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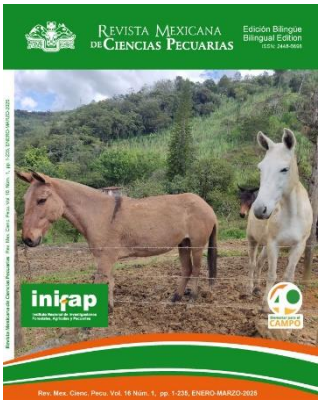

Mulares de trabajo en explotaciones agropecuarias de Antioquia, Colombia. Autores: **Lady Calixto, José Ramón Martínez y Andrés Felipe Castro.**

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REV. MEX. CIENC. PECU. VOL. 16 No. 1 ENERO-MARZO-2025

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Actualización: septiembre, 2024

NOTAS AL AUTOR

La **Revista Mexicana de Ciencias Pecuarias** se edita completa en dos idiomas (español e inglés) y publica tres categorías de trabajos: Artículos científicos, Notas de investigación y Revisiones bibliográficas.

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   Página del título
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   Resumen en inglés
   Texto
   Agradecimientos y conflicto de interés
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7. **Página del Título**. Solamente debe contener el título del trabajo, que debe ser conciso pero informativo; así como el título traducido al idioma inglés. En el manuscrito no se debe incluir información como nombres de autores, departamentos, instituciones, direcciones de correspondencia, etc., ya que estos datos tendrán que ser registrados durante el proceso de captura de la solicitud en la plataforma del OJS (revisar el Instrucctivo para envío de artículos en la dirección: http://ciencias pecuarias.inifap.gob.mx.

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Introducción
Material y Métodos
Resultados
Discusión
Conclusiones e implicaciones
Literatura citada

En los artículos largos puede ser necesario agregar subtítulos dentro de estas divisiones a fin de hacer más claro el contenido, tanto en Material y métodos como en las secciones de Resultados y de Discusión, las cuales también pueden presentarse como una sola sección.

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II) Stephano HA, Gay GM, Ramírez TC. Encephalomielitis, reproductive failure and corneal opacity (blue eye) in pigs associated with a paramyxovirus infection. Vet Rec 1988;(122):6-10.

III) Chupin D, Schuh H. Survey of present status of the use of artificial insemination in developing countries. World Anim Rev 1993;(74-75):26-35.

No se indica el autor.

IV) Cancer in South Africa [editorial]. S Afr Med J 1994;84:15.

Suplemento de revista.

V) Hall JB, Staigmiller RB, Short RE, Bellows RA, Bartlett SE. Body composition at puberty in beef heifers as influenced by nutrition and breed [abstract]. J Anim Sci 1998;71(Suppl 1):205.

Organización, como autor.

VI) The Cardiac Society of Australia and New Zealand. Clinical exercise stress testing. Safety and performance guidelines. Med J Aust 1996;(164):282-284.

En proceso de publicación.

VII) Scifres CJ, Kothmann MM. Differential grazing use of herbicide treated area by cattle. J Range Manage [in press] 2000.

Libros y otras monografías

Autor total.

VIII) Steel RGD, Torrie JH. Principles and procedures of statistics: A biometrical approach. 2nd ed. New York, USA: McGraw-Hill Book Co.; 1980.

Autor de capítulo.

IX) Roberts SJ. Equine abortion. In: Faulkner LLC editor. Abortion diseases of cattle. 1rst ed. Springfield, Illinois, USA: Thomas Books; 1968:158-179.

Memorias de reuniones.

X) Loeza LR, Angeles MAA, Cisneros GF. Alimentación de cerdos. En: Zúñiga GJL, Cruz BJA editores. Tercera reunión anual del centro de investigaciones forestales y agropecuarias del estado de Veracruz. Veracruz. 1990:51-56.

XI) Olea PR, Cuarón IJA, Ruiz LFJ, Villagómez AE. Concentración de insulina plasmática en cerdas alimentadas con melaza en la dieta durante la inducción de estro lactacional [resumen]. Reunión nacional de investigación pecuaria. Querétaro, Qro. 1998:13.

XII) Cunningham EP. Genetic diversity in domestic animals: strategies for conservation and development. In: Miller RH et al. editors. Proc XX VI eltsville Symposium: Biotechnology's role in genetic improvement of farm animals. USDA. 996:13.

Tesis.

XIII) Alvarez MJA. Inmunidad humoral en la anaplasmosis y babesiosis bovinas en becerros mantenidos en una zona endémica [tesis maestría]. México, DF: Universidad Nacional Autónoma de México; 1989.

XIV) Cairns RB. Infrared spectroscopic studies of solid oxigen [doctoral thesis]. Berkeley, California, USA: University of California; 1965.

Organización como autor.

XV) NRC. National Research Council. The nutrient requirements of beef cattle. 6th ed. Washington, DC, USA: National Academy Press; 1984.

XVI) SAGAR. Secretaría de Agricultura, Ganadería y Desarrollo Rural. Curso de actualización técnica para la aprobación de médicos veterinarios zootecnistas responsables de establecimientos destinados al sacrificio de animales. México. 1996.

XVII) AOAC. Official methods of analysis. 15th ed. Arlington, VA, USA: Association of Official Analytical Chemists. 1990.

XVIII) SAS. SAS/STAT User's Guide (Release 6.03). Cary NC, USA: SAS Inst. Inc. 1988.

XIX) SAS. SAS User´s Guide: Statistics (version 5 ed.). Cary NC, USA: SAS Inst. Inc. 1985.

Publicaciones electrónicas

XX) Jun Y, Ellis M. Effect of group size and feeder type on growth performance and feeding patterns in growing pigs. J Anim Sci 2001;79:803-813. http://jas.fass.org/cgi/reprint/79/4/803.pdf. Accessed Jul 30, 2003.

XXI) Villalobos GC, González VE, Ortega SJA. Técnicas para estimar la degradación de proteína y materia orgánica en el rumen y su importancia en rumiantes en pastoreo. Téc Pecu Méx 2000;38(2): 119-134. http://www.tecnicapecuaria.org/trabajos/20021217 5725.pdf. Consultado 30 Ago, 2003.

XXII) Sanh MV, Wiktorsson H, Ly LV. Effect of feeding level on milk production, body weight change, feed conversion and postpartum oestrus of crossbred lactating cows in tropical conditions. Livest Prod Sci 2002;27(2-3):331-338. http://www.sciencedirect. com/science/journal/03016226. Accessed Sep 12, 2003.

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cal caloría (s)
cm centímetro (s)
°C grado centígrado (s)
DL_{50} dosis letal 50%
g gramo (s)
ha hectárea (s)
h hora (s)
i.m. intramuscular (mente)
i.v. intravenosa (mente)
J joule (s)
kg kilogramo (s)
km kilómetro (s)
L litro (s)
log logaritmo decimal
Mcal megacaloría (s)
MJ megajoule (s)
m metro (s)
msnm metros sobre el nivel del mar
µg microgramo (s)
µl microlitro (s)
µm micrómetro (s)(micra(s))
mg miligramo (s)
ml mililitro (s)
mm milímetro(s)
min minuto (s)
ng nanogramo (s)
P probabilidad (estadística)
p página
PC proteína cruda
PCR reacción en cadena de la polimerasa
pp páginas
ppm partes por millón
% por ciento (con número)
rpm revoluciones por minuto
seg segundo (s)
t tonelada (s)
TND total de nutrientes digestibles
UA unidad animal
UI unidades internacionales
vs versus
xg gravedades

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4. Because it is a peer-reviewed journal, and to facilitate the work of the reviewers, all the lines of each page must be numbered continuously throughout the document; each page must also be numbered, including tables, illustrations, and graphs.

5. The articles will have a maximum length of 20 double-spaced pages, not including Title pages, and tables or figures (which must not exceed eight and must be included in the text). The Research Notes will have a maximum length of 15 pages and 6 tables or figures. Bibliographic Reviews a maximum length of 30 pages and 5 tables.

6. The manuscripts of the three categories of papers that are published in **Rev. Mex. Cienc. Pecu.** must contain the components indicated below, each of them starting on a separate page.

Title page
Abstract in Spanish
Abstract in English
Text
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7. **Title Page.** It must only contain the title of the work, which should be concise but informative, as well as the title translated into Spanish. The manuscript should not include information such as names of authors, departments, institutions, correspondence addresses, etc., since these data will have to be registered during the process of capturing the application on the OJS platform (look at the Instructions for submitting articles at the address: http://ciencias pecuarias.inifap.gob.mx.

8. **Abstract in Spanish.** On the second page, an abstract that does not exceed 250 words must be included. It will indicate the purposes of the study or research; the basic procedures and methodology used; the most important results found, and if possible, their statistical significance and main conclusions. After the abstract, and on a separate line, add, properly labeled, 3 to 8 key words or short phrases that help indexers classify the work, which will be published along with the abstract.

9. **Abstract in English.** Write down the title of the work in English and then write the "abstract" with the same instructions that were indicated for the abstract in Spanish. At the end, and on a separate line, the corresponding keywords must be written.

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a) Scientific Articles. They must be reports of original works derived from partial or final results of studies. The text of the Scientific Article is divided into sections that have these headings:

Introduction
Material y methods
Results
Discussion
Conclusions and implications
Literature cited

In long articles, it may be necessary to add subheadings within these divisions in order to clarify the content, both in Material and methods and in the Results and Discussion sections, which can also be presented as a single section.

b) Research Notes. They consist of modifications to techniques, reports of clinical cases of special interest, preliminary work or limited research, description of new grass varieties, as well as research results that, in the opinion of the editors, should be published. The text will contain the same information as the experimental method referred to in subparagraph (a) but will be worded continuously from the beginning to the end of the work; this does not mean that subheadings are only deleted, but that it is written in a continuous and consistent manner.

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Basic rules for Literature cited

Name of the authors, with capital letters only the initials, starting with the paternal surname, then initials of the maternal one and name (s). In case of compound surnames, a hyphen must be put between them, example: Elías-Calles E. Between the initials of an author, no punctuation mark, or separation should be placed; after each author, only a comma must be placed; after the last author, a period must be placed.

The title of the paper must be written in full (in its original language) then the abbreviated title of the journal where it was published, without any punctuation mark; immediately after, the year of publication, then the volume number, followed by the number (in parentheses) of the journal and finally the number of pages (this in the case of an ordinary journal article).

The list of references may include accepted articles, even if they are not yet published; indicate the journal and add "in press" (in square brackets).

In the case of books by a single author (or more than one, but all responsible for the total content of the book), after the name or names, the title of the book, the edition number, the country, the publishing house, and the year must be indicated.

When it comes to the chapter of a book by several authors, the name of the author of the chapter must be indicated, then the title of the chapter, then the name of the editors and the title of the book, followed by the country, the publishing house, year, and the pages covered by the chapter.

In the case of a thesis, the following must be indicated: name of the author, the title of the work, then the degree (bachelor's, master's, doctorate) in brackets, then the name of the city, state and, where appropriate, country, followed by the name of the University (not the school), and finally the year.

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Journals

Ordinary article, with volume and number. (Include the name of all authors when they are six or less; if there are seven or more, write down only the name of the first six and add "et al.").

I) Basurto GR, Garza FJD. Efecto de la inclusión de grasa o proteína de escape ruminal en el comportamiento de toretes Brahman en engorda. Téc Pecu Méx 1998;36(1):35-48.

Only the number without indicating the volume.

II) Stephano HA, Gay GM, Ramírez TC. Encephalomielitis, reproductive failure and corneal opacity (blue eye) in pigs associated with a paramyxovirus infection. Vet Rec 1988;(122):6-10.

III) Chupin D, Schuh H. Survey of present status of the use of artificial insemination in developing countries. World Anim Rev 1993;(74-75):26-35.

The author is not indicated.

IV) Cancer in South Africa [editorial]. S Afr Med J 1994;84:15.

Journal supplement.

V) Hall JB, Staigmiller RB, Short RE, Bellows RA, Bartlett SE. Body composition at puberty in beef heifers as influenced by nutrition and breed [abstract]. J Anim Sci 1998;71(Suppl 1):205.

Organization as author.

VI) The Cardiac Society of Australia and New Zealand. Clinical exercise stress testing. Safety and performance guidelines. Med J Aust 1996;(164):282-284.

In the process of publication.

VII) Scifres CJ, Kothmann MM. Differential grazing use of herbicide treated area by cattle. J Range Manage [in press] 2000.

Books and monographs

Total author.

VIII) Steel RGD, Torrie JH. Principles and procedures of statistics: A biometrical approach. 2nd ed. New York, USA: McGraw-Hill Book Co.; 1980.

Chapter author.

IX) Roberts SJ. Equine abortion. In: Faulkner LLC editor. Abortion diseases of cattle. 1rst ed. Springfield, Illinois, USA: Thomas Books; 1968:158-179.

Meeting proceedings.

X) Loeza LR, Angeles MAA, Cisneros GF. Alimentación de cerdos. En: Zúñiga GJL, Cruz BJA editores. Tercera reunión anual del centro de investigaciones forestales y agropecuarias del estado de Veracruz. Veracruz. 1990:51-56.

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13. **Tables, Graphs, and Illustrations.** It is preferable that they be few, concise, with the necessary data to be self-sufficient, that they be understood alone without having to read the text. For footnotes, conventional symbols should be used.

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18.

Frequently used abbreviations:

cal	calorie (s)
cm	centimeter (s)
°C	degree centigrade (s)
LD_{50}	lethal dose 50%
g	gram (s)
ha	hectare (s)
h	hour (s)
i.m.	intramuscular (-ly)
i.v.	intravenous (-ly)
J	joule (s)
kg	kilogram (s)
km	kilometer (s)
L	liter (s)
log	decimal logarithm
Mcal	megacalorie (s)
MJ	megajoule (s)
m	meter (s)
masl	meters above sea level
µg	microgram (s)
µL	microliter (s)
µm	micrometer (s)(micra(s))
mg	milligram (s)
mL	milliliter (s)
mm	millimeter (s)
min	minute (s)
ng	nanogram (s)
P	(statistical) probability
p	page
CP	crude protein
PCR	polymerase chain reaction
pp	pages
ppm	parts per million
%	per cent (with number)
rpm	revolutions per minute
sec	second (s)
t	tonne (s)
TDN	total digestible nutrients
AU	animal unit
IU	international units
vs	versus
xg	gravities

Any other abbreviation should be placed in parentheses immediately after the full word(s).

19. Scientific names and other Latin phrases must be written in italics

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Article

Dietary level of potato protein concentrate and its effect on cytokine and volatile fatty acid intestinal concentration in weaned piglets

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Abstract:

Weaning is a traumatic event for the piglet, since it implies changes that are responsible for gastrointestinal disturbs, a productivity decreases or even death. Bacterial resistance development on account of sub-therapeutic doses of antibiotics inclusion in starter diets has become an important public health matter, banning their inclusion on animal feed. Potato protein concentrate (PPC) has been considered an alternative to regulate intestinal inflammation and gut disorders due to its content of antimicrobial peptides which have beneficial effects on gut homeostasis. This study evaluated the effect of the inclusion level of PPC in an antibiotic free diet on the inflammatory markers concentration as interleukin-12p40 (IL-12p40) and tumor necrosis factor alpha (TNF-α) in ileal tissue and volatile fatty acid (VFA) concentration in colonic digesta. 90 piglets were assigned to three treatments: 1, basal diet (C) (diet without antibiotics nor PPC); 2, basal diet with 6% PPC (PPC 6%) and 3, basal diet with 8% PPC (PPC 8%). At 15 postweaning day, six piglets per treatment were

euthanized for sample collection. The PPC 8% group had the highest levels of VFA and the lowest concentration of inflammatory cytokines compared to the C group which had the lowest levels of VFA and the highest concentration of inflammatory markers. The inclusion of PPC on the starter diets of weaned piglets can be an effective alternative to regulate the gut dysbiosis during weaning.

Keywords: Potato proteins, Proinflammatory cytokines, Intestinal fermentation, Weaning.

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Introduction

Gastrointestinal cellular development, physiology, microbiota, and immunity adjust their functions according to the animal requirements, to nurture and confer protection against potentially pathogen microorganisms that could compromise the survival of the early weaned piglets, this due to the immaturity of their digestive and immunological activity[1,2]. Weaning of piglets is associated with an imbalance between immune and inflammation responses, leading to the development of pathological diseases by the secretion of pro-inflammatory cytokines[3]. Cytokines are a group of proteins and glycoproteins synthesized by different cell lines, responsible for the regulation of inflammatory and immune responses that act at the systemic level by modulating cellular activity through interaction with specific membrane receptors that trigger a chemical reaction downstream. The synthesis and release of cytokines can be stimulated by inappropriate inflammatory activation caused by feed, environment, bacteria, and metabolites, as well as the presence of other proinflammatory cytokines, including interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6), which induce the production of acute phase proteins[4,5]. The proinflammatory cytokine TNF-α acts as an acute inflammation mediator that increases the production of IL-1 and IL-6[6].

The gut microbiota is a complex population with hundreds of diverse microorganisms that contribute to the breakdown of nutrients and energetic metabolism, affecting the immune system and homeostasis[7]. Gut microorganisms in the large intestine are responsible for the fermentation of indigestible carbohydrates and protein in piglets diets to produce a series of metabolites denominated volatile fatty acids (VFA). Among the various metabolites, short

chain fatty acids (SCFA) have received extensive attention because of their positive effects on health[8].

Antibiotics have been widely used in the swine industry as growth promoters, which has led to bacterial resistance development and to the presence of antibiotic residue in animal products[9]. To face this problematic, the need to identify alternatives that replace the use of antibiotics while maintaining growth productive parameters has arisen. Potato protein concentrate has been considered as a valuable essential amino acid source that could replace the inclusion of animal protein in the piglet starter diets due to the presence of antimicrobial peptides (AMP) and the content of high digestible protein[10,11]. The antibacterial activity of PPC AMP is based primarily on the interaction of positively charged peptides with negatively charged components of the bacterial membrane such as phospholipids and teichoic acids of gram-positive bacteria or lipopolysaccharide of gram-negative bacteria, which leads to pore formation, membrane permeabilization, and cell lysis after re-localization in the cytosolic membrane[12].

PPC antimicrobial activity could be an interesting alternative to the use of antibiotic as growth promoters, altering the composition of the microbiota to reduce competition for nutrients, reduce pathogen, and control mucus, however the level of inclusion has not yet been determined for piglets nutrition, therefore, it is necessary to evaluate the effect of different levels of inclusion.

This study aim was to establish the optimum level of PPC inclusion to decrease the post weaning inflammatory process, defined by the presence of pro inflammatory markers, as well as VFA profile.

Material and methods

Animal management

All animal experimental procedures were conducted according to the guidelines of the Mexican Official Norm NOM-062-ZOO-1999 for production, care and use of animals for experimentation[13] and guidelines of the International Guiding Principles for Biomedical Research Involving Animals[14]. The experimental design and procedures in this study were reviewed and approved by the Bioethical Committee of the Faculty of Natural Sciences of the Autonomous University of Queretaro (approval number: 96FCN2021).

Ninety (90) hybrid piglets (Large white x Landrace) x PIC337 weaned at 21 ± 2 d and weighing 6.85 ± 0.93 kg were utilized in this study and assigned to three experimental diets according to litter origin and bodyweight, and were housed in six pens per diet, each with five piglets, making a total of 30 piglets per diet. Three experimental diets were formulated with a basal diet without antibiotics or potato protein concentrate (C) subsequently 6 % and 8 % of potato protein concentrate was added to the basal diet to establish the two other diets, namely PPC 6% and PPC 8% respectively, as described in Table 1. Pens were equipped with a feeder with six spaces and nipple drinkers in an environmentally controlled weaning room (32 ºC with -2 ºC during the first and second post weaning weeks).

Table 1: Ingredients and chemical percentage composition of the experimental diets

	Experimental diets		
Ingredients (%)	**C**	**PPC 6%**	**PPC 8%**
Maize	40.63	39.69	39.36
Soybean meal	15.00	15.00	15.00
Soybean protein isolate	11.55	6.98	5.46
Potato protein concentrate	-	6.00	8.00
Sweet whey powder	24.69	24.69	24.69
Maize oil	3.29	3.06	2.98
Lysine	0.41	0.29	0.24
AminoGut®*	0.80	0.80	0.80
Threonine	0.11	0.01	-
Methionine	0.22	0.17	0.15
Tryptophan	0.01	-	-
Sodium	0.50	0.50	0.50
Calcium carbonate	0.54	0.48	0.45
Dicalcium phosphate	1.64	1.71	1.74
Titanium dioxide	0.30	0.30	0.30
Vitamins**	0.07	0.07	0.07
Coline	0.14	0.14	0.14
Minerals***	0.10	0.10	0.10
Dry matter, %[a]	92.73	92.85	92.55
Crude protein, %[a]	23.12	22.99	23.45
ME, kcal/kg[b]	3,400	3,400	3,400

*L-glutamine, L-glutamic acid (1:1).**vitamins per kg of diet, A: 13,000 UI, E: 160 mg, K: 9 mg, thiamine: 4 mg, riboflavine: 12 mg, pyridoxine:6 mg, cianocobalamine: 0.07 mg, niacin:66 mg, panthotenate: 46 mg, folate: 5 mg, biotin: 0.67 mg, C: 266 mg. ***minerals per kg of diet, manganese: 32 mg, zinc: 120 mg, iron: 100 mg, copper: 12 mg, iodine 0.8 mg, selenium: 0.25 mg, cobaltum: 0.6 mg.

[a] Analyzed value, [b] Calculated value.

Samples collection

At 15 d post weaning, six piglets per diet group were euthanized for posterior sampling. Animals were tranquilized with 20 mg/kg azaperone (Sural® Chinoin, Mexico City, Mexico) and then euthanized with an overdose of sodium pentobarbital (Pisabental ®, PiSA Agropecuaria, Hidalgo, Mexico). Ileal tissue sections (10 cm) were sampled from 5 cm before the ileocecal valve, gently washed with distilled water, and then dissected and fixed in 10 % buffered formalin solution. Ileal sections were embedded in paraffin and cut into 5 µm slices. Colonical digest content was collected, frozen and stored for later analysis.

Cytokines analysis

The 5 µm slices were used to quantify TNF-α and IL-12p40 using an immunofluorescence technique adapted from that of Bautista-Marín *et al*[15]. Ileal slices were deparaffinized for 24 h at 60 °C in a dry-heat oven and then rehydrated in 100% xylol (10 min), 100% ethanol (5 min), 96% ethanol (5 min) and deionized water (10 min), in this order. Ileal slices were then soaked in saline sodium citrate buffer concentrate (Sigma Aldrich, USA) in a water bath at 80 ºC for 25 min .After that, ileal slices were blocked in 1% non-fat milk for 1 h, washed three times for 5 min each with 0.05% TRIS buffered saline plus Tween (TBST), and incubated for 2 h at 4 ºC with the following antibodies diluted in TBST: i) rabbit anti TNF-α (ab 6671) (Abcam, Cambridge, MA, USA) at 1:200 dilution, ii) rabbit anti IL-12p40 (ab 106270) (Abcam, Cambridge, MA, USA) at 1:100 dilution and incubated for 16 h .Ileal slices were then washed three times for 5 min each with 0.05% TBST and incubated for 2 h with the secondary antibody Alexa Fluor 488 chicken anti-rabbit IgG (H+L) (A-21441) (Invitrogen Molecular Probes Inc., Eugene, OR, USA) at 1:500 dilution. The intensity of the fluorescence (arbitrary fluorescence units (AFU)) was evaluated with the positive (primary and secondary antibodies) and negative (secondary antibodies only) controls considering the same size of area in ileal tissue under both conditions. For the analysis, fluorescence was visualized and measured with a fluorescence microscope (Eclipse E600, Nikon) and Image-Pro Plus version 6.1 in 10 intestinal villi per piglet and photographed at 40x magnification and then 10 sections from each intestinal villi were selected per photo to obtain 100 measurements per piglet.

Volatile fatty acid analysis

Approximately 2 g of colonical digest content were weighted and placed in previously identified centrifuge tubes, after that 3 ml of HPLC- grade water were added to the tubes and mixed with a vortex for 30 sec. Tubes were then placed in a Beckman Coulter centrifuge at 4 ºC for 30 min. With the assistance of a 3 ml syringe supernatant was extracted and emptied in an amber tube after passing through a 0.2-µm syringe filter. Concentrations of acetic, propionic, butyric, and valeric acids (SCFA); isobutyric, isovaleric, and isocaproic acids (BFA) were analyzed on an Agilent 6890 gas chromatograph equipped with a flame-ionization detector and DB-FFAP column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies, Wilmington, DE). The following gas chromatograph parameters were used: split mode, 20:1; inlet temperature, 220 °C; initial inlet pressure, 168 kPa; injection volume, 1 µL; constant column flow (He), 1.4 mL/min; and detector temperature, 250 °C.

Statistical analysis

Inflammatory marker concentration as well as volatile fatty acid concentration were analyzed using a completely randomized design, each piglet was considered an experimental unit[16]. ANOVA was utilized to analyze the differences between diets groups. Means were compared with the Tukey test using the GLM procedure of SAS and differences were considered statistically significant at $P<0.05$[17].

Results

Inflammatory cytokines concentration

The concentrations of TNF-α and IL-12p40 in the ileal villi of weaned piglets were affected ($P<0.0001$) by diet (Table 2). The piglets fed the C diet had the highest concentrations of all inflammatory markers. Animals fed the PPC 6% diet showed intermediate concentrations, and the PPC 8% group had the lowest concentrations ($P<0.0001$). There was the highest AFU signal for IL-12p40 compared with TNF-α in piglets fed all experimental diets, but particularly in those fed the C diet (Figure 1).

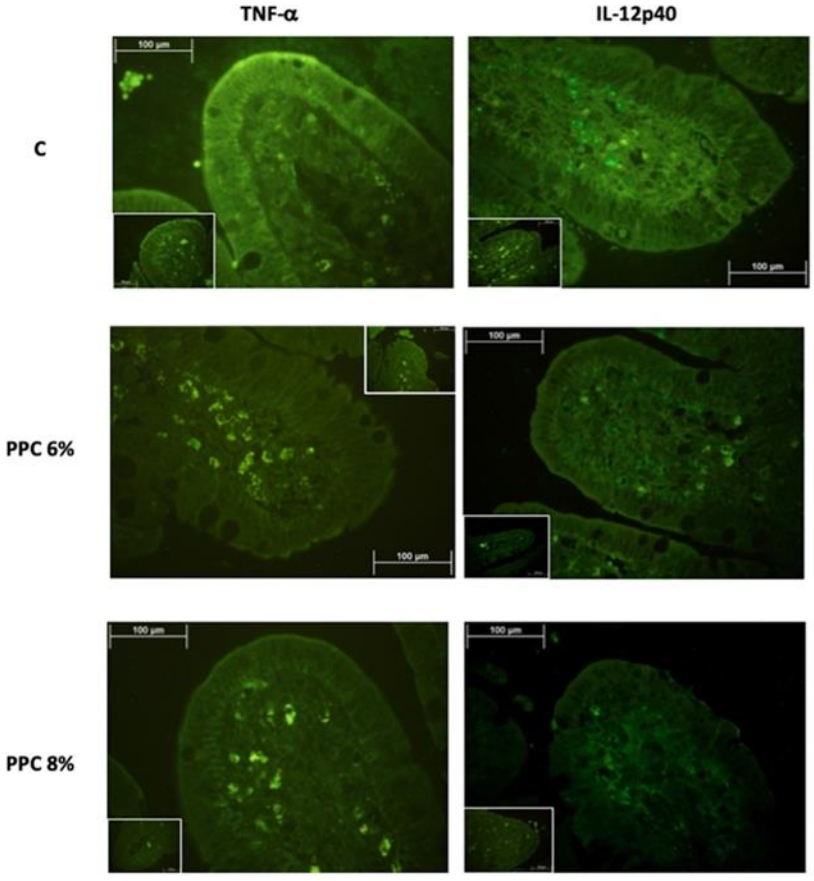
Table 2: Effect of potato protein concentrate (PPC) level of inclusion on pro inflammatory cytokines ileal concentration

Cytokines (AFU)	Experimental diets			*P*-value	SEM
	C	PPC 6%	PPC 8%		
TNF-α	6.26 [a]	4.33 [b]	3.55 [c]	***	0.0326
IL-12p40	20.65 [a]	12.54 [b]	8.17 [c]	***	0.0058

AFU= arbitrary fluorescence units; PPC= potato protein concentrate; C= diet without antibiotics nor PPC; PPC 6%= basal diet with 6% PPC; PPC 8%= basal diet with 8% PPC; P= probability. SEM= standard error of mean

[abc] Row means with different superscripts differ significantly at $P<0.05$. *** $P<0.0001$.

Figure 1: Concentration of TNF-α and IL-12p40 in the ileum of piglets fed with three experimental diets highlighted by immunofluorescence staining



The inset boxes show representatives of the controls without the primary antibody.

Volatile fatty acid concentration

Fermentation profiles in the colon were affected by diet (Table 3). Piglets fed C diet had lower concentration of total VFA (P<0.0001) than piglets fed diets with PPC at 6 and 8%. Consequently, the inclusion of PPC in both levels in the diet increased SCFA and branched fatty acids (BFA) production in the colon compared to the C diet (P<0.0001). Animals fed the PPC 8% diet had higher concentration of SCFA (P<0.0001) than animals fed the PPC 6% or C diet. Acetic was the most abundant SCFA in the digesta of all experimental diets, followed by butyric and propionic. Higher concentrations of BCFA were observed in the digesta of animals fed the PPC 8% diet than in those fed the PPC 6% and C diets (P<0.0001).

Table 3: PPC level of inclusion effect on colonic volatile fatty acid production

VFA (µmol/g)	Experimental diets			*P*-value	SEM
	C	PPC 6%	PPC 8%		
Acetic	77.86 ^{c}	95.38 ^{b}	124.68 ^{a}	***	0.5415
Propionic	22.96 ^{c}	31.88 ^{b}	55.46 ^{a}	***	0.2110
Butyric	32.13 ^{c}	36.45 ^{b}	41.00 ^{a}	***	0.2433
Isobutyric	1.33 ^{c}	1.66 ^{b}	2.26 ^{a}	***	0.0187
Valeric	1.85 ^{c}	2.41 ^{b}	2.56 ^{a}	***	0.0106
Isovaleric	1.20 ^{c}	1.56 ^{b}	1.86 ^{a}	***	0.0125
Isocaproic	0.50 ^{c}	0.71^{b}	0.90 ^{a}	***	0.0060
BFA	3.01 ^{c}	3.91^{b}	5.02 ^{a}	***	0.0271
SCFA	134.81 ^{c}	166.13 ^{b}	223.70 ^{a}	***	0.7813
Total	137.83 ^{c}	170.03 ^{b}	228.70 ^{a}	***	0.7787

PPC= potato protein concentrate; C= basal diet without antibiotic nor PPC; PPC 6%= basal diet with 6% PPC= PPC 8%= basal diet with 8% PPC; BFA= branched fatty acids; SCFA= short chain fatty acids. *P*= statistical significance; SEM= standard error of mean.

^{abc} Row means with different superscripts differ significantly at P<0.05. ***P<0.0001.

Discussion

Previous work had suggested that the results from the immunological markers in the ileum confirmed that cytokines play a central role in cell immunity and participate in maintaining tissue integrity. Alterations in intestinal cytokines concentrations were expected for weaning piglets due to severe modifications in dietary elements and environmental stressful factors during this period lead to important morphophysiological adaptations in the gastrointestinal

tract[1,4,5]. Microbial surface antigens in the intestinal lumen generate an early inflammatory response by modulating proinflammatory cytokines, which activate a signaling pathway, which leads to the release and nuclear translocation of nuclear factor-κB (NF-κB), serving as a transcription factor for the synthesis of IL-6 and TNF-α[18-20].

Interleukin 12 subunit p40 (IL-12p40) concentration arises when a chronic inflammation process takes place in the gastrointestinal tract[21]. The inflammation modulated by IL-12p40 is triggered through the Janus kinase/signal transducers and activators of transcription (JAK/STAT), in which it is dimerized and phosphorylated, translocating to the nucleus where IL-12p40 is transcribed[22].

The cross-talk between intestinal epithelial cells and underlying lamina propria cells transfers immune-related signals to the local adaptive immunity, which subsequently help to maintain gut immune homeostasis[23].

The disruption in the integrity of the intestinal mucosa generates an inflammatory process that activates NF-κB and subsequent production of proinflammatory cytokines[6]. The proinflammatory response is exacerbated when early weaned piglets are fed antibiotic-free diets[24].

Dietary supplementation of potato protein concentrate helped to regulate the synthesis of inflammatory markers TNF-α and IL-12p40, which concentration decreased of 43.31 % and 60.45 % respectively in PPC 8% diet in compare with C diet. The downregulation of cytokines by PPC antimicrobial peptides was confirmed by the current results, since TNF-α and IL-12p40 concentration decreased in piglets fed the PPC 6% and PPC 8% diet. The antimicrobial effect on the colon bacterial population probably downregulated the signaling pathway that induces the release of NF-κB[19], which was highly marked by the primary antibody used in the current study for immunofluorescence staining (Figure 1). LPS is a major component of Gram-negative bacterial outer membranes and can be recognized by host toll like receptor-4 (TLR4) and activates production of pro-inflammatory cytokines by immune cells via TLR4 signaling[25]. The anti-inflammatory functions of PPC are mainly due to LPS-neutralizing activity, which suppresses downstream TLR4 signaling pathways, such as mitogen-activated protein kinase [MAPK] and NF-κB signaling[26]. Furthermore, the PPC inclusion in the diet decreased IL-12p40 synthesis, and thus concentration, these attributed to the lack of stimulus generated by pathogen microorganisms and to the inhibition of the pathway mediated by JAK/STAT[21,22]. Therefore, dietary PPC supplementation regulates inflammatory response and indirectly protects the intestinal mucosa, decreasing the detrimental effects of weaning on the intestinal cells[27,28].

Volatile fatty acids also have a key role in the regulation of inflammatory mechanisms that confer a protector or causal effect by the modulation of inflammatory cytokines release and the recruitment of immune cells through surface receptors or by the enzymatic activity inhibition[29]. SCFAs are defined as groups of volatile fatty acids comprising less than six carbons, mainly acetate, propionate, and butyrate, which are known to be the main fuel source for colonocytes and are essential for maintaining the normal metabolism of colon mucosa, including colonocyte growth and proliferation[2]. These acids make possible the regulation of the pH of the colonic content, inhibiting the growth and development of potentially pathogen bacteria such as *E. coli*, *Clostridium* and *Salmonella*. VFA stimulate bicarbonate luminal secretion, which has an important buffer effect upon the luminal pH regulation while induces the absorption of sodium and water at the mucosal level[30].

The increase in SCFAs observed in piglets fed with PPC 6% and PPC 8%, compared to those produced by piglets fed a diet without antibiotic nor PPC, may probably be the outcome of intestinal environmental alterations favoring the establishment of beneficial bacteria and limiting the growth of pathogenic microbes[31]. In turn, acetic acid can be metabolized to butyric acid, which contributes between 70 % and 90 % of the energy necessary by colonocytes metabolism[31]. These changes may reduce the incidence and severity of postweaning diarrhea and improve piglet's growth[26]. SCFA promote an eubiotic intestinal environment by maintaining an acidic pH, which suppresses the growth of potentially pathogenic bacteria that require an alkaline pH to survive[32]. Beneficial microbiota proliferates through competitive exclusion, occupying binding sites on the intestinal mucosa that could otherwise be employed by pathogenic bacteria[33]. Establishment of beneficial microorganisms modulates pathogenic bacteria development through bacteriocin secretion, which destroys the bacterial cell wall mimicking an antibiotics mechanism[32,33]. SCFAs could act as histone deacetylase (HDAC) inhibitors and therefore attenuate inflammatory responses by blocking nuclear factor-κB (NF-κB) signaling[34]. In addition, SCFA also have a direct effect on microbiota-derived aryl hydrocarbon receptor (AHR) ligands which enhance regulatory T cells' (Tregs) immunosuppressive activities during inflammation, and indirectly influence neutrophil recruitment and activation[23]. Increased production of acetic, propionic and butyric acids are most likely related to the modulation of the intestinal microbial population through PPC antimicrobial activity.

The influence of dietary factors, through dietary interventions such as butyric acid and acid-producing bacteria, can increase the SCFA in the intestine, however, excessive SCFA in the hindgut could promote the development of metabolic syndrome via the gut microbiota–brain–β-cell axis[34]. An appropriate amount of SCFA in the intestine may be beneficial to early weaned piglet gut health. The content and ratio of VFA in the intestine are affected by the characteristics of proteins, like solubility and fermentability[35]. As for protein fermentation, proteolysis is the first step in the utilization of protein by bacteria, followed by deamination and decarboxylation of amino acids which limits their availability to the host

and yields several putrefactive compounds including ammonia, amines, branched fatty acids, indoles, phenols and sulfur-containing compounds[36]. These compounds may have toxic effects on the piglets and can affect function and diversity of the gut microbiota[37]. Therefore, there may be a threshold for SCFA and BFA in the intestine, even though PPC 8% had the highest levels of total VFA, particularly SCFA, it was also the diet with the highest level of BFA, suggesting that PPC 6% may be the optimum level of inclusion, in order to prevent harmful effects upon gut health, maintaining the immunomodulatory and anti-inflammatory properties of PPC inclusion.

The current results support the hypothesis that potato protein concentrate can modulate the levels of intestinal cytokines and increase the SCFA production. Piglets fed diets supplemented PPC had the lowest levels of inflammatory markers that can be comparable with those of piglets fed diets with antibiotics[15]. The immunomodulatory effect of PPC at the intestinal level is probably due to the inhibition of signaling pathways like NF-κB and JAK/STAT complex suppressing the synthesis of proinflammatory cytokines[38]. An additional benefit observed with the inclusion of PPC to antibiotic-free diets was the control of chronic inflammation associated with the increase in IL-12p40[39]. The efficiency of PPC observed in the present study had a positive effect in controlling acute and chronic intestinal inflammation.

Conclusions and implications

Inclusion of potato protein concentrate in antibiotic-free diets can improve intestinal health in the post-weaning period. Furthermore PPC 6% and 8% helped to decrease the inflammatory response of the measured pro-inflammatory markers, mainly in the IL-12p40 concentration, as well as it probably helped establishing an optimum intestinal environment for beneficial microbial fermentation and SCFA production having a potential influence in the proliferation of intestinal bacteria. However, PPC 6% may be the optimum level of inclusion, preventing BFA harmful effects upon gut health, maintaining the immunomodulatory and anti-inflammatory properties of PPC inclusion. Therefore, the inclusion of potato protein concentrate can be used as an effective feed additive in antibiotic-free diets. Economic considerations should be considered for the selection of the optimal level of inclusion of PPC in the piglet diet by the producer. The broad-spectrum antibacterial activities of PPC have been indirectly demonstrated, making them promising alternatives to antibiotics. The immunomodulatory properties of PPC mean they likely have similar performance compared to antibiotics in the swine industry. Future research is needed to elucidate its precise effect upon anti-inflammatory cytokines concentration and microbial intestinal population diversity to fully understand its action mechanism.

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Conflicts of Interest

The authors declare no conflict of interest.

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Article

Effect of *Moringa oleifera* in *in vitro* rumen fermentation tests and its impact on greenhouse gases

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Abstract:

The rumen environment has been biotechnologically manipulated to improve animal productivity and natural, healthy, and ecological alternatives have recently been sought for this purpose. *Moringa oleifera* has been tested as livestock feed because its leaves are rich in minerals, protein, and secondary metabolites. The objective was to evaluate the changes in rumen activity due to the presence of *M. oleifera*. Three treatments were tested: CA (control 100 % alfalfa), LM (15 % Moringa-85 % alfalfa), and HM (30 % Moringa-70 % alfalfa). The experiment was performed *in vitro* with sheep rumen fluid. No differences were observed ($P>0.05$) for pH and ammoniacal nitrogen ($N\text{-}NH_3$). Dry matter digestibility differed ($P<0.05$) between treatments. Gas production showed differences ($P<0.05$) among treatments. There were differences ($P<0.05$) for the concentration of volatile fatty acids (VFAs). CO_2 and CH_4 were different between treatments ($P<0.05$), with LM being the lowest for both variables. It is concluded that adding moringa to a ration of alfalfa has no effect on pH and $N\text{-}NH_3$; nevertheless, it increases the digestibility of the dry matter and decreases the concentration of VFAs and the digestibility of fibers. In addition, including 15 % of moringa

in a ration of alfalfa can reduce the production of greenhouse gases. It is recommended to continue evaluating this alternative for animal nutrition.

Keywords: *Moringa oleifera*, Rumen fermentation, *Ovis aries*, Greenhouse gases.

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Introduction

Global demand for animal products has increased in recent years[1]. Due to the constant increase in population, there has been a search for alternatives that produce a greater quantity of food of better protein quality and with greater efficiency. In extensive livestock farming, native grasses are used as the main source of food and during the dry period, it is necessary to use feed supplements because the quality of these grasses decreases, which increases feed costs. Therefore, farmers are looking for food sources that are accessible both in terms of their availability and low cost and that do not imply competition with humans[2].

On the other hand, ruminants produce greenhouse gases, such as methane (CH_4), which is emitted into the atmosphere[1,3]. The use of shrubs and trees in animal nutrition can be an alternative to increase digestibility, improve the nutritional value of the diet, and reduce methane production[4]. There is research focused on proving that the use of leaves from various plants, as well as the foliage of some trees, can serve as food, with adequate nutrients to achieve an economically and ecologically viable production that reduces the production of greenhouse gases[1]. Moringa (*Moringa oleifera*) forage is one of these plants[2], as it is characterized by its ease of propagation and low demand for nutrients and water in its growth[5]. It has been shown that it can be a good source of protein, which may represent an alternative to replace conventional protein inputs in ruminants[6].

Moringa oleifera is a thin, evergreen, deciduous tree native to India that has spread to other parts of the world. Worldwide, it is one of the fastest growing trees that has a high biomass yield, high crude protein content (>25 %), and a balance of other nutrients in its leaves[7]. It is also considered one of the most useful trees in the world as all its parts can be used as food, as medicine, or for industrial purposes[8,9,10]. The different parts of *M. oleifera* contain important minerals and are a good source of protein, vitamins, and amino acids. It has also been reported that it has beneficial agronomic properties, such as drought tolerance, high

biomass production, and high crude protein content, which makes it competitive with high-quality forages[8,11], in addition to the fact that its leaves contain many active compounds, such as flavonoids, saponins, tannins, and alkaloids[8]. *M. oleifera* has been used to feed cattle as the leaves are rich in minerals essential for weight gain and milk production, and because it is an excellent source of protein that can improve microbial protein synthesis in the rumen[12]. Its nutritional effect was also analyzed in sheep, where they had an improvement in milk production and quality[12]. However, no studies have been reported on the use of moringa grown in the state of Chihuahua and the impact of its use as feed on animal production. Therefore, this study aimed to evaluate the effect of *Moringa oleifera* leaves grown in the central region of the state of Chihuahua on different parameters of *in vitro* rumen fermentation in order to have a first approach to the viability of using this shrub as feed for cattle in this region.

Material and methods

This study was carried out at the Animal Nutrition Laboratory of the Faculty of Zootechnics and Ecology of the Autonomous University of Chihuahua (UACh, for its acronym in Spanish), located at Km 1 of the Periférico Francisco R. Almada in the city of Chihuahua, Chihuahua, Mexico (28°35′10.9″ N; 106°6′26.6″ W; altitude 1,440 m asl).

Moringa supplement

It was obtained from a plantation of *M. oleifera* trees located in the central region of the state of Chihuahua, Mexico. The leaves of the plant were collected, dried in the shade, and then macerated to obtain powdered feed[13].

Characterization of the bromatological profile

The bromatological characterization of *M. oleifera* leaves and alfalfa (control treatment) was carried out by means of a proximate analysis. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured using the method described by Van Soest *et al*[14] adapted for the Ankom 200 Fiber Analyzer equipment (Ankom Technology, Fairport NY). Crude protein (CP), dry matter (DM), ash, and EE were determined according to the standard

methods of the Association of Official Analytical Chemistry[15]. Minerals were analyzed by atomic absorption and the amino acid profile by chromatography.

Animals and diet

Rumen fluid was obtained from three rumen-fistulated sheep of the Pelibuey breed (45 ± 3 kg). Prior to the fistulation process, the animals were vaccinated (Lapibac; Lapisa®) and dewormed (Levax ADE; BioZoo®). They were then adapted to the diet (alfalfa forage) for 15 d. Feed was offered twice a day (0800 and 1700 h). Once the adaptation period was over, the animals were kept fasting for 24 h and then rumen fluid samples were taken from the three animals directly from the cannula to perform *in vitro* fermentation tests.

Treatments and experimental design

Once the bromatological profile of *M. oleifera* was characterized (Table 1), three treatments were established, looking for isoprotein values; CA (Control: 100 % alfalfa), LM (Low Moringa: 15 % moringa and 85 % alfalfa) and HM (High Moringa: 30 % moringa and 70 % alfalfa). A completely randomized design with repeated measurements over time was used in the *in vitro* fermentation test. There were three replications for each fermentation time (6, 12, 24, and 48 h). The gas production analysis was performed according to Theodorou's[16] technique; to this end, 2 g of the substrate was weighed directly in 100 mL glass tubes with butyl stopper and agraffe. To analyze the rest of the variables studied, 0.5 g of the substrate was weighed in FN57 (Ankom™) bags with a pore size of 25 μm and then each bag was sealed and placed in 250 mL bottles. The rumen microbial inoculum was prepared using two parts of a buffer solution[17] and one part of rumen fluid. Rumen fluid was collected from the three previously fistulated Pelibuey sheep, directly from the cannula before feeding. The inoculum was filtered with muslin and dispensed under CO_2 anaerobiosis conditions (15 mL for gas production and 40 ml for the rest of the parameters); it was immediately sealed and placed in an incubator with stirring at 120 rpm and controlled temperature at 39 °C. Three substrate-free replications were prepared as a control for gas production.

Total gas production was measured with a FESTO® pressure transducer (SIEMENS). The pH was measured with an electronic potentiometer immediately after sampling. The digestibility of NDF and ADF was evaluated using the IVTD - Daisy method (*in vitro* True Digestibility Method)[18]. NDF and ADF were determined at the end of each incubation time; processing was performed in the Ankom® 2000 fiber analyzer according to the methods

described by Van Soest *et al*[14]. N-NH_3 was calculated by spectrophotometry[19]. Volatile fatty acids (VFAs) were determined by gas chromatography with flame ionization detection. A Claurus 400® gas chromatograph (Perkin Elmer) adapted with a Varian CP-wax58 (FFAP) CB capillary column (15 m × 0.53 mm, 0.5 μm) was used[20]. The determination of CH_4 and CO_2 was calculated from the concentrations of VFAs using the equation method proposed by Wolin[21].

Statistical analysis

The data were analyzed with a completely randomized design using the GLM procedure (SAS ver. 9.4); repeated measurements over time were considered for the pH and GP variables and the analysis was performed using the Proc MIXED of SAS version 9.4[22].

Results and discussion

Bromatological analysis of Moringa supplement

The results of the bromatological analysis performed on the moringa supplement are described in Table 1.

Table 1: Bromatological analysis of *Moringa oleifera* supplement

Variable	%
Dry matter	93.87
Ash	15.68
Ethereal extract	5.99
ADF	14.85
NDF	26.42
Crude protein	16.97
Ca	3.23
P	0.27
Mg	0.8
K	1.35
Mn, ppm	120.4
Cu, ppm	18.64
Zn, ppm	20.63

The values obtained differ from what has been reported since the CP was lower and the EE content was higher (Table 1) than reported in other studies[23-27]. The CP content had an acceptable value (16.97 %) for inclusion in the diet of ruminants at different feeding stages (NRC), but it is lower compared to alfalfa[28], which is one of the main hayed forages used in dairy cattle diets. Nonetheless, in northern Mexico, it is common to use oat hay in beef cattle feed, suggesting that moringa may represent an alternative to the use of oat hay, which has a lower CP value[28]. The ash content was higher (15.68 %) than that reported in other studies[8,11,23]. This suggests that dried moringa leaves contain a considerable amount of them. Calcium, magnesium, copper, and potassium had higher values compared to those reported in the literature[25,27,29]. The value of calcium was even higher than that reported for oat and alfalfa hay[30]; this gives an important value to *M. oleifera* since this mineral is of great importance for the regulation of different processes[31], productive aspects, and for the maintenance of bone and dental structure[29].

The amino acid profile found in the moringa supplement is presented below (Table 2).

Table 2: Amino acid content profile of *Moringa oleifera* supplement

Amino acid	**g/100 g**
Alanine	0.87
Glycine	0.63
Valine	0.53
Leucine	0.56
Isoleucine	0.52
Threonine	0.54
Serine	0.76
Proline	0.69
Aspartate	1.93
Methionine	0.11
Glutamate	6.05
Phenylalanine	0.75
Lysine	0.43
Histidine	0.53
Tryptophan	0.27
Cysteine	0.04

Glutamate, aspartate, alanine, leucine, and serine had a higher concentration than reported[32]. On the other hand, the alanine content was similar and leucine content was lower than that found in the literature[32]. However, the data reported by other studies[1,5,8,25] are inconsistent. All amino acid values are below those reported by these authors, except for glutamate and aspartate; this could be due to the fact that the moringa they analyzed was grown in conditions

of humidity, altitude, and temperature very different from those found in the growing area of *M. oleifera* used in this study; therefore, the biochemical behavior of the plant could have generated different concentrations of these metabolites. Although these amino acids, with the exception of leucine, are non-essential, they do represent a good source of nitrogen for microbial metabolism and, therefore, for microbial protein synthesis[33].

Nutritional composition of treatments

The nutritional composition of the three treatments used in *in vitro* fermentation is shown in Table 3.

Table 3: Composition of diets used in *in vitro* fermentation tests

Variable (%)	CA	LM	HM
Dry matter	89.4	90.1	90.7
Ash	11	11.7	12.4
Ethereal extract	2.08	2.7	3.3
ADF	29	26.9	24.8
NDF	36.1	34.6	33.2
Crude protein	21.2	20.6	19.9
Ca	1.4	1.7	1.9
P	0.26	0.3	0.3
Mg	0.32	0.4	0.5
K	3.03	2.8	2.5

CA= control treatment (100 % alfalfa); LM= low moringa treatment (15 % moringa 85 % alfalfa); HM= high moringa treatment (30 % moringa 70 % alfalfa).

In vitro rumen fermentation

It was performed with the three treatments described above. The results of the variables evaluated from *in vitro* rumen fermentation are shown in Table 4.

Table 4: Variables evaluated in the *in vitro* fermentation tests

Variable	CA	LM	HM	*P*-Value
pH	6.74 [a]	6.86 [a]	6.76 [a]	≥ 0.05
DM digestibility, %	63.88 [a]	66.65 [ab]	67.87 [b]	<0.05
NDF, %	24.64 [a]	30.96 [ab]	32.09 [b]	<0.01
ADF, %	17.80 [a]	22.53 [ab]	23.54 [b]	<0.01
GP	49.06 [a]	51.18 [ab]	55.93 [b]	<0.05
Acetic acid, mmol/L	63.9 [a]	42.3 [b]	31.2 [c]	<0.05
Propionic acid, mmol/L	30.6 [a]	21.0 [b]	11.1 [c]	<0.05
Butyric acid, mmol/L	9.3 [a]	4.6 [b]	4.2 [b]	<0.05
TVFAs, mmol/L	103.9 [a]	67.9 [b]	46.5 [c]	<0.05
CO_2 production, % molar	51.7 [a]	48.5 [b]	51.9 [a]	<0.05
CH_4 production, % molar	28.6 [a]	26.0 [b]	32.3 [c]	<0.05
NH_3, mmol/ml	14.6 [a]	14.9 [a]	15.1[a]	>0.05

CA= control treatment (100 % alfalfa); LM= low moringa treatment (15 % moringa 85 % alfalfa); HM= high moringa treatment (30 % moringa 70 % alfalfa).

For pH, no differences were observed between treatments, time, or their interaction ($P \geq 0.05$; Table 4). This coincides with what was previously reported[34,35,36], where they evaluated the effect of moringa leaf extracts on different fermentation kinetics and observed no differences between treatments. These results guarantee the viability of the rumen microbiota[37]. On the other hand, for dry matter (DM) digestibility, there were differences between treatments ($P<0.05$); it was higher for the HM treatment (30 % moringa). This differs from what was reported by other researchers[38], who found a decrease in digestibility as the concentration of moringa in feed rations increases; nevertheless, it coincides with Morsy *et al*[6], who observed that, when the proportion of moringa extract increases, digestibility increases. This increase in digestibility is related to changes in the amount of NDF and ADF, which decreased as the level of moringa in the diet increased (Table 3), allowing to observe the impact that the addition of this ingredient has on a whole diet. This difference in dry matter digestibility may be related to differences in the composition of bacterial communities[6], which could have been impacted by the presence and increase of moringa in the diet. Gas production (GP) showed differences between treatments and was higher for HM treatment ($P<0.05$; Table 4); however, this increase did not have the desired impact on the end products of fermentation, where the total volatile fatty acids (TVFAs) were lower for the HM treatment (Figure 1). This is directly related to the production of CO_2 (Figure 2), which showed the same behavior as the TVFAs, decreasing as the level of moringa in the diet increased ($P<0.05$). On the other hand, methane (CH_4) production did not show the same behavior; it can be observed that the LM treatment was lower than the other treatments ($P<0.05$, Figure 3). Finally, for NH_3, there were no differences between treatments, time, or their interaction ($P>0.05$; Table 4).

Figure 1: Total production of volatile fatty acids

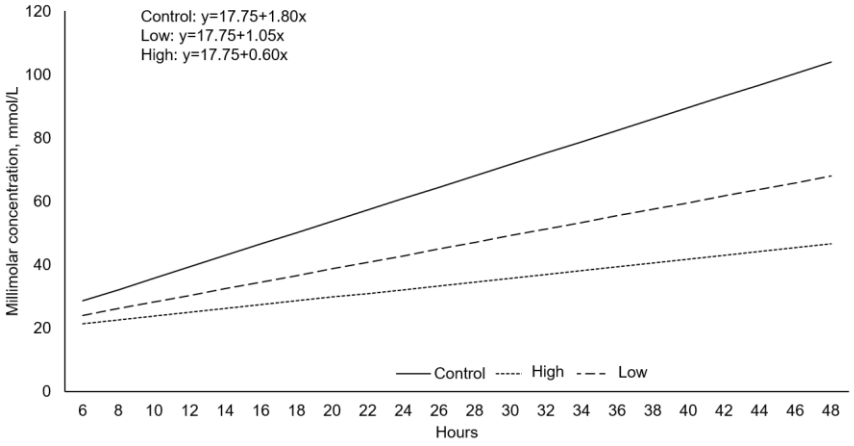


Figure 2: CO_2 production

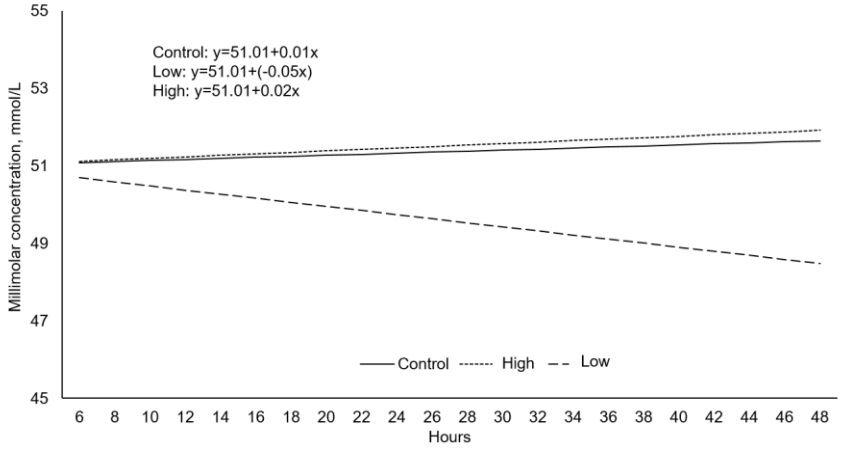
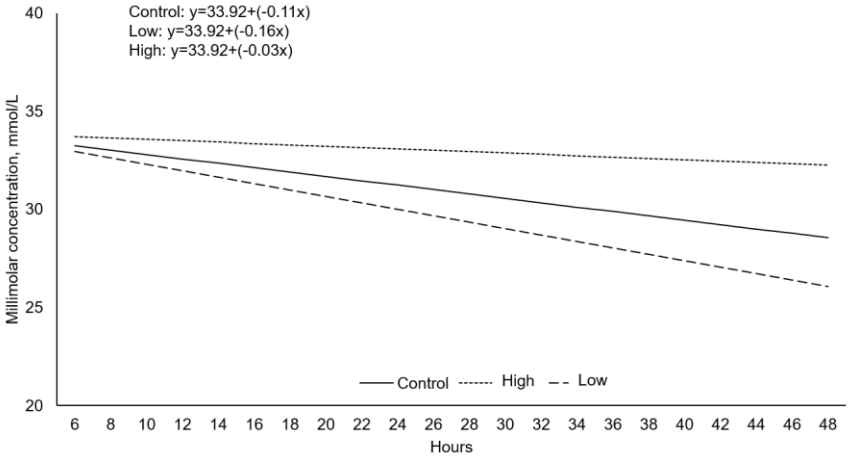


Figure 3: CH_4 production



CO_2 and CH_4 emissions during rumen fermentation cause an energy loss of between 2 and 12 %[36]. The reduction of methane due to the presence of *Moringa oleifera* has also been reported in other studies[24,35,39]. It is also interesting to note that, at higher levels of moringa, methane concentrations increase. It is well documented that secondary metabolites of some plant species can mitigate CH_4 production in rumen fermentation and *Moringa oleifera* is rich in secondary metabolites, such as tannins, saponins, and other phenolic compounds that have antimicrobial and antiprotozoal properties that, consequently, could be modifying the composition of the microbiota and thus the production of CH_4[6].

The molar percentage of the millimolar concentrations of the VFAs was also calculated for the three treatments (Table 5). The fermentation pattern of the three diets was mainly acetic, since this acid was the one that was found in greater proportion. This coincides with what has been reported in the literature, where it is mentioned that, in forage-based diets, the concentration of acetic acid is usually between 60 and 75 %[40]. Regarding propionic acid, the literature indicates that its proportion in this type of diet varies between 15 % and 19 %[40]. Nonetheless, a higher proportion was obtained in this experiment, with the lowest being 23.82 % in the HM treatment and the highest being 30.93 % in LM. The proportion of butyric acid also coincides with that reported in the literature[40]; however, it is at the lowest levels of the expected range, which varies between 8 and 16 %.

Table 5: Comparison of the concentration of volatile fatty acids (VFAs) in mmol/L and their conversion to molar percentage

	CA		**LM**		**HM**	
	mmol/L	**%**	**mmol/L**	**%**	**mmol/L**	**%**
Total VFA	103.9	100	67.9	100	46.6	100
Acetic acid	63.9	61.5	42.3	62.3	31.2	66.95
Propionic acid	30.6	29.45	21	30.93	11.1	23.82
Butyric acid	9.3	8.95	4.6	6.77	4.2	9.01

CA= control treatment (100 % alfalfa); LM= low moringa treatment (15 % moringa 85 % alfalfa); HM= high moringa treatment (30 % moringa 70 % alfalfa).

Conclusions and implications

Almost all fermentation parameters were affected by the presence of *Moringa oleifera*, except for pH and N-NH_3. Although the obtained concentrations of NDF, ADF, and TVFA were not as expected, they are in acceptable proportions for ruminant feed. Nevertheless, some of the variables had desirable behaviors, such as dry matter digestibility, production of gas, carbon dioxide and methane. It should be noted that both CO_2 and CH_4 had a decrease

in the treatment of moringa at 15 % (LM), which could be due to the presence of the secondary metabolites of this plant, which, in turn, affect the population of protozoa and methanogenic microorganisms. Similarly, increasing the amount of moringa to 30 % may also be increasing the antinutritional factors of *M. oleifera*, which can affect the rumen microbiota. Due to the above, it is proposed to perform rumen population analyses to identify the microorganisms present and the active metabolic pathways to find their relationship with these results.

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Conflicts of interest

All authors declare that there are no conflicts of interest of any kind in the publication of this document.

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Article

Effect of including pisonay (*Erythrina edulis*) meal on the hematological profile in guinea pigs (*Cavia porcellus*)

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Abstract:

This study aimed to evaluate the hematological profile of guinea pigs supplemented with *Erythrina edulis* leaf meal. *Erythrina edulis* leaves were collected at the regrowth ages of 4, 8, and 12 mo (A4, A8, A12), ground into meal, and included in the diets for guinea pigs at 10, 20 and 30 % (P10, P20, P30). A total of 80 improved and weaned male guinea pigs were randomly distributed for each diet. After 56 d, blood was collected directly from the jugular vein in EDTA tubes to analyze erythrocytes, mean corpuscular volume, hematocrit, hemoglobin, leukocytes, platelets, and mean platelet volume. Erythrocytes and hemoglobin were similar between diets. The factors of age and meal inclusion did not affect the values found in erythrocytes, mean corpuscular volume, hematocrit, and hemoglobin. The age factor (A4:8.55; A8:11.92; A12:10.14 x10^3/ul) and meal inclusion (P20:11.11; P30:12.08; P10:7.43 x 10^3/uL) caused differences in leukocytes. Platelets were affected by the age factor (A4:391.98; A12:400.67; A8:467.08 x 10^3/uL) and meal inclusion factor (P10:444.22; P20:443.05; P30:372.45 x 10^3/uL). The mean platelet volume showed variations due to the age factor (A8:11.39; A12:11.31; A4:11.90 fL). *Erythrina edulis* leaf meal in guinea pig diets has potential as a feed input without altering the hematological profile, which would indicate that age and inclusion factors would not cause toxicity.

Keywords: Erythrocytes, Hematocrit, Hemoglobin, Leukocytes, Platelets.

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Introduction

Guinea pig production systems in the Peruvian Sierra have changed from the family system, characterized by native guinea pigs fed on fodder, weeds, kitchen and harvest residues, which led to slow growth and the consumption or sale of guinea pigs at an age of 6 mo with average weights of 966 g[(1)], and the systems currently used are family-commercial and commercial, which have increased the production of guinea pigs by using improved guinea pigs, preferably the Peruvian breed, which receive mixed or complete foods, guinea pigs aged 30 to 72 d reached weights greater than 1,070 g[(2)]; in addition, guinea pig meat is recognized as a good quality exotic meat due to its low amount of lipids and its use in gastronomic traditions[(3)].

The versatile feeding of guinea pigs leads to the search for alternative foods to increase productivity; for example, the inclusion levels of *Arachis pintoi* meal in 5, 10, and 15 % to replace alfalfa hay in the guinea pig diets for 49 d lead to average final weights of 997 g and 74 % carcass yield[(4)]; likewise, the supplementation with tarwi meal in 18 % in the complete concentrate feed caused a positive response in body weight gain[(5)]. Another alternative is the use of tropical forage plants; *Erythrina poeppigiana* plus 15 g of feed for 56 d in the production process resulted in 8 g of daily weight gain and 66 % of carcass yield[(6)]. *Erythrina* sp. (pisonay), when included up to 50 % as fresh forage in the fattening phase of guinea pigs, allowed increasing body weight up to 1,221 g[(7)]. Furthermore, an increase in serum aminotransferase concentrations, a greater presence of liver pathologies, and a decrease in the liver/body weight ratio were observed with the inclusion of 50 and 100 % pisonay[(8)]; likewise, high serum creatinine and urea levels can cause disorders in the renal function of guinea pigs[(9)]. In rabbits, it has been observed that including *Moringa oleifera* meal causes variations in hematology[(10)].

The hematological profile in animals is performed to know the state of health and physiological variations[(11)] and to assess the toxicity caused by food consumption[(12)]; it is mentioned that plants and additives in feed inputs present toxic agents that would cause hemolytic anemia due to a decrease in erythrocytes, this behavior would help to diagnose diseases related to metabolism or eating disorders[(13)], as occurred with the toxicosis of *Ipomoea carnea* in guinea pigs, which caused normocytic hypochromic anemia due to a significant reduction in erythrocytes, hematocrits, and hemoglobin concentration after 20 d and non-regenerative anemia at 40 d[(14)]; in another study, the inclusion of *Curcuma longa* powder in guinea pig diets had a variable influence on the concentration of leukocytes, lymphocytes, and monocytes, which was caused by stimuli in the immune

system and antioxidant effects[15]. The present experiment aimed to assess the effect of including pisonay (*Erythrina edulis*) meal on the hematological parameters of guinea pigs (*Cavia porcellus*).

Material and methods

Foliage was collected from trees used in living fences and animal feeding from the sector located in Mosoccpampa, district of Tamburco, located at 2,880 m asl. Pisonay trees were selected for convenience; the foliage was cut after 4, 8, and 12 mo of regrowth; after drying the leaves in the shade for approximately 30 d, the meal was made in a hammer mill with a 2 mm diameter sieve.

Subsequently, the complete diets (Table 1) were processed into meal, considering 18 % protein and 3,000 kcal of digestible energy/kg of dry matter, and the diets were named D0, D1, D2, D3, D4, D5, D6, D7, D8, and D9; each diet was added 10 % (P10), 20 % (P20), and 30 % (P30) of pisonay meal for each age of regrowth: 4 mo (A4), 8 mo (A8), and 12 mo (A12), and a control diet (D0) with the inclusion of 20 % alfalfa meal.

The experimental complete diets were as follows:
D0: Complete diet, it includes 20 % alfalfa meal
D1: Complete diet, it includes 10 % meal of pisonay with a regrowth age of 4 mo
D2: Complete diet, it includes 20 % meal of pisonay with a regrowth age of 4 mo
D3: Complete diet, it includes 30 % meal of pisonay with a regrowth age of 4 mo
D4: Complete diet, it includes 10 % meal of pisonay with a regrowth age of 8 mo
D5: Complete diet, it includes 20 % meal of pisonay with a regrowth age of 8 mo
D6: Complete diet, it includes 30 % meal of pisonay with a regrowth age of 8 mo
D7: Complete diet, it includes 10 % meal of pisonay with a regrowth age of 12 mo
D8: Complete diet, it includes 20 % meal of pisonay with a regrowth age of 12 mo
D9: Complete diet, it includes 30 % meal of pisonay with a regrowth age of 12 mo

From 80 improved weaned male guinea pigs approximately 15 d-old, 8 guinea pigs were randomly distributed for each experimental diet, two replications per diet and 4 guinea pigs per replication were assigned; the guinea pigs were previously identified with numbered metal ear tags. The guinea pigs were placed in single-level mesh cages, with capacity for four guinea pigs, with dimensions of 0.9 x 0.9 x 0.4 m.

Table 1: Complete diets (%) used in the experiment

Inputs	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9
Pisonay meal		10.0	20.0	30.0	10.0	20.0	30.0	10.0	20.0	30.0
Alfalfa meal	20.0									
Wheat bran	46.0	58.1	39.9	22.0	58.2	40.3	22.6	59.2	42.2	25.4
Soybean cake	18.4	17.3	16.2	14.5	17.3	16.2	14.3	17.4	16.1	14.6
Corn	11.9	11.9	21.3	31.4	11.9	21.0	31.0	10.9	19.2	27.9
Dicalcium phosphate	1.4		1.2	1.0		1.2	1.0		1.2	1.0
Calcium carbonate	0.6	1.6	0.4		1.6	0.4		1.6	0.4	
Common salt	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Mycotoxin sequestrant	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Vitamins and minerals	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
DL Methionine	0.1	0.02	0.1	0.17	0.02	0.1	0.17	0.02	0.1	0.17

The guinea pigs received the complete diets for 56 d in hopper-type feeders and a 7-d adaptation phase was considered; in addition, each cage had bell-type drinkers where fresh water was added at will.

Once the experimental phase was completed, the blood sample was obtained. For the blood count, blood was collected directly from the jugular vein in EDTA tubes (1 mL). Two readings of the erythrocyte count (RBC), mean corpuscular volume (MCV), hematocrit (HCT), hemoglobin (Hb), leukocytes (WBC), platelets (Plat), and mean platelet volume (MPV) were taken with the automatic hematology analyzer (Urit 2900 Vet Plus, China). The differential count of leukocytes – neutrophils (N), lymphocytes (L), monocytes (M), and eosinophils (E) – was performed in duplicate by blood smear in 100 cells from each sample.

Data from the 10 diets were analyzed using a completely randomized design. The factors of age and meal inclusion were evaluated through the 3 x 3 factorial arrangement without considering the control diet (D0); the normality test was performed using the Shapiro-Wilk test and to compare the means, the homogeneity of variances test was previously applied through the Levene test. A significance level of 0.05 was considered. RBC, HCT, Hb, WBC, Plat, and MPV data were transformed by Arcsine. In addition, for the data found in M and E, the square root transformation $(Y + 0.5)^{1/2}$ was used.

Results and discussion

Erythrocytes (Table 2) were similar between diets and the factors of age and meal inclusion did not affect the values found, which ranged from 5.52 to 5.96 x 10^6/uL (P>0.05). Regarding mean corpuscular volume, the D3, D5, D6, D7, D8, and D9 diets

were different from the D0 diet (control diet) (P<0.05) and the factors of age and meal inclusion did not influence the indicators found, which remained between 71.06 and 72.14 fL (P>0.05).

The erythrocytes follow trends similar to those reported in the reference intervals indicated for 13/N[16], Dunkin-Hartley[17], and native guinea pigs[18]. The mean corpuscular volume was similar to the report in 13/N guinea pigs[16]. Djoumessi *et al*[15] mention that the decrease in erythrocytes would be related to inadequate feeding.

The hematocrit (Table 2) showed that D3 (42.99 %) was similar to D0 (44.98 %) and regarding the rest of the diets, they were different and decreased to 40.88 % (P<0.05); the factors of age and meal inclusion did not affect the indicators found, which were between 41.19 and 42.50 % (P>0.05).

The hematocrit was close to the minimum reference value indicated for 13/N[16] and Dunkin-Hartley[17] guinea pigs, unlike D0, which was similar to that reported in native guinea pigs[18]; this behavior has a direct relationship with the adequate amount and intake of water during the experiment.

The concentration of hemoglobin (Table 2) was similar between the diets and ranged from 14.11 to 14.96 g/dL (P>0.05); the factors of age and meal inclusion would not affect the indicators found, which were between 14.47 and 14.72 g/dL (P>0.05). Hemoglobin is similar to the reference intervals indicated for 13/N[16], Dunkin-Hartley[17], and native guinea pigs[18].

Leukocytes (Table 2) showed similarity between diets, except for D2, D4, and D7, which were different from D0 (P<0.05); the age factor would cause differences between A4 (8.55 x 10^3/uL) and A8 (11.92 x 10^3/uL) and they were similar to A12 (10.14 x 10^3/uL) (P<0.05); the meal inclusion factor induced a similarity between P20 (11.11 x 10^3/uL) and P30 (12.08 x 10^3/uL) and it decreased due to the effect of P10 (7.43 x 10^3/uL) (P<0.05).

The percentage of neutrophils (Table 2) was affected by the D3 and D4 diets, which were lower and different from the D0 diet (P<0.05). In lymphocytes, there were differences between D4 and D0 (P<0.05); in monocytes, D3, D6, D7 and D9 were different from D0 (P<0.05); and eosinophils showed differences between D6 and D9 when compared with D0 (P<0.05).

The age factor caused differences and an increase in neutrophils between A4 (43.31 %), A8 (50.20 %), and A12 (58.12 %) (P<0.05); in lymphocytes, a decrease was observed as the age of regrowth of A4 (51.45 %), A8 (44.85 %), and A12 (36.72 %) (P<0.05) increased; monocytes and eosinophils showed no differences (P>0.05) and remained between 2.93 to 3.27 % and from 0.85 to 1.27 %, respectively.

In neutrophils, the factor of meal inclusion induced a similarity between P20 (52.48 %) and P30 (53.93 %), which differed from P10 (45.22 %) (P<0.05); lymphocytes in P10 (49.06 %) were higher than P20 (42.46 %) and P30 (41.51 %), which were similar (P>0.05).

Monocytes and eosinophils showed similarity between P10 (2.88 and 0.64 %) and P20 (2.56 and 0.91 %), both indicators were increased by P30 (3.91 and 1.62 %, respectively) (P<0.05).

The number of leukocytes covered a heterogeneous amplitude compared to the references mentioned for 13/N[16] and Dunkin-Hartley[17] guinea pigs, unlike guinea pigs of the meat and early lines, which exhibited values from 3.47 to 14.94 x 10^3/uL[19]. These variations observed by the addition of pisonay meal in the complete feed for guinea pigs would not cause harmful consequences, as occurred with the inclusion of *Mucuna utilis* in the diet of rabbits, it did not cause disorders in hematological parameters[20]; a similar trend was observed with *Azadirachta indica*, which was not harmful for hematopoiesis[21] and it turns out to be contradictory with the leaf meal of *Morinda lucida* as an antimicrobial supplement in the diet for chickens, it stimulated the decrease in leukocytes[22].

The percentages of neutrophils, lymphocytes, monocytes, and eosinophils follow trends similar to the reference intervals indicated for 13/N[16] and Dunkin-Hartley[17] guinea pigs. Regarding monocytes and eosinophils, they showed similarity to the values reported for native guinea pigs[18]. Zimmerman *et al*[23] mention that toxicity would increase the number of neutrophils and their maturation would be accelerated. The stable percentages of leukocytes, lymphocytes, and monocytes would indicate the absence of inflammatory and infectious diseases in guinea pigs[24].

Pisonay meal would not cause an increase in neutrophils and eosinophils, therefore, the immune system would not be activated since pisonay would have a minimum amount of toxic compounds; this behavior was observed with the addition of meal from *Agave tequilana* stems in rabbits[25]; in addition, eosinophilia is an indicator of allergies and inflammatory processes, which were not observed in the guinea pigs; the addition of natural supplements as growth promoters in Broiler chickens preserved the normal percentage of eosinophils[26].

Platelets (Table 2) showed similarity between diets, except for D5, which was different from D0 (P<0.05); the age factor would cause similarity between A4 (391.98 x 10^3/uL) and A12 (400.67 x 10^3/uL), which were lower than A8 (467.08 x 10^3/uL) (P<0.05), and the meal inclusion factor induced a similarity between P10 (444.22 x 10^3/uL) and P20 (443.05 x 10^3/uL) and it decreased due to the effect of P30 (372.45 x 10^3/uL) (P<0.05).

The mean platelet volume (Table 2) in D6 and D7 differed from the D0 diet ($P<0.05$); the age factor produced similarity between A8 (11.39 fL) and A12 (11.31 fL), which decreased compared to A4 (11.90 fL) ($P<0.05$); and the meal inclusion factor induced similarity in the values found, which ranged from 11.42 to 11.63 fL ($P>0.05$).

Platelets were similar to the references mentioned for Dunkin-Hartley guinea pigs[17], except for D5, the effect of A8 and the effects of P10 and P20 were above the maximum value indicated for 13/N[16] guinea pigs; these variations would indicate that the complete diet for guinea pigs could induce chronic diseases[27]. The mean platelet volume reported in all cases was above the values reported for 13/N[16] and Dunkin-Hartley[17] guinea pigs. These variations could be a consequence of the altitude level; this was corroborated with values from 249 to 800 x 10^3/uL obtained in guinea pigs of the meat and early lines that were raised at 3,350 m altitude[19].

Nutrition is one of the factors influencing the hematological profile[28]; in the leaves of *Erythrina edulis*, there was a notable presence (++) of alkaloids, flavonoids, and saponins, and a mild presence (+) of sterols[29]; in another study, in two phenological stages, the antinutritional levels were below 2 % on a dry matter basis[30]; this would indicate the slight variation observed in some hematological indicators due to the effect of pisonay meal and would probably not represent a nutritional problem in guinea pigs. On the other hand, if one or more blood components are directly affected, it would be related to primary hematotoxicity, which will depend on the amount and time of exposure to an extrinsic substance[31].

Conclusions and implications

Erythrina edulis leaf meal in guinea pig diets has potential as a feed input without altering the number of erythrocytes, mean corpuscular volume, percentage of hematocrits, hemoglobin, leukocytes, platelets, and mean platelet volume, which would indicate that age and inclusion factors would not cause toxicity.

Table 2: Influence of pisonay meal on the hematological profile of guinea pigs

Parameters		D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	SEM
RBC, 10^6/uL	M	5.92[a]	5.80[a]	5.88[a]	5.96[a]	5.86[a]	5.70[a]	5.64[a]	5.89[a]	5.88[a]	5.52[a]	0.03
	m	5.89	5.81	5.88	5.97	5.73	5.73	5.63	5.89	5.85	5.49	
MCV, fL	M	75.25[a]	72.81[a]	71.91[a]	71.69[b]	72.48[a]	71.70[b]	71.51[b]	69.98[b]	71.68[b]	71.51[b]	0.29
	m	75.63	72.08	73.13	72.38	73.50	72.08	72.10	70.15	71.88	72.23	
HCT, %	M	44.98[a]	41.62[b]	41.56[b]	42.99[a]	41.61[b]	41.29[b]	40.98[b]	41.16[b]	40.88[b]	41.54[b]	0.22
	m	44.95	41.60	42.25	43.15	40.98	41.30	41.33	41.73	41.00	42.13	
Hb, g/dL	M	14.53[a]	14.54[a]	14.11[a]	14.76[a]	14.58[a]	14.62[a]	14.52[a]	14.96[a]	14.88[a]	14.32[a]	0.08
	m	14.63	14.58	15.08	14.60	14.48	14.58	14.53	14.95	14.83	14.30	
WBC, 10^3/uL	M	12.23[a]	8.98[a]	7.15[b]	9.52[a]	6.22[b]	13.63[a]	15.93[a]	7.10[b]	12.55[a]	10.76[a]	0.52
	m	12.63	9.05	7.73	9.55	5.85	15.48	14.38	6.78	12.53	10.60	
N, %	M	52.50[a]	44.25[a]	45.88[a]	39.81[b]	38.18[b]	51.31[a]	61.12[a]	53.25[a]	60.25[a]	60.88[a]	1.22
	m	52.85	44.35	42.85	43.85	36.85	49.50	64.35	52.55	61.35	61.35	
L, %	M	45.00[a]	50.38[a]	49.62[a]	54.38[a]	57.43[a]	42.38[a]	34.73[b]	39.38[a]	35.38[b]	35.43[a]	1.20
	m	45.50	49.00	53.50	51.50	58.00	42.00	32.25	38.50	33.00	35.50	
M, %	M	1.31[a]	2.18[a]	3.00[a]	3.62[b]	2.68[a]	2.68[a]	4.06[b]	3.75[b]	2.00[a]	4.06[b]	0.18
	m	1.25	1.75	2.50	4.25	2.50	2.00	3.75	3.75	1.75	4.00	
E, %	M	0.43[a]	0.43[a]	0.75[a]	1.38[a]	0.56[a]	1.00[a]	1.62[b]	0.93[a]	1.00[a]	1.88[b]	0.10
	m	0.50	0.25	0.50	1.00	0.25	0.50	1.50	1.00	0.50	1.50	
Plat, 10^3/uL	M	393.35[a]	383.40[a]	437.70[a]	354.85[a]	507.35[a]	527.54[b]	366.34[a]	441.92[a]	363.91[a]	396.18[a]	11.12
	m	392.75	351.85	431.70	359.48	527.83	533.50	365.50	424.78	360.25	398.73	
MPV, fL	M	12.68[a]	11.74[a]	12.05[a]	11.93[a]	11.54[a]	11.40[b]	11.25[b]	11.00[b]	11.45[b]	11.48[b]	0.10
	m	12.40	11.58	11.63	11.70	11.43	11.38	11.00	10.95	11.33	11.40	

RBC=erythrocytes; MCV= mean corpuscular volume; HCT= hematocrit; Hb= hemoglobin; WBC= leukocytes; N= neutrophils; L= lymphocytes; M= monocytes; E= eosinophils; Plat= platelets; MPV= mean platelet volume; M= mean; m= median; SEM= standard error of the mean.

[ab] Different letters in the rows indicate significant difference in means ($P \leq 0.05$).

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Article

Proximate chemical analysis of waste from craft brewing and its acceptance in backyard pigs (*Sus scrofa domesticus*)

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Abstract:

The items incurred in pig production determine about 50 % of the productive cost of the activity. Craft brewing produces solid waste, which, when treated, can be considered to be used as food; therefore, the objective of this research is to characterize the chemical components of the waste from the production of craft beer, where the following values were obtained: dry matter 84.77 %, ash 2.54 %, fat 1.98 %, crude fiber 4.85 %, protein 10.86 %, nitrogen-free extract 64.54 %, and total digestible nutrients 73.21 %. Next, a group of backyard pigs were adapted to feed on waste from craft beer production in a substitution of 40 % and 60 %, and the remaining was commercial balanced feed; where weight gains of 1.09 kg d^{-1}, feed conversions of 3.95, carcass yields of more than 80 %, and back fat thickness of 25.61 mm were achieved. Regarding apparent digestibility of

nutrients, the following was found: dry matter 77.09 %, crude ash 63.87 %, crude protein 69.20 %, crude fiber 46 %, fat 54.08 %, and gross energy 78.7 %; this determines the prospects of the partial use of this waste in feed and energy requirements within the backyard pig production.

Keywords: Feed, Yield, Productivity, Pig breed, Brewery waste.

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Introduction

Craft beer is one of the alcoholic beverages with a high growth in demand by consumers worldwide, which implies an increase in production[1]; 20 million t of solid waste are generated in this industry[2]. According to the Association of Craft Brewers of Ecuador "ASOCERV" (for its acronym in Spanish), in 2018, the production was 30,730 hectoliters, that is, approximately 614.6 t of waste in Ecuador.

Solid waste represents 85 % of total production[3]; this waste comes from separating the must in the filtration stage prior to the milling and maceration stages[4]. In addition, for every 100 L of craft beer, 20 kg of solid waste is generated[5]. Waste has high nutritional and functional properties such as proteins, fibers, lipids, carbohydrates, vitamins, phenolic compounds, and minerals[6]. Likewise, the high fiber and protein contents of some wastes can be used to feed humans and animals[7]. Therefore, these wastes constitute no-cost or low-cost potential raw material, rich in organic matter, with availability all year round for agroindustry use[8].

Digestibility in animal feed is considered one of the most important aspects since the nutritional quality of the inputs is assessed depending on their solubility, the extent of their chemical hydrolysis, and the enzymatic digestion in the intestine[9]. One of the main challenges in agribusiness is the recovery and valorization of this waste through the application of a circular economy model[10]. For this reason the present research aimed to determine the nutritional composition and digestibility of solid waste from craft brewing and to determine its acceptance in the pig diet.

Material and methods

The present research was carried out in two stages: the first was the laboratory analysis and the second corresponded to feeding craft beer waste to pigs. The samples required for the research were obtained from the craft brewing process of the Technical University of Manabí in the Laboratory of Agroindustrial Processes, located in the Chone Cantón on the Boyacá road km 2 1/2, Ánima site, in which 2 kg of the waste was collected, which was crushed and sieved (4 mm). The samples were stored in airtight bags with pressure closure at room temperature. Each analysis was performed in triplicate.

First phase: Proximate chemical analysis of solid waste from craft beer

Dry matter (DM) determination

Two grams of sample were taken and placed in a porcelain capsule to be put in an oven (Memmert) at 105 °C for 24 h, then it was placed in a drying capsule equipped with silica gel to cool the capsules with the sample and thus prevent them from becoming wet, until they were weighed on an Adams® analytical balance and a constant weight was achieved, in accordance with the standard[11].

Ash (A) determination

On porcelain capsules, 3 g of sample was weighed until it reached a constant weight, then the sample was pre-calcined using a grill and then taken to total calcination in a muffle at 700 °C for 2 h. Finally, the samples were cooled and weighed to determine the percentage of ash according to the current standard[12].

Crude protein (CP) determination

It was performed on a Kjeldahl Vapodest 50® equipment for analyzing total nitrogen according to the standard[13]. One gram of sample was used for digestion with 25 ml of H_2SO_4, then digestion was done with an automatic distiller. Subsequently, the sample was titrated with H_2SO_4 (0.1N) to determine the percentage of nitrogen present and quantify the protein content, multiplying by the factor of 6.25.

Fat (F) determination

Approximately 2 g of sample was weighed, which was previously dried at 60 °C and it was determined using the Soxhlet equipment with ethyl ether as solvent for 6 h; then, the fat was recovered in previously dried flasks at constant weight; subsequently, the rest of the ether was removed at 100 °C; then, the flask was weighed with the fat and the percentage of ethereal extract was obtained by difference in weights, in accordance with the regulations[14].

Crude fiber (CF) determination

The samples used were the defatted malt samples obtained in the determination of fat. Then, the following was carried out: acid digestion with H_2SO_4 (0.2N), washes with hot water, and basic digestion with NaOH (0.2N). The analysis of this parameter was carried out based on the weight of the ash in the digested sample, as indicated by the regulations[15].

Nitrogen-free extract (NFE) determination

The content of NFE was calculated with the following formula:

$$\% \text{ NFE} = 100 - (\% \text{ moisture} + \% \text{ ash} + \% \text{ fat} + \% \text{ protein} + \% \text{ crude fiber}$$

Total digestible nutrients (TDN) determination

It was calculated by adding all the organic compounds of the proximate analysis present in the food (crude proteins, ethereal extract, crude fiber, and nitrogen-free extract), multiplied by their digestibility coefficient using the following formula:

$$\text{TDN} = \text{protein } 80 + \text{NFE } 90 + \text{crude fiber } 50 + (\text{fat } 90\ 2.25)$$

The second phase of the research was carried out on the Tres Hermanos farm, located in the Bravos Grande site of the Chone Cantón in the province of Manabí; the climatic conditions are hot-dry, the temperature ranges between 23 and 34 °C, average relative humidity 38.24 %, and annual precipitation 900 mm.

Second phase: Performance test

The design used was completely randomized with four treatments (Table 1). Twenty-four (24) castrated males of the Landrace x Pietrain breed were used, which weighed 31.4 kg on average and were 80 d old, and they were distributed in each of the four treatments (6 pigs per treatment) and housed in individual pens; the experiment lasted 150 d. All the handling of the pigs for this study followed the guidelines established by the Ecuadorian Animal Welfare Regulations issued by the Agency for Phytosanitary and Zoosanitary Regulation and Control (AGROCALIDAD, for its acronym in Spanish), guaranteeing humane treatment, care, and welfare throughout the experimental period.

The amount of food fed to the animals was according to technical criteria[16]; the study factor of this research was the complement of balanced feed of solid waste from the production of craft beer in pig feeding (Table 1).

Table 1: Different harvest waste and balanced feed in pig feeding

Treatment	Type of feeding
T1, (control)	100 % balanced feed
T2, Treatment 2	80 % balanced feed + 20 % of CBW
T3, Treatment 3	70 % balanced feed + 30 % of CBW
T4, Treatment 4	60 % balanced feed + 40 % of CBW

T= treatments; CBW= craft beer waste.

The amount of balanced feed and harvest waste was calculated daily and divided into two rations per day, the first was at 0800 h and the second at 1600 h. Intake was determined by the following equation.

$$\text{Food intake} = \text{initial ration} - \text{waste}$$

The weight of the animals was recorded at 0900 h every 8 d using a generic brand industrial scale (digital type, maximum weight 150 kg and 110 v); the weight gain of the different treatments was determined by the difference in weights;

$$\text{Weight gain} = \text{initial weight} - \text{final weight}$$

Feed conversion was determined using the below formula:

$$\text{Feed conversion} = \frac{\text{feed consumed}}{\text{weight increase}}$$

The variable of carcass yield was obtained when the animals reached the commercial weight of approximately 100 kg. This variable was determined through the following formula:

$$\text{Carcass yield} = \frac{\text{live weight} - \text{viscera weight}}{\text{live weight}}\ 100$$

Back fat thickness was determined with a king's foot, between ribs 10 and 11; in this area, a cut 10 cm wide by 10 cm long by 10 cm deep was made, and a caliper was used to measure the amount of fat present in the cut.

Apparent nutrient digestibility

The apparent digestibility coefficients of protein, crude fiber, fat, ash, gross energy, calcium, and phosphorus were calculated as follows[17]:

$$\% \text{ Digestibility} = \frac{\text{nutrient consumed (g)} - \text{nutrient in feces (g)}}{\text{nutrient consumed (g)}} 100$$

The data were processed using the free version of the R statistical program. The effect of the treatments on each of the variables analyzed was evaluated through the mean difference using Fisher's LSD test ($P \leq 0.05$).

Results and discussion

Proximate chemical analysis of craft beer solid waste

The chemical analysis is shown in Table 2.

Table 2: Results of the proximate chemical analysis of solid waste from craft beer

Parameter	Average ± standard deviation (%)	Coefficient of variation (%)
Dry matter	84.77 ± 1.19	1.40
Ash	2.54 ± 0.08	3.05
Fat	1.98 ± 0.11	5.39
Crude fiber	4.85 ± 0.19	3.94
Protein	10.86 ± 0.10	0.88
NFE	64.72 ± 1.09	1.69
TDN	73.21 ± 1.03	1.40

NFE= nitrogen-free extract; TDN= total digestible nutrients.

The values of the proximate parameters show that the DM has an average value of 84.77 ± 1.19 %, similar to that reported for craft beer waste, with an average of 84.45 % DM[18]. The percentage of ash, from the same authors, reports an average value of 2.43 %, whereas in this study, it was 2.54 ± 0.08 %.

The fat of solid waste has an average of 1.98 % fat for this type of by-products of the craft brewing industry[19]. The fat content in this type of by-products is present in a minimal percentage, of which triglycerides have a presence of 67 % of the total composition of the extracts, followed by 18 % of fatty acids[18].

Chemical characterization studies of beer waste determine that the percentage of fiber found was 4.91 %; in contrast, an average value of 4.85 ± 0.19 % was obtained in this study; this allow to determine that these residues have a high concentration of dietary

fiber and could contribute to the intestinal transit of animals that are fed with these by-products of the brewing industry[19].

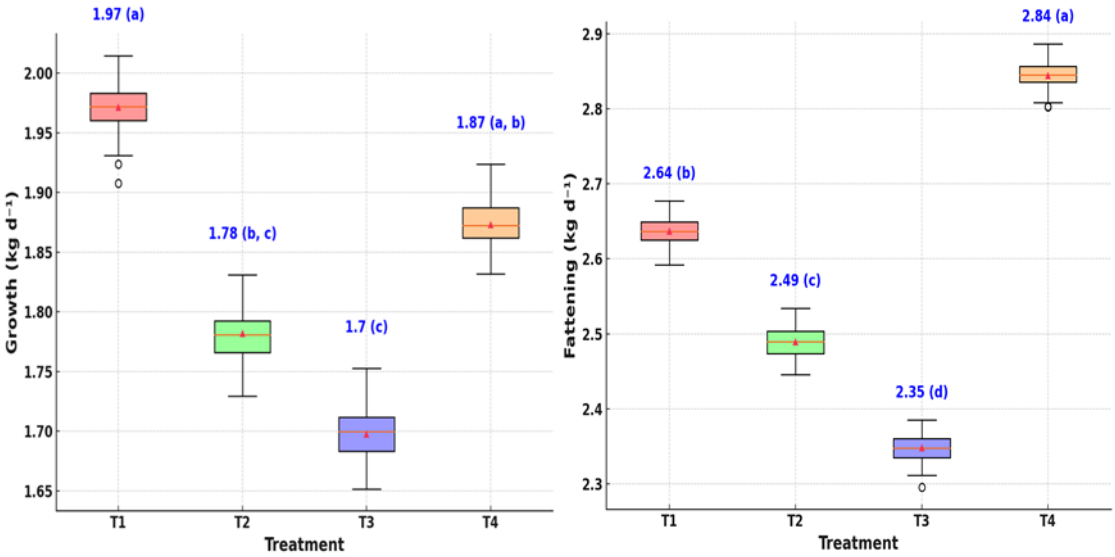
The percentage of proteins in the craft beer waste shows a low value (10.86 ± 0.10 %) compared to other studies that report protein levels around 20 % of DM[20]. Another study determines a protein range of 13.16 ± 0.05 % in the stages of the brewing process[21].

The values of NFE and TDN were 64.72 ± 1.09 and 73.21 ± 1.03 %, respectively; these data were similar (64.20 and 73.47 %) to another study, indicating that beer brewing demands carbohydrates[18].

Feed intake

Feed intake was higher in T1 and T4, whereas T2 and T3 had lower intakes (Figure 1); evidence shows that pigs in the growing stage need an intake of 1.81 kg d^{-1}[16]. These results are slightly higher than intakes of 1.66 kg d^{-1} for growth and 2.48 kg d^{-1} for fattening[22]. The recommendation is to partially replace the balanced feed at a percentage of 60 %[23].

Figure 1: Feed intake (kg d^{-1}) at the different supplementation concentrations; a) Growth and b) Fattening

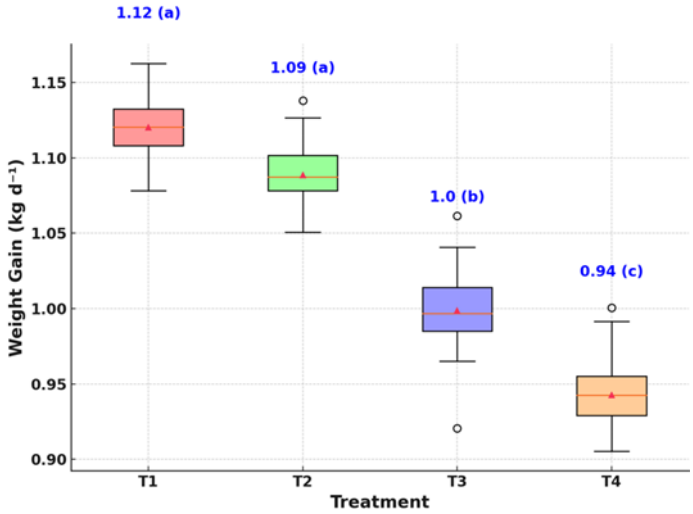


T1= control treatment (100 % balanced feed); T2= treatment 2 (80 % balanced feed + 20 % craft beer waste); T3= treatment 3 (70 % balanced feed + 30 % craft beer waste); T4= treatment 4 (60 % balanced feed + 40 % craft beer waste).

Weight gain

T1 and T2 had the highest weight gains (1.12 and 1.09 kg d^{-1}) in contrast to T3 and T4 (1 and 0.94 kg d^{-1}) (Figure 2). In this case, T1 and T2 did not show significant differences ($P>0.05$), whereas T3 and T4 did ($P\leq0.05$). The weight gain obtained in the present study is favorable to determine that craft beer waste contains proteins but it should be used as a partial substitute in pig diets[22].

Figure 2: Weight gain in pigs fed with craft beer waste for 10 weeks

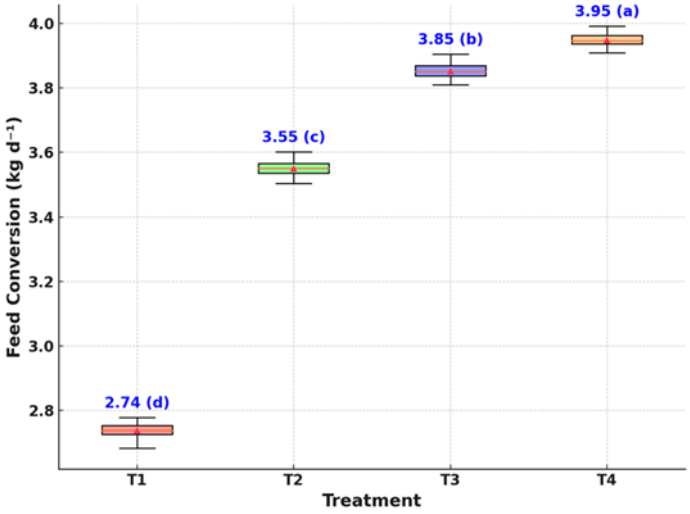


T1= control treatment (100 % balanced feed); T2= treatment 2 (80 % balanced feed + 20 % craft beer waste); T3= treatment 3 (70 % balanced feed + 30 % craft beer waste); T4= treatment 4 (60 % balanced feed + 40 % craft beer waste).

Feed conversion (FC)

The highest feed conversion was for T4 (3.95), followed by T3 and T2 with 3.85 and 3.55, respectively. The lowest value was for T1: 2.74. These values were higher than those reported in other studies where partial substitutions of corn for cassava meal were established for growing pigs[23]. Particularly, the formation of muscle tissue is promoted in the developmental stage[24]. Likewise, the values found in this study were higher when comparing the feed conversion of 3.4 with sweet potato as a partial substitute for balanced feed[22].

Figure 3: Feed conversion in pigs fed with craft beer waste for 10 weeks

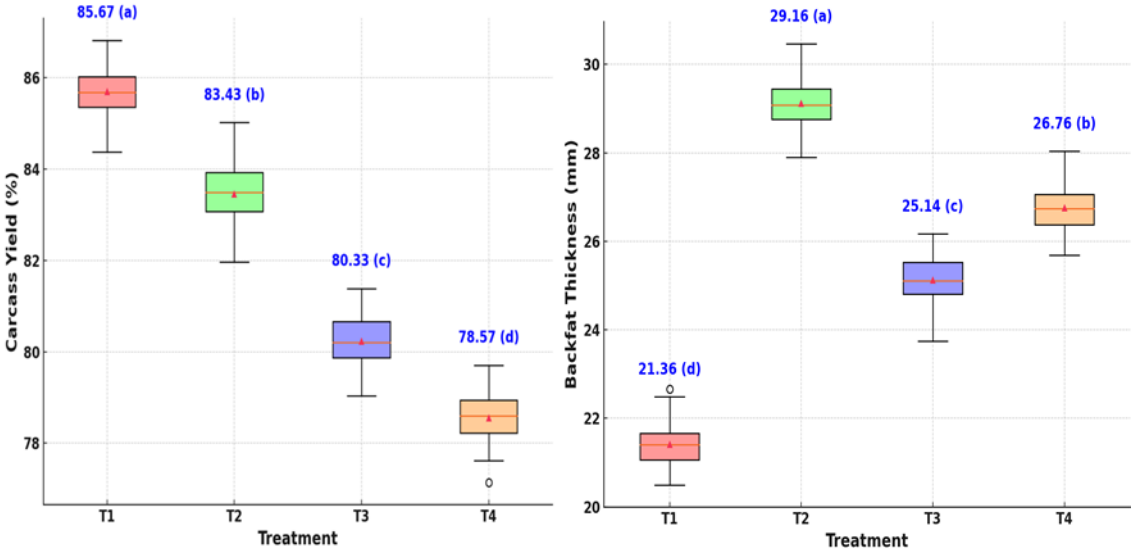


T1= control treatment (100 % balanced feed); T2= treatment 2 (80 % balanced feed + 20 % craft beer waste); T3= treatment 3 (70 % balanced feed + 30 % craft beer waste); T4= treatment 4 (60 % balanced feed + 40 % craft beer waste).

Carcass yield and back fat

The results are shown in Figure 4. Carcass yield was higher than reported in another study, indicating an average of 65.54[25]. In this study, back fat thickness was 25.61 mm, a value that is slightly higher than the standard reported of 25 mm[22] (Figure 4b).

Figure 4: a) Carcass yield in pigs fed with craft beer waste; b) Back fat (mm) in pigs fed with craft beer waste



T1= control treatment (100 % balanced feed); T2= treatment 2 (80 % balanced feed + 20 % craft beer waste); T3= treatment 3 (70 % balanced feed + 30 % craft beer waste); T4= treatment 4 (60 % balanced feed + 40 % craft beer waste).

Apparent nutrient digestibility

Table 3 shows the apparent digestibility coefficients of DM, crude ash, CP, crude fiber, fat, and gross energy of the four treatments analyzed in this study.

In DMaD, there were significant differences between T1, T2, and T3, whereas there were no differences between T1 and T4 ($P>0.05$); the DM digestibility of the waste from craft breweries was adequate; nevertheless, the results of this study were lower than those reported with tara meal in pigs of the Pig Program of the Amazonian State University (UEA, for its initialism in Spanish)[26]. Likewise, the CAaD presented significant differences between all the treatments evaluated ($P\leq0.05$), with the coefficient for T2 being higher, followed by T3, T4, and T1. These values were similar to the average apparent ash digestibility values of 61.12 % in a nutrient digestibility study in pigs[27].

Table 3: Average apparent digestibility coefficients (%) of dry matter (DMaD), crude ash (CAaD), crude protein (CPaD), crude fiber (CFaD), fat (FaD), and gross energy (GEaD)

Treatments	DMaD	CAaD	CPaD	CFaD	FaD	GEaD
T1	73.10^{c} (1.88)	57.48^{d} (0.89)	68.50^{b} (0.81)	45.63^{b} (0.43)	59.54^{a} (0.61)	78.52^{b} (1.74)
T2	79.13^{a} (1.99)	69.45^{a} (0.94)	68.38^{b} (0.87)	45.27^{b} (0.48)	54.75^{a} (0.67)	77.22^{c} (1.81)
T3	77.05^{b} (1.93)	61.56^{b} (1.06)	69.35^{a} (0.84)	46.42^{a} (0.41)	53.54^{b} (0.64)	78.65^{b} (1.76)
T4	75.11^{bc} (1.79)	60.61^{c} (0.42)	69.89^{a} (0.96)	46.32^{a} (0.46)	53.95^{ab} (0.68)	80.23^{a} (2.01)

T1= control treatment (100 % balanced feed); T2= treatment 2 (80 % balanced feed + 20 % craft beer waste); T3= treatment 3 (70 % balanced feed + 30 % craft beer waste); T4= treatment 4 (60 % balanced feed + 40 % craft beer waste).

^{abc} Values with different letters are different ($P<0.05$).

CPaD showed no significant differences ($P>0.05$) between T1 *vs* T2 and T3 *vs* T4; however, there were differences between T1 *vs* T2 ($P\leq0.05$). These results were possibly due to low excretions in the amount of fecal nitrogen, a product of the low or no presence of animal proteins in the diets studied[27]. CFaD behave the same as the protein, and it was determined that they are relatively low values; this is because the digestibility decreases whenever the fiber content increases[28].

FaD was different between T1, T2 *vs* T3 and T4 ($P\leq0.05$). On average, there was an apparent digestibility of 55.45 %, a figure that is below that obtained with foods based on poultry viscera[16].

GEaD showed significant differences between T1 *vs* T2 and T4 ($P \leq 0.05$); apparent digestibility was higher in T4, with 80.23 %; this result is related to the percentage of apparent digestibility of gross energy of diets composed of different levels of phytases, with a value of 78.42 %[16].

Conclusions and implications

Incorporating waste from craft brewing into backyard pig diets is a sustainable alternative that improves feed efficiency and production performance, while reducing costs and environmental impact. Although they should not be the only food source, their nutritional composition promotes optimal digestibility and benefits in carcass quality, highlighting their viability as a supplementary feeding practice in small and medium-scale pig production.

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Conflict of interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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Article

Multitrait analysis of growth traits for the optimization of breeding value prediction in Braunvieh cattle

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Abstract:

Currently, the genetic evaluations of growth traits **(**birth weight (BW), weaning weight (WW), and yearling weight (YW)) for the Mexican Braunvieh cattle are carried out in a univariate (for BW) and a bivariate (for WW and YW) models. Precision of genetic evaluations can be improved by a trivariate model. It was aimed to study bias in the univariate and bivariate evaluations due to the missing trait(s) in the analysis and the accuracy gain by the trivariate analysis. Pedigree and performance data were obtained from the Asociación Mexicana de Criadores de Ganado Suizo de Registro. After data edits, univariate, bivariate, and trivariate analyses were performed to make comparisons. A simple data pruning strategy was employed, considerably reducing the data size in the analyses. Animals excluded from the analyses were evaluated at a low computational cost from solutions of animals included in the analyses. The bivariate analysis showed biased WW and YW evaluations and genetic trends. The genetic trends underestimated young animals. Since the mid-1990s, all the traits showed a steady genetic progress. The bias was due to natural/artificial preselection on BW. The inclusion of BW in the trivariate analysis helped to consider the preselection information. The univariate BW evaluation and genetic trend were unbiased. Also, BW gained less accuracy from WW and YW than WW and YW from BW. Based on the results of this study, it is recommended the trivariate analysis of the traits with data pruning to lower the computational cost.

Keywords: Accuracy, Animal model, Bias, Breeding value, Braunvieh, Multitrait, Preselection, Pruning, Univariate.

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Introduction

The European Brown Swiss cattle was introduced to Mexico in the mid-19th century[1,2]. In 1967, the Asociación Mexicana de Criadores de Ganado Suizo de Registro (AMSGSR) was established, and in 1968, both dairy (American Brown Swiss) and dual-purpose (European Brown Swiss or Braunvieh) variants were registered[3]. The potentials of the breed, such as good fertility, hardiness, adaptability, and dairy and meat production, have positioned this breed as one of the favorite breeds to cross with Zebu in the Mexican tropics production

systems. During the last two decades, the Mexican Brauvieh cattle have gradually displaced *Bos indicus*[4].

The first national genetic evaluation of the Braunvieh breed was carried out in 2003 by AMGSR, with periodic evaluations and genetic trend monitoring since then[5]. Currently, birth weight (BW), weaning weight (WW), yearling weight (YW), and scrotal circumference (SC) are evaluated, while the American Brown Swiss also includes milk volume adjusted to 210 d in milk[6]. Although Braunvieh breeding has led to genetic gains, the levels of genetic gain were not as expected. Larios-Sarabia *et al*[7] reported declines in milk production genetic trend in Jersey and American Brown Swiss herds in Mexico, partly due to herds with different selection goals. They raised the need to revisit and restructure the national genetic improvement programs for those dairy populations.

Multitrait genetic evaluations have been very useful in the genetic improvement of animals[8,9]. Advantages of these models compared to univariate models have been reported, such as a greater magnitude of the estimated heritabilities[10], reduction of bias introduced by sequential selection[8,11], gain in the accuracy of breeding values[11,12], better estimator properties, especially for incomplete data[9]. Compared to univariate models, they better utilize the available information via the correlations among traits. Hence, more accurate evaluations are produced[11]. Multitrait evaluations come at a greater computational cost to construct the equation system, solve a large set of equations, and slow convergence (more and slower iterations) due to an increased number of non-zero off-diagonal elements of the coefficient matrix[11].

In multitrait models, phenotypes for one trait serve as phenotypes (with weighted importance) for the other traits. As a result, more breeding values are obtained, such as breeding values for traits measured later in life. For example, a newborn calf will receive breeding values for WW and YW based on its own BW phenotype and relatives' phenotypes for any of the three traits. Those breeding values are more accurate than the calf's parent (breeding value) averages for WW and YW.

AMSGSR regularly evaluates BW, WW, YW, and SC, and the genetic evaluation results are communicated to stakeholders and farmers. AMSGSR aims to increase the cattle's productivity, protect the interests of breeders, and promote the breed. Due to the large historical data and limited computational resources, these traits have been evaluated separately in univariate animal models for years. Since 2016, WW and YW have been evaluated with a bivariate animal model. The next step to this improvement might be evaluating the three growth traits together in a single multitrait animal model. That way, WW and YW evaluations get free from (natural/artificial) preselection for BW, and the three traits benefit from the additional information in the analysis.

The aims of this study were 1) to develop a multitrait model for the joint evaluation of BW, WW, and YW for the Mexican Braunvieh population in a constraint computational environment, 2) quantify bias in the current state of genetic evaluation for the growth traits (i.e., a univariate BW and a bivariate WW-YW evaluation), and 3) accuracy gain in analyzing the three traits simultaneously.

Material and methods

Data

Pedigree and performance data were obtained from AMSGSR. For each trait (BW, WW and YW), the herds were required to have a minimum of four performance records. Records from animals born from embryo transfer (due to the lack of identification of the recipient cows) or with both parents unknown were removed. WW phenotypes were limited to those taken in a range of 195 and 285 d of age and then adjusted to a target of 240 d of age. YW phenotypes were limited to those taken in a range of 320 and 410 d of age and then adjusted to a target of 365 d of age. Then, records outside the trait's mean ± 3 SD range were discarded. Phenotypes were also discarded if the age of the dam at the animal's birth was outside the 20 to 180-mo range. Contemporary groups were defined within trait by the herd (256), year (1901–2020), and season (rainy *vs* dry) of weighting. Contemporary groups were required to have a minimum size of three animals. Smaller contemporary groups were discarded, and 2,532 contemporary groups gathering 37,738 animals remained.

Data pruning

There were 193,442 animals in the pedigree born until 2020. A simple data pruning strategy was applied by upward pedigree extraction from animals with phenotype in at least one of the three traits (37,738). The extracted pedigree subset from those 37,738 animals contained 64,501 animals born from 1950 to 2020. This is expected to have a considerable effect on reducing computational time and demands. The excluded animals had no phenotype contribution. Breeding values and their accuracies (EBV and *r*) of the animals excluded from the analyses were estimated iteratively from parents' information ($EBV_{progeny} = (EBV_{sire} + EBV_{dam})/2$ and $r_{\text{progeny}} = 0.5 \times \sqrt{r_{sire}^2 + r_{dam}^2}$). The iterative procedure was:

1. Calculate breeding values and accuracies for animals with both parents (if known) in the pedigree subset, based on parents' breeding values and accuracies.
2. Append the pedigree rows for those animals to the pedigree subset.
3. Repeat steps 1 and 2 while there are animals to be added to the pedigree subset.

The pedigree subset contained 21,405 males, 43,096 females, 3,321 sires, and 29,700 dams. Breeding values and accuracies of 171,390 animals were calculated using the above iterative procedure. The 22,052 remaining animals were in pedigree trees that received no phenotype contribution. Those animals were not considered in the study. Regardless of the analysis, those receive a breeding value and an accuracy of 0.

The analyses were performed on a t2.medium AWS (Amazon Web Services) Ubuntu 20.04 LTS server with two CPUs and 4 GB of RAM.

Analyses

Following the current practice at AMGSR, BW was analyzed in a univariate model, and WW and YW in a bivariate model:

$$\mathbf{y}_{BW} = \mathbf{X}_{BW}\mathbf{b}_{BW} + \mathbf{Z}_{BW}\mathbf{u}_{BW} + \mathbf{e}_{BW},$$

$$\begin{bmatrix}\mathbf{y}_{WW}\\ \mathbf{y}_{YW}\end{bmatrix} = \begin{bmatrix}\mathbf{X}_{WW} & \mathbf{0}\\ \mathbf{0} & \mathbf{X}_{YW}\end{bmatrix}\begin{bmatrix}\mathbf{b}_{WW}\\ \mathbf{b}_{YW}\end{bmatrix} + \begin{bmatrix}\mathbf{Z}_{WW} & \mathbf{0}\\ \mathbf{0} & \mathbf{Z}_{YW}\end{bmatrix}\begin{bmatrix}\mathbf{u}_{WW}\\ \mathbf{u}_{YW}\end{bmatrix} + \begin{bmatrix}\mathbf{M}_{WW} & \mathbf{0}\\ \mathbf{0} & \mathbf{0}\end{bmatrix}\begin{bmatrix}\mathbf{m}_{WW}\\ \mathbf{0}\end{bmatrix} + \begin{bmatrix}\mathbf{W}_{WW} & \mathbf{0}\\ \mathbf{0} & \mathbf{0}\end{bmatrix}\begin{bmatrix}\mathbf{w}_{WW}\\ \mathbf{0}\end{bmatrix} + \begin{bmatrix}\mathbf{e}_{WW}\\ \mathbf{e}_{YW}\end{bmatrix},$$

where, **y**, **b**, **u**, **m**, **w**, and **e** are the vectors of phenotypes, fixed effects, direct genetic effect, maternal genetic effect, maternal permanent environmental effect, and residuals. Matrices **X** and **Z** relate phenotypes to fixed effects and animals, respectively. Matrices **M** and **W** relate phenotypes to dams. To Analyze all three traits jointly, the following model was used:

$$\begin{bmatrix}\mathbf{y}_{BW}\\ \mathbf{y}_{WW}\\ \mathbf{y}_{YW}\end{bmatrix} = \begin{bmatrix}\mathbf{X}_{BW} & \mathbf{0} & \mathbf{0}\\ \mathbf{0} & \mathbf{X}_{WW} & \mathbf{0}\\ \mathbf{0} & \mathbf{0} & \mathbf{X}_{YW}\end{bmatrix}\begin{bmatrix}\mathbf{b}_{BW}\\ \mathbf{b}_{WW}\\ \mathbf{b}_{YW}\end{bmatrix} + \begin{bmatrix}\mathbf{Z}_{BW} & \mathbf{0} & \mathbf{0}\\ \mathbf{0} & \mathbf{Z}_{WW} & \mathbf{0}\\ \mathbf{0} & \mathbf{0} & \mathbf{Z}_{YW}\end{bmatrix}\begin{bmatrix}\mathbf{u}_{BW}\\ \mathbf{u}_{WW}\\ \mathbf{u}_{YW}\end{bmatrix} + \begin{bmatrix}\mathbf{0} & \mathbf{0} & \mathbf{0}\\ \mathbf{0} & \mathbf{M}_{WW} & \mathbf{0}\\ \mathbf{0} & \mathbf{0} & \mathbf{0}\end{bmatrix}\begin{bmatrix}\mathbf{0}\\ \mathbf{m}_{WW}\\ \mathbf{0}\end{bmatrix} + \begin{bmatrix}\mathbf{0} & \mathbf{0} & \mathbf{0}\\ \mathbf{0} & \mathbf{W}_{WW} & \mathbf{0}\\ \mathbf{0} & \mathbf{0} & \mathbf{0}\end{bmatrix}\begin{bmatrix}\mathbf{0}\\ \mathbf{w}_{WW}\\ \mathbf{0}\end{bmatrix} + \begin{bmatrix}\mathbf{e}_{BW}\\ \mathbf{e}_{WW}\\ \mathbf{e}_{YW}\end{bmatrix}.$$

The variance component structures were:

$$V\begin{pmatrix}\mathbf{u}\\ \mathbf{m}\end{pmatrix} = \mathbf{A} \otimes \begin{pmatrix} \sigma^2_{u_{\mathrm{BW}}} & \sigma_{u_{\mathrm{BW,WW}}} & \sigma_{u_{\mathrm{BW,YW}}} & \sigma_{u_{\mathrm{BW}},m_{\mathrm{WW}}} \\ & \sigma^2_{u_{\mathrm{WW}}} & \sigma_{u_{\mathrm{WW,YW}}} & \sigma_{u_{\mathrm{WW}},m_{\mathrm{WW}}} \\ & & \sigma^2_{u_{\mathrm{YW}}} & 0 \\ & & & \sigma^2_{m_{\mathrm{WW}}} \end{pmatrix},$$

V(**e**) = **I**⊗**R**, and V($\mathbf{w}_{WW}$) = **I**σ^2_w. **A** is the (pedigree-based) numerator relationship matrix, and **R** is the trait × trait residual covariance matrix.

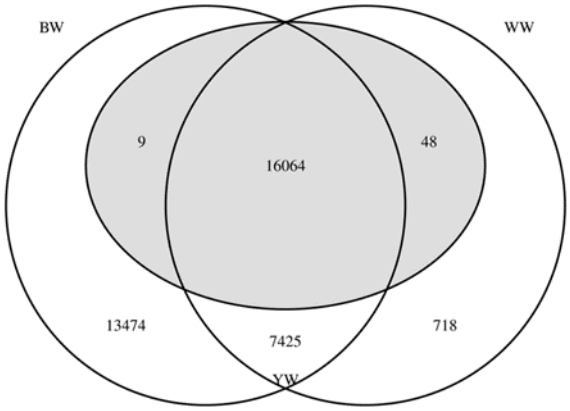
Fixed class effects of sex (all traits), milk feeding regimen (3 levels – WW only), post-weaning feed regime (3 levels – YW only), and contemporary group (all traits), as well as fixed regression effects of age of dam at birth (aod), aod^2, and percentage of Braunvieh purity, were included in the models. These effects are described in a previous study[13]. The purity covariate had a minimum, mean, and median of 0.880, 0.996, and 1, respectively. Only 10.7 % and 1.9 % of data rows had a purity less than 0.99 and 0.95, respectively.

The BLUPF90 family of programs[14] was used for the data analysis, including variance components estimation using the expectation–maximization to compute REML estimates (EM-REML) with acceleration, breeding value, and accuracy prediction. Schaeffer[11] explained the building of mixed model equations and the theory of multitrait animal models well.

Results and discussion

Figure 1 shows the Venn diagram for the number of animals with different combinations of available phenotypes. Among the 37,738 phenotyped animals kept for the analyses, 16,064 had phenotypes for all the traits, and 13,474 had phenotypes for only BW. Of the 16,121 animals phenotyped for YW, 48 were missing BW phenotype, 9 were missing WW phenotype, and none were missing both BW and WW phenotypes (Figure 1). Table 1 describes the phenotype data used in the analyses.

Figure 1: Number of animals with available phenotypes in different combinations of traits



(BW= birth weight (left circle), WW= weaning weight (right circle), YW= yearling weight (middle grey oval).

Table 1: Descriptive statistics of the phenotypes used in the study

Trait	Min.	Max.	Mean	SD	N (male)	N (female)
BW	23.0	53.0	38.21	4.79	18,120	18,852
WW	104.0	393.5	235.79	42.17	11,929	12,326
YW	146.7	526.7	323.63	55.86	8,065	8,056

BW= birth weight, WW= weaning weight, YW= yearling weight.

Heritabilities were estimated as $h^2 = \sigma_u^2/(\sigma_u^2 + \sigma_m^2 + \sigma_w^2 + \sigma_e^2)$, where σ_u^2, σ_m^2, σ_w^2, and σ_e^2 are the estimated variance components associated with the vectors **u**, **m**, **w**, and **e**, respectively. Where any of these effects are absent in the model, the corresponding variance equals 0. Heritability estimates from the trivariate model were similar to those from the univariate and bivariate models (Table 2). For WW, $h_m^2 = \sigma_m^2/(\sigma_u^2 + \sigma_m^2 + \sigma_w^2 + \sigma_e^2)$ decreased from the bivariate (0.021 ± 0.009) to the trivariate analysis (0.020 ± 0.008). From the univariate/bivariate analyses to the trivariate analysis, the genetic covariances changed from

$$\begin{array}{c} \\ \text{BW} \\ \text{WW} \\ \text{YW} \\ \text{WW}_m \end{array} \begin{array}{c} \begin{array}{cccc} \text{BW} & \text{WW} & \text{YW} & \text{WW}_m \end{array} \\ \begin{bmatrix} 2.57 & 0 & 0 & 0 \\ & 105.7 & 95.6 & -10.7 \\ & & 105.2 & 0 \\ & & & 12.25 \end{bmatrix} \end{array}$$

to

$$\begin{array}{c} \\ \text{BW} \\ \text{WW} \\ \text{YW} \\ \text{WW}_m \end{array} \begin{array}{c} \begin{array}{cccc} \text{BW} & \text{WW} & \text{YW} & \text{WW}_m \end{array} \\ \begin{bmatrix} 2.6 & 7.42 & 7.61 & 0.44 \\ & 103.7 & 93.7 & -8.75 \\ & & 105.5 & 0 \\ & & & 11.27 \end{bmatrix}, \end{array}$$

and the residual covariances changed from

$$\begin{array}{c} \\ \text{BW} \\ \text{WW} \\ \text{YW} \end{array} \begin{array}{c} \begin{array}{ccc} \text{BW} & \text{WW} & \text{YW} \end{array} \\ \begin{bmatrix} 8.46 & 0 & 0 \\ & 433.1 & 299.4 \\ & & 665.0 \end{bmatrix} \end{array}$$

to

$$\begin{array}{c} \\ \text{BW} \\ \text{WW} \\ \text{YW} \end{array} \begin{array}{c} \begin{array}{ccc} \text{BW} & \text{WW} & \text{YW} \end{array} \\ \begin{bmatrix} 8.44 & 8.02 & 8.53 \\ & 435.4 & 302 \\ & & 666 \end{bmatrix}, \end{array}$$

where WW_m is WW's maternal genetic effect. The changes in the genetic and residual correlations were small. WW's maternal permanent environment variance changed from 7.34 to 6.84 from the bivariate to the trivariate model.

Table 2: Estimated heritabilities from different analyses and traits

Evaluation	**Trait**	**h^2**
Current[1]	BW	0.233 ± 0.002
	WW	0.189 ± 0.018
	YW	0.136 ± 0.013
Trivariate	BW	0.235 ± 0.014
	WW	0.186 ± 0.018
	YW	0.136 ± 0.017

[1] Univariate for BW and bivariate for WW and YW.

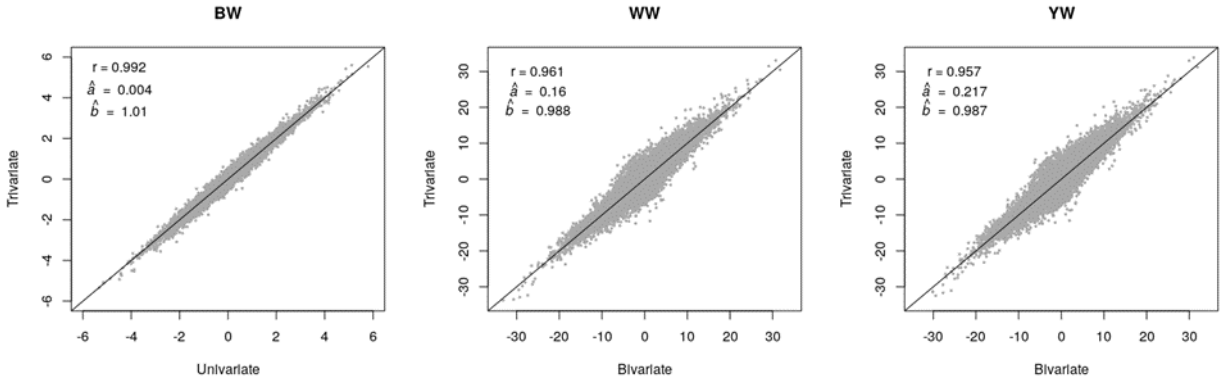
BW= birth weight, WW= weaning weight, YW= yearling weight.

Unlike the univariate and bivariate models, the trivariate model considers genetic and residual correlations between BW and the two other traits. It is based on the assumption that BW phenotypes may contribute to better genetic evaluations of WW and YW and vice versa. Also, if there is natural or artificial selection on BW, the trivariate model can remove that preselection bias from WW and YW genetic evaluations. Another important feature of the

trivariate model is that it provides the first estimates of WW and YW genetic merits for newborn calves.

Figure 2 shows the scatter plot of the univariate/bivariate vs the trivariate breeding values for different traits. The corresponding correlation and regression coefficients are also presented in the sub-figures (for each trait). YW showed the lowest correlation coefficient (r = 0.957), the largest deviation of the intercept from 0 ($\hat{a}$= 0.217), and the largest deviation of the regression coefficient (slope of the regression line) from 1 ($\hat{b}$= 0.987). On the other hand, BW showed the least deviations. Deviating the regression coefficient from 1 indicates bias, considering the true assumption that BW phenotypes do not introduce bias to WW and YW evaluations. The bias for WW and YW in the bivariate analysis was minor and upward ($\hat{b}$< 1), and it was due to natural/artificial preselection on BW.

Figure 2: Breeding values estimated via the univariate (BW) or the bivariate (WW and YW) *versus* those estimated via the trivariate (BW, WW, and YW) analysis

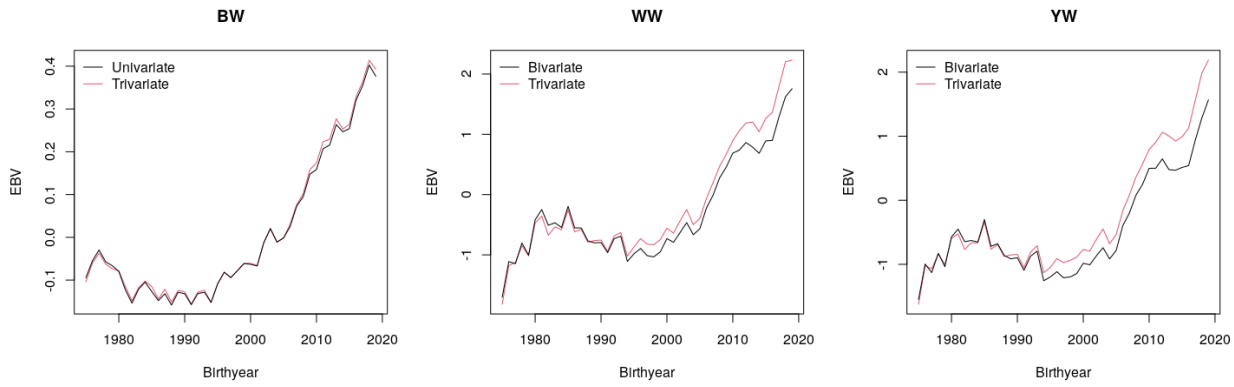


BW= birth weight, WW= weaning weight, YW= yearling weight.

BW had a higher expected rate of breeding value changes due to its higher heritability. However, BW breeding values were the least affected by the trivariate analysis (Figure 2). This is likely due to the lack of the preselection effect from WW and YW on BW. Though YW's heritability was lower than WW's heritability (Table 2), the rate of breeding value changes (i.e., correlation) was similar between the two traits. This is due to BW information directly and indirectly (via WW) influencing YW's evaluation. In a similar study, Ramírez-Valverde *et al*[8] recommended a univariate model for BW, and a bivariate model for WW & YW, for the Angus breed. They also recommended bivariate models for BW & WW, and WW & YW, for the Tropicarne breed.

Genetic trends from 1975 to 2019 for different traits and analyses (univariate, bivariate, and trivariate) are shown in Figure 3. There were only 20 animals born in 2020. Therefore, 2020 was not considered when studying genetic trends. The rate of genetic gain was slow or negative in the 1980s and the 1990s for all the traits. Since the mid-1990s, genetic gain accelerated for all the traits. The univariate vs trivariate analysis did not affect the genetic trend of BW, which supports the other finding ($\hat{b}$= 1.01 for BW, Figure 1) that the univariate BW evaluations are unbiased. On the other hand, the bivariate analysis showed bias in the genetic trends of WW and YW by underestimating young animals. Deviations between the breeding value averages were greater for animals born in recent years (Figure 3). This again showed the importance of BW data to avoid/reduce bias due to BW preselection for the evaluation of WW and YW.

Figure 3: Genetic trends estimated via the univariate (BW), bivariate (WW and YW), and trivariate (BW, WW, and YW) analyses

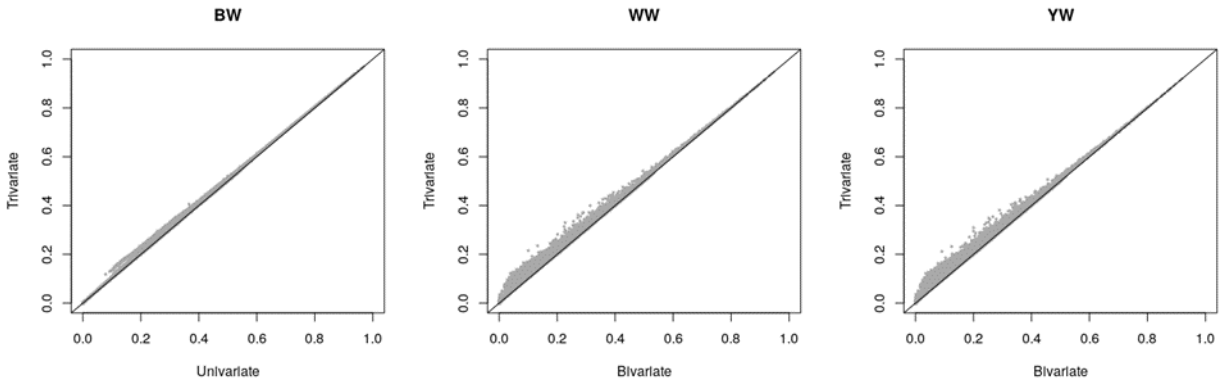


BW= birth weight, WW= weaning weight, YW= yearling weight.

Figure 4 shows the accuracy gain from the univariate (BW) and the bivariate (WW and YW) model to the trivariate model. The average accuracy gain was 0.006, 0.010, and 0.011 for BW, WW, and YW, respectively. Possible reasons for BW's low accuracy gain are: 1) There were only 766 animals with WW phenotype and without BW phenotype, of which 48 animals also had YW phenotype (Figure 1) 2) The heritabilities of WW and YW were lower than BW's heritability (Table 2). The average BW accuracy gain for those 766 animals with WW and without BW phenotypes was 0.028. However, it should be mentioned that accuracy gain is not all about own phenotype but also about phenotype contribution from all the relatives (weighted by the relationship coefficient and the heritability). WW and YW gained more accuracy from the presence of BW in the trivariate analysis. This is likely due to 1) greater BW's heritability and 2) many animals with BW phenotype and without WW and YW phenotypes (Figure 1; i.e., animals with little phenotype contribution for WW and YW receive phenotype contribution from the correlated trait BW). In fact, animals with low

accuracy gained more accuracy from the trivariate analysis (Figure 4). Those animals had low phenotype contribution (most likely no own phenotype) in the univariate (BW) or the bivariate (WW and YW) analysis but gained phenotype contribution (via own performance and/or relatives) by the additional trait(s) in the trivariate analysis. For example, the average WW accuracy gain for the 13,483 animals with BW and without WW phenotypes was 0.037. Similarly, the average YW accuracy gain for the 13,474 animals with BW and without WW and YW phenotypes was 0.040. The average YW accuracy gain for the 718 animals with WW and without BW and YW phenotypes was 0.004, and for the 7,425 animals with BW and WW phenotypes and without YW phenotype was 0.015. Breeding values with low accuracy tend to regress toward the founders' solution, which is 0.

Figure 4: Breeding value accuracies estimated via the univariate (BW) or the bivariate (WW and YW) analysis versus those estimated via the trivariate (BW, WW, and YW) analysis



BW= birth weight, WW= weaning weight, YW= yearling weight.

The greater the heritability of the traits and the absolute genetic correlations among them, the greater the accuracy gain. Also, animals with missing phenotypes are expected to gain more accuracy from the correlated trait phenotypes. Tong[15] studied the relationships between heritability, genetic correlation (r_g), and residual correlation (r_e) between traits in a multitrait animal model and found that: a) the greater the $|r_g - r_e|$, the greater the accuracy gain, b) for $|r_g| < |r_e|$, the trait with lower heritability gains more accuracy, and c) for $|r_g| > |r_e|$, the trait with higher heritability gains more accuracy.

Conclusions and implications

For years, due to limited computational resources, genetic evaluations of growth traits (BW, WW, and YW) for the Mexican Braunvieh cattle were carried out in univariate models. Since 2016, WW and YW have been evaluated in a bivariate model. The most important reasons for multitrait evaluations are better use of available data and accuracy gain via correlations among the traits and removing/reducing bias caused by selection[11]. The latter is more evident for traits measured and selected sequentially. The results showed unbiased univariate BW evaluations and sightly biased bivariate WW and YW evaluations caused by (non-random) selection on BW. Artificial selection on BW is presumably weak, and selection on other (correlated with BW) pre-weaning traits such as pre-weaning daily gain as well as natural selection on BW and those traits are involved. A multitrait model including all three traits is the solution to this problem, and we recommend its implementation to AMGSR. To tackle the increased computational cost by the trivariate model, it is proposed a data pruning strategy, reducing computational demands considerably. Pruned animals were then evaluated at a low computational cost using the solutions of animals in the analysis.

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Article

Association between retained fetal membranes, clinical endometritis, and reproductive performance of Holstein cows in the family dairy production system

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Abstract:

In the family dairy production system, the prevalence of assisted calving and retained fetal membranes (RFM) is high; however, the extent to which these conditions affect the prevalence of uterine diseases and reproductive performance has not been determined. This study aimed to assess the presence or absence of assisted calving and RFM on the prevalence of clinical endometritis (CE) and conception rate at the first postpartum artificial insemination (1SCR) of Holstein cows. An observational retrospective cohort study was conducted to examine the relationship between assisted calving, RFM, CE, and 1SCR. The data were analyzed using descriptive statistics and logistic regression. The prevalence of assisted calving, RFM, and CE was 4.8 %, 8.3 %, and 16.8 %, respectively, and the overall 1SCR was 58.5 %. Cows with RFM had a 5.6 times higher risk of developing CE (P=0.001), and cows with CE had a 5.4 times higher risk of not becoming pregnant at the first postpartum artificial insemination (P<0.001). The prevalence of assisted calving and RFM observed in this study was lower than typically reported in this production system. Nevertheless, it was confirmed that RFM is a significant risk factor for CE, and this uterine condition negatively impacts reproductive performance in the family dairy production system.

Keywords: Calving quality, Uterine disease, Conception rate.

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Introduction

The family dairy production system contributes to food security, job creation, and poverty alleviation, especially in families living in rural areas in developing countries[1,2]. In México, this system is characterized by the fact that operation on the farms and agricultural work to obtain forage for cattle are carried out by family members[3,4]. It has been estimated that this system contributes around 30 % of the bovine milk production in the country[5] and although it commonly faces socioeconomic and management problems, it has good potential for vertical growth based on the improvement of its production processes[6].

The low productivity observed in family dairy farms is partially associated with inadequate management of the reproductive process[7]. Recent studies have identified that reproductive performance in these dairy farms is affected by both animal-related factors (such as body

condition score) and herd-level factors like management practices[8,9]. Regardless of the production system, poor reproductive performance negatively impacts on milk production, increases costs for reproductive treatments, and leads to losses due to involuntary culling, all of which affect the profitability of dairy farms[10-13].

The conception rate after artificial insemination is one of the most important variables impacting the reproductive performance of cattle[14]. Studies indicate that assistance at calving and/or RFM can reduce the conception rate at first service[8,15]. In the family dairy production system, an increase in artificial insemination use[16,17], as well as a high prevalence of assisted calving and RFM (>10%) have been observed[8]. Both, assisted calving and RFM are indicators of low-quality calving, and this condition, increases the risk of developing clinical or subclinical uterine infections[18,19], which can affect the establishment and maintenance of pregnancy[20]. In intensive dairy farms, cows presenting clinical endometritis (CE) between days 20 and 33 postpartum have been reported to have less likely to become pregnant and more likely to be culled from the herd[21].

The high prevalence of assisted calving and RFM in the family dairy production system in Mexico would suggest the existence of a high rate of uterine diseases and low 1SCR. However, acceptable 1SCR rates have been reported for this production system[8], which appears contradictory. To the best of our knowledge, no studies have specifically examined the effect of calving assistance and RFM on the prevalence of uterine diseases and reproductive performance of cattle in such a system. Therefore, this study aimed to determine the effects of the presence or absence of assisted calving and RFM on the prevalence of clinical endometritis and conception rate at the first postpartum artificial insemination of Holstein cows in the family dairy production system.

Material and methods

A retrospective cohort observational study was conducted using a subset of a data from a previous experiment performed within this production system in Jalisco state[8]. Information regarding CE had not been analyzed previously[8], as it was beyond the scope of the original study. In this study, data on calving characteristics (assisted calving and RFM), the occurrence of CE, and the 1SCR of 241 Holstein cows across 22 farms were included.

Assisted calving was recorded when cows required help during the expulsion phase of their calves, regardless of the extent of help. RFM was recorded when cows failed to expel the placenta within 12 h after calving. CE was diagnosed by evaluating vaginal mucus between 28 and 35 d postpartum. Vaginal mucus was scored on a 0-to-3 scale, according to the

methodology described by Sheldon *et al*[22]. A score of 0 (clear and transparent mucus) refers to a cow with no CE problems. Scores of 1 (mucus with white or off-white flecks of pus), 2 (exudate containing ≤50 % white or off-white mucopurulent material), and 3 (exudate containing >50 % purulent material, usually off-white or yellow) indicate cows with different levels of CE.

Reproductive performance was assessed using 1SCR, calculated based on the number of pregnant cows at pregnancy diagnosis following artificial insemination, which was performed by the producers after estrus detection without using any hormonal protocol for synchronization. Only inseminations from the first postpartum service across the different farms were included. Pregnancy diagnosis was conducted between 35 and 42 d post-insemination, using ultrasonography with a UMS900 unit equipped with a 5 Mhz transducer (Universal Imaging, Bedford Hills, NY, USA).

All analyses were performed using SAS 9.3 program (SAS Institute Inc. Cary, NC, USA). Descriptive statistical analysis was conducted for assisted calving (yes/no), RFM (yes/no), 1SCR (yes/no), CE including the different levels of severity (0, 1, 2 and 3) and CE without including severity levels (yes/no). A Cochran-Mantel and Haenszel analysis was used to assess associations among the evaluated factors. Since no significant associations were found (P>0.1), a univariate logistic regression was conducted to determine the impact of assisted calving and RFM on CE (yes/no; without severity levels). The same analysis was performed to determine the impact of assisted calving (yes/no), RFM (yes/no), and CE (yes/no) on pregnancy failure following artificial insemination. In all cases, the LOGISTIC procedure of SAS was used to calculate odds ratios and confidence intervals. Statistical significance was established at the P≤0.05 level. None of the cows with CE at severity level 3 became pregnant, which limited the logistic regression analysis by severity level of this condition.

Results

The prevalence of assisted calving and RFM was 4.8 % (11/231) and 8.3 % (19/230), respectively. The overall prevalence of CE was 16.7 % (39/233), with severity levels of 6.4 % (15/233), 7.3 % (17/233), and 3.0 % (7/233) for grades 1, 2, and 3, respectively (Figure 1A). The 1SCR was 51.0 % (99/154), 27.3% (3/11), 38.3 % (5/13), and 0.0% (0/5) for cows with a severity level of CE 0, 1, 2, and 3, respectively (Figure 1B). The overall 1SCR was 58.5 % (107/183); 64.3 % (99/154) for cows that did not show CE and 27.6 % (8/29) for the cows that did show CE (P<0.001), regardless of severity (Figure 1C).

The results of the univariate logistic regression analysis are presented in Table 1. No statistically significant association was found between assisted calving and the presence of CE (P=0.235). However, RFM had a significant effect on the occurrence of CE (P<0.001). The odds ratio indicates that cows with RFM were 5.6 times more likely to develop CE compared to the cows without this condition.

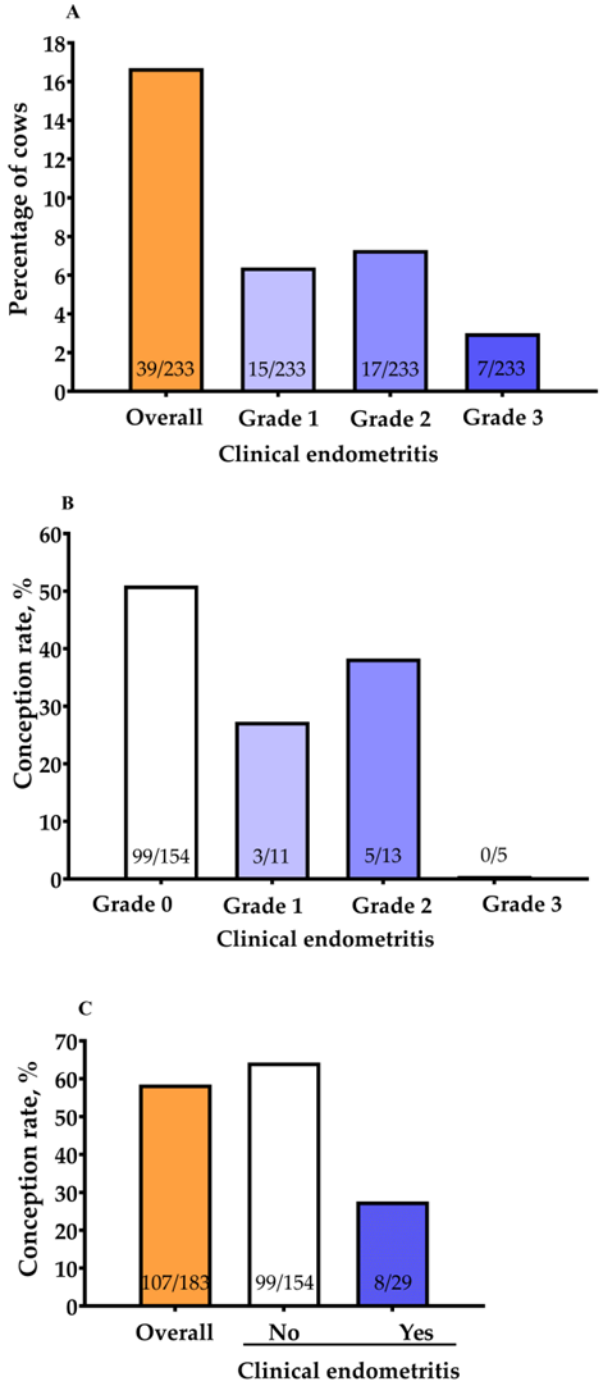
No statistically significant differences were observed for assisted calving (P=0.427) and RFM (P=0.259) in relation to pregnancy failure. However, there was a significant effect of CE, regardless of severity, on pregnancy failure following the first postpartum artificial insemination (P<0.001). Cows that showed CE had a 5.4 times higher risk of not becoming pregnant after artificial insemination than cows not experiencing such uterine condition (Table 1).

Table 1: Impact of analyzed factors on the risk of developing clinical endometritis and pregnancy failure following the first postpartum artificial insemination

Variable	**Risk factor**	**OR**	**95% CI**	***P*-value**
Clinical endometritis	Assisted calving (yes *vs* no)	2.3	0.57 - 9.5	0.235
	Retained fetal membranes (yes *vs* no)	5.6	2.0 - 15.7	0.001
Pregnancy failure	Assisted calving (yes *vs* no)	1.8	0.40 - 8.5	0.427
	Retained fetal membranes (yes *vs* no)	1.8	0.62 - 5.7	0.259
	Clinical endometritis (yes *vs* no)	5.4	2.17 - 13.6	0.001

OR= odds ratio; CI= confidence interval 95 %.

Figure 1. Prevalence of overall clinical endometritis and by severity level



(A), conception rate after the first postpartum artificial insemination according to the severity level of clinical endometritis (B), and overall conception rate after the first postpartum artificial insemination for cows with and without clinical endometritis (P<0.001), regardless of the degree of severity (C).

Discussion

It was hypothesized that in the family dairy production system in Mexico, the high frequency of assisted calving and RFM would result to an elevated prevalence of CE, subsequently reducing the reproductive performance of the cows. However, the prevalence of assisted calving and RFM was considerably lower than previously reported (14.1 % and 14.9 %, respectively) in this production system[23].

The 1SCR was similar to that reported in previous studies (~50 %) in farms under these production conditions[9,24] and higher than the rates observed in cows from intensive production systems (30-45 %) in México[25,26]. This difference is likely due to the lower production levels and nutritional demands of cows in family dairy production systems compared to those in intensive systems[27]. Additionally, it may be related to the fact that, in intensive production systems, artificial insemination is typically performed at a fixed time using ovulation synchronization protocols, whereas in the present study, inseminations were performed based on detected natural estrus, which has been linked to higher fertility rates[28].

The percentage of cows with CE in this study was in the lower end of the range observed in studies conducted in intensive production systems[21,29,30] and it was considerably lower than the prevalence reported in small-scale dairy farms (~70 %) in other countries[31]. Assisted calving and/or RFM have been identified as important risk factors for the development of CE[18,19,32]. In this study, the occurrence of CE was influenced by RFM but not by assisted calving, which could be due to the low frequency observed (4.8 %) and could have affected the statistical power of the analysis. Additionally, but particularly dystocia, the delay in the expulsion of fetal membranes, generates a favorable environment for the rapid growth of bacteria such as *Escherichia coli* and *Arcanobacterium pyogenes*[33], the primary microorganisms associated with postpartum uterine infections[34,35].

On the other hand, cows that showed CE had a higher risk of failing to establish pregnancy, consistent with the results from various studies[18,21,36]. It has been observed that cows showing CE have a lower 1SCR[18], a higher number of services per conception[36], and an increased probability of being culled from the herd for not becoming pregnant[21]. Postpartum uterine infections negatively impact reproductive performance by disrupting the hypothalamic-pituitary-ovarian-uterus reproductive axis[37]. For example, bacteria-associated molecules such as lipopolysaccharides can alter GnRH/LH production[38,39], reduce estradiol synthesis by granulosa cells[40,41], affect the mRNA profile in oocytes[42], and impair oocyte competence to develop into embryos[43]. Additionally, uterine infections induce tissue damage and trigger an inflammatory response, with perfusion of immune cells in the endometrium, which generates changes that affect its competence to establish and

maintain a pregnancy[37]. These results highlight the importance of preventive management in family dairy production systems to reduce the prevalence of uterine diseases and mitigate risk factors such as the RFM.

Conclusions and implications

Overall, the prevalence of assisted calving and RFM was lower than what is typically observed in the Mexican family dairy production farms. However, this study confirmed that RFM constitutes a risk factor for CE, and this uterine condition negatively impacts cows' reproductive performance in this production system. Further research with a larger sample size and including other high prevalence risk factors like inadequate body condition score of cows at calving is warranted to fully understand the influence of calving conditions on reproductive indicators in the Mexican family dairy production system.

Acknowledgments and conflicts of interest

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Article

Chemical composition and adaptation of tropical grass *Leersia hexandra* Sw. exposed to crude oil soil

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Abstract:

The purpose was to evaluate the chemical composition and adaptation potential of L. *hexandra* under crude oil stress conditions, through the rhizobacterial population, crude protein accumulation, neutral detergent fiber, acid detergent fiber, and lignin in foliage of young plants emerging from the main tillers of the plant at different growth ages (d 180 and 360), as well as the production of young plants in the tillering stage, and the aerial and root dry matter. The results showed that crude oil concentrations in the soil significantly affected the population of *Azotobacter* spp. (0.361*); however, those of *Azospirillum* spp. and *Pseudomonas* spp. were inhibited, while both populations increased with time extension (0.778*, 0.767*). Likewise, the synthesis of crude protein (0.551**) and lignin in the foliage

(0.354*) and the production of young plants in the tillering stage (0.465**), as well as of root dry matter (0.362*) were increased, indicating a strategy of *L. hexandra* to survive and adapt to soil contamination by crude oil. Nevertheless, the chemical composition was affected by the age of the grass, in which the percentage of neutral detergent fiber (0.832**), acid detergent fiber (0.741**), and lignin (0.661**) increased, while that of crude protein decreased (-0.497**).

Keywords: Rhizobacteria, Crude Protein, Lignin, Neutral Detergent Fiber.

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Introduction

Soils contaminated with crude oil have generated a toxic environment for plants and microbial activity in the rhizosphere of various plant species[1,2] due to petroleum's toxic, mutagenic, and carcinogenic nature[3]. Soil contamination with crude oil affects fertility in a negative way because crude oil decreases the availability of nutrients (nitrogen, phosphorus, and potassium), pH, and moisture content, and increases the electrical conductivity and organic carbon content of the soil[4] directly affecting plant growth and development[5]. In addition, the oil also forms a hydrophobic surface around the roots, which limits the absorption of water and nutrients from the soil[6]. In oiled soil, *Leersia hexandra* grows and produces forage[7]; it is a perennial grass that inhabits humid areas in tropical and subtropical regions; it propagates vegetatively by rhizomes, invading wetlands by the abundant accumulation of its foliage and roots on the soil and water[8] and is highly consumed by cattle in both dry and rainy seasons[9].

The rhizosphere is the soil-root interface, the volume of soil influenced by root activity[10]. Tropical grasses are characterized by an extended rhizosphere system due to the abundant fibrous root system that characterizes them, and have demonstrated adaptation to the stress conditions established in soils contaminated with crude oil[11]. Tropical grasses that have naturally evolved under stress conditions due to the accumulation of biogenic and petroleum carbon in the soil are able to mitigate stress by changing the chemical conditions in the rhizosphere and the proliferation of specific heterotrophic microorganisms[12,13]. The rhizobacteria are free-living heterotrophs associated with the plant root[14]; they stimulate

plant growth, provide plant tolerance to petroleum diesel stress[15], and are involved in the rhizodegradation processes of alkanes, cycloalkanes, and polycyclic aromatic hydrocarbons[16]. *Azotobacter*, *Azospirillum*, and *Pseudomonas* bacterial genera tolerate crude oil-induced stress[17,18]. Plant growth-promoting rhizobacteria that colonize the rhizosphere mineralize intermediate metabolites from the decomposition of organic carbon in petroleum[19], and the exoenzymes released by them reduce and oxidize nitrogen, phosphorus, and sulfate, but also fix nitrogen biologically[20]. The adaptation of grasses to crude oil soil suggests that it has a rhizosphere with roots that assimilate NO_3, H_2PO_4, and SO_4, and are used in an essential way in the primary metabolism inside the plant[21]. Grasses adapted to crude oil exposure are likely to respond in a similar manner to other plant species such as *Banksia seminuda* Rie., *Hakea prostrata* R. Br.[22], and *Secale cereal* L.[23] where abiotic stress modifies the development and expression of genes involved in cell synthesis in the roots and foliage[24]. The adaptation of plastids to stress by abiotic factors is based on the positive response of growth, production, and synthesis of secondary metabolites. In this regard, Orocio-Carrillo *et al*[13] report that the root and leaf protein content of *L. hexandra* has a hormetic response to doses of total petroleum hydrocarbons in the soil. Correa and Maranho[25] indicated that stem length, root biomass, and stomatal density increase in *Echinochloa polystachya* (Kunth) Hitchc. exposed to oil-containing soil; Habermann *et al*[26] indicate that water deficit stress and soil heating induced an increase in fiber and lignin content and reduced leaf protein in *Panicum maximum* Jacq; other authors[27,28] report that high temperatures lead to high detergent fiber and lignin content in the cell wall, and decreased protein synthesis in forage grasses. The objective of this study was to determine the rhizobacterial population, crude protein accumulation, neutral detergent fiber, acid detergent fiber, and lignin in the foliage, as well as the production of young *L. hexandra* plants in the tillering stage and in their aerial and root dry matter, to obtain a grass adapted to soils contaminated with crude oil for the Mexican humid tropics.

Material and methods

Soil and rhizome collection

Uncontaminated soil (Gleysol) was collected from the surface layer (0-30 cm) located in *Ejido* Blasillo 4th Section, Huimanguillo, Tabasco, Mexico (18° 05' 08.4" N and 93° 56' 50" W). The soil was dried under shade, ground, and sieved (5 mm mesh). The physical and chemical characteristics of the soil are shown in Table 1. *L. hexandra* rhizomes were collected from a wetland affected by a chronic oil spill, located two kilometers southwest of the "La

Venta" Gas Processing Complex. The cultivation of *L. hexandra* seedlings was similar to the procedure used by Orocio-Carrillo *et al*[13].

Table 1: Physical and chemical characteristics of soil and oil

Soil characteristics										
Texture	Sand	Silt	Clay	pH	OM	TN	SO_4^{2-}	PO_4-	EC	CEC
Loamy clayey sandy	45.2%	20.7%	34.1%	5.5	17.2%	0.66%	45 mg kg^{-1}	85 mg kg^{-1}	0.9 dS m^{-1}	9.6 cmol kg^{-1}
Characteristics of petroleum[a]										
API Gravity	Sulphur	Factions								
32°	1.8%	Saturated 61.2 %	Aromatic 24.8 %	Asphaltenes + Resins 14 %						

OM= organic matter; TN= total nitrogen; EC= electrical conductivity; CEC= cation exchange capacity.
[a]Hydrocarbon fractions using the Soxhlet and gravimetric methods.

Soil contamination and experimental design

32° API crude oil (CO) was obtained from the Ogarrio Field, Battery 2, in Huimanguillo, Tabasco, Mexico. The experiment was carried out with a completely randomized design and a 4x2 factorial arrangement: four concentrations of CO [0 (control), 30, 60, and 90 g kg^{-1} DW (dry weight)] and two exposure times of *L. hexandra* to CO= (d 180 and 360). A total of eight treatments with four replicates maintained at random locations in a microtunnel with an average temperature of 29 ± 6 °C and humidity at field capacity of 32 ± 5 %. The experimental unit was a plastic container with 4 kg of dry soil and one *L. hexandra* plant.

Rhizobacterial population

Azospirillum bacteria were grown on Congo red agar[29], *Azotobacter* bacteria on Asby agar(29), and *Pseudomonas* bacteria on cetrimide + glycerol agar[30]. Cultures were incubated at 28 °C for 72 h, and counts were expressed as colony forming units (CFU) per gram of soil.

Chemical composition of *L. hexandra*

Destructive sampling of plant tissue (leaves and stems) from young plants emerging from the main plant of *L. hexandra* that were 180 and 360 days old was performed, dried in a forced air oven at 60 °C for 72 h and ground for crude protein, neutral detergent fiber, acid detergent fiber, and lignin analysis[31,32].

Production analysis

The evaluation of young plants in the tillering stage, aerial dry matter, and root dry matter was similar to the procedure used by Orocio-Carrillo *et al*[33].

Statistical analyses

The data collected for all variables were subjected to an analysis of variance and a multiple comparison test of means with Tukey's method ($P \leq 0.05$), as well as to Pearson's bivariate correlation, using the SAS v.9.4 statistical software[34].

Results

Rhizobacterial population

Table 2 shows the changes in the mean values of the three groups of rhizobacteria due to the effect of crude oil on the soil and to the exposure time. The highest density of *Azotobacter* [438 and 132 x 103 CFU g^{-1} dry rhizosphere (d.r.)] was found in soil with 90 g kg^{-1} of crude oil, where it was 106.6 % and 40.4 % higher than the control at d 180 and 360 respectively. However, the densities of *Azospirillum* and *Pseudomonas* decreased. In general, the effect of crude oil contamination increases in the population of *Azotobacter* (81.7 %) and decreases in those of *Azospirillum* and *Pseudomonas* by 36 and 47.7 %, respectively. However, the effect of the time of evaluation induced a positive response in the density of *Azospirillum* and *Pseudomonas*, being 1.9 and 23.9 times higher at d 360 than at d 180.

Table 2: Changes in *Azospirillum*, *Azotobacter*, and *Pseudomonas* in the rhizosphere of *L. hexandra* exposed to crude oil at 180 and 360 days

Time/Crude oil (g kg^{-1})	*Azospirillum*	%	*Azotobacter*	%	*Pseudomonas*	%
	10^3 UFC g^{-1} r.s.				10^1 UFC g^{-1} r.s.	
180 days						
0	84 ^{c}		212 ^{c}		95 ^{c}	
30	67 ^{d}	−20.2	372 ^{b}	+75.5	58 ^{c}	−38.9
60	53 ^{e}	−36.9	482 ^{a}	+127.3	34 ^{c}	−64.2
90	21 ^{f}	−75.0	438 ^{a}	+106.6	74 ^{c}	−22.1
360 days						
0	144 ^{a}		94 ^{e}		2,427 ^{a}	
30	101 ^{b}	−29.9	85 ^{e}	−9.6	2,335 ^{a}	−3.8
60	102 ^{b}	−29.2	159 ^{d}	+69.1	572 ^{bc}	−76.4
90	95b ^{c}	−34.0	132 ^{de}	+40.4	889 ^{b}	−63.4
Contamination						
Without	114*		153		1,261*	
With	73	−36	278*	+81.7	660	−47.7
Time (days)						
180	56		376*		65	
360	110*		117		1,556*	

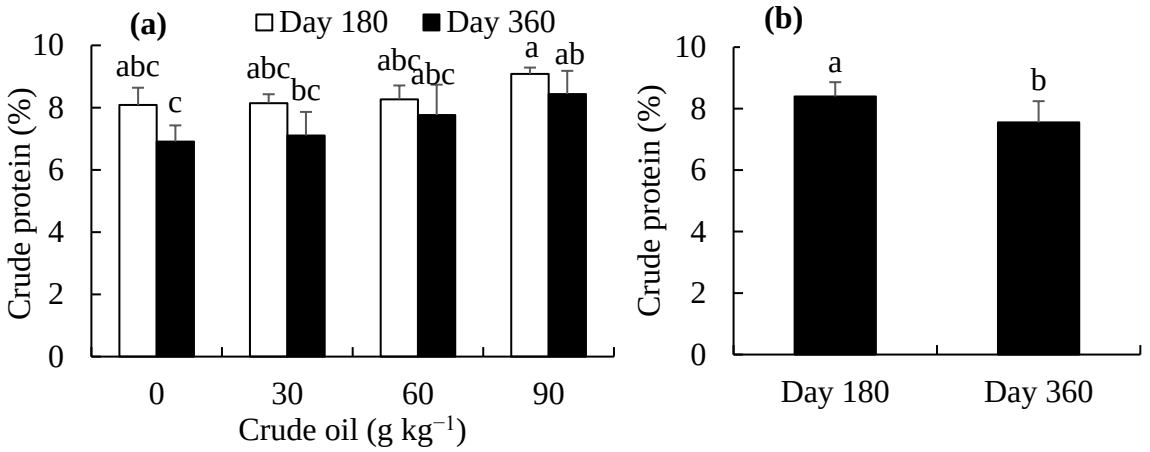
The symbol % (+) represents an increase, and % (–), a decrease of *Azospirillum*, *Azotobacter*, and *Pseudomonas* at 180 and 360 d with respect to the values of the control treatment (0 g kg^{-1} crude oil). ^{abcde} Different lowercase letters within a column indicate statistically different values ($P \leq 0.05$, n=4). * Statistically higher.

Chemical composition of *L. hexandra*

Figure 1 shows the tendency in crude protein content in the aerial dry matter of young *L. hexandra* plants by effect of crude oil dosage and exposure time. The highest crude protein content at d 180 (9.1 %) and 360 (8.4 %) was recorded at the 90 g kg^{-1} crude oil concentration, being 1 and 1.5 % higher than that of the control (Figure 1a). On the other hand, the

evaluation time decreased from 8.4 to 7.5 % as the age of the plant increased from 180 to 360 d (Figure 1b).

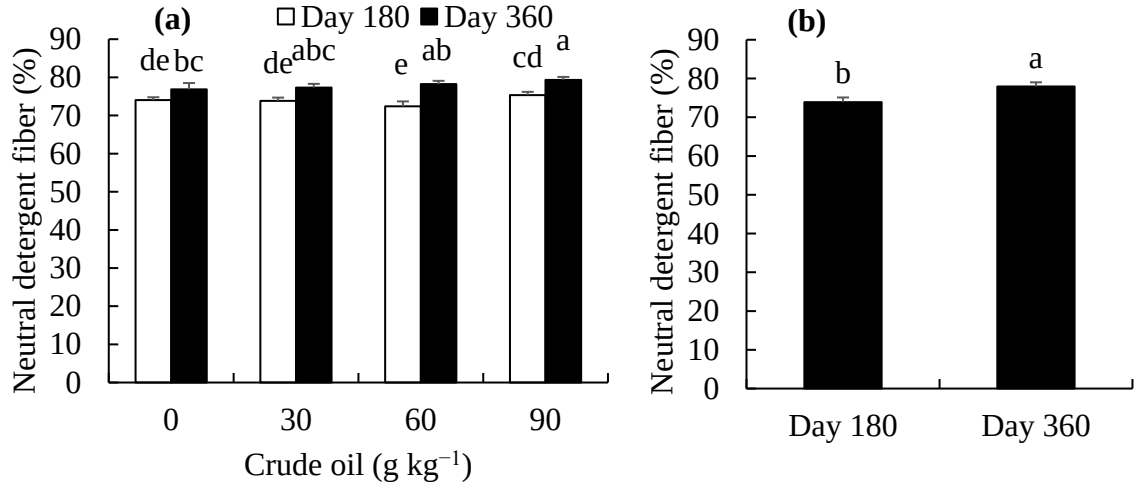
Figure 1: Effect of the crude oil in the soil (a) and exposure time (b) on the crude protein percentage in *L. hexandra*



[abcd] Different letters indicate different values ($P \leq 0.05$, n=4).

Figure 2 shows the performance in neutral detergent fiber. Significant differences were observed according to the oil dose and the evaluation time ($P \leq 0.05$). The dose of 90 g kg^{-1} of crude oil induced the highest neutral detergent fiber content at d 180 (75.3 %) and 360 (79.3 %), reaching an increase of 1.3 and 2.5 % over that of the control (Figure 2a). As for the evaluation time, it increased from 73.9 to 77.9 % as plant maturity increased from 180 to 360 d (Figure 2b).

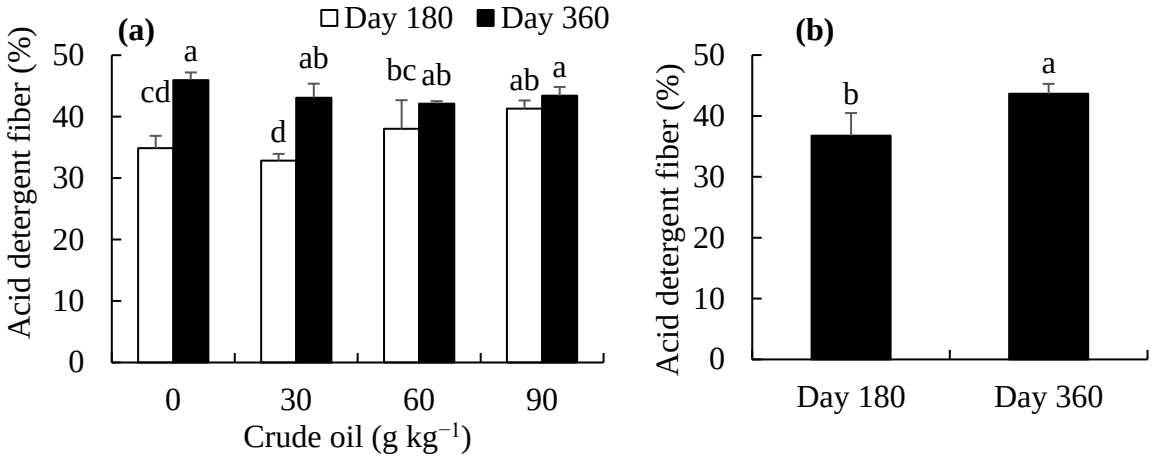
Figure 2: Effect of crude oil in the soil (a) and the exposure time on the neutral detergent fiber in *L. hexandra*



[abcd] Different letters indicate different values ($P \leq 0.05$, n=4).

Figure 3 shows the behavior in the content of acid detergent fiber. At day 180, the dose of crude oil was observed to stimulate the acid detergent fiber, which was higher (41.3 %) in soil with 90 g kg^{-1} of crude oil, increasing 6.4 % over that of the control (Figure 3a). At day 360, crude oil concentrations showed a statistically similar response to the control. As for the evaluation time, the acid detergent fiber was stimulated to increase from 36.8 to 43.6 % at day 180 and 360, respectively (Figure 3b).

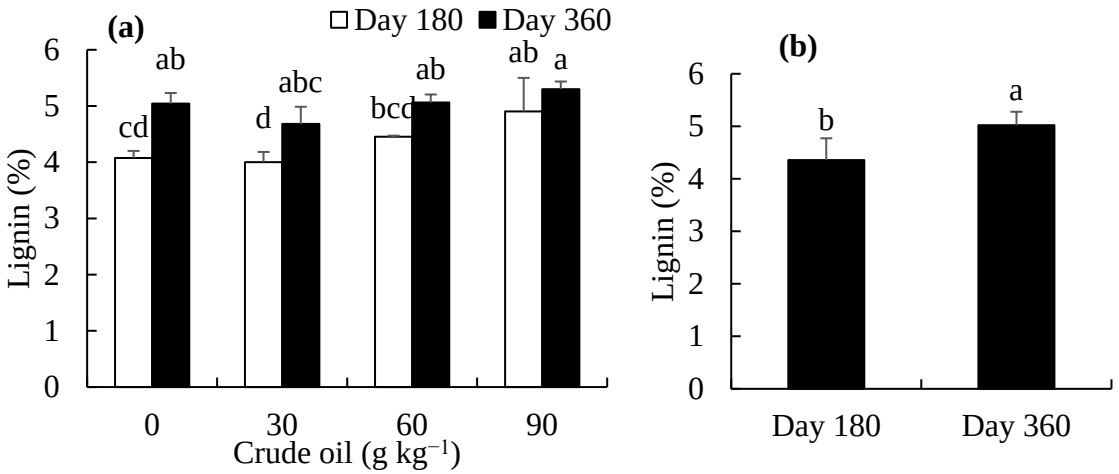
Figure 3: Effect of the crude oil on the soil (a) and time of exposure (b) on the acid detergent fiber in *L. hexandra*



[abcd] Different letters indicate different values ($P \leq 0.05$, n=4).

The effect of crude oil on the lignin content in *L. hexandra* harvested at different ages showed significant differences ($P \leq 0.05$) (Figure 4). At d 180, it is observed that high concentrations of oil stimulate lignin, being higher (4.9 %) in soil with 90 g kg^{-1} of crude oil, increasing 1 % with respect to the control (Figure 4a). On the other hand, the effect of evaluation time induced a positive response, reaching an increase of 4.3 to 5 % as plant age advanced (Figure 4b).

Figure 4: Effect of crude oil on the soil (a) and exposure time (b) on the percentage of lignin in *L. hexandra*



[abcd] Different letters indicate different values ($P \leq 0.05$, n=4).

L. hexandra production

The effects of crude oil doses and evaluation time on the means of young *L. hexandra* plants in the tillering stage and their aerial dry matter and root dry matter exhibited significant differences ($P<0.05$) (Table 3). Exposure to 90 g kg^{-1} of crude oil promoted an increase of up to 300 and 89.3 % of young plants in the tillering stage compared to the control soil at d 180 and 360 respectively. The results for root dry matter increased up to 203.7 and 169.7 % at d 180 and 360, respectively, in the most contaminated soil, with respect to the control soil. However, the aerial dry matter production of the grass decreased up to 25.9 and 18.9 % at day 180 and 360 with the highest dose. In general, the effect of crude oil contamination on young plants in the tillering stage increases by 78.6 % and radical dry matter 135.1 %. However, it decreased 13.4 % in the aerial dry matter, although this reduction was statistically equal to the control soil. The effect of the evaluation time induced a positive response in young *L. hexandra* plants in the tillering stage and the aerial and root dry matter, which were 210.8, 378.7, and 545 % higher at d 360 than at d 180.

Table 3: Changes in young plants in the tillering stage, the aerial dry matter and the root dry matter of *L. hexandra* exposed to crude oil at 180 and 360 days

Time/ Crude oil (g kg^{-1})	Young plants in tillering stage	%	Aerial dry matter (g)	%	Root dry matter (g)	%
180 days						
0	27±2.4 ^{f}		43.5±2.3 ^{d}		2.7±0.2 ^{d}	
30	52±5.1 ^{e}	+92.6	42.2±2.5 ^{d}	−2.9	6.2±0.3 ^{d}	+129.6
60	71±12.1 ^{e}	+163	36.2±4.2 ^{d}	−16.8	7.0±1.4 ^{d}	+159.3
90	108±4.2 ^{d}	+300	32.2±5.3 ^{d}	−25.9	8.2±0.3 ^{d}	+203.7
360 days						
0	140±4.3 ^{c}		204.2±8.1 ^{a}		19.5±2.1 ^{c}	
30	191±6.9 ^{b}	+36.4	188.6±9.3 ^{b}	−7.6	39.2±3.8 ^{b}	+101
60	210±8.7 ^{b}	+50	178.7±4.9 ^{bc}	−12.5	43.5±5.3 ^{b}	+123.1
90	265±17.8 ^{a}	+89.3	165.6±7.7^{c}	−18.9	52.6±2.8 ^{a}	+169.7
Contamination						
Without	84		123.9*		11.1	
With	150*	+78.6	107.3*	−13.4	26.1*	+135.1
Time (days)						
180	65		38.5		6.0	
360	202*	+210.8	184.3*	+378.7	38.7*	+545

The symbol % (+) represents an increase, and % (-) a decrease, of the aerial dry matter and root dry matter of young plants at d 180 and 360, compared to the values of the control treatment (0 g kg^{-1} of crude oil). ^{abcde} Different lowercase letters within each column represent different values ($P \leq 0.05$, n=4). *Statistically higher.

Correlation of variables are presented in Table 4.

Discussion

Rhizobacterial population

Reports indicate that the rhizosphere of grasses used for the removal of total petroleum hydrocarbons from soils contaminated with crude oil host intense microbial activity, including that of plant growth-promoting rhizobacteria[35,36]. The significant increase in the *Azotobacter* population (0.361*) (Table 2 and 4) in the rhizosphere of *L. hexandra* is similar

to that reported in other studies evaluating the same grass exposed to 60-180 g kg^{-1} of total petroleum hydrocarbons[20] and *Echinochloa polystachya* K. exposed to 65.89 g kg^{-1} of total petroleum hydrocarbons[37]. The increase in the *Azotobacter* population could be due to the adaptation of the bacterium through the secretion of extracellular enzymes essential for the initial degradation of high molecular weight substrates[38] such as petroleum hydrocarbons. Similarly, it may be an adaptation to a reduction in the availability of essential nutrients such as nitrogen due to the properties of crude oil, which tends to agglomerate in the soil[4;5]. In addition, it has been shown that under stress conditions plants increase root exudation[39], which become a source of nutrients and stimulating substances for the growth of microorganisms[40]. However, not all microorganisms can adapt quickly, probably because of the toxic hydrocarbons in crude oil, which make it difficult for some microbial species to grow and survive[41]. In addition, the presence of oil can modify the physicochemical properties of the soil[4], generating unfavorable conditions for microorganisms[42]. In contrast, the current study revealed that the population of *Azospirillum* and *Pseudomonas* decreased with increased crude oil, demonstrating the negative effects of this pollutant; at the same time, with the extension of time, the stimulation of both populations was promoted (0.778**, 0.767**), indicating that the bacteria need some time to acclimatize and achieve significant growth. Similar results were reported in other investigations[1,7], where they found a significant increase of rhizobacteria over time in the rhizosphere of *L. hexandra* and *Urochloa brizantha* Hochst exposed to oil.

Chemical composition of *L. hexandra*

The present study showed increases in crude protein content in shoots of *L. hexandra* planted in soil with crude oil (0.551**) relative to the control. At d 180 and 360 at doses of 60 and 90 g kg^{-1} of crude oil, the tendency is for the crude protein to increase with respect to the control, while at doses of 30 g kg^{-1} there was no statistical difference (Figure 1a). A similar effect was reported by Orocio-Carrillo *et al*[13], who found an increase in crude protein in *L. hexandra* exposed to 102 g kg^{-1} of crude oil. Likewise, the protein content in *Simmodsia chinensis* L. and *Vigna unguiculata* L. respectively has reportedly[43,44] increased due to the effect of crude oil in soil. Roa *et al*[45] report an increase in protein concentration in *Triticum aestivum* L. due to the effect of sulfur fertilization. Therefore, the increase in protein in this study could be due to the nitrogen and sulfur content present in the crude oil[46]. On the other hand, crude protein is diluted as the age of the grass increases (−0.497**). It has been mentioned[47] that the increase in the age of the grass results in a decrease in protein, as dry matter production increases. In this regard, this study found a negative and highly significant relationship between crude protein and aerial dry matter (−0.564**). As the maturity stage

of grasses increases, so does the content of structural carbohydrates and lignin, while the protein content decreases[48].

This study shows that the neutral detergent fiber at d 180 did not change in contaminated soils with respect to the control; however, at d 360, the tendency was to increase very slightly with high doses of crude oil (60 and 90 g kg^{-1}), but at 30 g kg^{-1} there was no statistical difference compared to the control. On the other hand, neutral detergent fiber increased due to the effect of the evaluation time (0.832**). Similar tendencies were reported by other researchers[49,50], who evaluated the percentages of neutral detergent fiber of fountaingrass at different harvesting ages and observed an increase in its concentration of 5.5 and 17.9 % between day 30 and 167 d, respectively. It has also been mentioned[51] that, as forage maturity increases, the concentrations of neutral detergent fiber in stems and leaves augment, reducing the voluntary consumption of forages.

The concentration of acid detergent fiber in this study shows that it increased at high doses of oil (60 and 90 g kg^{-1}) with respect to the control at d 180, but at doses of 30 g kg^{-1} the response was not statistically different from that of the control. At d 360, the tendency was to decrease at doses of 60 and 90 g kg^{-1}, while at doses of 30 g kg^{-1} there were no statistical differences in relation to the control. On the other hand, the concentration of acid detergent fiber augmented as the age of the grass increased (0.741**). Similar values were reported by Schnellmann *et al*[52] when evaluating the nutritional quality of *Megathyrsus maximus* Jacq., as they recorded values of 29.3 % at d 90 and 34.4 % at d 180. Similarly, Álvarez-Vázquez *et al*[50] found an increase of 40.36 % at d 33 and 58.5 % at d 180 when evaluating the chemical composition of *Cenchrus* sp. grass. Acid detergent fiber is reported to be an important component that regulates forage quality and is positively related to the crop's age or stage of development, with forage quality declining as fiber becomes a predominant component[53].

In the present study, one explanation for the increase in high doses of crude oil may be the mechanism of osmotic adjustment that favors the accumulation of compatible solutes, which are organic compounds that do not interfere with cell metabolism, even at high concentrations, and can act as antioxidants to minimize the impact of abiotic stress on the plant[24]. Several studies have shown that lignin increases in response to various environmental stresses[26,27], playing a role in the adaptation of plants to their environment[54]. On the other hand, the lignin content is stimulated (0.661**) as the age of maturity of the plant increases. Rosales and Pinzón[48] mentioned that, as the grass maturity stage increases, the proportion of cell wall components, including lignin, increases, reducing the digestibility of the grass.

L. hexandra production

In pastures, the addition of crude oil to the soil has been reported to induce a significant reduction of plant dry matter[55]. It should be noted that, at d 180, aerial dry matter production was reduced only by 2.9 % at a concentration of 30 g kg^{-1} of crude oil, compared to the control, demonstrating the high tolerance of the grass in these conditions; however, as the dose of crude oil in the soil increases (60 and 90 g kg^{-1}), the percentage reduction increases both at d 180 and at d 360 with respect to the control. The negative impact of crude oil on the aerial dry matter of *L. hexandra* has also been reported[13,56]. Crude oil leads to negative changes in soil properties such as reduced moisture absorption and retention capacity, water repellency and insufficient soil aeration[57,58], which consequently makes plant growth even more difficult. On the other hand, the production of young plants in the tillering stage and root dry matter were stimulated (0.465**, 0.362*). This behavior was also mentioned in other researches[13,56]. The increase in these variables may indicate a strategy of *L. hexandra* to survive and adapt to soil contamination by crude oil. The increase of young plants in the tillering stage and root dry matter in oiled soils could be explained by the increase in soil organic carbon content due to the degradation of crude oil[18]. Likewise, the increase in roots could be due to a response of reinforcing the root tissues in order to limit soil nutrient deficiency stress induced by crude oil[5]. Studies have reported increased root biomass in soils treated with petroleum hydrocarbons[22].

Conclusions and implications

The effect of high doses of crude oil in the soil induces in the rhizosphere of *L. hexandra* high populations of bacteria of the *Azotobacter* group; however, it caused the inhibition of *Azospirillum* and *Pseudomonas*. On the other hand, crude oil is positively correlated with crude protein and lignin synthesis, playing a role in grass adaptation to its environment. Likewise, high doses of crude oil induced a positive response in the production of young plants in the tillering stage and root dry matter, which indicates a strategy developed by the grass to survive and adapt to oil contamination. However, the chemical composition of the grass was affected by the age of harvest, in which the percentage of neutral detergent fiber, acid detergent fiber, and lignin increased, while the concentration of crude protein decreased. Therefore, even if the grass is able to adapt to the stress induced by crude oil, its protein content and, therefore, its nutritional value are affected, which can be detrimental to farmers and cause long-term deterioration of the affected property.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 4: Correlation of variables

Parameter	Time	PJM	ADM	RDM	CP	NDF	ADF	Lignin	AZP	AZT	PSE
CO	NS	0.465**	NS	0.362*	0.551**	NS	NS	0.354*	−0.558**	0.361*	−0.372*
Time		0.872**	0.987**	0.876**	−0.497**	0.832**	0.741**	0.661**	0.778**	−0.857**	0.767**
PJM			0.798**	0.966**	NS	0.860**	0.690**	0.739**	0.416*	−0.614**	0.485**
ADM				0.802**	−0.564**	0.789**	0.737**	0.632**	0.826**	−0.879**	0.829**
RDM					NS	0.828**	0.581**	0.656**	0.474**	−0.678**	0.454**
CP						NS	NS	NS	−0.627**	0.519**	−0.641**
NDF							0.646**	0.668**	0.520**	−0.740**	0.576**
ADF								0.635**	0.466**	−0.530**	0.667**
Lignin									NS	−0.475**	0.369*
AZP										−0.861**	0.743**
AZT											−0.745**

CO= crude oil; PJM= young plants in the tillering stage; ADM= aerial dry matter; RDM= root dry matter; CP= crude protein; NDF= neutral detergent fiber; ADF= acid detergent fiber; AZP= *Azospirillum*; AZT= *Azotobacter*; PSE= *Pseudomonas*.

* $P \leq 0.05$. ** $P \leq 0.01$. NS= non significant.

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Article

Forage preferences of bighorn sheep (*Ovis canadensis*, Shaw) in Baja California, Mexico

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Abstract:

This research aimed to analyze the food composition and preferences of bighorn sheep in Sierra Juárez and Sierra Santa Isabel during the wet and dry seasons of 2022-2023. To assess forage availability, 17 100-meter-long Canfield lines were implemented. Dietary composition was determined by micro histology of fecal samples, whereas forage and diet diversity were calculated using Shannon's index. Forage selection was evaluated with Ivlev's

index. It was observed that the most common biological forms in the habitat of bighorn sheep were trees and shrubs. In Sierra Juarez and Sierra Santa Isabel, 31 and 43 species of plants were identified in their diet, respectively; trees and shrubs were the most consumed. There were no differences in diet between times and sites. *Larrea tridentata* and *Hibiscus denudatus* were the most frequent in the diet, whereas the preferred ones included *Eriogonum inflatum*, *H. denudatus*, *Horsfordia newberryi*, *Justicia californica*, and *L. tridentata*. These results provide information to establish strategies for conservation and community management of bighorn sheep in Baja California.

Keywords: Wild sheep, Cordillera Molina, Matomí, Desert scrubland, Sierra Santa Isabel, Sierra Juárez.

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Introduction

The desert bighorn sheep (*Ovis canadensis*) is one of two wild sheep species with a natural distribution in North America[1]. It is currently found in the wild in the arid mountainous regions of the southwestern United States of America and northwestern Mexico[2]. Nevertheless, until the second half of the nineteenth century, its natural distribution area extended to the northeastern region of Mexico and included part of the states of Chihuahua, Coahuila, and Nuevo León[3]. This population decrease is due to habitat degradation, poaching, and disease transmission by domestic livestock[1,2]. As a result, the Mexican legislature has classified this species as subject to Special Protection (Pr) since 2010[4].

The bighorn sheep plays an important ecological role by directly influencing vegetation dynamics[5,6] and the nutrient cycle of the ecosystem[7]. Therefore, conservation efforts have focused on preserving its natural habitat to promote the development and establishment of its populations[8]. To achieve this objective, it is necessary to understand the use it makes of different types of forage, which allows to understand its adaptability capacity to variations in its availability and quality[9]. Likewise, knowledge about the diet also allows the identification of the key foraging areas of the species, which is important information for its conservation[8,9,10].

Several studies have been carried out in North America on the feeding habits of the bighorn sheep[8,11,12], in which it is described as an opportunistic consumer with a preference for shrubs and herbs depending on their availability[6,8,9]. However, in the Baja California

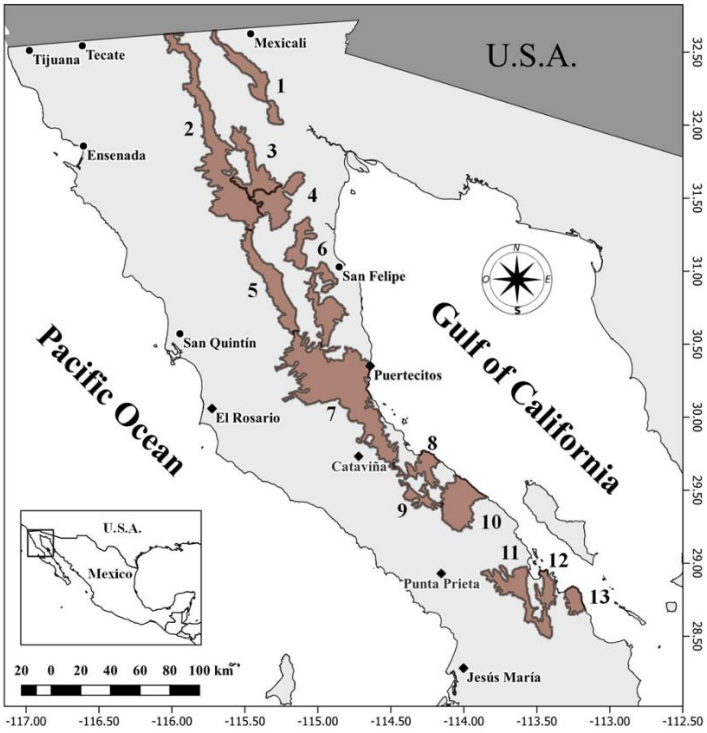
peninsula, its forage habits have been little studied and most studies are based on direct observation or analysis of stomach contents[13], which are not representative of total food consumption, but are valuable because they indicate a significant consumption of grasses in a highly arid region. Therefore, the present study aimed to identify and compare the composition of the diet of bighorn sheep through microhistological analyses in two mountain systems of Baja California, Mexico.

Material and methods

Description of the study area

The study was conducted from November 2021 to November 2022, in Sierra Juarez and Santa Isabel mountain ranges, in the northern and central regions of the state of Baja California, respectively (Figure 1). These areas represent a continuous habitat for bighorn sheep, as they include rugged topography, canyons, and steep slopes[14,15]. Sierra Juarez has an area of 42,364 ha and is located between the cities of Tecate and Mexicali; Santa Isabel covers an area of 65,961 ha on the coast of the Gulf of California, 90 km south of the port city of San Felipe[15]. These areas belong to the San Felipe desert ecoregion, where microphyllous desert scrubland predominates, characterized by plant species such as creosote bush (*Larrea tridentata*), white bursage (*Ambrosia dumosa*), buggywhip (*Fouquieria splendens*), desert agave (*Agave deserti*), and ironwood (*Olneya tesota*)[16]. The average annual temperature ranges between 12 and 22 °C. The average monthly rainfall ranges between 0.0 mm and 0.8 mm and occurs throughout the year; nevertheless, the winter months are the wettest[17].

Figure 1: Main mountain systems in the state of Baja California, Mexico (2. Sierra Juarez; 7. Sierra Santa Isabel)



Forage availability assessment

Forage availability was assessed by the frequency of each plant species by site and season of the year (dry and wet). To do this, the line intercept or Canfield method was used. To reduce the error and increase the representation of the sampling, an accumulation curve was generated to estimate the number of potential species in each ejido according to the Jackknife[18] and Chao 2[19] estimators, using the statistical package EstimateS V. 9.1.0. In total, the evaluation was carried out on 17 lines of 100 meters in length according to the accumulation curve: in Sierra Juarez, four lines were established during the dry season (May and August 2022) and four during the wet season (November 2022); and in Sierra Santa Isabel, four lines were placed in the dry season (April and November 2022) and five in the wet season (June and January 2022). In each one, all the plants that intersected the line were identified, counted, and classified according to their species, linear cover, height, and biological form (trees, shrubs, herbs, grasses, and succulents)[20]. The distribution of the lines was determined based on the information provided by the members of each ejido on the plant species consumed by the bighorn sheep, the identification of areas with topography associated with the presence of the bighorn sheep, and the direct observations generated during the prospecting tours carried out by the team of the Wildlife Management and Conservation Laboratory of the Autonomous University of Baja California. The data

collected made it possible to calculate the frequency of availability by plant species, study site, and season of the year using the following equation:

$$Availability\ of\ species\ i\ (\%) = \left(\frac{No.\ of\ lines\ that\ contain\ species\ i}{Total\ number\ of\ lines\ in\ the\ site}\right)\ x\ 100$$

Forage composition, diversity and selection

To identify the species that constitute the diet of bighorn sheep, the microhistological technique was used, which involves the identification of patterns of cellular structures of the plant epidermis in fecal samples[21]. A reference catalogue consisting of a collection of photographs of plant cell structures was created. To do this, plant samples including flowers, leaves, and stems were collected in the same places where the fecal samples were obtained. The collected plants were pressed and then taxonomically identified with the help of the herbarium collection of the Autonomous University of Baja California (BCMEX), the Baja California plant guide[22], the Naturalista platform, and consultations with experts. To prepare the reference catalog, the plants were processed in a Wiley mill, model Thomas tp4276 m004, with a mesh size 20 (1 mm); they were then rinsed with 20 mL of 5 % sodium hypochlorite (commercial chlorine) and mounted on slides. The slides were observed under a high-end digital microscope with a 10X objective to identify and photograph diagnostic cellular structures: trichomes, stomata, crystals, epidermis arrangement, etc.[21]

One hundred ninety-five (195) fresh fecal samples were randomly collected in both mountain ranges during the dry and wet seasons according to the methodology suggested by Anthony and Smith[23]. Prior to histological analysis of fecal samples, five subsamples were randomly taken at each site and sampling season to form composite samples. The composite samples were rinsed using the same procedure used for the plant samples and distributed on five slides per season and per site (20 in total). To ensure the homogeneity of the sample quantity in each slide, a metal slide with holes of 7 mm diameter was used[21,24]. In each slide, cell structures were identified and counted in 20 fields (400 in total) under a microscope. Finally, the identified species were classified according to their temporal variation (rainy or dry), growth form (shrub, tree, herb, grass, or succulent), and frequency of appearance[25,21]. These analyses were carried out in the Department of Zootechnics of the Autonomous University of Baja California Sur.

Data analysis

The composition of the diet was expressed in a matrix where the frequencies of each plant species were included according to the biological form, the time of year, and the site[26]. In

addition, the diversity of the diet by site and season of the year was evaluated with Shannon's[27] diversity index. To find out if there is a difference in diet diversity by time of year at each study site, the non-parametric Mann-Whitney U test ($\alpha \leq 0.05$) was applied using the statistical software PAST 4.0. The degree of selectivity of forage intake (FSI) was determined based on the availability and consumption of each plant species according to Ivlev's [28] selection index, using the following equation:

$$Ei = \frac{[r(i) - p(i)]}{[r(i) + p(i)]}$$

Where: *Ei* is Ivlev's selection index; *r(i)* is the relative frequency of species *i* in the diet; and *p(i)* is the relative frequency of species *i* in the habitat.

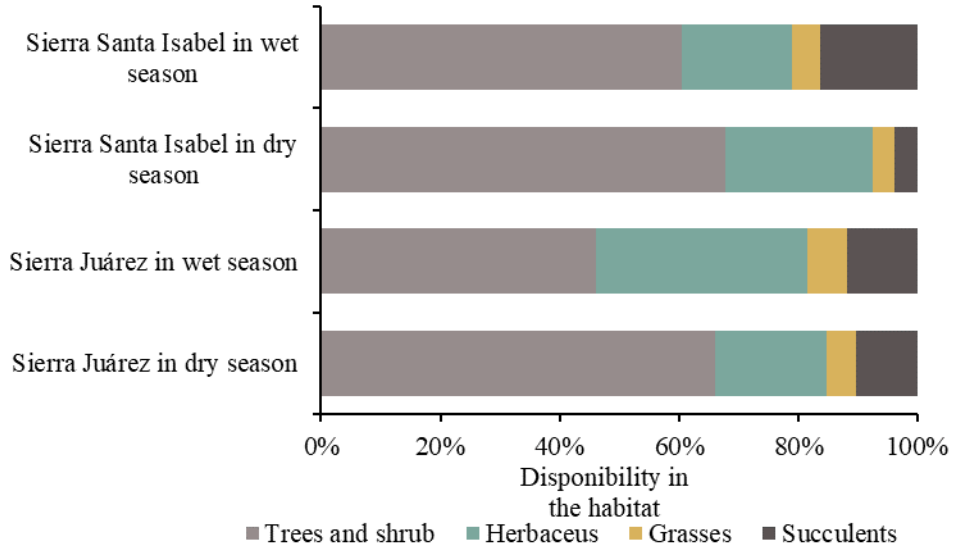
Ivlev's selection values range from -1 (rejection or negative selection of a species) to 1 (preference or positive selection), whereas a value of 0 implies random food consumption or in proportion to its availability. In this regard, Stuth[29] categorizes the values of this index as follows: species with an FSI greater than 0.35 were preferred over other available species; from -0.35 to 0.35, maintenance species or species consumed in proportion to their availability; finally, species avoided with an FSI less than -0.35.

Results and discussion

Vegetation cover assessment

In Sierra Juarez and Sierra Santa Isabel, the habitat of bighorn sheep was characterized by a high availability of tree and shrub species, and a lower frequency of grasses (Figure 2). Species availability varied by site and time of year ($P<0.05$). In Sierra Juarez, 52 species belonging to 23 taxonomic families were identified, the main ones were: *Asteraceae* (7), *Cactaceae* (5), *Fabaceae* (5), and *Asparagaceae* (4). The diversity of plant species in this mountain range was greater in the wet season ($H'= 3.69$) than in the dry season ($H'= 3.43$). In Santa Isabel, 55 species corresponding to 21 taxonomic families were identified, of which *Asteraceae* (9), *Cactaceae* (6), *Fabaceae* (6), and *Euphorbiaceae* (5) were the most common. Plant diversity in Sierra Santa Isabel in the wet season ($H'= 3.89$) was higher than in the dry season ($H'= 3.55$). Maintaining a high diversity of forage in the sheep's habitat is important because there is no single species that covers all nutritional requirements[27].

Figure 2: Seasonal variation in forage availability (%) in relation to site, time of year, and biological form of plant growth

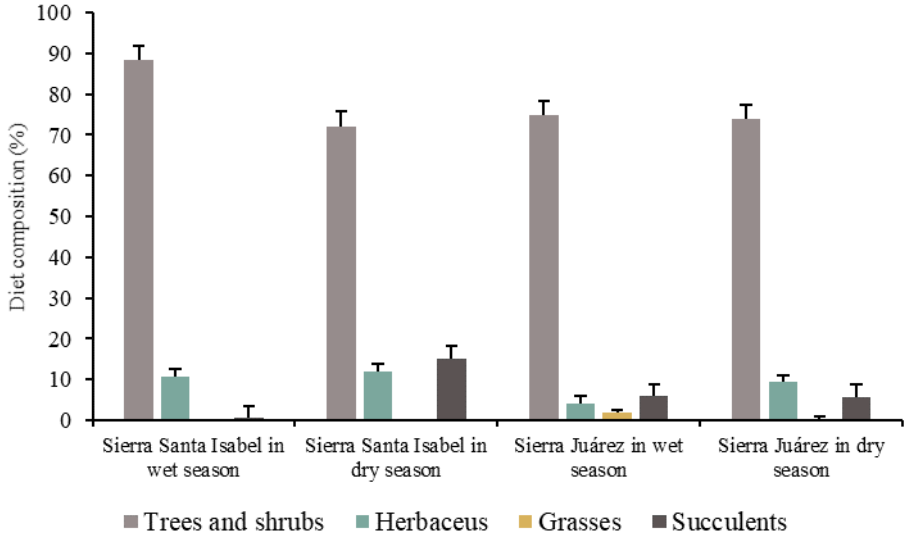


Fifty-two species were recorded in Sierra Juarez, but the Chao 2 and Jacknife diversity estimators predict a richness of 63 species (82.9 % effectiveness) and 69 species (75.7 % effectiveness), respectively. In Sierra Santa Isabel, 55 plant species were identified, but the Chao 2 estimator calculates a potential richness of 66 species (88.5 % effectiveness), whereas 70 species (78.4 % effectiveness) are estimated with the Jacknife estimator.

Diet composition and diversity

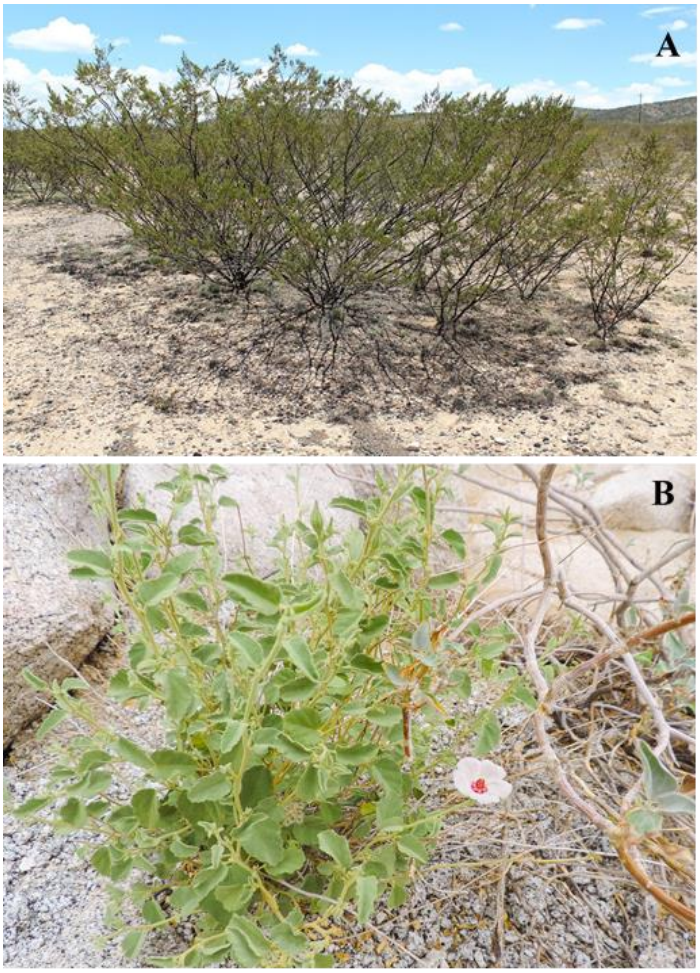
Wild herbivores are adapted to the consumption of a wide variety of forage types, depending on environmental gradients that influence food availability[30]. In this regard, 31 species that make up the diet of the bighorn sheep population in Sierra Juarez were recorded. Twenty-five (25) species were identified during the dry season, whereas the consumption amounted to 27 species in the wet season (Table 1). The biological forms with the highest consumption were trees and shrubs, with a frequency of consumption of 74.7 % and 73.8 % in the wet and dry seasons, respectively. Herbaceous plants represented 4.11 % of the diet in the wet season and 9.39 % in the dry season. The contribution of grasses to the diet was higher during the wet season (1.77 %), whereas that of succulents was more notable in the dry season (5.64 %; Figure 3). Although Shannon's diversity index showed higher values during the wet season (*H'*= 3.05) than during the dry season (*H'*= 2.97), no differences were found in dietary diversity between the two times of the year.

Figure 3: Composition of the bighorn sheep diet in relation to the site, time of year, and biological form of the forage (vertical lines on the bars indicate the standard error)



The bighorn sheep population in Sierra Santa Isabel fed on 43 species. The diet consisted of 36 species during the dry season, and the consumption was 29 species in the wet season. Tree and shrub species predominated in the diet during the two seasons of the year: 88.22 % in the wet season and 72.12 % in the dry season, respectively. In addition, 10.76 % were herbaceous species in the wet season and 12.12 % in the dry season. A difference of 15.15 % ($P<0.05$) was observed in the consumption of succulents between the wet and dry seasons (Figure 3). Shannon's diversity index showed similar values in the composition of the diet in the wet ($H'= 3.28$) and dry ($H'= 3.14$) seasons; this is mainly due to the fact that the highest percentage of consumption corresponds to perennial species. In both study sites, the species with the highest frequency in the diet were *Hibiscus denudatus* and *L. tridentata* (Figure 4).

Figure 4: Most frequent species in the diet of bighorn sheep in Sierra Juarez and Sierra Santa Isabel



A. Creosote bush (*Larrea tridentata*), of the family Zygophyllaceae (photograph by National Forestry Commission); B. paleface (*Hibiscus denudatus*), of the family Malvaceae (photograph by James Varnell). Both are species of perennial shrubs native to Mexico.

Studies on the feeding habits of bighorn sheep in North America reveal a high diversity of species in the diet of these animals. Monson and Sumner[31] point out that up to 110 species of plants have been identified in the diet of bighorn sheep in desert areas. In the United States of America, specifically in Arizona[32], they reported the presence of 58 species of plants in the diet, whereas in California[33], they found that sheep fed on 32 different species. In regions of the northern United States and southern Canada, it has been documented that the feeding of Rocky Mountain bighorn sheep (*O. c. canadensis*) comprises up to 200 plant species[33,34].

Sierra Juarez showed the lowest number of species in the diet of bighorn sheep compared to other studies carried out in the Sonoran Desert. In Sonora, 41 species were identified in Sierra El Viejo, Caborca[5]; another study[9] found 40 species in Sierra Noche Buena, Hermosillo; and O'Farril *et al*[35] reported 39 species of plants in the diet of the sheep from Isla Tiburon. In the Baja California Peninsula[36], it was documented that the diet of bighorn sheep in Sierra

San Pedro Mártir, Ensenada, is composed of 72 species of plants; an annual consumption of 47 species was documented in Sierra El Mechudo, Baja California Sur[8]. In Coahuila, Gastelum-Mendoza *et al*[6] found an annual consumption of 50 species. These results are similar to the results of this study in terms of the number of species consumed by bighorn sheep in Sierra Santa Isabel[43].

Variations in diet composition can be attributed to physiographic and climatic variations in the habitat. In this research work, it was observed that the places where bighorn sheep live are generally rocky, open, and with limited vegetation cover. This finding coincides with a study on the diet of mule deer in southern Arizona[37], where high temperatures and low rainfall were observed, which directly influenced the metabolism of the plants, causing their drying in a short time.

The intensity of herbivory on some species can have a negative impact on vegetation dynamics. In this regard, the species identified in the diet of bighorn sheep were classified as decreasing or basic (those with higher consumption that decrease their availability due to high herbivory pressure) and increasing or emergent (those with lower consumption that increase their availability due to low herbivory pressure)[30,38].

According to the composition of the diet of bighorn sheep in Sierra Juarez, *H. denudatus* and *L. tridentata* were classified as basic species, which together contributed 21.82 % of the annual diet. However, these species only represented 6.5 % of the total species richness that make up the diet. Likewise, 11 species were considered as emergent species, which together contributed 9.87 % of the diet and 35.5 % of the richness of species consumed. In the population of Sierra Santa Isabel, *H. denudatus*, *L. tridentata*, *Solanum hindsianum*, *Condea emoryi*, and *Eriogonum inflatum* were considered as basic species, which together represented 40.48 % of the annual diet, but only 11.63 % of the total richness of species that make up the diet. On the contrary, 15 species were considered to be increasing, since together they contributed 9.8 % of the annual diet, and 13 of them each contributed less than 1 % of the diet. These species can be considered emergent and are important in periods of low availability of basic species[6]. For example, *Amaranthus palmeri*, *Atriplex barclayana*, *Senegalia greggii*, and *Krameria erecta* (Table 1).

Tree and shrub species were the basis of the bighorn sheep's diet (Figure 3). This result coincides with other studies on the feeding habits of this species in North America[8,9,35]. The importance of shrub and tree species in the sheep's diet is due to the fact that they are forages available throughout the year[6]. Likewise, Bolen and Robinson[39] state that wild herbivores in arid areas prefer to browse shrubs and trees because they contain more digestible nutrients than other grass species. According to some researchers[40], shrubs in arid areas accumulate nutrient reserves during their growth for the formation of new tissues, resulting in a higher concentration of crude protein compared to some grasses and grasses.

Forage selection varied by site and time of year ($P<0.05$). In Sierra Juarez, during the dry season, the bighorn sheep preferred the consumption of *H. denudatus*, *L. tridentata*, and *E. inflatum*; in contrast, in the wet season, it preferred *Bebbia juncea* and *Justicia californica*. In the dry season, it avoided consuming *Cylindropuntia ramosissima*, *Ephedra californica*, and *Sphaeralcea ambigua*; and during the wet season, the species it avoided were *Neltuma glandulosa* and *Krameria bicolor* (Table 2). Some of these species are avoided because they have structures that prevent their optimal consumption, for example, thorns, pubescence, or high wax content in their leaves and stems[30]. It should be noted that uncommon species in the sheep diet were identified, such as *Washingtonia* sp. and *Typha domingensis* (Table 1), which grow in wet substrates around natural water sources.

A significant difference ($P<0.05$) was identified between the availability and consumption of herbaceous species. Although these were highly available, particularly in Sierra Juarez (Figure 2), they were not observed in high percentages of consumption (Figure 4). Herbaceous plants, especially annual species, tend to be less consumed by wild herbivores in arid areas[5,6,9] since their availability is closely linked to humidity and rainfall. As a result, their presence in vegetation cover is limited to short periods of the year. Although previous studies indicate that herbaceous plants are more common in the diet of herbivores during the wet season[6,30], in the present study, the highest percentages of occurrence in diets were detected during the dry season. This can be explained by the existence of oases in the study areas, which provide sufficient humidity for the growth of herbs throughout the year. Although their contribution to the overall diet was low, herbaceous species play a crucial role in the nutrition of bighorn sheep. These plants offer 35 to 40 % more energy, similar protein levels, and 40 to 45 % more phosphorus compared to shrubs in northern Mexico[12]. In addition, they are particularly relevant during the breeding season. Gastelum-Mendoza *et al*[9] reported that both males and females of bighorn sheep consumed mainly herbaceous species during this period in Sierra Noche Buena, Sonora, with a consumption of 38.6 and 47.6 %, respectively. Finally, although herbaceous plants were not highly consumed in general, a significant preference for *E. inflatum* was observed in Sierra Santa Isabel throughout the year (Table 3).

Although the digestive physiology of bighorn sheep is adapted for the digestion of grasses[41], which tend to have high fiber content and relatively low digestibility during most of the year[8,30], these were not significant components in the composition of their diet (Figure 3). In this regard, Brown *et al*[34] point out that wild mountain sheep populations in Nevada, USA, consume between 62 and 81 % of grasses compared to desert sheep populations. This is due to a greater availability of shrubs and trees in the Sonoran Desert[42]. This coincides with previous findings in Sierra El Mechudo, Baja California Sur, where only two species of grasses were identified in the diet[8]. Similarly, in the state of Sonora, studies conducted by Tarango *et al*[5] in Sierra El Viejo and by O'Farrill *et al*[35] on Tiburon Island reported that grasses represented only 5 % of the diet. Nonetheless, more recent research found a grass contribution of 26.8 % in Sierra Noche Buena, Sonora[9] and 17.21 % in a rosetophyllous desert scrubland in Coahuila[6]. It is also pointed out[30] that in the arid areas of northern

Mexico, grasses have a high content of cellulose and hemicellulose, which limits their nutritional value and reduces their consumption by wild herbivores. In this sense, it is mentioned[12] that *Aristida adscensionis* presents neutral detergent fiber values (composed of cellulose, hemicellulose, and lignin) of 61.3 % in 2010 and 79.6 % in 2011 in the diet of sheep from Sierra El Mechudo, Baja California Sur. These values suggest that it is a species of grass with low digestibility[43].

In Sierra Santa Isabel, the contribution of succulent species to the diet of bighorn sheep was greater than in Sierra Juarez (Figure 3) and varied depending on the time of year ($P<0.05$); with greater consumption of these plants in the dry season. Succulent species do not represent a high nutritional contribution for the sheep[12], but they are important elements in the diet as an important source of water in the dry season[5,6,30] when it requires a minimum water intake equivalent to 4 or 5 % of its body weight[42]. The relevance of nopales, magueys, and biznagas in the sheep's diet has been reported in different studies: in Sonora, in Sierra El Viejo, the frequency of succulent consumption was 18 %[5,9]; in Sierra El Mechudo, it was 0.2 %[8]; in Sierra San Pedro Mártir, Baja California, it was 12 %[44].

Conclusions and implications

In Sierra Juarez and Sierra Santa Isabel, bighorn sheep fed mainly on tree and shrub species. For their part, succulents were important species in the diet during the dry season. Although no significant differences in diet diversity were detected between the two mountain ranges, the key species in the bighorn sheep diet were different between sites. In Sierra Juarez, the main species consumed were *E. inflatum*, *H. denudatus*, *J. californica*, and *L. tridentata*; whereas in Sierra Santa Isabel, they were *E. inflatum*, *H. denudatus*, *Horsfordia newberryi*, and *L. tridentata*. These results are important to identify and delimit priority foraging areas for bighorn sheep populations, as well as to guide the design of community strategies for the management of forage species in the state of Baja California. In addition, it is recommended to complement these findings with a bromatological analysis of the main species consumed to assess their nutritional quality and their impact on the health of bighorn sheep populations.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

Table 1: Composition of the diet of bighorn sheep according to the relative frequency of the species identified in fecal samples, collection site, and time of year

Biological form	Family	Species	Sierra Santa Isabel		Sierra Juarez	
			Wet season (%)	Dry season (%)	Wet season (%)	Dry season (%)
Trees and shrubs	*Acanthaceae*	*Justicia californica*	3.08		7.06	0.47
	Amaranthaceae	*Amaranthus palmeri*	0.51			
	Amaranthaceae	*Atriplex barclayana*	0.51			
	Amaranthaceae	*Atriplex hymenelytra*	3.59			0.47
	Apocynaceae	*Asclepias subulata*		0.61		
	Arecaceae	*Washingtonia* sp.	2.07		1.18	1.41
	Asparagaceae	*Yucca* sp.			1.18	
	Asteraceae	*Ambrosia dumosa*	5.13	3.64	4.71	5.16
	Asteraceae	*Bebbia juncea*	3.08	3.03	7.06	3.29
	Asteraceae	*Encelia farinosa*	7.69	3.03	5.29	6.51
	Asteraceae	*Peucephyllum schottii*		0.61		
	Asteraceae	*Stephanomeria* sp.			1.76	0.47
	Burseraceae	*Bursera hindsiana*	1.03	3.03		
	Burseraceae	*Bursera microphylla*	3.59	2.42	1.76	4.23
	Ephedraceae	*Ephedra californica*	0.51	0.61	4.12	0.47
	Euphorbiaceae	*Croton californicus*	0.51	1.21		
	Euphorbiaceae	*Ditaxis lanceolata*	3.08	0.61	2.35	8.82
	Euphorbiaceae	*Euphorbia lomelii*		3.03		
	Fabaceae	*Astragalus* sp.	2.56		2.35	3.76
	Fabaceae	*Hoffmannseggia microphylla*	0.51	0.61		
	Fabaceae	*Neltuma glandulosa*	1.54	0.61	1.18	3.76
	Fabaceae	*Parkinsonia microphylla*	0.51	3.03	2.35	2.82
	Fabaceae	*Psorothamnus emoryi*		0.61		
	Fabaceae	*Senegalia greggii*	0.51			0.45
	Fouquieriaceae	*Fouquieria splendens*	3.59		4.12	
	Juncaceae	*Juncus acutus*	0.51	3.03		
	Krameriaceae	*Krameria bicolor*	2.05		1.76	

	Krameriaceae	*Krameria erecta*	0.51			
	Lamiaceae	*Condea emoryi*	8.21	7.27	5.29	4.63
	Lamiaceae	*Salvia apiana*	0.51			
	Lamiaceae	*Salvia* sp.			1.18	
	Malvaceae	*Hibiscus denudatus*	11.28	7.27	9.41	13.55
	Malvaceae	*Horsfordia newberryi*	3.59	6.67		
	Malvaceae	*Sphaeralcea ambigua*			2.35	0.47
	Polygonaceae	*Eriogonum sp.*	2.05			
	Resedaceae	*Oligomeris linifolia*	1.54	1.21	0.59	
	Simmondsiaceae	*Simmondsia chinensis*	1.03	0.61		
	Solanaceae	*Solanum hindsianum*	8.21	7.85		
	Typhaceae	*Typha domingensis*	1.03			
	Zygophyllaceae	*Larrea tridentata*	6.15	11.52	7.65	13.02
Herbs	*Polygonaceae*	*Eriogonum inflatum*	4.1	9.09	2.35	5.63
	Polygonaceae	*Eriogonum sp.*	2.05			
	Loasaceae	*Eucnide cordata*	0.51			
	Onagraceae	*Chylismia cardiophylla*	4.1	3.03	1.76	3.76
Grasses	*Poaceae*	*Aristida adscensionis*			1.18	
	Poaceae	*Cynodon dactylon*			0.59	0.47
Succulents	*Asparagaceae*	*Agave deserti*	0.51	5.45	4.12	1.41
	Asparagaceae	*Agave* sp.				0.47
	Cactaceae	*Cylindropuntia cholla*		3.64		
	Cactaceae	*Cylindropuntia ramosissima*		3.64		2.35
	Cactaceae	*Opuntia* sp.		2.42	1.76	1.41
Fragments no identified			0.51	0.61	13.54	10.74

Table 2: Types of use and selection of forage by bighorn sheep according to Ivlev's index, site, and time of year

Species	Season	Observed use	Expected use	Ivlev	Type of use
Sierra Juarez					
Cylindropuntia ramosissima	Dry	0.02	0.06	-0.48	A
Ditaxis lanceolata	Wet	0.02	0.01	0.28	P
Ditaxis lanceolata	Dry	0.02	0.01	0.25	P
Encelia farinosa	Wet	0.05	0.05	0	P
Encelia farinosa	Dry	0.07	0.05	0.19	P
Ephedra californica	Dry	0	0.01	-0.56	A
Eriogonum inflatum	Wet	0.02	0.01	0.28	P
Eriogonum inflatum	Dry	0.05	0.01	0.53	**S**
Fouquieria splendens	Wet	0.04	0.02	0.22	P
Hibiscus denudatus	Wet	0.09	0.05	0.28	P
Hibiscus denudatus	Dry	0.14	0.05	0.48	**S**
Justicia californica	Wet	0.07	0.02	0.45	**S**
Krameria bicolor	Wet	0.01	0.03	-0.38	A
Larrea tridentata	Wet	0.07	0.05	0.18	P
Larrea tridentata	Dry	0.15	0.03	0.63	**S**
Neltuma glandulosa	Wet	0.01	0.02	-0.38	A
Opuntia sp.	Dry	0.01	0.01	-0.08	P
Parkinsonia microphylla	Wet	0.02	0.02	-0.05	P
Sphaeralcea ambigua	Wet	0.02	0.01	0.28	P
Sphaeralcea ambigua	Dry	0	0.01	-0.56	A
Sierra Santa Isabel					
Ditaxis lanceolata	Wet	0.03	0.02	0.13	P
Ditaxis lanceolata	Dry	0	0.01	-0.51	A
Encelia farinosa	Wet	0.07	0.05	0.13	P
Encelia farinosa	Dry	0.03	0.05	-0.3	P
Eriogonum inflatum	Wet	0.04	0.01	0.55	**S**
Eriogonum inflatum	Dry	0.09	0.03	0.41	**S**
Fouquieria splendens	Wet	0.03	0.04	-0.12	P
Hibiscus denudatus	Wet	0.11	0.02	0.65	**S**
Hibiscus denudatus	Dry	0.07	0.05	0.12	P

Horsfordia newberryi	Wet	0.03	0.03	0.01	P
Horsfordia newberryi	Dry	0.06	0.01	0.55	**S**
Larrea tridentata	Wet	0.06	0.02	0.45	**S**
Larrea tridentata	Dry	0.11	0.05	0.34	P
Parkinsonia microphylla	Wet	0	0.04	-0.8	A
Parkinsonia microphylla	Dry	0.03	0.07	-0.42	A

Type of use: proportional (P), selected (S) and avoided (A).

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Article

Morning cortisol serum concentration in agricultural mules in the tropics

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Abstract:

This study evaluated the serum cortisol level and its relationship with the work type, age and sex of mules in tropical conditions. Each blood serum obtained from 97 mules was analyzed by a commercial ELISA kit specific to cortisol. The mean cortisol level was 96.3 ng/mL with no significant differences regarding the variables evaluated making it suitable for used as reference value. Also, this is lower than reported in previous studies in show and recreation horses from the same region (133.0 ng/mL). In summary, it is important to utilize species-specific values and conduct studies to determine the mules' ability to adapt and resist.

Key words: Well-being, Disease, Equines, Stress, Hybrids.

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Introduction

Mules are hybrids of horses and donkeys. There are produced worldwide and are known for their hardiness and effectiveness in agricultural work (e.g. dragging, hauling and loading). In Colombia, they are also used for riding, wrangling, showing, transport, tourism, equine therapy and even in policing[1,2]. Though widely used for many purposes, many aspects of mule disease epidemiology and general health are relatively unstudied compared to other equine species[2,3].

Cortisol is a hormone secreted by the adrenal gland cortex. It is a glucocorticoid with important functions in organic homeostasis: gluconeogenesis, proteolysis, lipolysis, hyperglycemia, and modulation of immunity and inflammation, among others[4,5]. It is also involved in pathological processes and is a valuable diagnostic tool in different equine gastrointestinal and respiratory disorders, as well as sepsis[6-9]. It is considered an adaptive hormone in stress-generating activities[10-13], and an indicator of physical conditioning and athletic performance in sport horses[14].

Cortisol is secreted in response to adrenocorticotropic hormone (ACTH), when the hypothalamic-pituitary-adrenal axis (HPA) is activated by physiological (glycemia, blood pressure), pathological, stress, environmental, climatological or seasonal factors[15,16]. ACTH is released in response to corticotropin-releasing hormone (CRH) from the hypothalamus, which acts on type 1 receptors located in the corticotrophs of the anterior pituitary; this, in turn, is activated by HPA and, simultaneously, is controlled by negative feedback[15].

The high variability in cortisol concentrations found in horses and donkeys is multifactorial[17-21]. As a result, reference values have been established according to physiological state, age group, activity type, and management and environmental conditions in different breeds. Cortisol has been extensively studied in horses, notably less so in donkeys, and almost not all in mules. The present study objective was to collect serum cortisol concentration data for a population of mules in the tropics, and relate it to age group, sex and agricultural activity.

Material and methods

The study design was approved by the Animal Experiments Ethical Committee of Antioquia University (Comité de Ética para la Experimentación Animal de la Universidad de Antioquia); Protocol No. 1222019. The sampled mules were from municipalities near the Medellín metropolitan area, Antioquia Department, Colombia. Average elevation in Antioquia Department is 2,055 m asl. Average annual rainfall ranges from 1,500 to 5,000

mm, with two dry and two rainy seasons. Relative humidity ranges from 63 to 73 %, and temperature from 18 to 28 °C. Based on the Köppen-Geiger system, climate is intertropical zone with tropical climates A (equatorial, monsoon and savannah climates) (IDEAM Cartografía Básica IGAC, 2018).

Animals

Experimental animals were 97 clinically healthy mules (65 males, 32 females), of 8.7 ± 4.4 yr of age, 290.5 ± 37.6 kg weight, and 5 ± 0.8 body condition[22]. They were grazed with supplementation with sugar cane byproducts, and were used in agricultural work such as hauling, wrangling and riding. The sampled mules were classified into three groups by age: young (<5 yr), adult (6-14 yr), and old (>15 yr); type of use was not considered in the classification. Each animal was physically examined and measurements taken of heart rate (HR), respiratory rate (RR), and body temperature (°C). Blood samples were taken to measure blood cortisol levels.

Blood samples

Blood samples were taken with minimal animal handling, in the morning (0700-1100 h) before their routine activities and after cleaning and sterilization of the puncture site. The samples were taken with a vacuum tube without anticoagulant, from the jugular vein. They were centrifuged at 210 xg for 10 min to separate out the serum, aliquots of which were placed in Eppendorf tubes and stored at -20° C until analysis.

Cortisol concentration

Cortisol quantification was done using an established protocol[23]. Briefly, a commercial sandwich ELISA kit was used (AccuBind®, Monobind Inc., USA), with a conventional 450-630 nm wavelength reader (Stat Fax 303® Plus Microstrip Reader, Awareness Technology Inc., USA). The kit was validated using six calibrators and three controls: Multiligan A: 07.7 ng/mL, Multiligan B: 97.4 ng/ml, and Multiligan C: 193.2 ng/mL (QSure® Multi-Ligand Control Tri-Level, Monobind Inc., USA). Serum cortisol concentrations were expressed as ng/mL. According to the manufacturer, the kit has 3.77 ng/ml sensitivity, and can detect concentrations from 4.0 to 950 ng/mL.

Statistical analyses

The number of individuals to evaluate was calculated using the conventional formula to find sample size in an infinite population, which generated a sample of 97 mules. The study design was descriptive and cross-sectional, taking a single sample from each individual. The resulting data were entered into Microsoft Excel®, and a Kolmogorov-Smirnov test run with the SAS® (ver. 9.2, USA) statistical software found the data not to have a normal distribution. The correlation analysis between age groups and activity was run using the Kruskal-Wallis test, and, for sex, the Mann-Whitney test was applied to identify significance level ($P<0.05$).

Results

Median serum cortisol concentration was 96.3 ng/mL, with a minimum of 6.8 ng/mL and a maximum of 248.2 ng/ mL; the 25% percentile distribution was 78.6 ng/mL and the 75% was 134.4 ng/mL (Table 1). In other words, serum cortisol concentration was 96.3 ng/mL (variance: 165.3 ng/mL) with no differences between sex, age group and activity ($P>0.05$). Additionally, 75 % of the population had a cortisol value less than or equal to 134.4 ng/mL, with a distribution range between 6.8 and 248.2 ng/mL.

Table 1: Serum cortisol concentration (median), by sex, age and activity in a sample of 97 mules

	N	Cortisol (ng/mL)			HR (bpm)	RR (bpm)	Temp (ºC)
		Mean	SD	*P*-value			
Age group[1]							
Young	17	93.1	36.5		40.0	20.0	37.6
Adult	67	80.0	41.7	0.368	38.0	24.0	37.6
Old	13	98.3	40.5		36.0	20.0	37.8
Sex[2]							
Female	32	98.4	33.9	1.0	37.5	20.5	37.7
Male	65	93.1	43.7		38.0	22.0	37.6
Activity[1]							
Hauling	63	108.2	45.6		39.0	21.0	37.4
Wrangling	28	82.0	24.4	0.564	38.0	27.0	37.5
Riding	6	105.9	29.2		41.0	22.0	37.7

SD= standard deviation; HR= heart rate; RR= respiratory rate.

[1]Kruskal-Wallis test, [2]Mann-Whitney test.

Discussion

Mules are known for their resistance and efficiency, and are therefore used, at times overexploited, in myriad activities[1,2]. The present study provides valuable data on serum cortisol levels in mules, but does not address diseases caused by abuse, neglect and others disorders related to animal welfare[24]. The mule specimens sampled in the present study were representative of the region.

In contrast to mules, cortisol concentrations have been widely studied in horses in different types of samples (plasma, saliva, tears, fecal matter and hair) using different laboratory techniques, and including variables such as physiological state, health condition, age, sex, breed, management strategy, zootechnical use, and stress response[25-29]. This has allowed comparisons to be made between environmental conditions and geographic region[18,19]. Though studied less than horses, reference values reported for donkeys are similar to horse baseline concentrations[30,31,32]. Unlike in horses, there are no reports of cortisol levels responding to climatic season[33], although, given the seasonality of ACTH production, some effect is likely to exist. No such studies have been done in mules, leaving no recourse other than to compare the present results with previous studies of other equine species.

The 96.3 ng/mL serum cortisol concentration observed here is higher than the 29.0 and 66.0 ng/mL reported for horses and donkeys in non-tropical countries[30-33]. However, it is lower than the 133.0 ± 74.0 ng/mL reported in Colombian Criollo Horses (CCC), in a study using the same commercial kit, and animals managed in similar climatic and topographical conditions, but under a different use regime (stabling and established exercise routines)[13]. These discrepancies may be explained by evolutionary differences between species, adaptation level, hardiness and degree of physiological response to stress factors and adversity.

The outlier serum cortisol concentrations observed here in mules (minimum: 6.8 ng/mL, maximum: 248.2 ng/mL) were lower in both instances than those reported in CCC (minimum: 42.0 ng, maximum: 481.0 ng)[13]. In the present results and the study of CCC, the animals were clinically normal (physiological constants within species parameters), suggesting that the higher cortisol levels in the CCC may be due more to factors inherent to each individual (temperament) and the effect of the tropics[26]. Of particular note is that, despite their working long and demanding days, with prolonged fasting, cortisol values in the studied mules were lower than in the CCC. Absence of stress or adaptation level cannot be inferred from a single cortisol value, without a control group and without characterizing animal workload, and would require morning and evening blood samples to calculate the cortisol index[17].

The lack of statistical difference in serum cortisol concentrations between males and females, age groups and type of activity, was also similar to that reported in the CCC[13]. However, this lack of difference was more homogeneous in the variables of the mules than in those of the CCC. This could indicate some level of stability in cortisol concentration, suggesting it might function as a reference value for mules, considering the individual and external responses reflected in the minimum and maximum values[17,34]. Any comparison of cortisol concentrations between mules and CCC would require simultaneous sampling of horses and mules from the same region and in similar environmental conditions.

Differences between techniques have been reported when determining cortisol concentration, especially between chemiluminescence and ELISA; differences of up to 15.7 ng/mL are reported, with the former being more accurate[7]. No such differences have been reported when quantifying cortisol concentration with radioimmunoassay and ELISA[8]. Sample type can also influence cortisol concentration results. Several studies have used different substrates to quantify cortisol concentration, some reporting no differences between substrates[14]. Serum was used in the present study since no values have been reported previously for this substrate. Measures were taken here to minimize results alteration from external factors, for example, minimal manipulation of animal when taking samples, and technique validation with commercial controls.

Conclusions and implications

Serum cortisol concentrations in mules (96.3 ng/mL) used in extensive agricultural activities under tropical conditions were lower than reported for horses in the same geographic region and under less stressful conditions. Further research is needed to confirm if these differences are due to adaptation and stress resistance.

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Article

The network of cattle mobilized in Mexico for slaughter 2017-2021

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Abstract:

Social-network analysis (SNA) offers an alternative way to study the market of cattle destined for slaughter, making it possible to come up with measurements for the purpose of analyzing both the sources and destinations of the said animals. The research described here set out to determine the network structure of Mexico's internal market of cattle mobilized for slaughter between 2017 and 2021. The structure of that market was analyzed using spatial-localization, economic-specialization, and network-density and specialization measurements. An average of 4.7 million cattle were mobilized each year in Mexico for slaughter, with 302 mobilization permits being applied for every day, each for an average of

42.7 heads of cattle. Relative market specialization was low, being higher for demand, but no different from that for supply (P>0.05). The market was characterized by intrastate trade, low network density, low input centrality, low output centrality and high levels of municipal market specialization. Hence, the national cattle-for-slaughter market has low specialization, high diversification and homophily.

Key words: Regional specialization, Market structure, Market cohesion, Livestock mobilization**.**

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Introduction

Cattle mobilization is important in Mexico, where the overall cattle market is composed of six sub-markets with an annual average of 8.9 million heads of cattle being mobilized nationwide — 53.5 % for slaughter, 44.5 % for fattening, 1.1 % for grazing, 0.5 % for breeding, 0.3 % for fairs, and 0.2 % for shows[(1)]. Of the 3.9 million calves that are the foundation of Mexico's meat production, 43.2 % were bred in the states of Chiapas and Veracruz, with one third of the nationwide total being mobilized to the states of Durango and San Luís Potosí[(2)]. Furthermore, in the period 2017-2022, Mexico exported 1.1 million calves and heifers to the USA[(3)], 40 % of which were from the state of Chihuahua[(4)].

Cattle are mobilized in Mexico for various reasons, the first being to supply the meat market, which is made up of four products: cows, bulls, bullocks and heifers[(5)]. The reason why cows enter the meat market are primarily due to culling[(6)], illness[(7)], and low financial yield[(8)], with it being common practice to slaughter pregnant cows[(9)]. Bulls enter the meat market for similar reasons as cows, but the likelihood increases with age. There is a 47.0 % chance of cows aged 10 yr or more being slaughtered, and a 73.3 % probability for bulls of the same age[(10)]. Bullocks and heifers are primarily destined for the meat market[(2)].

The availability of slaughterhouses in Mexico is another variable associated with the intrastate and inter-state mobilization of cattle. The country has 1,175 slaughterhouses for all animal species, a monthly installed capacity for the slaughter of 1'267,995 heads of cattle, and a 42.1 % utilized capacity[(5)]. Moreover, with the exception of Mexico City, there are

slaughterhouses for cattle in 31 of the 32 Mexican federal entities, with an installed capacity of 1.7 million heads per month[3].

The problem of having limited information about cattle for slaughter can be solved by triangulating data[11]. Attributes (i.e. the volume and structure of mobilizations) and the source-destination relationships between livestock species determine market structure[2]. Although Mexico has an important national cattle herd which amounted, in 2022, to 33.3 million heads[3], the National Cattle Census (NCC) reported 12.8 million heads of cattle more than the SIAP in 2021, and, in 2019, the National Agriculture-and-Livestock Survey (NALS) reported 1.5 million heads less than the NCC[12].

Social Network Analysis (SNA), an innovative tool for studying market structures, analyzes how linkage patterns assign resources within social systems, seeking to identify groups, central features and indirect links among network elements[13], while the structural variables of a network can be studied at four levels: i. total internal network, ii. individuals as nodes, iii. external networks, and iv. clusters within the network[14].

Besides the aforesaid variables, livestock activities are influenced by the availability of resources, the location of markets, workforce availability, soil, climate and technical conditions, all of which may rule out certain locations for specific productive activities[15]. Moreover, the quality and yield of beef decrease in keeping with the distance travelled before slaughter[16].

Hence, the research described here set out to determine the network structure of Mexico's internal market of cattle mobilized for slaughter between 2017 and 2021, positing that the number of heads per mobilization and market specialization determine the structure of the country's cattle-for-slaughter network.

Material and methods

Materials

The analyzed information was the number of heads of cattle mobilized each day from all Mexico's productive markets to all its consumer municipalities between 2017 and 2021. This information was obtained from the official register of all the Verification and Inspection Points (VIPs) of the National Agrofood Health, Innocuousness and Quality Service (Spanish abbreviation: SENASICA).

The observation units were the source and destination municipalities of mobilized cattle, and the measurement unit was the number of animals mobilized. Likewise, at the state level, the unit of observation was all the source and destination states of cattle mobilized for slaughter. To facilitate the handling of this information, the names of Mexico's 32 federal entities (i.e. 31 states + Mexico City) were abbreviated in accordance with the ISO 3166-2 norm[17].
The municipal data were set out in Matrix (A_{ij}), with i = 1, 2…,951 mobilized-cattle source municipalities and j =1, 2…,853 cattle-destination municipalities. The municipal data were set out in a national state-level matrix (E_{ij}), with i =1, 2…,32 mobilized-cattle source states and j =1, 2…,32 mobilized-cattle destination states. The elements of the main diagonal, A_{ii}, showed intrastate trade (a_{ii}) and the (a_{ij}) elements showed inter-state trade. For purposes of analysis, the A_{ij} and E_{ij} elements were converted into a dichotomy, with market relationships being assigned the number 1 ($a_{ij} = 1$) and non-market ones the number ($a_{ij} = 0$).

Methods

Two types of analysis were carried out, the first consisting in calculating the degree of cattle-mobilized-for-slaughter source-market and destination-market specialization based on location theory[1]. The aim was to identify the similarity between the state-level and national market structures. The source markets were the zones (X_{ij}) and the destination markets the regions, where i represented number of heads of cattle mobilized from the source market and j the number of heads of cattle received in the destination market.

The regional economic structure of state markets was analyzed using the location quotient (Q_{ij}) and the specialization coefficient (Q_R)[18]. The location quotient (Q_{ij}) measures relative or interregional specialization; a Q_{ij} greater than 1 indicates a specialized market because its relative size is larger than that of the national market, whereas a Q_{ij} less than 1 indicates a non-specialized market. The specialization coefficient (Q_R) measures market diversification, ranging from 0 to 1. A Q_R value close to 1 indicates a diversified market, while a value closer to 0 suggests the market resembles the national market.

$$\boldsymbol{Q_{ij}} = \frac{\frac{V_{ij}}{\sum_{i=1}^{n} V_{ij}}}{\frac{\sum_{j=1}^{n} V_{ij}}{\sum_{i=1,j=1}^{n} Vij}} \quad ; Q_R = \frac{1}{2}\sum_{I=1}^{N}\left|\frac{V_{ij}}{\sum_{i=1}^{n} V_{ij}} - \frac{\sum_{j=1}^{n} V_{ij}}{\sum_{i=1,j=1}^{n} Vij}\right|$$

where V_{ij} = livestock in the destination market j from the market of origin i.

The second analysis, based on the SNA-theory, consisted in examining the structure of the national network of cattle mobilized for slaughter by means of centrality and density measurements. The said calculations of density[19] and centrality[19] were carried out using Ucinet statistical software[20].

Density (D), which measures the market's social structure and evaluates the cohesion between source cattle-for-slaughter markets (M_i) and destination cattle-for-slaughter markets, is the coefficient of actual markets (AMs) divided by possible markets (PMs). Centrality (C) evaluates the individual social structure of exit markets (c_{ij}) and entry markets (c_{ji}): while c_{ij} is the total number of actual destination markets (x_j) and c_{ji}, was worked out in the same way. The bigger the number of actual markets, the more centrality there will be the more important the market will be within the national network.

The statistical analysis was descriptive, using average and standard-deviation statistics. The hypothesis was substantiated using t-student statistics with a 95% significance level. Meat prices were for the year 2022[3].

Results

Due to their location, only 34.1 % of Mexico's 32 state-level cattle-for-slaughter markets were found to be specialized (Q>1). With regard to supply, the four smallest markets had 100 % specialization in all the destination markets to which they mobilized cattle, with the four biggest markets having only 6.0 % specialization. When it came to demand, only two small markets had 100 % specialization in all the markets that received cattle, with the five main markets having only 8.0 % specialization.

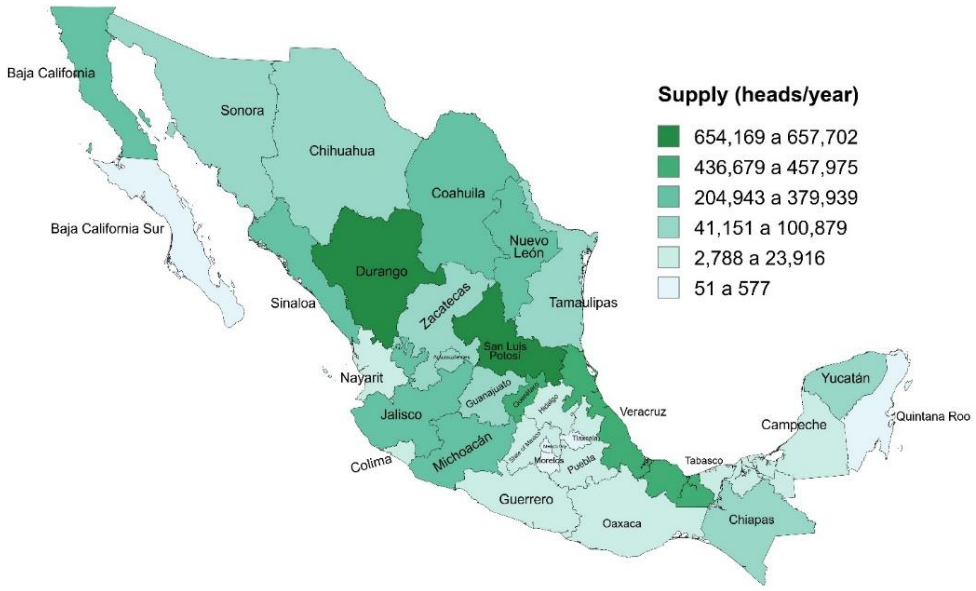
Likewise, regional economic structure shows a diversified market. The average specialization of the cattle market for slaughter was high, with supply at 80.7 ± 10.9 % and demand at 81.1 ± 11.4 %. However, the difference between supply and demand was not significant (P>0.05). Nevertheless, the economic structure was diversified for 62.5 % of the state-level supply markets and 46.9 % of the state-level demand markets. Furthermore, small markets were the most specialized ones in both supply and demand.

Supply

In the period 2017-2021, only 25.0 % of Mexico's 2,446 municipalities and 95.0 % of its 32 federal entities supplied cattle for slaughter. Average annual supply was for 4.7 ± 0.3 million heads of cattle, with an average 3.4 % annual growth, equivalent to 35.9 % of the monthly installed capacity of all the cattle slaughterhouses in Mexico. Average annual demand at the municipal level was for 4,763.3 ± 35,499.4 heads of cattle, and for 149,382.8 ± 199,516 heads of cattle at the state level. Moreover, the cattle-for-slaughter market was characterized by intrastate trade, with 71.8 % of all the cattle mobilized for that purpose being sent to local slaughterhouses, while 52.8 % of national supply was concentrated in six municipalities. Hence, the national supply of cattle for slaughter in municipal markets was extremely heterogeneous, with 5.9 % of all municipalities accounting for 83.0 % of all such cattle. The municipalities of Tamuín in the state of San Luis Potosí and Tlahualilo in the state of Durango respectively supplied 13.5 % and 10.6 % of all cattle for slaughter. Other important supply municipalities were Mexicali in the state of Baja California (7.7 %), Ezequiel Montes in the state of Queretaro, Vista Hermosa in the state of Michoacán (7.4 %) and Querétaro in the state of Querétaro (6.6 %), the first three of which were characterized as suppliers of finished cattle.

Based on the state supply of cattle for slaughter, it is possible to split the 32 state markets into six strata, two of them with high concentration levels. Of the markets, 25 % supplied up to 4,163 heads of cattle, 50 % up to 251,227 heads, and only one market up to 657,702 heads. The highest concentration of cattle was in the San Luis Potosí market (13.7 %) and the Durango market (13.7 %), both of which were characterized by intrastate trade (97.6 % and 69.3 % respectively) and finished cattle. The Veracruz market constituted 9.1 % of the national market, and the Jalisco with 4.5 %, while their intrastate trade made up 80.1 % and 8.1 % respectively. In other important markets such as Baja California, intrastate trade constituted 100 % of the state market, in Querétaro it constituted 15.4 %, and in Nuevo León it constituted 95.3 % (Figure 1).

Figure 1: Average annual state supply in Mexico of cattle for slaughter, 2017-2021



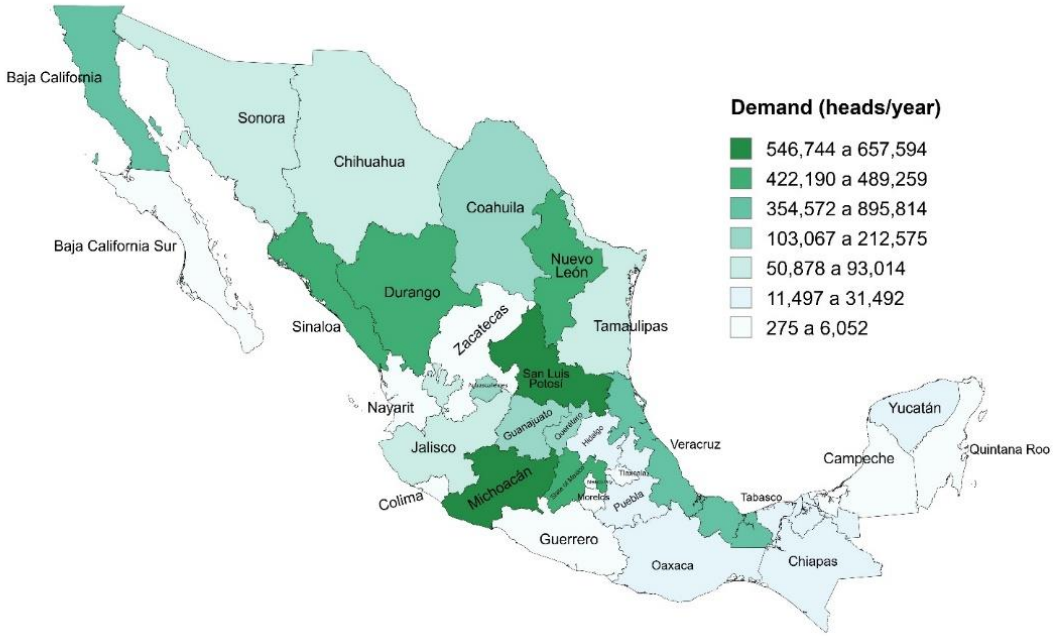
The dynamics of the sate supply of cattle for slaughter are attested to by the number of mobilization permits granted by SENASICA. An average of 306.4 permits per day were granted for the mobilization of 13,095 heads of cattle – i.e. 42.7 heads per permit. The market of the state of Queretaro was the most dynamic one, with 39.8 permits being granted each day for the mobilization of 1,254 heads of cattle – i.e. 31.5 animals per permit. In San Luis Potosí and Durango, the two most important markets, 16.4 and 27.1 permits per day were issued respectively. Finally, the market with the most interstate mobilization at the national level was Querétaro with 35.0 % of all the cattle mobilized for slaughter, followed by Durango with 18.7 % of all cattle mobilized for slaughter and Jalisco with 17.5 %.

Demand

Only 20.7 % of the 2,446 municipalities, and 100 % of the state-level markets, required cattle for slaughter in Mexico. Demand was very uneven, with 88.4 % of all cattle destined for slaughter being concentrated in a mere 3.0 % of all the municipal markets. The most important municipal markets were Tamuín in the state of San Luis Potosí and Vista Hermosa in the state of Michoacán, with respective concentrations of 13.6 % and 10.8 % of all the cattle destined for slaughter. Other important markets were Culiacán in the state of Sinaloa (9.0 %), Tlahualilo in the state of Durango (8.4 %) and Mexicali in the state of Baja California Norte (8.0 %). For the Tamuín, Tlahualilo and Mexicali markets, 95.0 % of all trade occurred at the intrastate level, while the figure for the other markets mentioned was lower (72.4 %).

Just as in the state supply markets, it was possible, based on the state demand for cows for slaughter, to split the 32 states into seven strata, two of which were characterized by high concentration levels. Of the 32 state-level markets, 31.8 % accounted for 86.3 % of all the mobilized cattle. The most important state markets were located in the north and center of the country: San Luis Potosí (13.8 %), Michoacán (11.4 %) and Durango (10.2 %). Intrastate mobilization constituted 90.0 % of all intrastate movement of cattle in San Luis Potosí and Durango, and 65.0 % of all such movement in Michoacán (Figure 2).

Figure 2: Average annual state demand in Mexico for cattle for slaughter, 2017-2021



In addition to the variables that made up the structure and dynamics of the interstate supply of cattle for slaughter in Mexico, the trade deficit was added to the demand. The said deficit constituted 18.5 % of all the cattle mobilized for slaughter, 49.6 % of which were moved to the State of Mexico, 21.5 % to state of Michoacán and 8.2 % to state of Sinaloa. These states accounted for 96.8 %, 34.7 %, and 16.2 % of the national demand respectively. Since 35.0 % of the national deficit was covered by the state of Querétaro market, 18.7 % by the state of Durango market, and 17.5 % by the state of Jalisco market, the most dynamic demand state markets were Querétaro with 37.4 cattle-mobilization permits for 41.8 heads of cattle per permit, while state of Michoacán issued 33.1 permits and 78.0 heads of cattle per permit.

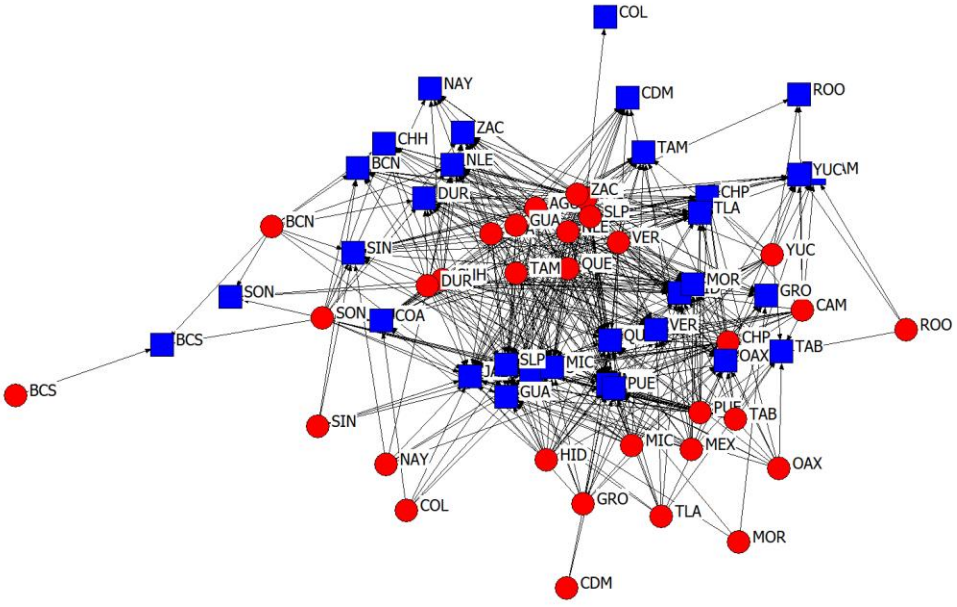
The interstate market mobilized equal amounts of male and female cattle, but the preference was different, with 50.9 % of the mobilization permits being exclusively for male animals, 25.45 % for both male and female animals, and 23.7 % exclusively for female animals. Of the male cattle, 16.3 % came from Querétaro, 14.6 % from San Luis Potosí, and 14.2 % from Durango, while 21.7 % of the female animals came from Nuevo León, 12.6 % from Durango,

and 10.4 % from Coahuila; 17.2 % of both male and female animals came from Baja California, 16.6 % from San Luis Potosí, and 13.6 % from Durango. On average, 54.1 % female cattle were mobilized per permit, 44.2 % male animals per permit, and 33.9 % both male and female cattle.

Networks

Figure 3 shows the interstate connections between the source markets (red circle) and the destination markets (blue square) pertaining to cattle for slaughter. The bigger the supply (source) and the demand (destination), the bigger the resulting network with the markets with the highest degree of centrality located in the center of the network, which was complete between 2017 and 2019, and incomplete between 2020 and 2021, when the Baja California Sur (BCS) market split off from the national network. The communication of information with a high level of homophily was continuous at 77.4 % of nodes, and it can also be seen that Sonora (SON) and Baja California Norte (BCN) served as the links between BCS and the national market.

Figure 3: Interstate distribution network in Mexico of cattle for slaughter 2017-2021



Aguascalientes (AGU), Baja California Norte (BCN), Baja California Sur (BCS), Campeche (CAM), Chiapas (CHP), Chihuahua (CHH), Ciudad de México (CDM), Coahuila (COA), Colima (COL), Durango (DUR), Estado de México (MEX), Guanajuato (GUA), Guerrero (GRO), Hidalgo (HID), Jalisco (JAL), Michoacán (MIC), Morelos (MOR), Nayarit (NAY), Nuevo León (NLE), Oaxaca (OAX), Puebla (PUE), Querétaro (QUE), Quintana Roo (ROO), Sinaloa (SIN), Sonora (SON), Tabasco (TAB), Tamaulipas (TAM), Tlaxcala (TLA), Veracruz (VER), VI (San Luis Potosí (SLP), Yucatán (YUC), Zacatecas (ZAC).

While the average annual density of the national cattle-for-slaughter network was low (34.2 %), an 8.0 % decrease in density was observed, from 36.9 % in 2017 to 31.0 % in 2021, showing a direct relationship between possible markets and feasible markets. Furthermore, the low standard deviation indicates that sources and destinations maintained their market preferences (i.e. homophily).

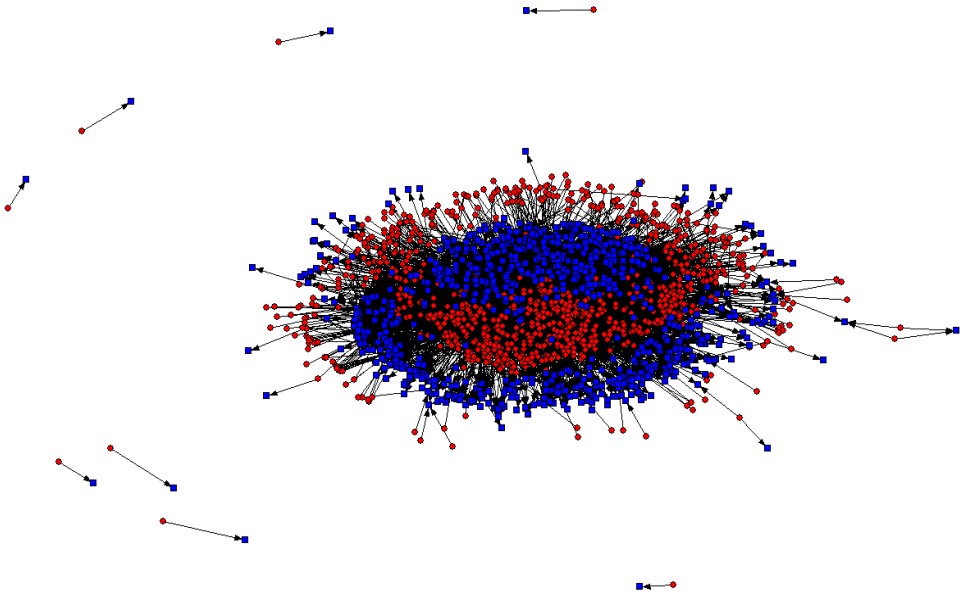
In-degree and out-degree centrality was low (32.7 %), confirming the homophily existing in the cattle-for-slaughter market. In terms of supply, Jalisco's market had the highest centrality (78.1 %), supplying cattle to 77.5 % of the 32 national markets; Zacatecas with 69.4 %, and Querétaro with 61.3 %, linked up with 68.8 % and 60.3 % of the national markets respectively. The three least centralized markets were Morelos, Quintana Roo and Baja California, which respectively linked up with 7.0 %, 3.1 % and 2.5 % of the national markets.

With regard to demand, average output density was low, and average input density was even lower, but with less homophily (10.1 ± 5.8 %). The State of Mexico market was the most central one (74.4 %), requiring cattle from 76.3 % of the state-level markets, while Aguascalientes and Querétaro required cattle from 63.8 % and 64.4 % of the state-level markets respectively. At the other extreme were the markets of Mexico City (output) and Colima (input), with the lowest level of influence (1.3 % and 3.8 % respectively).
Another indicator of centrality that measures the prestige of markets within the network is eigenvalue or characteristic value. The prestige of the national cattle-for-slaughter market was low (17.0 %) and stable (6.0 %). The markets with the highest levels of prestige were Jalisco (24.0 %), Zacatecas (23.0 %), and the State of Mexico (23.0 %), while those with the least influence were Colima (5.0 %), Quintana Roo (3.0 %), and Baja California Sur (1.0 %).

Municipal market network

The municipal supply network pertaining to cattle for slaughter was composed of 1,013 municipalities (41.4 % of all the municipalities in Mexico), while the municipal demand network was composed of 890 municipalities (36.4 % of all the municipalities in Mexico). It was an incomplete network, with eight markets being disconnected from the national network (Figure 4).

Figure 4: Municipal network pertaining to the mobilization in Mexico of cattle for slaughter 2017-2021



A high level of concentration was observed in the network. With 10 markets supplying 64.7 % of all the cattle for slaughter, the most important of which were Tamuín in San Luis Potosí (13.5 %), Tlahualilo in Durango (10.6 %), and Mexicali in Baja California (7.7 %); all characterized by an intensive meat-production system. While 68.4 % of all demand was concentrated in 10 municipalities, the most important of which were Tamuín in San Luis Potosí (13.6 %), Vista Hermosa in Michoacán (10.8 %) and Culiacán in Sinaloa (9.0 %).

The density of the national network of municipal markets was very low, with only 4,694 of 342, 421 markets being effective. Average exit density (0.98 %) was lower than average entry density (1.2 %), but not different (P<0.05). The most prestigious municipal markets were Ezequiel Montes in Querétaro and La Paz in State of Mexico.

Average municipal out-degree centrality was low (9.6 ± 15 %), with the most central municipal output markets being Ezequiel Montes in Querétaro (20.2 %), Tepatitlán de Morelos in Jalisco (14.6 %) and San Juan de Los Lagos in Jalisco (12.8 %). In-degree centrality was greater than output density (11.9 ± 23.2 markets), with the most central entry market being La Paz in State of Mexico with 301 effective markets of 1,013 possible markets. Other important destination markets were Ezequiel Montes in Querétaro (23.8 %) and Aguascalientes in Aguascalientes (20.3 %). Moreover, both annual out-degree and in-degree centrality remained unchanged, showing no significant differences in average centrality over the years (P>0.05).

Discussion

The analysis reveals the official situation of SENASICA with regard to the flow of cattle for slaughter in Mexico between 2017 and 2021. Veracruz and Jalisco are the most important states[3,21,22]. The findings show that San Luis Potosí and Durango had the first and second highest levels of supply respectively, while San Luís Potosí and Michoacán had the first and second highest levels of demand respectively, with Veracruz having the fourth highest level of supply and the eighth highest level of demand. Furthermore, it is the municipal markets that are significant, rather than the state-level ones. The most important supply municipalities were determined to be Tamuín in San Luis Potosí and Tlahualilo in Durango, while the most important demand municipalities were found to be Tamuín in San Luis Potosí and Vista Hermosa in Michoacán.

The analysis of market location shows that the markets remitting and receiving the smallest amounts of heads of cattle are the most specialized ones, due to the fact that they have the smallest share of the national herd and their interstate trade is in finished cattle. Furthermore, they have the lowest prices for cattle on the hoof and send their cull cows and bulls to local slaughterhouses.

In contrast, the lowest levels of specialization were found in the states with the biggest stocks, due to the proportions of cull animals and slaughterhouses. The largest inventories were recorded in Veracruz (13.5 %) and Jalisco (9.2 %), while the smallest inventory was recorded in nine states, which had a total of 3.2 % of the national herd[3].
The findings regarding market specialization coincide with those of Callejas and Rebollar[5], who report that 50.5 % of the cattle slaughtered in Mexico are steers and heifers killed for meat, while 34.8 % of all slaughtered cattle are cull cows and 13.8 % are cull bulls. Moreover, Callejas[2] found that 42.7 % of all beef cattle are sent to the most specialized markets – i.e. Tamuín in San Luís Potosí (15.2 %), Mexicali in Baja California (10.5 %), Durango (8.9 %), and Ezequiel Montes in Querétaro (8.1 %).

On the other hand, high concentration and intrastate trade are subject to variables such as pre-slaughter stress[23], low yield and financial loss[24], and disease reduction[25], although Hultgren *et al*[16] found no significant differences, in terms of animal welfare, between killing cattle in local slaughterhouses and killing them in mobile slaughterhouses.

The larger size of the intrastate market was due to the fact that the destination of mobilized cattle is associated with the installed capacity of the slaughterhouses. Mexico's slaughterhouses have a total monthly installed slaughtering capacity of 1'267,995 heads of

cattle and a used capacity of 52 %, added to which demand efficiency in the ten main cattle-for-slaughter states was 64.4 %[3].

The slaughterhouses located in San Luis Potosí have an annual installed slaughtering capacity of 1.2 million heads of cattle, but the average annual demand was for only 52.8 % of that capacity, while only 51.9 % of installed capacity was found in the state of Michoacán. Callejas *et al*[4] found that cattle slaughterhouses have an average efficiency level of 54.0 % of installed capacity, while the only slaughterhouses in Chiapas with 100 % efficiency were private ones. The "slaughterhouse", "price" and "distance-from-source-to-destination" variables explain why there is more intra-municipal trade than inter-municipal trade.

Moreover, a significant association between the number of entering trade connections and the price of cattle on the hoof[26]. Examination of a sample of prices of beef on the hoof from cows, bulls, bullocks and heifers in 20 municipal, 'federal-inspection-type' and private slaughterhouses during the period studied reveals that the average price was MXN$40.75 ± 6.82/kg. The highest price (MXN$54.90/kg) was paid in San Luis Potosí, and the lowest price (MXN$30.96/kg) in Campeche. These price differences represent opportunity costs, with San Luis Potosí being a small, specialized market, while Campeche is a big market with a low level of specialization.

The average opportunity cost in Mexico's cattle-for-slaughter market was MXN$14.58 ± 5.66/kg. In Campeche it was MXN $23.94/kg, although, even if the cost of mobilization had been added to this, a bigger profit would have been obtained if the cattle in question had been sold in San Luis Potosí. The second-best option was the State of Mexico, which had an opportunity cost of MXN$1.63/kg as against San Luis Potosí.
The density of the network pertaining to Mexico's cattle-for slaughter market shows that all the country's markets are interconnected, but with a low level of density that is directly related to slaughterhouse availability in the main productive municipalities, which makes the latter the main consumers. However, the high degree of concentration shows that market structure is determined by the amount of cattle mobilized for slaughter. The low degree of density also gives rise to —and conditions— the high level of homophily. Only five of the 32 markets had no intra-municipal trade, it being no coincidence that they were the smallest ones, so that 7.7 of every 10 cattle mobilizations had the same source and destination.

The low level of network centrality reveals a disperse market. Although Jalisco has more destination markets and the State of Mexico has more source markets, they both have low participation in the source and destination markets. Both the aforesaid markets share 68.8 % of all the markets, having weak links with the two most important ones. Although Jalisco shows a profit, it mainly slaughters cull cattle, while the State of Mexico has a deficit and slaughters finished cattle. Hence, the Jalisco market occupies the centermost position in the

network, with its strength residing in the fact that it has the highest number of slaughterhouses in the country.

Conclusions and implications

Social-network analysis offers an alternative way to study the cattle-for-slaughter market. The structure of Mexico's cattle-for-slaughter market is characterized by a high level of intra-municipal trade resulting from an important municipal supply of slaughterhouses with low used capacity, while intrastate trade is mainly due to the State of Mexico's large deficit. Therefore, Mexico's national cattle-for-slaughter network is complete and with a high level of homophily, but with low levels of density and centrality.

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Review

Control and bacterial dissemination associated to cell death in *Mycobacterium bovis* infection. Review

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Abstract:

One of the hallmarks of *Mycobacterium bovis* infection is cell death. The type of cell death occurring during the infection determines the persistence of mycobacterial diseases. The aim of this article is to provide a comprehensive review and draw the possible scenarios of cell death types in the pathogenesis of bovine tuberculosis. The current data suggest that: 1) the development of apoptosis and its different variants is related to mycobacterial control, 2) autophagy is a conserved mechanism that limits mycobacterium intracellular replication, 3) pyroptosis is an extreme mechanism that helps control *M. bovis* at the cost of damaging host tissue, and 4) necrosis will allow the escape and proliferation of mycobacteria.

Keywords: Cell death, Bovine tuberculosis, *Mycobacterium bovis*, Apoptosis, Autophagy, Pyroptosis.

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Introduction

The way a cell dies plays a crucial role in physiological processes. In mycobacterial infections, some types of cell death have been cataloged as defense mechanisms of the host but also as consequences of the pathogen's virulence factors[1,2,3]. The Nomenclature Committee on Cell Death (NCCD) 2018, proposes classifying the types of cell death based on the mechanistic and essential aspects of the process, categorizing the majority within the group of regulated cell death[4]. Of this large group, some types have been reported in mycobacterial infections, for example, apoptosis[5-9], pyroptosis[10], ferroptosis[11], and necroptosis[12].

Mycobacterium bovis (M. bovis) belongs to the *Mycobacterium tuberculosis* complex. This species is the causative agent of zoonotic tuberculosis and the main etiologic agent of bovine tuberculosis (bTB). *M. bovis* affects many animal species[13,14]; therefore, it is a problem for public health and the livestock sector[15,16].

M. bovis is mainly transmitted by air, through exhaled droplets from the respiratory system of infected animals. A cellular immune response is developed, which is considered the main immune mechanism against intracellular bacteria[17,18]. The dinamics between, macrophages, neutrophils, fibroblast, dendritics cells, B cells, γδ T cells, CD4+, CD8+ lymphocytes, and pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interferon gamma (IFN-γ), give rise to the formation of the characteristic defense structure against mycobacteria: the granuloma[19,20,21].

Granulomatous lesions are characteristic of bTB, and are found mainly in lymph nodes and the lungs[22]. Its development varies in different lymph nodes of the same animal[23] and in addition, there has been reported variation in the structural morphology of granulomas from calves and adults[24], therefore granulomas in bTB are considered to have a heterogeneous presentation.

Cell death is one of the determining mechanisms in the formation and evolution of the granuloma that drives the development of the infection and presentation of the disease[25]. As

a consequence of the persistent nature of *M. bovis* infection, several types of cell death may occur, highlighting apoptosis and necrosis[5-9,25]. However, other modalities, such as pyroptosis and autophagy, may also play a role in the infection[10,26,27,28]. This review aims to provide a comprehensive summary of the types of cell death that have been identified in *M. bovis* infection and highlight their impact on the host. To achieve this goal, we present the information divided in two main sections: 1) Pathogenesis and immune response in bTB and 2) cell death pathways in bTB.

Pathogenesis and immune response in bovine tuberculosis

Bovine tuberculosis is transmitted by direct contact with infected excretion products (urine, saliva, milk, semen, uterine discharges) or mycobacteria present in exhaled droplets from the respiratory system of infected animals[29]. The respiratory system is mainly affected, including the lungs and associated lymph nodes[24,30,31]. Lesions in the digestive system have been related with transmission by ingestion of contaminated food[32], and transplacental transmission occurs in calves born with congenital infection[33].

bTB can be subclinical for long periods, symptomatic (fever, weight loss, respiratory distress, and decreased milk production), or have an evolution towards a generalized presentation as a consequence of the lymphatic or hematogenous dissemination of the mycobacteria changing to the early and late phases of the infection[14]. Factors such as the localization of the disease, the evolution of the primary lesion, mycobacterial virulence factor, bacterial concentration, development of granulomatous lesions and immunocompetence of the host, are determinants for the presentation of clinical symptoms[15,21,29,34,35].

The immune response plays a crucial role in the evolution of the infection in acute and chronic phases[36]. In particular, the cell-mediated responses are vital[37]. Since the respiratory system is one of the most affected by *M. bovis*, transcriptional and functional studies have been carried out in different cell populations of this system.

Alveolar macrophages are among the first cell populations infected by inhaled mycobacteria; therefore, they have been studied using different approaches. Transcriptomic analyses have revealed that the changes in gene expression are contrasting. For instance, a decrease in the expression of genes relevant to the recognition of *M. bovis*[37,38], and a greater polarization of macrophages towards a more permissive-replicative M2 phenotype[39] have been reported. On the other hand, genes that encode chemokines, recognition receptors and

proinflammatory molecules showed an increase upon infection with *M. bovis*[40,41]. And finally, this approach has also identified genomic variation related to both susceptibility/resistance to infection[42]. Another study using composition and lipid metabolism analysis, identified significant differences in the lipid group between *M. tuberculosis* related to the formation of foamy macrophages and *M. bovis* with the inhibition of autophagy[43].

These findings related to the protective and non-protective function of alveolar macrophages against infection, accompanied by a response dependent on mycobacterial species, demonstrate the determining role played by the mycobacteria/alveolar macrophage interaction both in the acute phase and in the evolution of the infection.

Neutrophils are another cell population important in mycobacterial infection[44]. Bovine neutrophils function as regulatory cells mainly in the innate immunity of clinical healthy cattle, but also in infected conditions[45]. For instance, bovine neutrophils exposed to *M. bovis* increased phagocytosis, cellular activation, secretion of pro-inflammatory cytokines and intracellular replication[46]. These results suggest that *M. bovis* infection could modulate the response in bovine neutrophils.

β-defensin-5 is an antimicrobial peptide stored in bovine macrophages and neutrophil granules. Incubating recombinant β-defensin-5 with *M. bovis* evidenced its time-dependent antimicrobial effects; this peptide inhibited growth by 88 % and disrupted the mycobacterial wall at 72 h of incubation[47]. The immunoprotective role of recombinant β-defensin-5 was also demonstrated. Recombinant β-defensin-5 from bovine neutrophils was used in the immunization of mice, which were then infected with *M. bovis*. The results showed a reduction in inflammatory tissue and in the bacterial load in the lung and spleen, demonstrating the potential of its immunoprotective function[48].

The changes in the structure of neutrophil nuclei have been suggested as a complementary diagnostic method for bovine tuberculosis. In human neutrophils exposed to serum from Purified Protein Derivative from Mycobacterium (PPD+) cows, after 3 h, pyknocytosis was the most common change observed in cell nuclei[49]. Additionally, specific pattern of expression of IFN-inducible transcriptional genes, myeloperoxidase(MPO) and pentraxin-related protein pentraxin-inducible protein (PTX3) genes, from neutrophils showed their potential as diagnostic tools for *M. bovis* infection in cattle[50]. Despite the modulatory effect that *M. bovis* apparently exerts on neutrophils, the antimicrobial findings of some of its intracellular molecules evaluated in a recombinant manner, could represent a field of research for biotechnological development with the potential for application in diagnosis and therapeutics.

Considering the importance of dendritic cells (DCs) in innate and adaptive immunity, some research groups have studied their response against *M. bovis* infection. A comparative analysis between bovine DCs and macrophages, both infected with *M. bovis*, identified lower production of nitric oxide (NO) and up to 10 times lower secretion of pro-inflammatory cytokines (IL-1**β** and TNF-α) in dendritic cells compared to macrophages. Moreover, DCs had a lower antimicrobial response to IFN-γ than bovine macrophages[51]. NO was also measured in murine DCs exposed to *M. bovis* and *M. bovis* BCG; the results showed lower production of NO in the population infected with *M. bovis* compared to the one infected with BCG; however, NO production increased significantly when adding IFN-γ[52]. Overall, these results suggest that DCs from these two species are permissive to *M. bovis* infection; however, IFN-γ only rescued NO production in murine DCs, evidencing a species-specific response.

Another study addressed the influence of bone marrow-derived DCs on the T lymphocyte profile in *M. bovis* infection in murine. Analyses of transcription levels, histopathology, and secretion molecules were carried out ten times during 56 d post-infection. The main findings were as follows: 1) Influence of high levels of prostaglandin-2 (PGE2) and cyclooxygenase-2 (COX2) mRNA on the cytokine profile (IL-17/IL-23); 2) Naïve LTCD4 were stimulated for differentiation towards Th17 and Treg, and 3) High bacterial load and tissue damage was observed in *M. bovis* infection. Considering these results, the researchers suggested that the induction of the PGE-2/COX-2 axis during infection with *M. bovis* contributes to sustained over-inflammation and could be related to the higher tissue damage[53]. The greater permissiveness, higher response to external stimuli, and differentiation of T lymphocytes under *M. bovis* infection, could represent a key mechanism of very early immune modulation by the mycobacteria.

Lymphocyte function is important in *M. bovis* infection because TCD4+ lymphocytes produce IFN-γ that induces the microbicidal activity of macrophages and CD8+ T cells have also shown lytic activity on infected cells[54,55,56]. A recent transcriptomic study compared whole blood from uninfected cattle and cattle experimentally infected with *M. bovis* at 8 and 20 wk post-infection. This study found that *M. bovis* infection upregulated chemokine genes such as monocyte chemoattractant protein 2 and chemokine (C-C motif) receptor 8 (CCR8), which are related to chemotaxis of monocytes and T lymphocytes, respectively, and downregulated genes related to class I antigen presentation and chemokines of neutrophils. The granzyme B gene was notably upregulated in the early and late stages of the infection, suggesting it may function as an infection biomarker. Since the genetic profile found high expression of cellular chemotactic genes and granzyme B, these are likely the most relevant defense mechanisms during the infection. In addition, the sustained transcription of granzyme B suggests that *M. bovis* antigens are being recognized by the population of cytotoxic T lymphocytes[57].

Although IFN-γ is a key cytokine in *M. bovis* infection, other circulating cytokines have been related to specific T lymphocyte populations. For example, T CD4+ lymphocytes and γδ T cells were identified as the main sources of IL-17 and IL-22, respectively, and a small population of γδ T cells produced both cytokines[58], besides a study in an experimental infection model found that the development of granulomas was directly related to increased IL-17 expression and decreased IL-22 expression. Therefore, the authors proposed IL-17 as a possible biomarker of bovine tuberculosis[59].

γδ T cells are particularly interesting, due to their production of IL-17 and also because this population is highly present in the circulation of bovines (up to 70 % in calves) compared to other species like humans and mice[60]. The functions of γδ T cells in bovines include antigen presentation, IFN-γ production, cytotoxic activity, and regulation of the immune response has been reported[20,61]. The genes expressed in subset WC1.1/T of γδ T cells from cows naturally infected with *M. bovis* were related to cell proliferation, activation, chemotaxis, and cytotoxic activity, evidencing their function on inflammation in bTB[62]. A wider expression profile was described by quantifying mRNA from circulating γδ T cells and advanced-stage lung and lymph node granulomas. The analysis identified IFN-γ and IL-17 as the genes with the greatest differential expression between circulating γδ T cells of infected vs uninfected cattle. Furthermore, CCL2, IL-17, IL-10, and IFN-γ showed the greatest expression in the γδ T cells surrounding the granulomas[63]. Overall, the production of chemoattractants, pro-inflammatory, and anti-inflammatory factors by circulating γδ T cells and those located in the infection site demonstrates their importance in the initial response and in maintaining the structure of the granulomatous lesion.

The series of cellular and molecular events induced by infection leads to the formation of granulomas, which are considered defense mechanisms against mycobacterial infections[64]. In bTB, granulomas are classified into four stages[65] that have been used as a study reference[21,30]. Previous work showed that granulomatous lesions in lung and mediastinal lymph nodes from naturally infected calves were devoid of capsules and displayed more necrosis and mycobacterial antigens than granulomas from adult cows[24]. In addition, granulomatous tissue from calves showed more CD3+ positive cells and higher concentrations of TNF-α, IFN-γ, and inducible nitric oxide synthase (iNOS), as well as fewer γδ T cells compared to granulomas of adult cattle[66]. These data suggest that age is a determining factor in the pathogenesis and immune response to bTB.

The humoral response to bTB was evaluated in 6-mo-old calves infected with different strains of *M. bovis*. The results identified antibodies against the antigens early secretory antigenic target (ESAT-6), culture filtrate protein (CFP10), and protein MPB83; however, the response was highly variable among animals and was predominant at week 18 post-infection. Moreover, antibodies directed against MPB83 remained constant from week 4 post-infection, regardless of the strain used[67]. MPB83, MPB70, and ESAT-6/CFP10 were also evaluated

in a comparative serological characterization performed in cattle, bison, and buffaloes naturally infected with *M. bovis*. In cattle, the predominant response was towards MPB70/MPB83; in bison, the response was similar towards the two antigenic groups; and in buffalo, the response was very low. Unlike ESAT-6/CFP10, which exclusively induces the production of IgG antibodies, MPB70/MPB83 were recognized by IgM and IgG antibodies. These results highlight the heterogeneity of the humoral response between species. Furthermore, the researchers hypothesized that *M. bovis* antigens induce the two antibody isotypes by reactivation at different times throughout the disease, which would explain the simultaneous presence of IgG and IgM[68].

Although most immunological studies in bovine tuberculosis have been directed to evaluate the response against infection using different strains of *M. bovis*, co-infection with other microorganisms has also been reported. For example with viruses[69], with other bacteria like, *Brucella*[70], and parasites[71-73]. In most of the works where co-infection with *M. bovis* is reported, a statistical positive correlation with greater susceptibility and severity of bTB is suggested, however, studies with a functional approach at the cellular, molecular and tissue levels are necessary to elucidate the immunological dynamics and the effect on the evolution of bTB in the same host.

The diversity of immunological responses to *M. bovis in vitro* and *in vivo* models and the capacity of *M. bovis* to infect around 85 animal species[74] highlight its high capacity for adaptation and development of different immune evasion mechanisms. Considering all of the above, it suggests that these key variables strongly influence the outcome of the infection: 1) The age and breed of cattle; 2) The immune response to the infection, i.e., the greater permissiveness of some cells, the cell populations involved, the type of cell death, maturation stage of granulomatous lesions, and co-infection. Studying these variables through a comprehensive approach could generate more systematic knowledge to understand the high heterogeneity of bovine tuberculosis.

Cell death pathway in bovine tuberculosis

Apoptosis or programmed cell death

Apoptosis consists of a series of molecular processes known as programmed cell death[26]. This concept was previously reported in silk moths[75], and the term apoptosis was only used

until 1972[76]. Research in this field has identified the genes involved in its initiation and regulation, which led to the award of the 2002 Nobel Prize in physiology[77]. Currently, it is known that caspases (cysteine-aspartic acid proteases) are the initiating proteins of apoptosis in humans[78].

The morphological changes observed during apoptosis are cell shrinkage and a decrease in the nucleus size, characterized by Deoxyribonucleic acid (DNA) fragmentation, chromatin condensation, and detachment of cells from the surrounding tissue. Apoptotic bodies are also formed; these are phagocytosed by cells that arrive at the site due to the exposure of phosphatidylserine in the apoptotic cell membrane[79]. Depending on the stimulus and the balance between an extensive group of pro- apoptotic and anti-apoptotic molecules, apoptosis can take two pathways: the intrinsic pathway (triggered by perturbations of the cell microenvironment, in particular, the mitochondria and endoplasmic reticulum) and the extrinsic pathway (induced by disturbances of the extracellular microenvironment and mediated by receptors)[79].

Some stimuli that activate the intrinsic mitochondrial pathway are hormones, radiation, toxins, hypoxia, and viral infections. These stimuli affect the permeability of the mitochondrial intermembrane[80], resulting in the release of pro-apoptotic proteins and cytochrome C to the cytoplasm. The interaction between apoptosis protease-activating factor-1 (Apaf-1) and caspase-9 forms the apoptosome, which activates the effector caspase 3. Furthermore, the Second Mitochondrial Activator of Caspases/Direct IAP-Binding Protein with Low pI (SMAC/DIABLO) inactivates an inhibitor of apoptosis factor (IAP). All molecular dynamics are regulated by proteins of the BCL-2 family of pro-apoptotic or anti-apoptotic nature, which are found in the cytoplasm and the outer membrane of the mitochondria[81,82].

Endoplasmic reticulum (ER) stress is associated with apoptosis. ER stress may be caused by loss of intracellular calcium balance, accumulation of misfolded proteins in the lumen of the ER, and disturbed protein transport to the Golgi apparatus[83]. These conditions activate the unfolding protein response (UPR) system, composed of proteins such as inositol-requiring protein-1 (IRE1α) and protein kinase RNA (IPK-R)-like ER kinase (PERK), which activate accessory molecules or interact with each other to either restore balance or induce cell death[84]. During a prolonged period of ER stress, the expression of pro-apoptotic proteins increases, and they interact with other molecules to promote apoptosis. For example, IRE1α activates apoptotic signaling-regulating kinase-1 (ASK1), which initiates a cascade of reactions that lead to the activation of pro-apoptotic molecules (Bim) and inactivation of anti-apoptotic molecules (Bcl-2)[85,86,87].

The extrinsic pathway of apoptosis is induced by receptor-ligand interactions. The most important ligands and receptors for apoptosis belong to the Tumor Necrosis Factor superfamily. Ligands can interact with one or more receptors, and most receptors are

transmembrane proteins with an extracellular N-terminal that interacts with the ligand and an intracellular C-terminal that has a death domain[88]. The interaction with this death domain can activate effector caspases through several pathways. For example, the FAS/FASL interaction along with adapter proteins can bind to pro-caspases 8 and 10 and subsequently activate effector caspases by autocatalysis[89] or form protein complexes that activate or inhibit caspases, as occurs with the TNF receptor[90,91].

Apoptosis is an essential mechanism to maintain cellular homeostasis[92-98] and also represents a defense mechanism in the immune response, especially against intracellular pathogens[99].

Role of apoptosis in *Mycobacterium bovis* infection

In mycobacterial infections, apoptosis has been associated with reduced bacterial spread and viability[1,2]. However, virulent mycobacterial strains and antigens may inhibit apoptosis in cells infected previously[2,3].

Complete mycobacteria

One of the first publications reporting apoptosis in *M. bovis*-infected bovine macrophages showed that cell death occurred as early as 4 h post-infection using different multiplicities of infection (MOI). The authors concluded that apoptosis was time and MOI-dependent[5]. Using the same cell model, apoptosis was enhanced by IFN-γ/LPS and diminished by blocking TNF-α. In addition, in the presence of IL-10, mycobacterial intracellular replication was inversely related to apoptosis, suggesting that apoptosis plays a protective role against infection[6].

The Rodriguez group compared mice previously infected with attenuated *vs* virulent *M. bovis* strains. They identified that the virulent strain had a greater capacity to inhibit apoptosis in alveolar macrophages. In addition the apoptosis was decreased by IL-10 and increased by TNF-α[7]. The previous findings were carried out *in vivo* and *in vitro* in macrophages infected with *M. bovis*. This study demonstrates that mycobacteria modulate apoptosis through

cytokine production, level of virulence, and exposure dose. In Table 1, there are some of the most relevant findings of apoptosis in infection with the main causal agent of bTB.

Natural resistance against a disease is defined as the ability of the host to resist the development of a disease after the first exposure to the pathogen and without prior immunization[8]. Natural resistance to mycobacterial infections in cattle has been reported by several authors. For example, the Esquivel-Solis group[9], compared apoptosis and microbicidal activity in resistant and susceptible bovine macrophages infected with *M. bovis*. The findings indicate that apoptosis increased in macrophages with high NO levels, suggesting a relationship between apoptosis and microbicidal activity in the resistant phenotype[9]. These results coincide with those obtained from resistant macrophages infected with *M. tuberculosis*[100,101]. The effect of IL-4 was studied in bovine macrophages in both phenotypes. The results show a decrease in the expression of pro-inflammatory genes and a lower tendency towards apoptosis in resistant macrophages, evidencing that alternative activation by IL-4 increased susceptibility to infection in resistant macrophages[102]. The relationship between NO production, apoptosis, and intracellular survival of mycobacteria was also evaluated in dendritic cells in mice. Apoptosis (DNA fragmentation and caspases 3, 6 and 9) and bacterial concentration were quantified in the absence or presence of an iNOS inhibitor. Results from this study showed that: a) the population infected with BCG showed more apoptosis compared to *M. bovis*, b) in the presence of the inhibitor, apoptosis was significantly reduced in both infected populations, and c) *M. bovis* survived better than BCG in DCs. These results suggest that the reduced production of NO by dendritic cells due to the infection with *M. bovis* modulates the development of apoptosis and increases the possibility of mycobacterial survival[52]. These results highlight the role of nitric oxide in apoptosis in the early phases of infection.

Several research groups have focused on specific intracellular targets to understand the mechanisms and organelles involved in apoptosis. Vega, *et al*[103] in 2007 suggested an association between apoptosis and the nuclear translocation of Apoptosis-Inducing Factor (AIF) and mitochondrial membrane depolarization in macrophages exposed to an *M. bovis* protein extract[103]. This prompted the investigation of other apoptosis-associated components, for example, the impact of mitochondrial permeability on DNA fragmentation and mycobacterial viability in bovine macrophages infected with *M.bovis*. DNA fragmentation decreased independently of caspase activity when mitochondrial permeability was inhibited. Furthermore, the translocation of AIF and Endonuclease G (Endo-G) to the nucleus, measured by immunoblot, increased 15 and 43 times, respectively, and the viability of the intracellular mycobacteria increased by 26 %[104]. These results support the idea that the translocation of Endo-G to the nucleus is also involved in DNA fragmentation as a result of *M. bovis* infection by altering mitochondrial permeability. The identification of molecules such as Endo G and AIF in the nucleus and the decreased intracellular mycobacterial viability in the absence of activated caspases, suggest that caspase activation is not required for DNA

fragmentation and reveals the existence of different mechanisms in the development and modulation of apoptosis, especially during infection.

Mitochondrial stress induced by *M. bovis* infection was also evaluated in THP-1 cells. It was found that apoptotic caspases negatively modulate IFN-β production by reducing the nuclear translocation of p-IRF3 (Interferon Regulatory Factor 3)[105]. This represents a beneficial scenario for the host since a lower IFN-β in *M. bovis* infection has been associated with a better prognosis[106].

The endoplasmic reticulum stress induced by mycobacterial infection was investigated in murine macrophages previously infected with *M. bovis*. This study showed a higher intracellular survival of mycobacteria upon adding an ER stress inhibitor, which directly modulated the percentage of apoptotic cells[107]. The relationship between apoptosis and the functionality of the mitochondria and ER and its impact on intracellular mycobacterial viability highlights the protective effect of apoptosis against infection[108]. However, these organelles and pathways can also become targets for mycobacteria modulation.

Activated caspases have been used as the only marker of apoptosis in mycobacterial infections[109]. Nevertheless, caspase-independent apoptosis has been identified in cattle and buffaloes infected with *M. bovis*[103,110]. Furthermore, since apoptosis limits the intracellular growth of mycobacteria, the absence of caspase activation could represent a mechanism of evasion of cell death by *M. bovis*[107].

Most approaches to investigating apoptosis in mycobacterial infection have been carried out in cell models (mainly macrophages), allowing the study of the protective role of apoptosis in the acute phase. However, due to the persistent nature of the infection, apoptosis should also be studied in the chronic phase of mycobacterial infection. The Cherdantseva group reported that apoptotic cells corresponded to approximately 11 % of cells in lung granulomas of mice infected with *M. bovis*-BCG after 180 days of infection. These data suggest that, although apoptosis is induced at the tissue level, it is insufficient to eliminate the mycobacteria during the development of the pathological process[111].

Proteins from *M.bovis*

The dual role of apoptosis in mycobacterial infection has been observed in studies using individual *M. tuberculosis* antigens, in which the antigens are classified as pro-apoptotic or

anti-apoptotic. These studies suggest that mycobacteria modulate the cell death mechanism through dynamics of antigenic expression[2]. In this context, to identify specific *M. bovis* apoptosis-inducing proteins, bovine macrophages were exposed to different protein extract fractions. Caspase-independent apoptosis was induced by two *M. tuberculosis* recombinant proteins, hsp70 and heparin-binding haemagglutinin (HBHA), which have high homology with *M. bovis* in bovine macrophages[112]. In this regard, efforts have been made to determine the protein profile in the protein extracts of *M. bovis*. Using mass spectrophotometry, MPB70, MPB83, and 60-kDa chaperonin were identified as the main protein candidates that induce caspase-independent apoptosis[28].

M. bovis and *M tuberculosis* have a high genomic homology[113]. Therefore, investigating the modulatory effect exerted by highly homologous proteins in the two species could identify the new mechanisms in cell death. This knowledge would allow to understand the particularities of the infection and the general pathogenesis of bTB.

All together, the above mentioned results indicate that apoptosis is a multifactorial event involving characteristics of the bacteria (such as virulence, time and multiplicity of infection) and intrinsic characteristics of the affected host. However, despite the effect of these multiple variables, apoptosis and intracellular growth of mycobacteria in bovine macrophages are inversely correlated, which suggests that apoptosis in *M. bovis* infection represents a host defense mechanism.

Necrosis or accidental cell death

The term necrosis comes from the Greek "necro," which means corpse or death and “osis”, which means condition or state. Necrosis was used to describe the morphological death of cells resulting from infection, cell damage, noxious stimuli, or mechanical damage; therefore, necrosis was thought to be due to abrupt changes leading to accidental cell death[114]. Pathological diagnosis evaluates macroscopic and microscopic features in the affected tissue and classifies necrosis as coagulative, fibrinoid, hemorrhagic, and caseating[115].

In *M. bovis* infection, necrosis is present in the advanced stages of granulomatous lesions[65]. In addition, unregulated necrosis has been associated with a higher spread of mycobacterial infection[116]. An analysis of granulomas from cattle naturally infected with *M. bovis* showed large necrotic areas with central calcification, no connective tissue capsule, and few giant cells. Necrosis was the predominant cell death observed, and it was accompanied by more

mycobacterial antigens, which was mainly observed in calves[24]. Moreover, inducing necrosis using hydrogen peroxide in *M. tuberculosis*-infected macrophages favors the escape of the bacteria to the extracellular medium without affecting its viability[117]. In addition, other necrosis induction assays allow the exit and proliferation of mycobacteria[118] .

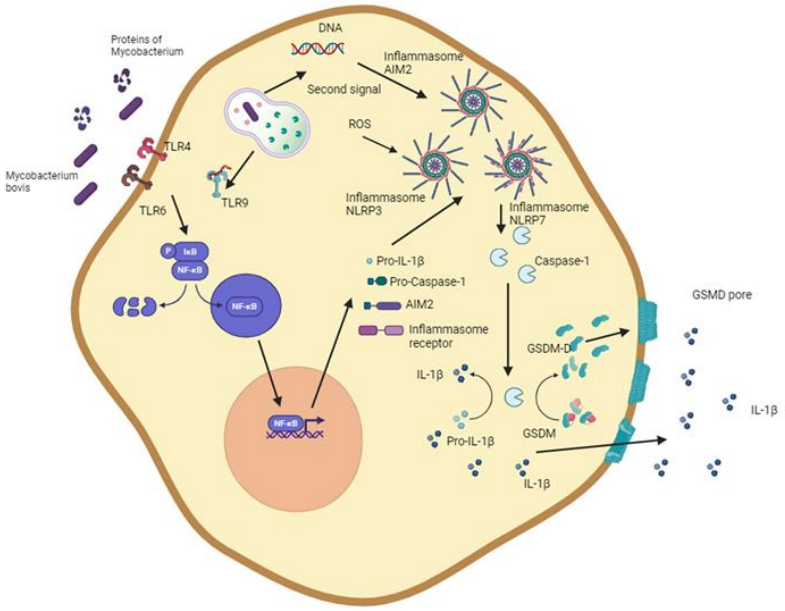
Different types of regulated cell death

In recent decades, biochemical and molecular advances have enabled the discovery of some types of necrosis that are not accidental, rather, they follow regulated signaling pathways that produce a necrotic morphology[99,100,119,120]. The description of these signaling pathways has helped define the diverse pathways of cell demise that lead to necrotic cell death. Among the different types of necrotic cell death are necroptosis, pyroptosis, among others[99,101,102,103,119,121,122,123].

Pyroptosis

The term pyroptosis is derived from the Greek “pyro” (fire, fever) and “ptosis” (falling)[104]. Pyroptosis was first described in *Salmonella* and *Shigella in vitro* infection models, in which caspase 1 initiated cell death[104,105,106,124,125,126]. Pyroptosis is an inflammatory cell death classically characterized by the inflammasome, caspase 1, gasdermin D (GSDMD), and the release of IL-1β and IL18 (Figure 1).

Figure 1: Routes of induction of pyroptosis by *Mycobacterium bovis*



The diagram shows the ability of *M. bovis* and mycobacterial proteins to activate NLRP3 and AIM2 inflammasomes. The activation of the NLRP3 inflammasome is initiated through pattern-recognition receptors and then by multiple stimuli such as the generation of reactive oxygen species, potassium efflux, or lysosomal components. Activation of the AIM2 inflammasome is initiated by bacterial DNA recognition. The assembly of the inflammasome leads to the maturation of IL-1β and the cleavage of gasdermin, forming gasdermin D which damages the cell membrane and results in necrotic cell death. This figure was created using BioRender.com.

The inflammasome, which becomes activated in pyroptosis, consists of multiprotein structures including a receptor of the NLR (nucleotide-binding oligomerization domain-like receptors) or AIM myeloma 2 (AIM2)-like receptors families, as well as the ASC (Apoptosis-associated speck-like protein containing a CARD) and pro-caspase 1[107,108,127,128]. However, less frequently, pyroptosis can be activated by an alternative pathway. Activation of the inflammasome leads to the activation of inflammatory caspases (caspase-1,-4,-5 in humans and caspase-1 and -11 in mice) and the cleavage of the interleukin-1 family and GSDMD. The active GSDMD can assemble to form pores in the cell membrane and generate an osmotic imbalance that leads to cell death under an inflammatory environment[109,110, 129,130]. *M. bovis* can induce pyroptosis in macrophage cells and macrophage-derived cell lines (Table 1). The strain of the bacterium, the multiplicity of infection, and the time after infection are among the factors that favor pyroptosis[10,131,132,133]. The main mechanisms that induce pyroptosis are related to the canonical activation of inflammasomes (Figure 1). NLRP7, which recognizes bacterial glycoproteins; AIM2, which recognizes double stranded DNA; and NLRP3, which is activated by various signals, such as potassium efflux, ROS, extracellular ATP, pore-forming toxins, and mediate pyroptosis associated to *M. bovis* infection[111,112,113,131,132,133]. The activation of inflammasomes affects the production of IL-1β, IL-18, and IL-33, generating an inflammatory environment that helps control the infection produced by mycobacteria[132].

Inflammasome NLRP3 activation requires two signals and generates an inflammatory environment. Stimulation of macrophages with LPS increases IL-1 β and nitric oxide, which may limit the intracellular growth of mycobacteria[9,10]. Activating the inflammasome by *M. bovis*-infected bovine macrophages decreases the intracellular growth of mycobacteria[10].
The inflammatory environment generated by pyroptosis can regulate the proliferation of bacteria, recruiting immune cells that help control bacterial infections. However, pyroptosis can cause tissue damage, therefore, it represents a strong mechanism that some host cells have to control bacterial intracellular growth. Of note, there is currently no information on which bacteria strains commonly induce pyroptosis. It is also unknown whether bacterial growth is controlled or whether some bacteria induce this type of cell death to escape from the cells and infect the surrounding tissues[12,134].

Autophagy

The term autophagy is derived from the Greek "*auto*" (self) and "*phagen"* (to eat). Autophagy is a highly conserved pathway that degrades cellular components using lysosomes[135]. Autophagy is a regulated mechanism that allows cells to survive under nutrient deprivation or adverse conditions. However, autophagy can also cause cell death (autophagy-cell death dependent). This mechanism can occur concomitantly with another type of cell death, such as apoptosis , or start as autophagy and trigger apoptosis[136].

Autophagy has been shown to limit intracellular bacteria. Some of the molecules involved in this process are myeloid-related protein 8/14 and interferon-γ inducible protein 204 (IFI204) that induces autophagy in peripheral blood mononuclear cells and THP1 cells in a ROS-dependent manner, which inhibits the intracellular growth of *Mycobacterium* BCG (Table 1)[137]. Moreover, IFI204 is a DNA sensor that activates the innate immune response, including autophagy and interferon-β production (IFN-β). IFI204 proteins are involved in IFN-β responses by recruiting STING to activate TBK-1-IRF3 pathways. Induction of autophagy by IFI204 induces phosphorylation of TBK-1 to inhibit *M. bovis* survival in macrophages[138].

Importantly, *M.bovis* can evade autophagy. One of the mechanisms consists in the specific inhibition of autophagy responsible for the control of intracellular organisms (xenophagy), for example, through the activation of the PINK1-PRKN/Parkin indicating pathway involved in mitophagy, which generates a competition of both pathways for p-TBK1 leading to a

decrease in xenophagy and the survival of the mycobacteria[139]. The role of the microRNA miR-199a was evaluated in macrophages derived from bone marrow, lung, and spleen of *M. bovis*-infected mice. The infection increased the expression of miR-199a, and this suppressed autophagy by blocking phagolysosome maturation through the interaction with TANK binding Kinase 1. These changes led to an increase in intracellular survival of the mycobacteria. These results provide a mechanism for *M. bovis* to evade elimination[137].

Although the development of autophagy participates in maintaining cellular balance, it may also function as an innate immune response mechanism that limits the growth of intracellular bacteria. In infections with *M. bovis*, autophagy is induced by low-virulence bacteria, suggesting that *M. bovis* may also modify processes involved in sustaining cellular homeostasis[140,141].

Conclusions

Regardless of the influence of different variables (such as virulence, time, species, and the host resistance phenotype) on apoptosis, experimental results suggests that cell death by apoptosis helps to control bacterial growth.
The bacterial inhibitory effect on apoptosis, the redirection of autophagy, and the induction of inflammatory cell death such as necrosis and pyroptosis may be bacterial mechanisms to evade the host immune response.

Although experimental conditions allow the detection of a specific type of cell death, the simultaneous activation of multiple types of cell death, known as PANoptosis, has also been observed in *M. tuberculosis* infection. This scenario opens the possibility of studying *M. bovis* infection in a global manner that considers all experimental variables and phases of the different cell death types.

The high adaptability of *M. bovis* and the key role of cell death in immune activation highlight the need for more studies on regulated and non-regulated cell death. These studies will increase the understanding of bovine infection and aid in developing new strategies to counteract bovine tuberculosis.

The most important points of this review can be numbered in: 1) Cell death by apoptosis helps to control bacterial growth. 2) Autophagy is a conserved mechanism that limits mycobacterium intracellular replication. 3) Pyroptosis is an extreme mechanism that helps

control *M. bovis* at the cost of damaging host tissue. 4) Necrosis will allow the escape and proliferation of mycobacteria.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 1: Cell death in *Mycobacterium bovis* infection

Strain	Protein	+Target molecule	Evaluation model	Mycobacterial load*	Remarks	Reference
Apoptosis						
M. bovis Wild type	---	Chromatin condensation, and fragmentation DNA	Macrophages derived from bovine monocytes	ND	Apoptosis induced for *M. bovis* infection	(5)
M.bovis ATCC 35723	---	Mono and oligonucleosomes in cell lysates	Macrophages derived from bovine monocytes	Decreased	Apoptosis, enhanced for IFN-γ and diminished for IL-10	(6)
M.bovis 9926	---	DNA fragmentation	Macrophages$^{(R)}$ derived from bovine monocytes	Decreased	Increased apoptosis in resistant macrophages	(9)
M.bovis C68004	---	Caspases 3 and 9	Murine macrophages and THP-1 cells	ND	Negative modulation of apoptotic caspases on IFN-β	(105)
M. bovis ATCC	---	Annexin V	Macrophages derived from BALB/C mice	Decreased	Virulent strain has a greater capacity to inhibit apoptosis	(7)
M.bovis Beijing	---	Caspases 3 and 9	Murine macrophages	Decreased	*M. bovis*-induced apoptosis depends in part on endoplasmic reticulum stress	(107)
M.bovis AN5	---	DNA fragmentation	Macrophages derived from bovine monocytes	Decreased	Translocation of Endo G to the nucleus in *M. bovis*-infected macrophages	(104)

M.bovis 9926	Protein extract	Chromatin condensation, fragmentation DNA and caspase 3, 8 and 9	Macrophages derived from bovine monocytes	ND	Translocation of AIF to the nucleus in *M. bovis*-infected macrophages	(103)
M.bovis AN5	Protein extract	DNA fragmentation and caspase 3	Macrophages derived from bovine monocytes	ND	Caspase-independent cell death by hsp70 and HBHA proteins	(112)
Pyroptosis and cell Death related with inflammasome						
M. bovis Beijing	---	AIM2 inflammasome markers, LDH released	J774A.1 macrophage cultures and bone-marrow derived macrophages (BMDMs)	ND	The activation process requires cytoplasmic potassium efflux, mycobacterial internalization.	(132)
M. bovis Beijing	---	LDH release, NLRP7, IL-1β	THP-1 cells	ND	NLRP7 is uniquely stimulated by microbial acetylated lipopeptides	(133)
M. bovis BCG strain *Moreau*	---	Caspasa-1, LDH release, IL-18, IL-1β	Human mononuclear cells	ND	Induction of IL-1β but not of IL-18, induces cell death with membrane damage	(142)
M.smegmatis transfected with sequence *M. bovis*	PPE13	NLRP3 inflammasome, markers	J774A.1, BMDMs and THP-1	Decreased	Enhanced-IFN-γ and diminished-IL-10	(143)
*M. bovis AN5/*CFPE	---	LDH release, NLRP3, IL-1β, PI	Macrophages derived from bovine monocytes	Decreased	Activation of NLRP3 inflammasome and gasdermin D cleavage	(10)

Autophagy						
M. bovis BCG	MRP8/14	Flow cytometry, LC3	THP-1	ND	MRP8/14 promoted autophagy in a ROSdependent manner	(140)
M. bovis C68004 strain	PP2Ac	LC3, AMPK pathway	Murine macrophages (BMDM and RAW264.7)	ND	TKI-induced AMPK activation was dependent on PP2Ac regulation	(144)
M. bovis BCG	LRG-47	LC3, Beclin-1,	RAW264.7	ND	IFN- induced autophagy in macrophages	(141)
M. bovis C68004 strain	—---	LC3, HSPD1, LAMP-1	J774.1 and BMDM C57BL/6 mice	ND	PINK1-PRKN/Parkin pathway is involved in the mitophagy induced by *M. bovis*	(139)

+Target molecule: molecule selected to evaluate cell death, (R): Resistance phenotype, ND: Not Determined, Mycobacterial load*: Quantified in the presence of the specific type of death concomitantly, AIF: Apoptosis Inductor Factor, HBHA: heparin-binding haemaglutinin, AIM2: absent in melanoma 2, LDH: lactate dehydrogenase, NLRP3: NOD, leucine-rich repeats and pyrin domain-containing protein 3, NOD, leucine-rich repeats and pyrin domain-containing protein 7, LC3: Microtubule-associated protein 1A/1B-light chain 3, LAMP-1: lysosomal associated membrane protein 1, HSPD1: heat shock 60-kDa protein 1, PPE: Pro-Glu motif-containing (PE) and Pro-Pro-Glu motif-containing (PPE) family proteins, BMDM: bone-marrow derived macrophages, MRP8/14: Myeloid-related proteins (MRPs) 8 and 14, LRG-47: IFN-inducible protein Irgm1.

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Technical note

Comparison of machine learning methods for predicting genomic breeding values for growth traits in Braunvieh cattle

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Abstract:

Machine Learning (ML) algorithms have proven advantageous in addressing challenges associated with the quantity and complexity of information, discovering patterns, performing efficient analyses, and serving as a decision-making tool. The objective of this study was to compare four ML methods —artificial neural networks (NN), regression trees (RT), random forests (RF), and support vector machines (SVM)— for predicting genomic value in European Swiss cattle using phenotypic records of birth weight (BW), weaning weight (WW) and yearling weight (YW), as well as genomic information. The results indicate that the predictive ability of the models varies according to the features and the amount of information available. NN, RF, and SVM exhibited similar performances, while RT underperformed. The SVM methodology stood out as the tool with the greatest potential, achieving the highest values of Pearson correlation between corrected phenotypes and

predicted genetic values for WW. Despite its higher computational cost, the NN performed reasonably well, especially for BW and YW. The selection of the final model depends on the specific requirements of the application, as well as on such practical factors as data availability, computational resources, and interpretability; however, in general, the NN and SVM emerged as solid choices in several categories.

Keywords: Neural networks, Predictive capacity, Random forests, Regression trees.

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Genomics has evolved in recent years thanks to advances in DNA sequencing technology. This progress has allowed the generation of large amounts of data at an unprecedented speed. However, the inherent complexity of genomic data, as well as its dimensionality, pose significant obstacles[1]. The diversity of genomic information, ranging from DNA sequences to associated phenotypic data, adds further complexity. In addition, variability in the quality and structure of genomic data can make it difficult to extract useful and meaningful insights. Within this context, machine learning (ML) methods emerge as valuable tools to address these challenges, offering the ability to process and analyze large volumes of data efficiently and accurately[2]. Their ability to identify complex patterns and nonlinear relationships in genomic and phenotypic data makes them a powerful tool for knowledge extraction[2,3].

The application of ML techniques allows for addressing such tasks as the identification of genes relevant to specific traits, prediction of gene functions, detection of genetic variants associated with particular traits, and classification of species based on genomic information[4,5,6]. Recently, ML has become attractive in genomic prediction because of its ability to handle large volumes of data, its flexibility in modeling nonlinear relationships, improving predictive accuracy, and continuous innovations in algorithms and techniques; nevertheless, research is needed to investigate how it compares in predicting genetic values with conventional GBLUP methods[7]. Combining genomic data with ML algorithms would lead the creation of reliable predictive and descriptive models, which in turn would have implications for selective breeding, species conservation, and the understanding of evolution[8,9].

Among the most commonly used ML methods are neural networks, support vector machines, decision trees, linear regression, and clustering methods[3,8-11]. The diversity of available approaches reflects the versatility of these methods in solving challenges involving genomic information, such as DNA sequence classification and protein structure prediction[12]. The

success of the application of these methods in animal genomics depends to a large extent on the availability of information[13], as well as on selecting the optimal ML method, given that several methods have been proposed, each with its own characteristics and specific predictive capabilities with different data sets and features[3,7].

Thus, the objective of this study was to compare the following ML methods —neural networks (NN), regression trees (RT), random forests (RF), and support vector machines (SVM)— to predict genomic breeding values using phenotypic records of birth, weaning and yearling weights, as well as genomic information of a population of Swiss European cattle in Mexico.

The information was drawn from the database of the Mexican Association of Registered Swiss Cattle Breeders (Asociación Mexicana de Criadores de Ganado Suizo de Registro, AMCGSR), which contains phenotypic records and animal identification, ranch of origin or owner, genealogy, and economically important traits such as birth weight (BW), weaning weight (WW) and yearling weight (YW). The data set used was previously analyzed by Valerio-Hernández *et al*[14,15] to fit other models, so that some of the results obtained here compare directly with those of the authors mentioned above. The treatment of phenotypic information for BW, WW, and YW followed the procedure described by Valerio-Hernández *et al*[14,15], i.e., individuals with missing information on maternal age, management, herd of origin, as well as individuals not genetically related were omitted. Contemporary groups (CG) were defined by combining the effects of herd, year, and time of birth. For WW, the CG were formed according to the feeding management given to the herd, as well as adjustment to specific days for weaning. CG with less than three individuals or with zero variance were discarded, according to the methodology cited above[14].

Genomic information was obtained through the analysis of hair samples collected from 300 animals from ranches belonging to the AMCGSR in Colima, Jalisco, and Veracruz. Genotyping was performed by GeneSeek (Lincoln, NE, USA), using the Genomic Profile Bovine LDv.4 chip, which has been used to genotype various *Bos indicus* and *Bos taurus* breeds. A total of 150 animals were genotyped with a chip containing 30,000 markers, and another 150 animals were genotyped using a chip with 50,000 SNP (Single Nucleotide Polymorphism) markers. A total of 12,835 SNP markers present in both chips were selected.

The recoding of additive genetic effects such as AA=0, AB=1, and BB=2 and the quality control of genotypic information carried out by Valerio-Hernández *et al*[15] were based on that performed by Jarquín *et al*[16]. For the imputation of missing genotypes in the present study, it was used the FImpute[17] software (version 2.2), this process yielded 1). A marker map (marker, chromosome, base-pair position), eliminating duplicate markers or markers with unknown positions, and 2) The pedigree of the individuals and their corresponding sex. Monomorphic markers and those with a minor allele frequency (MAF) lower than 0.04 were

eliminated. A total of 9,008 markers were obtained and used to build the genomic relationship matrix **G**; Table 1 shows the number of animals incorporated into the study for each trait after filtering.

Table 1: Number of animals from a Braunvieh cattle population genotyped and phenotyped for three growth traits

Group/Variable	BW	WW	YW
Genotyped	300	300	300
Phenotyped	330	267	232
Phenotyped in $\mathbf{G}^2$	232	218	191

BW= birth weight, WW= weaning weight, YW= yearling weight. $\mathbf{G}^2$ Animals with phenotypes and genomic information.

The genomic relationship matrix **G** was estimated using the methodology described by Pérez-Rodríguez *et al*[18], $\mathbf{G}=\mathbf{WW}^t/p$, where **W** is the centered and standardized marker matrix and p is the total number of markers. Additionally, the relationship matrix **H**, which combines information from the **G** matrix with information from the additive genetic relationship matrix **A**, obtained for pedigree individuals.

Linear mixed models (Base Models). Comparison of the results of predictive power for the BW, WW, and YW breeding values considers the sequence of models and results described by Valerio-Hernández *et al*[15] for linear mixed models versus machine learning models. In order to present all the pertinent information, the linear mixed model used by these authors is described below:

$$\boldsymbol{y} = \boldsymbol{Xb} + \boldsymbol{Z}_1\boldsymbol{c} + \boldsymbol{Z}_2\boldsymbol{a} + \boldsymbol{e}, \ldots (1)$$

where $\boldsymbol{y}$ is the phenotype vector, $\boldsymbol{X}$ is the incidence matrix for fixed effects —which for this study are sex of the animal, age of the mother of each animal, and the contemporaneous group, described above—, $\boldsymbol{b}$ is the vector of fixed effects, $\boldsymbol{Z}_1$ is an incidence matrix connecting phenotypes with contemporaneous groups, whose effects are assumed to be random and represent the variability in phenotypes due to differences between groups of individuals that are subject to the same environmental and management conditions, $\boldsymbol{c} \sim NM(\mathbf{0}, \sigma^2_{gc}\boldsymbol{I})$, where NM denotes the multivariate normal distribution, with mean **0** and associated variance parameter σ^2_{gc}, **I** the identity matrix, $\mathbf{Z_2}$ is an occurrence matrix connecting phenotypes with additive genetic effects which are assumed to be random effects, $\boldsymbol{a} \sim MN(\mathbf{0}, \sigma^2_a\boldsymbol{K})$, with $\boldsymbol{K} \in \{\boldsymbol{A}, \boldsymbol{G}, \boldsymbol{H}\}$, $\boldsymbol{e} \sim MN(\mathbf{0}, \sigma^2_e\boldsymbol{I})$ represents the random error vector, where σ^2_e denotes the variability associated with it. Depending on the data used, model (1) gives rise to three different models, denoted as follows: 1) BLUP, $\boldsymbol{K} = \boldsymbol{A}$, 2) GBLUP, $\boldsymbol{K} =$

$\boldsymbol{G}$, and 3) ssGBLUP (single-step GBLUP) with $\boldsymbol{K} = \boldsymbol{H}$. The linear mixed models described above were fitted by Valerio-Hernández *et al*[15] using the BGLR statistical package[19].

Machine learning models. The input variables for the ML algorithms were the genetic relationship matrix combining genomic information and pedigree information called **H**, as well as the effects of dam's age for each animal, indicator variables for sex, and contemporaneous group described above. In order to include the information of the **H** matrix in the learning models, a spectral decomposition of the matrix was performed, i.e., $\boldsymbol{H} = \boldsymbol{\Gamma\Lambda\Gamma}^{t}$, from which $\boldsymbol{X} = \boldsymbol{\Gamma\Lambda}^{\frac{1}{2}}$ (main components) were obtained and utilized as covariables (explanatory variables) in the models; this and other related computational strategies have been used by other authors in the past[20,21].

Artificial neural network. Neural networks (NN) were initially designed to emulate the functioning of the nervous system and which process input information through mathematical operators, generating output values or the final result[3,22]. Input variables affect model's performance and can generate overfitting if the amount of information is large; therefore, it is important to optimize these variables[23]. One of the advantages of neural networks is their ability to learn nonlinear patterns[3]. The model of an NN with an input layer with p predictors, a hidden layer with S neurons, and an output layer with a continuous response can be expressed as follows:

$$y_i = \beta_0 + \sum_{k=1}^{s} w_k \, g(\beta_0^{(k)} + \sum_{j=1}^{p} \beta_j^{(k)} x_{ij}) + e_i,$$

where $e_i \sim NIID(0, \sigma_e^2)$, with *NIID* denoted by normal, independent, identically distributed random variables; $k = 1, \ldots, S$ (neurons); $j = 1, \ldots, p$ (predictors); $i = 1, \ldots, n$ (observations), and $g(\cdot)$ represents the activation function, according to Bai *et al*[24] and Gianola *et al*[20], where, y_i is the response variable for the i^{th} individual, in this case, the growth weights (BW, WW, YW) of Braunvieh cattle that the network predicts as a function of inputs; β_0 is the bias term or the intercept, which can represent the predicted value when the inputs are equal to zero, and w_k are weights associated with each of the neurons and determine the contribution of each neuron to the final prediction. The hidden layer is an intermediate layer between the input layer and the output layer, it is where most of the processing and feature extraction of the dataset take place; it is composed of a specific number of neurons. (*S*) is a hyperparameter of the model that is adjusted during the training process. A higher value of *S* allows the neural network to capture greater complexity in the data, but may also increase the risk of overfitting. The NN adjusts the parameters $(\beta's, w's)$ during the training process to minimize the prediction error. The activation function, $g(\cdot)$, maps the real line entries to the bounded open interval (-1,1), as described by Pérez-Rodríguez *et al*[25], where $g(x) = 2/[1 + \exp(-2x)] - 1$ is known as the hyperbolic tangent activation function (htaf). The "brnn" function was used to fit the neural network model[26]

included in the package of the same name (version 0.9.3) in the statistical package R[27] (version 4.3.0).

Regression trees. This model is based on the one proposed by Breiman *et al*[28], $y_i = \sum_{j=1}^{J} y_j I(\boldsymbol{x}_i \epsilon\, R_j)$, where y_i is a response variable (BW, WW, and YW), y_j is the regression value associated with a "leaf", $\boldsymbol{x}_i$ is the set of characteristics of the observation, R_j is the region associated with "leaf *j*" defined by characteristics and cutoff values on the path from "root" to "leaf". $I(\cdot)$ is an indicator function that takes the value 1 if observation *i* belongs to the region R_j. The tree identifies the splits that minimize the error in each region and split recursively until a process-stopping criterion is reached, such as the maximum depth of the tree or the minimum number of cases in a leaf. The model fitting was performed with the "rpart" function[29] included in the library of functions of the same name (version 4.1.19) within the statistical package[27] (version 4.3.0).

Random forests. This model combines multiple RTs averaging the predictions of each to obtain a final optimized prediction, $y_i = \frac{1}{N}\sum_{j=1}^{N} y_{ij}$, where *N* is the number of trees in the random forest, y_i is an observed random variable (BW, WW, and YW), and y_{ij} is the prediction of the *j*th RF for the observation *i*. The random forests algorithm was implemented using the "randomForest" function[30] included in the library of functions of the same name (version 4.7-1.1) within the statistical package R[27] (version 4.3.0).

Support vector machine. The Support Vector Machine Model (SVM) was used for classification and regression[31]. Within the context of regression, given a data set $\{y_1, \boldsymbol{x}_1\}, \ldots, \{y_n, \boldsymbol{x}_n\}$, where y_i represents the value of the continuous response variable for the *i*th individual, and $\boldsymbol{x}_i$, the value of the associated covariates, the objective is to obtain a function $f(\boldsymbol{x})$ such that the distance with y is no larger than ε for each of the training points. According to Hastie et al.[32] the regression function is approximated in terms of basis functions $\{h_m(\boldsymbol{x})\}, m = 1, \ldots, M$ as follows:

$$f(\boldsymbol{x}) = \beta_0 + \sum_{m=1}^{M} \beta_m h_m(\boldsymbol{x}),$$

where $\beta = (\beta_0, \beta_1, \ldots, \beta_M)^t$ are coefficients obtained by minimizing: $Q(\boldsymbol{\beta}) = \sum_{i=1}^{n} L\big(y_i - f(\boldsymbol{x}_i)\big) + \frac{\lambda}{2}\sum \beta_m^2$, in which $L(\cdot)$ is called loss function (e.g. quadratic or absolute value), and λ is a positive regularization parameter. For any selection of $L(\cdot)$, the solution has the form: $\hat{f}(\boldsymbol{x}) = \sum_{i=1}^{n} \hat{a}_i K(\boldsymbol{x}, \boldsymbol{x}_i)$, with $K(\cdot,\cdot)$ known as kernel function. Kernels are fundamental components of the model; they serve as functions that allow transforming the data and generating a higher dimensional space; they help to model complex relationships in the data. The most common kernels are the linear $(\boldsymbol{x}_i^t \boldsymbol{x}_j)$, polynomial $(\boldsymbol{x}_i^t \boldsymbol{x}_j + coef_0)^d, d = 2,3, \ldots,$

radial $\left(e^{-\gamma\left|\left|x_i-x_j\right|\right|^2}\right)$, and sigmoid (htaf($\gamma \boldsymbol{x}_i^t \boldsymbol{x}_j$), $+coef_0$), where γ is known as the bandwidth that is adjusted in the training process through cross-validation, and $coef_0$ is a constant that can be adjusted during the model training process, although it is usually set to 1. The model was fitted using the "e1071" package[33] (version 1.7-13), with the help of the SVM function in the statistical package R[27] (version 4.3.0). Codes for model fitting are available upon request to the author for correspondence.

Cross-validation. Cross-validation is a widely used data re-sampling method to estimate the true prediction error of models and to adjust model parameters[20,34]. Therefore, in order to obtain the predictive capability of the models NN, RT, RF, and SVM, and thus make the comparison, the cross-validation was carried out using as a reference the procedures performed by Valerio-Hernández *et al*[15] These authors randomly divided the data into percentages, allotting 80 % to the training set and 20 % to the validation set, and the process was repeated 100 times. The ML models were fitted, and the correlations between the observed vs. predicted values were estimated by observing the values of the response variable corrected for fixed effects and other random effects. Pearson's correlation coefficient was estimated for the corrected phenotypes and predicted genetic values for each one of the partitions, and averages were obtained for each model.

Table 2 presents the averages of the 100 Pearson correlations (based on cross-validation) between corrected and predicted values for the BW, WW, and YW traits, using the four ML algorithms compared in the study. For WW, the SVM algorithm achieved the highest values for the Pearson's correlation coefficient between corrected and predicted values in the validation sets (WW= 0.256). By this method, the best fit for the three characteristics was obtained with the "Radial Kernel" by optimizing the hyperparameters γ (gamma) and cost (BW: 0.045 and 0.05; WW and YW: 0.05 and 0.01, respectively). Tests performed using the Artificial NN method determined the number of neurons in the hidden layer of the model to be 3 neurons for BW and 2 for WW and YW when appropriate parameter estimators of weights were obtained generating a parsimonious model. The best performance of this method was estimated at 0.402 for the BW and 0.195 for the YW.

For the RF method, tests were conducted with different numbers of "trees" as model parameters; 150 of these obtained optimal prediction values for BW and WW, and 250, for YW. The third-best performance values were predicted for WW and YW. The RT methodology showed lower predictive capacity for WW and YW in this study. Based on these results, the following set of hypotheses were proposed to test the significance of the estimated correlation coefficients: $H_0: \mu_r \leq 0$ *vs* $H_1: \mu_r > 0$, where μ_r is the mean of the distribution of the Pearson correlation coefficient and it was to test whether the association is positive or not. The set of hypotheses was tested using the 1-sample t-test, first verifying

the assumption of normality in each of the cases[35]; in all cases, it was concluded that the assumption of normality is appropriate ($P - value > 0.05$).

Table 2: Average Pearson's correlation estimators and standard deviation between corrected phenotypes and predicted genetic values with the 100 cross-validations for the three growth characteristics and the compared algorithms

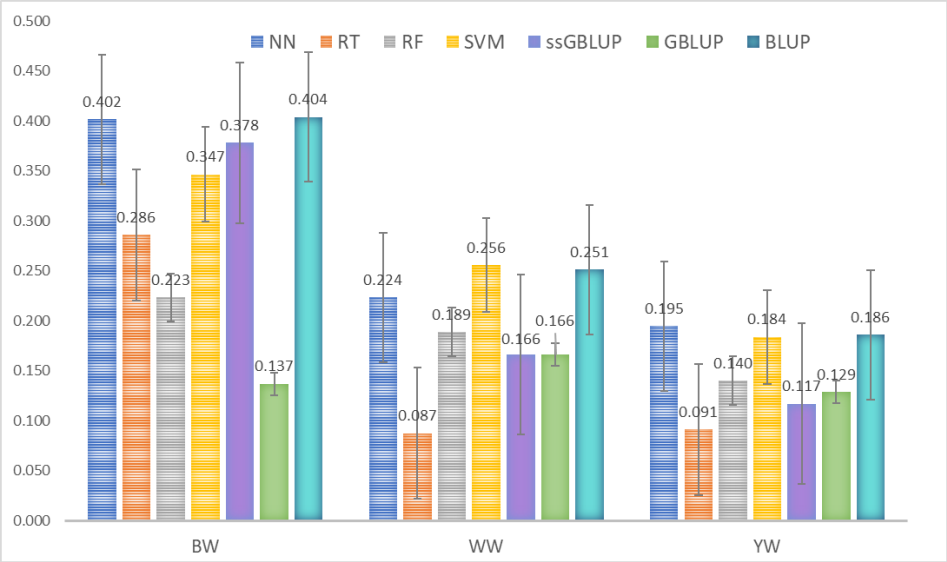
Characteristic	Algorithm	PCC	SD
BW	Neural network	0.402	0.160
	Regression tree	0.286	0.153
	Random forests	0.223	0.163
	Support Vector Machine	0.347	0.129
WW	Neural network	0.224	0.126
	Regression tree	0.087	0.163
	Random forests	0.189	0.117
	Support Vector Machine	0.256	0.144
YW	Neural network	0.195	0.152
	Regression tree	0.091	0.178
	Random forests	0.140	0.128
	Support Vector Machine	0.184	0.160

BW = birth weight, WW = weaning weight, YW = yearling weight; PCC = Pearson correlation coefficient; SD= standard deviation of the 100 correlation estimators for randomly selected partitions.

To determine the predictive ability of the ML models, Pearson correlation coefficient estimators between corrected phenotypes and predicted genetic values were compared with those obtained from the analyzed models[36] in the test sets for each characteristic of the cross-validation methodology described above; unlike the previous studies, this study maintained consistency in the data used in the analyses. This ensures consistency in the comparisons made and provides a solid basis for evaluating the relative performance of traditional methods and ML algorithms. The problem of inferring genetic values and predicting phenotypes for quantitative traits governed by complex forms of gene interactions is difficult to solve using the routinely used linear mixed models[37,38], Therefore, the use of ML algorithms is an alternative to model complex functions by identifying nonlinear relationships between the covariates and the response variable[20]. The correlations between corrected phenotypes and predicted values with the methodologies used made it possible to evaluate the NN, RT, RF, and SVM machine learning algorithms for the growth characteristics BW, WW, and YW in bovines. Figure 1 illustrates that the NN, RF, and SVM algorithms generally showed a similar predictive performance to that of the methodologies assessed by Valerio-Hernandez *et al*[15] using the same variables. In a study comparing the predictive capacity of nonlinear neural networks (NLNN) with linear models, these were found to be potentially useful to predict complex characteristics based on genomic information, a situation in which the number of

parameters to be estimated usually exceeds the sample size[20]. Rodríguez-Alcántar[3] compared ML algorithms using different sets of SNPs generated from chromosomes with a high number of QTLs associated with high milk production. This author found that classification accuracy ranged between 90.9 and 94.5 % with decision trees, and between 79.0 and 87.3 % with neural networks. The author concludes that both the neural network method for binary classification and decision trees are efficient tools for the early identification of highly producing dairy cows.

Figure 1: Comparison of correlation coefficients (average of 100 validations) of corrected phenotypes and predicted genetic values



Genetic values obtained with machine learning methods, artificial neural networks (NN), regression trees (RT), random forests (RF), and support vector machines (SVM) with the methodologies applied by Valerio-Hernández *et al*[15], best linear unbiased predictor (BLUP), genomic BLUP (GBLUP) and single-step GBLUP (ssGBLUP) for birth weight (BW), weaning weight (WW) and yearling weight (YW) of a population of Braunvieh cattle.

The results suggest that the performance of the models varies according to the feature and the amount of information[20], among other factors. This suggests that better results can be obtained with these models by including more variable and covariate information to fit the training model[39,40]; despite the low correlations and large variances of the predictions, these can be attributed to several genetic and methodological factors. Consistently with the findings of Cuyabano *et al*[41], it is important to consider genetic differences between reference and target populations when calculating the accuracy of predictions. Furthermore, it is suggested that there is a theoretical upper limit to the accuracy of these predictions, determined by the square root of the heritability. Zhang *et al*[42] mention that various factors can influence the accuracy of genomic breeding value predictions; heritability (using the model described as BLUP, Valerio-Hernandez *et al*[15] report 0.260 for BW; 0.223 for WW, and 0.231 for YW), the density of genetic markers, the minor allele frequency (MAF) utilized during the data

cleaning process, and the statistical model used are just some factors that can affect the accuracy of genomic breeding value predictions. This poses significant challenges in the prediction of complex traits.

The SVM, NN, and RF methodologies showed similar performance in terms of Pearson correlation coefficients of corrected phenotypes and predicted values for the three growth characteristics used; these results were subsequently compared with the values obtained by Valerio-Hernández *et al*[15] using traditional BLUP, GBLUP, and ssGBLUP methodologies. The computational cost of the BW was higher than that of the other three compared algorithms; it was determined by measuring the runtime required to train and validate each one of the algorithms on this training and test data sets, recording the time elapsed from the start of the training to the completion of the validation process. This result is similar to that reported by Zhao *et al*[43], who mentioned that NR adjustment is more complicated and time-consuming. The SVM algorithm stood out as a promising tool for prediction based on genomic information, considering the amount of information and the parameters used with this methodology. Like the Kernel[31], this algorithm contributes to ML applications for the analysis of datasets derived from genetic and genomic information[44,45].

The results obtained in this study prove that ML algorithms have the potential to generate useful predictions even under constrained information conditions, such as a small sample size and low density of genetic markers. This finding highlights their applicability in practical scenarios where resources are limited. Nevertheless, significant challenges were identified, such as high computational cost and dependence on sufficient quality data to maximize predictive capability. Despite these limitations, algorithms such as NN and SVM showed consistent performance, suggesting that they may be valuable tools for genomic analysis. These results not only provide practical insights on the use of ML algorithms, but also open the door to future research focused on evaluating their behavior with larger and more detailed databases, optimizing both their implementation and their predictive capacity within different contexts.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Technical note

Preliminary analysis of the development of a breeding program of the Peruvian Paso horse in field conditions

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Abstract:

The genetic parameters of overreach, term and acuteness in Peruvian Paso horses (PPH) have not been determined to date. It is important to estimate these parameters for application in PPH breeding, therefore, the aim of this study was to estimate the heritability, repeatability, and genetic correlations in field conditions of overreach, term, and acuteness of PPH. The study included 134, 137 and 134 stallion and mare records for the traits overreach, term and acuteness, respectively. All measurements were recorded in MP4 video format with a

resolution of 1,920 x 1,080 megapixels and at 60 frames per second. All traits were measured three to five times (once per stride), and each trait was analyzed. KINOVEA software version 0.9.5 was used to analyze the measurements. A multivariate repeated measures animal model with sex effect was used to estimate the variance components for each trait using WOMBAT software. The results showed heritability of 0.411, 0.476 and 0.405, for the traits of overreach, term, and acuteness, respectively. Repeatability was high in all traits (> 0.70). Genetic additive correlations ranged from -0.30 to 0.49. It can be concluded that overreach and term have high heritability values, which allows these traits to respond better in a selection process, unlike acuteness, which has a moderate heritability value.

Keywords: Heritability, Genetic correlations, Repeatability, Overreach, Term, Acuteness.

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The Peruvian Paso horse (PPH) is an equine breed native to Peru. Since 1947 the National Association of Breeders and Owners of Peruvian Paso Horses (ANCPCPP) of Peru has made great efforts in the conservation, breeding and selection of this breed[1]. This breed is considered a gaited horse with a symmetrical four-beat rhythm and lateral footfall sequence during the *paso llano* gait[2-4]. The ANCPCPP[5] defines the *paso llano* as when the horse breaks the ambling gait on the sides into 4 steps. Other breeds have similar gaits, although with some differences, including the classic *Fino*, the curly rack, the coon rack, the fox trot, the *marcha picada*, the mountain pleasure rack, the rocky mountain rack, the road gait, the *sobreandando* and the toelt[4]. In PPH, the smoothness and harmony of the movement arises from the combination of execution modalities during the *paso llano* (or "*ambladura rota*")[2]. The main traits involved are term, acuteness[6-9] and overreach[10-13] (Figure 1). More optimal values of these three traits used as part of the criteria to evaluate the performance of each animal are desired. These traits were selected because they directly influence the smoothness, harmony and efficiency of the horse's movement, resulting in a smooth gait[7], which is the characteristic that distinguishes this breed from others in the world and is of utmost importance in horse selection by breeders. The evaluation of these characteristics during movement allows identifying animals with the most favorable movements and, therefore, with greater potential to be used in genetic improvement programs. When evaluating horses, the measurement of traits by the human eye can be challenging, due to subjectivity and limited precision[9,14]. Several studies on equine sports medicine have focused on kinematics

in horses, identifying changes in athletic performance and health[8,15,16]. To measure the functional traits of a horse, it is recommended to estimate objectively measurable kinematic variables[17]. Therefore, the aim of this study was to estimate the heritability, repeatability, and genetic correlations in field conditions of overreach, term, and acuteness in PPH.

In order to achieve the proposed objectives, 140 animals were phenotyped, and of these, only records that could be analyzed were used. Records not considered in the analysis were discarded due to recording problems related to the video recorder lens. Horses that did not move parallel to the camera, that limped, had a very slow speed, handler blocking camera view or mares with foals at their sides during the gait that prevented the identification of the marks during the *paso llano*, were not included in the study after debugging the videos. Finally, the records of 134, 137, and 134 animals, with a higher proportion of females than males (80 % and 20 %, approximately), were used to study the traits of overreach, term, and acuteness, respectively. The mean age of the horses was 7.67 ± 2.61 yr, ranging from 5 to 11 yr (median 7.05 yr). The same animals were used for the analysis of the three traits. In accordance with each trait (overreach, term and acuteness), a total of 1,615, 1,641 and 1,615 individuals, respectively, that could be traced back 21 generations, were included in the database. Information related to pedigree analysis, including the number of animals traced, related phenotypes and inbreeding coefficients, are presented in Table 1. The generational interval of the entire population tracked was 8.76 ± 4.53 yr. This study was approved by institutional Committee of Ethics in Research with Animals and Biodiversity of the *Universidad Cientifica del Sur* (Cod. 028-2021-PRO99) and permission was obtained from the owners of the animals for data collection. Only healthy animals, examined by a registered veterinarian, with no signs of lameness in one or more legs, were included in the study.

Table 1: Pedigree structure for each trait

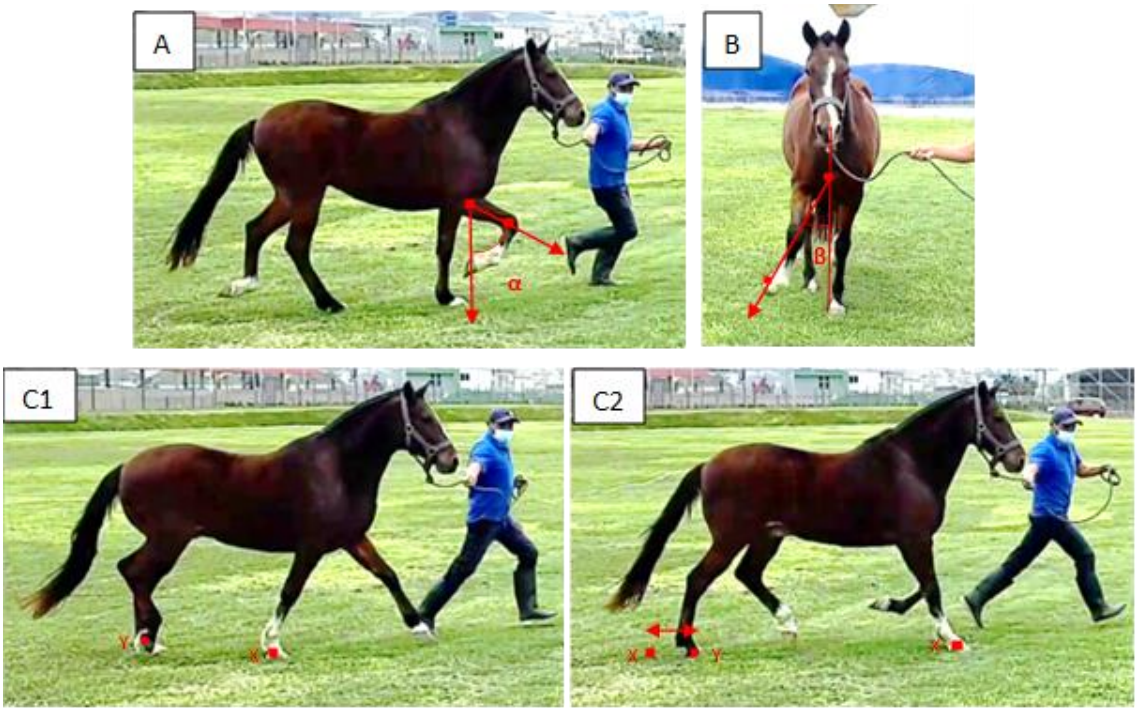
		Overreach	Term	Acuteness
Animals in pedigree file		1,615	1,641	1,615
Animals with records		134	137	134
Animals with	3 records	90	100	86
	4 records	2	12	8
	5 records	42	25	40
Animals with	unknown sire	79	81	79
	unknown dam	211	211	211
	both parents unknown	56	57	56
Animals without offspring		123	126	123
Animals with offspring		1,234	1,257	1,234
Animals with offspring and records		11	11	11
Sires		458	467	458
Sires with progeny in data		51	52	51
Sires with records and progeny in data		3	3	3
Dams		774	788	774
Dams with progeny in data		102	104	102
Dams with records and progeny in data		8	8	8
Average inbreeding coefficient, %		5.42	5.43	5.44
Amongst inbreed animals, %		8.51	8.41	8.45
Average inbreeding coefficient amongst animals phenotyped, %		8.31	8.29	8.30

All animals were evaluated in their breeding facilities. These consisted of a flat, dry, unobstructed grassy fields. Start and end points were determined, which were perpendicular to the location of the camera lens, through which each animal moved during the recording of overreach and acuteness. During the recording each horse travelled a straight distance of 50 m from a start to a finish point located in front of the camera lens. Each horse was evaluated on different days, with groups of three to ten horses assessed per day, depending on the availability of the breeders. Each breeder used an experienced handler to record the video recordings.

To identify the reference points for measuring each trait, a 4 x 4 cm tape was attached to the areas marked in Figure 1. All measurements were recorded in video MP4 format with a resolution of 1,920 X 1,080 megapixels and at 60 frames/sec[18]. The animals were placed on a flat surface and were pulled by an operator at a *paso llano* with an approximate speed of

between 2.5 and 4 m/sec, covering 50 m. The performance of the horse was recorded by filming with video camera on a tripod with a fixed position positioned horizontally (confirmed with a level) at a height of 1.3 m and 12 m from the middle of the line of motion, recording the movement of each animal laterally. All traits were measured by three to five technical replicates (once per stride) and were included in the model for analysis. The description of the measurement of each trait is detailed in Figure 1. KINOVEA software version 0.9.5 (http://www.kinovea.org/) was used to analyze the measurements[19].

Figure 1: Genetic parameters of overreach, term and acuteness in Peruvian Paso horses



Acuteness (A): representation of the maximum α angle of the acuteness of the right forelimb measured from the orientation connecting the knee to the elbow with respect to the vertical, in the sagittal plane (9). Term (B): representation of the maximum β angle of the term of the right forelimb measured from the lateral hoof wall with respect to the vertical in the coronal plane of the horse (9). Overreach (C1 and C2): representation of overreach, measured as the distance between X and Y, where X is the footfall of the right forelimb hoof position during maximum protraction and retraction onto the ground and Y is the footfall of the right hind limb hoof position during maximum protraction and retraction onto the ground when X is exceeded in the next stride.

For statistical analysis, all the traits were subjected to descriptive statistical analysis and normality analysis using the Anderson Darling test with $P>0.05$ indicating that the trait met the normal distribution. JASP software was used for these analyses. Heritability is expressed by $h^2 = \sigma_a^2 / \sigma_p^2$, where σ_a^2 is the additive genetic variance, and σ_p^2 is the phenotypic variance[20]. The phenotypic records of three traits were fitted to the repeated measures multivariate animal model with a fixed sex effect to estimate the variance components of

each of the three traits using the average information (AI) algorithm for restricted maximum likelihood[21]. WOMBAT software was used for all procedures (http://didgeridoo.une.edu.au/km/wombat.php)[22]. The model used is expressed as:

$$Y_{ijk} = \mu + Sex_i + Animal_j + Horse_k + e_{ijk},$$

Being

$\mathbf{Y_{ij}}$ the phenotypic value for each trait;
μ the population mean;
$\mathbf{Sex_i}$ the fixed effect of sex (2 levels);
$\mathbf{Animal_j}$ the random effect of the jth animal $\sim ND(0, A\sigma_a^2)$, A denotes the numerator relationship matrix among animals and σ_a^2 the additive variance;
$\mathbf{Horse_k}$ is the random effect of the kth measure of the individual (3 to 5 levels) $\sim ND(0, I\sigma_{pe}^2)$, where I is the identity matrix, σ_{pe}^2 is the permanent environment variance;
$\mathbf{e_{ijk}}$ the residual random effect$\sim ND(0, I\sigma_e^2)$.

The pedigree structure was used for the estimation of additive variances and covariances of the random effect for the calculation of heritability, repeatability and genetic correlations.

Repeatability can be estimated as the proportion of the phenotypic variance explained by the additive variance and the permanent environmental variance[20]. Nonadditive contributions to consistent among-individual differences are normally referred to as "permanent environment effects". If a trait has repeated measures, then it is necessary to model permanent environment effects in an animal model to prevent upward bias in additive variance[22]. Therefore, among-individual variance is given by the *Animal$_j$* component, while the residual component represents within-individual variance[22]. For calculation of repeatability, three records (3 levels) per animal per trait were used to estimate these variance components. Repeatability (R) was calculated from: $R = \left(\sigma_a^2 + \sigma_{pe}^2\right) \Big/ \left(\sigma_a^2 + \sigma_{pe}^2 + \sigma_e^2\right)$. Phenotypic and additive genetic correlations were calculated with the same records used for the heritability calculations. All traits were subjected to the Anderson-Darling normality test and showed normal distribution with a significance value of $P>0.05$ for all traits; therefore, the use of the proposed animal model is appropriate.

According to the results, overreach was found to have high[23] heritability, in line with the findings of Molina *et al*[24] for stride length. In contrast, Sole *et al*[10] reported an overreach heritability in the Lusitano horse that was considerably lower than observation in this study . In addition, it was observed that overreach in this study significantly differed from that

reported in other studies, particularly in the trot gait[10]. However, some studies[12,13] reported positive overreaches in Andalusian horses. The heritability of term, also proved to be high. Regarding acuteness, its high heritability was similar to that found in Lusitano horses[10], albeit for a related, yet different, trait. The discrepancy in results compared to Molina *et al*[24] could be attributed to factors, such as the horse's training level[25] and the omission of the sex effect in their study, since they only assessed males.

As could be observed, the heritability values of all the traits analyzed were high (greater than 0.40). These values can be explained by the non-inclusion of an external factor that allows free gait of the horse, such as a rider that can alter the rhythm and movement of the animal during the flat gait[26]. These movements are performed freely and are more homogeneous without the external effect and are therefore more heritable[27]. Another reason that might explain the high heritability values is that there may have been more specialization in PPH contests[27], or better use of the selection process in the breed[23], although it may also be because this population was more homogeneous due to the higher number of mares (~80 %) analyzed in this study and only adult animals (5 to 11 yr old) were included[27]. One way to achieve greater genetic progress may be with higher selection intensity, as well as higher heritability values[28]. Furthermore, it should also be taken into account that improvement in these traits is the result of a complex combination of conformational, physiological and behavioral traits[29]. Efficiency in genetic selection for bio-kinematic variables can be more efficient than selection based on animal performance, and this can be translated into higher heritability values[30].

As a criterion for categorizing correlations, the Quinnipiac University scale[31] was used to classify correlations less than or equal to 0.20 as weak, greater than 20 and less than 0.40 as moderate and greater than 0.40 as strong[27,32,33]. The additive genetic correlations found in the present study ranked between absolute values of 0.301 (standard error= 0.432) and 0.697 (standard error= 0.374), similar to other studies conducted under field conditions[30], and the phenotypic correlations ranked between absolute values of 0.183 (standard error= 0.081) and 0.213 (standard error= 0.079). Although it is true that genetic correlations provide information about the relationship between traits, they are not always as useful as phenotypic correlations at the time of evaluation during performance[32] possibly due to training time at the time of the assessment, rider experience or other environmental factors. Their main utility can be applied to the construction of selection indices or to predict correlated response to selection[34].

Regarding repeatability values, these were greater than 0.70 for all the traits, which is considered excellent[35]. Similar results in kinematic traits were found in trained dressage and bullfighting horses (over 0.50), and in Swedish Warmblood horses in scored gaits with values between 0.75 and 0.77[36]. The low standard error values close to 0.02 indicate high precision

in all traits (0.898, 0.842 and 0.901 for overreach, term and acuteness, respectively), indicating that this parameter has little effect on the temporal environment of the three traits[20]. This can also be corroborated by the c^2 values obtained in this study, with values ranging from moderate to high, indicating that a considerable proportion of the phenotypic variance is explained by additive variance and permanent environmental variance, rather than temporal environmental variance[20], although this is not concise with the term trait, which may be mainly affected by the additive genetic effect. Taking into account the methodology of Sepulveda *et al*[35], who found higher repeatability values in daily than in weekly observations, it can be suggested that observations made with minute differences could be even higher, as found in this study. This can be corroborated in a study conducted in horses of different breeds[37] in which the repeatability of head and pelvis position asymmetry presented values between 0.89 and 0.95. Furthermore, these results corroborate that trait with high repeatability require few measurements (3 in this study) to obtain higher precision, and an increase in the number of measurements may be irrelevant for parameter estimation. Taking into account the high repeatability values found, this parameter can be used as an indicator of how effective the selection process can be, considering its relationship with heritability, due to the inclusion of permanent environmental variance (within-individual variance) in its estimation[20,38].

This research presents several positive findings, with promising heritability values indicating that overreach, term and acuteness have high potential for improvement in a selection plan. The positive genetic correlations found between overreach and acuteness suggest that both traits can be improved simultaneously through specific breeding strategies. Furthermore, high repeatability values with high precision indicate that the number of measurements required for these traits can be reduced, simplifying the evaluation process.

However, certain limitations in the study. The overall accuracy of the traits was somewhat limited, probably due to the small sample size. The complexity of performing kinematic measurements and the time required to travel between different breeding centers were the main reasons for this limitation. However, despite the small number of animals phenotyped, the estimation of genetic parameters is justified as preliminary values and are useful for later references, as has been observed in other studies with similar sample sizes (100 to 362)[29]. The population of animals included in this study was small, but the average total inbreeding coefficient (~5.43 %) was comparable to the studies of Larrea *et al*[39] and Montenegro *et al*[40] (5.97 % and 5.44 %, respectively). This suggests that the results obtained can be interpreted as a reference for the general population of PPH. All horses were tested with the same device under field conditions, ensuring that any potential bias was consistent across subjects.

In conclusion, findings of this study provide a valuable reference for the genetic improvement of PPH, despite the noted limitations. The results indicate that traits, such as overreach, term and acuteness, exhibit high heritability and can be effectively targeted in breeding programs. The preliminary genetic parameters of the study and the comparability of inbreeding coefficients with other research support the relevance and applicability of this results.

Conflicts of interest

The authors state that records of eight animals owned by José Dextre were used. It is also stated that José Dextre was Chairman of the Board of the Universidad Científica del Sur during the development of the methodological phase of this research.

Annex 1: Descriptive statistics per trait

		Overreach (cm)	Term (degree)	Acuteness (degree)
Animals	Stallions	28	28	28
	Mares	106	109	106
Median		25.9	25.3	72
Mean		25.338	25.111	71.512
Records		500	481	500
Standard error of mean		0.909	0.288	0.308
95% CI, mean upper		29.119	25.675	72.117
95% CI, mean lower		25.556	24.546	70.907
Standard deviation		20.33	6.315	6.902
Coefficient of variation, %		74.40	25.10	9.70
Skewness		0.219	0.177	-0.298
Kurtosis		-0.445	0.038	0.087
Minimum		-18.080	6.800	49.000
Maximum		86.180	44.200	88.600
P-value		0.881	0.814	0.897

CI= confidence interval.

Annex 2: Estimates of heritability (h^2), ratio of the permanent environment variance to phenotype variance (c^2) and repeatability (R) (on diagonal), phenotypic correlations (bellow diagonal) and additive genetic correlations (above diagonal)

	Overreach	Term	Acuteness
Overreach	h^2 = 0.411 (0.199) c^2 = 0.436 (0.193) R = 0.847 (0.022)	-0.697 (0.374)	0.493 (0.360)
Term	-0.213 (0.079)	h^2 = 0.476 (0.197) c^2 = 0.287 (0.187) R = 0.763 (0.032)	-0.301 (0.432)
Acuteness	0.189 (0.081)	0.183 (0.081)	h^2 = 0.405 (0.224) c^2 = 0.447 (0.217) R = 0.851 (0.021)

Standard error in brackets.

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Technical note

Forage yield and nutritional value of silage from alternative and traditional autumn-winter forages

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Abstract:

The objective was to evaluate the forage yield and nutritional value of alternative and traditional forage silages. It was assessed the effect of the species on dry matter (DM) yield,

fermentation (pH and N-ammoniacal [NH_3-NT]) and nutritional value (crude protein [CP], neutral detergent fiber [NDF], non-fibrous carbohydrates [NFC]), nutritional quality (total digestible nutrients [TDN]), nutritional value (net energy for lactation [NE_L], *in situ* digestibility of DM [DMD], and NDF [NDFD]) of silages. Oats had the highest DM yield (9,784 kg ha^{-1}) and safflower, the lowest (6,998 kg ha^{-1}), but there were no differences between rapeseed (8,937 kg ha^{-1}), beetroot (8,828 kg ha^{-1}), barley (9,784 kg ha^{-1}), and triticale (9,355 kg ha^{-1}). Fermentation indicated a similar pH among the silages evaluated, but NH_3-NT was higher in beetroot and safflower silages than in the other silages. CP was higher in rapeseed, beetroot, and safflower silages (17.8 to 19.5 %) than in oats, barley, and triticale silages (13.7 to 15.0 %; $P<0.0001$), but the NDF was higher in the latter (49.7 to 53.4 %; $P<0.0001$). Rapeseed silage had more NFC, TDN, and NE_L and only beetroot silage could match it. The DMD was higher in rapeseed (80.52 %) and beetroot (84.55 %) silages than in oats (62.24 %), barley (58.90 %), and triticale silages (62.79 %; $P<0.0001$). However, NDFD was similar among all silages.

Keywords: Rapeseed, Beetroot, Safflower, Digestibility.

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Intensive bovine milk production systems in Mexico demand forage of high nutritional value to maintain current production levels of cows. Including forages of high nutritional value maximizes the profit per area of sown land[1], increases income over feed cost[2] and improves the productive life of cows in the long term[3]. Nevertheless, the production of high nutritional value forages at the farm level is often affected by adverse climatic conditions, availability and quality of agricultural soil, agronomic management, and limited use of forage species. Under these conditions, it is necessary to expand the number of forage crops in the current traditional forage patterns in dairy farms.

In Mexico's main dairy basins, forage production is based on few forage options. Corn and sorghum as energy sources are established in the spring-summer production cycles[4], alfalfa as a perennial protein crop[5] and oats[6,7] or other small-grain autumn-winter cereals, such as triticale[7] and barley[8,7], which provide protein and fiber when hayed or ensiled. The latter can provide forage with a high protein content and low fiber concentrations; however, its harvest must be carried out in the booting state. This implies sacrificing the forage yield per hectare. Nonetheless, the harvest of cereals can occur until the formation of grain to obtain a better yield per hectare, but its nutritional value declines.

One option that can increase the yield and nutritional value of small-grain cereals during the autumn-winter production cycle is the use of alternative forages. Among these alternative forages are rapeseed, safflower, and fodder beetroot. These forages have been satisfactorily adapted to the climate and soil characteristic of milk production systems located in northern Mexico[9,10]. In addition, these forage crops have demonstrated good DM yields per hectare and a high nutritional value either as fresh forage or preserved as silage[11-15]. Therefore, it is important to know the forage yield and nutritional value of these alternative forages preserved as silage so that they can be incorporated into the traditional autumn-winter forage pattern in dairy farms. This study aimed to evaluate the forage yield and nutritional value of silages of alternative forages, such as rapeseed, beetroot, and safflower, and that of traditional forages, such as oats, barley, and triticale, during autumn-winter. The hypothesis was that there are similarities in forage yield and nutritional value between rapeseed, safflower, beetroot, oats, barley, and triticale silages.

The experiment was carried out in the autumn-winter 2018-2019 production cycle, at the La Laguna Experimental Field of the National Institute of Forestry, Agricultural, and Livestock Research (INIFAP, for its acronym in Spanish), located in Matamoros, Coahuila, Mexico (25° 32' N, 103° 14' W, and 1,150 masl). The soil at the experimental site has a clayey loam texture, an organic matter content of 1.6 %, and a pH of 8.3.

The study consisted of evaluating the DM yield of forage at harvest, as well as the pH, percentage of NH_3-NT and the nutritional value in rapeseed, beetroot, safflower, oats, barley, and triticale silages. The varieties used were Ortegón rapeseed, Starmon beetroot, CD868 safflower, Cuauhtémoc oats, Narro-95 barley, and Río Nazas triticale. Each experimental plot was established in 20 rows with a distance between rows of 18 cm and 6 m in length (21.6 m^2). The plots were randomly distributed in the field under an experimental design of randomized complete blocks with four replications.

The preparation of the land consisted of a fallow, double harrowing, and leveling with a scraper. Sowing was done manually on dry soil on October 12, 2018, and irrigation was applied a day after sowing. The sowing densities per hectare were 12 kg for rapeseed, 40 kg for beetroot, 40 kg for safflower, 100 kg for oats and barley, and 120 kg for triticale. In rapeseed, beetroot, and safflower, plant thinning was carried out 25 d after sowing (das) to leave a population density of 120 plants m^{-2}. The fertilization dose for N and P was estimated considering the extraction capacity of the crop: 250 and 80 kg of N, P_2O_5 ha^{-1}, respectively. Before sowing, 50 kg of N ha^{-1} and 80 kg of P_2O_5 were applied, using granulated ammonium sulfate and monoammonium phosphate as sources. The rest of the N dose was applied equally before the first and second supplemental irrigation in all crops. Potassium fertilizer was not applied because the soils in the region have a high content of available potassium (3,030 kg ha^{-1} at 0.30 m depth)[9]. Oats and triticale required six supplemental irrigations; five supplemental irrigations were applied in beetroot, and four supplemental irrigations were

applied in barley, rapeseed, and safflower. In total, 870 mm irrigation sheets were applied in oats and triticale, 750 mm in beetroot, and 630 mm in barley, rapeseed, and safflower.

All crops were manually harvested; in the booting stage in oats (115 das), barley (104 das), and triticale (114 das); at the beginning of flowering in safflower (129 das) and rapeseed (123 das); and in the vegetative stage in beetroot (129 das). The useful plot was 5 m long of the 10 central furrows (9 m^2). The fresh forage of each useful plot was weighed to estimate the forage production on a green basis per hectare. In each useful plot, a forage sample of 0.4 m^2 was randomly taken to determine the DM content, for which 0.74 m of the three central furrows in each useful plot were sampled. The forage sample was weighed fresh and then dehydrated inside a greenhouse for 5 d. The samples were then dried at 60 °C in a forced-air oven for 72 h. The DM percentage of the forage and forage production on a green basis were used to estimate the forage production based on DM. The rest of the forage was left to dehydrate in the field to make the silage once the optimal DM percentage (between 35 and 40 %) was reached.

To make the silage, it was necessary to regularly determine the DM content of the dehydrated forage in the field by using a microwave oven[16] until it reached a DM percentage between 35 and 40 %. Once the forage reached the desired DM, it was removed from the field to be taken to the area where the silages were made. The dehydrated forage from each treatment was processed to a theoretical particle size of 3.5 to 12 mm using a mill (Model JF5; Terramark, JF Máquinas Agrícolas). The forages were placed inside each mini-silo constructed with PVC pipes (10.5 cm diameter x 18 cm long) sealed at the top and bottom with an insertion plug of the same material[17]. In the central part of the lower plug of each mini-silo, a hole was made with a 2.78 mm drill bit to allow runoff when compacting the forage.

The forage of each treatment was packed using a density of 240 kg m^{-3} DM[18]. The amount of forage that was placed in each mini-silo to achieve the desired packing density was calculated using the DM content value of each chopped forage and the volume of each mini-silo. The volume of each mini-silo was calculated as: $V= \pi r^2 \times h$, where r is the radius and h is the height of each mini-silo. The compaction of the chopped forage in each mini-silo was carried out using a manual press, which is composed of a metal arm fixed at the top that enters the mini-silo and a 4 t hydraulic jack that generates the pressure by lifting the mini-silo. Finally, the mini-silos were plugged, sealed with adhesive tape, and transported to the laboratory to ferment for 60 d. The experimental design used for the silages was randomized complete blocks with four replications.

After opening the mini-silos, the first 5 cm of forage from the top were discarded; two samples of 20 g of fresh silage were taken from each. Two hundred (200) milliliters of deionized water were added to one of the samples and mixed for 30 sec using a high-speed

blender. The mixture was filtered through three layers of cheese cloth; the resulting liquid phase was used to determine pH using a portable potentiometer (OHAUS Model ST2100, Parsippany, NJ, USA.)[19]. The second sample of 20 g of fresh silage was used to determine the N-ammoniacal content of each sample using the Kjeldahl procedure according to the AOAC[20] methods. Approximately 500 g of sample was taken from the remaining material of each mini-silo and dried at 60 °C in a forced-air oven for 72 h for subsequent bromatological analysis. The dried samples were ground to pass a 1 mm sieve in a Wiley mill (Arthur T. Thomas, Swedesboro, NJ.). In each ground sample, the total N content was determined with the Dumas method by dry combustion (Leco FP-528, St. Joseph, MO) and the percentage of CP was calculated as total N × 6.25. The fiber analysis was performed sequentially starting with the determination of NDF in 0.5 g of sample, which was introduced into filter bags with porosity of 25 µ (F57, Ankom Tech., Macedonia, NY) and using thermostable α-amylase and sodium sulfite in the fiber analyzer (A200, Ankom Tech., Macedonia, NY); after the bags were dried and the weight was recorded, the ADF was determined with CTAB and H_2SO_4 in the same fiber analyzer. Finally, using the same bags, the lignin content was determined using 72 % H_2SO_4. The ash content was determined by incinerating 2.0 g of dry sample placed in crucibles, which were placed in a muffle at 550 ºC for 6 h. The non-fibrous carbohydrate content (NFC) was obtained by difference as: NFC (%) = 100 – (% CP + % NDF + % Ash + % EE), where the EE (ethereal extract) was assumed to be 2.8 % for all samples[21]. The estimation of TDN and NE_L was calculated in the NRC[21] model with equations 2-5 and 2-11, respectively, using the results of the bromatological analyses obtained in each sample.

For digestibility analysis, 4.5 g of the dry sample was used and placed in a 10 × 20 cm bag with porosity of 50 µ (R1020, Ankom Tech., Macedonia, NY) to be incubated in duplicate for 120 and 30 h in the ventral sac of two rumen-fistulated cows (ENLS, Zapotlanejo, Jal.). First, the samples to be incubated were introduced for 120 h to determine the potentially digestible NDF ($pdNDF_{120}$) and undigestible NDF at 120 h ($uNDF_{120}$ = 100 - pdNDF); in contrast, the samples to determine the digestibility of NDF at 30 h ($NDFD_{30}$) were introduced 30 h before the 120 h of incubation. All the bags were removed from the rumen simultaneously and immersed for 10 min in a bucket with cold water at 4 ºC. Subsequently, all the samples were rinsed until clear water was obtained. The bags were then left to drain and placed in a forced-air oven to be dried at 55 ºC for 48 h and calculate the digestible DM by difference of the initial weight and the final weight. At the end, approximately 0.5 g of remnant sample was extracted and placed in F57 bags (Ankom Tech., Macedonia, NY) to determine residual NDF and calculate $pdNDF_{120}$ and $NDFD_{30}$.

Forage production on a dry basis at harvest, fermentation indicators, nutritional value, and silage digestibility were analyzed using a one-way analysis of variance according to the completely randomized block design using the PROC MIXED of SAS version 9.3 (SAS Institute Inc., Cary, NC. USA).

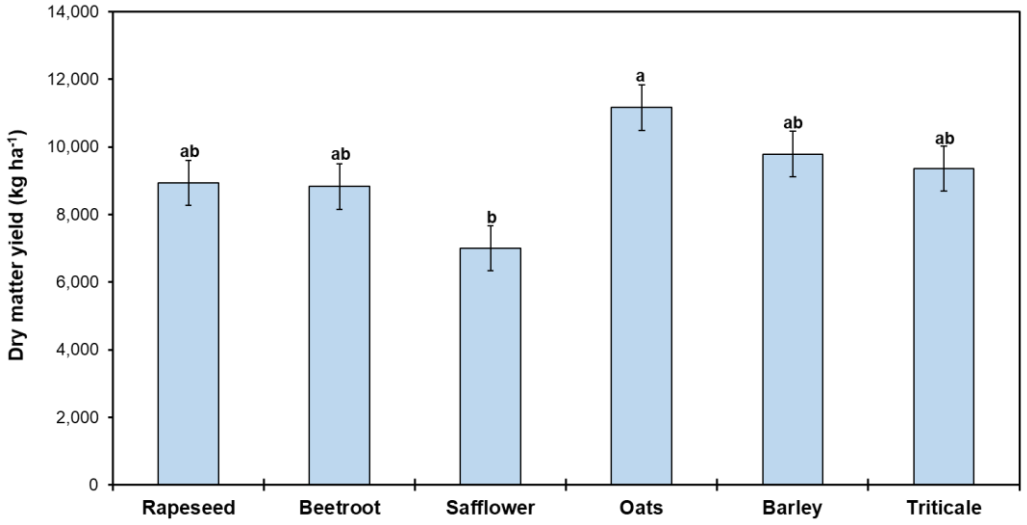
The model used was:

$$Y_{ijk} = \mu + T_i + B_j + e_{ijk}$$

Where: Y_{ijk} is the dependent variable representing the values of production, fermentation, nutritional value, and digestibility, μ is the general mean, T_i is the treatment effect (i = 1 to 6), B_j is the random effect of the block (j = 1 to 4) and e_{ijk} is the random residual error. The Tukey-Kramer test was used to separate the means of the treatments, declaring a statistical difference in all variables at a value of $P \leq 0.05$.

Forage yields (DM) are shown in Figure 1. Oats was the crop with the highest DM yield (11,161 kg ha^{-1}). Barley (9,784 kg ha^{-1}), triticale (9,355 kg ha^{-1}), rapeseed (8,937 kg ha^{-1}), and beetroot (8,828 kg ha^{-1}) had a yield that was intermediate and equal among them. Safflower, on the other hand, was the crop that showed the lowest DM yield (6,998 kg ha^{-1}) among all the forages evaluated. It is possible that the sowing date did not favor safflower since the best DM production in this crop has been obtained when sowing is carried out between the end of November and the beginning of December[10]. In another study[22], they found similar DM yields per hectare between beetroot (7,884 kg), rapeseed (7,396 kg), safflower (8,179 kg), triticale (7,245 kg), and barley (7,384 kg). The DM yields of rapeseed and beetroot in the present study are higher than those observed by other authors[23] in oats (7,346 kg), barley (7,263 kg), and triticale (7,972 kg) harvested in a milky-doughy grain maturity stage. DM production is one of the main factors to consider when it is intended to introduce a new forage to an existing traditional forage pattern. Feed scarcity is one of the main factors limiting milk production and is usually attributed to low quality forage production and limited diversification of forage species[24]. So, due to their productive behavior, the DM yield of rapeseed and beetroot in the present study may contribute to improving the production of quality forages in autumn-winter.

Figure 1: DM yield at harvest of rapeseed, beetroot, safflower, oats, barley, and triticale during autumn-winter



[ab] Means with distinct letters are different ($P \leq 0.05$; SE= 670.59 kg ha^{-1}).

Table 1 shows two parameters of fermentation and the nutritional value of rapeseed, beetroot, safflower, oats, barley, and triticale silages. Regarding fermentation, no significant difference was observed in pH between the silages evaluated. In the production of N-ammoniacal, it was observed that beetroot (15.98 %) and safflower (15.95 %) silages had higher concentrations of NH_3-NT than oats (11.48 %), barley (14.36 %), and triticale (13.93 %) silages. Only rapeseed silage showed a similar concentration of NH_3-NT (15.10 %) as oats, barley, and triticale silage (P=0.05). The pH of all silages in this study was slightly higher than the pH suggested for legume silages (4.3 to 5.0)[25]. This reference value was taken from legume silages because both the traditional and alternative forages of the present study have high CP contents, which give greater neutralizing capacity to the crops, so the pH does not decrease markedly as in crops with lower crude protein content[25].

Microbial fermentation and protein degradation during the silage fermentation process increases the amount of N-ammoniacal, which should not exceed 10 to 15 % of the total N. Higher concentrations of N-ammoniacal have been associated with excessive protein degradation during silo storage, which may be linked to a slow pH drop or to the proteolytic activity of clostridia[25,26]. So, considering the pH and the N-ammoniacal observed in the present study, it can be considered that the evaluated silages had a poor to regular fermentation during storage in the silo.

Table 1: Fermentation and nutritional value of rapeseed, beetroot, safflower, oats, barley, and triticale silages during autumn-winter

Item	Rapeseed	Beetroot	Safflower	Oats	Barley	Triticale	SE	*P*-value
Fermentation								
pH	5.13	5.15	4.97	4.75	5.03	5.00	0.19	0.73
NH_3-NT (% total N)	15.10 ^{ab}	15.98 ^{a}	15.95 ^{a}	11.48 ^{b}	14.36^{ab}	13.93^{ab}	1.00	0.05
Chemical composition[1]								
DM (% of the silage)	39.32	41.95	44.81	38.80	37.09	39.57	1.73	0.07
CP	19.51^{a}	18.17 ^{a}	17.88 ^{a}	13.76 ^{b}	14.78 ^{b}	15.06 ^{b}	0.60	<.0001
NDF	30.27 ^{bc}	23.74 ^{c}	37.10 ^{b}	52.02 ^{a}	53.40 ^{a}	49.79 ^{a}	1.54	<.0001
ADF	27.92 ^{a}	18.16 ^{b}	28.45 ^{a}	32.20 ^{a}	34.07 ^{a}	31.17 ^{a}	1.44	<.0001
Lignin	5.12 ^{c}	7.48 ^{b}	10.11^{a}	4.26 ^{c}	4.78 ^{c}	4.43 ^{c}	0.45	<.0001
LNDF[2], % NDF	16.89 ^{c}	31.54 ^{a}	27.20 ^{b}	8.12 ^{d}	8.92 ^{d}	9.11^{d}	0.98	<.0001
Ash	14.12 ^{b}	28.06 ^{a}	19.12 ^{b}	12.84 ^{b}	13.72 ^{b}	14.95 ^{b}	1.70	<.0001
NFC	33.61^{a}	27.54 ^{ab}	23.96 ^{bc}	18.87 ^{bc}	15.60 ^{c}	17.70 ^{c}	2.01	<.0001
TDN	71.65 ^{a}	62.12 ^{bc}	65.70 ^{ab}	60.42 ^{bc}	56.23 ^{c}	59.47 ^{bc}	1.87	0.0005
NE_L, Mcal/kg DM	1.76 ^{a}	1.47 ^{bc}	1.57 ^{ab}	1.38 ^{bc}	1.26 ^{c}	1.36 ^{bc}	0.06	0.0004

^{abc} Means with distinct letters within each row are different at the indicated probability level. SE= standard error.

NH_3-NT= N-ammoniacal as a percentage of total nitrogen; DM= dry matter; CP= crude protein; NDF= neutral detergent fiber; ADF= acid detergent fiber; NFC= non-fibrous carbohydrates; TDN= total digestible nutrients; NE_L= net energy for lactation.

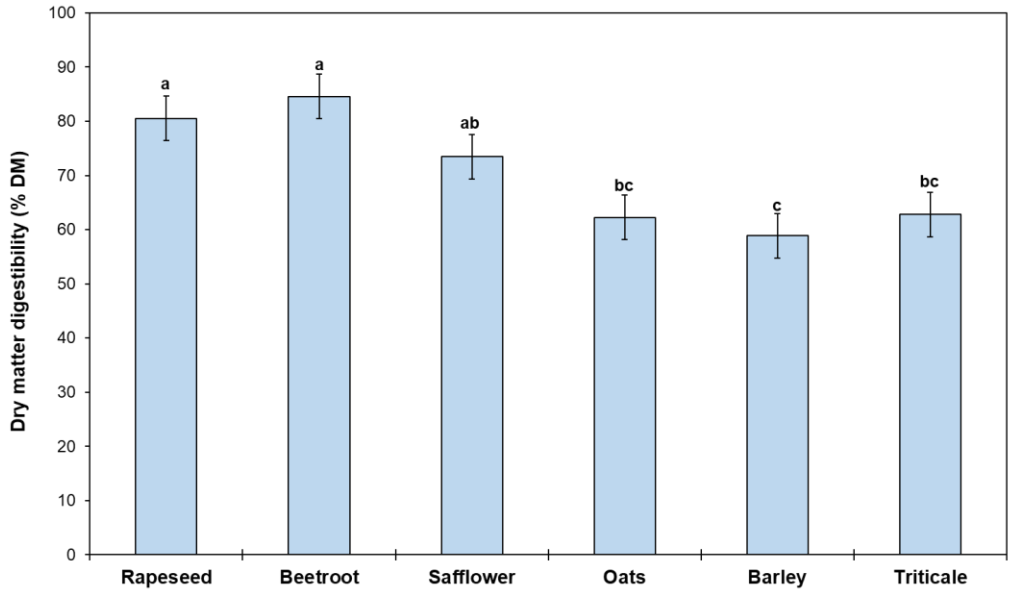
[1] Chemical composition expressed as a percentage of dry matter (DM), unless otherwise stated.

[2] LNDF= lignified NDF calculated as 100 x (% Lignin / % NDF).

Regarding the nutritional value, it was observed that the DM of beetroot (41.95 %) and rapeseed (44.81 %) silages tended to be higher (P=0.07) than the rest of the evaluated silages (37.09 - 39.57 %). It is possible that the high DM contents of the forages in the present study also contributed to the high pH of the silages. The above is because it has been found that the lack of moisture affects the growth of lactic acid bacteria[27], which are responsible for acidifying silage through the production of lactic acid. The CP concentrations of rapeseed (19.5 %), beetroot (18.1 %), and safflower (17.8 %) silages were higher (P<0.0001) than those observed in oats (13.7 %), barley (14.7 %), and triticale (15.0 %) silages. In addition, the NDF concentration was higher in oats, barley, and triticale silages (49.7 to 53.4 %) compared to rapeseed, beetroot, and safflower silages (23.7 to 37.1 %; P<0.0001). A higher CP content and a low NDF concentration have been considered as two of the most important parameters to classify high-quality forages[28], which significantly affect feed intake and productivity in dairy cows. In an evaluation of different species of alternative and traditional forages, higher CP contents were found in beetroot (25.6 %), rapeseed (24.9 %), and safflower (22.8 %) compared to triticale and barley (9.2 %)[21]. These authors[21] also reported higher concentrations of NDF in barley (60 %) and triticale (53.5 %) forage compared to those observed in rapeseed (34.5 %), beetroot (22.4 %), and safflower (41.8 %). Although the values of protein and NDF reported in this study[21] are higher than those of the present study, these differences may be due to the harvest stage; however, alternative forage crops have better nutritional value than traditional ones in both studies. Other authors[15] found that rapeseed silage produced on a commercial scale had an average of 4 to 5 % more CP and 20 to 25 % less NDF than oats and triticale silage. In the ADF concentration, only beetroot silage presented values lower than those of the other silages evaluated. This implies that rapeseed, beetroot and safflower silages can be considered as a viable option to produce protein forages with low fibrous content in the autumn-winter cycle.

Rapeseed silage had the highest values of NFC, TDN, and NE_L and only beetroot silage could match it in NFC and safflower silage in TDN and NE_L (Table 1). Higher NE_L values (1.76 Mcal kg-1 DM) in rapeseed silage in the present study are consistent with those found in oats, barley, and triticale silages (0.60-1.06 Mcal kg^{-1} DM)[29,30]. The *in situ* digestibility of DM at 30 h of incubation is presented in Figure 2. The highest DM digestibility was observed in rapeseed (80.5 %) and beetroot (84.5 %) silages. This was followed by safflower (73.4 %), oats (62.2 %) and triticale (62.7 %) silages and finally, by the silage obtained with barley silage (58.9 %).

Figure 2: *In situ* digestibility of DM at 30 hours of incubation of rapeseed, beetroot, safflower, oats, barley, and triticale silages during autumn-winter



[ab] Means with distinct letters are different (P<0.0001; SE= 4.11 %).

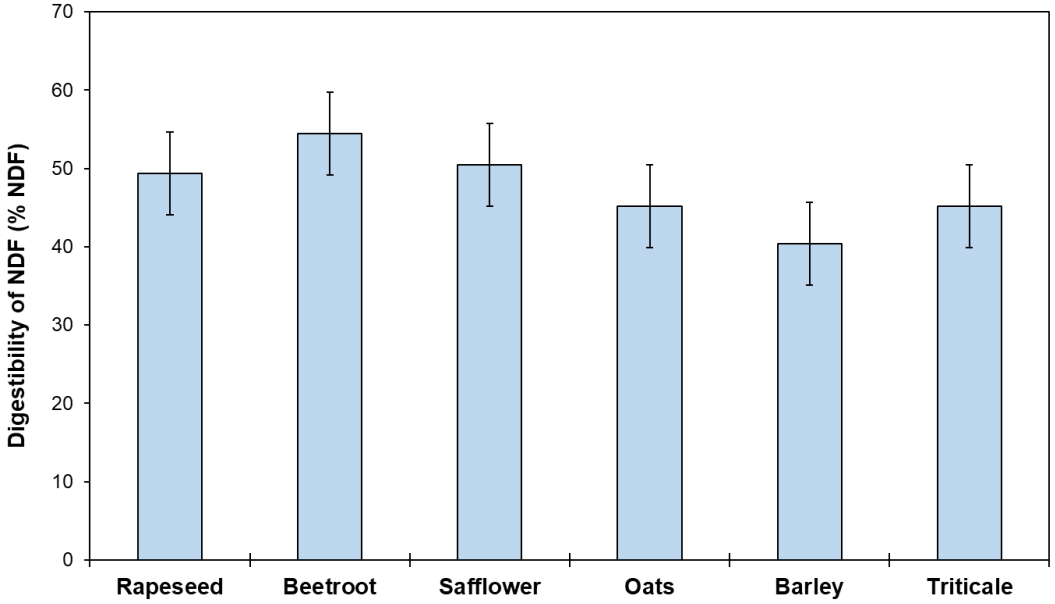
This is associated with a higher NDF content in oats, barley, and triticale silages compared to that contained in rapeseed and beetroot silages. The higher DM digestibility of alternative forage silages compared to that of traditional forages observed in the present study is consistent with previous research as there are reports of higher digestibility in rapeseed (84 %)[31], beetroot (76 %)[14], and safflower (65 %)[32] than in oats (64 %), barley (58 %), and triticale (59 %)[22].

The proportion of more soluble nutrients, such as protein and carbohydrates, compared to fibrous components, also contribute to increasing the digestibility of forages[33,34]. In the present study, rapeseed and beetroot silages had on average 4.31 % and 13.2 % more CP and NFC, respectively, than oats, barley, and triticale silages. Feeding with highly digestible forages improves the animal's consumption and productive behavior. In steers fed with a mixture of grass and alfalfa silage, an increase in consumption of 23 g DM per kilogram of silage was found when its *in situ* digestibility increased by 4.6 percentage units[35].

The *in situ* digestibility of NDF at 30 h of incubation ($NDFD_{30}$) was similar among the different silages evaluated (Figure 3). Although rapeseed, beetroot, and safflower silages contained less NDF, the lignification of NDF (LNDF) was higher in them (rapeseed= 16.8 %, beetroot= 31.5 %, and safflower= 27.2 %) than in oats (8.1 %), barley (8.9 %), and triticale silages (9.1 %; Table 1). This led to a lower or higher fraction of NDF being $pdNDF_{120}$ (potentially digestible NDF at 120 h of incubation) or $uNDF_{120}$ (undigestible NDF at 120 h of incubation), respectively, in rapeseed, beetroot, and safflower silages (Figure 4).

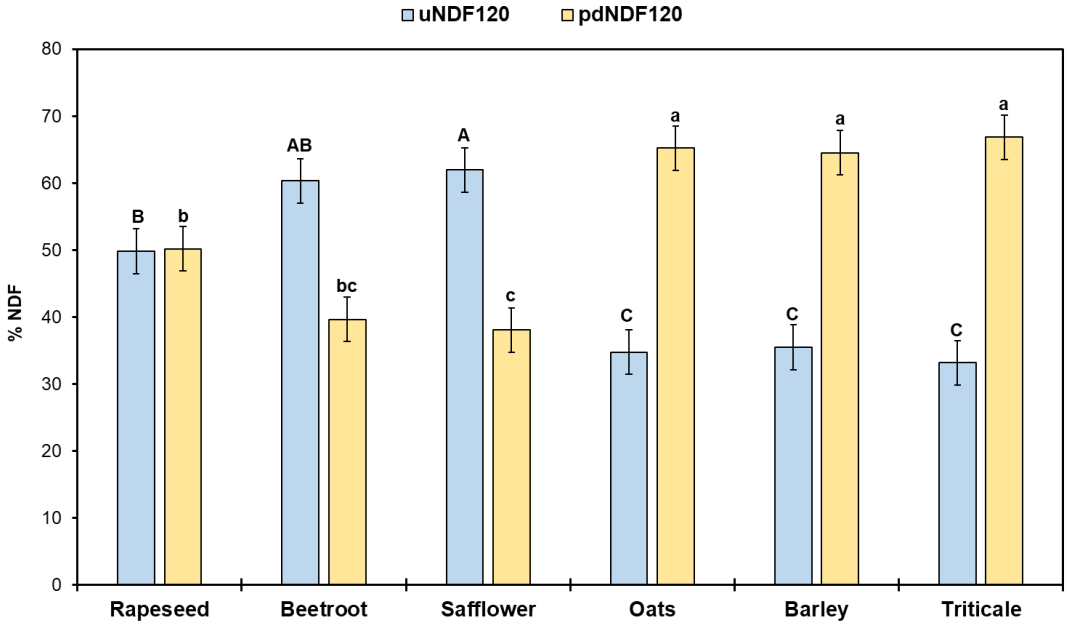
This clearly explains the similar $NDFD_{30}$ despite differences in NDF values between rapeseed, beetroot, safflower, oats, barley, and triticale silages. There is not enough literature documenting $NDFD_{30}$ in rapeseed, beetroot, and safflower; in contrast, the results for oats, barley, and triticale forages are consistent with those reported by other authors[36]. Although there were significant differences in DM digestibility between the silages evaluated in the present study, it is important to evaluate their effect on the animal's consumption and productive behavior. This is because forages high in uNDF have been linked to a longer intestinal retention and filling time in dairy cows[37], which can negatively affect fiber digestibility and potential intake in the animal[38].

Figure 3: *In situ* digestibility of NDF at 30 hours of incubation of rapeseed, beetroot, safflower, oats, barley, and triticale silages during autumn-winter



[ab] Means with distinct letters are different (P=0.33, SE= 5.28%).

Figure 4: Potentially digestible NDF ($pdNDF_{120}$) and undigestible NDF ($uNDF_{120}$) at 120 hours of incubation of rapeseed, beetroot, safflower, oats, barley, and triticale silages during autumn-winter



Means with distinct letters within each category are statistically different ($pdNDF_{120}$ [P<0.0001, SE= 3.32 %]; $uNDF_{120}$ [P<0.0001, SE= 3.32 %]).

In general, oats had the highest DM yield and safflower the lowest, but there were no differences between rapeseed, beetroot, barley, and triticale. The pH of the silages was high, with no differences between the forages evaluated, but the N-ammoniacal was higher in beetroot and safflower than in the rest of the silages. This was due to the high DM of the silages and the high protein content of the forages, respectively. Rapeseed, beetroot, and safflower silages have lower NDF and higher CP than oats, barley, and triticale silages. In addition, rapeseed and beetroot silages have higher *in situ* digestibility of DM than the rest of the silages, which is associated with their lower proportion of fiber and higher soluble components, such as protein and carbohydrates. The *in situ* digestibility of NDF was similar between silages, but undigestible NDF was higher in rapeseed, beetroot, and safflower as a result of increased lignification of the fiber in these forages. It is concluded that rapeseed, beetroot, and safflower silages represent an alternative to expand the production pattern of traditional autumn-winter forages. Nevertheless, *in vivo* studies are required to measure the nutritional value of these forages in livestock.

Acknowledgements

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Technical note

Classification of beekeepers in Chihuahua, Mexico

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Abstract:

Beekeeping is socioeconomically and ecologically important as it generates income and jobs while benefiting agriculture and the environment. A classification and description of beekeepers in the state of Chihuahua, Mexico, was done by analyzing the results of a beekeeper survey. Sixty beekeepers from twelve municipalities in the state were surveyed

from November 2021 to May 2022. Principal component factor analysis (PCA), hierarchical and K-means cluster analysis (CA), and discriminant analysis (DA) were applied to the results using eight descriptive variables and five indices. Three groups of beekeepers were identified with the CA: small beekeepers (47 % of total), medium beekeepers (42 %), and large beekeepers (11 %). These were distinguished mainly by the number of apiaries and hives owned by producers. No significant differences were observed in a technical management index, but values in indices representing basic and specialized management, and genetic and nutrition were higher than those in other honey-producing regions in Mexico. The proposed beekeeper classification is potentially useful in designing strategies and actions to strengthen and promote beekeeping in Chihuahua.

Key words: Beekeeping, *Apis mellifera*, Technological index, Productive Capacity, Technical management.

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Beekeeping contributes to biodiversity preservation and can effectively improve income in rural areas[1]. In 2020, Mexico was the tenth largest honey-exporting country in the world, with more than sixty thousand tons harvested annually[2], in five regions: Altiplano, Pacific, Gulf, North and Yucatan Peninsula[3]. The state of Chihuahua is part of the Mexican Altiplano region[4], and in 2021 it was nationally ranked nineteenth in honey production. In addition to honey, beekeeping generates an economic impact through other derived products; for example, Chihuahua produces 15,000 reproductive queen bees sold throughout Mexico, which contribute to controlling Africanization and increasing production[5]. The state is also the main national producer of crops (e.g. alfalfa, cottonseed and apple) pollinated by bees and other pollinators[6].

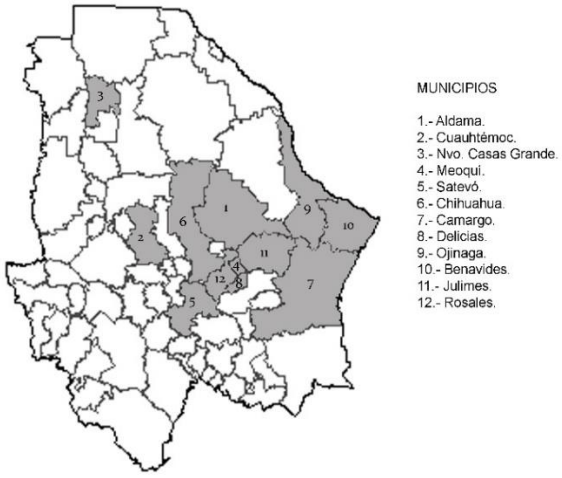
Beekeeping practices vary throughout Mexico in response to regional biological variation and ecosystem diversity. This, in turn, influences economic and productive organization in the country's beekeeping regions. Minimal data is available on beekeeping in Chihuahua and the physical, socioeconomic and technical differences among its beekeepers and their production units. Research describing and classifying producers aids in optimizing public resource allocation, as well as proposing strategies to improve beekeeping. Multivariate techniques are most often used to classify producers[7]. This is because they provide the advantage of classification based on production unit similarities and differences *versus* a set

of classification criteria[8]. Some classifications of beekeepers and their production systems consider the diversity of physical, socioeconomic and technical factors that cause beekeepers in different regions to have specific characteristics and challenges[8,9,10].

Beekeeping in Mexico, and particularly in Chihuahua, faces the challenge of an increasingly competitive and demanding market[11], specifically in terms of food safety and traceability. This is why having data on the beekeeping systems in a given area, and the production processes and factors that influence them, is vital for proposing intervention policies or recommendations. The present study objective was to classify and characterize beekeepers in the state of Chihuahua, Mexico, to increase information on this productive activity.

The study area included twelve municipalities in the state of Chihuahua (Figure 1). Located in northwest Mexico, Chihuahua is bordered to the north by the state of New Mexico and to the east by the state of Texas, both in the United States; in Mexico, it is bordered to the southeast by the state of Coahuila, to the south by the state of Durango, to the southwest by the state of Sinaloa and to the west by the state of Sonora. Chihuahua is the largest state in the country, representing 12.6 % of its area. Its population is 3.74 million inhabitants, 87 % of whom live in urban areas and 13 % in rural areas[12].

Figure 1: Municipalities in the state of Chihuahua where beekeeper surveys were applied



Data was collected through surveys applied to beekeepers from November 2021 to May 2022. The questionnaire included open and closed questions about beekeepers, apiary activities, sales and the market, organization and production costs.

The sample size (n= 60) was calculated by simple random sampling without replacement, considering maximum variance[13,14]. The population (N) was 187 beekeepers (a figure from Chihuahua state government reports)[15,16], the confidence level was 90 % and maximum permissible error was 9 %.

$$n = \frac{Z^2 Npq}{(N-1)e^2 + Z^2 pq}$$

Eight original variables were included in the multivariate analysis: beekeeper age, years of beekeeping experience, and education level; number of hives, number of apiaries, number of honey-producing hives, annual hive maintenance costs, and market shares. Five synthetic variables were also included in the analysis. These were technological indices[17], with values estimated based on field data:

1) Basic management index. Encompasses hive space management practices, frame and hive repair, area cleaning, changing wax in frames, queen replacement, hive inspection, and producer participation in the national beekeeping system.

2) Specialized management index. Includes hive identification activities, production and financial records, colony division, hive replacement, production of byproducts (pollen, propolis, royal jelly and wax), weighing honey, frame casting and painting hives.

3) Genetic index. Includes queen replacement from producer hives, queen replacement with queens produced in-state, queen replacement with queens from Ministry of Agriculture-certified producers, and application of a genetic improvement program.

4) Nutrition index. Based on whether beekeepers provide maintenance feed and stimulation feed.

5) Health index. Includes pest, varroa, and disease control activities, and producer participation in anti-varroa campaign.

In estimating the indices, each practice and technology was assigned a value of 1 or 0, where (1) indicated that the beekeeper did it and (0) that they did not[10,17]. The mathematical formula was:

$$I_{ij} = \sum_{ij}^{n} \frac{\delta_{in}}{\delta_{i...n}}$$

Where I_{ij} is the technological index *i* for beekeeper *j*; δ_{in} is the real sum the beekeeper attains based on the number of practices and technologies implemented; and $\delta i...n$ is the maximum sum of *n* practices or technologies that beekeeper *j* can undertake per index *i*. Index values were within a $0 \geq I_{ij} \leq 1$ interval. An estimate was also generated for a total technological index IT_j, with a value interval of $0 \geq IT_j \leq 5$, using the formula[10]:

$$IT_j = \sum_{i=1}^{5} I_{ij}$$

The first step in the statistical analysis was a principal component analysis (PCA) using a varimax rotation to reduce the variables to components explaining the greatest variance. Analysis feasibility was corroborated with the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy and Bartlett's sphericity test, which tests the hypothesis that the correlation matrix is an identity matrix. As a second step, a hierarchical cluster analysis (CA) was run based on Ward's algorithm to graphically identify the number of groups; the beekeeper groups were then identified using the K-means cluster analysis. A discriminant analysis (DA) was used to evaluate classification and assignment of each individual to a group[18]. Within the DA, stepwise variable selection was used to identify the independent variables that most distinguished the groups, and verify that group formation was robust. An ANOVA was run to identify differences and compare between groups. Statistical analyses were done using the SPSS 27.0 software[19].

Five components were identified in the PCA, explaining a total of 71.7 % of total variance: the first contributed 20.7 %, the second 17.9 %, the third 13.0 %, the fourth 10.1 % and the fifth 10.0 %. Both the KMO (0.62) and Bartlett sphericity test (P<0.000), confirmed PCA feasibility. The first component included variables related to the number of colonies in a production unit and was labelled "productive capacity". The second encompassed the basic management, nutrition, specialized management and genetic indices and was labelled "technical management". The third, consisting of producer age and years of experience, was labelled "producer capabilities". The fourth included annual hive maintenance costs and the health index, and was labelled "health status". The fifth grouped education and market participation variables and was labelled "management potential".

The CA identified three groups, and the DA correctly classified 98.3 % of the respondents. The Wilks' Lambda statistic (0.132) indicated that the groups were statistically different. Both the Wilks' Lambda (P<0.05) and F statistic values (>3.84) for each factor confirmed that the factors that most contributed to group definition were productive capacity, producer capabilities, health status and management capacity; technical management did not contribute to group definition (P= 0.408, F= 0.912). This was corroborated with a completely random ANOVA of the technical management variables. In other words, no differences were identified between beekeeper groups in the indices for basic management (F= 2.093; gl= 2, 57; P= 0.133), specialized management (F= 2.583; gl= 2, 57; P= 0.084), nutrition (F= 0.887; gl= 2, 57; P= 0.417) and genetics (F= 1.484; gl= 2, 57; P= 0.235). It is therefore probable that the producers applied similar technical management practices.

The first group, "small beekeepers," consisted of 28 beekeepers and accounted for 47 % of respondents. This group included the youngest and least experienced beekeepers, with a high school or university education level. In terms of productive capacity, they had an average of three apiaries and 62 colonies, and a honey yield of 18.4 kg per hive per year (Table 1). Annual maintenance costs were low compared to the other two groups. Sales were focused on local, state and national markets, although 18 % had exported honey. Their relatively lower production capacity allowed them to be self-employed, and their income from beekeeping represented less than 50 % of family income.

Table 1: Mean and standard deviation of descriptive variables

Factor	Variable	Beekeepers		
		Small	**Medium**	**Large**
1. Production capacity	Number of hives	62±38	98±76	465±162
	Number of honey-producing hives	51±37	76±61	379±217
	Number of apiaries	3±2	8±7	16±7
2. Technical management	Basic management index	0.81±0.21	0.89±0.07	0.89±0.09
	Nutrition index	0.89±0.28	0.94±0.22	0.79±0.39
	Specialized management index	0.51±0.22	0.58±0.24	0.71±0.13
	Genetic index	0.42±0.19	0.44±0.24	0.57±0.28
3. Producer capabilities	Age	43±15	57±13	45±9
	Years' experience	11±8	23±16	28±16
4. Health condition	Annual hive maintenance costs	879±449	1,382±1,042	1,418±871
	Health index	0.88±0.32	0.67±0.31	0.96±0.09
5. Management capacity	Education level	12±3	14±3	10±3
	Market share	0.28±0.22	0.17±0.08	0.29±0.16
	Kilos honey per hive	18.4±11.2	14.8±8.4	23.15±15.3
	Total technological index	3.49.0±0.86	3.49±0.68	3.9±0.75
	Family jobs	1±1	1±1	2±2
	Contribution to household income	<50 %	< 50 %	> 75 %

The second group, "medium beekeepers" consisted of 25 beekeepers, representing 42 % of the sample. It included older beekeepers with more experience in beekeeping, and a high school or university education level (Table 1). They had an average of 98 colonies distributed in eight apiaries, and an average production of 14.8 kg honey per hive. Their total technological index was 3.49, with particular emphasis on nutrition practices such as maintenance and stimulation feeding, as well as basic management activities such as colony inspection, area management and cleaning, frame and hive repair, changing of old combs and queen replacement. Most of those in this group applied control measures for varroa (84 %), pests (wax moth) (68 %), and other diseases (nosemosis and European foulbrood) (56 %). These producers were self-employed and generated less than 50 % of their household income from beekeeping. Their market share was local and statewide.

The third group, "large beekeepers", included seven producers and represented 11 % of respondents. These were the most experienced in honey production, with 45 yr average age and a secondary or high school education level. On average, they had 379 colonies for honey production, and average production per hive was 23.15 kg. They generated an average of

two-family jobs and had a slightly higher technological index than the other groups, since they used more genetic and specialized management activities; annual hive maintenance costs were consequently higher. These producers generally kept financial and production records, replaced hives, divided colonies, and produced honey and wax. They also rented hives for pollination of crops such as apple, watermelon, melon, cotton, cucumber and green tomato. Their market share was local, state and national, and more than 75 % of their household income came from beekeeping.

Classification of beekeepers in Chihuahua was based on unit productive capacity, which is related to production unit size. This coincides with the small, medium and large classifications reported in a similar study done in the state of Morelos[10]. Production unit size in Chihuahua was smaller in terms of number of colonies and honey production than in regions such as Morelos and the Gulf[9,10]. In these regions, small or traditional beekeepers had an average of 80 colonies, medium beekeepers had 157 colonies, and large or commercial beekeepers 426 colonies. In Chihuahua, small beekeepers had 62 colonies, medium ones had 98, and large ones 379. This is a relevant metric because in beekeeping production systems colonies represent capital and are associated with a producer's economic dependence on beekeeping.

The nutrition, basic management, specialized management and genetic indices did not differ between the three groups. However, compared to these indices for beekeepers in Morelos[10], the index values in all three groups in Chihuahua were higher, especially in the genetic and nutrition indices, highlighting different management practices between the two regions. One notable difference is that 90 % of the surveyed beekeepers in Chihuahua employed maintenance feeding and stimulation practices in an effort to increase honey production and guarantee colony survival through the winter[20], a particular concern in the highlands where flowering occurs suddenly and is short-lived[21]. Another discrepancy is that producers in Morelos largely replaced queens from their own hives or from in-state producers, with only large beekeepers replacing them with queens from certified producers. In Chihuahua, by contrast, 85 % of large and 60 % of small producers replaced them with queens from certified suppliers, and 64 % of medium producers used queens from in state. Compared to the total technological index results in the Morelos study[10], the index value for the studied Chihuahua producers indicates they are at an intermediate technological level.

Productive capacities were another important component in classifying the beekeepers in Chihuahua. These were similar to those reported in the states of Jalisco[22] and Morelos[10], and in the Gulf region[9]. Average age in the groups ranged from 43 to 56 yr, with the middle-aged beekeepers being the most experienced.

Hive health status index values were high (0.7) in all three groups of beekeepers in Chihuahua, and higher than reported in Morelos[10]. Beekeepers in Chihuahua clearly valued hive health since 92.5 % engaged in activities related to this index. Even though it increases annual hive maintenance costs, they invested in hive health because it reduces the risk of losses both in bee populations and income.

In contrast to other honey-producing regions in Mexico[10,23], beekeepers in Chihuahua generally had a higher educational level, allowing them to access information and connect with markets. These are important aspects of the marketing process, since the market is dynamic, requiring sales strategies and clear definition of distribution channels[24]. For example, in the present results, 81.7 % of respondents sold honey in the local market, 8.3 % in the national market and 10% exported to countries such as the United States and Germany.

For the small and medium producers, beekeeping was a secondary economic activity representing less than 50 % of household income. They supplemented it with agricultural activities, another business or a job. This is similar to the type of beekeeping done in the state of Yucatan[25], where it is considered a complementary household income source, but still contributes to regional development and sustainability. The large beekeepers, for whom beekeeping generates most of their income, applied technological innovations in technical management, much like producers with more than 500 hives in the state of Jalisco[22]. Studies done in Mexico[26], Argentina[8], and Brazil[17], highlight the importance of beekeeping in generating jobs and household income, and emphasize its worldwide social and economic importance. It also has the added advantage of generating favorable environmental impacts[27].

The present analysis of beekeepers in the state of Chihuahua identified three types of beekeepers: small, medium and large. Production unit productive capacity (e.g. number of apiaries and colonies) was the main factor in effectively classifying them. The three types did not differ in terms of technical management practices, although, compared to other honey-producing regions in Mexico, the Chihuahua beekeepers implement management practices that place them in an intermediate technological index value. All three groups were concerned with maintaining hive health condition and exhibited management capacity when accessing different markets. For the small and medium beekeepers, it is a secondary activity, while for large producers it is a primary activity that generates jobs.

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