: a clinical examination, libido assessment, and semen quality evaluation (Páez-Barón & Corredor-Camargo, 2014). Specifically, in the case of semen quality, the assessment should consider factors such as color, volume, motility, morphology, viability, and sperm concentration. However, according to Boggio (2007), it is also important to conduct blood sampling to rule out the presence of infectious diseases that may affect reproduction, such as bovine viral diarrhea, brucellosis, infectious bovine rhinotracheitis, leptospirosis, neosporosis, among others.

The tropical region, which includes the Costa Chica region of the state of Guerrero, is known for its livestock activity under dual-purpose systems (meat and milk production). However, this can be affected by nutritional factors, viral agents, parasitic, and infectious agents (Avilés-Ruíz, 2022), which negatively impact reproductive factors, leading to economic losses for producers. This region is representative of tropical and subtropical areas where the presence of *Anaplasma marginale* causes economic losses (Olvera, 2021). *A. marginale* is a type of Rickettsia that parasitizes mature erythrocytes in cattle, causing hemolytic anemia, weight loss, reduced milk production, abortions, and even death (Corona *et al.*, 2014; Cora-Ibarra *et al.*, 2021). Although its effect on semen is not clear, it is believed to reduce semen quality by decreasing motility and sperm concentration. The objective was to determine the association between the presence of *A. marginale* and the percentage of individual motility and sperm concentration in male cattle from the Costa Chica region, under the hypothesis that animals that test positive for *Anaplasma* spp. will have lower sperm concentration and motility compared to negative animals.

MATERIALS AND METHODS

Description of the Study Area

This study was conducted in the municipalities of Cuajinicuilapa (16° 27’ 60” N, 98° 24’ 60” E) and Ometepec (16° 40’ 57” N, 98° 24’ 43” E), both located in the Costa Chica region of the state of Guerrero, Mexico. Both municipalities are situated between 0 and 50 meters above sea level, with a predominantly warm subhumid climate and summer rainfall (1,200 mm of annual average precipitation). Average temperatures range from 22 to 28 °C.

Animals

A total of 92 male cattle, either bulls or potential bulls, all older than 16 months, without defects or pathologies in the reproductive organs, and in good general health, were evaluated. All animals were maintained under a semi-intensive grazing system with access

to mineral salts and water *ad libitum*. At night, the animals were housed in individual pens with access to clean, fresh water. Prior to the start of the study, the animals were dewormed and vaccinated according to regional recommendations.

The animals were classified according to their genetic group as zebu, european, and crossbred animals (Zebu×European) based on their phenotype and information provided by the bull owner. For their evaluation, all animals were placed in a handling chute, without sedation, minimizing any source of stress (Grandin, 2019). The animal management practices and the study design were approved by the Institutional Bioethics Committee of the Universidad Autónoma de Guerrero.

Semen Evaluation

Semen samples were collected using an electroejaculator (Standar Precision, USA). Spermatozoa were observed under a microscope (i4 Infinity, LW Scientific, USA). The percentage of individual motility (%IM) was evaluated using the technique and parameters established by Koziol & Armstrong (2018), and classified as suitable (>60% IM) or unsuitable (<60% IM). Sperm concentration (SCo, millions of spermatozoa mL^{-1}) was evaluated by counting in a Neubauer Bright line chamber following the methodology of the World Health Organization (2021), and categorized as suitable (>250 million spermatozoa mL^{-1}) or unsuitable (<250 million spermatozoa mL^{-1}, Koziol & Armstrong, 2018).

Determination of *Anaplasma marginale*

In the evaluated cattle, a blood sample was taken by puncturing the coccygeal vein using a polypropylene SARSTEDT tube with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (Monovette 4.9). The blood sample was homogenized with slow movements and allowed to rest for 15 minutes at room temperature. It was then stored at 5 °C until processing in the laboratory.

The diagnosis of *A. marginale* was performed through antibody determination. The plate was sensitized with Ag-2012 at 1.07 μg/200 μl of protein/well, and incubated overnight at 4 °C. Afterward, the plates were washed three times with PBS pH 7.2-Tween-20 at 0.05% (PBS-T20) and blocked with 5% skim milk in PBS-T20 pH 7.2 for 30 minutes. After three washes with PBS-T20, the control serum samples, diluted 1:100 in PBS-T20, were run in duplicate or triplicate. The plates were incubated for 1 hour at 37 °C and washed three times with PBS-T20. Rabbit anti-bovine IgG conjugated (Salinas *et al.*, 2022) with alkaline phosphatase (Jackson ImmunoResearch Laboratories, Lot: 112108), diluted 1:10,000 in PBS-T20, was added to each well. The plates were incubated for 60 minutes at 37 °C and washed as described. Finally, p-nitrophenylphosphate at 0.075% in Tris buffer pH 9.5 was added and incubated for 30 minutes at 37 °C.

The plates were read at 405 nm using a microplate absorbance reader (BioRad, iMark™), and optical density (OD) values were recorded. Two wells containing all components except the serum were used as blanks. The average absorbance of these blank wells was subtracted from the absorbance values of all other wells on the plate. The mean and standard deviation of the replicas and the positive and negative serum controls were also calculated.

Statistical analysis

To study the association between *A. marginale* and other risk factors (origin and racial group), the data were analyzed using logistic regression (Hosmer *et al.*, 2013). The dependent variables (SCo and %IM) were analyzed using a binary logistic model, as the responses were considered dichotomous (Y=0, unsuitable; 1, suitable), using the SPSS statistical software (IBM®, 2023).

The model used to analyze the data can be represented as:

$$Y_{ijkl} \sim binary\ dichotomus\ outcome\left(P_{ijkl}\right)$$

$$Logit\left(P_{ijkl}\right) = \beta_0 + \beta_1 origin_j + \beta_2 group_k + \beta_3 presence\ of\ anaplasma_l + e_{ijkl}$$

Where: $Logit\left(P_{ijkl}\right)$=Probability that the *i-th* animal will present a suitable SCo or %IM, given that it is from the *j-th* municipality of origin, belongs to the *k-th* racial group, and shows the *l-th* presence of *A. marginale*; β_0=Independent term; $\beta_1 origin_j$ =Effect of the *j-th* municipality of origin (1=Cuajinicuilapa, 2=Ometepec); $\beta_2 group_k$=Effect of the *k-th* racial group of the individual (1=zebu, 2=european, 3=crossbreed); $\beta_3 presence\ of\ anaplasma_l$=Effect of the *l-th* presence of *A. marginale* (0=healthy individual, 1=individual with *A. marginale*); e_{ijkl}=Residual random effect.

RESULTS AND DISCUSSION

Table 1 shows the relative frequency of the observed values for the evaluated variables. Most of the sampled bulls originated from the municipality of Ometepec. Regarding the racial group, 75% of the sampled animals were zebu, classified by their phenotypic appearance and the information provided by the owners. Due to the tropical-subhumid and monsoonal climatic conditions (Köppen classification Aw) (INEGI, 2020), zebu animals are preferred by producers because of their adaptive characteristics to the adverse conditions of temperature, humidity, and presence of parasites (Rubio *et al.*, 2021).

The incidence of *A. marginale* was 50%, lower than the 67.1% reported by Mattos-Ferreira *et al.* (2022) in Mexico; however, it remains one of the highest in Latin America, comparable to Ecuador, where Muñoz-Guarniz *et al.* (2017) reported an incidence

Table 1. Frequency of independent variables related to seminal characteristics in the Costa Chica region of the state of Guerrero.

Variable	Nivel	(n) %
Origin	Cuajinicuilapa	31 (0.34)
	Ometepec	61 (0.66)
Racial Group	Cebuino	69 (0.75)
	Europeo	6 (0.07)
	Cruzas	17 (0.18)
Anaplasma marginale	Negativo	46 (0.50)
	Positivo	46 (0.50)

of *A. marginale* of 49.5%. The high incidence in both countries may be related to the abundance and poor control of its transmitting agent (Mattos-Ferreira *et al.*, 2022).

The results of the logistic model for SCo and %IM are presented in Tables 2 and 3. The risk factors that might influence the studied variables are more closely related to the municipality of origin and the racial group of the individuals ($p<0.05$), whereas the presence of *A. marginale* was not a significant risk factor for SCo ($p=0.324$) or %IM ($p=0.384$). The seminal quality of the bulls can be affected by multiple causes, both infectious and non-infectious, as well as various environmental factors, breed, age, nutritional factors, stress, and collection system, among others (Bhave *et al.*, 2020, Butler *et al.*, 2020).

There is a negative association between the municipality of origin of the animals and the values of SCo and %IM considering the values of the parameters β_i (-1.855 to -1.940) (Tables 2 and 3). The variable *municipality of origin* had OR values <1, suggesting that the environmental differences between municipalities do not result in a decrease in seminal characteristics, indicating that the municipalities of origin share similar climatological factors (humidity, rainfall, and temperature), as well as management within the production units. On the other hand, a greater availability of sampled animals in Ometepec may be related to the sampling period, which may have coincided with the time of greatest reproductive activity of the bulls, reflecting in the quality of semen samples from recently worked bulls or those with little rest.

In the case of the racial group, the crossbreed between zebu and european cattle is positively associated with SCo and %IM ($\beta_i=1.511$ to 1.903), showing 4.53 to 6.70 (OR) times higher chances of achieving better SCo and %IM characteristics compared to European-type animals ($OR<1$ and $p>0.183$). While the productive traits of european-type animals are desirable in tropical regions, the use of crossbred animals (Zebu×European) can be an option to increase reproductive efficiency. This alternative reduces the negative effect that high temperatures have on european animals, as developing above their thermoneutral zone can lead to alterations in metabolic activity, general health status, and hormonal concentration, ultimately resulting in reproductive deterioration (Façanha *et al.* 2019). Under tropical conditions, bulls of european origin suffer from heat stress, which may be related to thermoregulation failures and cause damage to testicular tissue (Sabés-Alsina *et al.* 2019). This can contribute to the production of heat shock proteins (Rahman *et al.* 2018), affecting spermatogenesis and leading to lower semen quality. The association between heat stress and reduced sperm viability is explained by the production of reactive oxygen species (ROS) in spermatozoa, which can lead to oxidative stress and DNA damage, affecting viability and fertilization capacity. According to studies involving metabolomics and metagenomics, alterations in taurine metabolism reduce the expression of genes linked to oxidative phosphorylation, decreasing the ability to generate adenosine triphosphate (ATP) and provide energy for sperm motility and functionality (Rao *et al.*, 2022). However, in the present study, crossbred animals (Zebu×European) show a higher likelihood of achieving better semen characteristics due to the advantages conferred by crossbreeding —the adaptability of zebu cattle to high temperatures and the greater semen production and quality inherited from european breeds.

Although the presence of *A. marginale* had a negative relationship with SC (β_i=−0.476, OR=0.621, Table 2) and %IM (β_i=−1.561, OR=0.348, Table 3), its effect on these variables was not significant (p>0.324). While there is evidence that *A. marginale* reduces the percentage of normal spermatozoa, sperm motility, and even scrotal circumference in bulls due to the fever and anemia caused by the infection —affecting spermatogenesis both at the cellular and tissue levels (Lovett *et al.*, 2022)— some studies have found no association between the high prevalence of *A. marginale* and the results of breeding soundness examinations (BSE) (Johnson *et al.*, 2018; Jablinski *et al.*, 2023).

Perspectives

Just as the febrile process affects semen viability (Rao *et al.*, 2022), anemia can cause hypoxia in the reproductive tissue, leading to oxidative stress, which may interfere with spermatogenesis and directly with mature spermatozoa (Esmaeilnejad *et al.*, 2018). Therefore, studies considering animals of different breeds in different climatic conditions will help clarify the relationship between the prevalence of *A. marginale* and negative results in BSE examinations, taking into account the underlying environmental mechanisms. It should be considered that bulls that survive *A. marginale* infection may return to their reproductive potential (Lovett *et al.*, 2022; Jablinski *et al.*, 2023). It is recommended to perform a new evaluation at least 61 days after the infection has been controlled, as this is the necessary time to complete the spermatogenesis process (Staub & Johnson, 2018). However, depending on the severity of the disease, a longer period may be needed before

Table 2. Risk factors associated with sperm concentration in bulls from the Costa Chica, Guerrero, Mexico.

Variable	$\gamma\beta$	$SE_{\beta\gamma}$	OR	CI (95%)	*P*
Constant	1.406	0.531	-	-	0.008
Origin	−1.855	0.549	0.157	0.053 - 0.459	<0.001
Racial Group	-	-	-	-	0.024
European	−0.433	0.985	0.649	0.094 - 4.474	0.660
Crossbreed	1.903	0.712	6.703	1.660 - 27.067	0.008
A. marginale	−0.476	0.483	0.621	0.241 - 1.602	0.324

β=Estimator coefficient; SE_β=Standard error of the estimator; OR=Odds ratio; CI=Confidence interval.

Table 3. Risk factors associated with the % of individual motility in bulls from the Costa Chica, the state of Guerrero, Mexico.

Variable	$\gamma\beta$	$SE_{\beta\gamma}$	OR	CI (95%)	P
Constant	1.561	0.583	-	-	0.007
Origin	−1.940	0.616	0.144	0.043 - 0.481	0.002
Racial Group	-	-	-	-	0.043
European	−1.331	1.001	0.264	0.037 - 1.879	0.183
Crossbreed	1.511	0.721	4.532	1.104 - 18.614	0.036
A. marginale	−1.561	0.491	0.348	0.606 - 4.150	0.384

β=Estimator coefficient; SE_β=Standard error of the estimator; OR=Odds ratio; CI=Confidence interval.

performing the semen quality test. Additionally, it should be assessed whether there is damage to the testes and if sperm production is economically viable to decide whether the bull should be slaughtered or retained as a breeder in the production unit. Finally, bulls that survive remain asymptomatic carriers, so it must be considered that they may be a source of infection for other animals in the herd.

CONCLUSIONS

Under the conditions of the present study, the presence of *A. marginale* is not a risk factor that could modify the seminal characteristics of the cattle; however, it does decrease the likelihood of obtaining semen samples with adequate sperm concentration and individual motility percentage for bulls to develop their reproductive function properly.

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Bromatological characterization of three types of semi-industrial chocolates

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ABSTRACT

Objective: To determine the physical and chemical characteristics of three types of semi-industrial chocolate.

Design/Methodology/Approach: Three types of semi-industrial chocolate made from dry fermented cocoa were analyzed in triplicate. Five nutrients were evaluated: total fat, protein, crude fiber, moisture, and carbohydrates. A completely randomized experimental design was applied. Significant differences between treatments were identified using Analysis of Variance (ANOVA) at a 95% confidence level, followed by Tukey's multiple mean comparison test.

Results: Milk chocolate contained 6.37% protein, 48% carbohydrates, 38.99% fat, 3.38% fiber, and 1.55% moisture. Semi-dark chocolate showed 6.83% protein, 36.06% carbohydrates, 50.25% fat, 3.27% fiber, and 1.91% moisture. Dark chocolate exhibited 7.74% protein, 35.51% carbohydrates, 48.42% fat, 3.95% fiber, and 2.29% moisture. Significant differences were observed in protein, fat, carbohydrates, and fiber, except for moisture.

Study Limitations/Implications: The study was limited to a single type of cocoa cultivated under conventional agricultural practices. Consequently, the findings are applicable primarily to producers combining traditional and modern techniques in cocoa cultivation.

Findings/Conclusions: The nutrient analysis of semi-industrial chocolates highlights their nutritional quality, supporting their marketing potential, labeling with origin traceability, and disclosure of nutritional content. Chocolates with higher protein, cocoa fat, and cocoa percentages were identified as nutritious options for consumers.

Keywords: chocolate, characterization, standards, cocoa paste.

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INTRODUCTION

In Mexico, cacao (*Theobroma cacao* L.) is a millenary, ancestral, medicinal, and cultural food that has been intertwined with the traditions and customs of Indigenous peoples in the Americas, including Ecuador, Brazil, Venezuela, Colombia, Peru, and Mexico (Battcock *et al*., 2022; Fins *et al*., 2013). Since pre-Hispanic times, cacao has been used in human nutrition. Following the Spanish arrival in Mexico, cacao consumption expanded to Europe (Huamanchumo, 2017; Coe & Coe, 2013). The *per capita* consumption of chocolate

in Europe is 10 kg, while in Mexico it increased from 600 to 700 g between 2016 and 2023 (Camacho-Gómez, 2017), with milk chocolate being the consumers' favorite due to its flavor and aroma (Arvelo-Sánchez, 2017). Today, consumers seek high-quality chocolates with good nutritional content (Brambila, 2008). Chocolate with a higher cocoa content, at least 70%, is considered beneficial to human health (Córdova *et al.*, 2023). However, micro-enterprises producing chocolate face several limitations in their production processes, such as challenges accessing markets, lack of nutritional labeling for their products, and insufficient training in technological innovation for adding value. There is also a lack of financing, subsidies, and specialized advice for developing individual and collective brands. These factors highlight the need for bromatological analysis to comply with regulatory standards (Martínez-Salvador & Martínez-Salvador, 2020). Considering these challenges, the aim of this research is to analyze the nutritional content of the chocolates produced at the chocolate school/factory of the Colegio de Postgraduados Campus Tabasco, with the aim of transferring this innovation model to cacao producers in Tabasco.

MATERIALS AND METHODS

Location of the study area

The research was carried out in the chocolate school laboratory of the Tabasco Campus, College of Postgraduates, located in the Ranchería Río Seco second section of Huimanguillo, Tabasco (17° 58' 34" N, 93° 23' 11" W).

Preparation process of the three types of chocolates

The cocoa was purchased from a producer in the José María Morelos y Pavón Ejido, located in Cárdenas, Tabasco. Its quality was determined based on NMX-FF-118, 2014, using a cut test conducted at the chocolate school, classifying it as extra-fine cocoa, fermented and sun-dried, of the Patastillo variety (NMX-FF-118, 2014; Aguilar, 2016). The moisture content was 6%, measured with a DICKEY-John (GAC500 XT, United States) device.

The cocoa beans were selected by removing impurities, broken beans, insect-damaged beans, moldy beans, and beans without cotyledons (CXS 87-1981, 2022). The selected cocoa was roasted in a Micron 5 kg roaster. The grinding and shelling process was carried out using a CITLALI sheller (CACDES003, Mexico). The grinding was performed with a Pulvex 9 Hp mill (200, Mexico), resulting in cocoa paste, which was stored in a cooler at 5° C for use the following day. For the preparation of the chocolate types used in the bromatological characterization, a completely randomized design with three repetitions was applied. The first treatment consisted of milk chocolate with 30% cocoa paste; the second treatment was semi-dark chocolate with 50% cocoa paste; and the third treatment was dark chocolate with 70% cocoa paste. The treatments were prepared according to the proportions of fat, milk, sugar, cinnamon, and vanilla (Table 1).

Each sample was refined, tempered, molded, crystallized. The crystallized bars and tablets were disassembled for wrapping, bagging, sealing, labeling and coding for our analysis.

Table 1. Semi-industrial chocolate treatments.

Type of semi-industrial chocolate			
Ingredients	**T1 With milk**	**T2 Semi-bitter**	**T3 Bitter**
Cocoa paste	30%	50%	70%
Brown sugar	28%	30%	20%
Cocoa butter	20%	20%	10%
Whole milk powder	20%	-	-
Natural vanilla extract	2%	-	-

Methods and techniques of analysis

The techniques used to measure the five attributes were as follows: total fats were determined using the SOXHLET method (NMX-F-615-NORMEX-2004); protein content was measured using the assay method (NMX-F-608-NORMEX-2011); crude fiber was analyzed using the general test method (NMX-F-613-NORMEX-2017); moisture was determined through the desiccation method (NMX-F-030-1985); and carbohydrate content was calculated.

Statistical analysis: model

The analysis model for the completely randomized design was as follows:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

Where: Y_{ij} represents the observation in the i-th experimental unit assigned to the j-th treatment. μ is the general or overall mean of all observations. τ_i is the effect of the i-th treatment. ε_{ij} is the random error or residual, representing the variation not explained by the treatments.

Hypothesis

H_0: $T_1=T_2=...=T_9$, meaning there is no interaction between the effect of cocoa concentration on the nutrients in chocolate.

H_1: $Ti \neq Tj$, meaning there is interaction, indicating that different cocoa concentrations result in varying nutrient levels. The analyzed nutrients included protein, carbohydrates, fats, fiber, and moisture. An Analysis of Variance (ANOVA) was conducted at a 95% confidence level, followed by Tukey's mean comparison test to identify significant differences in the nutritional content among the types of chocolate.

RESULTS AND DISCUSSION

Of the analyzed nutrients, milk chocolate (30% cocoa) contained 6.37% protein, 48% carbohydrates, 38.99% fat, 3.38% fiber, and 1.55% moisture. Semi-dark chocolate (50% cocoa) had 6.83% protein, 36.06% carbohydrates, 50.25% fat, 3.27% fiber, and 1.91% moisture. Dark chocolate (70% cocoa) showed 7.74% protein, 35.51% carbohydrates, 48.42% fat, 3.95% fiber, and 2.29% moisture. Significant differences were observed in

protein, fats, carbohydrates, and fiber, while no significant differences were found in moisture (see Table 2).

Protein: As observed in terms of protein, the null hypothesis was rejected, and H_1 was accepted, as the p-value (0.05) was similar to the α value. The results showed a gradual increase in protein content with higher cocoa concentrations, with dark chocolate (70% cocoa) having the highest protein content. Protein values ranged between 6% and 7%, aligning with the findings of Enríquez-Estrella and El Salous (2022), who reported a protein content of 9.26%, and Sánchez *et al.* (2016), who observed 8.75% protein in homemade dark chocolates from the Chontalpa region for pure 100% cocoa chocolates. It is worth noting that the analyzed chocolates were made using cocoa paste, whereas large multinational companies use cocoa with vegetable fat, as permitted by regulations.

Carbohydrates: Regarding carbohydrates, the p-value (0.002) was less than α (0.05), leading to the rejection of the null hypothesis (H_0) and acceptance of H_1. The results indicate a gradual decrease in carbohydrate content as the cocoa percentage increases. This is attributed to the formulation, as milk chocolate (30% cocoa) contains a higher amount of cane sugar, resulting in a greater carbohydrate content compared to dark chocolate. Clará-Nolasco (2017) reported a carbohydrate content of 88% in a chocolate bar with a high sucrose formulation (78% sugar), which corroborates the findings observed in this study.

Total fats: For total fats, the p-value (0.00662) was less than α (0.05), leading to the acceptance of H_1, indicating that different cocoa concentrations result in changes in fat content. It was observed that semi-dark chocolate (50% cocoa) contained slightly more fat on average compared to the other types, while milk chocolate (30% cocoa) had the lowest fat content. Fat content ranged between 40% and 50%, aligning with the findings of Quiróz-Martínez *et al.* (2023), who reported 39.68% fat in goat milk chocolate, and Sánchez *et al.* (2016), who found fat percentages ranging from 10-30% in milk chocolates, 30-40% in semi-dark chocolates, and similar values for dark chocolates at 40%. Cocoa fat, or cocoa butter, contains approximately 35% oleic acid, 35% stearic acid, and 25% palmitic acid, with the remaining 5% composed of various short-chain fatty acids (Valenzuela B., 2007). This composition provides cardiovascular health benefits, which are absent in chocolates that replace cocoa butter with other vegetable fats.

Fiber: In terms of crude fiber, differences were observed based on cocoa concentration, as the p-value (0.004) was less than α (0.05). This leads to the rejection of the null hypothesis (H_0) and acceptance of H_1. Dark chocolate, with a fiber content of 3.95%, had the highest

Table 2. Difference in attributes according to cocoa concentration.

Treatment	Protein(%)	Carbohydrates(%)	Fat(%)	Fiber(%)	Moisture (%)
T1 With milk	6.36±0.60a	47.99±0.96a	38.99±0.42a	3.38±0.08a	1.55 ±0.74a
T2 Semi-bitter	6.83±0.715ab	36.06±4.68b	50.25±4.63a	3.27±0.25b	1.91 ±1.60a
T3 Bitter	7.74±0.09b	35.51±1.27b	48.42±1.92b	3.95±0.07b	2.29 ±0.65a
p-value	0.0536	0.00281	0.00662	0.00425	0.719

* Mean values ± standard deviation. Means with the same letter in the same column are not significantly different according to Tukey's test (α=0.05).

value, aligning with Ropero (n.d.), who reported fiber contents of 0.8%, 3.9%, and 3.1% for milk chocolate, milk and almond chocolate, and milk chocolate with polyalcohols, respectively, as well as 9.1% for dark chocolate with almonds. These findings confirm that higher cocoa concentrations result in greater fiber content in the final product. Fiber is essential for maintaining a healthy digestive tract and serves as an ally in preventing metabolic diseases (Almeida-Alvarado *et al.*, 2014).

Moisture: Finally, with a p-value (0.7) greater than α (0.05), moisture levels across the three cocoa concentrations were statistically similar, ranging between 1.55% and 2.3%. These values are consistent with findings by Quiróz-Martínez *et al.* (2023), Enríquez-Estrella and Salous (2022), and Clará-Nolasco (2017), who reported moisture levels of 1.59%, 1.81%, and 2.74%, respectively.

CONCLUSIONS

It is concluded that dark chocolate, with a higher cocoa concentration, offers superior nutritional benefits in terms of protein and fiber. Conversely, chocolates with lower cocoa concentrations tend to have higher carbohydrate content due to increased added sugar. Overall, the results highlight the importance of considering cocoa concentration when evaluating the nutritional properties of chocolate, which are vital for consumer health and well-being. These benefits are particularly evident when opting for chocolates with a high cocoa concentration.

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Laboratory evaluation of meal supplements made of overripe mango flour and parota pod flour

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ABSTRACT

Objective: to evaluate the chemical content [crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF)], and *in vitro* fermentative characteristics [biogas and CH_4 cumulative production, metabolizable energy (ME), dry matter degradation (DMD) and neutral detergent fiber degradation (NDFD)] of three supplements based on overripe mango flour (HMD) and parota pod flour (HVP) through a grazing simulation in the laboratory.

Design/Methodology/Approach: supplements S1=40% HMD+60% HVP, S2=60% HMD+40% HVP, and S3=80% HMD+20% HVP; Treatments to simulate grazing T1=70% cobra grass (PCo)+30% S1, T2=70% PCo+30% S2, and T3=70% PCo+30% S3.

Results: S3 had lower DMD, NDFD and ME ($p \leq 0.05$). T1 had higher CP, DMD, NDFD and ME; and T3 showed lower NDF ($p \leq 0.05$).

Limitations/Implications of the study: this study was implemented in laboratory, so there were some climatological aspects and physiological issues of ruminants that were not considered.

Findings/Conclusions: S1 improved the fermentative characteristics of cobra grass when grazing was lab simulated.

Keywords: alternative supplementation, regional ingredients, *Enterolobium cyclocarpum* (a.k.a; guanacaste), grazing, ruminants, tropics.

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INTRODUCTION

Tropical regions are important for producing grazing cattle, because of the forage biomass produced per hectare. However, grazing ruminant production has low net productivity because tropical grasses provide mainly fibrous carbohydrates. So one strategy to improve productivity is to use supplementation; since grass-based feed does not satisfy the nutrient requirements of animals (protein, energy, and minerals). Due to this, supplementation eliminates deficiencies, stimulates meal consumption, increases digestibility and animal performance (Estrada *et al*., 2019; Souza *et al*., 2021; Cazzuli *et al*., 2023).

Methods of supplementation vary from offering the same amount of supplement for a given period, to offering a certain amount of supplement based on a partcular parameter in ruminant production, such as milk yield (Hills *et al.*, 2015). Supplements can account for up to 70% of production costs. In addition, the lack of technical knowledge, evaluation of forage availability and quality, and basic nutritional requirements, all added encourage producers to use grain-based supplements (Esparza-Jiménez *et al.*, 2021). This is expensive without considering unconventional ingredients which are available in the region that could reduce costs for producers. In that regard, information is required to produce such supplements.

The *in vitro* technique of rumen fluid fermentation offers a fast, low-cost alternative for feed evaluation (Rodríguez *et al.*, 2017; Sobalvarro-Mena *et al.*, 2020). This is complemented with studies on chemical composition. In addition, *in vitro* technique can replace tests *in vivo* and allows for greater control over experimental conditions (Sobalvarro-Mena *et al.*, 2020). However, it is a batch technique, so fermentation products do accumulate. In its implementation, equal amounts of dry matter (DM) or organic matter (OM) from treatments are incubated to be compared and those indicators that are measured are expressed per incubated units of DM or OM (Rodríguez *et al.*, 2017). Thus, the objective was to evaluate the chemical-bromatological content, production and composition of biogas and fermentative characteristics of three supplements based on overripe mango flours and parota pod fluors; as well as to determine these variables also in a grazing simulation implemented in the laboratory.

MATERIALS AND METHODS

Study site

The study was carried out in the Animal Nutrition Laboratory, of the Faculty of Veterinary Medicine and Zootechnics No. 2, under the Autonomous University of Guerrero, located at km 197 of the roadway Acapulco-Pinotepa Nacional, Cuajinicuilapa (Guerrero), Mexico.

Supplements

Pods of the tree *Enterolobium cyclocarpum* (named 'parota' by the locals) was collected in the months of April and May 2020 in lands of the Cuajinicuilapa municipality (Guerrero), Mexico. Four branches were randomly selected from four trees and all physiologically mature pods were harvested; these were placed in paper bags and transferred to the Animal Nutrition laboratory for analysis. The overripe mango flour was processed by collecting overripe mangoes from four trees in the same municipality, they were placed in a 20 L plastic canisters and moved to the laboratory. Mangoes and pods were ground in a mixed mill (M.A.GRO® TR-3500, Mexico) with a 2.54 cm diameter sieve and dehydrated at 55 °C to a constant weight in an oven (Riossa® HCF-41, Mexico). They were then ground in a Thomas-Wiley Mill (Thomas Scientific®, Swedesboro, NJ, USA) with a 1 mm sieve. The supplements made were S1=40% overripe mango flour and 60% parota pod flour; S2=60% overripe mango flour and 40% parota pod flour; S3=80% overripe mango flour and 20% parota pod flour.

Grazing simulation

At the laboratory level, a grazing simulation was done, where a consumption of 70% of cobra grass (*Brachiaria* spp.) 60 d of regrowth and 30% of one of the 3 supplements made was simulated. The treatments in the simulation were: T1=70% grass+30% of S1; T2=70% grass+30% S2; T3=70% grass+30% S3.

Chemical-bromatological analysis

Dry matter (DM), crude protein (CP), ash (A) and organic matter (OM) were determined according to the methodology described by AOAC (2005). In addition, neutral detergent fiber (NDF) and acid detergent fiber (ADF) with the Van Soest *et al.* (1991) methodology, using the ANKOM® Technology Method.

In vitro gas production

The components of the culture medium were 30% clarified rumen fluid [fresh bovine rumen fluid centrifuged 10 min at 12.857 × *g* then sterilized (All American® 1941X, USA) 15 min at 121 °C and 15 psi], 5% 1st mineral solution [6 g K_2HPO_4 (J. T. Baker®) in 1 L of distilled water], 5% 2nd mineral solution [6 g K_2HPO_4 (J. T. Baker®)+6 g $(NH_4)_2SO_4$ (J. T. Baker®)+12 g NaCl (Meyer®)+2.45 g $MgSO_4$ (Meyer®)+1.6 g CaCl-2H_2O (Meyer®) in 1 L of distilled water], 0.1% resarzurine to 0.1% (Sigma-Aldrich®), 0.2% soy peptone (MCDLab®), 0.1% yeast extract (BD Bioxon®), 4% cysteine-sulfide solution [2.5 g L-cysteine (Sigma-Aldrich®) in 15 mL of 2N NaOH (Meyer®)+2.5 g of Na_2S-9H_2O (Meyer®) measured in 100 mL of distilled water], 5% solution to 8% Na_2CO_3 (J. T. Baker®) and 50.6% distilled water. The medium was sterilized for 15 min in an autoclave at 121 °C and 15 psi according to the Cobos & Yokoyama (1995) methodology, modified by Sánchez-Santillán *et al.* (2020).

A serological vial (120 mL) with 0.5 g of sample and 45 mL of culture medium was considered as a biodigester. The vials were kept under anaerobic conditions with CO_2, hermetically sealed with a neoprene cap (20 mm Ø) secured with an aluminum ring.

Table 1. Chemical composition and *in vitro* fermentative characteristics of ingredients.

Variables	Mango flour	Parota pod flour	Cobra grass with 60 d regrowth
Organic matter (g kg^{-1} DM)	972.1	959.5	878.6
Crude protein (g kg^{-1} DM)	55.9	197.2	78.2
Neutral detergent fiber (g kg^{-1} DM)	289.5	389.2	674.3
Acid detergent fiber (g kg^{-1} DM)	156.5	208.4	357.8
Ashes (g kg^{-1} DM)	27.9	40.5	121.4
Biogas production at 72 h (mL g^{-1} DM)	238.68	193.34	136.23
Methane production at 72 h (mL g^{-1} DM)	68.51	60.18	51.19
Dry matter degradation (g kg^{-1} DM)	754.6	712.1	493.2
Neutral detergent fiber degradation (g kg^{-1} DM)	226.9	349.8	326.5
Metabolizable energy (Mcal kg^{-1})	2.80	2.64	1.83

Biodigesters were sterilized at 121 °C and 15 psi for 15 min, and incubated for 24 h at 39 °C to verify sterile condition (Herrera-Pérez *et al.*, 2018). Then, biodigesters were inoculated with 5 mL of total rumen bacteria obtained from the rumen fluid of a Suiz-bu cow; The cow grazed on pangola grass (*Digitaria eriantha*) meadows before taking the rumen fluid sample. Rumen fluid was centrifuged 1.157 × *g* for 3 min to precipitate protozoa and fiber particles (Torres-Salado *et al.*, 2023).

In vitro biogas production was measured by displacing the plunger of a glass syringe (50 mL; BD Yale, Brazil) at 0, 3, 6, 9, 12, 24, 36, 48 and 72 h. The biogas production rate at each incubation time was obtained by estimating the partial production at each time span divided by the incubation hours. Cumulative biogas production was reported at 72 h of incubation (10 independent samples).

In the estimation of methane (CH_4) production, a Taygon® hose (2.38 mm Ø internal and 45 cm in length) was used, at the ends hypodermic needles (20 G × 32mm) were used to couple the biodigester with a trap vial of NaOH solution (2N). The trap vial was placed in reverse in a modified specimen that served to collect the CH_4-displaced NaOH solution produced during incubation by a hypodermic needle placed as an outlet valve (Torres-Salado *et al.*, 2018). CH_4 production was measured at 24, 48 and 72 h. The rate of CH_4 production at each measured time was obtained by estimating the partial production at each time span between the incubation hours. Cumulative CH_4 production was reported at 72 h of incubation (10 independent samples).

At the end of the 72 h of incubation, the biodigesters were used to determine ammonia nitrogen ($N\text{-}NH_3$), dry matter degradation (DMD) and neutral detergent fiber degradation (NDFD). For $N\text{-}NH_3$, 1 mL of the medium contained in the biodigester was taken, then mixed with 0.25 mL of metaphosphoric acid (Meyer®) at 25% (4:1 ratio) then centrifuged for 25 min at 3500 × *g* and the supernatant was restored in 2 mL vials. A volume of 20 μL of this supernatant was mixed with 1 mL of phenol solution [10 mg of $Na_2(NO)Fe(CN)_5$. H_2O (Meyer®) + 10 g of phenol (Meyer®) crystals diluted to the 1 L mark in distilled water] and 1 mL of hypochlorite solution [7.5 g of NaOH (Reasol®) + 21.3 g of Na_2HPO_4 (Meyer®) + 15 mL of hypochlorite (5%; Reasol®) diluted to the 1 L mark in distilled water]. The mixture was incubated for 30 min at 37 °C in a warm water bath. Subsequently, 5 mL of distilled water was added to dilute and stir on a vortex (Genie 2 G-560, USA). Absorbance was measured at 630 nm in a UV/VIS spectrophotometer (Jenway® 6850, USA) calibrated (r^2=0.9994) with a method of ammonia nitrogen concentration according to the technique described by McCullough (1967).

The residual sample from the biodigester was filtered into ANKOM® F57 bags at constant weight. The sample bags were dried at 60 °C for 24 h in a drying oven. The DMD was calculated with the formula:

$$DMD(\%) = (initial\ sample - residual\ sample \mid initial\ sample) * 100$$

(Hernández-Morales *et al.*, 2018). ANKOM® bags were heat-sealed and NDF content determined (Van Soest *et al.*, 1991). The percentage of NDF degradation (% NDFD) was calculated using the formula:

$$NDFD(\%) = (initial\ NDF - residual\ NDF \,/\, initial\ NDF) * 100$$

(Hernández-Morales *et al.*, 2018).

Determination of metabolizable energy (ME) was done with the DMD values; first, the equation described in (NRC, 1982) was used to obtain the digestible energy (DE),

$$DE\left(Mcal\ kg^{-1}\right) = (DMD\,/\,1000) * 4.525$$

Subsequently, the equation described by INRA (1989) was used to estimate the value of ME;

$$ME\left(Mcal\ kg^{-1}\right) = DE * 0.82$$

Statistical analysis

The chemical and bromatological analysis, biogas and CH_4 production rates at different incubation hours, cumulative biogas and CH_4 production, and fermentative characteristics of supplements by a laboratory grazing simulation were analyzed in a completely randomized design. Data were analyzed using the GLM procedure in SAS© (SAS Institute Inc., 2011). Mean differences were compared using Tukey's multiple means test (α, $p \leq 0.05$).

RESULTS AND DISCUSSION

Supplements

The supplements were characterized by a decrease in parota pod flour, which led to ash content (A) and crude protein (CP) decreased as the mango flour content increased. In contrast, organic matter content (OM) increased ($p \leq 0.05$). Neutral detergent fiber content (NDF) showed no differences among supplements ($p > 0.05$); while S3 showed the lowest content of acid detergent fiber (ADF, $p \leq 0.05$) (Table 2). Thus, the nutrient content of the supplements was related to the rate of biogas production and composition (Sobalvarro-Mena *et al.*, 2020).

The rate of biogas production made it possible to indirectly identify potentially fermentable carbohydrates at given times (Amanzougarene & Fondevila, 2020); since, the fermentation process of other nutrients such as proteins and fats are not expressed in the in vitro proportion of gas (Posada & Noguera, 2005; Sobalvarro-Mena *et al.*, 2020). Thus, the rate of biogas production in the different periods, up to 48 h of incubation, did not show differences among supplements ($p > 0.05$), which averaged (mL g^{-1} DM h^{-1}) 12.0, 26.2, 5.0, 5.3, 5.2 y 2.0, at times 0-3, 3-6, 6-9, 9-12, 12-24 y 24-48 h, respectively. In the period 48-72 h, S3 presented the lowest rate ($p \leq 0.05$; Table 2). In the case of CH_4 production rate, the periods were shorter, 24 h, so that supplements evaluated in this study did not present differences at 24, 48 and 72 h of incubation ($p > 0.05$); but they averaged 1.7, 0.6 and 0.3 mL g^{-1} DM h^{-1}, respectively (Table 2).

In cumulative biogas production, S3 had the lowest production ($p \leq 0.05$); in contrast, cumulative CH_4 production at 72 h showed no differences among supplements ($p > 0.05$). Now, the fermentation of carbohydrates from a sample using the *in vitro* gas production technique produces CO_2 and CH_4, since the production of other gases are only traces undetectable with this technique (Amanzougarene & Fondevila, 2020). Therefore, CH_4 represented 21.9%, 22% and 21.5% of the total biogas production produced by S1, S2 and S3, respectively (Table 2). This occurs because, when mainly propionate is fermented, the H_2 ions that form CH_4 are reduced by the proportion of non-structural carbohydrates, thus improving the microbial growth rate (Miranda-Romero *et al.*, 2020).

Rumen fermentation of carbohydrates produces short-chain fatty acids (acetate, propionate, and butyrate), succinate, formate, lactate, ethanol, CH_4, CO_2, and H_2. So, non-fibrous carbohydrates (starch, pectin and glucans) are fermented as part of the soluble components in the first 24 h (Posada & Noguera, 2005; Sobalvarro-Mena *et al.*, 2020; Souza *et al.*, 2021); then, the fermentation of insoluble carbohydrates begins. In both cases there are hydration and colonization by rumen microorganisms. So, biogas production and rate provide information on the degradation and kinetics of fermentation. Thus, the production rates and cumulative production of biogas from supplements can be explained by their chemical and bromatological composition. Also, by the energy content of the foods evaluated (Sobalvarro-Mena *et al.*, 2020), and their interactions and successive changes in microorganisms (Miranda-Romero *et al.*, 2020).

Regarding the rate of production and cumulative production of CH_4 it is assumed that methanogenic archaea use CO_2 and H_2 within their metabolic pathway, generating CH_4 as a product of their fermentation (Torres-Salado *et al.*, 2018). Because of the rate, proportion and amount of CH_4 it can be assumed that the supplements contained mainly non-structural carbohydrates that increased the production of propionate and the H^+ ions that form CH_4, so their production was stoichiometrically reduced (Miranda-Romero *et al.*, 2020).

In fermentative characteristics, S3 had the lowest content of metabolizable energy (ME), and nitrogen ammonia ($N\text{-}NH_3$) in the culture medium at 72 h; As well as the lowest degradation of dry matter (DMD) and neutral detergent fiber degradation (NDFD) ($p \leq 0.05$; Table 2). The *in vitro* degradation of CP is given by the bacteria and protozoa that were used as inoculum, which degraded it into peptides and free amino acids by enzymes, while ammonia was released; this was determined by the $N\text{-}NH_3$ content of the culture medium (Mejía & Mejía, 2007). The $N\text{-}NH_3$ content of S3 can be assumed by supplement composition, since the proportion of parota pod flour was reduced, thereby reducing CP; whereas in S1 and S2 PC content was higher (Bargo *et al.*, 2003) (Table 2).

Neutral detergent fiber (NDF) is an important nutritional factor due to its effect on rumen filling and rumminal digestion. Thus, the supplements in this study can be classified as highly degradable; and the expected increase in degradation occurred when grazing was simulated in laboratory (Baudracco *et al.*, 2010)2010. This is because DMD values were higher than 60%, which indicated that NDF content was lower than 40% (Table 2). In turn, this met a requirement for supplement production, that it contains low concentrations of detergent fibers (Hernández-Morales *et al.*, 2018).

Rojas-García *et al.* (2020) evaluated *in vitro* a supplement made with 50% pumpkin pulp & peel flour and 50% parota pod flour; they reported contents of 166 g kg^{-1} of CP, 409 g kg^{-1} of NDF, 241 g kg^{-1} of ADF (values greater than S1 in this study) and 924 g kg^{-1} of OM (a smaller value than S1 in this study). Regarding the *in vitro* fermentative characteristics, those authors reported values in DMD (776 g kg^{-1}), NDFD (549 g kg^{-1}), N-NH_3 content in the culture medium (9 mg dL^{-1}), cumulative biogas production (149 mL g^{-1}) and CH_4 production (56.2 mL g^{-1}), which are lower than those we consistently obtained with S1 in this study (Table 2).

Grazing simulation

Grazing simulation in the laboratory aimed to establish whether grass fermentation is improved when supplemented. Thus, the content of A, OM and ADF did not present

Table 2. Chemical composition and *in vitro* fermentative characteristics of supplements made of overripe mango flour and parota pod flour.

Variable	S1	S2	S3	SEM
Chemical composition (g kg^{-1} DM)				
Ashes	34.8 a	32.2 b	30.0 c	0.7
Crude protein	138.8 a	111.6 b	79.2 c	9.0
Neutral detergent fiber	332.8	334.9	314.8	4.4
Acid detergent fiber	192.2 a	191.9 a	172.6 b	3.6
Organic matter	965.2 c	967.8 b	970.0 a	0.7
Rate of biogas production (mL g^{-1} DM h^{-1})				
0-3 h	12.36	10.87	12.76	0.45
3-6 h	26.94	25.87	25.92	0.83
6-9 h	4.72	4.13	6.24	0.41
9-12 h	6.53	4.82	4.61	0.37
12-24 h	5.49	4.96	5.09	0.1
24-48 h	2.14	1.96	1.90	0.07
48-72 h	1.18 a	1.17 a	0.83 b	0.06
CH_4 production rate (mL g^{-1} DM h^{-1})				
0-24 h	1.79	1.67	1.63	0.05
24-48 h	0.66	0.53	0.59	0.03
48-72 h	0.27	0.29	0.24	0.01
Biogas production at 72 h (mL g^{-1} DM)	297.1 a	271.7 b	275.2 b	4.18
CH_4 production at 72 h (mL g^{-1} DM)	65.20	59.87	59.24	1.54
DM degradation at 72 h (g kg^{-1} DM)	832.3 a	821.3 a	759.8 b	1.14
NDF degradation at 72 h (g kg^{-1} DM)	592.4 a	572.4 a	505.8 b	1.38
Average ammoniacal nitrogen (mg dL^{-1})	21.26 a	16.15 a	14.52 b	0.97
Metabolizable energy (Mcal kg^{-1})	3.09 a	3.05 a	2.82 b	0.04

a,b: Variables with different letter per row show statistical difference ($p \leq 0.05$). S1=40% overripe mango flour - 60% parota pod flour; S2=60% overripe mango flour - 40% parota pod flour; S3=80% overripe mango flour - 20% parota pod flour; NDF=neutral detergent fiber; CH_4=methane; DM=dry matter; SEM=standard error of the mean.

differences among treatments ($p>0.05$), with an average of 91.1, 908.9 and 308.4 g kg^{-1} DM, respectively. The CP content showed the same trend as in the supplements, since the parota pod flour content decreased. So, T1 was 13.3% higher than T2 and 32.2% higher than T3 ($p\leq0.05$). The lowest NDF content was presented by T3 ($p\leq0.05$); T1 and T3 were both 4.5% higher than T3 (Table 3).

The biogas production rate showed that in the first 3 h of incubation, T1 had the lowest rate ($p\leq0.05$), 44.0% lower than T1 and T2; at 3-6 h, T1 was 30.2% higher than T2 ($p\leq0.05$); at 6-9 h, T3 was 34.4% higher than T1. But, at 9-12 h, 12-24 h and 24-48 h T1 was 103%, 90.8% and 49.4% higher than T3 ($p\leq0.05$). T2 showed no differences with both treatments ($p>0.05$; Table 3) in the same times. The 48-72 h rate showed no difference among treatments ($p>0.05$), and averaged 1.2 mL g^{-1} DM h^{-1}. The CH_4 production rate did not show differences in the measured times ($p>0.05$), but averaged a rate of 1.1,

Table 3. Chemical composition and *in vitro* fermentative characteristics obtained in a grazing simulation in laboratory; cobra grass supplemented with 30% meal supplements made of overripe mango flour and parota pod flour.

Variable	T1	T2	T3	SEM
Chemical composition (g kg^{-1} DM)				
Ashes	90.9	90.2	92.3	0.5
Crude protein	100.6 a	88.8 b	76.1 c	3.8
Neutral detergent fiber	594.4 a	590.9 a	567.0 b	4.7
Acid detergent fiber	308.5	320.7	295.9	4.8
Organic matter	909.1	909.8	907.7	0.5
Rate of biogas production (mL g^{-1} DM h^{-1})				
0-3 h	8.93 b	13.39 a	12.33 a	0.68
3-6 h	9.61 a	7.38 b	7.58 ab	0.4
6-9 h	3.43 b	3.56 ab	4.61 a	0.21
9-12 h	4.67 a	3.55 ab	2.30 b	0.37
12-24 h	2.71 a	1.98 ab	1.42 b	0.21
24-48 h	3.57 a	2.44 ab	2.39 b	0.22
48-72 h	1.38	1.07	1.08	0.08
CH_4 production rate (mL g^{-1} DM h^{-1})				
0-24 h	1.13	1.09	1.10	0.02
24-48 h	0.67	0.73	0.74	0.06
48-72 h	0.41	0.44	0.43	0.03
Biogas production at 72 h (mL g^{-1} DM)	231.16 a	191.93 ab	180.90 b	8.25
CH_4 production at 72 h (mL g^{-1} DM)	53.16	54.33	54.66	1.35
DM degradation at 72 h (g kg^{-1} DM)	705.1 a	640.9 b	637.7 b	11.2
NDF degradation at 72 h (g kg^{-1} DM)	652.9 a	538.3 b	503.9 b	22.9
Average ammoniacal nitrogen (mg dL^{-1})	18.07 a	13.91 b	13.08 b	0.77
Metabolizable energy (Mcal kg^{-1})	2.62 a	2.38 b	2.37 b	0.04

a,b: Variables with the same letter per row are statistically equal ($p>0.05$). T1=70% grass+30% S1; T2=70% grass+30% S2; T3=70% grass+30% S3; NDF=neutral detergent fiber; CH_4=methane; DM=dry matter; SEM=standard error of the mean.

0.7 and 0.4 mL g^{-1} DM h^{-1} in the periods 0-24 h, 24-48 h and 48-72 h incubation, respectively (Table 3).

The cumulative biogas production of T1 was 27.8% higher than T3 ($p \leq 0.05$), with no differences with T2 ($p > 0.05$). In contrast, CH_4 production showed no differences among treatments ($p > 0.05$). However, CH_4 production accounted for 23%, 28.3% and 30.2% of the biogas produced at 72 h. The biogas production rates of the simulation showed that the soluble components were rapidly fermented in the first six hours. This was given by the supplements composition, as it was correlated with carbohydrate content in cells and subsequently with the fibrous components (Sobalvarro-Mena *et al.*, 2020).

In order to explain the behavior of CH_4 in the simulation, two factors are assumed. 1) the fermentation of low-quality forages increases the production of acetate, H^+ ions; therefore, production rates and the accumulated production of CH_4 follow (Miranda-Romero *et al.*, 2020); 2) on the differences in the proportion we assumed that parota flour contained secondary metabolites acting as 'defaunators' that inhibited methanogenic archaea, thus decreasing their production. Thus, as in T1 we had the higher amount of parota pod flour, the proportion of CH_4 was lower compared to the rest of the treatments.

T1 showed 10.6%, 29.6%, 10.3% and 38.1% more DMD, NDFD, ME and N-NH_3 of the medium than T2 and T3 ($p \leq 0.05$). T1 showed a higher content of potentially degradable dry matter; we consider that it will present a better biological performance when offered to the animal. The CP content of S1 was directly related to both degradation levels, and N-NH_3. This follows their importance in the growth and synthesis of rumen microorganisms, which degrade dietary components and release nutrients for absorption (Souza *et al.*, 2021).

Thus, the best fermentative characteristics of S1 in the grazing simulation in laboratory were the result of the interaction of all its nutrients. This shows the benefit of its addition to a grazing rumen diet; DMD, NDFD and ME were improved by 211.9 g kg^{-1}, 326.4 g kg^{-1} and 0.79 Mcal kg^{-1}, respectively.

The trend of increasing N-NH_3 content as the CP content in the supplement increased is similar to that reported by Bargo *et al.* (2003) when they increased soy flour in their supplements for an *in vivo* test. Therefore, this type of *in vitro* studies should be continued; and their limitations considered, because they are useful to understand the potential of the products that can be used afterwards, for *in vivo* grazing trials.

CONCLUSIONS

Supplementation meals in the tropics, using regional products such as overripe mango flour and parota pod flour, represent a viable supplementation alternative for grazing ruminants. The tested supplement with 40% overripe mango flour and 60% parota pod flour improved all the *in vitro* fermentative characteristics of cobra grass in a grazing simulation in laboratory.

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Behavior of Nitrogen Use Efficiency in Maize (*Zea mays* L.) Under Different Management Conditions in Veracruz, Mexico

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ABSTRACT

Currently, technological packages primarily aim to achieve high economic performance without considering environmental impact. This approach promotes the use of agrochemicals, leading to high production costs and negative environmental consequences, while disregarding the efficiency of agro-productive systems.

The objective of this research was to determine dry matter production, nitrogen (N) absorption, and nitrogen use efficiency (NUE) under different maize (*Zea mays* L.) management conditions. The experiment was conducted at the Colegio de Postgraduados, Campus Veracruz.

Using 300-gauge black polyethylene bags, 16 treatments were evaluated, resulting from the combination of the following factors: soil type (crumbly-sandy and loamy), genotype (the hybrid "Orca®" seed from Monsanto and the native variety "Olotillo"), nitrogen application (0 and 140 kg N ha^{-1}), and the presence or absence of agricultural lime. A Taguchi L8 orthogonal array was used in a randomized block design with four replications.

For the variables leaf dry weight and root dry weight, significant statistical differences were observed in favor of nitrogen application. The percentage of nitrogen absorption by the plant showed no significant differences for any of the factors or their interactions, whereas nitrogen absorption in grams was higher in fertilized plants. Finally, nitrogen use efficiency (NUE) was higher in unfertilized plants, and the interaction between soil texture and nitrogen concentration was significant.

Keywords: *Zea mays* L., nutritional efficiency, agricultural lime.

INTRODUCTION

Modern crop production is related to the modification and control of management factors that determine higher yields, where the primary criterion favors the combination

of factors that maximize economic performance. However, the efficiency of agro-productive systems is rarely analyzed, particularly in terms of the combination of various management factors.

Considering that agriculture must meet the demand for food and raw materials from a growing population while minimizing environmental contamination risks, sustainable production systems must be implemented to preserve resources for future generations. A key aspect of achieving agroecosystem sustainability is the efficient use of both soil nutrients and applied fertilizers (Salazar *et al.*, 2021).

One of the current challenges in mineral nutrition is the efficiency with which plants utilize nutrients, particularly in the context of the continuously rising cost of fertilizers. Thus, the efficient use of fertilizers applied to crops is one of the most important factors in maintaining food production at a level sufficient to meet population needs, reducing environmental pollution and production costs, while increasing agricultural profitability.

Fageria *et al.* (2011) state that nutrient efficiency in crops refers to the ability to produce a greater amount of dry biomass per unit of applied or absorbed mineral. Differences in crop yield are attributed both to the efficiency of nutrient acquisition by the roots and to the plant's effective utilization of these nutrients.

Regarding nutrient use efficiency, the following findings have been reported: 1) It is a plant adaptation mechanism to low-fertility conditions (Retuerto & Woodward, 2003); 2) In maize varieties and hybrids, differences in yield and efficiency have been observed (Delgado, 2002); 3) Higher-yielding plants are not always the most efficient (Sosa & García, 2018); and, 4) In maize, nitrogen use efficiency varies from 44% to 143% (González *et al.*, 2014).

Gallais and Hirel (2004) and Xu *et al.* (2012) suggest that through a combination of management practices, technologies, and genetic improvement, efficiencies above 85% can be achieved. Furthermore, each improvement in nitrogen use efficiency (NUE) reduces nitrogen losses, and by increasing its absorption, it proportionally enhances economic returns, particularly under high fertilizer cost conditions.

Nitrogen is an essential element for photosynthesis. In other words, for plants to fix carbon from the air, accumulate dry matter, and achieve economically attractive yields, a sufficient supply of this element is required. However, nitrogen is often a limiting nutrient in agricultural soils (Delgado, 2002; Zenteno *et al.*, 2019).

Nitrogen use efficiency (NUE) depends on several factors, including soil pH, soil type, crop species, agroecosystem type, type of fertilizer applied, and management practices (Smith, 2020).

The Taguchi experimental design is a factorial arrangement used in exploratory studies. It offers advantages such as reducing the number of treatments, experimental units, and overall experiment costs. This design is based on the confounding of effects, which results in a loss of precision when analyzing high-order interactions. However, these interactions are generally of low importance and difficult to interpret. This loss is compensated by an increase in the precision of the main effects (Padrón, 1996).

Each variety within the same species can also exhibit particular characteristics of behavior and production, reflected in different capacities for nutrient absorption and

utilization. Therefore, it is important to evaluate different maize genotypes in order to identify efficient materials and incorporate them into agroproductive systems, which will bring economic benefits to producers and ecological benefits due to better fertilizer use (Bertsch, 2005; Herrera *et al.*, 2022).

Finally, understanding the physiological and genetic foundations of nitrogen use efficiency is necessary. Thus, the objective of this research was to evaluate dry matter production, nitrogen absorption, and nitrogen use efficiency (NUE) in maize under different management conditions, based on the hypothesis that management factors modify these variables.

MATERIALS AND METHODS

Study Area

The experiment was conducted in 300-gauge black bags from February 24 to April 7, 2007, on the grounds of the Colegio de Postgraduados, Veracruz Campus, in the "Tepetates" property, located in the municipality of Manlio Fabio Altamirano, at coordinates 19° 16' North Latitude and 96° 16' West Longitude, at an altitude of 40 m (García, 2004).

Treatments and Experimental Design

Sixteen treatments were evaluated, resulting from the combination of the following factors: soil texture (crumb-sandy and loamy), genotype (hybrid seed "Orca®" from Monsanto and the native variety "Olotillo"), nitrogen (0 and 140 kg N ha^{-1}), and with or without agricultural lime. The factors under study and their levels are presented in Table 1.

A Taguchi L 8^{-1} orthogonal arrangement (Stuart, 1993) was used in a randomized block experimental design with 16 treatments and four replications per treatment. The assignment of 300-gauge polyethylene black bags in the field was done randomly, with each bag representing an experimental unit placed randomly. A light variation gradient was identified based on the sun's path, so the randomized blocks were positioned perpendicular to this gradient.

Soil Characteristics Under Study

Soil from two maize-producing regions in the municipality of Acayucan, Veracruz, was used: La Colonia Hidalgo (Soil A) and La Colonia Agrícola Michapan (Soil B). The soil analyses were carried out at the Soil Laboratory of the Colegio de Postgraduados Veracruz Campus, and their physical and chemical characteristics are presented in Table 2.

Table 1. Factors and Levels Under Study.

Factors	Level 1	Level 2
Type of soil	Sandy loam (S1)	Loam (S2)
Genotype	Olotillo (G1)	Orca (G2)
Nitrogen	0 kg ha^{-1} (N1)	140 kg ha^{-1} (N2)
Lime	Without agricultural lime (C1)	With agricultural lime (C2)

Table 2. Physical and Chemical Characteristics of the Soils used in the Study.

Characteristic	Soil A	Soil B
Texture	Sandy loam	Loam
Sand	76.7%	41.8%
Silt	7.0%	31.8%
Clay	16.3%	26.4%
pH-H_2O	4.7	5.5
Organic matter	2.3% (medium)	2.9% (medium)
Total nitrogen	0.11% (medium)	0.14% (medium)
Phosphorus	1 ppm (very low)	4 ppm (low)
Potassium	50 ppm (low)	100 ppm (medium)
Calcium	960 ppm (low)	2,160 ppm (moderately high)
Magnesium	37 ppm (medium)	487 ppm (very high)

Sowing

Sowing was carried out in black polyethylene nursery bags, measuring 30 cm × 40 cm in width and length, respectively. In each black bag containing the corresponding soil type, three seeds were placed at the center of the bag, at a depth of 3 cm. Once germinated, one plant per bag was selected, and the others were removed. The bags were arranged so that the distances between plants and rows were 20 cm and 40 cm, respectively.

Fertilization

The fertilization formula applied was equivalent to 140-60-60 kilograms of nitrogen (N), phosphorus (P_2O_5), and potassium (K_2O) per hectare, respectively. The dose was split into two parts: all of the P and K, along with half of the N, were applied at planting, and the other half of the N was applied 20 days after sowing (das). The sources used were urea, triple superphosphate, and potassium chloride.

Lime Application

Titration tests with sodium hydroxide (NaOH) were performed in the laboratory to determine the lime requirements and adjust the pH of the soils used, from 4.7 and 5.5 to 7.0, following the methodology described by Ortega (1981). For liming the experimental units, dolomitic lime with a purity of 78% was used, at doses of 2.7 t ha^{-1} and 0.9 t ha^{-1} for the crumb-sandy and loamy soils, respectively, and was applied at planting.

Irrigation

Light and frequent irrigations were scheduled according to the water requirements of the treatments, applied using a watering can. The field capacity (FC) and the permanent wilting point (PWP) of each soil were considered, and both FC and PWP were calculated using the gravimetric method. Soil moisture was maintained within a range of 60 to 75% of available moisture.

Sample Handling

Based on a study by Delgado (2002) and Yato *et al.* (2015), which found a significant correlation ($P \leq 0.01$) ($r = 0.92$) between the kilograms of dry matter (DM) and the kilograms of nitrogen absorbed per hectare at 30 days after planting, the decision was made to collect samples at 35 days after sowing (dds). Pruning shears were used to cut the above-ground parts of the maize plants (leaves and stems) into fractions, and these were placed in pre-labeled paper bags with the corresponding treatment and replication numbers. The roots were collected and any soil fractions were removed using running water with a hose. These were also placed in labeled paper bags. All samples were then dried in a forced-air oven at a temperature of 60 °C until a constant weight was achieved.

Chemical Analysis of Samples

The analysis of the leaf and stem samples was carried out at the High Technology Laboratory of Orizaba S. C. (LATO). The methods and procedures used were as follows: dry weight of leaves and stems was determined by gravimetry, and nitrogen (N) was measured using the Kjeldahl method. Additional analyses for other elements were conducted; for this, the samples were first incinerated at 550 °C, and the dissolution of the ash allowed for the determination of calcium (Ca), magnesium (Mg), and potassium (K) through atomic absorption. Phosphorus (P) was determined by UV-VIS spectrophotometry.

Registered Variables

The dry weight of leaves and stems (DWLaS) and roots (DWR) were determined using a granatary balance. The dry weight of leaves and stems in kg DM/ha and nitrogen absorption in kg N/ha were obtained through data transformation, considering a population density of 62,500 plants per hectare. Nitrogen absorption in percentage was calculated from laboratory results, and nitrogen absorption in grams per plant was determined based on the dry weight of each plant. The nitrogen use efficiency (NUE) was defined by the following mathematical formula:

$$NUE = dry\ weight\ of\ leaves\ and\ stems (DWLaS)\ in\ grams \mid grams\ of\ N\ absorbed$$

Statistical analysis

For the studied variables, analysis of variance and mean comparison tests were conducted using the Tukey method, $p \leq 0.05$, with the Statistica software. Interactions that were statistically significant were graphed and interpreted (Gómez and Gómez, 1983). For the variable nitrogen absorption as a percentage, a square root transformation was applied to the original data.

RESULTS AND DISCUSSION

Dry weight of leaves and stems (DWLaS)

The analysis of variance for the dry weight of leaves and stems (DWLaS) revealed a statistical difference ($p \leq 0.01$) only for the nitrogen factor, which was confounded with the interaction of soil × genotype × lime (Table 3). To determine which of these two

Table 3. Analysis of variance for the variables dry weight of leaves and stems (DWLaS) and dry weight of roots (DWR).

FV	DF	SM (PSHyT)	SM (PSR)
Blocks = soils × genotypes × N × lime	3	125.37*	56.93
Soils = genotype × nitrogen × lime	1	0.34	14.44
Genotypes = soils × nitrogen × lime	1	43.89	2.25
Nitrogen = soils × genotypes × lime	1	933.12**	283.81**
Soils × genotypes = nitrogen × limel	1	7.84	0.05
Soils × nitrogen = genotypes × lime	1	2.88	2.36
Genotypes × N = soils × lime	1	0.91	87.44
Soils × genotypes × nitrogen = limel	1	69.08	60.77
Error	21	650.41	41.35
Total	31	2084.64	1490.40

*.** Tukey's significance levels at p≤0.05 (*) and p≤0.01 (**) probability levels, respectively.

effects showed statistical significance, the mean comparison for nitrogen (Table 4) was examined, showing values of 14.35 and 25.15 grams per plant for the 0 and 140 kg of nitrogen per ha doses, respectively. This difference provides sufficient evidence to favor nitrogen application. When the DWLaS values were transformed to kg DM/ha for the 0 and 140 kg N/ha doses (Table 4), the observed values were 807.2 and 1414.7, respectively. These values closely match those obtained by Delgado *et al.* (2004) for maize in Venezuela, with 806 and 1469 kg DM/ha for the 0 and 120 kg N/ha doses, respectively, 30 days after planting. Delgado (2002) and Naiz *et al.* (2015) indicate that the crop growth rate increases with higher nitrogen doses. Moreover, dry matter production is closely related to the amount of nitrogen absorbed, which in turn depends on the available nitrogen. The evidence of higher photosynthesis rates under higher nitrogen absorption conditions could explain the greater dry matter production in the treatments with higher nitrogen doses compared to the unfertilized treatments.

Table 4. Mean comparison for the variables dry weight of leaves and stems (DWLaS) and dry weight of roots (DWR).

Factors	Dry weight of leaves and stem (g planta^{-1})	Dry weight of leaves and stem (kg MS ha^{-1})	Dry weight of roots (g planta^{-1})
Creole variety	19.65a[1]	1105.3a	18.20a[1]
Hybrid	19.86a	1117.1a	16.85a
Sandy loam soil	18.58a	1045.1a	17.79a
Loam soil	20.92a	1176.7a	17.26a
Without nitrogen	14.35b	807.2b	14.55b
With nitrogen	25.15a	1414.7a	20.50a
Without agricultural lime	19.21a	1080.6a	17.23a
With agricultural lime	21.13a	1188.6a	18.05a

[1] Means with the same letters within factors indicate no statistical difference between the levels (Tukey, 0.05).

Dry Weight of Roots (PSR)

Regarding the variable dry weight of roots (Cuadro 3), a highly significant difference ($p \leq 0.01$) was found for the nitrogen factor, with values of 14.35 and 25.15 g for the doses of 0 and 140 kg N ha^{-1}, respectively (Cuadro 4).

Nitrogen absorption percentage (%)

The analysis of variance for the variable nitrogen absorption percentage did not show statistical differences for any of the factors or their interactions (Table 5). That is, regardless of the plant size, the nitrogen content percentage remained constant.

Nitrogen Use Efficiency (NUE)

For NUE, statistical differences were found for the nitrogen factor ($p \leq 0.01$) and for the nitrogen × soil interaction ($p \leq 0.05$), as indicated in Table 5. The means for NUE were 72.0 and 53.3 g DM per g of N absorbed for the 0 and 140 kg N/ha doses (Table 6). These values fall within the ranges reported by Delgado (2002), which are from 60 to 90 g DM per kg of N absorbed at 90 days after planting. When comparing NUE in soils without and with nitrogen application, an inverse relationship was found between NUE and the N dose applied, meaning that NUE was higher in the treatment where no nitrogen was applied. This can be explained by the fact that under stress conditions, plants have compensatory functions, and the increase in physiological efficiency is a mechanism of adaptation to low fertility conditions (Shi *et al.*, 2001).

Nitrogen absorption (g $plant^{-1}$)

The analysis of variance for the variable nitrogen absorption (g $plant^{-1}$) only showed a statistical difference for the nitrogen factor, with values of 0.25 g and 0.50 g for the levels of

Table 5. Analysis of variance for the variables: nitrogen absorption (%), nitrogen absorption (grams), and nitrogen use efficiency (NUE).

FV	DF	MS Absorption of N (%) $^{\forall}$	MS Absorption of N (g)	MS NUE (kg MS kg N absorbido $^{-1}$)
Blocks = soils × genotypes × N × lime	3	0.37	0.09*	643.96
Soils = genotypes × nitrogen × lime	1	0.08	0.004	362.67
Genotypes = soils × nitrogen × lime	1	0.002	0.01	25.25
Nitrogen = soils × genotypes × lime	1	1.15	0.5**	2554.25**
Soils × genotypes = nitrogen × lime	1	0.37	0.03	235.07
Soils × nitrogen = genotypes × lime	1	1.12	0.03	1685.93*
Genotypes × N = soils × limel	1	0.17	0.009	274.67
Soils × genotypes × Nitrogen = lime	1	0.007	0.042	28.26
Error	21	6.43	0.43	9251.77
Total	31	10.48	1.34	16340.40

*.** Significant at Tukey levels $p \leq 0.05$ and $p \leq 0.01$ probability, respectively. Square root transformation was applied to the original data.

Table 6. Comparison of means for the variables: nitrogen absorption percentage, nitrogen absorption in grams, and nitrogen use efficiency (NUE).

Factors	Absorption of N (%)† ɣ	Absorption of N (g/plant)	Absorption of N (kg N/ha)	NUE (kg DM/ kg N absorbed)
Creole variety	1.85 a	0.38 a	21.4 a	59.6 a[1]
Hbrid	1.74 a	0.36 a	20.2 a	65.7 a
Sandy soil	1.79 a	0.35 a	19.7 a	63.8 a
Clayed soil	1.80 a	0.39 a	30.0 a	61.5 a
Without nitrogen	1.60 a	0.25 b	14.1 b	72.0 a
With nitrogen	1.90 a	0.50 a	28.1 a	53.3 b
Without agricultural lime	1.65 a	0.35 a	19.7 a	63.3 a
With agricultural lime	1.68 a	0.40 a	22.5 a	65.7 a

[1] Means with the same letters within factors indicate no statistical difference between the levels (Tukey, 0.05).
† The square root transformation was applied to the original data.

0 and 140 kg N ha^{-1}, respectively (Table 6). This is due to the close relationship between absorbed N and available N in the soil, which has been documented by Ding *et al.* (2005), who indicate a linear relationship between both variables.

Nitrogen Use Efficiency (NUE)

For NUE, statistical differences were found for the nitrogen factor ($p \leq 0.01$) and for the soil by nitrogen interaction ($p \leq 0.05$), as indicated in Table 5. The means for NUE were 72.0 and 53.3 g DM g N absorbed[1] for the 0 and 140 kg N/ha doses, respectively (Table 6). These values fall within the ranges reported by Delgado (2002), from 60 to 90 kg of DM per kg of N absorbed at 90 days after planting. When comparing NUE in soils with and without nitrogen application, an inverse relationship was found between NUE and the nitrogen dose applied, *i.e.*, NUE was higher in the treatment where no nitrogen was applied. This can be explained by considering that, under stress conditions, plants exhibit compensatory functions. Furthermore, an increase in physiological efficiency is a mechanism by which plants adapt to low fertility conditions (Shi *et al.*, 2001).

Soil Texture by Nitrogen Interaction

Since the first-order interactions of soil × nitrogen and genotypes × lime were confounded (Table 5), and given that a statistical difference was observed as a main effect for the nitrogen factor, it was considered that the significant interaction is the one involving nitrogen. Therefore, the significant interaction was determined to be soil texture × nitrogen. The highest NUE was obtained in treatments where no nitrogen was applied, especially in the sandy-loam soil. These differences are attributed to both the efficiency of nutrient acquisition by the roots and the proper utilization of nitrogen by the plant, confirming that plants under stress conditions develop compensatory mechanisms that increase efficiency in physiological processes, in this case, nitrogen use efficiency in low-fertility soils (Retuerto and Woodward, 2003).

CONCLUSIONS

For the variables dry weight of leaves and dry weight of roots, only statistically significant differences were observed in favor of nitrogen application. Nitrogen absorption percentage showed no differences for any of the factors studied, nor their interactions, while nitrogen absorption in grams was higher in fertilized plants. Nitrogen use efficiency was higher in unfertilized plants due to the homeostatic adaptation mechanism present in all species. The soil × nitrogen interaction was statistically significant due to the deficient nature of the soil and the absorption capacity of the plants.

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Inclusion of ginger (*Zingiber officinale* Roscoe) in the diet of rams and its effect on sperm quality

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ABSTRACT

Objective: To compare changes in sperm quality in adult rams when ginger pellets were included in the diet during the breeding season.

Design/methodology/approach: The study was conducted for 16 weeks with 31 adult rams (eight Pelibuey, eight East Friesian, eight Damara and seven Dorper) at the Sheep and Goat Reproduction Laboratory (LaROCa) of the Colegio de Postgraduados, Campus Montecillo, located at 19° LN (north latitude) during short days (September-December). During the first 8 weeks, the rams received a diet based on commercial concentrate, minerals, lucerne, oats and water ad libitum; during the following 8 weeks (from 9 to 16), 2 g of dehydrated ginger pellets per day per animal were added to the diet. Weekly measurements were made of scrotal circumference, body weight and semen variables (volume, sperm concentration, mass motility, individual progressive motility, percentage of live sperm and normality).

Results: An increase in body weight and scrotal circumference was observed in all four breeds during the experiment, with increases of 4.94 kg and 2.41 cm, 12.85 kg and 7.48 cm, 10.88 kg and 5.9 cm and 6.48 kg and 3.77 cm in Pelibuey, East Friesian, Damara and Dorper breeds respectively. A treatment effect ($p<0.05$) was found on sperm volume, live sperm percentage, sperm normality and sperm concentration. Differences ($p<0.05$) were found in the breed effect for the variables time to ejaculate, volume, mass motility, percentage of live sperm, normality and sperm concentration and in the breed by treatment interaction for the variables volume, mass motility, individual motility, percentage of live sperm, normality and sperm concentration.

Limitations on study/implications: The study was carried out in only one season of the year (breeding season), it would be of great interest to carry out evaluations throughout the year (breeding *vs.* non-breeding season).

Findings/conclusions: Including ginger in the diet of sheep during the breeding season is a viable management option to improve sperm quality.

Keywords: Sheep, ginger, sperm quality, breeding season.

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INTRODUCTION

The hypothalamic-pituitary-testicular axis is influenced by nutrition through metabolic signals triggered by nutrients in the diet (Blache *et al.*, 2000; Ungerfeld, 2020), and therefore different strategies are being developed to include nutritional supplements and according

to the physiological state of the animal (Martin *et al*., 2004). In sheep production systems, the increase of lambs per year is influenced by the reproductive capacity of females and males (Akhlaghi *et al*., 2014).

Ginger (*Zingiber officinale*) a natural supplement with antioxidant, anti-inflammatory, and anti-apoptotic properties, has demonstrated positive effects on sperm quality across species, improving volume, concentration, motility, and viability while enhancing semen's antioxidant capacity, leading to increased fertility rates (Ali *et al*., 2008; Jorsaraei *et al*., 2008; Khaki *et al*., 2009; Saeid *et al*., 2011; Oyewo *et al*., 2012; Akhlaghi *et al*., 2014). However, its effects in sheep under production conditions remain largely unexplored. Given that rams exhibit seasonal variations in reproductive activity, supplementation strategies that optimize sperm production during the breeding season merit investigation.

Reproductive patterns vary among sheep breeds, particularly in photoperiod sensitivity. While Pelibuey, Dorper, and Damara breeds maintain prolonged reproductive activity with short anestrus periods, the East Friesian breed has a restricted breeding season (Porras *et al*., 2003; Abecia *et al*., 2024). Thus, evaluating ginger supplementation during the breeding season could enhance semen quality and reproductive efficiency in sheep (Rubianes and Ungerfeld, 2002).

This study assessed the impact of dehydrated ginger pellet supplementation on semen quality in rams of four breeds (Pelibuey, East Friesian, Damara, and Dorper) during the breeding season. We hypothesized that ginger supplementation would improve seminal parameters compared to a non-supplemented diet.

MATERIALS AND METHODS

Study site

The experiment was carried out at the Sheep and Goat Reproduction Laboratory (LaROCa) of the Colegio de Postgraduados, Campus Montecillo, located at Km. 36.5 Carretera México-Texcoco, Montecillo, Texcoco, Estado de México (19° 27' 38" N, 98° 54' 11" W) at an altitude of 2243 m according to Köppen, modified by García (2004), during the months of September to December (reproductive season, 16 weeks).

Animals and feeding

Thirty-one adult rams were used, eight Pelibuey, eight East Friesian, eight Damara and seven Dorper, weighing approximately 70 kg for Pelibuey and Damara, 80 kg for East Friesian and Dorper; aged between one and four years; clinically healthy, previously wormed, hoofless and vitaminised. For 16 weeks (September-December; breeding season) the rams were divided into four groups according to breed. The first eight weeks corresponded to the control period, they were fed a basic diet of 2 kg animal^{-1} day^{-1} based on commercial concentrate and minerals (Fosforysal Borrego® purine) from Ovina Reproductores 14, dehydrated lucerne, oats and water ad libitum, covering the nutritional requirements (NRC, 2007); the following eight weeks (from 9 to 16) corresponded to the experimental period, where 40 g of pelleted feed was added to the basic diet with a ratio of 1: 20 dehydrated ginger (2 g) animal^{-1} day^{-1}, given in the morning on an empty stomach.

Scrotal circumference and determination of body weight

Scrotal circumference was measured weekly using a flexible tape measure and weighed on a weighing machine with a capacity of 250 kg ± 100 g (Braunker model YP200S).

Semen collection and evaluation

Weekly semen collections were performed using an artificial vagina, and ejaculated volume, time of ejaculation, mass motility, percentage of live sperm, normal sperm (normality) and sperm concentration were determined according to the method described by Cortez-Romero & Gallegos-Sánchez (2014). A Carl ZEISS microscope, Primo Star, CP1145K06, Microimaging GmbH 37081, Göttingen, Germany, was used for the analysis of sperm concentration, normality and percentage of live spermatozoa. Series no: 3125001511; and for individual and progressive motility variables, an LW Scientific LW200, No. 506227 microscope equipped with a thermoplate (Vari-Warm LW Scientific) maintained at 37 °C was used.

Statistical analysis

A 2×4 factorial analysis was used for statistical analysis, where the factors were diet with and without ginger and the 4 breeds.

For analysis of repeated measure over time, the following model was used:

$$Y_{ijk} = \mu + \beta_j + T_i + (T\beta)_{ij} + \varepsilon_{ijk}$$

Where: y_{ijk}=Response variable; μ=Mean effect; β=effect of level A (Control diet and diet containing ginger); T_i=Effect of level B (breeds, Pelibuey, East Friesian, Damara and Dorper); $(T\beta)_{ij}$=Interaction effect; ε_{ijk}=Random error. Both models were run in SAS 9.1 (2003).

RESULTS AND DISCUSSION

Body Weight and Scrotal Circumference

The values obtained for body weight and scrotal circumference during the experiment are presented in Table 1. In the four evaluated breeds, a progressive increase in body weight was observed from week 1 to week 16, with values ranging from 66.2±4.9 kg to 71.1±4.9 kg in Pelibuey, 66.1±4.9 kg to 78.9±4.9 kg in Frisian East, 72.60±5.30 kg to 83.48±4.96 kg in Damara, and 64.26±5.30 kg to 70.7±5.3 kg in Dorper. Similarly, scrotal circumference increased, with values ranging from 31.5±0.9 cm to 33.9±0.9 cm in Pelibuey, 28.46±0.91 cm to 35.94±0.91 cm in East Friesian, 31.94±0.98 cm to 37.84±0.91 cm in Damara, and 28.16±0.98 cm to 31.93±0.98 cm in Dorper. These results are consistent with previous reports for these breeds (Chi *et al.*, 2009; Arellano-Lezama, 2015; Calderón-Leyva *et al.*, 2018).

Variations in body weight and scrotal circumference are indicators of sexual maturity and reflect neuroendocrine changes throughout the year (Lincoln, 1998). Environmental factors, such as photoperiod and nutrition, have been documented to influence these parameters (Martin *et al.*, 1994; Blache *et al.*, 2000). During the reproductive season, an

Table 1. Means and standard errors (X±SE) of the variables body weight and scrotal circumference evaluated in sheep of the Pelibuey, East Friesian, Damara and Dorper breeds.

Variable	Breed / Week	Scrotal circumference				Body weight			
		Damara	Dorper	East Friesian	Pelibuey	Damara	Dorper	East Friesian	Pelibuey
Control	1	31.94±0.98	28.16±0.98	28.46±0.91	31.54±0.91	72.6±5.3	64.26±5.3	66.08±4.96	66.16±4.96
	2	32.96±1.02	29.4±1.24	29.81±1.35	31.69±0.15	76.1±3.5	65.03±0.77	68.58±2.5	66.55±0.39
	3	32.94±0.02	29.83±0.43	30.53±0.71	31.74±0.05	76.55±0.45	65.94±0.91	69.08±0.5	67±0.45
	4	33.43±0.49	29.3±0.53	31.05±0.53	31.86±0.13	77.15±0.6	66.23±0.29	69.68±0.6	67.39±0.39
	5	33.89±0.46	28.84±0.46	31.4±0.35	31.55±0.31	77.85±0.7	66.29±0.06	71.03±1.35	67.49±0.1
	6	34.23±0.34	29.51±0.67	31.53±0.13	32.2±0.65	78.48±0.63	66.6±0.31	71.58±0.55	67.73±0.24
	7	34.04±0.19	29.57±0.06	31.7±0.18	32.73±0.52	78.45±0.02	67.26±0.66	72.29±0.71	67.91±0.19
	8	34.55±0.51	28.81±0.76	31.91±0.21	33±0.27	78.58±0.13	67.57±0.31	73.15±0.86	68.03±0.11
Exp(ginger)	9	34.58±0.03	29.9±1.09	32.2±0.29	32.9±0.1	79.3±0.72	67.69±0.11	73.75±0.6	68.11±0.09
	10	35.13±0.55	30.07±0.17	32.56±0.36	32.5±0.4	79.13±0.17	67.77±0.09	74.35±0.6	68.26±0.15
	11	35.23±0.1	30.51±0.44	32.71±0.15	33.21±0.71	79.58±0.45	69.51±1.74	74.78±0.43	69.05±0.79
	12	35.18±0.05	30.54±0.03	33.09±0.38	33.18±0.04	79.88±0.3	69.51±0	75.38±0.6	70.25±1.2
	13	35.63±0.45	30.51±0.03	33.69±0.6	32.73±0.45	79.63±0.25	68.91±0.6	75.63±0.25	70.43±0.17
	14	36.33±0.7	30.74±0.23	33.94±0.25	32.43±0.3	80.5±0.88	69.77±0.86	76.18±0.55	70.69±0.26
	15	36.64±0.31	31.21±0.47	34.69±0.75	32.86±0.44	81.15±0.65	69.83±0.06	76.94±0.76	70.94±0.25
	16	37.84±1.2	31.93±0.71	35.94±1.25	33.95±1.09	83.48±2.32	70.74±0.91	78.93±1.99	71.1±0.16

increase in Sertoli cell volume promotes spermatogenesis, resulting in increased testicular size (Lincoln, 1998). In this study, the increase in scrotal circumference and body weight suggests greater reproductive activity, which may be related to the improvement in the evaluated seminal variables. The analysis of variance showed a significant effect of treatment ($p<0.05$) on ejaculate volume, percentage of live sperm, sperm normality, and sperm concentration. A significant effect of breed ($p<0.05$) was also detected on ejaculation time, ejaculate volume, mass motility, percentage of live sperm, sperm normality, and sperm concentration. Furthermore, the breed x treatment interaction had a significant effect ($p<0.05$) on ejaculate volume, mass motility, individual motility, percentage of live sperm, sperm normality, and sperm concentration (Table 2). No significant differences ($p>0.05$) were found between weeks in the temporal analysis.

During week 8 of the experiment, the highest values for all evaluated seminal variables were recorded. These findings suggest that supplementation with ginger pellets should be maintained for at least 60 days to maximize the reproductive response, in accordance with previous studies demonstrating that prolonged nutritional supplementation in males positively impacts spermatogenesis (Martin *et al.*, 2004).

Ejaculate volume

A significant interaction effect between treatment and breed ($p<0.05$) was observed in ejaculate volume. A decrease was recorded in Pelibuey, Frisian East, and Dorper with

ginger administration, although the recorded values remained within previously reported ranges (Arellano-Lezama, 2015). Studies in domestic birds have not shown changes in ejaculate volume following ginger supplementation (Akhlaghi *et al.*, 2014), suggesting a species-specific response. However, in this study, the reduction in semen volume was accompanied by an increase in sperm concentration, indicating greater efficiency in sperm production.

Sperm concentration

Ginger supplementation significantly increased ($p<0.05$) sperm concentration, from 381.87×10^6 mL^{-1} to 451.88×10^6 mL^{-1}. This result aligns with findings in animal models such as mice (Khaki *et al.*, 2009; Oyewo *et al.*, 2012) and broiler chickens (Saeid *et al.*, 2011; Shanoon, 2011). The positive effect of ginger on spermatogenesis may be attributed to the presence of bioactive compounds such as gingerols, paradols, and shogaols (Faivre *et al.*, 2006), which possess antioxidant properties and reduce oxidative stress in testicular cells (Agarwal *et al.*, 2014). Additionally, ginger has been associated with modulation of glutamate receptors in the brain (Ali *et al.*, 2008; Kuete, 2017), influencing GnRH secretion and, consequently, LH and FSH production (Maffucci and Gore, 2009).

Mass motility and individual progressive motility

A significant interaction effect ($p<0.05$) between treatment and breed was detected in mass and individual motility. During the control period, the Frisian East breed exhibited the highest mass motility (4.82 ± 0.09), while Pelibuey recorded the lowest values. After ginger supplementation, mass motility values were 4.36 ± 0.09, 4.40 ± 0.09, 4.67 ± 0.09, and 4.74 ± 0.10 in Pelibuey, Frisian East, Damara, and Dorper, respectively, exceeding values documented in previous studies (Karagiannidis *et al.*, 2000; Cárdenas-Gallegos *et al.*, 2012; Arellano-Lezama, 2015). These findings support the hypothesis that the bioactive compounds in ginger can enhance sperm motility through antioxidant and hormonal mechanisms, highlighting its potential as a supplement to optimize seminal quality in sheep.

Table 2. Means and standard errors (X±EE) of the variables ejaculate time (ET), ejaculate volume (Vol); mass motility (Mass Mot); individual motility (Ind Mot); live sperm count (Live); normality (Norm) and sperm concentration (Con) evaluated in sheep of the Pelibuey, East Friesian, Damara and Dorper breeds.

Var	Period		EEM	Breed				EEM	P>F		
	Control	Exp(ginger)		Pelibuey	EF	Damara	Dorper		Control	Breed	Per* Breed
ET	0.71	0.52	0.10	0.51^{ab}	0.34^{b}	0.88^{a}	0.74^{ab}	0.14	0.2094	0.0266	0.4985
Vol	0.90^{a}	0.76^{b}	0.21	0.82	0.78	0.83	0.89	0.03	<0.0001	0.0051	0.0006
Mass Mot	5.53	4.55	0.05	4.19^{b}	4.61^{a}	4.72^{a}	4.66^{a}	0.10	0.9081	<0.0001	0.0033
Ind Mot	95.94	96.18	0.61	94.67^{b}	96.13^{a}	98.34^{a}	95.00^{b}	0.88	0.5989	0.0815	0.0009
Live	66.38^{b}	69.40^{a}	0.65	74.59^{a}	68.48^{b}	61.92^{c}	66.32^{b}	0.93	0.0021	<0.0001	0.0100
Norm	91.78^{b}	93.52^{a}	0.40	92.46^{ab}	93.63^{a}	93.66^{a}	90.61^{b}	0.56	0.0017	0.0007	0.0059
Con	381.87^{b}	451.88^{a}	6.80	394.08^{b}	394.33^{b}	451.4^{a}	429.9^{a}	9.71	<0.0001	<0.0001	0.0002

Means with different literals in the same row are different ($p<0.05$).

Percentage of live spermatozoa

When analysing the variable percentage of live spermatozoa, a treatment effect was observed ($p<0.05$) that increased the percentage of live spermatozoa per ejaculate, a similar effect has been previously reported in rat (Khaki *et al.*, 2009; Oyewo *et al.*, 2012) and broilers (Saeid *et al.*, 2011; Shanoon, 2011). The improvement recorded in the percentage of live spermatozoa in the present experiment is probably due to its antioxidant property, due to the presence of gingerols, paradols and shogaols in ginger (Faivre *et al.*, 2006). This antioxidant property helps to protect the sperm membrane and counteracts the peroxidation that occurs in semen over time, thereby increasing the percentage of live sperm. At the brain level, ginger has been shown to have effects on glutamate receptors (Ali *et al.*, 2008; Kuete, 2017), which in turn influence GnRH secretion (Maffucci and Gore, 2009), so it can be suggested that ginger influences the increased production of LH and FSH, leading to an increase in sperm and testosterone production; not surprisingly, an increase in sperm concentration was reported in this study. It is therefore suggested that a 60-day course of ginger pellets will increase the percentage of live sperm per ejaculate.

Sperm normality

When analysing the sperm normality variables, a treatment effect was observed ($p<0.05$). This effect of ginger intake has been reported in rat (Khaki *et al.*, 2009; Oyewo *et al.*, 2012) and broilers (Saeid *et al.*, 2011; Shanoon, 2011). The sperm normality in sheep reported in the present study during the period with ginger was 93.52%, a value higher than those previously reported with other diets (Arellano-Lezama, 2015; Cadena-Villegas, 2015). This increase in sperm normality could be due to the presence of gingerols, paradols and shogaols, which support spermatogenesis (Faivre *et al.*, 2006).

When analysing the interaction effect between treatment and breed, it was found that there were significant differences ($p<0.05$) in the interaction for the normality variable. The Pelibuey breed showed the highest increase in normality (94.63 ± 0.77), which may be due to the fact that it is a breed that is less susceptible to the effect of photoperiod (Porras *et al.*, 2003) and may be a breed that is more responsive to changes in the amount of nutrients supplied (De Waal and Combrinck, 2000). However, it is important to carry out further studies on this subject, including the reproductive rest period, in order to be able to recommend the use of ginger during the rest period and to be able to maintain a constant semen quality throughout the year or whenever the producer wishes to breed.

CONCLUSIONS

The inclusion of ginger in the diet of rams at a rate of 2 g per day per animal has a positive effect ($p<0.05$) on the variables volume, percentage of live spermatozoa, sperm normality and sperm concentration. Therefore, we can conclude that the inclusion of ginger in the diet of rams during the reproductive season is a viable option to improve sperm quality. It would be interesting to repeat this experiment during the inactive reproductive period and to compare the results between the two periods.

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