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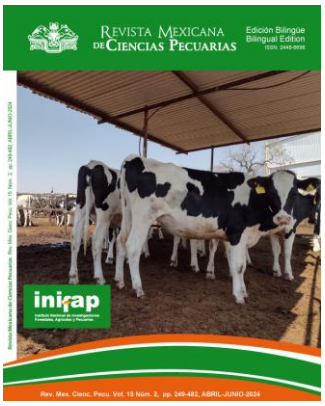

Establo lechero San Cristóbal en Tepatitlán, Jalisco; con promedio de producción de 31 lts/vaca/dia bajo sistema familiar.
Autor: Adriana García Ruíz

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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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   Resumen en español
   Resumen en inglés
   Texto
   Agradecimientos y conflicto de interés
   Literatura citada

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 MétodosResultados
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.

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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. Oficial 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)
DL50 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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7. **Title page.** It should only contain the title of the work, which should be concise but informative; as well as the title translated into English language. In the manuscript is not necessary information as names of authors, departments, institutions and correspondence addresses, etc.; as these data will have to be registered during the capture of the application process on the OJS platform (http://cienciaspecuarias.inifap.gob.mx).

8. **Abstract.** On the second page a summary of no more than 250 words should be included. This abstract should start with a clear statement of the objectives and must include basic procedures and methodology. The more significant results and their statistical value and the main conclusions should be elaborated briefly. At the end of the abstract, and on a separate line, a list of up to 10 key words or short phrases that best describe the nature of the research should be stated.

9. **Text.** The three categories of articles which are published in **Revista Mexicana de Ciencias Pecuarias** are the following:

   a) Research Articles. They should originate in primary works and may show partial or final results of research. The text of the article must include the following parts:

   Introduction
   Materials and Methods
   Results
   Discussion
   Conclusions and implications
   Literature cited

   In lengthy articles, it may be necessary to add other sections to make the content clearer. Results and Discussion can be shown as a single section if considered appropriate.

   b) Technical Notes. They should be brief and be evidence for technical changes, reports of clinical cases of special interest, complete description of a limited investigation, or research results which should be published as a note in the opinion of the editors. The text will contain the same

information presented in the sections of the research article but without section titles.

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    People which collaborated in the article may be named, adding their function or contribution; for example: "scientific advisor", "critical review", "data collection", etc.

11. **Literature cited.** All references should be quoted in their original language. They should be numbered consecutively in the order in which they are first mentioned in the text. Text, tables and figure references should be identified by means of Arabic numbers. Avoid, whenever possible, mentioning in the text the name of the authors. Abstain from using abstracts as references. Also, "unpublished observations" and "personal communications" should not be used as references, although they can be inserted in the text (inside brackets).

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    a. The names of the authors should be quoted beginning with the last name spelt with initial capitals, followed by the initials of the first and middle name(s). In the presence of compound last names, add a dash between both, i.e. Elias-Calles E. Do not use any punctuation sign, nor separation between the initials of an author; separate each author with a comma, even after the last but one.

    b. The title of the paper should be written in full, followed by the abbreviated title of the journal without any punctuation sign; then the year of the publication, after that the number of the volume, followed by the number (in brackets) of the journal and finally the number of pages (this in the event of ordinary article).

    c. Accepted articles, even if still not published, can be included in the list of references, as long as the journal is specified and followed by "in press" (in brackets).

    d. In the case of a single author's book (or more than one, but all responsible for the book's contents), the title of the book should be indicated after the names(s), the number of the edition, the country, the printing house and the year.

    e. When a reference is made of a chapter of book written by several authors; the name of the author(s) of the chapter should be quoted, followed by the title of the chapter, the editors and the title of the book, the country, the printing house, the year, and the initial and final pages.

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Standard journal article (List the first six authors followed by *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.

Issue with no 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.

No author given

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 press

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

Books and other monographs

Author(s)

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

Chapter in a book

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

Conference paper

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 Beltsville Symposium: Biotechnology's role in genetic improvement of farm animals. USDA. 1996:13.

Thesis

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.

Organization as author

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.

Electronic publications

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. Accesed 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 Jul, 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/030 16226. Accesed Sep 12, 2003.

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17. **List of abbreviations:**

cal	calorie (s)
cm	centimeter (s)
°C	degree Celsius
DL50	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	mega calorie (s)
MJ	mega joule (s)
m	meter (s)
µl	micro liter (s)
µm	micro meter (s)
mg	milligram (s)
ml	milliliter (s)
mm	millimeter (s)
min	minute (s)
ng	nanogram (s)
P	probability (statistic)
p	page
CP	crude protein
PCR	polymerase chain reaction
pp	pages
ppm	parts per million
%	percent (with number)
rpm	revolutions per minute
sec	second (s)
t	metric ton (s)
TDN	total digestible nutrients
AU	animal unit
IU	international units
vs	*versus*
xg	gravidity

The full term for which an abbreviation stands should precede its first use in the text.

18. Scientific names and other Latin terms should be written in italics.

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Article

Study of the Genetic Structure and Diversity of Holstein cattle in the small holder system in Mexico

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Abstract:

The objective was to know the population structure of Holstein animals in the family dairy system, to identify possible origins of the genetic material, to know the degree of inbreeding and to identify possible traces of selection in the genome, which allow glimpses of the traits that have been improved over the years. The study included 270 animals genotyped with the GGP-50K® chip. After genotype quality control, 43,548 autosomal SNPs were included. To know the population structure, analyses of mixtures and principal components (PCs) were performed. To know genomic inbreeding and detect traces of selection, information on runs of homozygosity (ROH) was used. Mixture analysis was performed with the Admixture software, and PC, ROH and inbreeding analyses were performed with SVS-v7.6.8. Mixture

analysis showed evidence of six components, all linked to Holstein bulls families with different country of origin. The PCs did not show stratification of the population by herd. The mean inbreeding coefficient was 0.59 ± 0.53 %. In the regions of the genome with ROHs most frequent in the population (≥20 animals), numerous associations, QTLs and genes related to milk production and composition, fertility parameters, susceptibility to diseases, body conformation, feed efficiency and some characteristics of carcass composition have been reported. The results reflect the existence of a wide genetic diversity in this population and the possibility of carrying out genetic improvement work through selection without affecting inbreeding levels.

Keywords: Genetic diversity, Traces of selection, Inbreeding, Family dairy system.

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Introduction

The cattle dairy industry in Mexico produced around 11.489 billion liters of milk nationwide in 2020 (SIAP, 2020)[(1)], of which more than 30 % of the volume was produced in the family dairy system (FDS), which includes approximately 78 % of the farms[(2)]. In the FDS farms, Holstein animals predominate, although Brown Swiss animals and their crosses can be found[(3)]. Currently, in this system there is little information on production records by animal and on rare occasions genealogical information can be collected, which makes it unfeasible to carry out genetic evaluations of the animals in this system. The genetic improvement of these animals has been carried out by the selection performed by the cattle farmer within their herd, or by the introduction of genetic material, but there is no evidence of directed mating for a specific genetic purpose.

The use of genomic information has made it possible to describe the structure of populations that do not have genealogical information or records. The study of these populations or animals has been carried out from the study of single nucleotide polymorphism (SNP) markers or their clustering pattern, such as for example, runs of homozygosity (ROHs), which are homozygous segments in the genome, identical by descent, which can be used to study population structure, demographic history and to decipher the genetic structure of complex diseases[(4)]. ROHs are the result of crossbreeding between related individuals[(5)] from populations with a high level of selection intensity, influenced by the availability of

replacements and the adoption of technological and reproductive tools[6], or low rates of recombination[7]. Their distribution and length depend on the intensity of selection, being more frequent and more extensive when it is higher[8], when mating between close relatives is frequent or when the size of populations is small[4].

The potential of ROHs to help the genetic improvement of production animals is big due to the fact that they contain a large number of genes that encode traits of interest[9]. In addition, the identification of ROHs can help to visualize and recognize haplotype patterns characteristic of breeds or species[7], allowing the identification of genomic regions with possible traces of selection for the breed[10] and the calculation of individual inbreeding levels. The latter is done by evaluating the portion of the genome covered by ROH segments, especially since there is a high probability of detecting genomic information from ancient relationships[11]. This is a useful tool for populations that do not have genealogical information[12].

Traces of selection are regions of the genome that have been conserved for generations in populations due to natural or artificial selection. These sequences of genetic material are related to functionally important traits[13] and their detection helps to identify candidate genes that have been favored in the selection processes to which populations have been exposed, and to identify beneficial mutations. In addition, they help to understand the molecular pathways related to phenotypic traits[14,15].

With SNP marker analyses, it is also possible to know the population structure through mixture analysis and to know the most influential origins in a population. In addition, through information-reductive methods, such as principal component analyses, it is possible to determine patterns of population structure, important information for establishing the basis for a genetic improvement program.

The objective of this study was to know the population structure, identify possible origins of the genetic material, know the degree of inbreeding, and identify possible traces of selection in the genome that allow us to glimpse the characteristics that have been improved over the years by the decisions of cattle farmers in family production systems in Mexico.

Material and methods

A total of 270 Holstein cow genotypes were used, randomly chosen from the population present in three FDS herds located in the region of Tepatitlán, Jalisco, Mexico. The animals were genotyped with the GeneSeek Genomic Profiler Bovine GGP 50K® chip. Quality

control of genomic information consisted of excluding animals with a call rate <0.90, excluding SNPs with minor allele frequency (MAF) < 0.02, or with a call rate < 0.95, or with a Hardy Weinberg *P*-value < 0.0001[16,17]. After quality control, 43,548 autosomal SNPs were included.

In order to know the structure and main population origins, a mixture analysis was carried out through estimation based on likelihood models that define the structure of ancestry in unrelated individuals, a methodology implemented in the Admixture V 1.3.0[18] software. On the other hand, Principal Component (PC) analyses were performed to identify possible population groupings by herd. To estimate inbreeding with genomic information and traces of selection in the population, ROHs were searched in the genome. To define ROHs, runs with a minimum length of 500 kb and a minimum number of 25 SNPs, with a minimum density of 1 marker every 50 kb and a maximum gap between contiguous homozygous markers of 500 Kb were included. With the aforementioned parameters, the risk of including very short ROHs was avoided, a common case due to linkage disequilibrium (LD)[17]. LD is associated with the presence of linked genes, so when they are inherited from parents to children, they do so jointly, affecting the frequency of recombination (less than 50 %) and the presence of ROHs in the genome[19]. In addition, 1 heterozygous SNP and 5 genotypes lost per run were allowed[20,21].

ROH analysis was performed using the bioinformatics platform called SNP & Variation Suite v7.6.8 Win64 (Golden Helix, Bozeman, MT, USA)[22], while the analyses of the data obtained were carried out with SAS Institute 9.3.[23]. To analyze the distribution of L_{ROH}, six classes were defined according to their length, which were 0.5 to 4, >4 to 8, >8 to 12, >12 to 16, >16 to 20, and >20 Mb[24].

Traces of selection were detected through the loss of genetic variation, using the ROHs identified in the genome, using the most frequent in the population (in at least 10 % of the animals). According to their physical position in the genome, annotations, or regions previously related to genes, QTLs or traits that have been made in other populations and that are reported in the Animal QTLdb Release 43 database[25] were identified. In addition, traces of selection previously reported in the Bovine Genome Variation database (BGVD) were searched in order to find genomic information that helps to know possible characteristics that have been selected in the population of the Family Dairy System (FDS) in Mexico.

For the calculation of the coefficient of inbreeding by runs (FROH), the methodology proposed by Mcquillan *et al*[27] was used, who defined it as $F^{i}_{ROH} = \Sigma L^{i}_{ROH} / L_{auto}$, where F^{i}_{ROH} is the endogamy coefficient of individual i calculated by ROH, ΣL^{i}_{ROH} is the total sum of the ROH segments of an individual i above a specified minimum length, in this case > 500 kb, and L_{auto} is the length of the autosomal genome covered by SNPs including centromeres.

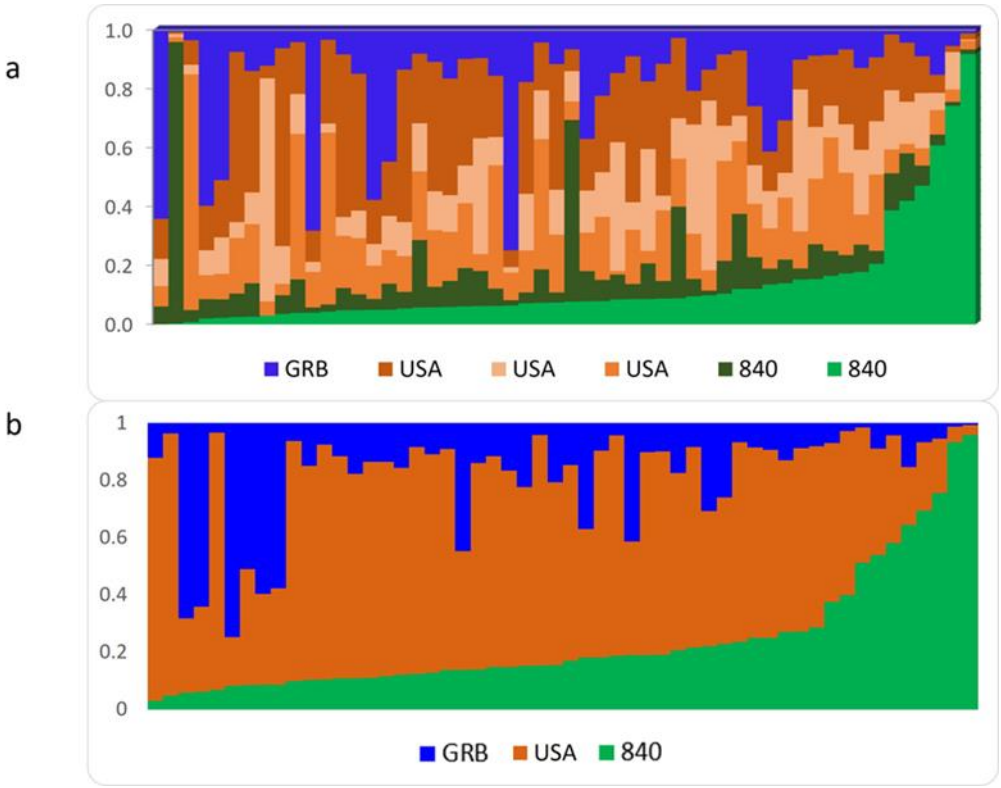
As the year of birth of the animals is unknown, the inbreeding trend by lactation number of the animals at the time of sampling was calculated.

Additionally, the calculation of the inbreeding coefficient was carried out through observed and expected homozygous markers (FHOE) for all animals, which has been reported to have a correlation of 0.96 ± 0.001 with the genomic relationship matrix in Holstein cattle[28], the values of FHOE can range from −1 to +1. The negative numbers refer to the exogamy present in mating between individuals from different populations and the positive values indicate the level of endogamy of individuals from the same population; the calculation was performed through the method referenced by Ferenčaković *et al*[11] with the program called SNP & Variation Suite v7.6.8 Win64 (Golden Helix, Bozeman, MT, USA)[22].

Results and discussion

In the analysis of mixtures, the value that best defined the number of ancestral populations (K) was six and according to the information collected from some cow parents, six large families were identified, defined mainly by the country of origin of the bulls. Figure 1-a shows the population structure linked to the six main groups by country of origin of the bulls, although some of these families share the same country of origin. Therefore, the groups that shared the same origin were combined, leaving only three large groups represented, two representing the United States of America and one representing the United Kingdom (USA, 840 and GBR, respectively) (Figure 1-b). The origins USA and 840 correspond to the United States of America, only that the 840 is assigned to animals that use radio frequency identifications (RFID), devices issued by the International Committee for Animal Recording (ICAR), while animals registered with the USA country of origin do not carry an RFID and the use of genetic material is locally or more limited than those of the 840[29]. The results of this study show the genetic dependence of the family system in Mexico on foreign material, mainly from the United States, since more than 80 % of the origins were linked to families with origins from this country, either from local trade or from those registered internationally.

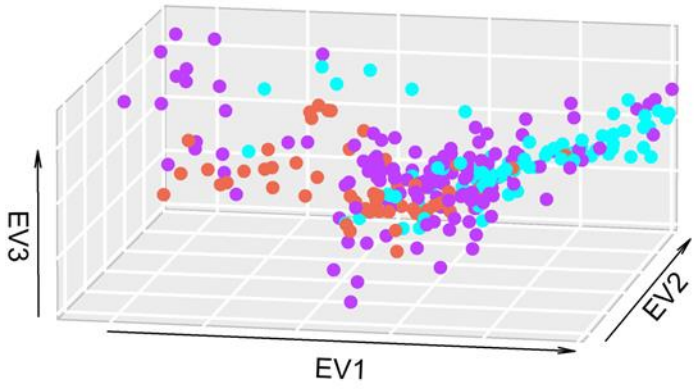
Figure 1: Population structure of Holstein cattle in the family system a) including the six origins attributed to bull families and b) grouped by country of origin



Although another study[29] had reported the influence of other breeds of dairy cattle on the family system, the present study found no evidence of the use or crossbreeding with other breeds. These results could suggest that cattle farmers have followed a more directed mating system, and that they limit themselves to using animals of the same breed in services.

In the PC analyses (Figure 2), no stratification was found by country of origin of the bull, and when the herd of origin was evaluated, a homogeneous herd (purple) was observed in the population, and a difference was observed between the animals of the other herds (red and blue). The percentage of variability associated with each component was 2.9, 2.0, and 1.8 for components or eigenvalues (EV) 1, 2, and 3, respectively.

Figure 2: Principal component analysis of the family system population with Holstein phenotype, defined by herd of origin



The total number of ROHs (N_{ROH}) found in the studied population was 15,695, with an average length (L_{ROH}) of 4.79 Mb, a minimum and maximum length of 0.5 and 91.49 Mb, respectively. The average length of the genome covered by ROH was 278.76 Mb, with a minimum and maximum of 13.28 and 535.83 Mb, respectively. According to the frequency of ROHs in the population, they were identified as unique (in a single animal) or repeated, the latter with the same length (identical) or of variable length. Thirty-five point eight six (35.86) percent of the ROHs were unique (Table 1), while 64.14 % (10,067) were repeated.

Table 1: Number and percentage of unique runs of homozygosity (ROHs) and repeated ROHs with the same start and end position, as well as variable positions

Type of ROH	Length	Number of ROHs	Percentage
Unique	-	5,628	35.86
	Identical	5,663	36.08
Repeated	Variable	4,404	28.06

The N_{ROH} was lower compared to that reported in animals that come from specialized production systems, which could be attributed to a lower intensity of selection since the loss of genetic variation or the formation of ROHs in the genome is influenced, among other factors, by the level of intensity of selection in the populations, which in turn is determined

by the availability of replacements and adoption of technological and reproductive tools, such as artificial insemination (AI) and embryo transfer (ET). In dairy cattle, the intensity of selection is very high and the selection of genetic material is influenced by a limited number of parent families, so the mating of related individuals may be common[30]. In Holstein cattle from the specialized production system in Mexico, the N_{ROH} was 88,529, with a larger population size (~4,500 animals) and L_{ROH} was greater than 8.95 Mb[24]. In other studies in Holstein cattle from the specialized system, the L_{ROH} reported are even higher; for example, in the US it is 299.6 Mb[31] and in Italy it is 297 Mb[32].

The average number of ROHs per animal was 58.13 ± 11.89, with a maximum and minimum of 92 and 10, which is a high value compared to the results of Holstein cattle from the specialized system in Mexico[24], reported on average at 20.07 ROHs per animal, with a maximum of 283 and a minimum of 1. Studies in other Holstein populations of intensive production have reported around 82.3 ± 9.83 ROHs per animal in Holstein cattle from the US[31] and 81.7 ± 9.7 runs per animal in Holstein cattle from Italy[32]. These differences may be due to the high degree of selection in the populations of specialized production systems in both the US and Italy, as well as the availability of genetic material from highly selected bulls compared to the FDS that was analyzed, where the selection objectives are not so defined.

According to the classification of L_{ROH}, the most frequent runs were the shortest (0.5 to 4 Mb) with 64.42 %, followed by those from 4 to 8 Mb with 20.45 %, and the least frequent were the longest runs (>16 to 20 and >20; Table 2). The lengths of the ROHs provide information on the number of generations in which the common ancestor is shared, with the longest being those formed in recent generations[21], so the length of the ROHs found in this population reflects recent and low inbreeding.

Table 2: Frequency of runs of homozygosity (ROH) at different lengths

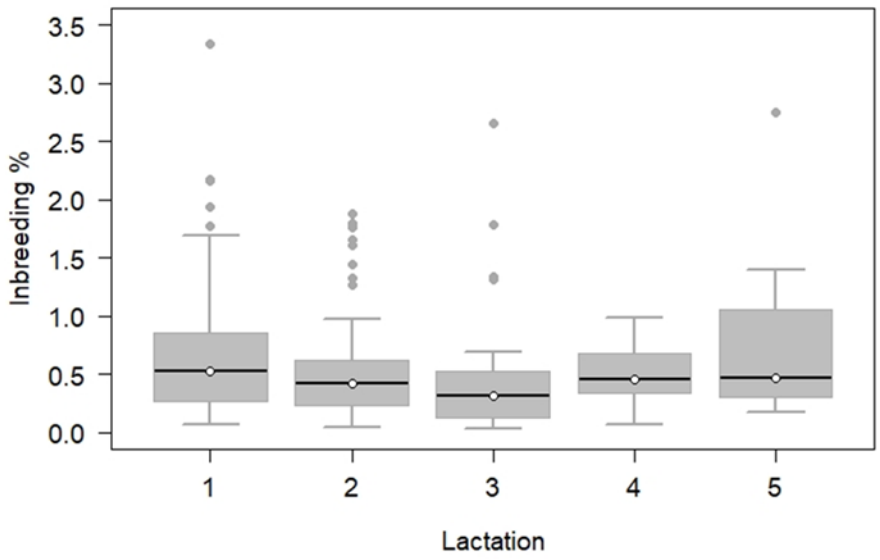
Length (Mb)	Number	Percentage
0.5 to 4	10,110	64.42
>4 to 8	3,209	20.45
>8 to 12	1,131	7.21
>12 to 16	539	3.43
>16 to 20	296	1.89
>20	410	2.61

Mb= Megabases.

The average inbreeding coefficient (FROH) in the population was 0.59 ± 0.53 %, with a maximum of 3.35 % and a minimum of 0.034 %. The results are consistent with the small

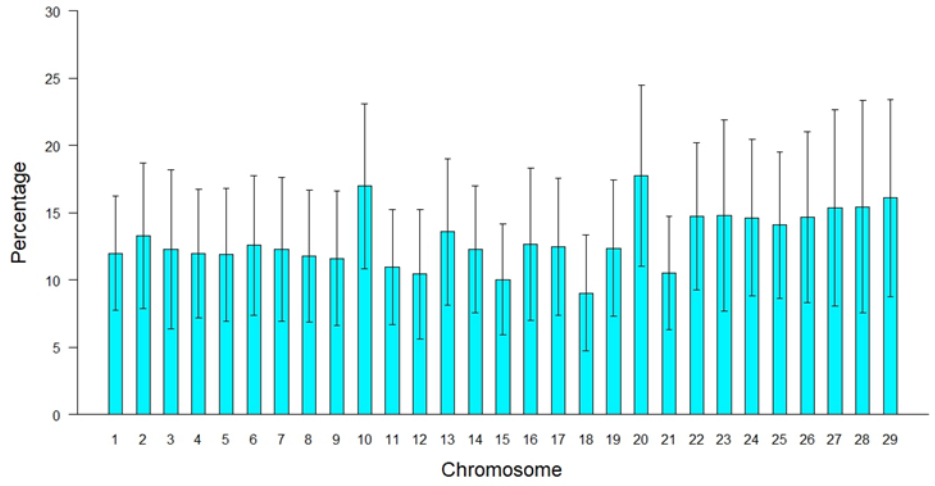
number of ROHs found and the short average length. These values were well below what was reported in other highly selected Holstein cattle populations; for example, 4.2 % in the US[33]. Although the inbreeding found in this population was insignificant, a value that is confirmed by the values calculated for FHOE, which were -0.02 ± 0.08, when reviewing the averages by number of births, a slight increase in FROH was found in recent generations, which could indicate the beginning of an unfavorable trend for the group studied (Figure 3).

Figure 3: Genomic inbreeding (FROH) percentages by lactation number at the time of sampling



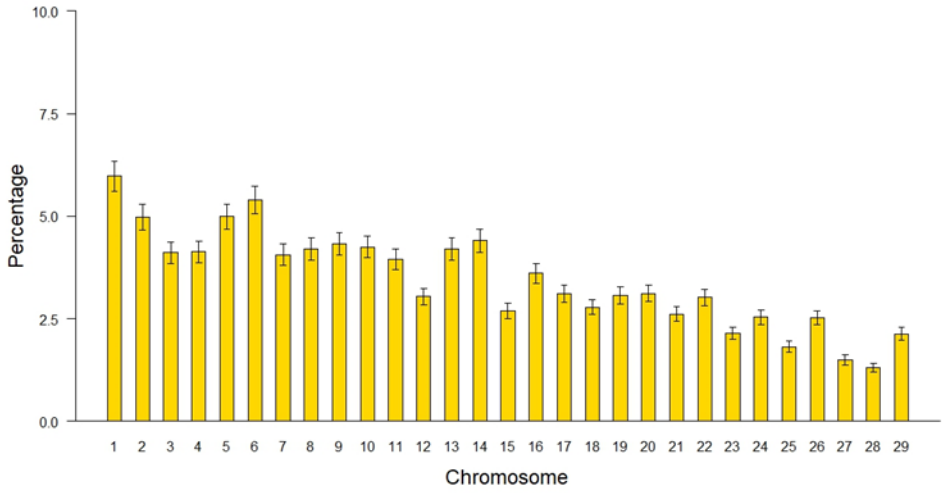
To identify traces of selection throughout the genome, it was searched for specific chromosomes and regions in the location and distribution of ROHs. The presence of ROHs occurred to a greater extent in the long chromosomes than in the short ones, although the latter presented a higher proportion of the genome covered by homozygous regions, as was the case of chromosomes 10 and 20, which presented 10 and 20 with 16.98 % and 17.76 % (Figure 4), behavior similar to what was reported by Szmatola *et al*[34] in Holstein cows from Poland, suggesting that these regions have been subject to greater selection, due to association with traits of economic interest. The percentages of homozygosity per chromosome are higher than the average FROH value because the length of the chromosome is taken as one hundred percent and not the total length of the genome covered by the SNPs; this gives a better perception of the length of the chromosome covered by ROHs.

Figure 4: Percentage of chromosome covered by runs of homozygosity (ROH)



Throughout the genome, a positive relationship was observed between chromosome size and the number of ROHs detected on that chromosome, but this was not the case for the percentage of chromosome length covered by ROHs since short chromosomes showed a higher proportion covered by ROHs (Figure 5), this is because the average length of ROHs was greater in short chromosomes than in long chromosomes, because long chromosomes have more recombination than short chromosomes[8]. Of the total ROHs determined in the population, chromosomes 1 and 6 were the ones with the highest number of ROHs (5.98 and 5.39 %) and the chromosomes with the lowest number were chromosomes 28 and 27 (1.31 and 1.50 %), results that are similar to those of Purfield *et al*[17], who also reported a higher number of ROHs on the long chromosomes than on the short ones.

Figure 5: Percentage of runs of homozygosity (ROH) on each chromosome



According to the frequency of repeated ROHs in the population, only 35 were found in 10 or more animals and were distributed on chromosomes 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 17, 19, 21, 22, 23, 26, and 29. The most frequent ROHs were found on chromosomes 2 and 22, in 27 and 23 animals, respectively, with the length in these runs being 1.82 Mb and 1.61 Mb. In the same position as the runs found on chromosome 2 (83.84-85.66 Mb), Cole *et al*[35] reported QTL (Quantitative Trait Loci) related to hip width and height in Holstein cattle from the US; Cai *et al*[36] reported QTLs associated with milk fat production in Holstein cattle from Nordic countries, which may indicate traces of selection on these chromosomes[37].

Of the total ROHs of variable length, only 37 were found in 10 or more animals, distributed throughout the genome, except on chromosome 8. In the region where the most frequent ROHs in the population were found (≥20 animals), numerous associations, QTLs and genes related to milk production and composition, fertility parameters, susceptibility to diseases, body conformation, feed efficiency and some characteristics of carcass composition (Table 3) have been reported. The results show that, although the ROH lengths in this population (~4.79 Mb) suggest crossbreeding of animals related approximately 16 generations ago[11], the conserved regions could indicate that selection in this population is aimed at improving milk production, composition, fertility, and health, as could be expected in milk production systems. On chromosomes 1 and 2, in addition to associations with characteristics of interest in dairy cattle, associations with carcass characteristics are observed, findings that could suggest possible crosses with other breeds.

To search for ROH annotations with variable length, the shortest ROH with respect to the final position was used as a reference (Table 3), to avoid providing information outside the region common to all animals with a specific ROH.

Table 3: Genome annotations found in regions where the most frequent runs of homozygosity (ROH) in the family dairy population were detected

BTA	Start position	Length (pb)	NoA	Associations /QTL reported	Genes NCBI	Traces of selection
13	389,736	1,593,318	38	CALEASE, PTAT, FY, MY, NM, PY, UHT, SB, STA, FANG, FTLEG, UA, RLEGR, RLEGS, RTPL, SCS, MRCT, FSC, CONCEPT, MBCASP, MPFRAT, DYF, DYST, TPL, TLGTH, UDPTH, PP, FP, HTINT.	Associated: 287026.	TMX4, PLCB1, MIR2285M-1.
1	761,316	1,116,706	32	PP, MKCASP, CONCRATE, MUGKCASP, BTBS, SCS, FATTH, PY, RFI.	Associated: 506426. Candidate: 282257.	ATP5O, ITSN1, CRYZL1, DONSON, SON, GART, DNAJC28, TMEM50B, IFNGR2, IFNAR1, LOC104970778, IL10RB, IFNAR2, LOC526226, OLIG1, OLIG2.
17	67,686	1,209,677	32	FY, PY, CONCRATE, CONCEPT.		TMEM192, KLHL2, MSMO1, CPE, LOC101903170.
2	83,841,602	1,794,112	31	FSC, NRR, CONCRATE, CONCEPT, MUGKCASP, MSPD, BTBS, FY, BD, CALEASE, PTAT, FTPL, UA, NM, PL, RTPL, UHT, RUMWD, SCS, SB, STA, STR, UC, UDPTH, LMY, EY, BW, BVDV.	Candidate: 526800 Candidate: 19122 Gene: 521004.	SLC39A10, DNAH7, STK17B, LOC531691.
7	153,780	811,014	31	CALEASE, SB, FANG, FTLEG, PTAT, FTPL, UA, NM, PL, RLEGR, RLEGS, UHT, SCS, STA, UC, UDPTH, MBCASP.	Gene: 338031.	LOC107131408, LOC100125913, LOC101902704, FLT4, CNOT6, GFPT2, MAPK9, RASGEF1C.

BTA= chromosome, NoA= number of animals.

Associations /QTLs reported. CALEASE= calving ease, PTAT= conformation final score, FY= milk fat yield, MY= milk yield, NM= net merit, PY= milk protein yield, UHT=udder attachment height,

SB=stillbirths, STA= stature, FANG= foot angle, FTLEG= foot and leg conformation, UA=udder attachment, RLEGR= rear leg placement - rear view, RLEGS= rear legs- side view, RTPL= rear teat placement, SCS= somatic cell score, MRCT= milk rennet coagulation time, FSC= first service conception, CONCEPT= number of inseminations per conception, MBCASP= milk B-casein percentage, MPFRAT= milk protein-to-fat ratio, DYF= dairy character, DYST= dystocia, TPL= teat placement, TLGTH= teat length, UDPTH=udder depth, PP= milk protein percentage, FP= milk fat percentage, HTINT= heat intensity, MKCASP= milk Kappa casein percentage, CONCRATE= conception rate, MUGKCASP= milk non-glycosylated kappa casein percentage, BTBS= bovine tuberculosis susceptibility, FATTH= fat thickness in the 12th rib, RFI= residual feed intake, NRR= non-return rate, MSPD=milking speed, BD= Body depth, FTPL=front teat placement, PL= productive life span, RUMWD= rump width, STR=milk strength, UC=udder cleft, LMY=lean meat yield, EY= energy of milk yield, BW= birth body weight, BVDV= bovine viral diarrhea susceptibility.

NCBI genes. 287026= phospholipase C beta 1, 506426= crystallin zeta protein encoder, 282257= subunit 1 of interferon alpha and beta receptor, 526800= ankyrin repeat domain 44, 19122= prion protein, 521004= solute carrier family 39 member 10, 338031= FMS-related receptor tyrosine kinase 4.

Traces of selection (protein-coding genes). TMX4= thioredoxin-related transmembrane protein 4, PLCB1= phospholipase C beta 1, MIR2285M-1= microRNAs involved in post-transcriptional regulation of gene expression, ATP5O= ATP synthase peripheral stalk subunit, OSCP= ATP synthase peripheral stalk subunit, ITSN1= intersection 1, CRYZL1= crystallin zeta protein encoder, DONSON= DNA replication fork stabilization factor, SON= DNA and RNA-binding protein, GART=phosphoribosylglycinamide formyltransferase synthetase, Phosphoribosylaminoimidazole synthetase, DNAJC28= heat shock protein family, TMEM50B= transmembrane protein 50B, IFNGR2=interferon gamma receptor 2, IFNAR1= interferon alpha and beta receptor subunit 1, LOC104970778= uncharacterized RNA gene, IL10RB= interleukin receptor subunit beta, IFNAR2= interferon alpha and beta receptor subunit 2, LOC526226= histone H4, OLIG1= oligodendrocyte transcription factor 1, OLIG2= oligodendrocyte transcription factor 2, TMEM192= transmembrane protein 192, KLHL2= kelch of family 2, MSMO1= methylsterol monooxygenase 1, CPE= carboxypeptidase E, LOC101903170= uncharacterized RNA gene, SLC39A10= solute carrier family 39, DNAH7= dynein axonemal heavy chain 7, STK17B=serine/threonine kinase 17b, LOC531691= HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2, LOC107131408= olfactory receptor family 5 subfamily W member 39, LOC100125913= uncharacterized gene, LOC101902704= C-type lectin domain family, 7, A, FLT4= fms-related receptor tyrosine kinase 4, CNOT6= CCR4-NOT transcription complex subunit 6, GFPT2= glutamine-fructose-6-phosphate transaminase 2, MAPK9= mitogen-activated protein kinase 9, RASGEF1C= RasGEF domain family member 1C.

In the study population, ROHs that have been maintained as a result of the selection process of the family system population were identified. In these conserved regions, associations of SNP markers, QTL and gene are found, which are mostly related to characteristics of economic interest in the dairy industry, such as milk production and composition, fertility parameters, susceptibility to diseases, body conformation, feed efficiency and some other characteristics such as carcass composition, which could be taken as traces of selection (Table 3). These results show the traits that have been included in the selection processes in the population, either intentionally because of the selection made by cattle farmers or unintentionally because of the availability of genetic material in the market, since, by using AI, the choice of bulls guides the cattle farmer to modify the genetics of their animals in the way that AI companies do.

Conclusions and implications

The dairy cattle from the FDS have ancestral origins from countries that are suppliers of genetic material internationally, such as the US and GBR, showing no evidence of recent crossbreeding with other dairy breeds. Within the studied population, it can be observed genetically homogeneous, with a lower number and length of ROHs than animals in specialized production systems, reflecting a wide genetic variation caused by a low intensity of selection. In this work, ROHs that have been maintained as a result of the selection process were identified, which are mostly related to characteristics of economic interest in the dairy industry. The results of this study reflect the existence of a low level of inbreeding in the population and a greater genetic diversity in this population compared to those found in specialized systems, so it is possible to carry out genetic improvement work aimed at the characteristics of interest of the producers through selection, without inbreeding compromising the productivity and health of the population.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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https://doi.org/10.22319/rmcp.v15i2.6420

Article

Effect of various castration protocols on production indicators in pigs: meta-analysis

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Abstract:

The objective was to evaluate the effect of various castration protocols through a meta-analysis of indicators of daily feed intake, feed conversion, daily weight gain, slaughter

weight, hot carcass weight, and carcass yield. 179 publications from three electronic sources (Scopus, PubMed, and Web of Science) were reviewed over a 24-year period, out of which the 26 studies that met the inclusion criteria were selected. The effect was analyzed with six comparisons: C1=surgical castration *vs* whole; C2=standard immunocastration *vs* whole; C3= standard immunocastration *vs* surgical castration; C4= alternative immunocastration *vs* whole; C5= alternative immunocastration *vs* surgical castration, and C6= alternative immunocastration *vs* standard immunocastration. Averages were estimated for feed intake (kg) (0.23, 0.23, -0.05, 0.32, 0.11, -0.09), feed conversion (kg:kg) (0.27, 0.05, -0.16, 0.11, 0.11, 0.11, -0.19), daily weight gain (g) (-9. 54, 39.08, 40.70, 107.63, -53.0, 69.14), carcass weight (kg) (-9.54, 39.08, 40.70, 107.63, -53.0, 69.14), and hot carcass weight (kg) (1.23, 0.85, 0.46, 1.03, 1.02, -0.42) respectively. The indicators of feed consumption, feed conversion, daily weight gain, slaughter weight, and hot carcass weight proved different ($P<0.05$); only the carcass yield variable exhibited no difference ($P>0.05$). The conclusion is that immunocastration improves performance in production and carcass indicators; surgical castration improves carcass yield; whole pigs have better feed conversion, and standard and alternative immunocastration differ in their response in terms of production and carcass measurement indicators.

Keywords: Immunocastration, GnRH, Meta-analysis, Carcass, Stockbreeding.

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Introduction

In whole pigs for slaughter, the sexual taste and odor in the meat is perceptible[1]. Therefore, at early ages, it is preferable to castrate pigs surgically; this technique is the most commonly used, while it is also invasive[2]. Various methods of castration were addressed in the 1990s[3], among which the results of the application of immunization against GnRH on sexual odor, the response in production and carcass indicators were most prominent[4,5].

The standard immunization protocol consists of two subcutaneous doses at 12 and 16 wk of age[6]. The former allows for immune recognition, antibody production, and anchoring to gonadotrophs. The second dose increases the immune response, causing gonadal atrophy and eliminating androstenone as a sexual odor precursor[7].

An increase in daily weight gain, higher carcass weight at slaughter and a reduction in back fat thickness have been reported to occur with immunocastration[8,9]; however, other authors report the opposite, observing a lower carcass yield and a lower daily weight gain[10,11].

Studies have been published in which different GnRH immunization protocols involving age, interval, and number of doses have been applied. Some of the results observed in these studies in the protocols that had 4- and 6-wk intervals between doses were improvements in feed conversion and carcass weight[12] and improved growth performance indicators in late immunocastration and prepubertal protocols[13], while the standard and delayed immunization protocols resulted in better feed efficiency[14].

The results of the different studies on castration methods have been analyzed with the statistical tool of meta-analysis. An example of this can be seen in the 2012 paper by Batorek *et al*[15], where the comparison between immunocastration *versus* surgical castration and whole pigs showed that immunocastrated pigs had longer carcasses compared to surgically castrated and whole pigs, as well as a faster growth rate and a better feed conversion than whole pigs.

In another 2018 study[16], immunocastration in pigs produced a higher daily weight gain and a lower feed conversion rate than surgical castration. On the other hand, higher daily weight gain, feed intake, trace weight and back fat were observed in immunocastrated sows compared to whole sows[17].

The objective of the present meta-analysis was to evaluate the effect of the different castration protocols used until 2020, with an emphasis on the analysis of the castration technique, as well as the effect on the age of application of the immunocastration protocol, and to compare between standard and alternative immunization application, through the production indicators daily weight gain, daily feed consumption, feed conversion, and carcass evaluation based on live slaughter weight, hot carcass weight, and carcass yield.

Material and methods

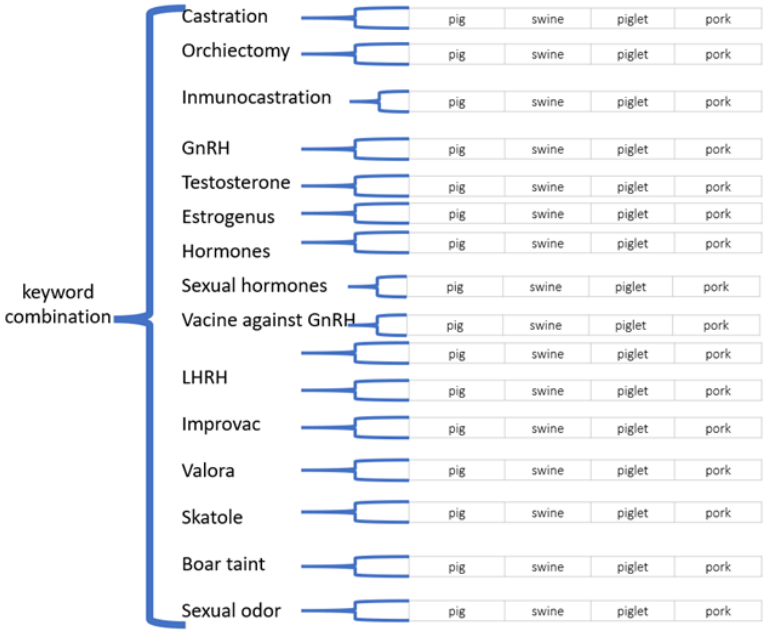
The development of the present work included a literature review of the publications related to the different castration protocols (surgical castration, standard GnRH immunization, and alternative immunocastration) and also considered whole pigs. The methodological procedure was: information search; systematic review; quantitative synthesis; categories of analysis, and statistical analysis.

Information search

The search began with the approach and the research question of this work, which brought the focus of the literature review to those publications that studied the effect of castration protocols, either surgical or immunological, on the production process of pigs for slaughter during breeding and the subsequent output of finished pigs to the slaughterhouse, as well as the collection and measurement of the carcass. The search for information began in 1994, when the first results on this subject were reported, up to 2020. In the systematic review, the electronic meta-search engines Scopus, PubMed, and Web of Science were used to search for papers.

The primary search was performed by mixing keywords related to the topic (Figure 1). The available publications in the fields of biological sciences, animal production sciences, and meat science and technology were segregated, and the corresponding results, mean values, and dispersion measures were published.

Figure 1: Keyword combinations for article searches



Systematic review

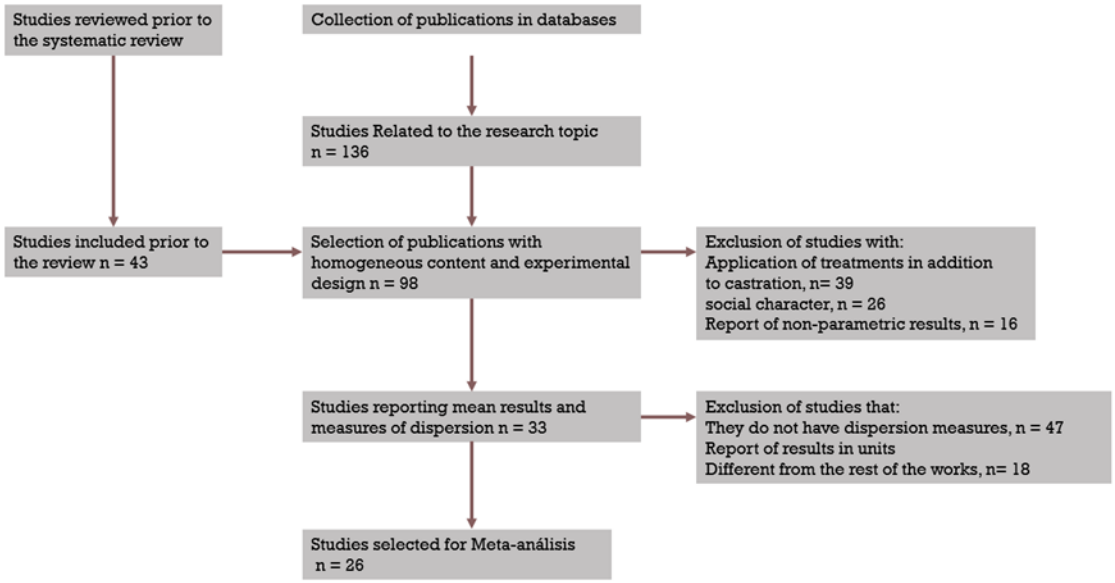
The selection of the papers was applied to 179 articles by two team members, who determined the criteria for selection and exclusion of the articles based on the initial research

question and approach (Figure 2). Once these criteria were established, the selection process resulted in 26 articles for analysis (Figure 3).

Figure 2: Inclusion and exclusion criteria for article selection

Inclusion	Exclusion
• GnRH immunization, surgical castration and use of whole pigs. • Use of pigs for slaughter and consumption as research subjects. • Pigs with a terminal genetic basis in the conformation of different local genetic lines. • Work with results on growth, development and finishing indicators. • Papers with results on indicators of interest in meat science and technology. • All with results in the chemical and nutritional composition of the meat. • Works by various nationalities. • Works by different institutes and educational or governmental entities.	• To have any other treatment in addition to surgical and immunological castration protocols. • Subjects of research not intended for consumption, slaughter or production processes. • Work directed to a social field to which no corresponding results were reported for pork production, procurement, or consumption processes. • Papers that reported nonparametric results, as well as those whose results were ranges or subjective values. • Observational studies with data reported by qualitative indicators.

Figure 3: Information selection flow



Quantitative summary

Table 1 shows the characteristics of the works included in the analysis in terms of technique and method of castration.

Table 1: Details of publications selected for statistical analysis

Author	Gentics	Sex	Experimental groups
Andersson *et al*[19]	Yorkshire x Landrace	m, h	Q, Ie, aI, W
Bonneau *et al*[3]	Large white x Pietrain	h	Q, Ie, W
Channon *et al*[20]	Large White x (Landrace x Duroc x Largewhite)	m	sI, W
Daza *et al*[11]	Duroc x (Landrace x Large white)	m, h	Q, sI, W
Di Martino *et al*[21]	Terminal	h	sI, W
Dunshea *et al*[8]	Large white x Landrace	m	Q, sI, sI, W
Font *et al*[22]	Terminal	m, h	Q, sI, W
Galleos *et al*[23]	Terminal	m	sI, Ia
Gamero *et al*[24]	Ibérico x Duroc	h	Q, sI, W
Gogic *et al*[25]	Swallow-bellied Mangalitsa	m	sI, W
Grela *et al*[9]	Polish Zloynika	m	Q, sI, aI, W
Laeliifano *et al*[12]	Large White x Landrace	m	sI, aI, W
Morales *et al*[26]	Terminal	m	Q, sI, W
Oliviero *et al*[27]	Landrace	m	Q, W,
Pauly *et al*[28]	Large white	m	Q, sI, W
Rikard-Bell *et al*[29]	Terminal	m	sI, W
Rodriguez *et al*[30]	Terminal	h	sI, W
Skrelp *et al*[31]	Cerdos gordos eslovacos x Duroc	m	Q, sI, W
Skrelp *et al*[32]	Large white x Landrace x Duroc	m	Q, sI, W
Stupka *et al*[33]	Duroc x (Large White x Landrace)	m, h	Q, sI, W
Turkstra *et al*[10]	(Deutch Landrace x Finnish Landrace) x Large White	m	Q, sI, aI, W
Van den Broeke *et al*[34]	Terminal	m, h	Q, sI, aI, W
Weiler *et al*[35]	Terminal	m, h	Q, sI, W
Yuan *et al*[36]	Duroc·(Landrace·x Large White)	m	Q, sI
Zoels *et al*[14]	Piétrain × Large White x Landrace	m	Q, sI, aI, W

*sex: f=females, m=males; Experimental group: Q= surgical castration, sI= standard immunocastration, aI= alternative immunocastration; W=whole pigs.

The groups for analysis were the following:

Whole: pigs that remained without any type of intervention during the production period.
Surgical castration: pigs that had their testicles surgically removed before 7 d of age.
Standard immunocastration: pigs that underwent the immunization protocol as indicated by the manufacturer, two doses subcutaneously, at approximately 12 and 16 wk of age.
Alternative immunocastration: pigs that received the immunization protocol at ages other than the standard or with a longer interval between doses.

Categories of analysis

The analysis of the information was divided into two stages:
a) Production period: described by indicators of feed intake, feed conversion ratio, and daily weight gain.
b) Carcass measurement process: described by the indicators slaughter weight, hot carcass weight, and carcass yield.

From the identification and description of the categories of analysis, the following comparisons were established for the analysis of the information in each of the aforementioned indicators:

C1: surgical castration
C2: standard immunocastration *vs* whole.
C3: standard immunocastration *vs* surgical castration.
C4: alternative immunocastration *vs* whole.
C5: alternative immunocastration *vs* surgical castration.
C6: alternative immunocastration *vs* standard immunocastration.

Statistical analysis

The statistical analysis used to synthesize the results of various studies on the effect of castration protocols on the indicators of the productive period and the carcass measurement process was carried out with the NCSS® software (NCSS Statistical System for Windows, Kaysville, UT: Number Cruncher Statistical Systems, 2021). Dispersion averages were estimated based on the values of the mean, standard deviation, and number of observations for each indicator under study. A random effects model was utilized to test the hypothesis of

heterogeneity, the average standard difference of the effect, and its confidence interval (α= 0.05); this decision was supported by Chi-square tests[18].

Results

Table 2 shows the comparisons, the result of the differences between the means points to the first analysis protocol for each of them.

Production period indicators

For the feed intake indicator, the alternate immunocastration (C4 and C5), surgical castration (C1 and C3) and standard immunization (C2 and C6) protocols obtained the highest feed intake (Table 2). Animals within the two immunization protocols had a higher intake than castrated animals (C3 and C5). Finally, among the immunization protocols, the standard protocol was the one that had the highest intake.

For feed conversion (Table 2), the whole pig protocol obtained the best value (C1, C2, and C4), followed by the standard immunocastration (C3 and C6) and surgical castration protocols (C5).

For daily weight gain (Table 2), the standard immunization protocol exhibited the best results (C2, C3 and C6), followed by the alternate immunization (C4 and C5) and whole pigs (C1) protocols.

Indicators of the carcass measurement process

Table 2: Results of the analysis of the indicators for each comparison

C1 **Surgical castration *vs* whole**					**C4** **Alternative immunocastration *vs* whole**				
Variable	n	Difference between means ± SW	Coefficient limits (95%)	*P*	Variable	n	Difference between means ± SW	Coefficient limits (95%)	*P*
Feed intake, kg	16	0.23 ± 0.03	(0.16; 0.29)	0.01	Feed intake, kg	8	0.32 ± 0.09	(0.13; 0.51)	0.01
Feed conversion, kg:kg	13	0.27 ± 0.04	(0.20; 0.34)	0.01	Feed conversion, kg:kg	5	0.11 ± 0.15	(-0.18; 0.40)	0.01
Daily weight gain, g	15	-9.54 ± 16.62	(-42.12; 23.03)	0.01	Daily weight gain, g	8	107.63 ± 58.75	(-7.52; 222.78)	0.01
Slaughter weight, kg	14	4.11 ± 3.35	(-2.45; 10.68)	0.01	Slaughter weight kg	5	0.09 ± 1.36	(-2.56; 2.76)	0.01
Hot carcass weight, kg	17	1.23 ± 0.51	(0.22; 2.24)	0.01	Hot carcass weight, kg	6	1.03 ± 0.86	(-0.66; 2.72)	0.01
Carcass yield, %	9	0.33 ± 0.52	(-0.69; 1.35)	0.97	Carcass yield %	6	-0.22 ± 0.63	(-1.46; 1.01)	0.94
C2 **Standard immunocastration *vs* whole**					C5 **Alternative immunocastration *vs* surgical castration**				
Feed intake, kg	17	0.23 ± 0.08	(0.08; 0.38)	0.01	Feed intake, kg	5	0.11 ± 0.12	(-0.13; 0.34)	0.01
Feed conversion, kg:kg	15	0.05 ± 0.04	(-0.0; 0.13)	0.01	Feed conversion, kg:kg	5	-0.19 ± 0.14	(-0.56; 0.08)	0.01
Daily weight gain, g	21	39.08 ± 11.37	(16.79; 61.34)	0.01	Daily weight gain, g	6	69.14 ± 32.67	(5.10; 133.18)	0.01
Slaughter weight, kg	21	1.24 ± 0.59	(0.07; 2.42)	0.01	Slaughter weight, kg	7	1.28 ± 0.50	(0.29; 2.27)	0.01
Hot carcass weight, kg	25	0.85 ± 0.47	(-0.06; 1.76)	0.01	Hot carcass weight, kg	7	-0.42 ± 0.44	(-1.28; 0.44)	0.01
Carcass yield, %	12	-0.68 ± 0.46	(-1.58; 0.22)	0.99	Carcass yield, %	4	-0.10 ± 0.75	(-1.58, 1.37)	0.89
C3 **Standard immunocastration *vs* surgical castration**					**C6** **Alternative immunocastration *vs* standard immunocastration**				
Feed intake, kg	12	-0.05 ± 0.07	(-0.19; 0.09)	0.01	Feed intake, kg	6	- 0.09 ± 0.11	(-0.31; 0.12)	0.01
Feed conversion, kg:kg	10	-0.16 ± 0.03	(-0.23; -0.09)	0.01	Feed conversion, kg:kg	7	0.11 ± 0.07	(-0.03; 0.24)	0.01

Daily weight gain, g	14	40.70 ± 12.18	(17.03; 64.77)	0.01	Daily weight gain, g	12	-53.00 ± 24.32	(-100.66; -5.34)	0.01
Slaughter weight, kg	14	1.21 ± 0.53	(0.16; 2.25)	0.01	Slaughter weight, kg	9	0.47 ± 2.07	(-3.58; 4.53)	0.01
Hot carcass weight, kg	17	0.46 ± 0.69	(-0.90; 1.81)	0.01	Hot carcass weight, kg	9	1.02 ± 1.67	(-2.25; 4.30)	0.01
Carcass yield, %	9	-0.66 ± 0.52	(-1.68; 0.35)	0.45	Carcass yield, %	9	0.29 ± 0.52	(-0.72; 1.31)	0.76

Final weight showed favorable indicators for alternative immunization at C4, C5, and C6 (Table 2), followed by standard immunization at C2 and C3, and surgical castration (C1).When analyzing the hot carcass weight, the highest value was obtained with the standard immunization protocol (C2 and C3), followed by the alternative immunization protocol (C4 and C6), and surgical castration (C1 and C5).

Contrary to the final weight and the hot carcass weight, the response observed in the carcass yield analysis showed that the surgical castration protocol obtained higher percentages in C1, C3, and C5. On the other hand, whole pigs show higher performance at C2 and C4, leaving only the alternative immunization protocol with a favorable indicator at C6.

Figures 4 and 5 represent the heterogeneous behavior ($P<0.05$) in the analysis of the alternative immunocastration protocol at C4, C5, and C6. In both cases, an increase in favorable results was observed with the application of the alternative immunization.

Discussion

The response of the analyzed indicators is explained on the basis of the results of a whole animal, considered as the ideal productive animal for its metabolic qualities and their effect on the carcass[37].

Production-period indicators

Feed intake

The amount of food consumed is regulated by the satiety center, which responds to serum concentrations of leptins, produced by adipocytes. Several factors can modify feed intake such as: the presentation and formulation of the food, the physical and physiological state of the individual, and the physical and social activity within the group[38].

The physical and physiological state of the animal modifies the feed intake; therefore, surgical castration or immunocastration are protocols that alter this indicator. Surgical castration increases feed intake due to the increase in the serum concentration of leptins, 2.97 ng/ml — as a consequence of the redistribution and increase of adipose tissue after the removal of the gonads[39,40]—, saturating and inhibiting the satiety center located in the central nervous system[41]. In immunocastrated pigs, serum leptin concentrations remain similar to those of whole pigs (2.68 ng/ml); therefore, satiety is not altered[15].

Likewise, the interruption of the hypothalamic-pituitary-gonadal axis when the gonads are removed affects the consumption and satiety habits, also influenced by sex hormones, especially by estrogens, which when the testes are removed are not aromatized; this causes the estrogen to be produced by the adipose tissue in low concentrations, of 0.34 pg/ml, and, therefore, the pig exhibits resistance to glucose and a higher feed intake[42]. Estradiol concentration decreases in immunized pigs, 0.37 pg/ml; however, its effect may vary depending on the age at which the second dose is applied[43].

Another element that modifies the intake is the physical and social activity within the group. While whole pigs show greater sexual activity, dominance and competitiveness for food, whereby their feed intake diminishes[37], immunocastration and surgical castration reduce the presence of androgens and, therefore, the pigs' sexual activity and dominance, increasing their feed intake[15].

With respect to immunization protocols, studies report no differences, contrary to the findings of this study, where variability in the timing of alternative immunizations may alter the feed intake response[23].

Feed conversion

The best feed conversion is established as the one with the lowest feed intake in relation to the production of one kilogram of meat; among the factors that can modify it are the physical and physiological state of the pig.

Somatotropin is one of the elements required for muscle development, which is altered if the physical state changes[41] as a consequence of the reduction of the blood IGF-1 concentration in castrated pigs to 256 ng/ml, related to the reduction of estradiol, whereby the mechanisms of muscle growth and development become altered[43]. In immunized pigs the concentration of IGF-1 reaches 332 ng/ml, compared to its value in whole pigs, which is 459 ng/ml[43].

In late alternative immunization protocol, the feed conversion value increases with the second dose; therefore, the age of application is a relevant factor in allowing the physiological mechanisms to remain for a longer period of time[15,44].

Daily weight gain

The physiological mechanisms and state, and the physical and social activity of the pigs are some of the factors that influence weight gain. The utilization of nutrients in the diet promotes the growth and development of tissues. An example of this is the response of the muscle masses to the presence of lysine, which causes a retention of nitrogen in muscle of 31.24 g/d[45,46]. Surgical castration allows a retention of 25.51 g/d of nitrogen, while immunocastrated pigs retain only 22.95 g/d[45,47,48].

The physical activity of whole pigs is more dynamic due to the hierarchy within groups, as well as the competition in feed intake, also considering that, as the age of puberty approaches, the incidence of aggressions increases[37].

In the case of surgically castrated pigs, physical activity and energy demand are lower; however, competition for feed intake prevails and, in some cases, increases because of alterations in the satiety centers. In immunized pigs, the increase in this indicator responds to the reduction of aggressions and dominance attitudes among pigs, which allows a homogeneous feed consumption and reduces the need to heal wounds and injuries[41].

Indicators of the carcass measurement process

Slaughter weight

At the end of the production cycle, the final weight of the animals will depend on the physical condition and age at slaughter; it should be noted that the result is related to the feed conversion rate and daily weight gain, as well as to the metabolic mechanisms of these indicators. The production cycle of pigs for slaughter usually lasts approximately 20 to 22 wk, during which time the whole pig reaches an average weight of 110 kg, attributed to its metabolic efficiency, considering that a large part of this value corresponds to muscle mass[38].

Surgically castrated pigs eventually gain weight due to the redistribution and growth of adipose tissue, especially subcutaneous fat[49]. The weight of immunocastrated pigs involves a similar muscle development to that of whole pigs, a thicker fat cover —without reaching those of the surgical castration protocol—, homogeneous feed consumption, metabolic utilization, and a higher weight gain due to docile behavior[41].

Hot carcass weight

After slaughtering and the process of removing the head and viscera, the carcass is obtained; its weight is determined by the morphology of the pig, as well as the amount of fat it contains and its relation to the muscles. The carcass of whole pigs is leaner because it has little fat cover and intermuscular fat, in addition to the 1.4 % reduction in weight corresponding to the testicles[50], which coincides with the results of the meta-analysis performed in 2012[15].

In surgically castrated pig carcasses, the amount of fat cover and intermuscular fat increases the weight, and the difference in final weight is not affected by the removal of the reproductive tract[41].

In immunized pigs, the fat cover is thicker than that of whole pigs and thinner than that of surgically castrated pigs; they have a better metabolic response after the second dose and allow similar muscle growth and development to those of a whole pig[51]. Alternative immunization protocols have a better response, especially in late immunization or with a longer interval between the first and second dose[19].

Carcass yield

The ratio between live weight and carcass weight, as well as the number of cuts that can be obtained and their weight, determine the carcass yield, which is influenced by the amount of inter- and intramuscular fat, as well as the ratio between the volume of the meat pieces and the covering fat[52]. It is important to consider that certain external factors may influence the percentage expression of yield, such as fasting prior to slaughter, transportation, and travel time.

In the case of surgically castrated pigs, there is a greater content of intermuscular adipose tissue, giving more weight to the cuts, despite the fact that the number of cuts in whole pigs is larger due to the length of the carcass[53].

The volume of meat of pigs immunized with either one of the application protocols is lower than that of whole pigs, as well as of the meat and intermuscular fat developed by surgically castrated pigs, and, therefore, their performance is lower[15,16].

Conclusions and implications

In conclusion, the meta-analysis of various castration protocols in pigs under experimental conditions shows that immunocastrated pigs, with both the standard and alternative application protocols, are more efficient in terms of the indicators of consumption and weight gain, as well as of live weight and hot carcass weight. A higher carcass yield is observed in surgically castrated pigs. Whole pigs have better feed conversion. There are differences in production indicators and carcass measurement between the standard and the alternative immunization protocols.

It is important to consider that the age of immunocastration modifies the results in the production indicators. Castration protocols exhibit different effects on pig production, depending on the type and age at which they are applied. Absence of gonadotropin-releasing hormone and androgen concentration modifies the physiological response to productive performance. Standard immunocastration enhances the response of pig production process

traits. Alternative immunocastration enhances carcass trait response; further research on the age of application of this protocol and its potential effects is required.

Acknowledgments and conflict of interest

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Article

Dry matter accumulation, yield, and nutritional quality of forage of corn hybrids harvested at different days after sowing

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Abstract:

The objective was to evaluate the dry matter (DM) accumulation by component, yield, and nutritional composition of forage of four corn hybrids harvested at 121, 128, 135 and 142 d after sowing. In each harvest, five plants were randomly taken and separated into their components (stem, leaves, grain, cob, bracts, and tassel) and DM was determined; chemical

composition and *in-situ* digestibility were analyzed in a composite sample of a whole plant. The accumulation of grain in the total DM increased from 35.8 to 43.9 % from 121 to 142 d to harvest, respectively, and diluted the other components, especially the proportion of stem and leaves, which decreased inversely proportional to the accumulation of grain. Total DM content differed between hybrids, from 3.8 and up to 8.3 percentage units on the same days to harvest. Nonetheless, the hybrid did not affect DM yield or grain production, increasing by 2.1 and 1.4 t ha^{-1} between harvests, respectively. NDF content decreased and starch increased (both linearly), affecting net energy for lactation, which increased from 1.49 to 1.56 Mcal kg^{-1} from 121 to 142 d to harvest, respectively. The interaction between days to harvest and hybrid affected starch content, which was 5.2 units higher in a hybrid with similar NFC and NDF content than its counterparts. DM, NDF and starch digestibilities were affected by the hybrid, but not by the days to harvest.

Keywords: Starch, Corn silage, Dairy cow.

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Introduction

In northern and central Mexico, corn forage is widely used as silage in dairy cow diets[1], where it represents between 40 and 60 % of the dry basis of the diet[2]. The level of inclusion of corn silage in the ration is a function of the yield and nutrient quality of the forage[3]. In Mexico, in the last 10 yr, the yield per hectare of irrigated forage corn (fresh) has remained relatively unchanged[4]. This is mainly associated with inadequate selection of hybrids and early harvests[2,5], which reduces dry matter (DM) yield and grain content, which is where the highest energy value of forage is concentrated[6].

In central Mexico, the dairy basins of Aguascalientes and Altos-Norte de Jalisco share similar agroclimatic characteristics, contribute 9 and 19 % of national milk production, and cultivate around 15,000 and 45,000 ha of irrigated forage corn, respectively[4]. In this region, water scarcity, growing demand for corn silage and high costs of grains and concentrates make it necessary to make corn production more efficient per unit area and per m^3 of water used[2]. Therefore, increasing forage yield and quality is essential to achieve more sustainable milk production[1,2,7].

The use of outstanding hybrids is the first step to high yield and nutrient quality of forage[8]. Selecting the hybrid and harvesting it at an optimal stage of maturity is essential to achieve maximum accumulation of DM in the grain in a reasonable time[8,9]. Corn silage with better nutritional quality and locally produced can displace imported corn kernel from the diet and reduce feed costs. As the days to harvest are delayed, there is a greater accumulation of grain in the plant, increasing the energy value of the as the proportion of other components in the plant with less digestibility dissolve[9]. Nevertheless, increasing the number of days to harvest to favor grain accumulation decreases the digestibility of neutral detergent fiber (NDF), which negatively affects feed consumption in dairy cattle[6,9]. Thus, the hypothesis of the present study was that delaying the days to harvest increases the yield of DM and grain accumulation without affecting the digestibility of DM and NDF, which are mostly influenced by the hybrid effect. Therefore, the objective was to determine the response of four hybrids harvested at 121, 128, 135 or 142 d in DM accumulation by component, total DM yield, bromatological composition and digestibility of DM, NDF and starch.

Material and methods

Area of study and experimental design

The study was carried out under surface drip irrigation conditions in the SS-2019 cycle in a farm located in San Juan de los Lagos, Jalisco (21º17'40" N and 102º18'01" W) at 1,838 m asl; where the climate is semi-dry temperate with an average rainfall of 600 mm. The soil is alkaline (pH 7.8) with 1.9 % organic matter content and 71 mg kg^{-1} inorganic N. A randomized block design was used with an arrangement in split plots with four replications, where the large plot was the hybrid and the small plots the days to harvest. The hybrids used were DK-4018 (H1, Dekalb®), Noble (H2, Aspros®), Antílope (H3, Asgrow®) and XR-49 (H4, Ceres®), which were selected for having a yield above the average from a local assessment in the previous year. All hybrids were intermediate cycle and semi-toothed white grain. Harvesting was carried out at 121, 128, 135 and 142 d after sowing, which corresponded approximately to a grain maturity stage of approximately R2, R3, R4 and R5, respectively. The experimental plot consisted of four furrows 5.0 m long and 0.75 m wide, and the useful plot consisted of the two central furrows. Sowing was carried out on May 30 in moist soil, depositing the seed manually at a distance of 15 cm at the bottom of the furrow; the population density to harvest averaged 93,211 ± 2,090 plants ha^{-1}.

Agronomic management, data collection and sampling

Prior to sowing, between the first and second harrow pass, 4 t ha^{-1} of cattle compost and poultry manure with a concentration of 1.1 % N and 0.8 % P were applied; additionally, 200 kg of ammonium nitrate was applied between vegetative stages V3 and V6. The total N available in the soil for the crop was estimated at 340 kg ha^{-1}. There were no diseases and only one application for fall armyworm (*Spodoptera frugiperda*) in stage V3 was necessary, which was controlled with an application of chlorantraniliprole (Coragen, FMC®, Mobile, AL). Precipitation and minimum and maximum temperatures were recorded at a weather station (Em50, Meter Group Inc., Pullman, WA) located 80 m from the experimental site. Growing degree-days (GDDs) were calculated as the difference between the average temperature and the base temperature of corn (10 °C). Flowering was recorded when 50 % of the experimental plot exhibited male inflorescence (tassels releasing pollen) and female inflorescence (stigmas on young ears).

At 121, 128, 135 and 142 d after sowing (DAS), all the plants in the useful plot were harvested at 15 cm above ground level and the total fresh weight was recorded. A random sample of five whole plants was separated into five components: ears of corn, stem, leaves, tassel, and bracts. Each component was weighed fresh and placed in paper bags to dry at 55 °C to constant weight to determine DM. After drying, the ear of corn was separated into cob and grain, and samples of each component were ground (SR300 Retsch®, Staufen, Germany) to pass a 1 mm sieve; subsequently, a composite sample of 100 g (dry weight) of whole plant was made in proportion of each component to the total dry weight. The whole sample was used to perform bromatological and *in-situ* digestibility analyses.

Bromatological and digestibility analyses

Chemical analyses were carried out in the forage laboratory of Unión de Cooperativas de Consumo Alteñas S.C. de R.L. (UCCA, San Juan de los Lagos, Jal.). Ash content was determined by introducing 1.0 g of sample into a crucible and incinerating at 550 °C for 6 h in a muffle furnace. The content of NDF and ADF was determined sequentially in 0.5 g of sample introduced into a F-57 bag in the fiber analyzer (A200, Ankom Tech., Macedonia, NY); first, the determination of NDF was performed using alpha-amylase and sodium sulfite, followed by the determination of ADF in CTAB and H_2SO_4 solution. The total nitrogen (N) concentration was determined using the Dumas dry combustion procedure (Leco FP-528, St. Joseph, MO) and the crude protein (CP) content was calculated as % N × 6.25. The starch content was determined using the enzymatic-colorimetric procedure[10]. Initially, glucose was

released by incubating 1.0 g of sample at 100 ºC for 1 h in 30 ml of 100 mM acetate buffer solution at pH 5.0 and 100 µL of alpha-amylase (Megazyme Ltd., Wicklow, Ireland) was added, then the reaction was incubated for 2 h at 50 ºC in 3 ml of GOPOD solution (Megazyme Ltd., Wicklow, Ireland) and then the absorbance was determined at 505 nm in a visible-light spectrophotometer (Genesys 10S, Thermo Sci., Madison, WI). Finally, the content of ethereal extract (EE) was analyzed with the gravimetric method in the Golfish equipment (Novatech GF-6, Tlaquepaque, Jal.) using hexane as a solvent.

Digestibility was determined *in situ* using two rumen-fistulated cows between 70 and 95 d in milk (ENLS, Zapotlanejo, Jal.) fed a fully mixed ration composed of 50 % corn silage, 25 % ground corn grain and 25 % protein-mineral core. First, 4.5 g of sample was placed in 10 × 20 cm dacron bags (R1020, Ankom Tech., Macedonia, NY) and they were secured with a strap. Duplicate samples were introduced into the ventral sac of the rumen to determine DM digestibility, NDF digestibility (NDFD) at 48 h, non-digestible fraction of NDF (uNDF) at 120 h, and starch digestibility at 12 and 24 h. All samples were removed simultaneously and rinsed in a 12 min cycle in a washing machine until clear water was obtained. Subsequently, the bags were dried at 55 ºC to constant weight to calculate the digestibility of the DM by initial *vs* final weight difference; NDFD, uNDF and starch digestibility were calculated by analyzing the bag residue with the procedures already described for NDF and starch.

Statistical analysis

All data were analyzed in the R statistical program (R Studio Inc., Boston, MA) using the *agricolae* package and the *aov* instruction for analysis of variance (ANOVA) with the following model:

$$Y = \mu + A_i + H_j + \delta_{ij} + D_k + (H \times D)_{jk} + E_{ijk}$$

Where:
Y is the response variable,
μ is the overall average,
A is the random effect of replication i (i= 1 to 4),
H is the fixed effect of the j-th hybrid (j= 1 to 4),
δ is the experimental error associated with the large plot (hybrid),
D is the fixed effect of the k-th day to harvest (k= 1 to 4),
(*D* × *H*) is the interaction between hybrid and days to harvest
E*ijk* is the residual error. For digestibility data, the random effect of the cow (l= 1 to 2) was included using the model described above.

All data are least squares means and statistical significance was stated at $P \leq 0.05$. Hybrid and day-to-harvest means, when a linear or quadratic effect was detected, were separated using Tukey's HSD test.

Results and discussion

Flowering, days to harvest, and GDD accumulation

The number of days to flowering was 73 for hybrids H1, H2 and H3 and 71 for hybrid H4. Days to harvest were September 29 (121 d), October 6 (128 d), October 13 (135 d) and October 20 (142 d); for each date, 1,266, 1,329, 1,397 and 1,470 GDDs accumulated, respectively. In the first 34 d of the crop, the mean temperature averaged 25 °C and then fluctuated between 19 and 23 °C.

DM accumulation by component

As shown in Table 1, the analysis of variance did not detect significant interactions between DAS and hybrid in five plant components, except for percentage of cob (P=0.01), where the differences were minimal. The components with the lowest proportion were tassel, cob, and bracts, which remained relatively similar in proportion in the four harvests and together accounted for about 14.5 % of the total DM. In contrast, the components with the highest proportion were stem, leaves, and grain, with the percentage of the latter increasing as the days to harvest progressed.

Table 1: Dry matter (DM) accumulation by plant component, DM content, and whole plant yield of four hybrids harvested at different days after sowing (DAS)

	Component, % of total DM						Whole plant	
	Stem	**Leaves**	**Tassel**	**Grain**	**Cob**	**Bracts**	**DM, %**	**DM, t/ha**
DAS[1]								
121	20.4^{A}	28.3^{A}	0.8	35.8^{C}	6.7	8.0^{A}	25.8^{D}	24.9^{C}
128	18.6^{B}	26.2^{B}	0.8	40.2^{B}	6.6	7.6^{B}	29.5^{C}	25.7^{C}
135	18.7^{B}	25.6^{B}	0.8	41.7^{B}	6.4	6.8^{BC}	34.6^{B}	30.3^{B}
142	19.3^{B}	23.3^{C}	0.6	43.8^{A}	6.4	6.6^{C}	37.8^{A}	33.3^{A}
Hybrid[2]								
H1	19.7^{b}	25.8	0.7^{b}	39.9^{b}	6.2	7.7^{a}	32.3^{b}	29.0
H2	19.2^{b}	26.7	0.6^{b}	39.7^{b}	7.0	6.8^{b}	29.0^{c}	28.1
H3	17.3^{c}	25.3	0.7^{b}	42.0^{a}	6.7	8.0^{a}	35.2^{a}	28.9
H4	20.7^{a}	25.6	1.0^{a}	40.0^{b}	6.1	6.6^{b}	31.2^{b}	28.3
SEM	0.354	0.451	0.025	0.481	0.074	0.163	0.885	0.791
DAS	Q**	L**	NS	Q*	NS	L**	L**	L**
Hybrid	< 0.01	0.184	< 0.01	< 0.01	NS	< 0.01	< 0.01	0.805
D × H	0.662	0.089	0.468	0.226	0.013	0.061	0.014	0.865

[1]Sowing date: May 30, 2019.

[2]Hybrid: (H1: DK-4018; H2: Noble; H3: Antílope; H4: XR-49).

SEM= standard error of the mean; DAS= response of linear (L) or quadratic (Q) days to harvest denoted by: *0.01 < $P \leq 0.05$ and **($P < 0.01$), D × H= interaction between DAS and hybrid, NS= not significant.

^{ABC} Means with different uppercase literals differ in DAS ($P \leq 0.05$)

^{abc} Means with different lowercase literals differ between hybrids ($P \leq 0.05$).

The percentage of stem showed a quadratic response ($P<0.01$), decreasing from 121 to 128 d to harvest and from which it remained without significant changes. In addition, it was observed that the hybrid affected the stem percentage ($P<0.01$), H4 surpassed H1, H2 and H3 by 1.1, 1.6 and 3.5 units, respectively. The percentage of leaves decreased linearly ($P<0.01$), decreasing 1.2 percentage units between harvests, but no hybrid effects were detected in this component. The percentage of grain increased quadratically ($P=0.02$) with the days to harvest, increasing by 4.5 percentage units from 121 to 128 d, and between 0.6 and 2.2 units from 135 and 142 d, respectively. Hybrid H3 outperformed hybrids H1, H2 and H4 in grain percentage by 2.1, 2.3 and 2.1 units, respectively. The largest increase in grain ratio from 121 to 128 corresponded to a decrease in stem percentage over the same period.

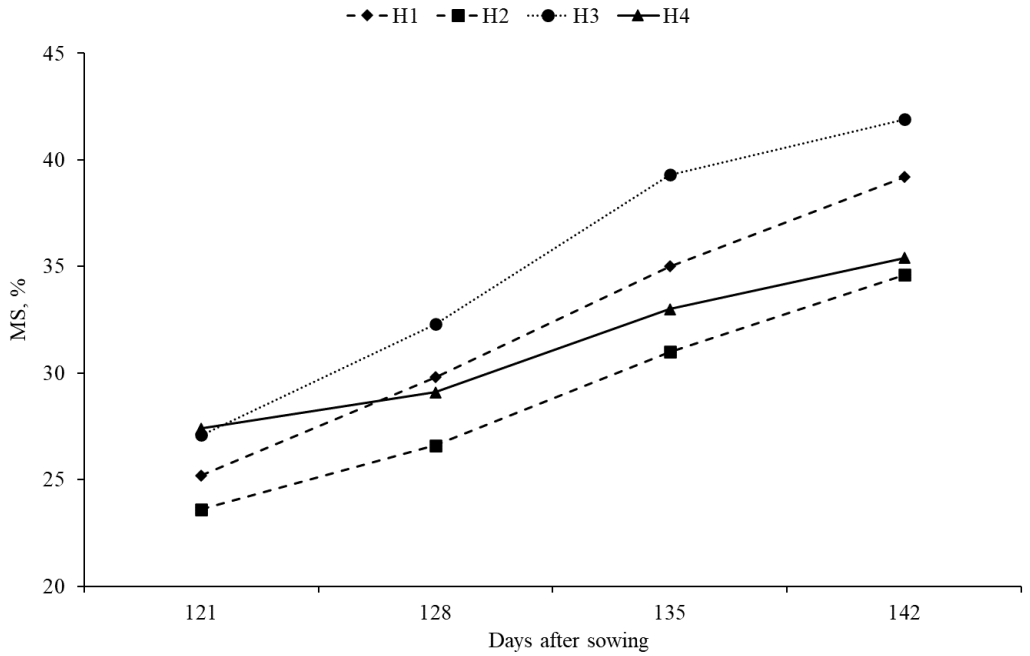
The proportion of tassel was not affected by the days to harvest, but differences between hybrids ($P<0.01$) were detected, which may not be important in the total composition of the plant due to its low proportion to the total DM. The percentage of cob was not affected by the days to harvest or hybrid and remained relatively constant, averaging 6.5 ± 0.3 % of the total DM. The proportion of bracts exhibited a linear response ($P<0.01$), decreasing at a rate

of 0.3 percentage units between harvests; a difference was also detected between hybrids (P<0.01) and it was higher in H1 and H3 compared to hybrids H2 and H4 (7.7 and 8.1 *vs* 6.8 and 66 %, respectively). Hybrids H1 and H3, with the highest proportion of bracts, also had higher grain accumulation.

Whole plant DM content and yield

The interaction between hybrid and days to harvest affected DM content (P<0.01), as shown in Figure 1. Hybrids H3 and H2 had the highest and lowest DM content in the four harvests, respectively; on the other hand, hybrid H1 exhibited a DM accumulation almost linear and intermediate between H3 and H2. In contrast, hybrid H4 showed higher variation in DM accumulation between harvests. These discrepancies may be mostly associated with grain accumulation, but it could also be that the stay-green trait of each hybrid to conserve moisture (mainly in the stems) affects the DM content[11]. The accumulation of DM increased linearly (P<0.01) at a rate of 3 weekly percentage units, equivalent to 0.4 % per day (Table 1). DM accumulation has been reported to be around 0.7 to 1.0 % per day under temperate conditions[12,13]. In the present study, drip irrigation and the regular distribution of rainfall recorded in the cycle could have helped to keep soil moisture constant and reduce plant moisture loss.

Figure 1: Dry matter (DM, %) content in forage of four corn hybrids (H1=DK-4018, H2=Noble, H3=Antilope, and H4=XR-49) grown under irrigated conditions and harvested at 121, 128, 135 and 142 days after sowing



[abcdfghi] Means with different literals differ statistically ($P \leq 0.05$).

DM production increased linearly (P<0.01) at a rate of 2.1 t ha^{-1} per week, but there was no hybrid effect despite the interaction detected in DM accumulation. The increase in DM production was mainly due to grain accumulation as it was the only component that increased in proportion to the total DM with days to harvest. Grain production (t ha^{-1}) increased linearly with days to harvest (P<0.01) and was 8.9, 10.3, 12.6 and 14.6 t ha^{-1} at 121, 128, 135 and 142 d to harvest, respectively, but no hybrid effect or its interaction with days to harvest was detected.

Chemical composition

Except for CP and EE contents, the other bromatological variables were affected by the days to harvest and hybrid (Table 2). CP and EE contents remained within normal and relatively stable ranges, with significant differences between hybrids, but these were minimal. In contrast, the values of NDF, ADF, NFC and starch differed to a greater degree between days to harvest and between hybrids. NDF content decreased linearly (P<0.01) at a rate of 1.6 percentage units between harvests, while the proportion of ADF increased linearly (P<0.01) at a rate of 0.9 percentage units per week. Differences were also detected between hybrids for NDF and ADF contents (both P<0.01), where hybrids H3 and H4 accumulated lower percentages of NDF and ADF than H1 and H2 (Table 2). These findings differ from those reported in a local study in which NDF and ADF decreased by about 3.1 and 1.0 percentage units, respectively, over a 10-d period[14]. In another study in which four harvests were carried out at similar DM content, a reduction in NDF and ADF was also reported; this was attributed to the dilution of these components due to the increase in grain percentage[9]. In the present work, it is speculated that the low cut height (15 cm) at which the harvest was carried out may have caused more cellulose at the expense of hemi-cellulose; this has been documented in other studies in which a shorter stem accumulates more ADF and lignin[15,16].

Table 2: Dry matter (DM) chemical composition in whole corn plant of four hybrids harvested at different days after sowing (DAS)

	% DM[1]							
	CP	**NDF**	**ADF**	**STA**	**NFC**	**EE**	**ASH**	**NEL Mcal kg^{-1}**
DAS[2]								
121	8.0	52.1^{A}	22.5^{D}	19.8^{D}	30.9^{D}	2.4	6.6^{A}	1.49^{D}
128	7.8	49.9^{B}	24.2^{C}	20.9^{C}	33.4^{C}	2.6	6.3^{A}	1.51^{C}
135	7.5	48.3^{C}	24.8^{B}	23.2^{B}	35.1^{B}	2.8	6.3^{A}	1.52^{B}
142	7.9	45.6^{D}	25.9^{A}	25.4^{A}	38.1^{A}	2.8	5.6^{B}	1.56^{A}
Hybrid[3]								
H1	7.6^{c}	49.5^{a}	25.1^{a}	20.6^{d}	33.6^{b}	2.8^{b}	6.5^{a}	1.51^{b}
H2	7.3^{d}	49.4^{a}	25.0^{a}	21.5^{c}	34.5^{ab}	2.2^{d}	6.6^{a}	1.50^{c}
H3	7.9^{b}	48.7^{b}	23.7^{b}	25.2^{a}	34.5^{a}	3.0^{a}	5.9^{b}	1.54^{a}
H4	8.5^{a}	48.2^{b}	23.7^{b}	22.0^{b}	34.7^{a}	2.7^{c}	5.9^{b}	1.53^{a}
SEM	0.020	0.246	0.062	0.034	0.286	0.022	0.064	0.006
DAS	NS	L**	L**	L**	L**	NS	Q*	L*
Hybrid	< 0.01	< 0.01	< 0.01	< 0.01	0.042	< 0.01	< 0.01	< 0.01
D × H	0.610	0.054	0.201	< 0.01	0.072	< 0.01	< 0.01	0.060

[1]Expressed as % of total dry matter (DM) of whole plant, unless otherwise indicated.

CP= crude protein, NDF= neutral detergent fiber, ADF= acid detergent fiber, STA= starch, NFC= non-fibrous carbohydrates, EE= ethereal extract, ASH= ashes; NEL= net energy for lactation calculated with the chemical composition presented here and digestibility of NDF at 48 h (Eq. 2.11; NRC, 2001).

[2]Sowing date: May 30, 2019.

[3]Hybrid: (H1= DK-4018; H2= Noble; H3= Antílope; H4= XR-49).

SEM= standard error of the mean; DAS= response of linear (L) or quadratic (Q) days to harvest denoted by: *0.01 < $P \leq 0.05$ and **($P < 0.01$), D × H= interaction between DAS and hybrid, NS= not significant.

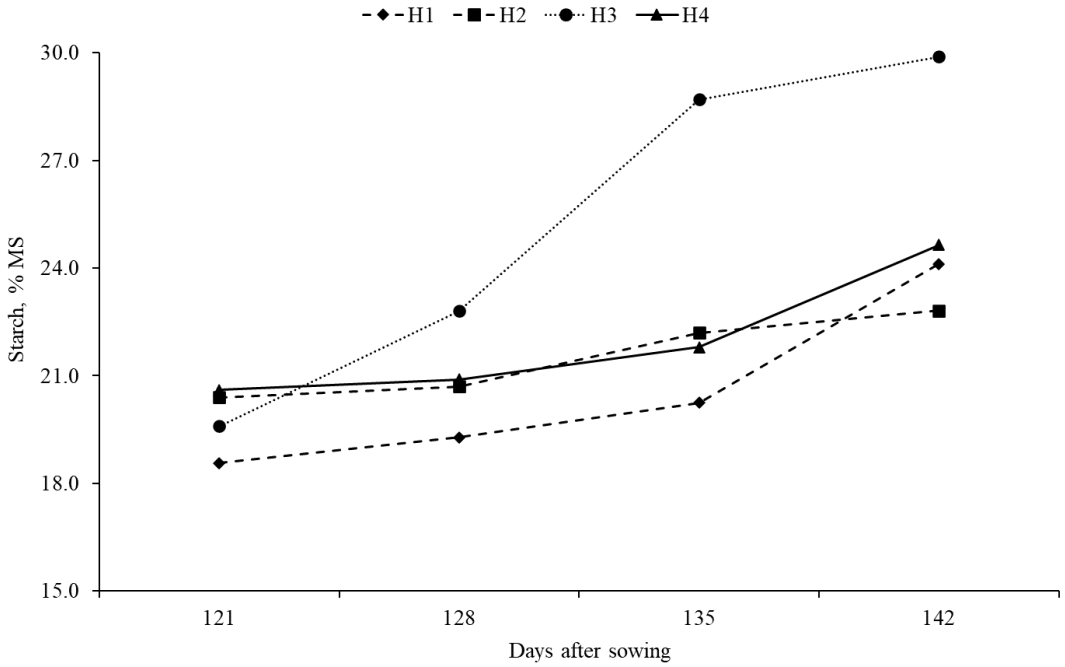
[ABC] Means with different uppercase literals differ in DAS ($P \leq 0.05$)

[abc] Means with different lowercase literals differ between hybrids ($P \leq 0.05$).

NFC content increased linearly ($P<0.01$) at a rate of 1.8 percentage units per week. The increase in NFC was inversely proportional to the decrease in NDF. Although there were differences in NFC between hybrids ($P<0.01$), these were minimal, from 0.9 to 1.1 %, and only hybrid H1 differed with the lowest NFC content. In the present study, the values obtained for NFC at 135 and/or 142 d to harvest were lower than those reported in other studies at similar days to harvest[9,14]. Starch accumulation was affected by the interaction between days to harvest and hybrid ($P<0.01$); hybrid H3 consistently outperformed the other materials at 128, 135 and 142 d to harvest, except at 121 d to harvest, when starch content differed slightly between hybrids (Figure 2). This could be related to the variability observed in DM and grain accumulation, which affects starch synthesis in grain[17]. Regarding days to

harvest, a linear effect (P<0.01) was detected in starch accumulation, which increased at a rate of 1.4 units between harvests.

Figure 2: Starch content in the dry matter (DM) of forage of four corn hybrids (H1= DK-4018, H2= Noble, H3= Antílope, and H4= XR-49) grown under irrigated conditions and harvested at 121, 128, 135 and 142 days after sowing



[abcdfg] Means with different literals differ statistically (P≤0.05).

Digestibility

Table 3 shows the digestibility parameters evaluated at different incubation times *in situ*. No interactions between hybrid and days to harvest were detected for any of the parameters evaluated. Contrary to expectations, the days to harvest did not affect DM digestibility, NDF digestibility (NDFD) or starch digestibility. These findings differ from those reported in other studies in which NDFD at 36 or 48 h decreases by delaying the days to harvest and the maturity of the plant[14,18]. On the other hand, the NDFD values reported here are lower than those reported in other local studies using the same *in-situ* method and incubation time[14,19]. On the other hand, DM digestibility and NDFD were affected by the hybrid (P=0.02 and P=0.01, respectively). Hybrid H1, which had the highest DM digestibility, also obtained the superior NDFD. The increase in DMD is associated with grain accumulation, while the decrease is attributed to a lower NDFD[9,20]. However, in the present study, the higher grain accumulation of hybrid H3 did not compensate for its lower NDF digestibility.

Table 3: *In-situ* digestibility of dry matter, neutral detergent fiber and starch of four corn hybrids harvested at different days after sowing (DAS)

	***In-situ* digestibility**				
	DMD_{48}	**$NDFD_{48}$**	**$uNDF_{120}$**	**STD_{12}**	**STD_{24}**
	% DM	**% NDF**	**% NDF**	**% starch**	**% starch**
DAS[1]					
121	59.6	32.2	47.3	46.9	96.7
128	59.0	30.1	47.6	47.0	95.6
135	60.2	30.2	48.8	46.6	94.5
142	60.7	30.0	49.3	44.6	94.4
Hybrid[2]					
H1	62.0[a]	34.2[a]	47.1	43.2[c]	96.9
H2	57.9[c]	30.5[ab]	49.6	48.6[a]	95.3
H3	60.0[b]	29.6[b]	49.2	46.8[b]	93.8
H4	59.5[b]	28.2[b]	48.2	46.5[b]	95.1
SEM	1.600	1.130	1.530	1.230	1.620
DAS	NS	NS	NS	NS	NS
Hybrid	0.021	0.041	0.257	0.014	0.265
D × H	0.140	0.072	0.128	0.124	0.202

[1]Sowing date: May 30, 2019.

[2]Hybrid: (H1: DK-4018; H2: Noble; H3: Antílope; H4: XR-49).

DMD_{48}= dry matter (DM) digestibility at 48 h of incubation, $NDFD_{48}$= neutral detergent fiber digestibility (NDF) at 48 h of incubation, $uNDF_{120}$= non-digestible NDF at 120 h of incubation, STD_{12}= starch digestibility at 12 h of incubation, STD_{24}= starch digestibility at 24 h of incubation.

SEM= standard error of the mean; DAS= linear (L) or quadratic (Q) effects of days to harvest denoted by: *$0.01 < P \leq 0.05$ and **($P<0.01$), D × H= interaction between DAS and hybrid.

[abc] Means with different literals differs ($P \leq 0.05$).

The fraction of non-digestible NDF (uNDF) at 120 h did not differ between days to harvest or between hybrids, and the means were 48.2 ± 0.9 and 48.2 ±1.4 %, respectively. In the present study, uNDF values were up to 10 units higher than those reported in other studies[18,19]. A high value of uNDF is associated with lignified fiber fractions, mainly from the base of the stem, where more lignin accumulates with plant senescence and increase in DM content[19,20,21]. Thus, it is possible that the low values of uNDF found in this study are associated with the low cut height used in the present study, compared to the aforementioned studies (15 *vs* 25 cm, respectively) and another local study using up to 40 cm of cut height[22]. Finally, starch digestibility at 12 or 24 h was not affected by the advance of days to harvest, and only a hybrid effect (P=0.01) was detected on starch digestibility at 12 h. Although all hybrids used were semi-toothed grains, it is possible that the vitreosity gradient at grain maturation affected starch digestibility at 12 h and this was without effect at 24 h[23].

Conclusions and implications

In the present study, grain accumulation and DM content increased by delaying the days to harvest and were influenced by the hybrid effect. Nonetheless, DM yield was not affected by the hybrid and only increased with days to harvest. The NDF content decreased and the starch content increased as the days to harvest progressed, but this factor did not affect the digestibility parameters evaluated. In general, DM yield and grain accumulation can be maximized by delaying harvest by up to 142 d without affecting NDF digestibility, but some agronomic strategies to harvest need to be explored to reduce the value of uNDF.

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Article

Electron microscopy and X-ray diffraction analysis of equine enteroliths from the Aburrá Valley in Antioquia, Colombia

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Abstract:

The objective of this study was to determine the mineralogical composition of equine enteroliths from the Aburrá Valley in Antioquia, Colombia. Samples of eight enteroliths from eight horses were subjected to semi-quantitative X-ray diffraction (XRD) and transmission and scanning electron microscopy (TEM/SEM) analysis. The TEM/SEM of the analyzed enteroliths reported the presence of carbon, oxygen, phosphorus, magnesium, calcium, silicon, potassium, bromine, iron, sulfur, and aluminum. The XRD identified struvite, newberyte, kyanite, low quartz, actinolite, nitratine, cordierite, and vivianite. Both techniques used in the analysis of the enteroliths were correlated by matching the mineral compounds with the detected chemical elements. The main mineral components of the enteroliths were magnesium phosphates, struvite and newberyte being the most common.

Keywords: Colic, Enterolithiasis, Equine, Struvite.

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Introduction

Enteroliths are concretions derived from mineral precipitations around a nucleus or nidus of organic or inorganic material, located in the gastrointestinal tract[1,2]. These foreign bodies have different shapes, among which the most common are those of spherical or tetrahedral and irregular conformation, with different sizes and weights[3]. Geographic regions with a high predisposition to enterolith formation due to specific mineral components of the soil, water, and plant species have been identified[2-8].

Risk factors such as water sources and high consumption of alfalfa hay with high levels of magnesium, nitrogen, and phosphorus in the diet may contribute to the formation of enteroliths, as the struvite formed by these minerals predisposes to their formation[9]. Alfalfa facilitates the formation of magnesium oxide by promoting an alkaline pH, which favors conditions for the deposition and formation of enteroliths; hence, this legume in the diet of horses is described as a potential risk factor. Among other factors involved, the environment, intestinal pH, hypomotility, and the presence of nuclei are reported to make the formation of these foreign bodies possible[1,8,10].

In addition to exogenous predisposing factors, endogenous factors such as breed, sex, age, and physiological particularities of horses are described for the presentation of enteroliths and phytobezoars[4,9]. For example, 15-yr-old horses have been found to have enterolithiasis in the major colon, and 13-yr-old horses, in the minor colon[3]; however, this condition is also reported in animals of all ages[2,4], beings less common in young animals because of the time required for its development[7].

The speed of enterolith formation in the intestinal tract is variable, as it is related to particularities of the luminal microenvironment of the colon, type of feed —mainly concentrate—, and management in confinement[8,9,10], growth form from the nucleus, and presence of minerals and trace elements[11]. Alterations in intestinal pH can contribute to both the formation and dissolution of enteroliths, thus affecting the time of formation[1]. In these situations, studies are needed to identify and determine the involvement of predisposing factors in order to establish appropriate preventive measures and avoid surgical solution as a last resort[12]. Therefore, the objective of this study was to evaluate the mineralogical composition by electron microscopy (chemical elements) and X-ray diffraction (chemical compounds) of enteroliths obtained from horses in Colombia.

Material and methods

It was used enteroliths collected (by surgical extraction and spontaneous excretion) from horses (Colombian Criollo and Argentine Silla) aged 12 to 16 yr, fed with commercial concentrate, Angleton hay (*Dichantium aristatum*), salt, and water at will, in the Aburrá Valley, in Antioquia, Colombia. Once photographically registered, the enteroliths were weighed and classified by appearance, shape, and size, and later fragmented with an electric saw, allowing the identification of their nidus or central nucleus. The slices facilitated the evaluation of color and internal architectural features such as texture and porosity. Eight samples of enteroliths from an equal number of

horses were analyzed in laboratories specialized in mineralogy, crystallography, or characterization of materials; by X-ray diffraction and transmission and scanning electron microscopy (TEM/SEM).

Fragments of enteroliths were pulverized and subsequently placed on the quartz crystal for mineralogical composition analysis, through the semi quantitative X-ray diffraction (XRD) technique (Empyrean® Series II - Alpha 1, Model 2012, Madrid, Spain). The analysis of crystalline phases and quantification was performed with the HighScore Plus software and the ICDD/PDF-4-2012 database for phase identification, with standard reflection configuration, angle 2^{θ} - 5-80°, step: 0.0263°, time: 46.359 sec. Mineral identification was obtained by comparing the diffraction patterns of the enterolith samples with the standard patterns.

On the other hand, samples of the enteroliths were cut into slices that were polished on both sides, subsequently dehydrated on a hot plate, and prepared according to the routine procedure for examination with TEM/SEM (FEI Tecnai® G2 F20), along with a dispersive X-ray spectroscopy for the scanning of the study material.

The data were tabulated and systematized in MS Excel spreadsheets, analyzed with descriptive statistics, and presented in frequency tables with reports in percentages of the elements and mineral compounds in the composition of each of the enterolith samples. This study was approved by the Ethics Committee for Animal Experimentation (CEEA, Spanish acronym) of the University of Antioquia, Medellin - Colombia (protocol No. 1062016).

Results

Figure 1 shows the size, shape and texture of the collected enteroliths. Spherical, polyhedral and irregular shapes with smooth, rough, and porous surfaces were predominant. The figure also shows the macroscopic and electron microscopic texture of some of the enteroliths. The enteroliths ranged in size from 5 to 15 cm, and weighed 664.14 ± 385.01 g (maximum 1,157 g; minimum 127 g). On the other hand, material of plant origin (fiber and seeds) was identified in all the cores of the enteroliths studied, when they were fragmented with the saw.

Figure 1: Enteroliths obtained from equines

a) Shape, size and texture of the enteroliths. b) Electron microscopy image of enteroliths by texture and conformation of struvite crystals.

Table 1 shows the chemical elements in compositional percentages reported during the analysis of each enterolith by TEM/SEM. The elements with the highest percentage were carbon (C), 46.06 %; oxygen (O), 26.85 %; phosphorus (P), 11.55 %; magnesium (Mg), 5.97 %, and calcium (Ca), 3.71 %, with minerals such as silicon (Si), 2.74 %; potassium (K), 1.24 %; bromine (Br), 0.35 %; iron (Fe), 0.71 %; sulfur (S), 0.61 %, and aluminum (Al), 0.17 %. The presence of these elements —especially those considered as trace elements— varied among the enteroliths.

Table 1: Compositional percentages of mineral elements in enteroliths from eight horses from Valle de Aburrá in Antioquia, Colombia, analyzed by transmission and scanning electron microscopy (TEM/SEM)

Enterolith	Element (%)										
	C	O	P	Mg	Ca	Si	K	Br	Fe	S	Al
1	31.86	21.07	32.46	12.32	0	0	2.29	0	0	0	0
2	38.07	30.61	11.32	2.56	13.96	2.88	0.60	0	0	0	0
3	57.46	24.00	3.27	1.27	4.12	4.37	1.35	0	1.98	1.48	0.71
4	27.16	27.22	30.18	13.29	0	0	2.15	0	0	0	0
5	61.81	23.79	1.18	0	1.18	9.37	0	2.66	0	0	0
6	32.56	30,45	7.13	11.23	5.21	3,56	2.34	0	3.45	3.40	0.67
7	63.45	27.04	2.34	4.23	1.56	1.23	0	0.15	0	0	0
8	56.12	30.67	4.56	2.89	3.67	0.56	1.23	0	0.30	0	0

Table 2 shows the chemical compounds detected in each enterolith by XRD analysis. The mineral compounds with the highest concentration were struvite (magnesium ammonium phosphate hexahydrate [MgNH4PO4-6H20]), 78.68 %; newberyte (magnesium acid phosphate), 11.23 %; kyanite (aluminum silicate), 3. 18 %; low quartz (silicon oxide), 2.36 %; actinolite (inosilicate), 2.15 %; nitratine (sodium nitrate), 1.45 %; cordierite, (magnesium cyclocyclicate), 0.46 %, and vivianite, (hydrated iron phosphate) 0.45 %. None of the samples contained more than five of these compounds.

Table 2: Concentration percentages of mineral compounds in horse enteroliths from the Aburrá Valley in Antioquia, Colombia, analyzed by X-ray diffraction (XRD)

Enterolith	Compound (%)							
	Struvite	Nitratin	Newberyte	Low quartz	Cordierite	Actinolite	Vivianite	Kyanite
1	83.8	7.8	1.1	6.9	0.3	0	0	0
2	99.6	0	0.4	0	0	0	0	0
3	81.9	3.3	3.1	4.0	0	7.8	0	0
4	81.5	0	18.1	0.1	0	0.3	0	0
5	96.1	0	0.5	0	3.4	0	0	0
6	96.4	0	0	0	0	0	3.6	0
7	55.7	0	33.3	7.9	0	3.1	0	0
8	34.5	0.5	33.4	0	0	6.0	0	25.5

Discussion

The literature reports that equine enteroliths are formed mainly by the precipitation of struvite, with increased presence of Mg, nitrates, phosphates, and high concentrations of cations within an alkaline environment in the colon[5,7,13]. In addition, high Mg concentrations in the equine colon have been associated with alfalfa-based diets (> 50 %) and are considered to predispose the formation of enteroliths. However, not all horses fed alfalfa develop enterolithiasis; this indicates the existence of other factors that may induce the formation of these concretions, such as individual issues, hypomotility, bacterial flora, diets, buffering capacity, and water quality, which may influence the intestinal pH and the colonic mineral content[6,9,10].

This study did not analyze the predisposing factors of enterolith formation or the evolution of the clinical pictures of horses diagnosed with enterolithiasis. This is a recognized limitation of this work, as it does not allow to infer the participation of these factors in the formation of enteroliths; only the composition of these factors is described. However, the enteroliths come from a geographical area of the department of Antioquia, Colombia, where it is unusual to feed horses with alfalfa and there is no desert context, in contrast with previous reports where regions of the world with a high alfalfa supply and sandy soils have been reported to have the highest frequencies of occurrence of enterolithiasis[1-4,6,7], indicating that the genesis of enteroliths may be multifactorial.

The variety of shape, size and texture, and configuration of the nidi were similar to those of other reports[13]. However, unlike in other studies, all nidi were identified, being the predominant plant material, in contrast with other studies that have described materials other than plant material[1,9]. It was not possible to verify the single or multiple presence of enteroliths; spherical enteroliths are interpreted as single presence of foreign bodies, and polyhedral enteroliths, as multiple presence[14,15], as complete information on the medical history of the equines was not available.

Struvite is identified as the predominant mineral compound in enteroliths as in other studies[6,7,13]. Likewise, the presence of vivianite, although in a lower proportion in the composition, was similar to that reported by Hassel *et al*[13]. Conversely, the presence of

newberyte, kyanite, low quartz, actinolite, nitratine, and cordierite —mineral elements and trace minerals determined by TEM/SEM (Table 1)— has not been reported; however, it cannot be inferred that this is a characteristic of enteroliths obtained from animals from this geographic region, given the low number of samples. Particularly noteworthy is the finding of more than three compounds in most of the enteroliths, with the exception of the one constituted by struvite and vivianite.

Although the presence of eight compounds was determined in the group of selected enteroliths, the presence of apatite (Ca phosphate) was not found, in consonance with the study by Hassel *et al*[13], although a larger number of samples are required to confirm this finding. However, in canines and felines, struvite urinary stones may be accompanied by apatite stones[16,17], indicating special conditions and interaction or substitution of ions that can influence the crystallization of apatite, as is the case of K and Mg[18].

As for the major elements, the concentrations of P, Mg, K, Ca and organic C and trace elements such as Fe, were similar to those reported in the petrographic and mineralogical studies carried out by Rouff et al. [11] in samples of enteroliths from different geographic regions. On the other hand, the present study reported concentrations of S, Si, Br, and Al, but did not detect the presence of Zn or Mn. In addition, copper (Cu) was not detected in any of the studies, despite being found in the nutritional analysis of equine feed carried out by the same authors[11]. Therefore, it is possible that the precipitation and crystallization of mineral compounds depends not only on saturation but also on the interaction of ions and pH conditions in the colonic fluid[18]. Based on the above, it is possible to hypothesize that the difference in contexts and feeding systems of the equines may affect the ionic saturation in the colonic fluid, which could partly explain the amount of major and trace elements determined in this work.

Despite the type of food supplied to the horses and the presence of certain minerals in their colonic fluid, these do not form compounds, or such compounds are not detected in the composition of the enteroliths, a fact that reinforces the hypothesis of the existence of other predisposing factors involved in their formation and growth[11,13,18]. However, it is interesting that struvite is the major component of the enteroliths analyzed in several parts of the world, which might suggest the existence of a potential analogy with the formation of struvite urinary calculi, in which there is evidence of microbial metabolism rather than mineral saturation[19,20]. However, this process is complex, and there is still no evidence that it occurs in the equine colon[9,21].

The recognition of trace elements and organic impurities in the composition of struvite is important, as the higher the concentration of these elements, the greater the susceptibility to decomposition[11]. In addition, canine and feline apatite stones are more resistant than struvite stones[16,17]; however, they are absent in equine enterolithiasis. Therefore, it is possible to consider medical treatments to dissolve the enteroliths and diet manipulation strategies to prevent their formation, given that the mineralogical analyses showed high impurities of organic material and trace elements that make them susceptible to disintegration and, depending on the composition, solvents like carbonated beverages such as Coca-Cola® may be utilized for this purpose [12].

Conclusions and implications

Both techniques (TEM/SEM and XRD) used in the analysis of the enteroliths were correlated by matching the mineral compounds with the detected chemical elements. In sum, the main mineral components of the analyzed enteroliths were Mg phosphates, the most common of which are struvite and newberyte, unlike vivianite which was also detected, but in a lower proportion than previously reported[13]. Other compounds were also reported to be distributed in all the analyzed samples; however, studies with a larger number of samples and with relevant information on the management, feeding, and clinical condition associated with enterolithiasis of the animals are required to determine the association with the mineralogical composition of the enteroliths.

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Article

Prevalence and risk factors associated with *Cryptosporidium* spp. in dairy cattle in Chiquinquirá (Colombia)

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Abstract:

Cryptosporidiosis is a disease characterized by episodes of diarrhea in cattle worldwide, caused by a protozoan parasite of the genus *Cryptosporidium* spp. of the phylum Apicomplexa and Family Cryptosporiidae. It is responsible for important economic losses, and, in addition to this, it generates an impact on human health, as it can parasitize humans. The objective of the study was to determine the prevalence of and risk factors associated with *Cryptosporidium* spp. in cattle in Chiquinquirá (Colombia). A descriptive cross-sectional study with simple random sampling was carried out, with a sample size of 1,044 head of cattle, including males and females of different breeds and age groups, using the WinEpi statistical software. Fecal samples were taken directly from the rectum and processed with the modified Ziehl-Neelsen (ZN) technique for the identification of parasite oocysts using a 100X objective. The data were processed with the Epi Info® statistical software. An overall

prevalence of 7.3 % (73/1000) was found; females, 2 to 4-yr-old bovines, and crossbred cattle were the most prevalent. No significant statistical association was found between breed, age, and sex of the individuals evaluated, and protozoan positivity ($P \geq 0.05$). The purchase of animals and larger productions were considered risk factors for parasitosis. Protozoan prevention and control plans should be designed and implemented based on sanitary practices to prevent the dissemination of oocysts found in fecal matter.

Keywords: *Crypstoporidium* spp., Cryptosporidiosis, Cattle.

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Introduction

Cryptosporidium spp. is a protozoan, coccidian, zoonotic, obligate intracellular parasite that is part of the phylum Apicomplexa and the family Cryptosporiidae; it is distributed across the world[1-5]. The parasite affects the gastrointestinal tract of vertebrate species such as cattle, birds, small ruminants, rodents, canines, felines, rabbits, squirrels, and even humans[6-9]. Recent reports indicate that more than 40 species of *Cryptosporidium* spp. have been described, among which *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni* are routinely found in cattle[5].

Parasites of this genus cause a serious gastrointestinal disease known as cryptosporidiosis[7,8] that impacts both human health and animal health[1,5,6]. Cattle, especially calves, have been identified as one of the most common reservoirs of this protist[1,4], which is one of the main causes of morbidity and mortality in calves aged 1 mo or less worldwide[10]. However, there is a wide variety of hosts that can act as reservoirs of the parasite, favoring the persistence of *Cryptosporidium* spp. in the environment for long periods of time as oocysts and, therefore, increasing the risk of their transmission to susceptible hosts[6,7].

Cryptosporidium spp. infections constitute a substantial public health burden and are responsible for economic losses in livestock herds worldwide[11]. Therefore, the reduction of disease and shedding of *Cryptosporidium* spp. oocysts is considered an important objective in livestock productions, by inhibiting the transmission of the protist through direct contact with infected animals, or ingestion of feed and water contaminated with animal feces[9]. The diagnosis of the protozoan is based on the identification of oocysts at the laboratory, where

it is a common practice to carry out a microscopic observation of the oocysts applying a Ziehl-Neelsen (ZN) stain with an acid alcohol solution, auramine with phenol, or immunofluorescent stain to fecal smears[12].

The therapeutic options available to treat cryptosporidiosis are limited[11]. Despite the substantial interest in this type of parasite, progress in terms of treatment development and understanding of most of the life cycle of this unusual organism is scarce[7]. So far, in the Department of Boyacá there are no recent studies on the identification of parasite oocysts in fecal material by microscopy, nor the analysis of different variables[13]. Therefore, the objective of this research was to determine the prevalence and risk factors associated with *Cryptosporidium* spp. in cattle in Chiquinquirá (Colombia).

Material and methods

Geographical location

Boyacá has four municipalities specialized in milk production (Chiquinquirá, Caldas, San Miguel de Sema, and Saboyá), reaching a volume of 70,000 L per day derived from approximately 50,000 cows destined for milk production[14]. According to national government data, livestock farming in Chiquinquirá represents an important part of the economy of the municipality, which is located at 5°36'48" N and of 0°15'21" W of the meridian of Bogotá, at an altitude of 2,000 to 3,200 meters above sea level, having an average temperature of 15 ºC[15].

Sample size

In the year 2022, Chiquinquirá reported 33 398 head of cattle, according to the National Livestock Census of the Colombian Agricultural Institute (*ICA*)[16]. Based on the reported data and following the formula obtained from the WinEpi statistical program, a sample size of 947 female and 47 male cattle of various age groups and breeds with dairy potential was determined. In addition, a confidence interval of 95%, an accepted error of 5%, a sampling fraction of 1.15% and an expected prevalence of 50% were considered.

$$n = \left(\frac{Z_{\frac{a}{2\sqrt{p(1-p)}}}}{E} \right) = \frac{Z^2 \alpha/2 \cdot p(1-p)}{E^2}$$

Where: n= sample size; E= accepted error; p= expected value of the ratio; α= queuing probability.

Sample collection and processing

A total of 2 to 5 g of fecal material were taken directly from the rectum by rectal palpation. The samples were labeled and stored in refrigeration coolers to be transported to the Veterinary Parasitology Laboratory of the Pedagogic and Technological University of Colombia (Universidad Pedagógica y Tecnológica de Colombia, UPTC) for processing. For the identification of *Cyptosporidium* spp. oocysts in bovine feces, the modified Ziehl-Neelsen (ZN) or Kinyoun cold staining technique was utilized. A thin smear of fecal material was made on the slide and allowed to air dry. The slides were then placed in staining racks where they were stained for 10 min with ZN fuchsin. The slides were then placed in staining racks where they were stained for 10 min with ZN fuchsin. The slides were examined microscopically using a 100x objective with immersion oil. In these samples, *Cryptosporidium* spp. oocysts stained bright red were considered positive[17].

Statistical analysis

The identification of *Cryptosporidium* spp. oocysts in bovine fecal material and the data obtained in the epidemiological survey were consolidated and filtered. Among the evaluated factors, it is important to mention that reference is made to the absence or presence of management practices; large herds were those with more than 10 animals in production, while small herds were those with 10 animals or less. In terms of the water sources, the only one that provided potable and treated water was the aqueduct. The results were analyzed with the Epi Info® statistical software, version 7.2.4.0.

The proportion of individuals affected by *Cryptosporidium* spp. and exposed to the factors evaluated in the study were compared with the same proportion of a population not exposed to that factor to estimate prevalence ratios (PR). The PR was employed to measure the association between cryptosporidiosis and the hypothesized causal factors, as well as the significance of these associations using Fisher's exact test[18].

PR values above 1 (lower 95% confidence interval < 1) and with $P<0.05$ were considered risk factors, whereas PR values below 1 (upper 95% confidence interval < 1) and with $P<0.05$ were regarded as protective factors. The dependent variable included the modified ZN results, while the independent variables were all the determinant variables established in the epidemiological survey applied during sampling. Once these factors were established, a logistic regression was performed[19].

Ethical considerations

The study was conducted per Resolution 8430 of the Colombian Ministry of Health and Social Protection and the 1989 Law No. 84. These set out the standards that are appropriate for the welfare of animals during research. In addition, before blood sampling, an informed consent form was signed by the owners of the cattle.

Results

An overall prevalence of 7.3 % (73/1000) was determined in the municipality of Chiquinquirá. Females were more prevalent than males, with a prevalence rate of 7.39 (70/947) and 6.98 % (3/43), respectively. Cattle aged 2 to 4 yr and crossbred cattle had a higher presence of *Cryptosporidium* spp. oocysts (Table 1). No significant statistical association was found between the breed, age, or gender of the individuals evaluated and protozoan's positivity ($P\geq0.05$).

Table 1: Prevalence of *Cryptosporidium* spp. by age group and breed in cattle in the municipality of Chiquinquirá, Boyacá

Variable	N	Positive *Cryptosporidium* spp.	Prevalence (%)
		Age groups	
< 2 years	304	20	6.58
2-4 years	84	10	11.90
> 4 years	612	43	7.03
		Breeds	
Ayrshire	138	11	7.97
Crossbreed	95	9	9.47
Holstein	767	53	6.91

Regarding the assessed variables, management practices such as the presence of cattle belonging to other owners (*P*=0.0018), pasture leasing (*P*=0.0010), and the purchase of animals (*P*=0.0062) were statistically significantly associated with the occurrence of the parasite in the evaluated cattle (Table 2).

Table 2: Analysis of management practices as potential risk factors associated with *Cryptosporidium* spp. infections

Variable	Category	PR	Confidence interval (95%)	*P*-value
Management practices	Pen	0.9769	0.9416 - 1.0135	0.1234
	Other owners' livestock	0.9427	0.9136 - 1.0729	0.0018
	Other species	0.9352	0.8351 - 1.0474	0.1113
	Lease of pastures	0.9455	0.9138 - 1.0783	0.0013
	Purchase of animals	1.0472	1.0118 - 1.0839	0.0062
	Damaged fences	1.0056	0.9696 - 1.0430	0.4254
	Deworming	0.9352	0.8351 - 1.0474	0.1113

The results are presented as prevalence ratio (PR) and 95% confidence interval (CI).

The association between diarrhea and the presence of *Cryptosporidium* spp. oocysts in the analyzed fecal samples was statistically significant. Herd size too was statistically significant: large herds were considered as a potential risk factor, while small herds were established as a preventive factor against the occurrence of the parasite. On the other hand, when analyzing the source of drinking water, the aqueduct and the stream exhibited a statistically significant association with the positivity of the protozoan, and the stream was established as a potential risk factor, while the aqueduct was a protective factor (Table 3).

Table 3: Analysis of clinical manifestations, herd size, and drinking water source as potential risk factors associated with *Cryptosporidium* spp. infections

Variable	Category	PR	Confidence interval (95%)	*P*-value
Clinical manifestations	Diarrhea	0.9552	0.9208 - 1.0909	0.0078
	Fever	0.9699	0.9366 - 1.0044	0.0545
Herd size	Large herd	1.051	1.0169 - 1.0863	0.0081
	Small herd	0.9515	0.9206 - 0.9834	0.0072
Water source	Aqueduct	0.9496	0.9175 - 0.9829	0.0023
	Cistern	0.9694	0.9364 - 1.0035	0.0657
	Gully	1.0589	1.0122 - 1.1078	0.0041

The results are presented as prevalence ratio (PR) and 95% confidence interval (CI).

The analysis of the variables that were determined as potential risk factors through logistic regression allowed determining that the purchase of animals and production units with more than 10 animals as risk factors for the occurrence of *Cryptosporidium* spp. oocysts in the evaluated cattle (Table 4).

Table 4: Analysis of variables as potential risk factors associated with infections by *Cryptosporidium* spp.

Variable	Odds ratio	LCI	UCS	*P*-value
Purchase of animals	2.252	1.3358	3.7965	0.0023
Large herd	2.6677	1.2593	5.651	0.0104
Gully	1.5773	0.9484	2.6232	0.0791

LCI= lower confidence interval; UCS= upper confidence interval.

Discussion

Enteric protozoan infection in cattle can pose a threat to the productivity and survival of the animals, resulting in negative impacts on the livestock industry[20]. Within this group of pathogens affecting animal health, *Cryptosporidium* spp. is an obligate intracellular parasite transmitted by the fecal-oral route after ingestion of oocysts that can contaminate, persist, and resist disinfection in the water and food[21]. The published literature on the parasite is extensive, providing details of its distribution in most regions of the world[22]. Prevalence rates of 52.2 % in Algeria[10], 16.2 % in Ethiopia[4], 53 % in Latvia[23], and 64 % in cattle sampled in the Lagoon region of Mexico have been reported[24].

Similarly, at the national level, there are prevalence rates of 22 % in the Central Savannah province (Cundinamarca)[25], 22 % and 7 % in Chiquinquirá[26,27], and 48 % in bovines in Boyacá[28]; microscopic diagnosis revealed that 115 calves (26.6 %) from 44 farms (59.5 %) in a central area of Colombia (Antioquia, Boyacá, Cundinamarca, and Meta) tested positive[29], these rates being higher than those found in the present study. The reported variations may be caused by different environmental conditions, management practices, and the number of animals in the farms; therefore, the role of the environment in direct and indirect contamination should be considered, mainly the accumulation of oocysts having occurred previously in animals of the herd, which facilitates the fecal-oral transmission route among the cattle and can thus modify the prevalence of infection by the parasite[30].

In the present study, cattle aged 2 to 4 yr had the highest prevalence of the parasite, unlike in Africa[6,10], Asia[20], and South America[29], where a higher infection rate was detected in young cattle compared to adult animals. No significant statistical association was found

between cattle age ($P \geq 0.05$) and the prevalence of *Cryptosporidium* infection in cattle from central Ethiopia[31]; however, in cattle from dairy farms in Colombia[28], United States[32], and India[2], reports have detected an association between the age of the bovines and the excretion of oocysts in fecal matter. Although age was not considered a risk factor for the occurrence of the protozoan in the present study, bovines aged <12 mo were associated with the excretion of *Cryptosporidium* spp. oocysts in Colombia[28]. In this regard, it should be taken into account that nursing calves are more predisposed to acquire infection by the parasite; in addition, the clinical disease may be influenced by the immune status of the host[33].

Das *et al*[2] report that there is statistical significance between positivity to *Cryptosporidium* spp. and the sex of the cattle, which was not evident in the present study; however, similarly to our results, in Ethiopia[31] and Nigeria[6] no significant statistical differences in the prevalence of *Cryptosporidium* infection were found between males and females. On the other hand, crossbreeds had the highest oocyst excretion of the protozoan, with no statistical association between cattle breed and parasite occurrence ($P \geq 0.05$). Likewise, in Addis Ababa and its surroundings[31], the prevalence of infection is likely due to the potential occurrence of the coccidian in beef and dairy cattle[2] regardless of the breed of the animals.

The risk factors for *Cryptosporidium* spp. are mainly associated with the handling and sanitary condition of the animals[31]. The presence in the herds of bovines belonging to other owners, the leasing of pastures, and the purchase of animals whose health and deworming history was unknown were associated with the presence of oocysts in the evaluated samples ($P \leq 0.05$). This is because the transmission of cryptosporidiosis is mainly due to management practices that allow the dissemination that oocysts found in the environment or in diseased animals or susceptible hosts. Similarly, the purchase of animals was identified as a risk factor for the presence of the parasite, possibly because *Cryptosporidium* spp. is not specific to the host. Thus, an environment contaminated with oocysts during an outbreak in cattle can lead to the infection of other species that subsequently use the same grazing area; the unknown health history of individuals can also increase the potential transmission of the protozoan[2].

Herd size was associated with *Cryptosporidium* spp. oocyst excretion, where smaller herd sizes were considered a protective factor, and larger herds were established as a risk factor for infection, consistently with the positive association between higher cattle population density and fecal excretion of *Cryptosporidium* spp. in Africa[31], Asia[20], Europe[34], and North America[32]. Likewise, individual calf rearing reduces the potentiality of infection by the protozoan by approximately 2.5 times compared to group calf rearing[31], as the rate of oocyst shedding differs between housing systems, exhibiting a higher prevalence in calves kept as a group compared to the individual system. However, this will depend on the age of the animals[34] which demonstrates the importance of the facilities used in intensive farms with higher animal densities[29].

The aqueduct differed significantly ($P \leq 0.05$) from the stream as a source of drinking water, the aqueduct having been established as a protective factor against parasite positivity. Farms with drinking water sources such as wells, rivers, or streams acquired 2.4 and 2.9 times more *Cryptosporidium* than farms using tap water to provide water to cattle[31]. Likewise, herds that dispose of wastewater in the field compared to farms that discharge wastewater into nearby wells may also be more likely to be infected with the protozoan[31]. Infection by *Cryptosporidium* spp. is also significantly associated with the symptoms of the infected animals[2], as in the case of the presence of diarrhea in the evaluated individuals ($P \leq 0.05$). However, previous studies showed no association between the presence of diarrhea and oocyst shedding[6,34,35], as well as only a slightly higher prevalence in diarrheic cattle compared to non-diarrheic cattle[6]. Despite this, the rate of infection by *Cryptosporidium* spp. in Colombia[27,28] and Algeria[10] is higher in animals with diarrhea compared to those that do not have it, consistently with the findings in Chiquinquirá, which highlight that the risk of occurrence of this symptom in bovines may decrease as they reach adulthood[23].

There is currently no vaccine or drug in the market for the treatment and control of cryptosporidiosis in ruminants, which makes its prevention difficult. In this sense, it is necessary to implement strategies to reduce the spread of infection in herds, including good disease management practices, such as the separation of cattle with diarrhea; cleaning and disinfecting the facilities before introducing animals; the removal and disposal of fecal matter or wet garbage; good hygiene of the feed and water troughs, and adequate supply of colostrum to newborns, as well as the development of strategies to reduce humidity in the herds[36].

Conclusions and implications

A moderate prevalence of infection by *Cryptoporidium* spp. was found in cattle in Chiquinquirá, where females, cattle aged 2 to 4 yr, and crossbreeds were the most prevalent. Although infection by the protozoan occurs more frequently in calves, adults can become a source of dissemination of the parasite; therefore, its prevention and control in herds should be paramount. The purchase of animals and larger productions were considered as risk factors for parasitosis; in this sense, sanitary and management practices should be adjusted to minimize the excretion of oocysts in fecal matter in extensive systems and in those where animals whose sanitary history is unknown are allowed to enter.

Conflict of interest

The authors of this article declare that they have neither conflict of interest nor any economic, personal, political, financial, or academic relationship that might influence their judgment.

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Article

Influence of the type of container and traditional methods on the long-term storage of honey produced by stingless *Scaptotrigona mexicana*: bioactive compounds and antioxidant properties

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Abstract:

Scaptotrigona mexicana honey is characterized by its nutritional and antioxidant properties, but it has a high moisture content that affects its stability during storage. The objective of this work was to evaluate the physicochemical and antioxidant properties by UV-Visible spectroscopy, profile of phenolic compounds by ultra-high performance liquid chromatography coupled to mass spectrometry and fatty acids and volatile compounds by

gas chromatography coupled to mass spectrometry, minerals by microwave plasma atomic emission spectroscopy, from honey stored in different containers that, along with traditional methods, are commonly used to increase its stability. Most physicochemical and antioxidant properties were not significantly different from those of freshly harvested honey. The results suggest that the packaging with an exhaust check valve has a significant effect on the decrease in moisture content and water activity, but not on the physicochemical and antioxidant properties for at least 2 yr of storage. These results suggest that the type of container should be considered when storing honey as it significantly ($P<0.05$) affects its properties and quality.

Keywords*:* Antioxidant activity; Container; Honey; *Scaptotrigona mexicana*; Meliponine stingless bees.

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Introduction

Stingless bee honey is highly demanded by consumers, due to its healing properties and quality[1]. However, this type of honey is characterized by a higher moisture content, which increases the probability of its deterioration. In addition, it has been reported that excess water can be a negative quality attribute since it creates a high risk of inducing fermentation processes and consequently altering the organoleptic properties of honey[2]. Various methodologies have been reported to maintain the nutritional and sensory properties of honey, increase its stability, and improve its handling and marketing conditions to obtain a safe product for the consumer[3], such as the use of dehydration in plastic trays using controlled-temperature ovens[4]. However, exposure to high temperatures produces an increase in the content of hydroxymethylfurfural[5]. It has been reported that heating to boiling temperature eliminates yeasts and reduces moisture content and that certain containers, such as unglazed clay pots, produce a reduction in moisture content of up to 20 %, increasing the shelf life of honey[6]. However, clay containers have the disadvantage of being fragile, of low capacity and not functional for transport. In a traditional method used in the Totonacapan region, Veracruz, Mexico, beans are added to honey since, according to the inhabitants, these seeds absorb moisture from the honey, making it more stable. Another traditional technique is the use of vacuum packing to avoid the entry of air. Therefore, the objective of this research was to investigate the effect of storage in different plastic containers on the physicochemical properties, antioxidants, phenolic compounds and fatty acids profile, minerals and volatile compounds in honey during storage.

Material and methods

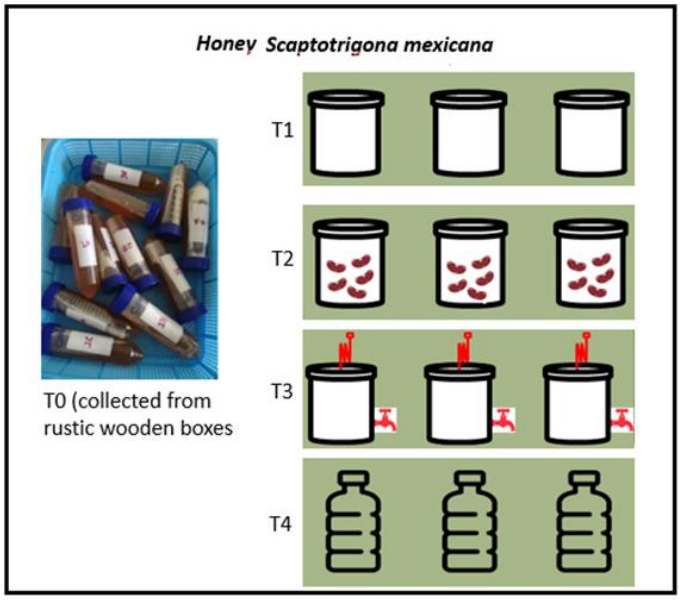
Chemicals

2,2´-Diphenyl-1-picrylhydrazyl (DPPH), Trolox (6-hydroxy- gallic acid, quercetin, Folin–Ciocalteu reagent and 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ) were purchased from Sigma (St. Louis, MO, USA).

Sample collection

Twenty-four (24) liters of honey were provided by the company Chasseurs De Saveurs S.A. C.V. They were collected from the Zozocolco region, Veracruz, Mexico. Samples were collected from hives in rustic wooden boxes that meliponicultors keep in their homes. The extraction was carried out during the month of May 2018 using a 20 mL syringe. The 24-L batch of honey collected was divided into four batches (6 L per batch). The honey from each batch was divided into three 2-L containers (Figure 1). T0 (control) was immediately used for the analysis of the evaluated parameters and collected from rustic wooden boxes. Batches T1 to T4 were placed in plastic containers commonly used for the commercialization of honey. The description of the treatments is presented below: T1: honey stored in an opaque plastic tray (high density polyethylene), T2: honey stored in an opaque plastic tray added with five bean seeds, T3: honey stored in an opaque plastic tray with an exhaust check valve (ZAZOLYNE, China) used in the fermentation of wines, T4: honey stored in a transparent plastic container (polyethylene terephthalate, 1). The honey samples were placed in a room with a temperature of 25 ºC and were analyzed at the beginning and after 2 yr of storage. All the determinations were made by triplicate.

Figure 1: Packaging treatments for honey *Scaptotrigona mexicana*



T0= control; T1= honey stored in an opaque plastic tray; T2= honey stored in an opaque plastic tray added with five bean seeds; T3= honey stored in an opaque plastic tray with an exhaust check valve; T4= honey stored in a transparent plastic container.

Physicochemical properties

The moisture, electrical conductivity, pH, and titratable acidity of the honey samples were determined, using the appropriate analytical standard procedures[7]. Electrical conductivity was determined using a conductivity meter (Mettler Toledo, ME 226 model, Pittsburgh, USA) and water activity was measured using a water activity meter (AquaLab, Model 4TE, Meter group, Inc, USA). The color was measured with a colorimeter (ColorFlex V1–72 SNHCX 1115, Hunter Lab, USA) using parameters CIE L*,a*, b* and total color change, browning index and Chroma were calculated.

Total phenolic compounds content, vitamin C and antioxidant activity

The content of total phenolic compounds and the antioxidant activity: DPPH (2,2-Diphenyl-1-picrylhydrazyl), FRAP (Ferric reducing ability of plasma) and ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) assays were determined using methanolic honey extract with a 1:100 dilution[8].

The total phenolic compounds were determined by the Folin-Ciocalteu method with some modifications[9]. Thirty microliters of each sample and 30 µL of Folin-Ciocalteu were mixed and incubated for 2 min (40 °C). After 240 µL of Na_2CO_3 (5%) were added, they were incubated for 20 min (40 °C). After that time, the absorbance was read (λ= 765 nm). Vitamin C content was determined using a standard curve made with L-ascorbic acid (99% purity; Sigma Rec. 84272, St. Louis, Missouri, USA) at a concentration of 0–50 mg and the results were expressed as mg equivalents of ascorbic acid (AAE) per gram.

The percentage of inhibition of the DPPH radical was determined by mixing 30 µL of each sample with 270 µL of DPPH reagent, then incubated for 30 min, subsequently the absorbance was read (λ= 517 nm, Multiskan FC, model IVD, Finland). ABTS was determined, 30 µL of extract and 270 µL of ABTS reagent were mixed and then incubated for 30 min (25 °C). Then the absorbance (λ= 734 nm, Multiskan FC, model IVD, Finland) was measured. Finally, FRAP was determined, 30 µL of extract and 270 µL of FRAP reagent were mixed, then incubated for 30 min (37 °C) and absorbance was measured (λ= 593 nm, Multiskan FC, model IVD, Finland). For the two technics 0.1–1 mg/mL Trolox calibration curve was performed[9]. The results are expressed in miligrams of Trolox equivalents per gram of dry weight of each sample.

UPLC-MS analysis

For the honey extracts, one gram of honey was weighed, 10 mL of methanol was added, and it was subjected to ultrasonication (Sonics Materials VCX 750 ultrasonic microprocessor, Connecticut, USA) for 10 min, this process was repeated until exhaustion. Subsequently, the solvent was evaporated to dryness in a rotary evaporator (Rotavapor R-100, Büchi, Flawil, Switzerland). Then, the honey extract (10 mg) was re-dissolved in 1.0 mL of MeOH with 0.1% of formic acid (Both MS grade, Sigma-Aldrich), filtered and placed in a 1.5 mL Ultra High-Performance Liquid Chromatography (UPLC) vial. The identification and quantitation of individual phenolic compounds was performed with an UPLC coupled to a triple quadrupole mass spectrometer (Agilent Technologies 1290-6460, Santa Clara, California, USA). Chromatographic conditions were: flow 0.3 mL/min, injection volume 2 µL, and column temperature 40 °C. The gradient started at 1% B, then changed to 50% B in 30 min, then 99 % B in 4 min followed by an isocratic step to 99 % B for 4 min. Subsequently, a gradient to 1% B in 1 min followed by an isocratic step for 5 min. The mass spectrometry conditions were electrospray ionization in positive and negative modes, temperature (T) of the gas 300 °C and T of the sheath gas 250 °C with flows of 5 and 11 L/min, respectively. The nebulizer pressure was 45 Psi and the capillary and nozzle voltages were 3,500 and 500 V, respectively. Forty-eight (48) compounds were searched: shikimic acid, gallic acid, L-

phenylalanine, protocatechuic acid, 4-hydroxybenzoic acid, gentisic acid, 4-hydroxyphenylacetic acid, (-)-epigallocatechin, (+)-catechin, vanillic acid, scopoline, chlorogenic acid, caffeic acid, malvin, kuromanin, procyanidin B2, vanillin, keracyanin, (-)-epicatechin, 4-coumaric acid, mangiferin, umbelliferone, (-)-gallocatechin gallate, scopoletin, ferulic acid, quercetin 3,4-di-O-glucoside, 3-coumaric acid, salicylic acid, sinapic acid, epicatechin gallate, ellagic acid, myricitrin, pelargonidin, quercetin 3-D-galactoside, rutin, p-anisic acid, quercetin 3-glucoside, luteolin 7-O-glucoside, malvidin, 2,4-dimethoxy-6-methylbenzoic acid, penta-O-galloyl-B-D-glucose, kaempferol 3-O-glucoside, quercitrin, naringin, rosmarinic acid, trans-cinnamic acid, luteolin, and kaempferide. Each compound was identified using a dynamic multiple reaction monitoring method and quantified using calibration curves from 0.25 to 19 µM, with a coefficient of determination greater than 0.99[9].

GC-MS compounds

Volatile compounds (esters, aldehydes, ketones and terpenes that are characteristic of this type of sample) were determined in 3.0 g of honey stored. The honey was placed in a vial sealed with a PTFE / Teflon cap and heated to 100 °C, then the sample was injected using an Agilent Technologies Head-space model 7694E and a gas chromatograph (Agilent Technologies™, model 6890 N, Net Work GC system, Santa Clara, California, USA) equipped with a DB-5 capillary column (60 m × 0.25mm id × 0.25 µm film thickness) was used. The GC conditions were initial temperature: 45 °C for 5 min, heating ramp: 15 °C/min up to 280 °C, for 1 min. Helium at a flow of 1 mL/min, injector temperature of 250 °C. Identification of volatile compounds was performed by mass spectrometry using the Agilent Technologies™ Model 5975 inert XL mass spectrometer, mass spectra were obtained by electron impact ionization at 70 eV. For identification, the mass spectra obtained for each compound were compared with a database (HP Chemstation-NIST 05 Mass Spectral search program, version 2.0d).

Fatty acid profile

Oily material was extracted from honey using a Soxhlet extractor with hexane (60–80 °C) for 6 h. The oily extract was filtered and concentrated under vacuum (Büchi, Flawil, Switzerland) to get crude extracts. Methyl Esters of Fatty Acids (FAMEs) were obtained through an esterification process and analyzed by gas chromatography coupled to mass spectrometry (GC-MS)[10]. A gas chromatograph (Agilent Technologies™, model 6890 N,

Net Work GC system, Santa Clara, California, USA) equipped with a DB-5 column (5% methylpolysiloxane, cat-1225082, J&W Scientific, USA) was used. The GC conditions were: initial temperature: 150 °C for 5 min, heating ramp: 30 °C/min to a temperature of 210 °C, 1 °C/min to 213 °C for 40 min, finally 20 °C/min up to 280 °C for 40 min. Helium at a flow of 1 mL/min, injector temperature of 250 °C. Identification of fatty acids was performed by mass spectrometry using the Agilent Technologies™ Model 5975 XL Inert Mass Spectrometer. The identity of each fatty acid was assigned using an external standard (FAMEs mix, C8:C22, cat no. 18920-1AMP, Sigma-Aldrich) which contained: octanoic acid, decanoic acid, undecanoic acid, dodecanoic acid, tridecanoic acid, tetradecanoic acid, pentadecanoic acid, 9-hexadecenoic acid, hexadecanoic acid, *cis*-9,12, heptadecanoic acid, octadecadienoic acid, *cis*-9-octadecaenoic acid, heptadecanoic acid, eicosanoic acid, 11-eicosenoic acid.

Minerals analysis

For mineral extraction, honey (1 g) was digested in digester tubes with a nitric acid solution (5%) in a 1:10 (p:v) ratio. The tubes were placed in a Kjeldahl digester (Speed Digester K-439, Büchi, Flawil, Switzerland) and digested at 170 °C for 2 h until an almost clear solution was obtained. This solution was filtered and later transferred to a 50 mL volumetric flask and diluted with 5% HNO_3 to finally be injected. The determination was performed with an Agilent MP 4100 MP-AES (Santa Clara, California, USA) consisting of a One Neb inert nebulizer, a double-pass glass cyclonic spray chamber, and a charge-coupled detector (CCD) of solid state. The plasma gas flow was 20 L/min and the makeup gas flow 1.5 L/min. A calibration curve was made from a mixture of 27 elements (Ag, Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Si, Sr, Ti, Tl, V, Zn) with eight points (concentrations: 100, 75, 50, 25, 10, 7.5, 5.0 and 1 ppm). The coefficient of determination was greater than 0.98 for each element. The conditions of the equipment for the analysis were: capture time 13 sec, plasma stabilization time with sample aspiration 15 sec, reading time 3 sec (reading in triplicate) and washing time 20 sec.

Microbiological analysis

The count of total mesophilic aerobic bacteria and molds and yeasts was carried out by weighing 1 g of honey that was mixed with 9 mL of PBS buffer. Subsequently, serial dilutions were made until the 10^{-9} dilution was obtained. Finally, 1 mL of each dilution was seeded in Petri dishes and plate count agar (Difco™, BD Detroit US) and PDA agar (potato

dextrose agar, BD™ Difco™ plate count agar) for aerobics were poured bacteria and molds and yeasts, respectively. Finally, they were incubated for 48 h and 5 d to 35 °C, and the colonies were counted.

Statistical analysis

Treatment and analysis were performed in triplicate, and the values were expressed as mean ± SD (standard deviation). All data were analyzed using one way of variance (ANOVA), followed by a Tukey's test with a significance level of 5% ($P<0.05$) using Minitab 16 statistical software (Minitab Inc. State College, PA, USA)[6].

Results

Physicochemical property analysis

Table 1 shows the physicochemical properties of honey stored in different packages. The moisture content of the analysed samples varied from 20.60 to 23.40 %, while the water activity varied from 0.663 to 0.675 after 2 yr of storage in the different treatments. Honey stored in a container with an anaerobic valve showed the greatest decrease in moisture content (20.60 %) and in water activity (0.667) compared to the control treatment, followed by honey stored in a transparent plastic container (moisture content: 22.80 %, a_w= 0.675). High moisture content is related to the environment in which the flowers from which the bees collect nectar are found. In addition, it must be considered that honey obtained from stingless bees contains a greater amount of water, so the effect that the type of container and its permeability has a greater effect on its stability compared to commercial honey obtained from *Apis mellifera*[3,5].

The color parameters of the honey exhibited slight changes during storage; these changes were reflected in the chroma values and in the total color change. The sample stored in transparent containers (T4) exhibited a greater total color change (8.08).

The pH of the samples varied slightly from 3.23 to 3.66. The values of pH obtained in the samples were in the range reported for this type of honey[11]. The total acidity of the samples stored in different containers (T1-T4) ranged from 85.66 to 87.33 meq/kg. The samples stored in the different types of containers (T2–T4) were not significantly different from each

other but were different from the control sample (73.66 meq/kg). The acidity values for treatments T1–T4 were similar to those reported for stingless bee honey of 85 meq/100 g[12]. The hydroxymethylfurfural (HMF) values of the samples stored in different containers (4.00–4.78 mg/kg) were not significantly different from those for the control treatment (4.09 mg/kg).

Antioxidant activity analysis

Table 2 shows that the content of total phenolic compounds, vitamin C and antioxidant compounds of honey stored in different containers were not significantly different *(P>0.05)* from that of the control treatment, except for vitamin C and DPPH radical inhibition. The content of total phenolic compounds varied from 12.55 to 14.31 mg GAE/100 g honey and that of vitamin C from 86.86 to 114.17 mg AA/g. Consistent with these values, the antioxidant activity determined by the DPPH radical scavenging activity presented high inhibition values (75.57–94.70 %). Similarly, the range of values determined by the FRAP (2.23-3.70 mg TE/g) and ABTS (0.61-1.13 mg TE/g) tests for the different types of containers show that honey contains compounds with a high capacity to reduce ferric ions and that they are stable during storage. These results are consistent with those reported for other types of honey[13] and the opposite to those reported for honey subjected to a temperature of 22–40 °C after 90 d of storage[14].

Table 2: Antioxidant properties of the honey recently harvested (T0-control), stored after two years in different containers (T1-T4)

Property	T0	T1	T2	T3	T4
Vitamin C, mg AAE/g	91.40 ± 5.24^{a,b}	87.50±8.26^{a}	86.86±1.02^{a}	114.17±2.16^{d}	106.64±1.35^{c}
Total phenolic compounds, mg GAE /g	12.55 ± 3.24^{a}	13.46±3.19^{a}	14.06±3.70^{a}	11.82±3.50^{a}	14.31± 2.63^{a}
DPPH inhibition, %	87.25 ± 2.65^{b}	94.05±2.69^{c}	75.57±5.65^{a}	94.70±1.88^{c}	84.71±4.65^{b}
FRAP, mg TE/g	2.97 ± 0.95^{a}	3.70±1.87^{a}	2.94±1.05^{a}	2.23±0.98^{a}	2.94±1.60^{a}
ABTS, mg TE/g	0.68 ± 0.18^{a}	0.61±0.22^{a}	0.72±0.31^{a}	0.69±0.24^{a}	1.13±0.76^{a}

The values are shown as the mean ± SD (n=3). AAE: Ascorbic acid equivalents. GAE: Gallic acid equivalents, TE: Trolox equivalents.
Different letters in each row indicate significant differences (P<0.05).

Phenolics identification and quantification by UPLC-MS

Table 3 shows the analysis of phenolic compounds present in freshly harvested honey and honey stored in different containers. A total of 17 phenolics plus two precursors (shikimic acid and *L*-phenylalanine) were identified in the honey stored in the different containers. Shikimic acid (35511–38504.90 µg/g dry extract), 4-hydroxybenzoic acid (2781.36–2996.87 µg/g dry extract), 4-hydroxyphenylacetic acid (1685.49–2294.62 µg/g dry extract) and *L*-phenylalanine (2917.68–3004.45 µg/g dry extract) were the major compounds in the samples. No significant differences ($P>0.05$) were found in most of the phenolics and precursors of the samples stored in the different containers after 2 yr of storage. The phenolics gentisic acid, 4-hydroxyphenylacetic acid, *p*-anisic acid and the precursor shikimic acid exhibited significant differences ($P<0.05$) in the samples stored in different containers, mainly in T4 (honey stored in a transparent plastic container). The profile of phenolic compounds found was similar to that reported for stingless honey by other authors[8], however, variations were found in relation to concentration, these differences in concentration have been attributed to floral and geographical variation and a collection time[8].

Volatile compounds

Table 4 shows that 18 volatile compounds were found in honey in the different treatments, ethyl acetate (20.20–30.24 %), *cis*-linalool oxide (30.05–34.73 %), *trans*-linalool oxide (12.97–15.75 %), and 1,5,7-octatrien-3-ol, 3,7-dimethyl (12.55-14.67 %) were the major compounds, representing approximately 50 % of the volatile compounds present in the samples. Alcohol derivatives were the predominant ones found in honey during storage.

Table 4: Volatile compounds (%) determined in the honey recently harvested (T0-control), stored after two years in different containers (T1-T4)

N	Compound name	RT (min)	T0	T1	T2	T3	T4
1	Ethyl acetate	5.46	-	20.20^{b}	22.25^{b}	30.24^{c}	28.67^{c}
2	3-Methyl butanal	5.66	-	-	-	-	-
3	Hexane, 3 methyl	7.46	-	1.79^{a}	1.77^{a}	2.18^{b}	1.91^{b}
4	2-Hexene, 3 methyl	8.64	-	0.240^{b}	0.236^{b}	0.272^{c}	0.16^{a}
5	Propanoic acid, 2-hydroxy-, ethyl ester	8.83	8.85^{c}	1.49^{a}	1.54^{a}	1.78^{b}	1.66^{b}
6	Furfural	9.60	-	1.71^{b}	1.87^{b}	0.96^{a}	0.68^{a}
7	D-Limonene	10.90	0.37	-	-	-	-
8	2-Heptanal acetate	10.93	1.38	-	-	-	-
9	Lilac alcohol B	11.05	-	0.14^{b}	0.15^{b}	0.110^{a}	0.112^{a}
10	Lilac alcohol C	11.19	0.09^{a}	0.11^{a}	0.10^{a}	0.09^{a}	0.09^{a}

11	Benzaldehyde	11.68	0.87[a]	1.76[b]	1.98[b]	1.99[b]	2.47[c]
12	*trans*-γ-Caryophyllene	11.89	18.72	-	-	-	-
13	Benzeneacetaldehyde	12.53	-	6.93[b]	6.19[b]	3.24[a]	3.70[a]
14	*cis*-Linalool oxide	12.71	48.07[c]	34.09[b]	34.73[b]	30.05[b]	31.51[b]
15	*trans*-Linalool oxide	12.90	21.648[b]	15.75[a]	14.81[a]	12.97[a]	13.44[a]
16	1,5,7-Octatrien-3-ol, 3,7-dimethyl	13.11	-	13.67[a]	12.55[a]	14.67[a]	13.44[a]
17	Nerol oxide	13.67	-	1.52[c]	1.25[b]	0.85[a]	1.51[c]
18	Linalool oxide	14.00	-	0.61[a]	0.62[a]	0.69[a]	0.69[a]

Results are expressed as the mean ± SD (n=3).

RT= retention time.

[ab] Different letters in the same row are significantly different (P<0.05). --Not present.

Fatty acids present in the honey

Analysis of the hexane extract of the honey samples revealed the presence of eight fatty acids (Table 5). Hexadecanoic acid (31.12–49.65 %), octadecanoic acid (21.48–26.86 %) and *cis*-9-octadecadienoic acid (14.31–40.04 %) were the major compounds found in the different stored samples.

Table 5: Relative area (%) of fatty acids in the hexane extract in the honey recently harvested (T0-control) and stored after two years in different containers (T1-T4)

Compound name	**RT (min)**	**T0**	**T1**	**T2**	**T3**	**T4**
Decanoic acid	6.42	-	0.47[d]	0.25[b]	0.22[b]	0.19[a]
Dodecanoic acid	8.34	29.72[c]	1.96[a]	7.02[b]	2.27[a]	1.90[a]
Tetradecanoic acid	10.49	1.91[c]	2.07[b]	1.15[a]	2.48[b]	1.19[a]
9-Hexadecenoic acid	12.21	-	0.65[a]	0.75[a]	0.55[a]	0.68[a]
Hexadecanoic acid	12.41	22.27[a]	43.08[c]	34.33[b]	49.65[c]	31.12[b]
cis-9,12, Octadecadienoic acid	13.79	1.41[a]	2.96[b]	3.24[b]	3.71[b]	3.41[b]
cis-9-Octadecaenoid acid	13.83	15.90[a]	22.36[b]	32.23[c]	14.31[a]	40.04[d]
Octadecanoic acid	13.98	28.80[b]	26.47[b]	21.44[a]	26.86[b]	21.48[a]

Results are expressed as the mean ± SD (n=3).

RT= retention time.

[abcd] Different letters in the same row are significantly different (P<0.05). -Not present.

Mineral content analysis

The mineral content remained constant during storage, and it descended in the following order: K > Mg > Ca > Na > Si, for honey stored in the different containers (Table 6). The

concentration of As, Be, Cd, Mo, Ni, Pb, Sb, Ti, Tl and V was similar to that reported by Villacrés-Granda *et al*[2]. K (109.36–125.68 mg/100 g DW) and Mg (31.60–100.49 mg/100 g DW) were found in higher concentrations compared to the other minerals present. Potassium was the majority mineral, representing a third of the total content and exceeding that of other minerals by approximately 10 times. No statistically significant differences were found for the minerals investigated in most samples evaluated during storage.

Table 6: Mineral and trace elements (mg / 100 g DW) in the honey recently harvested (T0-control) and stored after two years in different containers (T1-T4)

Mineral	T0	T1	T2	T3	T4
Al	1.378±0.01^{a}	1.352±0.04^{a}	1.451±0.10^{a}	1.221±0.07^{a}	1.235±0.00^{a}
As	-	-	-	-	-
B	1.670±0.02^{b}	1.208±0.03^{a}	1.456±0.10^{a,b}	1.765±0.09^{b}	2.089±0.00^{c}
Ba	-	-	-	-	-
Be	-	-	-	-	-
Ca	76.090±0.10^{b}	43.172±0.5^{a}	95.441±0.90^{c}	96.662±0.95^{c}	61.210±1.00^{b}
Cd	-	-	-	-	-
Co	-	-	-	-	-
Cr	-	-	-	-	-
Cu	0.578±0.07^{b}	0.476±0.03^{b}	0.554±0.03^{b}	0.753±0.02^{c}	0.159±0.01^{a}
Fe	0.970±0.08^{b}	0.740±0.02^{b}	1.179±0.09^{c}	0.815±0.03^{b}	0.613±0.06^{a}
K	115.90±3.00^{a}	125.686±2.76^{b}	109.97±2.00^{a}	109.361±3.09^{a}	122.840±2.67^{b}
Mg	84.32±1.00^{b}	100.490±1.98^{b}	31.608±1.65^{a}	96.608±1.49^{b}	87.096±1.34^{b}
Mn	0.11±0.00^{a}	0.123±0.00^{a}	0.14±0.00^{a}	0.113±0.00^{a}	0.189±0.00^{b}
Mo	-	-	-	-	-
Na	28.65±1.02^{b}	35.794±1.17^{c}	20.562±1.07^{a}	26.655±0.45^{b}	21.470±0.98^{a}
Ni	-	-	-	-	-
Pb	-	-	-	-	-
Sb	-	-	-	-	-
Se	4.870±0.98^{a}	4.589±0.76^{a}	4.892±0.49^{a}	4.891±0.29^{a}	5.494±0.57^{a}
Si	45.88±1.00^{a}	46.798±1.06^{a}	40.195±0.30^{a}	53.352±0.69^{a}	51.485±0.70^{a}
Sr	-	-	-	-	-
Ti	-	-	-	-	-
Tl	-	-	-	-	-
V	-	-	-	-	-
Zn	0.678±0.05^{a}	0.814±0.06^{b}	0.972±0.05^{b}	0.349±0.06^{a}	0.995±0.04^{b}

These values are the average of three determinations.

^{ab} Different letters in the same row are significantly different ($P<0.05$). -: no detectable.

Microbiological analysis

The results showed a higher number of microorganisms in the initial samples for total aerobic mesophilic bacteria (1.50 x 10^2 CFU/g) and molds, and yeast (2.30 x 10^2 CFU/g), compared to the samples in different containers stored for two years (Table 7). The results obtained for the analysis of microorganisms for the samples at the beginning of storage ranged from 0.78 x 10^2 - 0.98 x 10^2 CFU/g of sample for aerobic mesophiles and 0.03 x 10^2- 0.32 x 10^2 CFU/g of sample for molds, and yeasts.

Table 7: Total aerobic mesophilic bacteria, and molds, and yeast present in samples at the beginning and after two years of storage in different containers

Treatment code	Total Aerobic mesophilic count (CFU/g)	Total molds and yeast count (CFU/g)
T0 (initial storage)	1.50 x 10^{2a}	2.30 x 10^{2a}
T1	0.89 x 10^{2b}	0.32 x 10^{2b}
T2	0.98 x 10^{2b}	0.15 x 10^{2b}
T3	0.95 x 10^{2b}	0.08 x 10^{2b}
T4	0.78 x 10^{2b}	0.03 x 10^{2b}

These values are the average of five determinations (n=5).

[ab] Different letters in the same column are significantly different (P<0.05).

Discussion

Determination of the physicochemical properties, such as moisture, pH, acidity, and Brix degrees allow the evaluation of honey quality. The moisture of honey favors the growth of bacteria and fungi present in honey. The range of moisture values obtained for the different treatments was consistent with those reported for the honey from Ecuadorian stingless bees[(2)]. It was found that the sample stored in the container with an escape check valve showed a significant reduction in moisture content compared to the initial treatment, which it is possibly due to the gases produced by fermentation dragging the moisture present into the headspace of the container, preventing the moisture from returning. At the same time, color changes in honey are related to its botanical origin, mineral content, the content of phenolic compounds, antioxidant properties, room temperature, and storage time. The change in honey stored in the transparent container is possibly because light could affect the components of the honey such as carotenoids and flavonoids[(15)].

The values of pH and the total acidity, play an important role in the quality of the honey[(11)]. The acidity values show that this honey stored for 2 yr has a higher acidity. The increase in

acidity value during storage may be related to the fermentation of honey and to its antimicrobial properties, but it may also result in an undesirable vinegar taste because of acetic acid production. The acidity of honey is related to the glucose content. Glucose is converted by the action of the enzyme *D*-glucose oxidase into gluconic acid. This process produces hydrogen peroxide which is a component of the antimicrobial action of honey[16]. The production of acids occurs not only by enzymatic action but also by fermentation of the microorganisms present in the matrix. Increased acidity may also be associated with the transformation of sugars from honey into alcohols and then into organic acids by osmophilic yeasts. It is also necessary to consider that when the moisture content is high, the bacteria grow and ferment the sugars, producing compounds such as acetic acid that can affect the taste of honey[17].

A very important quality factor in honey is the HMF concentration, since it is an indicator of the quality, freshness, and aging of honey. Under conditions such as processing or aging, mainly influenced by temperature fluctuation, pH, storage conditions, and floral origin, may help bring about its presence[18].

The antioxidant activity of honey depends on its floral origin and the processing conditions and is closely related to the chemical compounds it possesses. The components of honey – phenolic compounds, flavonoids, and phenolic acids, as well as chlorophyll, carotenoids, and vitamin C – contribute to its antioxidant activity[15], coupled with the fact that the antioxidant properties are related to its color and the moisture content.

The antioxidant activity of honey is due, among other factors, to the presence of phenolic compounds, which are produced in plants as a protection system and are entrained in the nectar extracted by bees. UPLC analysis revealed the presence of shikimic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid and L-phenylalanine, among others. These compounds confer antioxidant activity to honey since they possess delocalized electrons, which cause free radical scavenging activity[19]. The radical scavenging activity of phenolics mainly depends on the number and position of hydroxyl groups in the molecules. The presence of these compounds is explained by the fact that shikimic acid is a precursor of aromatic metabolic intermediates, within which are flavonoids such as luteolin[20]. The presence of these phenolic compounds may suggest possible anti-inflammatory and antimicrobial activity, among other properties. Consistent with the analysis of total phenolic compounds, the concentration of most of the quantified phenolic compounds decreased in the sample subjected to heat treatment, compared to the control. This helps to explain the decrease in antioxidant activity.

Identifying volatile compounds plays a crucial role in assessing the quality of honey. These compounds, linked to flower nectar, geographical origin, and overall stability, offer insights into the honey's unique characteristics[21]. When honey is stored in various containers, there

is an increase in volatile compounds over the initial storage day. Notably, esters, aldehydes, ketones, and alcohols become predominant after 2 yr, contributing significantly to the honey's odor and flavor. The appearance and rise of specific volatile compounds may be linked to the fermentation process, with packaging type influencing oxygen availability and anaerobic respiration enhancement. Moisture content further affects fermentation, favoring the production of alcohol, carbon dioxide, and acetic acid, all influencing the concentration of volatile compounds in honey[17].

Free fatty acids, akin to volatile compounds, serve as lipid markers reflecting the floral origin of honey and can be crucial authenticity indicators[22]. Changes in the concentration of certain volatile compounds in honey stored in containers are likely due to the container material's permeability. Plastics, in particular, may retain some volatile compounds, facilitating their transfer between honey and the container material. This involves the adsorption or retention of volatile compounds in honey, causing shifts in their concentration. Additionally, some volatile compounds might be lost or absorbed, affecting the honey's aromatic profile and consequently altering its taste and aroma. Fatty acids such as hexadecanoic acid increased in proportion, while the proportion of dodecanoic acid decreased, and others like decanoic acid and 9-hexadecenoic acid emerged during storage. These changes may be related to variations in water activity and the permeability of different containers used for storage. Another factor is that honey crystallization can impact the mobility and availability of fatty acids, influencing their proportion.

Mineral content is another quality factor for honey. The analysis shows that the type of minerals and their concentration does not vary during storage in the evaluated containers, and it was similar to those reported by other authors regarding other types of honey; Consistent with other reports, potassium was the most abundant mineral, this is considered the most quantitatively important mineral in the honey, accounting for around 50 % of the total mineral content. The presence of Al, Ba, Si, and Co is mainly since these minerals are naturally present in the environment, demonstrated that the honey is a very good environmental indicator so reflects the content of toxic elements in the surrounding water, soil, and air[23]. Honey can contribute to the diet with elements such as Mg, Ca, and K. Mg and K are important micronutrients for the human body since they are involved in many physiological processes and are essential for the maintenance of the normal function of cells and organs, by which they make an important contribution to health[24]. These results are also consistent with those reported in a study on honey from stingless bees from Brazil, where it was found that these minerals are the most important quantitatively[8].

Honey can contain microorganisms from different resources, such as pollen digestive tracts dust, air soil, and nectar, or due to handling and processing. The presence of these microorganisms can affect the quality of honey during storage, so an analysis of the total count of aerobic microorganisms and molds, and yeasts were performed at the beginning and

end of storage in different containers. These values were below the limit reported by other authors and that established for *Apis mellifera* honey, which may be due to proper handling in harvesting and the presence of phenolic compounds, organic acids, and other bioactive compounds present in the honey that has an inhibitory effect on this type of microorganism. The concentration of total aerobic microorganisms and molds and yeasts decreased in honey during storage, which is consistent with the reduction in water activity and moisture. This could be attributed to various factors such as sugar crystallization or water evaporation due to plastic permeability. The decrease in microorganism concentration is a positive factor that ensures the quality of honey during its storage.

Conclusions and implications

In this study, a comparison was made between plastic containers used commercially, since the use of other types of containers, such as glass or metal, are more expensive for the producer. The study demonstrated that storing honey in traditional plastic containers (high-density polyethylene and polyethylene terephthalate) and using certain traditional methodologies provide significant differences in the moisture content of honey during storage, with the moisture content being minor in honey stored in the container with an escape check valve (T3). It was also found that, in general, storage for 2 yr does not produce major changes in the physicochemical properties and in the content of phenolic compounds, which are associated with a decrease in antioxidant properties and volatile compounds that together can affect the honey quality. Furthermore, storage had a positive effect on the microbiological analysis of the honey. Finally, the evaluation of these parameters suggests that treatment T3 would be the most suitable for storing honey since it presented a total color change of less than 3, an important quality parameter for consumers.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 1: Physicochemical properties of the honey recently harvested (T0-control), stored after two years in different containers (T1-T4)

Properties	**T0**	**T1**	**T2**	**T3**	**T4**
Moisture, gH_20/ 100 g d.m.	25.70 ± 0.47^{b}	23.23 ± 0.28^{ab}	23.40 ± 0.09^{b}	20.60 ± 1.33^{a}	22.80 ± 0.39^{a}
Water activity (25 °C)	0.733 ± 0.006^{b}	0.663 ± 0.001^{a}	0.671 ± 0.007^{a}	0.667 ± 0.006^{a}	0.6759 ± 0.003^{a}
*L**	18.33 ± 0.37^{a}	20.80 ± 1.85^{a}	21.67 ± 3.59^{a}	18.27 ± 0.43^{a}	25.64 ± 2.55^{b}
*a**	2.84 ± 0.75^{a}	6.66 ± 0.64^{b,c}	6.27 ± 0.87^{b,c}	5.40 ± 0.64^{b}	8.91 ± 1.82^{c}
*b**	5.39 ± 0.23^{a}	7.69 ± 2.34^{b}	8.41 ± 4.58^{b}	4.63 ± 0.08^{a}	7.26 ± 3.45^{b}
Chroma	6.11 ± 1.53^{a}	10.17 ± 2.21^{a}	10.49 ± 4.22^{a}	7.11 ± 0.51^{a}	10.02 ± 3.54^{a}
Total color change	-	4.81 ± 0.54^{b}	5.32 ± 0.38^{b}	2.69 ± 0.45^{a}	8.08 ± 0.37^{c}
Browning index	-	0.38 ± 0.09^{a}	0.42 ± 0.05^{a}	0.42 ± 0.03^{a}	0.39 ± 0.02^{a}
pH	3.66 ± 0.05^{a}	3.23 ± 0.05^{a}	3.26 ± 0.05^{a}	3.40 ± 0.06^{a}	3.43 ± 0.05^{a}
Brix (º)	71.66 ± 0.57^{a}	71.90 ± 0.55^{a}	72.03 ± 0.55^{a}	72.10 ± 0.26^{a}	72.76 ± 0.37^{a}
Electric conductivity, mS/cm	293.33 ± 11.54^{b}	320.25 ± 20.00^{a}	293.33 ± 15.27^{b}	303.33 ± 5.77^{b}	296.00 ± 15.16^{b}
Density, g/mL	1.40 ± 0.02^{b}	1.36 ± 0.01^{a}	1.36 ± 0.00^{a}	1.36 ± 0.02^{a}	1.36 ± 0.01^{a}
Hydroxymethylfurfural, mg /kg	4.09 ± 0.53^{a}	4.33 ± 0.20^{a}	4.00 ± 0.39^{a}	4.23 ± 0.22^{a}	4.78 ± 0.52^{a}
Titratable acidity, meq/kg d.m.	73.66 ± 0.57^{a}	87.33 ± 6.65^{b}	87.33 ± 0.57^{b}	87.33 ± 2.08^{b}	85.66 ± 1.15^{b}

Data represent the average of three replicates or measurements ± standard deviation.

^{abcd} Different letters in the same row indicate significant differences ($P<0.05$).

-- Not present.

Table 3: Phenolic compounds (µg/g dry extract) of the honey recently harvested (T0-control), stored after two years in different containers (T1-T4)

Phenolic compound	T0	T1	T2	T3	T4
Gallic acid	126.78 ± 10.00[a]	136.17 ± 16.20[a]	137.88 ± 4.91[a]	143.32 ± 5.97[a]	135.25 ± 8.16[a]
4-Hydroxybenzoic acid	2996.87 ± 135.76[a]	2847.66 ± 207.18[a]	2906.04 ± 118.78[a]	2934.22 ± 105.08[a]	2781.36 ± 146.05[a]
Protocatechuic acid	637.87 ± 21.79[a]	660.19 ± 22.55[a]	666.90 ± 13.40[a]	648.85 ± 24.07[a]	634.87 ± 34.48[a]
Vanillic acid	654.89 ± 38.33[a]	612.57 ± 59.27[a]	645.12 ± 39.07[a]	677.20 ± 29.77[a]	633.24 ± 27.82[a]
Gentisic acid	312.90 ± 89.87[a]	312.82 ± 36.07[a]	280.94 ± 29.34[a]	294.31 ± 10.82[a]	541.69 ± 48.62[b]
4-Hydroxyphenylacetic acid	2198 ± 129.88[a]	2294.62 ± 115.55[b]	1702.48 ± 195.65[a]	2036.27 ± 133.77[b]	1685.49 ± 103.27[a]
Sinapic acid	1677.33 ± 87.99[a]	1677.70 ± 81.08[a]	1636.52 ± 23.01[a]	1663.18 ± 57.32[a]	1565.35 ± 80.53[a]
Salicylic acid	1244.11 ± 199.55[a]	1240.45 ± 409.54[a]	1053.87 ± 54.18[a]	1074.40 ± 30.10[a]	1098.68 ± 32.97[a]
p-Anisic acid	399.97 ± 29.73[b]	317.57 ± 26.29[a]	455.27 ± 39.95[c]	384.97 ± 38.77[b]	456.20 ± 52.69[c]
Rosmarinic acid	297.45 ± 12.95[a]	275.61 ± 20.76[a]	220.68 ± 58.40[a]	248.44 ± 16.14[a]	276.69 ± 67.38[a]
4-Coumaric acid	301.86± 38.26[a]	291.65 ± 37.60[a]	320.81 ± 13.12[a]	323.82 ± 7.57[a]	271.41 ± 53.92[a]
Trans-cinnamic acid	139.87 ± 9.41[a]	124.02 ± 7.43[a]	123.18 ± 4.77[a]	122.89 ± 3.74[a]	127.99 ± 3.83[a]
Luteolin	289.56 ± 29.44[a]	273.83 ± 38.24[a]	325.69 ± 104.80[a]	315.66 ± 45.43[a]	320.41 ± 62.01[a]
Scopoletin	689.85 ± 58.33[a]	673.76 ±72.47[a]	720.55 ± 26.69[a]	740.75 ± 17.46[a]	619.54 ± 120.49[a]
Ferulic acid	132.63 ± 27.82[a]	127.52 ± 21.48[a]	136.69 ± 20.29[a]	150.77 ± 40.86[a]	111.18 ± 27.34[a]
Caffeic acid	478.86 ± 96.43[a]	410.22 ± 122.14[a]	570.36 ± 24.02[a]	590.65 ± 27.56[a]	452.59 ± 120.71[a]
Shikimic acid	36986.87±3999.20[a]	38200.95±3974.40[a]	37735.48±2688.39[a]	38504.90±2817.73[a]	35511.01±5741.62[a]
Vanillin	97.45 ± 6.98[a]	96.17±5.16[a]	97.36 ± 16.74[a]	85.01 ± 12.63[a]	98.26 ± 12.83[a]
L-phenylalanine	2930.99 ± 120.89[a]	2934.82±100.09[a]	2886.78 ±115.55[a]	3004.45 ± 130.22[a]	2917.68 ± 118.89[a]

Results are expressed as the mean ± SD (n=3).

[ab] Different letters in the same row are significantly different ($P<0.05$).

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Article

A novel effect of aqueous extract of *Pimpinella anisum* seeds on ticks of domestic dogs (*Canis lupus familiaris*)

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Abstract:

Synthetic pesticides used to combat ticks lose their effectiveness against certain species and can affect human health. The present study evaluated *in vitro* and *in vivo* the effect of the aqueous extract of *Pimpinella anisum* (*P. anisum*) seeds against *Rhipicephalus sanguineus* and *Ixodes affinis*, domestic dog ticks. In the *in vitro* evaluations, concentrations of 1.25, 2.5, 5, 10, 25, 50, 75 and 100 % of the aqueous extract of *P. anisum* were applied directly to ticks. The concentrations that had the highest effectiveness in immobilization were 50 %, 75 % and 100 %, but the latter caused immobilization for a longer time (55.89 ± 0.16 min). In the *in vivo* evaluation, the concentrated aqueous extract was applied to ticks attached to the skin of domestic dogs. Amitraz, a commercial tickicide, was used as a positive control. Both the concentrated aqueous extract and Amitraz caused 100 % of tick detaching. Nonetheless, concentrated aqueous extract of *P. anisum* seeds was more effective in reducing the average time of tick detaching (60.81 ± 3.17 min) compared to the commercial tickicide Amitraz (145.12 ± 15.97 min). This research suggests that the p-anisaldehyde identified in the aqueous extract could be linked to the immobilization and detaching of *R. sanguineus* and *I. affinis* from domestic dogs, suggesting that this extract could be used as a biopesticide to control ticks in domestic dogs.

Keywords: Biopesticide, Ticks, Immobilization, Domestic dogs, *In vitro*, *In vivo.*

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Introduction

Ticks of the family Ixodidae are hematophagous arthropods of worldwide distribution, which parasitize various species of mammals, birds, and reptiles. When these ectoparasites feed on their host (various domestic and wild animals, including humans), they can transmit pathogenic microorganisms such as bacteria, viruses, protozoa, and helminths[1,2].

Ticks are pests that are considered economically harmful in livestock and other animal species as they can cause severe anemia and weight loss[3]. Dogs that inhabit rural and urban areas are hosts of the species *R. sanguineus*. These ticks can be found in tropical and subtropical regions, adapting to indoor conditions[4]. *Ixodes affinis* is a species that is also common in dogs and cats worldwide[5].

The use of synthetic pesticides causes damage to the environment and is a health hazard[6,7]; however, in recent years, alternatives have been sought to control pests without polluting the environment, one of them is the use of plants with insect or mite control capabilities.

The use of medicinal plants is a practice that has carried out since ancient times and has contributed to the origin of modern medicine[8]. Numerous medicinal plants contain secondary metabolites and pigments, among other components that are toxic against various microorganisms. It has been reported that phytochemicals isolated from medicinal plants are key to the generation of biopesticides; they are also considered less toxic and easily degraded[9,10]. In recent years, the applications of extracts and essential oils from plant species have become new alternatives as environmentally friendly pesticides, with the purpose of limiting the use of synthetic pesticides in the agricultural sector[7,11].

Among the plant species used to combat pests is *Pimpinella anisum*[12], commonly known as anise, green anise or badian[13], it belongs to the family Umbelliferae, currently called Apiaceae, and has been used in traditional medicine as a carminative, aromatic, disinfectant and galactagogue. *P. anisum* seeds have antimicrobial, antifungal, antiviral, antioxidant and anticonvulsant activities and muscle relaxing, analgesic, and hypoglycemic effects[14], and have also been reported to have insecticidal activity[15].The essential oil of the seeds of this plant species has been shown to have a lethal effect against *Tribolium castaneum*[12], repellent activity against adults of *Culex pipiens*[16] and is toxic against *Daphnia magna*[17]. Recent studies have shown that *P. anisum* seed oil has acaricidal activity against *Tetranychus urticae*[18], while aqueous and methanolic extracts have antimicrobial activity against *Candida albicans*[19] and *Escherichia coli*[20], respectively. Compounds such as *trans*-anethole, methyl chavicol, anisaldehyde, estragole, and γ-hymachalen[14] have been identified in the seeds of *P. anisum*, and p-anisaldehyde[14] has been identified in the essential oil, which is a compound that causes immobilization, repellency, and mortality effects in insects such as *Haematobia irritans irritans* (L.) and *Musca domestica* L., and the response of its application depends on the stage of development in these insects[21,22,23]. According to the background and effects caused by the species *P. anisum* against other insects, this study aimed to evaluate the effect of the aqueous extract of *P. anisum* seeds against ticks that commonly attack domestic dogs, in order to reduce the use of synthetic insecticides that are not friendly to the environment.

Material and methods

Biological material

The seeds of *P. anisum* were obtained from the commercial house Granos y semillas Yael, located at Calle 52 No. 540 and 67, Centro, Mérida, Yucatan, which were dehydrated.

Preparation of aqueous extract of *P. anisum* seeds

The concentrated aqueous extract of *P. anisum* seeds was obtained from 50 g of seeds, which were crushed in a manual mill (Del Rey brand) to obtain particles of 1.5 mm in diameter on average. The seeds were decocted using 12.5 g in 1 L of purified H_2O (Bonafont® brand) at 90 °C. Cooking time was 20 min. Finally, the aqueous extract obtained was kept in amber bottles and preserved under refrigerated conditions at 4 °C until use. Subsequently, the concentrated aqueous extract was diluted with purified H_2O (Bonafont®) to prepare concentrations of 1.25, 2.5, 5, 10, 25, 50, 75 % and the 100 % concentrated aqueous extract. Purified H_2O was used as a negative control and the commercial compound Combatick® (12.5 % Amitraz), an insecticidal and acaricide solution, was used as positive control, which was prepared and applied according to the indications on the product label (2 ml of the solution per 1 L of H_2O).

Toxicity bioassay

To determine the toxicity of the aqueous extract of *P. anisum* seeds, a toxicity test was performed on *Artemia salina* (White Mountain, Great Salt Lake, Utah, USA); the cysts were incubated in filtered seawater for 24 h, at a temperature of 29 ± 4 °C, with constant aeration[24].

Bioassays were performed on 24-well plates. Four concentrations (0.0005, 0.05, 5, 500 mg/ml) of the aqueous extract of *P. anisum* seeds were prepared by serial dilution. Ten nauplii of *A. salina* were placed in each well. Filtered sea H_2O without extract was used as a negative control. All treatments were analyzed fivefold. The plates were incubated at 29 ± 4 °C for 24 h; after this time, they were observed under a stereo microscope (SMZ800, Nikon) and the number of live nauplii was counted. Mortality was considered when no movement

was observed after 10 sec. Mortality percentage and median lethal dose (LC_{50}) were calculated. To consider whether the plant extract is toxic, the toxicity criteria proposed by Clarkson *et al*[25] were followed: non-toxic when LC_{50} >1,000 μg/mL, low toxicity 500 < LC_{50} <1,000 μg/mL, moderate toxicity 100 < LC_{50} <500 μg/mL, and highly toxic 0 < LC_{50} <100 μg/mL.

Tick collection on domestic dogs

A total of 270 adult ticks were collected from 10 naturally infested domestic dogs of different breeds, ages, and sexes from the municipalities of Ticul (20°23′43″N, 89°32′02″W) and Oxkutzcab (20°18′10″N 89°25′06″W), Yucatan, Mexico; these animals received no previous treatment. The ticks were placed in glass bottles with perforated lids and stored in the laboratory at 29 ± 4 °C for 24 h.

In vitro evaluation in ticks

For the *in vitro* toxicity evaluation, was used the concentrated aqueous extract of *P. anisum* seeds and seven dilutions of this same extract (1.25, 2.5, 5, 10, 25, 50, 75 %) and purified H_2O as a negative control. For each of the treatments, 30 ticks and 0.5 ml of the solution were used; the solution was applied by spraying it on the ticks. After 30 min, the percentage of immobilized ticks (% I) was calculated using the formula:

$$\% I = (N_i/N_T) \times 100$$

Where: % I= percentage of immobilized ticks; N_i= number of ticks immobilized; N_T= total number of ticks treated.

In vivo evaluation in domestic dogs

The concentrated extract was used to evaluate the effect of the aqueous extract of *P. anisum* seeds on ticks in domestic dogs. For this test, the positive control was Amitraz, which is a commercial miticide and insecticide, and purified water of the Bonafont® brand was used as a negative control. For the evaluation, 12 domestic dogs of different breeds, sexes and ages that presented problems with the presence of ticks on their body were included and they were divided into three groups of four canine specimens each for the application of the product to

be evaluated and the count of the number of ticks present in each individual (Tables 1, 2 and 3). For each dog, a volume of 0.5 ml of concentrated extract of *P. anisum* was used and applied to the left ear, tail, armpits and on the back of the animal where the ticks were. Amitraz was applied in accordance with the commercial producer's instructions. To determine the time it took for each treatment to detach the ticks, we waited until the last tick became detached by application area.

Table 1: Breeds of dogs infested with ticks in different areas, treated with the negative control (purified H_2O)

	Number of ticks by area				
Breed	**Left ear**	**Tail**	**Armpits**	**Back**	**Total number of ticks**
Chihuahua	6	12	5	12	35
Maltese dog	5	15	11	11	42
German shepherd	5	15	11	11	42
Mixed breed	13	7	12	3	35

Table 2: Breeds of dogs infested with ticks in different areas, treated with concentrated aqueous extract of *P. anisum*

	Number of ticks by area				
Breed	**Left ear**	**Tail**	**Armpits**	**Back**	**Number of ticks by area**
Chihuahua	7	6	11	7	31
Maltese dog	15	15	12	7	49
German shepherd	7	7	8	9	31
Mixed breed	5	16	15	5	41

Table 3: Breeds of dogs infested with ticks in different areas, treated with the positive control (Amitraz)

	Number of ticks by area				
Breed	**Left ear**	**Tail**	**Armpits**	**Back**	**Number of ticks by area**
Chihuahua	10	6	7	8	31
Maltese dog	13	5	14	11	43
German shepherd	7	7	8	9	31
Mixed breed	5	16	15	5	41

Identification of tick species in domestic dogs

The identification of the 100 randomly selected ticks, treated in the laboratory, as well as those that became detached from domestic dogs after the application of the aqueous extract and Amitraz, was by morphological characteristics, the shape of the hypostome, capitulum, and pedipalp of each of the ectoparasites. The ticks were placed in a 70 % ethanol solution for 8 min, during which time the ticks remained motionless. They were then observed with a stereo microscope (Stemi 305, Zeiss). The identification of the species was carried out using the images of ticks published by Lord CC[26] and Solís Hernández[27] as a reference.

Identification of p-anisaldehyde in the aqueous extract of *P. anisum*

To determine the presence of p-anisaldehyde, 5 mL of the concentrated extract was used, which was filtered through 0.22 µM nylon membranes (Thermo Scientific Cat. No. 726-2520). Subsequently, the solution was frozen in a deep freezer for 3 h (Thermo Fisher Scientific Inc., Model-TSX400D, No. 144DT0B01A) and lyophilized (LABCONCO-No. cat 77540-00) for 24 h. The lyophilized product was resuspended in 5 mL MeOH (TEDIA-MS1922-001), stirred (Vortex-genie-Serial No G-560) and centrifuged again for 5 min (Galaxy mini centrifuge – Serial No. 1204), obtaining the supernatant. For sample analysis, 2 µL of the supernatant was injected into a gas chromatograph coupled to mass spectrometry (Agilent 7890A, Wilmington, Delaware USA), equipped with a hydrogen flame ionization detector for compound identification. Compound separation was performed with an HP5MS column (Agilent Technologies, 30 m × 0.250 mm, 0.25 µm, Cat. No. 190915-435, USA). The injector temperature was set to 250 °C and the initial oven temperature was 70 °C for 3 min, increasing by 5 °C/min to 250 °C.

Statistical analysis

The data obtained in the *in vitro* and *in vivo* treatments were analyzed with one-way ANOVA with the Holm-Sidak test using the Sigma Plot version 12 program.

Results

Toxicity bioassay

The toxicity results of the aqueous extract of *P. anisum* seeds showed that at a concentration of 500 mg/mL, the highest mortality percentage (14.3 %) was obtained, while with the other concentrations no mortality was observed (Table 4). The median lethal dose (LC_{50}) was 4645 µg/mL. With the toxicity and LC_{50} results obtained and, according to the toxicity criteria proposed by Clarkson *et al*[25], the aqueous extract of *P. anisum* seeds is considered to be non-toxic for use in canines.

Table 4: Percentage of mortality of *Artemia salina* in the presence of the aqueous extract of *P. anisum* seeds

Concentration (mg/mL)	Mortality (%)
Control	0.0
0.0005	2.0
0.05	0.0
5	0.0
500	14.3

Immobilizing effect of aqueous extract of *P. anisum* seeds *in vitro*

In the *in vitro* treatment, the aqueous extract of *P. anisum* seeds was sprayed on the ticks, and after 30 min it was observed that the concentration of 1.25 % of the aqueous extract had no effect on tick mobility. At the concentration of 2.5 %, an immobilization percentage of 16.7 ± 5.8 was observed. In the higher concentrations, the percentage of immobilization increased significantly; with 25 %, more than 96 % of the total number of ticks evaluated were immobilized, and with concentrations of 50, 75 and 100 % of extract, 100 % of the treated ticks were immobilized, with no significant difference observed in the last four treatments (Table 5).

The average time of tick immobilization differed depending on each concentration of the aqueous extract of *P. anisum* seeds. It was observed that the 2.5 % aqueous extract caused the ticks to remain motionless for 3.00 ± 0.04 min; with 10 % of the aqueous extract the time was 14.9 ± 0.21 min; with 50 %, the immobilization time was 45.14 ± 0.07 min. The 100 %

concentrated extract caused the ticks to remain motionless for more than 50 min (55.89 ± 0.16 min). Immobilization time was statistically different between each treatment (Table 5).

Table 5: Percentage of immobilized ticks and immobilization time caused by the effect of the aqueous extract of *P. anisum* seeds under *in vitro* conditions

Aqueous extract concentration (%)	Immobilized ticks (%)	Immobilization time (min)
0	0^{a}	0^{a}
1.25	0^{a}	0^{a}
2.5	16.7 ± 5.8^{b}	3 ± 0.04^{b}
5	43.3 ± 5.8^{c}	10.07 ± 0.13^{c}
10	60 ± 00^{d}	14.9 ± 0.21^{d}
25	96.7 ± 5.8^{e}	28.07 ± 0.07^{e}
50	100 ± 00^{e}	45.14 ± 0.07^{f}
75	100 ± 00^{e}	50.14 ± 0.07^{g}
100	100 ± 00^{e}	55.89 ± 0.16^{h}

^{abcd} Different letters, placed as a superscript, indicate significant differences. One-way ANOVA ($P<0.005$).

In vivo effect of aqueous extract of *P. anisum* seeds on ticks attached to domestic dogs

In order to demonstrate the effect of *P. anisum* seeds on ticks attached to domestic dogs, the concentrated aqueous extract was evaluated, which, under *in vitro* conditions, caused ticks to be immobilized for longer than dilutions (1.25, 2.5, 5, 10, 25, 50, and 75 %).

In the comparison of the effects of the negative control of purified H_20 (Bonafont®), the aqueous extract of *P. anisum* seeds and Amitraz as a positive control, it was shown that the purified water did not detach ticks from the skin of domestic dogs; in contrast, the aqueous extract of *P. anisum* seeds caused all ticks to detach in an average time of 60.81 ± 3.17 min, while Amitraz required 145.12 ± 15.97 min, observing that the average time of tick immobilization of these treatments was statistically different (Table 6). After the detachment of the ticks from the domestic dogs, the concentrated aqueous extract maintained the effect of immobilizing the ticks in the ground for 14.625 ± 1.36 min. On the other hand, Amitraz did so for 44.93 ± 2.38 min (Table 7), with a significant difference in the time of immobilization of ticks on the ground.

Table 6: Effect of aqueous extract of *P. anisum* seeds on the detaching time of ticks attached to domestic dogs

Treatments	Detached ticks (%)	Total time of tick detaching (min)
Purified water	0	0
Aqueous extract (100%)	100+00	60.813 ± 3.17^{a}
Amitraz	100±00	145.125 ± 15.97^{b}

[ab] Different letters, placed as a superscript, indicate significant differences. One-way ANOVA (P<0.005).

Table 7: Immobilization time of ticks after detachment in domestic dogs, due to the effect of the aqueous extract of *P. anisum* seeds

Treatments	Average immobilization time of the total number of ticks (min)
Purified water	0
Aqueous extract (100%)	14.625 ± 1.36^{a}
Amitraz	44.938 ± 2.38^{b}

[ab] Different letters, placed as a superscript, indicate significant differences. One-way ANOVA (P<0.005).

Morphology of ticks evaluated

Morphological analyses suggest that ticks that were immobilized *in vitro*, as well as those that became detached from domestic dogs when the aqueous extract of *P. anisum* and Amitraz were applied, vary in shape and size of the hypostome, pedipalp, capitulum shape, and color. In all the ticks that were evaluated, four pairs of legs were counted, observing 80 % *R. sanguineus* and 20 % *I. affinis*.

Identification of compounds of the aqueous extract of *P. anisum* seeds

Nine compounds were identified in the aqueous extract of *P. anisum* seeds: p-anisaldehyde (4-methoxybenzaldehyde), butanoic acid, benzyl alcohol, and falcarinol (compounds with antimicrobial activity), phenol (antioxidant property), 2-myristynoyl pantetheine (aromatic compound), paromomycin (antileishmaniasis property), 10-heptadecen-8-ynoic acid, methyl ester, (E)- and *d*-mannose (anti-inflammatory activity) (Table 8).

Table 8: Compounds identified in the aqueous extract of *P. anisum* seeds by gas chromatography coupled to mass spectrometry

N	Compound name	Formula	Area (%)	Reported biological activity
1	4-methoxybenzaldehyde	$C_8H_8O_2$	0.55	Immobilizing and repellent effect[21,22,23] Antifungal activity[28]
2	Butanoic acid	$C_4H_8O_2$	6.31	Antibacterial activity[29]
3	Phenol	C_6H_6O	0.96	Antioxidant property[30]
4	Paromomycin	$C_{23}H_{45}N_5O_{14}$	0.04	*Leishmania amazonensis* treatment[31]
5	2-myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	0.02	Sensory property[32]
6	Benzyl alcohol	$C_8H_{10}O_2$	2.62	It inhibits the reproduction of β-hemolytic *Streptococcus* and *Proteus* spp[33]
7	Falcarinol	$C_{17}H_{24}O$	0.49	Antimycobacterial activity[34]
8	10-heptadecen-8-ynoic acid, methyl ester, (E)-	$C_{18}H_{30}O_2$	0.03	Anti-inflammatory[35]
9	*d*-Mannose	$C_6H_{12}O_6$	0.02	Anti-inflammatory[36]

Discussion

In the evaluation of the aqueous extract of *P. anisum* seeds under *in vitro* conditions, the main effect was observed to be the immobilization of ticks in domestic dogs. The percentage of ticks that were immobilized and the duration of the effect depended on the increase in the concentrations of the aqueous extract of *P. anisum* seeds. The effect of tick detachment and immobilization when *P. anisum* extract concentrate is applied could be due to the compound identified as p-anisaldehyde (4-methoxybenzaldehyde).

Likewise, it was shown that the correlation between the aqueous extract and its effect in this study agrees with published results, Showler and Harlien[21], where they evaluated the activity of p-anisaldehyde powder at 98 % purity of the sigma brand, observing that by increasing the concentration of this product from 0.125 to 2.5 %, the number of immobilized adults of *Haematobia irritans irritans* (L) increased. Showler and Harlien[22,23] reported that p-anisaldehyde has lethal and repellent effects on *Musca domestica*. It has also been shown that the increase in the concentration of p-anisaldehyde powder causes greater mortality of

Amblyomma americanum larvae; nevertheless, the effect it generated was in accordance with the application technique[37]. Considering this background, the present study suggests that the effect of tick immobilization could be due to the presence of p-anisaldehyde in the aqueous extract of *P. anisum* seeds, which the less concentrated it is, the shorter the duration of its immobilization effect.

When the aqueous extract was applied to ticks attached to domestic dogs, it caused them to detach, suggesting that the aqueous extract of *P. anisum* seeds generates immobilization; this effect causes ticks to detach from the skin of domestic dogs, as does Amitraz (positive control in this study). Amitraz is a widely used product for the treatment of ticks in domestic animals; unfortunately, the extensive use of this product has caused certain species of ticks such as *Rhipicephalus microplus* to become resistant[38]; it is also a product that can cause poisoning by inhalation and dermal contact[39].

In this research, it was found that, under the applied conditions, the aqueous extract of *P. anisum* seeds detaches adult ticks from the skin of domestic dogs by 100 %, having similar effects to the commercial product Amitraz. This means that the effect generated by this extract could be related to the concentration used, or the application technique.

Likewise, it was found that the aqueous extract of *P. anisum* seeds formulated with 50 g of seeds per liter of purified water is not toxic to the person who applies it, according to the studies carried out with *A. salina* and with the criteria of Clarkson *et al*[25]; in addition, the aqueous extract of seeds did not cause irritation or redness of the skin of domestic dogs.

According to morphological analyses, ticks with an elongated, brown body, with short hypostome and pedipalp, and a hexagonal shape of their capitulum belong to the species of *R. sanguineus*, which are characteristics that coincide with data published by Lord CC[26]. On the other hand, ticks with a round body, with long hypostome and pedipalp, and a triangular shape of their capitulum and dorsal shield indicate that they belong to the genus *Ixodes* or to the species of *I. affinis*, characteristic data that coincide with specimens of *I. affinis* published by Solís-Hernández *et al*[27].

Although the oily extract has been used in some publications, this does not limit the evaluation of the use of the aqueous extract of *P. anisum* seeds as a sustainable and economical alternative. The purpose of this research was to demonstrate that the aqueous extract of *P. anisum* seeds can also work for the treatment of ticks, in addition to being prepared in a simple manner and at a lower cost than extracting the essential oil from the seeds.

In this work, it was observed that the aqueous extract of *P. anisum* seeds has potential as a commercial use for tick control and is also affordable for domestic consumers. This study is

one of the first to be published in which the aqueous extract of *Pimpinella anisum* seeds is evaluated.

Conclusions and implications

Aqueous extract of *P. anisum* seeds can be a sustainable alternative for the treatment of ticks in domestic dogs; this extract has been shown to have a more effective detaching time than Amitraz, in addition to having an effect of 100 % ectoparasite detaching from the skin of domestic dogs and keeping them immobilized for a certain amount of time, although shorter than Amitraz. It was shown that the number of seeds used per liter of water for the production of the aqueous extract makes it non-toxic. In addition, this extract did not cause irritation in the area of application. It is expected that the results of this research will provide the basis for future research on the aqueous extract made from the seeds of *Pimpinella anisum* and that it will be applied to other pests that afflict living beings.

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Article

Socio-ecological knowledge of the beekeeping activity in the Costa Chica region of Guerrero, Mexico

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Abstract:

Beekeepers need to identify melliferous flora (MF) in those areas where apiaries are established because bees (*Apis mellifera*) depend on these floral resources for food and honey production. The objective of the study was to analyze the socio-ecological aspects of beekeeping, considering the knowledge of the melliferous flora by the producers in the Costa Chica region of Guerrero (GCC), Mexico. A non-probabilistic convenience sampling was

carried out. The final sample consisted of 75 surveyed beekeepers. Descriptive statistics and cross-tabulations were used for data analysis; botanical collections were made to identify the species cited. Beekeeping is traditional (5-50 hives), the average age was 48 yr, with 10 years of schooling and 12 yr of experience. Producers mentioned 33 MF species (26 native and seven cultivated) belonging to 16 botanical families. In addition, they classified them by their use as nectapolliniferous (14 species), polliniferous (10), and nectariferous (9). It was recorded that native species flower during the winter (herbaceous) and spring (trees), coinciding with the honey harvest season, while cultivated species flower during the rainy season (summer) and are an important resource during the post-harvest season. GCC beekeepers registered low knowledge of the vegetation surrounding their apiaries, but have a high knowledge of the main MF species, finding that the older they are, the more knowledge they have about the MF species that bees use in their food (nectar or pollen).

Keywords: Beekeeper, Floral resources, Traditional knowledge, Vegetation.

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Introduction

Beekeeping is an activity that is directly linked to the sustainable management of natural resources, as beekeepers depend on areas with native or introduced flora to install their hives and thus provide bees with food sources (nectar or pollen) for the production of honey[1,2]. This activity is compatible with biodiversity conservation and with the surrounding traditional crops, as they contribute to pollination and food sovereignty[3]. Beekeeping requires little investment and provides an important income for the economic stability of the producers in the rural communities where it is practiced[4].

In Mexico, beekeeping is a livestock activity that influences socioeconomic and ecological aspects because it generates a significant foreign exchange[5]. The country ranks ninth in the world in terms of production volume and eleventh in terms of number of beehives; at the continental level, it ranks third in both areas[6]. Despite being among the main honey producers in the world, Mexico has shown a downward trend in honey volume and hive inventories for the last two decades[7]. This decline in production is due to multiple factors, such as pests and diseases (varroasis, foulbrood, small hive beetle), technical-social issues (lack of training and organization, middlemen, and competition in the international market),

and ecological issues (variations in phenology and floral synchronization)[8]. These obstacles have caused instability in the Mexican beekeeping sector, mainly in the beekeeping regions of the country (North, Central Highlands, Pacific, Gulf, and Yucatan Peninsula)[5].

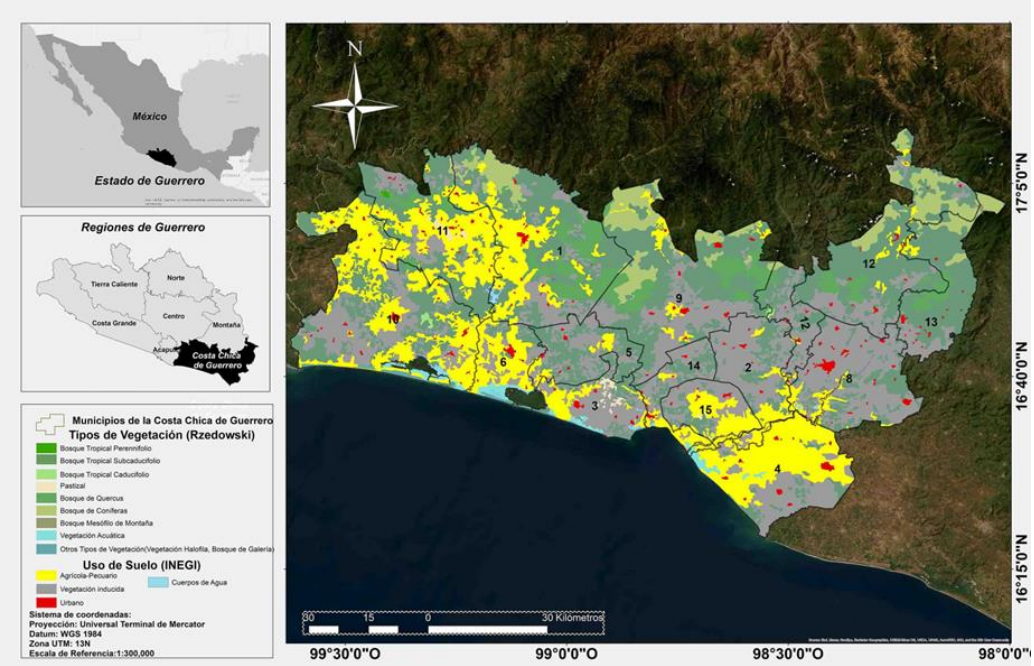
Beekeeping in Guerrero is favored by its geographic location and its diversity of climates and natural resources. The state is located in two beekeeping regions (Central Highlands and Pacific), which results in a high honey-production potential for the seven regions of the state (Acapulco, Costa Chica, Costa Grande, Centro, La Montaña, Norte, and Tierra Caliente)[10]. In 2021, Guerrero ranked as the tenth largest honey producer in the country, with 2,081 t; there has been a decline in this state's production in recent years[7] due to deforestation, land use change, and insecurity, which limits the development of this activity[10]. Costa Chica in Guerrero is the main honey-producing region at the state level, as it has a more extensive coverage of vegetation in low and medium tropical forests, where the supply of floral resources is more constant than in other regions; therefore, it is still necessary to expand the knowledge related to the melliferous flora (MF). This information can be useful to learn about the most important plant species for beekeeping, as well as to maintain established colonies and increase their development[11].

In order to assess the experience and knowledge generated by beekeepers in a specific area in regard to the vegetation and the diversity of MF species, with their flowering periods and their food utility (pollen and nectar) for bees, it is necessary to have the observations of the producers, information that is collected and validated through interviews, questionnaires or field studies, and which allows to know the flora of interest for honey production[12]. For this reason, the objective of the study was to analyze the socio-ecological aspects of the beekeeping activity, based on the knowledge of the MF of the producers of the Costa Chica de Guerrero (GCC), Mexico, in order to have updated information on the panorama of the beekeeping activity in this region.

Material and methods

The GCC region is made up of 15 municipalities: Ayutla de los Libres, Azoyú, Copala, Cuautepec, Cuajinicuilapa, Florencio Villarreal, Igualapa, Juchitán, Marquelia, Ometepec, San Luis Acatlán, San Marcos, Tecoanapa, Tlacoachistlahuaca, and Xochistlahuaca; the GCC is bordered to the north by the La Montaña and Central regions; to the south, by the Pacific Ocean; to the east, by the state of Oaxaca (Costa Chica region of Oaxaca), and to the west, by the Acapulco region[13] (Figure 1).

Figure 1: Municipalities where interviews with beekeepers were conducted in the Costa Chica region of Guerrero, Mexico



1= Ayutla de los Libres, 2= Azoyú, 3= Copala, 4= Cuajinicuilapa, 5= Cuautepec, 6= Florencio Villarreal, 7= Igualapa, 8= Juchitán, 9= Marquelia, 10= Ometepec, 11= San Luis Acatlán, 12= San Marcos, 13= Tecoanapa, 14= Tlacoachistlahuaca, 15= Xochistlahuaca.

The GCC has a predominantly warm-sub-humid climate, with temperatures ranging between 20 and 29 °C and rainfall of 1,100 to 2,200 mm from June to October. The topography varies from hilly terrain, in the municipalities of San Luis Acatlán and Ometepec, to flat or semi-flat, in the municipality of Marquelia. The vegetation is composed of a third of low and medium deciduous forests, and pine and oak forests in the areas near the Montain region[13].

A non-probabilistic convenience sampling[14] was carried out, where individuals were selected for their willingness to provide detailed information on beekeepers' knowledge and perception of MF in the GCC. A questionnaire was designed to collect the information, and a survey was administered during meetings of beekeepers' cooperatives and associations. The final sample consisted of 75 beekeepers surveyed during the period January to December 2021.

The questionnaire consisted of two sections: I) General data on the beekeeper (age, schooling, time in this activity, and main occupation) and on the beekeeping unit (land tenure, transhumance). II) Ecological knowledge of the flora of the region (acquisition of knowledge of the MF, reforestation, types of vegetation in the area surrounding the apiaries, main species close to their apiary and the contribution of nectar and pollen of the MF of the region); in

order to determine the beekeepers' knowledge of the flora, they were asked, of the total (100 %) of the plants in their region, what percentage they consider that they know.

For the purpose of identifying the MF cited by beekeepers, 10 random walks were conducted in low and medium tropical rainforests in the study area, guided by a key beekeeper. Species identification was based on a joint analysis between researchers and beekeepers and documentary research available for the area[9,15,16]. Species that could not be identified in the field were collected according to the described technique[17] and were sent to the María Agustina Batalla Herbarium at the Faculty of Sciences (FCME) of the National Autonomous University of Mexico (Universidad Nacional Autónoma de México, UNAM), for identification.

Data analysis

Descriptive statistics were used for data analysis, and the information was processed using the SPSS statistical software, version 19. Frequency analysis and cross-table analysis[18] were performed to compare the means of the indicators between the two sections of the survey and to determine whether or not there is a relationship between the social variables and the ecological variables. The Chi-square test was used to detect the association of the variable age and knowledge of vegetation, experience, and land tenure with reforestation. Beekeepers were classified into three categories, according to the number of hives they own: 1) traditional, from 10 to 50 hives, 2) semi-technified, from 51 to 200 hives, and 3) technified, > 200 hives.

Results

General characteristics of beekeepers

The average age of the beekeepers was 48 yr, and their average experience in beekeeping was 12 yr (Table 1); their average schooling was 10 yr (Table 2). According to the classification, traditional beekeeping engaged the largest number of beekeepers (58.7 %); semi-technified beekeeping employed 45.3 %, and technified beekeeping, a mere 4 %), while the latter have more experience (17 yr) and age (59 yr).

Table 1: Main socio-demographic characteristics of beekeepers

Type of beekeeper	Experience (years)	Age (years)	Education level			
			Basic	High-school	Higher	Total
Traditional	9 ± 1.02	43 ± 1.1	18	13	7	38
Semitechnified	15 ± 1.44	53 ± 1.31	20	9	5	34
Technified	17 ± 2.01	59 ± 6.1	2	0	1	3
Average	12	48	-	-	-	-
Number of beekeepers	-	-	40	22	13	75
Total %			53.4	29.3	17.3	100

Fifty-six percent of the beekeepers said that their apiaries are located on land that they legally own, and the remaining 44 % indicated that their apiaries are located on borrowed or rented land (Table 2). Technified beekeepers (4 %) have more than 200 hives and need to rent land to establish their apiaries; therefore, most of them practice transhumant beekeeping, which is not very deeply rooted in this region: only 12 % of the beekeepers practice it, generally moving to areas in the "La Montaña" region.

Table 2: Economic activities of beekeepers and apiary ownership

Type of beekeeper	Land tenure		Main activity		
	Rented	Private	Beekeeper	Farmer	Salaried employee
Traditional	19	19	10	11	17
Semitechnified	13	21	6	20	8
Technified	1	2	0	2	1
Number of beekeepers	33	42	16	33	26
Total %	44.0	56.0	21.3	44.0	34.7

With respect to the main activity of the beekeepers, it was observed that 21.3 % are exclusively dedicated to beekeeping, which means that beekeeping is a complementary activity to other agricultural and livestock farming activities. However, technified beekeepers were considered entrepreneurs, as they add value to beekeeping and diversify their economic activities.

Melliferous flora

Beekeepers identified 31 MF species, composed of 16 botanical families, of which Fabaceae had the highest number of species[14], followed by Boraginaceae and Malpighiaceae, with two, respectively, while the other 13 families had only one species; two species could not be identified (Table 3).

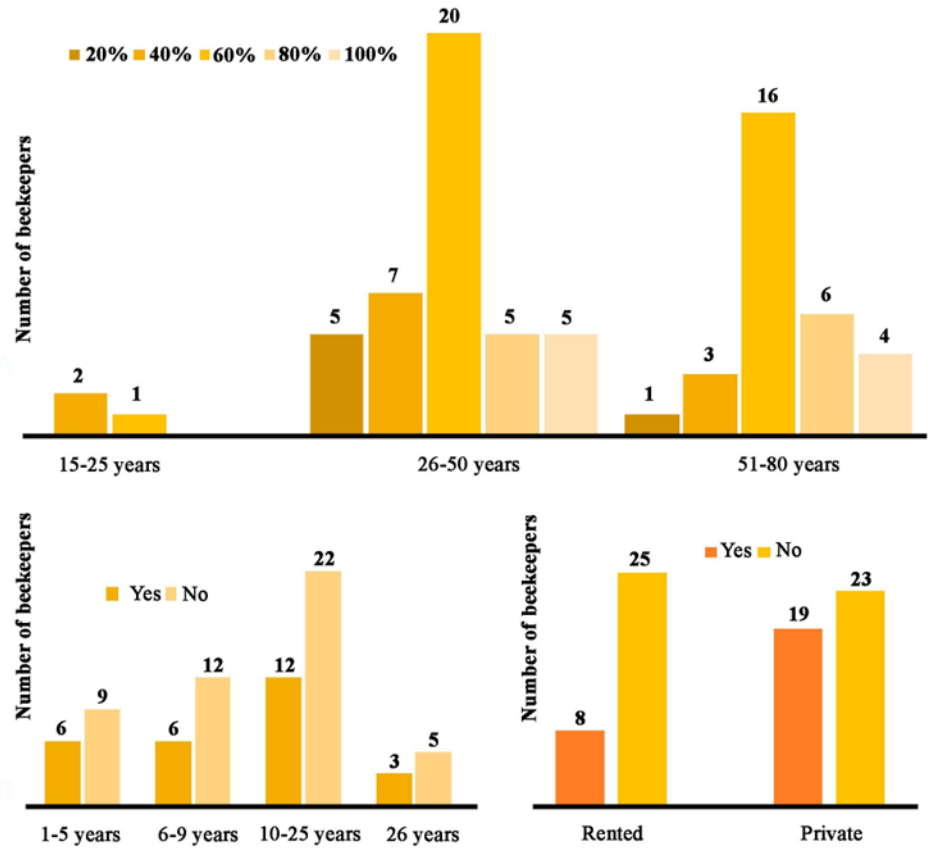
According to the knowledge of beekeepers, 14 species are considered nectariferous, 11 produce nectar and pollen, and 8, pollen; of all these species, 26 are wild and 6 are cultivated, the most prominent being *Mangifera indica* L., *Citrus* × *aurantiaca* (L.) Swingle and *Cocos nucifera* L., as they are widely cultivated in the region (Figure 2). Although sesame (*Sesamum indicum* L) is another important crop in the region, only two beekeepers mentioned it.

Figure 2: Vegetation and melliferous flora

A) Secondary vegetation, Vista Hermosa, Ometepec. B) *Ceiba pentandra* (L.) Gaertn. C) *Enterolobium cyclocarpum* (Jacq.) Griseb. D) *Pithecellobium unguis-cati* (L.) Benth. E) *Bauhinia pauletia* Pers. F) Vista panorámica, Selva mediana subcaducifolia. G) *Senna mollissima* (Humb. & Bonpl. ex Willd.) H.S. Irwin & Barneby.

In order to know the perception of the MF of the region, cross tabulations were generated with age and knowledge of the vegetation, finding that 24 % of the beekeepers identify or know 40 % of the vegetation, and 12 % of the beekeepers know between 80 % and 100 % of the vegetation (Figure 3). Knowledge of the flora increases with age; beekeepers in the 26-50 age group know 60 % of the region's flora, while the 15-25 age group knows only 30 % of the flora.

Figure 3: Cross-tablulation. **A.** Between age and knowledge of the flora of the region. **B.** Experience and reforestation. **C.** Land tenure and reforestation



* The Chi-square test showed that there was no correlation (P>0.05) between these three cross-tabulations

As for the beekeepers' years of experience and practice on reforestation, 40 % were shown to have 1 to 5 yr of experience, while only 33 % had between 6 and 9 yr of experience in reforesting. Likewise, it was observed that 45 % of the beekeepers with legal ownership of the land reforested, and 76 % who rented land did not do so.

Discussion

According to the historical national average number of hives per beekeeper[19], beekeeping in Mexico can be classified as traditional (1-50), semi-technified (51-200), or technified (more than 201).

The average age of beekeepers in the region was 48 yr, with an interval of 23 to 75 yr, similar to that recorded in other entities such as Yucatán[20]; the central-southern region of Jalisco[21], with 49 yr, and Campeche[22], with 57 yr. Consequently, the average age of beekeepers in the study region suggests that a generational changeover is occurring in the beekeeping activity; this is an advantage because older and more experienced beekeepers are less willing to change their traditional forms of production and to learn new techniques, compared to younger beekeepers[23].

The experience of beekeepers at the national level varies between 21 and 23 yr; these data are similar to those reported in the State of Campeche[20], where a value of 21 yr in the activity was reported; an average of 22 yr of experience was found in the Sierra Centro-Norte region of Veracruz[24], while a value identical to that of this study, of 23 yr of experience, was found in the south-central region of Jalisco[23]. The similarity of these results indicates that, at the national level, beekeepers have acquired knowledge, skills, and competencies for the practice of this activity.

The average schooling of the surveyed beekeepers was 10 yr, which is equivalent to the first year of high school, similarly to the average schooling of beekeepers in Jalisco[21], of 9 yr, but higher than the average schooling (5 yr) registered in Yucatán[25]. The low level of education is one of the main factors why field records or logs are not kept, a fact that limits the possibility of managing information, maintaining traditional practices, and applying new technologies[21].

Land ownership conditions are very different in the study region, since 44 % of the beekeepers rent the land where the apiary is established —compared, for example, to the state of Yucatán[22], where 74 % are privately owned and only 26 % are on rented land. This reflects regional and intergenerational contrasts in social land ownership and is related to the changes brought about by the 1992 agrarian reform, which encouraged the fragmentation of communal lands and led to disruptions among deeply rooted indigenous and peasant cultures[26]. This phenomenon of forest fragmentation forces beekeepers to move their hives to places with preserved vegetation in search of suitable species.

In this regard, transhumant beekeeping was carried out by only 12 % of beekeepers in the GCC. These results are similar to those recorded in the Central and Northern Region of Veracruz, where beekeepers with more than 150 hives (20 %) are the ones who practice transhumance[24]. On the other hand, the inadequate location of the apiary causes a lower honey yield per hive; therefore, transhumance implies maximizing the productive efficiency in function of the MF density, reducing the bee's foraging route and counteracting the investment of economic resources[27].

Beekeeping in the GCC is a complementary activity for traditional and semi-technified beekeepers. In the state of Yucatán, beekeeping is the main economic activity for 19 % of beekeepers; the percentage rises to 25 % if the apiary has between 50 and 100 hives[25]. The greater the number of apiaries, the more beekeepers perceive beekeeping as their main economic activity.

Community participation is a way to obtain reliable and useful results to solve issues and improve situations or the collective knowledge of their region[28]. This knowledge of the region's flora, especially that which has melliferous potential, serves as a tool for the beekeepers themselves, allowing them to better manage their apiaries, decide when to supplement the bees' nutrition or change their apiaries to places with adequate MF for the bees to forage for pollen and nectar, which contributes to the production of quality honey[1].

Mention by the surveyed beekeepers of certain species that are not important for the beekeeping activity (*Tamarindus indica* L., *Ehretia tinifolia* L., and *Persea americana* Mill.) confirms that beekeepers' perceptions are biased in favor of culturally influenced landscape species, both cultivated and wild[29]. The 72 % of beekeepers are familiar with 80 % of the vegetation in their surroundings; such familiarity with these landscapes makes it difficult to identify other types of species present in the vegetation of the forests.

Beekeepers in the municipality of Hopelchén, Campeche, recorded 50 species, three subspecies, and three varieties of MF, distributed in 26 botanical families[12] —a higher number than that found in this study, where 33 species of MF were recorded.

In regions with a strong change in land use and where agricultural landscapes predominate, the remnants of natural vegetation are mainly dominated by tree species that become important for beekeeping[30]. Similar values to those of this study were registered in a tropical dry forest in Ecuador[31], where 28 MF species were identified by the beekeepers. However, these questions addressed only those species they consider important for the bees, and not all vegetation in general. Similarly, another study carried out in Nicaragua[32] identified 89 species, but without specifying whether or not all of them correspond to MF.

Also, of the 33 species recorded in this study, seven are cultivated; however, the flowering period for bees is concentrated from November to May, while during the rest of the year, according to certain authors, nectar and pollen resources are harvested from monoculture plots[33]. In the study region, plantations such as sesame, citrus, hibiscus, coconut, mango, etc., play an important role as floral resources for beekeeping, due to their established surface area and to flowering periods that are not simultaneous with those of the wild vegetation. A noteworthy fact is that the beekeepers did not mention any introduced grass species, despite the fact that these are abundant in the region's tropical dry forests. This is because beekeepers associate grasslands with pollen production and do not consider them as an important floral resource for bees. However, in other parts of the Caribbean, such as the Dominican Republic[29], beekeepers have identified such invasive species as *Leucaena leucocephala* (Lam.) de Wit, *Syzygium jambos* (L.) Alston and *Prosopis juliflora* (Sw.) DC. as plants of beekeeping interest.

Finally, when asked about the level of knowledge of the flora around the apiaries, only 12 % of the beekeepers considered that they knew between 80 % and 100 % of the MF, a lower value than that registered in Campeche[12], where they found that 60 % are familiar with the vegetation of their apiaries as a result of the transmission of knowledge through generations.

Conclusions and implications

GCC beekeepers find it easier to adopt new technologies and diversify hive products due to their average age, which is lower than the national average. Transhumant beekeeping is carried out by technified beekeepers. Younger people have little knowledge of the environment and MF, but identify specific flora that provide nectar or pollen for bees, acknowledge that agricultural crops are important for bee activity, and recognize the importance of the environment for bee activity. On the other hand, the tenure of the land where the apiaries are located influences their reforestation, and because a high percentage of the land is rented, this practice is not carried out. Beekeepers' perception of the resources utilized by bees is an important source of knowledge about the flora of beekeeping interest, which increases with age and is an invaluable source of information.

Acknowledgments

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beekeeping in the Costa Chica region of Guerrero), within the framework of the "2022 Call for Proposals on Basic Science or Frontier Science, Modality: Paradigms and Controversies of Science".

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Table 3: Species cited by beekeepers in the Costa Chica region of Guerrero

Family	Taxa	FR	QFR	SC	F	M	A	M	J	J	A	S	O	N	D	RSDH	NMSB
Anacardiaceae	*Mangifera indica* L.	P	Re	X	X	-	-	-	-	-	-	-	-	-	-	---	17
Arecaceae	*Cocos nucifera* L.	N-P	Re	-	-	-	-	-	-	X	X	X	X	X	-	---	2
Bignoniaceae	*Tabebuia rosea* (Bertol.) DC.	N-P	Ab	-	-	X	X	-	-	-	-	-	-	-	-	L. Alaniz *et al.* 1008 (FCME)	3
Boraginaceae	*Cordia dentata* Poir.	N-P	Re	X	X	X	-	-	-	-	-	-	-	-	-	L. Alaniz *et al.* 289 (FCME)	4
Combretaceae	*Combretum fruticosum* (Loefl.) Stuntz	N-P	Re	-	-	X	X	X	-	-	-	-	-	-	-	L. Alaniz *et al.* 715 (FCME)	5
Convolvulaceae	*Ipomoea trifida* (Kunth) G. Don	N	Ab	X	-	-	-	-	-	-	-	-	-	-	X	L. Alaniz *et al.* 611 (FCME)	30
Dilleniaceae	*Curatella americana* L.	N	Ab	X	-	-	-	-	-	-	-	-	-	-	X	L. Alaniz *et al.* 805 (FCME)	11
Fabaceae	*Andira inermis* (W. Wright) Kunth ex DC.	N	Ab	-	X	X	X	-	-	-	-	-	-	-	-	L. Alaniz *et al.* 1000 (FCME)	36
Fabaceae	*Enterolobium cyclocarpum* (Jacq.) Griseb.	N	Ab	-	-	-	X	X	-	-	-	-	-	-	-	L. Alaniz *et al.* 1002 (FCME)	2
Fabaceae	*Gliricidia sepium* (Jacq.) Kunth ex Walp.	N	Ab	X	X	-	-	-	-	-	-	-	-	-	X	L. Alaniz *et al.* 1003 (FCME)	29
Fabaceae	*Hymenaea courbaril* L.	N	Ab	-	-	X	X	X	-	-	-	-	-	-	-	L. Alaniz *et al.* 1004 (FCME)	46
Fabaceae	*Pterocarpus orbiculatus* DC.	N-P	Ab	X	-	-	-	-	-	-	-	-	-	-	X	L. Alaniz *et al.* 771 (FCME)	6
Fabaceae	*Bauhinia pauletia* Pers.	P	Re	-	-	-	X	X	X	-	-	-	-	-	-	L. Alaniz *et al.* 730 (FCME)	3

Fabaceae	*Senna mollissima* (Humb. & Bonpl. ex Willd.) H.S. Irwin & Barneby.	P	Re	X	X	-	-	-	-	-	-	-	-	-	-	L. Alaniz *et al.* 538 (FCME)	3
Fabaceae	*Tamarindus indica* L.	P	Re	-	-	-	X	X	-	-	-	-	-	-	-	---	2
Fabaceae	*Vachellia farnesiana* (L.) Wight & Arn.	P	Re	-	-	-	X	X	-	-	-	-	-	-	-	L. Alaniz *et al.* 547 (FCME)	2
Lauraceae	*Persea americana* Mill.	N	Ab	-	-	-	-	X	X	X	-	-	-	-	-	---	2
Malpighiaceae	*Byrsonima crassifolia* (L.) Kunth	N	Ab	-	-	-	X	X	X	-	-	-	-	-	-	L. Alaniz *et al.* 1001 (FCME)	15
Malpighiaceae	*Malpighia ovata* Rose	N-P	Ab	-	-	-	-	X	X	X	-	-	-	-	-	L. Alaniz *et al.* 1007 (FCME)	10
ND	*Gusanillo* (common name)	N	Ab	X	X	-	-	-	-	-	-	-	-	-	-	---	3
ND	*Tanalocote* (common name)	N	Ab	-	X	X	-	-	-	-	-	-	-	-	-	---	7
Pedaliaceae	*Sesamum indicum* L.	N	Ab	-	-	-	-	-	-	X	X	-	-	-	-	---	2
Polygonaceae	*Coccoloba barbadensis* Jacq.	N	Ab	-	X	X	-	X	X	-	-	-	-	-	-	L. Alaniz *et al.* 520 (FCME)	21
Rutaceae	*Citrus* × *aurantiaca* (L.) Swingle	N	Ab	-	-	-	-	-	-	X	X	X	X	-	-	---	7

FR= Floral resource: N= nectar, P= pollen, N-P= nectar-pollen. QFR= quantity of the floral resource: AB= abundant, SC= scarce, RE= regular. Floral calendar with months of the year (January-December). RSDH= representative specimen deposited in the FCME herbarium (based on Alaniz *et al.*, collection number). NMSB= number of mentions of the species by beekeepers.

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Article

Prevalence of *Fasciola hepatica* and *Calicophoron* spp. in extensively reared cattle in the Florida district (Amazonas), Peru

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Abstract:

The present study determines the prevalence of eggs of *Fasciola hepatica* and *Calicophoron* spp. and of mixed infection in grazing cattle from six cattle ranches in the district of Florida, Department of Amazonas (Peru). Using the natural sedimentation technique, 358 fecal samples were examined. The prevalence of *F. hepatica* was 69.83 % (95% CI 65.08 - 74.59), followed by *Calicophoron* spp. 60.34 % (95% CI 55.27 - 65.40) and a prevalence of mixed infection 41.62 % (95% CI 36.51 - 46.73). The presence of *F. hepatica* eggs did not differ among farms, breeds, and age groups ($P>0.05$). The presence of *Calicophoron* spp. and mixed infection with *F. hepatica* showed differences between towns and breeds ($P<0.05$), unlike the age groups, which were statistically similar ($P>0.05$). A high prevalence of fecal eggs of *F. hepatica* and spp. was found, a situation that could be due to the environmental conditions that allow the optimal development of the intermediate host and the cattle grazing system.

Keywords: Prevalence, Coprology, Extensive breeding, Liver fluke, Rumen fluke.

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Introduction

Parasitic infections are considered one of the most frequent and important health issues in grazing animals. Parasites are an obstacle to profitable livestock farming, causing reduced production and economic losses due to the costs of control, treatment, and mortality[1,2].

Fasciolosis is a disease of veterinary and public health importance that develops from the ingestion of *Fasciola hepatica* metacercariae in feed or drinking water[3,4]. The parasite is located in the bile ducts and gallbladder, causes severe traumatic hepatitis during the migratory and biliary stages, and can lead to loss of liver function as a result of damage to liver parenchyma and bile ducts triggering liver fibrosis[5,6]. In various regions worldwide, it is considered a reemerging disease and a growing threat, mainly due to the rapid evolution of human activities[7-9].

On the other hand, paramphistomosis, a disease caused by rumen trematodes of the Paramphistomidae family, has been associated with significant morbidity and severe

pathological disorders such as enteritis and anemia, caused especially by the activity of juvenile trematodes in the intestine of the definitive host, the ruminant[10,11]. In acute infections, the immature forms can cause the death of the animal[12]. Adult parasites cause rumenitis, acute catarrhal diarrhea, hemorrhage, detachment of rumen papillae, and fibrosis, as well as the occurrence of areas with reticulum acanthosis, edema, ulceration, etc.[13-15]. As in the case of *F. hepatica*, ruminants become infected by ingesting metacercariae encysted in forage or in water[16].

Both parasitizes are distributed across the world, mainly in tropical and subtropical regions[17,18]. Because they share the same intermediate host (snails of the family *Lymnaeidae*), co-infections are possible in both the intermediate host and the definitive host[19]. The presence of these parasites is exacerbated under favorable conditions such as wet soils, high rainfall, extensive farming systems, and fresh water bodies that host snails[20,21]. On the other hand, they cause great negative economic impact on the livestock industry, affecting growth rate, feed conversion efficiency, reproductive performance, carcasses in poor condition, animals experience reductions in milk production and quality[22-25].

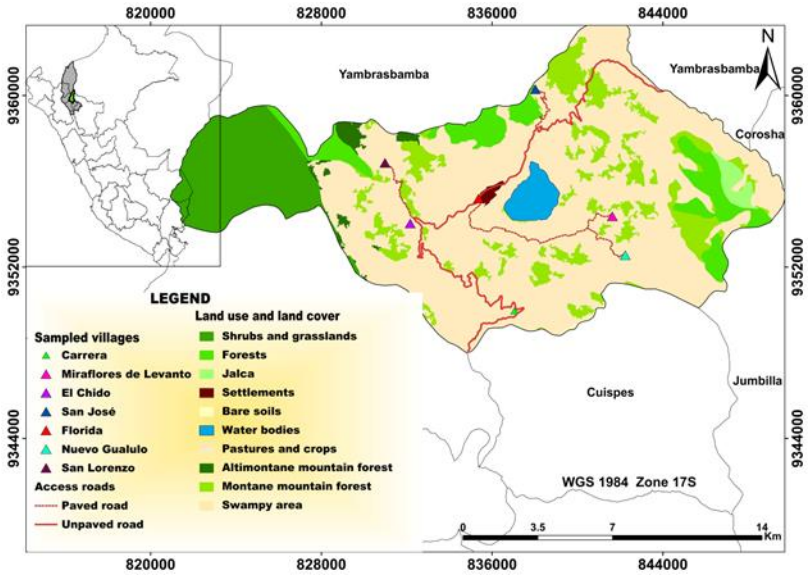
The lack of knowledge about the proper control of animal health issues and the low level of education of farmers, particularly in small production systems, could partly explain the high prevalence of bovine fasciolosis in certain scenarios[26]. Given the frequent reports of rumen and liver parasites found in cattle slaughtered for human consumption, the importance for public health and the high economic costs involved in pharmacological treatments, the present study determines the prevalence of *F. hepatica* and *Calicophoron* spp. in grazing cattle in six annexes of the Florida district of the Department of Amazonas (in Peru). In this way, it was seek to understand and achieve a more accurate picture of the presence of both trematodes in cattle in the study area, with the consequent adoption of preventive and prophylactic measures.

Material and methods

Study area

The study covered six villages in the district of Florida (Figure 1), located in the province of Bongará, Department of Amazonas, in the northeastern Peruvian Amazon. The study area has a humid tropical climate with frequent rainfall throughout the year and an average annual temperature of 16 °C.

Figure 1: Location map of the sectors in the study area. The towns are located at an altitude ranging between 2,280 and 2,750 m asl and have a relative humidity of 70 to 95 %



Animal selection and feces sampling

The sample size (n= 358) was estimated based on a population of 5,200 cattle (previous census), an expected proportion of 0.5, a 95 % confidence level, and a precision level of 5%. A stratified sampling with allocation proportional to the number of cattle determined the number of samples to be considered for each sector. Female cattle over 2 yr of age and of any breed were considered. Identification and age were taken from the ear tags. The animals were raised in extensive rearing systems, fed rye grass (*Lolium multiflorum*), clover (*Trifolium repens*), Kikuyu (*Pennisetum clandestinum*), and other native grasses (Figure 2).

Figure 2: Evaluated Brown Swiss cattle raised in open fields and fed green forage

Fecal samples (approximately 100 g) were collected directly from the rectum of the animals using sterile obstetric gloves. Each animal was restrained by the owner with the help of a rope, trying to cause the less pain as possible, with hands covered with latex gloves and the perianal region was washed with soap and water. The samples were transported to the Immunology Laboratory of the Faculty of Veterinary Sciences, National University of Cajamarca (Universidad Nacional de Cajamarca) in an expanded polystyrene box with cooling gels (2 to 4 °C). The transfer time lasted between 8 and 10 h. In the laboratory, they were kept refrigerated at 4 °C until processing after 24 h. Clean and labeled materials were used to avoid cross-contamination.

Analysis of the samples

Samples were processed by natural sedimentation[27]. Eggs were observed under a stereoscope with halogenated light at 5X (Nikon SMZ 745 - USA), and identification was based on the morphological characteristics of the egg of each parasite[28-31].

Cattle breeders' attitude towards parasites in cattle

According to the observations made during the fecal sample collection process, it was found that farmers in the evaluated areas lacked knowledge about mechanisms for the prevention

and control of trematodes in their animals. No control or prevention measures were identified, such as the proper management of excreta, the management of drinking troughs, the implementation of drainage systems on farms, the adoption of technified irrigation practices, or the implementation of strategies aimed at controlling the intermediate host, among others. In addition, no consistent information was obtained regarding the existence of deworming programs; in fact, farmers were unaware of the presence of rumen trematodes in their animals.

According to cattle ranchers, they sometimes perform deworming with albendazole-based chemicals to control *F. hepatica*, known locally as Fasciola, Liver fluke, *Alicuya*, Lunguash, Liver slug, Dystoma, and Coca Leaf. This process was carried out during the rainy season (December to April) or when the animals presented persistent diarrhea or showed signs of decay, without the supervision of a livestock professional, and in the absence of a parasitological laboratory diagnosis by observation of fecal eggs or by any other method.

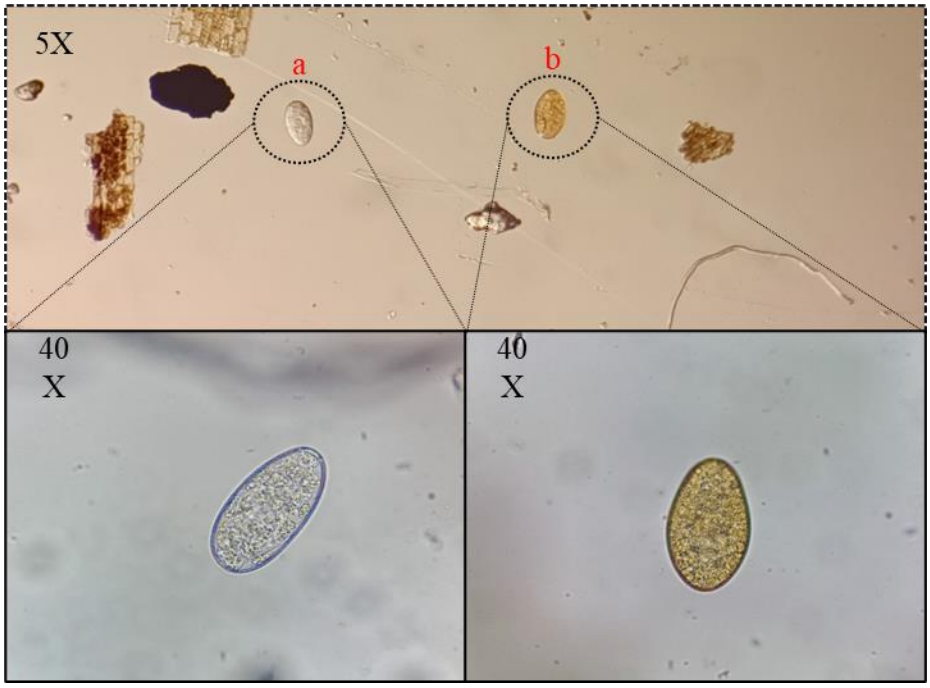
Statistical analysis

Data were processed using descriptive statistics. Positive cases were expressed as percentages with 95% confidence intervals[32]. The degree of association between prevalence by sector, race, and age group was determined using the nonparametric Kruskal-Wallis test. The Phi correlation function was used to determine the degree of the linear relationship between the mixed prevalence of trematodes.

Results

Brown Swiss and Fleckvieh cows raised on pasture, ranging from 2 to more than 6 yr of age, were found in the six farms of the Florida district. Eggs of *Fasciola hepatica* and *Calicophoron* spp. were observed in all sectors (Figure 3). *F. hepatica* eggs were observed in 69.83 % (95% CI: 65.08-74.59) of the animals, followed by *Calicophoron* spp. 60.34 % (95% CI: 55.27-65.40) and a prevalence of mixed infection of 41.62 % (95% CI: 36.51-46.73) (Table 1).

Figure 3: *Calicophoron* spp. eggs (a) and *Fasciola hepatica* (b). Stereomicroscopic view (top) and microscopic view (bottom)



Discussion

The trematode *Fasciola hepatica* outnumbered *Calicophoron* spp. by less than a tenth (9.12 %), with an overall mixed infection prevalence of 41.16 % (95% CI 36.09 - 46.23). The presence of *F. hepatica* eggs did not differ between sectors, breeds, or age groups ($P>0.05$). The prevalence of *Calicophoron* spp. and mixed infection with *F. hepatica* showed differences between sectors and breeds ($P<0.05$), unlike the age groups, among which they were statistically similar ($P>0.05$). The Phi correlation showed variable results of the associated occurrence of both parasites in the animals.

The high presence of parasite eggs may be due to the good development of the intermediate host in optimal environmental conditions, humid areas, and a variety of temperatures and altitudinal levels, for example. The locations evaluated range from 11 to 20 ºC and have a relative humidity of 60 to 95 %. Since both trematodes share the same intermediate host, freshwater pulmonate mollusks of the family *Lymnaeidae*[33,34], this condition would facilitate co-infection in both the intermediate and definitive host.

The areas where the sampling was carried out are located between 1,300 and 2,750 m asl, ideal ranges for the development of parasitoses. Parasitic forms of *F. hepatica* have been

reported even in intermediate hosts below 400 m asl[35] and up to 4,500 m[36]. *Calicophoron microbothrioides* can be found below 200 m and also in mountainous areas above 3,000 m, where there is stagnant water available for the cycle of the intermediate hosts, in areas used for livestock farming, and in the areas without water for the cycle of the intermediate hosts[37].

Climate influences the rate of parasitic infection in livestock[38,39]. As shown in Figure 1, the study area includes large vegetation and bodies of water, —favorable conditions for the development of the intermediate host. In general, the province has a humid tropical climate with frequent rainfall throughout the year and an average annual temperature of 16 °C. In an area close to the study area, researchers found that water sources, mainly streams, irrigation ditches and rivers, are risk factors for *F. hepatica*[40].

The breed of cattle has been reported as a risk factor in several studies. Purebreds are more susceptible to infection than crossbreds[9]. In a study conducted in Amazonas (Peru), it was determined that the Brown Swiss breed is more susceptible to infection by *F. hepatica* and other parasites[40]. However, it should be noted that, in their study, the sample size for this breed was larger than for other breeds. In the present research, a higher prevalence of trematodes was found in the Fleckvieh breed. Despite an evident higher number of Brown Swiss (n= 203) *versus* Fleckvieh (n= 155) cattle, the prevalence was statistically equal; therefore, the results do not consolidate the breed as a risk factor.

Similarly, another study reported that the Simmental breed was a risk factor for *F. hepatica* infection compared to Brown Swiss and other breeds[41]. Although the sample size of the Simmental breed was larger (as in the present study), the results were statistically similar to those of Brown Swiss, only differing with the Jersey, Holstein and crossbred breeds, although the sample size of these breeds was very low. Similar to the present study, several authors have not reported conclusive results in which breed is a risk factor, but rather that the presence of parasites is influenced by a higher population of a certain race in a certain place[14,42,43].

The age of the definitive host is also closely related to infection[15]. As in other reports, the presence of parasites was higher in older animals. Most authors show that the prevalence of trematodes is higher in animals older than 2.5 yr[21,43,44]. No association has been found between age and rumen trematode infection[14]. Infected animals have an age limit for becoming infected, since the life cycle of Paramphistomidae lasts at least 6 to 8 mo[9]; therefore, animals 12 to 24 mo of age may be at higher risk of infection. In addition, the animals are raised under grazing conditions from birth until they leave the herd.

Several studies have indicated that extensive animal husbandry is a factor in parasite infection[40,43]. Cattle managed in extensive or semi-extensive regimes where access to

pasture occurs almost year-round throughout the animal's life are more predisposed to grazing than those managed in an intensive or semi-intensive system[45].

The high prevalence of *F. hepatica* in cattle has been reported within the same Amazonas region, with a prevalence of 45.6 %[40], and 59.5 %[41]. The presence of trematodes in cattle has also been described in other regions of Peru. In three districts of the province of Oxapampa (Pasco - Peru), by rapid sedimentation of 408 samples of dairy cattle, a prevalence of 10.0 ± 2.9 % of *F. hepatica* and 28.4 ± 4.4 % of a digenean of the Paramphistomidae family were found[46].

Although studies on rumen trematodes in cattle in Peru are scarce, *C. microbothrioides* has been reported in Amazonas[31]. Trematode eggs of the Paramphistomidae family were identified in the Loreto region (Peruvian jungle)[47]. In San Martin (Peruvian jungle region) *Cotylophoron* sp. has been reported in cattle[48]. Both parasites have been identified in different parts of the world. In South America, *Cotylophoron cotylophorum* has been described in Colombia[49], *Cotylophoron marajoensis* n. sp. in Brazil[49] and *C. microbothrioides* in Chile[37]. As well as in the Americas, the presence of both trematodes has been reported in European[50,51,52], African[34,26,38], Asiatic countries[53], etc. These regions have similar conditions to those of the present study —extensive breeding, climatic conditions, age, breed, etc.—, as risk factors. Climate change and globalization contribute to the distribution of parasites in a territory where the intermediate host has adapted[19].

Due to the high prevalence of trematode eggs identified in the sampled areas, it is suggested that this situation may be attributed to the conditions in which cattle are raised, where no formal programs for the control and prevention of parasitoses are available. Despite the possible use of albendazole for the management of *F. hepatica*, no local studies of chemical-based antiparasitic efficacy have been reported.

Conclusions and implications

A high prevalence of fecal eggs of *Fasciola hepatica* and *Calicophoron* spp. was detected. Climatic and geographic conditions, in addition to the grazing system and the absence of control and prevention programs, predispose to the high presence of both trematodes. However, further studies are needed to evaluate drainage systems, pasture, and watering trough management practices in the control and prevention of trematodes, as well as evaluations of antiparasitic resistance and comprehensive studies from a One Health approach.

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Conflict of interest

The authors declare that they have no conflict of interest that could have interfered with the results of this research.

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Table 1: Prevalence (%) of trematodes found in grazing cattle in the district of Florida, Amazon (Peru)

Variable	Category	No	*Fasciola hepatica*		*Calicophoron* spp.		Association		Phi Coefficient
			Positive	% (95% CI)	Positive	% (95% CI)	Positive	% (95% CI)	
Hamlet	El Chido	41	26	63.41 (48.67 - 78.16)[a]	19	46.34 (31.08 - 61.61)[a]	11	26.83 (13.27 - 40.39)[a]	-0.106
	Florida	145	96	66.21 (58.51 - 73.91)[a]	100	68.97 (61.44 - 76.50)[b]	67	46.21 (38.09 - 54.32)[ab]	0.025
	Miraflores de Levanto	41	30	73.17 (59.61 - 86.73)[a]	27	65.85 (51.34 - 80.37)[ab]	18	43.90 (28.71 - 59.09)[ab]	-0.204
	Nuevo Gualulo	58	45	77.59 (66.85 - 88.32)[a]	42	72.41 (60.91 - 83.92)[b]	32	55.17 (42.37 - 67.97)[b]	-0.054
	San José	15	13	86.67 (69.46 - 100)[a]	5	33.33 (9.48 - 57.19)[a]	5	33.33 (9.48 - 57.19)[ab]	0.277
	San Lorenzo	58	40	68.97 (57.06 - 80.87)[a]	23	39.66 (27.07 - 52.24)[a]	16	27.59 (16.08 - 39.09)[a]	0.011
	P value			*0.347*		*<0.001*		*0.013*	
Race	Brown Swiss	203	141	69.46 (63.12 - 75.79)[a]	142	69.95 (63.64 - 76.26)[b]	99	48.77 (41.89 - 55.64)[b]	0.009
	Fleckvieh	155	109	70.32 (63.13 - 77.51)[a]	74	47.74 (39.88 - 55.61)[a]	50	32.26 (24.90 - 39.62)[a]	-0.058
	P value			*0.860*		*<0.001*		*0.002*	
Age group	2 - 4 years	244	167	68.44 (62.61 - 74.27)[a]	148	60.66 (54.53 - 66.79)[a]	101	41.39 (35.21 - 47.57)[a]	0.007
	> 4 - 6 years	72	52	72.22 (61.88 - 82.57)[a]	41	56.94 (45.51 - 68.38)[a]	27	37.50 (26.32 - 48.68)[a]	-0.073
	> 6 years	42	31	73.81 (60.51 - 87.11)[a]	27	64.29 (49.79 - 78.78)[a]	21	50.00 (34.88 - 65.12)[a]	-0.137
	P value			*0.693*		*0.192*		*0.424*	
	Total	358	250	69.83% (65.08 - 74.59)	216	60.34 (55.27 - 65.40)	149	41.62 (36.51 - 46.73)	

[ab] For each variable, the different letters between their levels are significant differences in each factor (Kruskall-Wallis, $P<0.05$).

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Article

Influence of feedlot living space on production variables, carcass and meat quality traits in Holstein steers

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Abstract:

A determination of how the amount of allotted feedlot living space influences both production indicators as well as carcass and meat quality traits obtained from Holstein steers was performed by forming two treatment groups, T14: 65 steers/pen (14 m^2/head of space allowances) and T16: 57 steers/pen (16 m^2/head of space allowances), with five replications each treatment. The average arrival weight 238 ± 0.74 kg. During the fattening period the cattle was feed twice a day with commercial diets. The steers were slaughtered after a 261-d period. At the moment of the first reimplant a greater average body weight was found in T16 *vs* T14 (384.25 *vs* 378.38 kg; $P<0.05$) and the difference

continued until day 261 (612.35 *vs* 595.54 kg; *P*<0.05); regarding ADG, hot carcass weight and cold carcass weight the result were: 1.50 *vs* 1.46 kg (*P*<0.05), of ADG kg/d; 367.34 *vs* 360.35 kg (*P*<0.05) and 366.68 *vs* 358.78 kg (*P*<0.05). No difference between treatments were found in dorsal fat, marbling, pH and meat color. The results suggest that an increase from 14 m^2/animal to 16 m^2/animal improves the production results as well as the hot and cold carcass weight, with no effect on the quality traits of the carcass and beef.

Keywords: Living space, Holstein steers, Feedlot, Carcasses, Meat quality.

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Introduction

During their stay in the pen beef cattle require enough space to express its natural behavior[1]. According to Lagos *et al*[2] it is necessary to provide at least 18.5 m^2/head to ensure the ideal conditions of space for each animal however in case that during the fattening period increases it is recommended that additional space is provided based on the increase in body weight, for cattle with a weight up to 300 kg, the recommended space is 15 m^2/head, for cattle with weights higher than 400 kg a 20 m^2 area is suggested. In Mexico, the manual of good practices for intensive beef cattle production published by the Agriculture Secretary (SAGARPA)[3] estimates that a space between 12 and 12.5 m^2/animal is enough for cattle to display its natural behavior.

Holstein calves have become an important input for feedlot beef production[4], so that it accounts for 20 % of the total amount of cattle fatten in the United States of America[5], a similar situation is now being observed in northern Mexico. Holstein steers offer certain advantages since show desirable carcass traits like a superior distribution of intramuscular fat and better dorsal fat width[6]. It has been reported that adult Holstein cattle fatten in feedlots exhibit an unpredictable and aggressive behavior[7], and for this reason this race of cattle requires a larger amount of space than the beef producing races. Another fact to take into consideration is that Holstein cattle more and more often so that the ground condition in the pens is not good[8,9]. Taking into consideration what has been above stated an increase in the feedlot vital space per animal would have a positive impact the cattle's welfare and thus better beef production results[10].

The objective of this study was to evaluate the effect that pen space had on the production variables, as well as on the quality traits of carcass and meat obtained from Holstein steers.

Material and methods

This study was review and approved by Veterinary Sciences Research Institute ethics committee, with the project number 201/2399.

Geographical location

This study was carried out in Mexicali, Mexico, which is found at 32° 32´00 N, 115° 12’41 W. The region is characterized by a dry desert climate with an average temperature of 34.7 °C (-5 °C winter and 50 °C summer), with an annual rainfall of 37 mm, and a relative humidity above 50 %[11].

Animals and design of the study

The study was performed using castrated Holstein calves between the ages of 7 and 8 mo, with an average weight of 238 ± 0.74 kg. Twenty four hours after the cattle arrived to the feedlot they were vaccinated, dewormed and implanted with a product that contained trembolone acetate, estradiol and tilosine. On arrival during spring (April-June) the animals were assigned to one of two groups so that two treatments may be established. Each treatment included five pens. The first treatment included 65 Holstein steers, in this case each animal had a space allowance of 14 m^2/per animal (T14), in the second treatment a 16 m^2/animal (T16) was allocated to each of 57 Holstein steers. The cattle were fed twice a day using a feeding program that included three different diets given during the fattening and finalization periods. In different proportions the ingredients of all diets were: sudangrass, wheat hay, tallow, dried distillers grains (DDGs) and a premix minerals.

After a 261 fattening period the steers were slaughtered, the average weight of the group was 604 ± 5.67 kg. On the day the steers were slaughter they were transported 36 km by truck to the slaughter house where they were put in waiting pens for 3.5 h, during this time only water was provided. The steers were slaughter in a Federal Inspection Type slaughter house (FIT) following the procedure described in the Mexican Official Norm NOM-033-SAG/ZOO-2014, “Slaughter methods to be used in domestic and wild animal”

Production behavior

The following production result: initial weight, weight after first reimplant, weight after second reimplant, final weight, average daily gain (ADG) and food conversion, were obtained from the company´s records. Each of the animals slaughter weight was obtained in the stunning box.

Carcass and meat evaluation

Carcasses from both treatments were chilled at 2 °C for 24 h and ribbed between the 12th and 13th ribs to collect additional carcass data. A total of 178 carcasses from T14 and 176 carcasses from the T16 were available by the slaughterhouse to be considered for the study of all the variables. The measurements of hot carcass weight (HCW) and cold carcass weight (CCW), dorsal fat , marbling, ribeye area, pH and color of each carcass were taken. Dorsal fat was measured in mm using a metric ruler. The ribeye area was evaluated using a plastic grid method suggested by Iowa State University and the marbling score (scale of slight; small; modest; moderate; slight abundant; moderately abundant), were both evaluated following the methodology described by AMSA[12]. The pH was determined using a potenciometer (HANNAH INSTRUMENTS Inc. pH 101), the color values (L*, a*, b*, C*, H*) were measured on the surface of the cut from the *Longissimus dorsi* muscle between the twelfth and thirteenth intercostal space using a MINOLTA CM-2002 spectrophotometer (Minolta camera, Co., Ltd., Japan) with a specular component included (SCI), a D65 illuminant, and a 10° observer, where L* is the index of luminosity, a* is the red color intensity and b* is the yellow color intensity and C* measure color saturation.

Statistical analysis

Productive data was analyzed using the following statistical linear model: $Y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij}$ where Y_{ij} is the response variable, μ is the true mean effect, τ_i is the fixed treatment effect, β_j is the fixed pen effect and ε_{ij} is the random residual error iid N (0, σ_e^2). The hypothesis that treatment effects do not differ, was performed by F test statistic in the ANOVA. Differences between treatments were declared when $P \leq 0.05$.

Carcass and meat quality data were analyzed as a randomized complete block design with sampling, with pen as the experimental unit and carcass as the observational unit. The statistical linear model was as follows: $Y_{ijk} = \mu + \tau_i + \beta_j + \varepsilon_{ij} + \delta_{ijk}$, where Y_{ijk} is the response variable, μ is the true mean effect, τ_i is the fixed treatment effect, β_j is the fixed

pen effect, ε_{ij} is the random residual error iid N (0, σ_e^2) and δ_{ijk} is the random sampling error iid N (0, σ_d^2). The hypothesis that treatment effects do not differ, was performed using an F test statistic in the ANOVA. Differences between treatments were declared when $P \leq 0.05$.

The hypothesis that treatment effects do not differ for proportions within each marbling class was done using a Chi-square test statistic in one frequency table. Differences between treatments were declared when $P \leq 0.05$. The analysis was made using the MIXED and FREQ procedures of the SAS 9.4 (TS1M7) statistical package.

Results and discussion

Production results

A relevant finding of this study was that steers with a larger pen space had a higher weight during all the fattening period; these results are presented in Table 1 and show that after receiving the first reimplant (day 94 after arrival to the feedlot), the steers from T16 showed an average higher weight when compared to the animals in T14 ($P<0.05$); this same result was observed after the second reimplant and through all the fattening period ($P<0.05$); the observed weight difference between the groups was 16 %. Similar results regarding weight differences have been reported by other authors[13], who found a higher final weight in Hanwoo steers when they were provided with a larger pen space.

Table 1: Holstein steers Median weight values ± SEM per treatment

Variable	Treatment		SEM	Pr>F
	14 m^2	16 m^2		
Initial weight, kg	238.57	237.62	0.74	0.2000
Weight at 1st reimplant, kg	378.38^b	384.25^a	1.65	0.0004
Weight at 2^d reimplant, kg	506.73^b	515.21^a	2.52	0.0008
Final weight, kg	595.54^b	612.35^a	5.67	0.0032

SEM= standard error of the mean.

^{a,b} Different letter indicates differences between treatments ($P<0.05$).

Table 2 shows the production results for both groups of steers. It was found that weight gain was higher for the steers in T16, however no difference was found in feed conversion and feed intake. Similarly, to this study Kim *et al*[14], observed that Holstein steers 20 mo of age that were provided with 16 m^2/animal, reached a 750.39 kg final weight and daily weight gain of 1.36 kg. A study in Holstein steers that did not considered the amount of living space per animal as a variable have reported a final weight between 613.3 a 631.4 kg, a 1.41 to 1.46 kg/d of ADG[15], while a study carried out in Mexico found that Holstein steers reached a final weight of 604.9 kg with a daily gain of 1.46 kg and a feed

consumption of 8.41 kg per day[16], another study performed by Carvalho *et al*[17] found that Holstein steers gained daily 1.73 kg/d with a final weight of 598 kg. Although in Mexico the federal norm[3] establishes that pen space for an animal under 400 kg should be 12 m^2 and for one above 400 kg 20 m^2 [2]. It may be expected that the world trend to reduce the space allowance per animal in cattle feedlot[18] is impacting Mexico, so it is likely that welfare and production variables will be affected because of smaller allowed space for feedlot cattle.

Carcass and meat evaluation

Table 2: Median production results ± SEM per treatment

Variable	Treatment		SEM	Pr>F
	14 m^2	16 m^2		
Daily weight gain, kg	1.46[b]	1.50[a]	0.01	0.0327
Feed conversion	7.51	7.17	0.17	0.1260
Feed consumption, kg	10.80	10.62	0.15	0.2967

SEM= standard error of the mean.

[a,b] Different letter indicates differences between treatments ($P<0.05$).

The group of steers that was provided with the largest living space showed a difference of 7 kg both in the hot and cold carcass weight ($P<0.05$), these results are shown in Table 3 and correspond with it was reported by Ha *et al*[13] who provided a greater living space to steers that were in the finalization period. A similar study[19] reported a larger hot carcass weight for feedlot steers which were provided with 16 m^2/animal, when compared with two other groups of animals that had a living space of 10.6 and 8 m^2/animal.

Table 3: Carcass median production results ± SEM per treatment

Variable	Treatment		SEM	Pr>F
	14 m^2	16 m^2		
Hot carcass weight, kg	360.35[b]	367.34[a]	2.98	0.0196
Cold carcass weight, kg	358.78[b]	366.68[a]	2.96	0.0079
Dorsal fat, mm	9.1	9.3	0.83	0.1939
Ribeye area, cm^2	96.14	98.66	2.31	0.9277

SEM= standard error of the mean.

[a,b] Different letter indicates differences between treatments ($P<0.05$).

In the present study dorsal fat and ribeye space showed no statistical difference between groups ($P>0.05$), this result corresponds to what is reported in Hanwoo cattle carcasses[19]. In contrast with this study, researchers[20] found no differences ($P>0.05$) between Hanwoo carcasses obtained from animals that were provided with different living spaces. Other authors have reported lower dorsal fat numbers, 5.15 mm[14]; 5.8 mm[17,21]; while Carvalho *et al*[15] reported a dorsal fat measurement between 8.6 and 9.3

mm, Torrentera *et al*[16] observed a dorsal fat depth of 10.9 mm results that are similar to what was observed in the present study.

Authors have found that dairy cattle tend to deposit greater amounts of fat in the abdominal cavity and to accumulate less subcutaneous fat[22], in this context bovine races that are bigger and take more time to mature have a larger proportion of inter and intramuscular fat when compared with smaller races which mature earlier[23].

In the case of ribeye area, the present study found that they were larger than the ones reported by Ha *et al*[13] for Hanwoo steers (91.0 and 94.6 cm^2 for 10 and 16.7 m^2 of living space) likewise other studies in Holstein steers reported ribeye areas of 72.36 cm^2 [17]; 73.7 cm^2 [21]; 74.9-82.5 cm^2 [15]; 77.21 cm^2 [14]; 81.22 cm^2 [16].

Regarding the amount of intramuscular fat in the meat (Table 4) the results indicate that there is no difference between the groups, however the findings support the reports from other researchers that in the case of Holstein steers choice beef is the grade that is observed[16,17,21]. In this study, 130 of the steer's carcasses produce beef that was classified as small while a second group of 159 carcasses yielded modest beef.

Table 4: Marbling score per treatment

Variable	Treatment		Pr>χ^2
	14 m^2 n = 178	**16 m^2 n = 177**	
Slight	10	14	0.4142
Small	57	73	0.1605
Modest	87	72	0.2342
Moderate	23	17	0.3428
Lightly abundant	1	1	---

Table 5 show both groups physicochemical results, it was found that in the case of pH, L*, a* y C* no differences were observed ($P>0.05$), and although the values for b* y H* showed differences ($P<0.05$), this dissimilarity does not result in noticeable differences in color between treatments.

Table 5: Meat physicochemical median results ± SEM per treatment

Variable	Treatment		SEM	Pr>F
	14 m^2	**16 m^2**		
pH	5.67	5.60	0.06	pH
L*	29.97	31.96	0.85	L*
a*	17.08	16.79	1.24	a*
b*	15.45[a]	15.02[b]	0.83	b*
C*	23.08	22.59	1.47	C*

SEM= standard error of the mean.

[a,b] Different letter indicates differences between treatments ($P<0.05$).

In regard to pH, values between 5.5 and 5.8 are considered as normal for bovine meat[24]; so, the results obtained by the present study may be viewed as typical. Similar pH values and have been reported in studies done with Holstein by other authors[6,25]. In the case of meat color, based in what has been reported by others authors[24], the meat obtained from both groups is considered as dark cutting, another research have reported similar results (L* =37.50, a*=14.69 y b*=12.39)[26] and (L*= 38.02, a*=19.86, b*=8.19, C*=21.49)[14]; the reason for this may be explained by the pre slaughter stress that the animals were submitted to, which depleted blood glycogen and affected the beef´s color[27]. Authors have informed that the way animals are handled, the novelty of environment and fatigue, are factors that contribute to stress[28].

Conclusions and implications

It is very important that feedlot cattle is provided by sufficient living space during the whole fattening period and considering that there is a trend to reduce the space allowance per animal, it is very important to better understand the negative impact that a reduce pen space has on the animal welfare and how this impacts beef production. As suggested by the results of the present study a relatively small increase of living space has a positive impact on carcass weight which at the end will translate into an increase of income.

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Conflict of interests

The authors declare that they have no conflict of interest.

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Review

Common duckweed (*Lemna minor*): food and environmental potential. Review

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Abstract:

Common duckweeds are flowering plants of the family Araceae, comprising the smallest angiosperms of the plant kingdom, a species of aquatic algae of universal distribution, found on the surface of freshwater bodies, mainly in puddles, swamps, lakes, and calm rivers. Recently, different research has been carried out on its potential and usefulness. Due to its nutritional composition, protein contribution, high fiber content and low fat and carbohydrate content, it would be an adequate input to generate products of high nutritional value, characteristics that make it interesting compared to other species. It is used as a complement to commercial diets in a wide variety of animals such as birds, ruminants, non-ruminants, crustaceans, and fish, reducing feed costs by up to 50 %. Likewise, used in remediation processes of a wide range of chemical contaminants with a high elimination rate, they can absorb some dissolved substances and provide oxygen through photosynthesis. It has been indicated that they are low cost of construction, maintenance, easy to operate, have a wide tolerance to growing conditions, are generally easy to harvest, and do not compete with farmland. In the environmental field, it is important to find alternative and innovative raw materials, even without the need to use growth media or fertilizers, however, their acceptance as a food source needs extensive research regarding their nutritional value, large-scale yield, economic market supply and analysis of antinutritive components for human food.

Keywords: Common duckweed, *Lemna minor*, Nutritional profile, Environmental remediation, Human and animal food.

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Introduction

In recent decades, the rapid growth of the world's population and the climate crisis have become a serious problem that threatens the food and feed supply, generating dietary patterns deficient in proteins and vitamins, the development of malnutrition due to excessive consumption of simple sugars and stigmatization of nutrients[1]. In this sense, common duckweeds have taken center stage in recent research in the search for new foods that provide healthy alternatives, pharmaceutical products that are sustainable and profitable on a large scale of production.

These small aquatic plants comprise a group that float on the surface of bodies of water with little movement, with a great capacity for reproduction and accelerated growth. Traditionally, they have been used as remediation agents for water body pollution due to their ability to absorb minerals, salts, nitrogenous substances, and heavy metals in water bodies[2].

From an ecological point of view, it can be seen that, given its interactions with other species, it can be considered as a keystone species in its habitat, although it has a small size, due to its rapid growth, high tolerance to pollution and capacity to absorb nitrogen and phosphorus, *Lemna minor* has previously been used for wastewater treatment[3,4].

In Asian countries and recently in Western countries, they are being included in plant mixtures for the raising of farm animals and fish cultures, showing favorable results in the development and growth of these animals, reducing feeding costs[2].

Common duckweeds are known to contain essential nutrients such as protein, carbohydrates, and fats. They also contain a variety of secondary metabolites that are beneficial to humans. Therefore, consideration of common duckweed cultivation methods is vital for their best utilization in various industrial applications. A number of reports have been generated on common duckweed utilization, metabolites, and cultivation; these should be reviewed and summarized as fundamental information to improve the application of common

duckweeds[5,6]. It is important to note that if they are used as pollution remediation agents, their use in human and animal food should be evaluated.

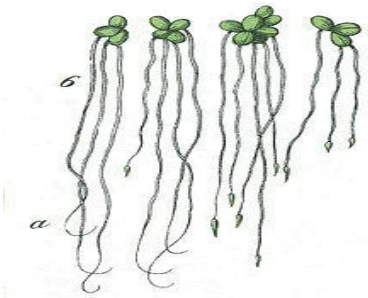
This paper aims to provide a global overview of common duckweeds, especially the species *Lemna minor*, through a bibliographic review from taxonomic description to their use as an alternative for inclusion in animal and human diet, considering their nutritional composition and current lifestyle patterns. In addition, environmental impact as biomarkers, environmental remediators, agricultural amendments in crops, biofuel sources and pathogenesis models is studied.

Common duckweed (*Lemna minor*)

Common duckweed (*Lemna minor*) is the world's fastest-growing, free-floating aquatic angiosperm plant that grows in immobile waters, usually in freshwater or wetlands in most parts of the world and is characterized by its small size and great reproductive capacity, which allows it to occupy large aquatic spaces in a truly short time[7,8]. They are generally described as aquatic or floating grasses of quite simple structure, lacking stem, or leaves, occasionally with small thread-like roots on their underside. It is an aquatic plant that can often be seen floating or just below the surface or moving very slowly[9].

Lemna minor has a thalloid-shaped vegetative body, characteristic of some plants in which it is not possible to differentiate the leaves from the stems, small in size with a flat structure, green coloration in its leaves and a single thin white root. According to the description of some authors, this characteristic is associated with a modified leaf that fulfills the functions of the stem, leaf, and axis to support flowers, as shown in Figure 1[10,11].

Figure 1: Illustration of the vegetative body and roots of common duckweed (*Lemna minor*)



Source: Landolt, E[11].

On the other hand, roots, generally related to the nutrient uptake aspect of the plant, seem to have a slightly different function in these species. Some researchers have reported that the consumption of nutrients via roots is little or non-existent, functioning as a stabilizing organ in the body of water; however, the common duckweed, *Lemna minor*, has been shown to acquire significant amounts of inorganic nitrogen through the root. The plant grows at different temperatures varying between 5 and 30 °C, growing optimally between 15 and 18 °C. It adjusts favorably to any lighting conditions. It grows rapidly in calm, nutrient-rich locations with high levels of nitrogen and phosphates. Iron often limits the proper development of this species. It is also tolerant to a wide pH range, between 4.5 and 7[12,13].

Cultivation of common duckweed (*Lemna minor*)

Common duckweed crops can be produced quite easily and inexpensively, even without the need to use growth media or fertilizers, as they are characterized by a high relative growth rate (RGR). This means that they are able to produce large amounts of biomass in a short time and in relatively small ponds, filled with a few centimeters of natural water (30 to 50 cm deep). The control and monitoring of the aquatic medium in which the plants grow is particularly important. The productivity of common duckweed increases more if the optimal ecological conditions for growth are respected, however, they are generally broad. These, although varying slightly from species to species, generally consist of moderately warm, sunny, nutrient-rich waters, as documented in ecological studies on some common duckweed species of the genus *Lemna*.

In recent decades, it has become common to grow them outdoors, but it can be difficult to optimize and control operationally. However, common duckweeds also represent a suitable crop for indoor farming, with most species, due to their flat structure particularly suitable for cultivation in multi-level (stacked) systems that use indoor floor space efficiently. Indoor cultivation also expands the scope of crop managing and allows for pest-free conditions and even sterile conditions. However, the technical and operational parameters required for large-scale effective interior have received little attention in the literature[14].

It is important to note that sporadic common duckweed growth causes serious damage to aquatic resources, with several economic implications. The dense and extensive blanket created by the plant in the block of surface water and water channels makes activities such as water flow, sailing, canoeing, swimming, and fishing impossible. It also affects irrigation, floods canals, can clog hydroelectric turbines and disturbs rice fields. A dense layer of common duckweeds shuts outs and inhibits competing aquatic plants, including algae that

require sunlight. For this reason, it is important to have integrated control or strategies that allow their exploitation.

According to various objectives and targets, optimal cultivation of common duckweeds will be necessary from an economic and industrial point of view. Various cultivation methods using various types of bioreactors and conditions for their use as food resources, pharmaceuticals, phytoremediators, and biofuels must be employed. Aquaponics combining aquaculture and hydroponics could be a sustainable production system for plants[15]. Currently, the technology enables the mass production of good quality by controlling environmental conditions, such as irrigation irradiation, atmospheric pressure, wind, speed, temperature, and humidity.

Nutritional composition

In recent decades, several scientific studies have highlighted the nutritional value of common duckweeds, which, due to their supply, could improve the quality of food in the future. However, according to some studies, the metabolism of the plant and, therefore, its nutritional composition depends a lot on the nutrients found on the surface of the body of water in which it is found. These extremely important factors capable of influencing the nutritional composition of the plant are reflected in the different results obtained in each study[16].

Proteins

The high-quality protein content reaches 20 to 35 % in dry matter, when grown under optimal conditions, higher than the protein present in cereals[6]. This means that common duckweed biomass can be considered as an ingredient for animal or human food and can contribute to improving food security through the development of sustainable methods of producing food with high nutritional value[17]. It has been documented that the protein production of common duckweeds per harvested area was higher than that of soybeans, rice, and corn; therefore, it could solve the problem of the scarcity of arable land to produce food[5].

The amino acid profile of common duckweeds stands out from some plant-based protein sources currently known in the human diet, an aspect that some authors have come to relate to an amino acid profile more similar to animal protein[18,19]. Recent studies analyzing the nutritional composition of several common duckweed crops showed, unlike previous analyses, a content of amino acids, such as isoleucine, leucine, cysteine, methionine,

threonine, and valine, similar to the recommendations for consumption for the population (Table 1); it can also be noted that the amounts were not lower than those recommended by the WHO, 2007[20,21]. Jahreis *et al*[20] found that the amino acid composition of common duckweeds is comparable to that of legume meals, such as chickpeas, lupins, or peas. Clinical nutrition studies have shown that the essential amino acids and vitamin B_{12} content of common duckweeds are comparable to peas and cheese[5].

In addition, their source of proteins, which could replace soybean meal, is expected to be used as a substitute to reduce environmental pollution created by the expansion of soybean cultivation[21]. Consumption of plant protein instead of animal protein could reduce energy use and greenhouse gases and alleviate the negative aspects of feed production[5].

Table 1: Amino acid content of common duckweed (*Lemna minor*)

Amino acids		**G / 100 g protein**
Cysteine	CYS	0.9
Methionine	MET	1.6
Asparagine	ASP	8.2
Threonine	THR	4.0
Serine	SER	4.1
Glutamine	GLU	9.8
Glycine	GLY	4.6
Alanine	ALA	5.1
Valine	VAL	4.6
Isoleucine	ILEU	3.7
Leucine	LEU	7.3
Tyrosine	TYR	3.1
Phenylalanine	PHE	4.4
Lysine	LYS	5.0
Histidine	HIS	1.5
Arginine	ARG	4.8
Proline	PRO	3.8

Source: Appenroth, *et al*[7].

Fats

Fats play an important role in the human diet; despite being stigmatized as nutrients harmful to health, in the last decade some studies have shown the great impact they have by contributing as protective factors against degenerative diseases such as Alzheimer's,

reducing the risk of cardiovascular accidents and even, mortality. Authors report a content of 4 to 6 % fat content per dry weight[6].

An approximate content of 30 % saturated fatty acids, specifically high levels of palmitic acid, is highlighted. Recently, in terms of fatty acid contribution by common duckweeds, it has been shown that it adapts very well to the requirements of the human population, with a low fat intake and a good ratio of polyunsaturated to monounsaturated fatty acids, generally close to or more than half of the total fatty acid content, ranging from 55 to 63 %[22]. It is also important to highlight the presence of n-3 class polyunsaturated fatty acids (alpha-linolenic, eicosapentaenoic, and docosahexapentaenoic acids), which are important in human metabolism and act as anti-inflammatories[23]. Other authors have described that 48 to 71 % of fats are polyunsaturated fatty acids and the ratio of omega-6 to omega-3 fatty acids is 0.5 or less[6].

Carbohydrates and fiber

Several studies with common duckweeds have concluded that starches or carbohydrates are fairly underrepresented in the analysis of the nutritional composition of these plants; in the best of cases, carbohydrates represent approximately 10 % of the total nutritional value of common duckweeds[24].

Some authors have shown that the growth environment, genetics of the species, nutrients of the medium, temperature range, time, and intensity of sunlight cause differences in the biochemical components (crude protein, ash, cellulose, water, fats, and minerals). It has been documented that the protein content of common duckweed depends primarily on the nutrient content in the water body, while the accumulation of minerals in common duckweed tissue depends primarily on the water conditions in the growing environment. During artificial cultivation, the starch, lipid, and protein content in common duckweeds can be controlled by changing factors affecting the common duckweed growing environment, such as pH value, temperature, medium structure, etc.[25].

On the other hand, the fiber content, unlike carbohydrates, is considerably high; common duckweeds can contain up to 25 % of the total nutritional value in fiber[26]. This high fiber content represents an excellent option for inclusion in the human diet, which, together with a large contribution of protein and a small contribution of fats and carbohydrates, would be, according to several authors, completely beneficial in healthy lifestyles[27].

Minerals and trace elements

The nutritional composition in terms of minerals in common duckweeds is characterized by being plants rich in potassium and iron, and poor in sodium; in contrast, in terms of trace elements, the content of manganese, zinc, copper, among others, stands out (Table 2). On the other hand, the total ash content is moderately high, with ranges of up to 18 % in some studies, however, these values may vary according to the composition of the medium[6,26].

Table 2: Contribution of trace elements from common duckweeds in mg per kg of total edible part

Trace elements		**mg / kg**
Magnesium	Mg	2850 ± 710
Iron	Fe	230 ± 90
Manganese	Mn	230 ± 98
Iodine	I	0.39 ± 0.19
Cadmium	Cd	0.076 ± 0.145

Source: Ziegler P, *et al*[26].

Vitamins

Regarding vitamins, there are few studies on common duckweeds that have focused on assessing the nutritional composition and presence of these micronutrients. The vitamins found in the highest amount in common duckweeds are carotenoids, precursors of vitamin A; the dominant carotenoid in this plant is lutein, followed by β-carotene. Other carotenoids are found in much lower amounts, such as α-tocopherol and zeaxanthin[28,29] (Table 3).

Table 3: Carotenoid content in common duckweeds

Nutrients carotenoids		**Contribution**
Lutein	mg/100 g	40 – 80
β-carotene	mg/100 g	10 – 30
α-tocopherol	mg/100 g	0.5 – 13
Zeaxanthin	mg/100 g	0.8 - 10

Source: Sree K, *et al*[28].

Human nutrition

In a study carried out by the Panel on Nutrition, Novel Foods, and Food Allergens (NDA) on the safety of the complete plant material of *Lemna minor* and *Lemna gibba* as a novel food in accordance with Regulation (EU) 2015/2283 in 2022[17] for consumption as a vegetable, toxicological, nutritional, microbiological analyses of powder from common duckweeds grown in greenhouses under controlled conditions were carried. Based on the proposed uses, expected intake and compositional data, the intake of heavy metals, microcystins and micronutrients, except manganese, it does not pose safety concerns for consumption as a novel food. However, the findings on neurotoxicity and the possible higher susceptibility of some subgroups of the general population, oral exposure to manganese beyond that normally present in foods and beverages could pose a risk of adverse health effects without evidence of any health benefit. On the other hand, the likelihood that the product may trigger allergic reactions in humans is similar to that of other leafy vegetables, and therefore the level of risk is considered low.

In this research, there were two human trials: a randomized crossover trial and a parallel controlled trial with healthy subjects. The commission's panel noted that the human studies provided were primarily designed to research putative beneficial effects and addressed only a limited number of safety-relevant evaluation criteria. The Panel considers that no adverse events related to consumption were reported, however, it is noted that no conclusions can be drawn from these studies about the safety of the product.

In some parts of Southeast Asia, such as Laos, Thailand and Myanmar, its consumption is normal in preparations such as salads, soups, curries or omelets as a source of vegetable protein, however, it has not been included as part of the diet in Western countries[16].

Common duckweed has been shown to have an amino acid profile that favors the diet of aquatic and terrestrial animals, a contribution of vitamins and minerals that contribute to its palatability, a concentration of fats (4 to 7 %) and starches (4 to 10 %) adequate when compared to other plant-based foods such as dried legumes; in addition, knowing the poverty rates in some populations of the world, and the high need for nutritional supplementation in populations with limited access to healthy food, it could be of great relevance in human nutrition[16,30].

In the case of Thailand, common duckweeds, in their variety of species, are marketed in vegetable markets and their acceptance in the population such as Khai Nam, Khai Pum, and Khai Phae (generally translated as "water eggs") stands out in the preparation of traditional local dishes such as salads, curried vegetables, and omelets[31].

Israeli studies seeking to compare the postprandial and nocturnal glycemic response by using dairy shakes from common duckweeds of the species *Wolffia globosa* observe in these plants a great opportunity in human nutrition, especially in population groups with difficulties in carbohydrate metabolism. According to these studies, this species of algae could serve as an emerging alternative plant protein source with potential beneficial postprandial glycemic effects, however, no scientific information has been found with the species *Lemna minor*[29].

Considering the nutritional contribution of common duckweeds, especially their outstanding contribution of proteins, fats, beta-carotenes, minerals, and low carbohydrate contribution, in addition to the absence of antinutritional substances, common duckweeds (*Lemna minor*) could represent an excellent option for consumption as a supplement in the dietary pattern of needy communities throughout the world[32]. However, in cases of uncontrolled growing conditions, and particularly when fertilizers, pesticides and other organic contaminants are present in large quantities at cultivation sites or in cases of water pollution by algae or microbes, the high concentration of contaminants or toxins in those plants may pose a potential risk to human health that should be considered[17]. On the other hand, Appenroth *et al*[6] indicate that *Wolffiella hyalina* and *Wolffia microscopica* are suitable for human nutrition, even compared to other common duckweed species, regarding amino acid composition and fatty acid distribution.

Animal nutrition

The high cost of feed for animal raising has led to the constant search for alternatives to improve animal production. At present, soybean meal is one of the most widely used alternative ingredients to replace fishmeal in animal feed due to its high protein content and relatively well-balanced amino acid profile, which can generally meet the requirements of many fish species. However, soybean meal is already in high demand in the human food chain, both directly and indirectly in feed for farmed terrestrial animals. This competition means that soybean meal is an expensive ingredient and this may limit its use as an ingredient to meet future fish feed demands. Therefore, there is a constant need to find other ingredients (insects, vegetables, algae, byproducts of aquatic organisms) to replace both fishmeal and its main substitute, soybean meal, in farmed fish feed. Ideally, such ingredients should be unconventional in order to avoid or minimize competition with other animal feed sectors[33].

Among these alternatives, common duckweed represents a great opportunity given its accelerated growth and great ability to adapt to the environment in which it grows; this, without leaving aside the source of raw vegetable protein that it represents, its contributions

of minerals, xanthophylls and amino acids such as lysine, threonine, and valine[18,34]. Hence the great opportunity from an economic point of view that it can represent.

Under experimental conditions, the production rate can be close to 183 extrapolated metric tonnes/ha/year of dry matter, although yields are closer to 10-20 tonnes DM/ha/year under real conditions. Its use has mostly occurred in a wide variety of animals of social interest such as farmed birds, ruminants, non-ruminants, and farmed fish[35]. In which, through different inclusion models, it has been shown that it can be a good complement to the food diet of livestock and fish[36,37].

Models implemented in small farms in the Asian continent focused on the recovery of nutrient flow from animal waste have used the resulting common duckweed biomass as a fresh feed for ducks, farmed fish and pigs; all this evidencing the contribution to the correct nutrition of these animals and reducing feed costs[7,38,39].

Common duckweed has similar or superior characteristics to plant-based proteins, such as legumes, however, it is rich in some essential amino acids. In Asian and Latin American countries, there are reports of the use of common duckweed in the diet of farmed pigs, with an inclusion of up to 10 % of the total feed consumption, showing excellent results in their reproductive response[37].

In Latin American countries such as Mexico and Venezuela, common duckweed is used to feed pregnant sows and piglets, replacing 80 % of protein from soybean cake or fishmeal as a whole, with exceptionally good results in production[8]. According to some authors, common duckweed reaches protein levels of up to 38 % of its biomass. This protein contribution and its ease of cultivation has allowed trials as feed for domestic ducks, obtaining results in weight gain and egg production comparable to the usual protein supplement, with the advantage of a 25 % decrease in feed costs in Asian countries[33].

Other agricultural models have implemented common duckweed as a fodder crop for livestock raising, considering that common duckweed biomass has a protein content of more than 30 % of dry weight, representing an excellent complement in the feed of farmed animals, environmental sustainability, and cost reduction[34]. When it is used as the only source of nutrition, at a rate that should not exceed 6 % of body weight (dry basis), the results are much lower than those obtained with conventional diets, at which point they cease to be potentially beneficial; however, experiences in polycultures have shown that common duckweed supplementation increases production per hectare[8]. In this way, for more than 50 years, science has studied the different alternatives that common duckweed represents in the nutrition of different species of animals for consumption, yielding promising results as it is a rich and sustainable source of protein.

Now, some research on feeding dry common duckweed, *Lemna minor*, as a protein source in the diet of common carp fry, has shown that there are no significant differences in the growth and development of fish that are fed diets supplemented with up to 20 % common duckweed versus commonly used fish protein fodder. Showing that a diet consisting of up to 20 % common duckweed content could be used as a complete replacement for commercial feed in the formulation of the diet for common carp fry, allowing cost reduction[40].

According to research carried out by Goswani *et al*[41], when evaluating the impact of protein from dry common duckweeds (*L. minor*) compared to the impact of the standard and commercial diets of the fry of rohu *Labeo rohita* (carp native to the rivers of India and Asian regions), slight modifications in the digestive enzymatic activity of fish were identified. The diet with protein from common duckweed stimulates amylase, trypsin, and chymotrypsin activities, which were significantly higher compared to other diets, but without altering or modifying the growth rate of the fish. In this sense, the inclusion of raw common duckweed in the feed, replacing amounts of up to 30 % of fishmeal in the diet, can be well tolerated by farmed fish without affecting growth[42].

In the case of farmed fish for human consumption, recent studies have researched the transfer of toxic heavy metals, such as cadmium, from common duckweed (*Lemna minor*) to freshwater tilapia (*Oreochromis mossambicus*). Through regression analysis, significantly positive correlations were found between the concentration of cadmium in common duckweed and freshwater tilapia meat, concentrations that were especially found in greater quantity in the tissues of intestine, edible muscle and remains. From this perspective, the analyses suggest the assessment of toxicity risks[40]. Other studies have researched the potential of common duckweed as an animal feed through the fermentation process with the addition of two probiotic strains, *Bacillus strains*, and *B. subtilis*, which have demonstrated health benefits for poultry, demonstrating that common duckweed is a promising alternative resource and has the opportunity to become a valuable resource in multiple industries such as that of foods, biofuels, pharmaceuticals, and wastewater phytoremediation. With the potential to increase sustainability, food security and reduce environmental impact[43].

Environmental impact of common duckweeds

With industrialization and the increase in the production of needs on a large scale by society, the pollution of both surface and underground water bodies has become a major problem with environmental and social impact[44]. The accumulation of numerous toxic substances in waters has led to the search for inexpensive and reachable options that allow to identify the level of toxicity that these natural spaces may have. Thus, the recent use of common

duckweed (*Lemna minor* and *Lemna gibba*) crops has made it widely possible to analyze the toxicity transmitted by water to organisms higher in the biological chain (animals and humans)[45].

Other studies where growth parameters and assessment criteria, such as pigment content, peroxidase activity, lipid peroxidation and alkaline comet assay, were used to detect the toxic and genotoxic effects of surface water samples in common duckweed plants were able to indicate the ability of selected biomarkers to predict the phytotoxic and genotoxic effects of complex water mixtures on living organisms, as well as the relevance of common duckweed as a sensitive indicator of water quality[46].

The inhibition of growth and reduction in the photosynthetic pigment of this plant when growing in polluted water environments have allowed its use as an effective biomarker in the non-specific detection of toxic components in water bodies. However, it should be recognized that although it is a good indicator of water pollution, common duckweed does not allow the nature of the agents or substances responsible for such toxicity to be determined by itself[47].

Recent studies, despite the impossibility of identifying these toxic agents from common duckweed, have managed to document the adaptive capacity they have to metabolize some of these substances, such as nickel and ammonia, bringing the quality of water bodies to acceptable levels in a prudent time, a process known as phytoremediation of water bodies[48].

Aquatic plants have been shown to be highly efficient in removing organic and inorganic pollutants[49]. *Lemna minor* has been widely applied for the remediation of various chemical contaminants. The plant is used separately or in combination with other aquatic macrophytes as an ecologically based pollution treatment technology[50]. *L. minor* has been reported as a floating microphyte highly successful for the phytoremediation of organic pollutants; it was the most effective plant in the treatment of wastewater for the remediation of municipal effluents. There was 98.8 % removal for total nitrogen and phosphorus, with a higher level of oxygen dissolution due to an improvement in nutrient loading by common duckweed[51]. Common duckweed has shown great potential for phytoremediation of organic pollutants, heavy metals, agrochemicals, pharmaceuticals, personal care products, radioactive waste, nanomaterials, petroleum hydrocarbons, dyes, toxins, and related contaminants[50]. Substances that pose a serious risk to the environment and all forms of life because they can be persistent, are easily transported through the media, and can cause poisoning of tissues and organs[52,53,54]. Tufaner[55] reported more than 90 % removal of heavy metalloids (chromium, zinc, aluminum, arsenic, cadmium, cobalt, copper, lead, and nickel), while 83 % for mercury in a mixture of wetland with *L. minor*. On the other hand, *Lemna minor* shows an increase in chromium absorption percentage of 6.1, 26.5, 20.5, and 20.2 % at a different exposure concentration of chromium stress[56,57]. In addition, research conducted in relation

to agricultural chemicals such as fertilizers, pesticides, herbicides, and fungicides shows that common duckweeds can accumulate and degrade these agrochemicals[58,59,60]. Common duckweed has the ability to conserve nature by acting as a hyperaccumulator. The wide application of the plant is due to its ubiquitous nature, invasive mechanism, sporadic reproductive capacity, bioaccumulation potentials and resilience in polluted environments[61].

Toxicity and antinutritional substances

Studies have highlighted the presence of antinutritional components, substances, or factors in common duckweed[3]. After several toxicity tests, it was discovered that common duckweeds are extremely sensitive to triazines, sulfonylureas and pyridines, compounds currently classified as toxic with a great polluting impact on the environment and that, given the way of nutrition of this plant, they can absorb them. However, many authors point out that the amounts of these compounds in this plant are small, and that they could be susceptible to denaturation when subjected to heat treatments[62].

Antinutritional factors are substances or compounds that have the ability to interfere with the biological use or exploitation of a food or nutrient, affecting a person's health and some or more of the physiological processes of the body. Some authors have reported the presence of tannins and phytic acid in common duckweeds in concentrations of 0.02 and 0.09 %, respectively[63].

Likewise, other studies showed concentrations of trypsin inhibitors at 1.47 %, calcium oxalates at 3.5 % and tannins in concentrations much higher than previously cited studies, 0.9 %[64]. However, recent research has highlighted low concentrations of cyanide at 0.15 %, phytic acid 0.58 %, and tannins 0.48 % when analyzing a wide variety of common duckweed strains; likewise, these samples were subjected to heat treatments where the deactivation or inhibition of these substances was evidenced, thus eliminating the toxicity that could be implied by the consumption of the plant[65].

According to research carried out by Sree *et al*[66] to determine the cytotoxic effects and antiproliferative activity in human cell lines of several common duckweed species, including *L. minor*, it was found that whole plant extracts do not have any detectable adverse effect in human cell lines, which is a step towards ensuring the global use of common duckweed as a component of human nutrition.

Conclusions

In recent years, common duckweed has taken on a prominent role in biotechnology and agricultural applications. It could potentially be an important resource as an alternative source of food for humans and animals. It has been used as raw or processed feed for meal production, making it interesting in the animal feed industry, aquaculture, health supplements, biofertilizers, biofuels, and emerging human food products.

It has demonstrated its strong potential for phytoremediation of organic pollutants, heavy metals, agrochemicals, pharmaceuticals, personal care products, radioactive wastes, nanomaterials, petroleum hydrocarbons, dyes, toxins, and related contaminants. The wide application of the plant is due to its ubiquitous nature, invasive mechanism, sporadic reproductive capacity, bioaccumulation potentials, and resilience in polluted environments.

The nutrients in the water in which it is grown critically affect its nutritional value, so it will likely need to be decontaminated before feeding the animals if there are heavy metals in the water, since common duckweed concentrates them. In this sense, it is important to highlight the scarcity of studies on the use of these plants in human nutrition; therefore, it is necessary to continue research to determine the role they could take and be included in the human diet and the safety associated with their continuous consumption, large-scale yield, economic market supply and sustainability.

Despite the challenges and knowledge gaps, there are realistic opportunities to develop and operate controlled, autonomous, high-capacity common duckweed crops under indoor conditions, for a wide range of purposes that ensure the characteristics of the final product. It should be noted that accelerated growth, the impact of climate change, the decrease in arable land, the depletion of soil, nutrients and water supply make it increasingly difficult to obtain quality food in the quantities required.

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Review

Implication of Fusariotoxins in poultry production. Review

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Abstract:

Mycotoxins are secondary metabolites produced by fungi of various genera. Among the most important mycotoxins are those produced by fungi of the genus *Fusarium* sp., which can be divided into several groups for study, which are the groups of trichothecenes (and T-2 toxin), fumonisins, mainly fumonisin B1 (B1, B2, B3, B4, A1, and A2), and zearalenone with estrogenic effects. Although fusariotoxins cause similar effects because they share the same mechanism of action, by altering protein synthesis in intoxicated poultry, it is important to mention the incidence as well as the characteristics between each of them. Therefore, the characteristics of each group mentioned are described in each section.

Keywords: Mycotoxins, Fusariotoxins, Poultry, Fungi.

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Introduction

Within livestock production, feed accounts for 65 to 70 % of the total cost of production; however, despite constant efforts to achieve the safety of food intended for animal production, there are still factors that diminish its quality, such as biological pathogens (such as *Salmonella* spp., *E. coli*, *Listeria* spp., *Campylobacter* spp.), chemical substances (fungicides, herbicides, and insecticides), and presence of fungi (Fusarium, Penicillium, Mucor etc.)[(1)]. Fungal contamination can occur under various conditions associated with both environmental factors and factors associated with the fungus, such as size and species[(2)].

Mycotoxins are secondary metabolites produced by filamentous fungi[(3)], considered toxic substances that present themselves as organic compounds of low molecular weight, which is why they do not have immunogenicity characteristics. The production of mycotoxins depends on a series of environmental factors such as humidity, temperature, ventilation, constitution of the substrate in which the fungus develops, damage to the integrity of the grains, and interaction between the various fungi present in the substrates[(4)]. It is mentioned that 25 % of crops worldwide are contaminated with mycotoxins[(5)].

Fusariotoxins

Among the toxins reported with the highest incidence within animal production are those produced by different species of fungi of the genus *Fusarium* sp, which are capable of inducing acute effects, as well as chronic effects, depending on the type of mycotoxin, the level and duration of exposure, and the species and age of the animal.

Avian species are considered resistant to the occurrence of fusariotoxin intoxication, which has been explained either by low sensitivity to toxicity mechanisms or by differences in toxicokinetic properties[(6)]. These fungal species have been classified within the group of field fungi, with grains requiring a high percentage of moisture (approximately 20 to 22 %) for the production of mycotoxins. The main substrate where the production of fusariotoxins is observed is corn; nevertheless, their growth has also been reported in other substrates such as sorghum, wheat, oats, barley, and soybeans. Fusariotoxins, in turn, are classified into three

groups for study, among which are the trichothecenes group (which in turn is classified according to the presence or absence of a macrocyclic ring in their chemical structure), the zearalenone group and the fumonisin group (Table 1).

Table 1: Mycotoxins produced by fungi of the genus *Fusarium* sp.

Mycotoxins produced	**Species of producing fungi**
• Trichothecenes	*Fusarium tricinctum, F. sporotrichioides, F. graminearum, F. culmorum, F. poae, Fusarium cephalosporium, F. myrothecium, F. acuminatum, F. nivale, F. oxysporum, F. solani*
• Zearalenone	*Fusarium graminearum, F. culmorum, F. poae, F. roseum, F. moniliforme, F. avenaceum, F. equiseti, F. nivale*
• Fumonisins	*Fusarium proliferatum, F. verticillioides, F. oxysporum*

Adapted from[11,94,95].

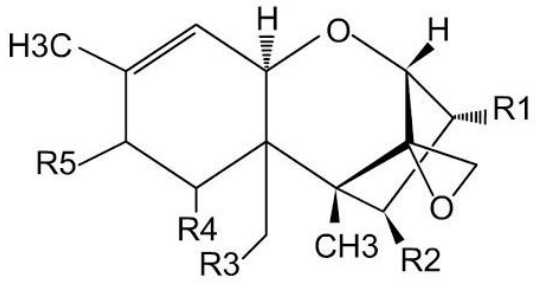
Experimental studies have agreed that the main effect of the toxins produced by *Fusarium* sp. has a direct impact on intestinal integrity (increased intestinal permeability), an effect correlated in turn with a decrease in the animal's immune response (alteration in the production of cells involved in the immune response)[7,8]. Both effects have their origin in the alteration of protein synthesis that takes place in the different metabolic processes in the animal[9,10]. It is important to mention that regardless of the mycotoxin or mycotoxins that are present in the feed, the cells of the digestive tract are the first to be in contact with the mycotoxins; this means that, possibly, the entire intestinal epithelium can be compromised even before the absorption of the feed begins, and in turn, it can also be affected by mycotoxins that have a low absorption rate, such as fumonisins and deoxynivalenol. Although fusariotoxins may have similar mechanisms of action among them, it is important to mention the individual characteristics of each[11].

Trichothecenes generalities

More than 150 different types of trichothecenes have been identified, and it has been established that, based on their occurrence in food, the most prevalent are T-2 toxin, deoxynivalenol (DON or vomitoxin), and diacetoxyscirpenol (anguidine), which in their chemical structure consist of a tetracyclic sesquiterpene skeleton, an oxane ring, and a stable epoxide group (12,13-epoxytrichothecene) that confers its toxicity (Table 2)[7]. The main substrates where can be find the presence of trichothecenes are mainly corn, wheat, barley,

oats, rice, and soybeans. Broadly speaking, trichothecenes are considered to be cytotoxic, immunosuppressive and inhibitors of protein synthesis[8]. Although trichothecenes can generally cause gastrointestinal, dermatotoxic, immunotoxic, and genotoxic effects, it has been reported that, under acute intoxication, they can be identified by evident skin inflammation, diarrhea, edema, dermal necrosis, hemorrhages in the mucosa of the gastrointestinal tract, and negative alterations in productive parameters[12,13,14].

Table 2: Structural differences in trichothecenes present in poultry production



Trichothecenes	R1	R2	R3	R4	R5
Type A					
HT-2 toxin	OH	OH	OAc	H	OCOCH2CH(CH3)2
T-2 toxin	OH	OAc	OAc	H	OCOCH2CH(CH3)2
Diacetoxyscirpentriol	OH	OAc	OAc	H	H
Type B					
Deoxynivalenol	OH	H	OH	OH	O
3-acetyl-deoxynivalenol	OAc	H	OH	OH	O
15-acetyl-deoxynivalenol	OH	H	OAc	OH	O
Nivalenol	OH	OH	OH	OH	O
Fusarenon X	OH	OAc	OH	OH	O

Adapted from[18].

T-2 toxin

Main characteristics and chemical structure

The T-2 toxin, belonging to group "A" of trichothecenes, contains in its tetracyclic chemical structure a sesquiterpenoid system and an epoxy-trichothecene group in C-12 and C-13. It can mainly be found in corn, wheat, oats, and barley[15,16].

It has been determined that the main fungus producing this mycotoxin is *Fusarium tricinctum*, however, it can be produced in turn by *Fusarium acuminatum*, *nivale*, *oxysporum*, *poae*, *solani* and *sporotrichioides*. In relation to this, the environmental conditions required for the production of the fungus are an average environmental temperature of 18 to 30° C, as well as a relative humidity of around 95 % and a water activity (AW) value greater than 0.88 (which must be 0.91 for the fungus to produce the mycotoxin)[17]. The chemical structure of this mycotoxin makes it a non-volatile compound soluble in acetone, chloroform and ethyl alcohol; it is of low molecular weight (approximately 466.52 g/mol) and is described as a mycotoxin highly resistant to heat (200-210 °C) and UV radiation, therefore, it is not easily inactivated in stages during feed processing; nevertheless, it has been reported that the addition of sodium hypochlorite or hydroxide for a period of 4 h can work as an inactivation method[18].

It has been reported that the maximum levels allowed within the European regulation in finished feed for T2 toxin range from 0.2 to 2 mg/kg of finished feed[19,20] and a concentration of 4.97 mg/kg of feed has been established as LD_{50}[1] and 10 mg/kg as LD_{PV}[2][18]. It should be considered that T-2 toxin can generate interactions in the presence of other mycotoxins, which will be additive in the presence of deoxynivalenol (DON), ochratoxin A (OTA) and fumonisin B1 and synergistic in the presence of nivalenol and aflatoxins[21-25].

Metabolism

The main pathways by which T-2 toxin will be metabolized in poultry are mainly de-epoxidation and de-acetylation[26]. In the case of de-epoxidation, which is the most common pathway, the result will be the loss of the "epoxy" group, which confers toxicity to the mycotoxin, while during the loss of the "acetyl" group, the result will be the obtaining of secondary metabolites of the toxin, such as HT-2 and T2-tetraol[6].

Mechanism of action and main effects in poultry

T-2 toxin and its derivatives base their toxicity on the negative alteration it will cause on protein, DNA and RNA synthesis, as well as on the cell cycle in different types of cells and

its ability to induce apoptosis and necrosis, as well as lipid peroxidation, mainly in labile or actively producing cells. It interacts with the peptidyl transferase of the ribosomal 60s subunit, inhibiting the formation of new peptide bonds[27]. Apoptosis caused by T-2 toxin was evidenced in cell lines such as Vero and human hepatocarcinogenic[28]. In addition, it has been reported that T2 toxin reduces production parameters, with oral exposure to the toxin being the main route of access to the body[18].

Effects on the immune system

In general, T-2 toxin has a time/dose-dependent immunosuppressive effect, either high concentrations for a short period of time or low concentrations continuously[29]. The presence of leukopenia has been reported, leading to an increased susceptibility to secondary infections (*Listeria monocytogenes* and *Salmonella* sp). T-2 toxin has also been associated with a decrease in the amount of antibodies against Newcastle disease and infection of the bursa of Fabricius[18]. In addition, it has been described that it can act as an immunostimulant by increasing IgA levels; this is related to the abnormal and transient activation of genes involved in the inflammatory response[26,27]. It is also mentioned that it can alter the maturation of antigen-presenting cells by modifying antibody levels by lymphocyte proliferation, leading to an increase in susceptibility to infectious agents[30].

Effects on the digestive system

Although T-2 toxin has a rapid absorption in the gastrointestinal tract and an elimination of approximately 80 to 90 %[19], its toxicity is mainly effected during the enterohepatic circulation, and the negative effect it has on the liver is the decrease of the enzymatic activity necessary for the metabolism of toxic substances and the induction of lipoperoxidation, which will consequently result in the formation of free radicals[18,31,32]. It is important to mention that the alteration on mucous membranes will be an evident characteristic related to the diagnosis of mycotoxicosis caused by trichothecenes, especially by T-2 toxin, this related to the caustic effect of the toxin, and it will be visible particularly in the oral cavity, being observed as dermonecrotic lesions that will go from a whitish coloration at the beginning of exposure to a black coloration in chronic exposures. Necrotic lesions may also be observed in gizzards, intestinal mucosa, proventriculus and liver[33,34].

Effect on the nervous system and productive performance

The effect on the nervous system occurs due to the inhibition of protein synthesis, which leads to an increase in the concentration of the amino acid tryptophan, a precursor of serotonin, which in turn increases the concentration of serotonin, thus causing the activation of serotonergic neurons[18,35]. The synergistic presence of DON with T-2 toxin can increase this effect, and anorexia, locomotor problems and vomiting can be observed[36,37]; lesions

present in the oral cavity should also be considered, which will contribute to the decrease in consumption, thus affecting body weight at the end of the cycle, also observing a deficient uniformity in the flock[19,38,39]. In the case of laying hens, a decrease in egg production, as well as in shell quality and hatchability, can be observed[38].

Deoxynivalenol

Deoxynivalenol, also known as DON or vomitoxin, is a common fusariotoxin in production poultry, which may be more resistant than other consumption species[24,21,40]. Deoxynivalenol can be found in finished products such as pasta, bread, cookies, and beer. Although it is considered a teratogenic mycotoxin, it has not been classified as carcinogenic, mutagenic, or genotoxic[41].

Characteristics and metabolism

DON is a stable organic polar compound with high resistance to acidic pH media and high temperatures (up to 180 °C). Its structure contains three free hydroxyl groups associated with its toxicity[42]. The lower susceptibility of poultry has been related to their low percentage of intestinal absorption, which is approximately 5 to 20 %, while their excretion is 78.6 to 98.5 %, mainly through the bile duct, in a period of 24 to 72 h[43,44]. In the case of poultry, the main metabolic pathways of DON are sulfation, glucuronidation, and de-epoxidation[45]. When DON is metabolized through acetylation processes, compounds such as 3-acetyl-DON and 15-acetyl-DON will be obtained, which can also be found in feeds, and their importance lies in the fact that they can easily reverse DON and recover its toxicity[46]. Other compounds derived from deoxynivalenol are DON-3-glucoside, as well as masked compounds that have gained importance for their ability to recirculate in the body, recovering their toxicity characteristic; in addition, they have been recognized as having the capacity to produce mixed toxic effects, causing adverse effects on animal welfare and productivity[15,47,48].

Inhibition of protein synthesis

Inhibition in protein synthesis occurs when DON forms a bond through the binding of its epoxide group with the ribosomal 60s fraction, causing an alteration in the binding of the latter with the 40s fraction, preventing the translation of messenger RNA and preventing the binding of amino acids to the polypeptide chain, either in elongation or during the termination of protein synthesis, also generating an increase in the amount of polyribosomes (80s), since the uncoupling of messenger RNA and the release of the peptide chain are inhibited[46]. It has also been reported that it can alter the activity of the enzyme ribosomal peptidyl transferase by forming peptide bonds between amino acids[46,49]. It is worth mentioning that changes in

the conformation of ribosomes can induce a stress response, thus leading to the activation of mitogen-activated protein kinases (MAPKs)[50].

Effect on the immune system

An increase in serum IgA concentration has been observed, which has been related to the inhibition of protein synthesis of protein components necessary for the transport of IgA to the hepatobiliary system[11]. Alterations in humoral immunity due to DON intoxication have been determined by evaluating the response to vaccination, mainly against Newcastle disease virus and infectious bronchitis virus, and a decrease in antibody titers has been observed. In the case of cellular immunity, induction of apoptosis in leukocyte cells such as T and B lymphocytes and macrophages, alteration of the activation of CD4 and CD8 T lymphocytes to CD4+ and CD8+, and a reduction in the serum concentration of TNF-α[51] have been observed. DON has the ability to alter the expression of genes that code for the production of pro-inflammatory cytokines and chemokines[52]. This has also been related to the activation of genes that also code for the activation of cyclo-oxygenase-2 (COX-2) and nuclear factor Kappa of activated B cells (NF-KB)[26].

Effect on intestinal health

Deoxynivalenol is known to be a potent anorexic and emetic compound due to the alteration in the regulation of several signaling pathways. These alterations can affect the secretion of anorexigenic or orexigenic hormones, such as serotonin, released by enterochromaffin or Kulchitsky cells. It has also been observed that DON, by causing severe suppression of cytokine signaling processes, generates the activation of pro-inflammatory cytokines, affecting growth hormone signaling by suppression of two proteins, which are the hepatic acid-labile subunit as insulin-like growth factor and insulin-like growth factor I[52]. In addition, an increase in transepithelial electrical resistance (TEER) in jejunum is mentioned, mainly attributed to the decrease in space between the tight junctions and a decrease in paracellular permeability to ions[53].

Effect of DON on productivity

In the case of laying hens, it has been reported that under a concentration of 2 to 3 mg DON/kg of feed, a slight decrease in egg production can be observed; however, fertility and hatchability can be maintained unchanged at this same concentration[45,46]. It is important to mention that the effects may vary according to the dose ingested, and with the consumption of high concentrations (5 to 10 mg/kg of feed) of deoxynivalenol, diarrhea may be observed, preventing the animal from correctly assimilating the nutrients, delaying its growth, and causing unevenness in the flock[54-56]. In the case of consumption of low concentrations, anorexia and stunted growth have been observed[6,46].

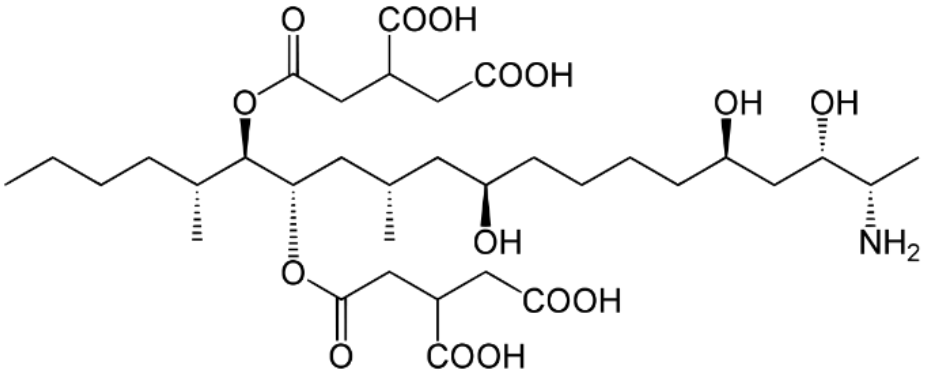
Fumonisins

Fumonisins are a group of toxins produced by several species of fungi (Table 1), within which the main producers of fumonisins are: *Fusarium verticillioides* and *F. proliferatum*[57]. These fungi will be found as common contaminants of substrates such as corn, rice, sorghum, barley, peanuts, and cotton, where their optimal growth will be in temperatures between 22.2 and 27.5 °C with an AW of 0.97-0.98 for the production of the toxin[58-60].

Main features and metabolism

There are six types of fumonisins, B1, B2, B3, B4, A1 and A2 (Table 3); nevertheless, the most important due to their level of incidence and toxicity are B1 and B2. The basic structure of fumonisins consists of a 20-carbon alkylamine, with one or two hydroxyl groups and one or more methyl groups or esterified tricarballylic acid[61]. Fumonisins are polar compounds that are soluble in water and organic compounds such as methanol and acetonitrile, but are insoluble in non-polar compounds, which in turn facilitates their elimination from the body[6]. In the case of poultry, it is now known that, like pigs, acetylated or hydrolyzed derivatives (HFB1) are also generated as a product of acetylation after hydrolysis, as part of the toxicity mechanisms associated with their metabolism in the body[62-65].

Table 3: Basic chemical structure of fumonisins B1, B2, B3, and B4



Fumonisin B1	R1=OH; R2=OH; R3=OH
Fumonisin B2	R1=OH; R2=OH; R3=H
Fumonisin B3	R1=H; R2=OH; R3=OH
Fumonisin B4	R1=H; R2=OH; R3=H

Adapted from [66,96-98].

Mechanism of action of fumonisins

The mechanism of action of fumonisins is based on the interference in the metabolism of sphingolipids by competitive inhibition with sphinganine from *de novo* synthesis and

sphingosine from sphingolipid turnover, generating an accumulation of sphingoid bases, blocking the synthesis of complex sphingolipids through the inhibition of the enzyme ceramide synthase[15,66,67]. In addition, sphingosine and sphinganine have pro-apoptotic, cytotoxic, and growth-inhibiting effects[68]. Sphingolipids can act as first and second messengers in a variety of signaling pathways and play a vital role in the formation of membrane microdomains called lipid rafts[69]. Ceramide is involved in the processes of cell differentiation, senescence, and death. Sphingosine 1-phosphate (S1-P), on the other hand, promotes cell survival and proliferation[66,67]. The immediate consequence of the inhibition of ceramide synthase is the accumulation of the sphingoid base that functions as a substrate, (Sa) sphinganine and, to a lesser extent, (So) sphingosine[66]. The liver and kidneys are the main target organs, although variations have been observed depending on the species, dose, and sex[70-74]. The gastrointestinal tract can also be a target organ for fumonisins since glycosphingolipids bind to sites for microbial pathogens and their toxins, through the inhibition of ceramide synthase in the digestive tract, it can alter the expression of glycosphingolipid binding sites or the transport of microbial toxins, and consequently the sensitivity of animals to infectious agents[17].

The different effects of fumonisins on different productive species have been reported, and it has been observed that they can vary from alterations in productive variables to changes in biochemical parameters and immune response.

Effect on production parameters

Although the negative effect on productivity has generally been observed in high concentrations, it has also been reported that, in concentrations around 5 ppm, it can cause low uniformity in production parameters, mainly on body weight at the end of the cycle in broilers[75].

Morphological and blood biochemistry alterations

The morphological alterations that have been observed in the case of production poultry are a decrease or increase in relative weight in organs (heart, liver, spleen, bursa of Fabricius, proventriculus)[76-78], hydropericardium, fatty liver or friable liver, hyperplasia of bile ducts, cardiac degeneration and necrosis, and loss of tonicity in gizzard and proventriculus[77,79]. In addition, an increase in serum calcium and cholesterol values and a decrease in liver enzyme values (aspartate amino transferase, alanine amino transferase, lactate dehydrogenase, γ glutamyl transferase) have been observed, suggesting an injury to hepatic metabolism[80,81].

Effect on immune response

Among the lesions that have been observed as part of the damage of fumonisins to the immune system in poultry, hemorrhages, leukocyte infiltrations, fat infiltration, necrotic lesions, fibrosis in the liver, kidneys, lung, heart, intestine, gizzard, bursa of Fabricius and pancreas, as well as edema and hemorrhages in the brain are described. Cortical atrophy in the thymus, multifocal hepatic necrosis, and biliary hyperplasia have also been observed, leading to lymphoid depletion[82]. On the other hand, a reduction in the size of the spleen may occur along with depletion of white pulp, thinning of cardiac myocytes, lymphoid cell depletion in the bursa follicles, and renal tubular nephrosis at dosages exceeding 150 mg/kg of feed[83-85]. Studies on the effect of fumonisins have been conducted using high concentrations of fumonisin; however, it has been reported that, on average, a concentration of between 3 and 5 mg/kg of fumonisin B1 has been found in the main components of diets intended for animal feed, such as corn[86].

Zearalenone

Zearalenone (previously known as F-2 toxin) is a non-steroidal estrogenic (mainly in pigs), hematotoxic and genotoxic (rodents) fusariotoxin. The main fungi that produce zearalenone are *F. graminearum*, *F. oxysporum*, *F. roseum*, which require temperatures of between 21 and 25 °C and an AW of approximately 0.87 for the production of the toxin. In particular, it can be found contaminating corn, barley, rice, and soybeans, as well as finished products such as meals and beer[87]. In the case of poultry, it is considered that the consumption of a high concentration is required to observe negative effects on production; in any case, it has been reported that turkeys and ducks are the most susceptible species in poultry, as is the case with other mycotoxins[88].

Metabolism and chemical structure

The characteristics of zearalenone allow it to be a compound of rapid biotransformation and excretion. This means that it is not easy to find zearalenone in poultry products[89]. Zearalenone has a percentage of absorption by the digestive tract of approximately 10 % in poultry, and a high percentage of elimination of both zearalenone and its conjugation metabolites (approximately 65 %)[90,91]. The structure of zearalenone is composed of a resorcyclic lactone ring like its main derivative, zearalenol (α-zearalenol), which will be a derivative with a higher degree of toxicity than zearalenone. In the case of poultry, the main pathways involved in zearalenone metabolism are sulfation and glucuronidation[6].

Mechanism of action

The main mechanism of action used by this mycotoxin is the disruption of endocrine metabolisms, this is done through interaction with nuclear estrogen receptors (ERs), with a direct effect on transcription (which is estrogen dependent) in the nucleus (mechanisms of competition with the same estrogens); although the structure of zearalenone is not similar to that of estrogens, it can place an OH group belonging to its lactone ring in an advantageous position for its interaction with estrogen receptors[92].

Alterations caused by zearalenone in poultry

In the case of roosters, a reduction in the size of the testicles can be observed, which can microscopically show fatty degeneration and atrophy of the germinal epithelium. Finally, production parameters can also be observed to be decreased, related to a decrease in feed consumption[93].

Conclusions

It is important to correlate the effects produced experimentally, which are generally based on the use of concentrations much higher than those found naturally in the feed, and generally continuously for long periods, and not to underestimate the presence of more than one mycotoxin that can enhance the individual effect of each of them. It should also be considered that, in the case of poultry, pathways of absorption, distribution and metabolism of toxins may be different from other species. As already described, fusariotoxins can lead to large economic losses in production, either due to the alteration of production parameters or their alternate effect on intestinal integrity and immune response, therefore, it is necessary to make a correct diagnosis if the presence of mycotoxins in the feed is suspected.

Conflict of interest

The authors declare no conflict of interest.

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Review

Contribution of forage grasses to biological nitrogen fixation and their response to diazotroph inoculation. Review

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Abstract:

The use of chemical inputs has led to the loss of microbial diversity involved in the N cycle, such as diazotrophic bacteria, which are inhibited by saturation of the receptors responsible for activating nitrogenase. Biological nitrogen fixation (BNF) in forage grasses can be used as an ecosystem service. The aim of this review was to analyze the contribution of forage grasses to BNF and their response to inoculation of non-symbiotic diazotrophs in order to find study opportunities. The analysis of the information was carried out using the prisma methodology of systematic reviews and meta-analyses. It should be noted that the main forage species that contribute to BNF are *Brachiaria* sp. and *Pennisetum* sp. The inoculation of *Azospirillum* sp. has generated a growth-promoting effect in grasses, but the response of

the inoculated forage depends mainly on the synergy between plant and bacteria, showing neutral, antagonistic, and positive effects.

Keywords: Fertilization, Nitrogen fixation, Forage, Nitrogenase, Pastures.

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Introduction

In livestock systems, animal feed is economically viable when the ration is mainly made up of forage. Nonetheless, it is necessary to produce grass in an eco-efficient scenario to compensate for the environmental footprint caused by livestock farming, considering that in Colombia it occupies 80 % of agricultural land[(1)]. The proposed strategies include the use of improved forage species, diversification of the system[(1)] and the utilization of natural phenomena such as biological nitrogen fixation (BNF)[(2)]. This is a process in which diazotrophs transform atmospheric nitrogen (N) into ammonium from the nitrogenase enzyme complex, and contributes about 62 %, which is equivalent to 11.29 million tonnes (Mt) of nitrogen per year, which enters the Latin American agricultural ecosystem, while chemical fertilization contributes approximately 6.81 Mt N per year[(3)]. BNF is a resource that can be used as a technological tool to reduce the application of nitrogen fertilizers of synthetic origin that have low efficiency (approximately 40-50 %) and contribute to the emission of greenhouse gases (ammonium, ammonia, and nitrous oxide)[(4)] and soil salinization[(5)]. Nevertheless, little is known about the contribution of forage grasses to BNF and the bacterial species with the best productive effect. Therefore, this paper aimed to analyze the contribution of forage grasses to BNF and their response to the application of biofertilizers constituted by non-symbiotic diazotrophic bacteria, pure and in consortium, based on a systematic review of literature to find study opportunities.

The prisma methodology of systematic reviews[(6)] was used; the databases consulted were Scopus and Web of Science; for the search for information, the following criteria were established: a) specificity, based on the use of Boolean operators, b) sensitivity, with CAB descriptors; c) comprehensiveness, through the verification of descriptors of interest. The search strategy was based on the following routes: TITLE-ABS-KEY (“Biofertilizer”) and TITLE-ABS-KEY (“Biofertilizer and Grass”). With the general search, a total of 6,813 records were found between the Scopus (n= 4,621) and Web of Science (n= 2,192) databases. The search was limited to the Boolean connectors “Biofertilizer and Grass” from which 128 records were found (Scopus: 84 records and Web of Science: 44 records), which were

imported into the Mendeley software and grouped by years; the analysis was limited to the period 2012-2022 (n= 80 records), then duplicate documents were removed (n= 2 records). Articles evaluating the effect of the application of biofertilizers on forages or the contribution of nitrogen fixed by these plants were included in the analysis. Publications with a title outside the search of interest (n= 5) and with only descriptive information that did not meet the inclusion criteria (n= 13 records) were excluded. Each record was independently reviewed by all authors for a total of 50 studies included within the review. The results of the analysis were defined as: a) Nitrogen fixed by forage grasses, b) Biofertilizers applied and their effect on forage grasses. The data of interest in the study (fixed nitrogen and plant effect) were tabulated and grouped by topic to measure their effect. A nonlinear regression analysis was performed with the number of records obtained from the sigmoidal models 3,4, Gompertz 3, and Hill 3. The models with the highest fit were selected based on the significance value and fit of the coefficient of determination to establish the overall trend of the area of interest.

Biological nitrogen fixation in forage grasses

In this review, it was identified that the test of choice for determining nitrogen fixed by forage grasses is natural abundance of ^{15}N[7]. In the main studies reporting N fixed by forage, it is highlighted that the rate of N fixation differs between species (Table 1). This has a direct relationship with the populations of diazotrophic bacteria that interact with each type of forage, in *Brachiaria* sp., approximately 10^2 to 10^8 CFU g^{-1} soil are estimated[8]. On the other hand, in *Pennisetum* sp., the diazotrophic bacterial population is reported to be 10^2 to 10^6 CFU g^{-1} soil[9].

Table 1: Some reports of forage species contributing to biological nitrogen fixation according to the review analysis

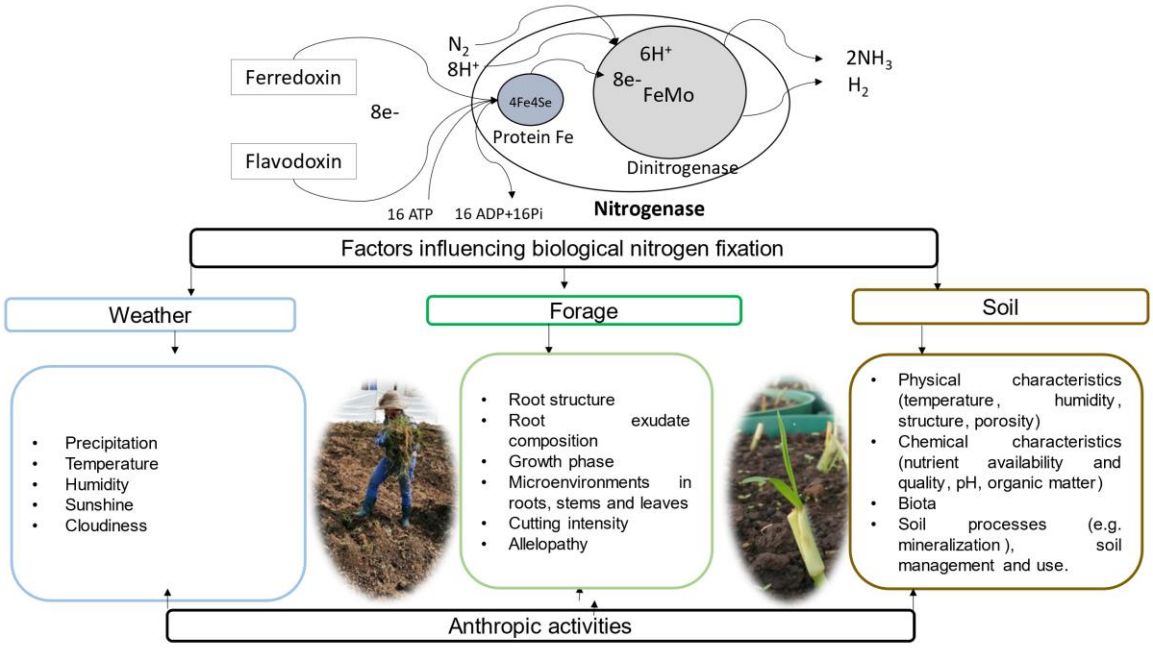
Crop	N fixed (%)	Source
Aristida laevis	36	Marques AC, *et al*[2]
Pennisetum purpureum	18-70	De-Morais RF, *et al*[10]
Megathyrsus maximus sp.	16 - 39	De-Carvalho EX, *et al*[11]
Brachiaria sp.	5.1 – 45	Leite RDC, *et al*[12]
Miscanthus giganteus	16	Leite RC, *et al*[13]

Source: prepared based on the indicated citations.

It was found that the main bacterial genera that persist in the rhizosphere and plant tissue of *Brachiaria* sp., *Pennisetum* sp., *Megathyrsus* sp., and *Panicum* sp., correspond to *Enterobacter* sp. (6 %)[10], *Azospirillum* sp. (25 %)[12,13,14], *Azotobacter* sp., *Bacillus* sp. (14 %)[2,15], *Herbaspirillum* sp. (11 %), *Burkholderia* sp. (8 %)[14], *Bradyrhizobium* sp. (6 %), *Klebsiella* sp. (5 %)[11,16], *Sphingomonas* sp. (4 %)[17], other (2 %). However, their distribution in roots, leaves and stems varies by forage species, locality, and soil type[18].

These microorganisms do not cause structural modifications in the plant and are encoded by the *nifH*[4] gene. The BNF process is carried out in sites with lower oxygen saturation to avoid nitrogenase inactivation, such as in clays, or through a reduction in intracellular oxygen concentration through an increase in cellular respiration[19]. During the reaction, eight electrons are pumped at high speed from a donor agent (ferredoxin or flavodoxin) to the nitrogenase enzyme complex consisting of the metalloenzymes dinitrogenase reductase or protein Fe encoded by the *nifH* gene and the dinitrogenase metalloenzyme encoded by the *nifD* and *nifK* genes[20]. Dinitrogenase reductase transfers each electron to dinitrogenase and they are stored in the FeMo cofactor, the binding site of N until it is reduced to NH_3, thus consuming 16 ATP, and producing 2 mol of ammonium and 1 mol of H_2 for each fixed N molecule[21]. As a result of the review, it was found that the differences in fixed nitrogen ranges between forages and species of the same genus are mainly determined by the factors: plant, soil, anthropogenic activities, and climate (Figure 1).

Figure 1: Factors influencing biological N fixation in forage grasses



Source: prepared based on citations[2,5,11,18,21,22].

Effect of climate on forage BNF

Although there are few studies analyzing the effect of climate on the BNF process, it is highlighted that cloudiness has a negative influence on this process due to the lower availability of photoassimilates that are produced in the leaves and distributed to the roots for the formation of rhizo-exudates[23]. The increased production of photoassimilates seems to have a direct relationship with the persistence of inoculated diazotrophs, which favors their

effect; for example, with the application of *Azospirillum brasilense* in *Urochloa brizantha*, it has been observed that at the beginning of the dry season in which solar radiation increases, the mass of the roots of inoculated plants was 27 % higher than in non-inoculated plants, and although during the transition period the production of grass decreased, in inoculated plants, it decreased by only 7 % and its height increased by 16 % compared to non-inoculated plants, due to the greater absorption of nutrients[12]. Similar responses are reported with the application of *Bacillus* sp. on *Megathyrsus maximus*[24].

Ensuring the persistence of diazotrophic communities can reduce dependence on nitrogen fertilization[13]; however, in the rainy season, N fixation due to bacterial effect may decrease perhaps due to the entrainment of microorganisms[1,9]. This may explain why it is reported that at the end of the wet season, root biomass decreases by 15 % in inoculated plants and there is a lower N content in the leaves compared to plants fertilized with N[12].

In N-deficient environments, BNF increases as a control response when there are low mineralization rates[4,25]. Thus, higher accumulated N is reported in autumn than in spring due to the effect of a lower temperature in the forages *Axonopus affinis* (37.6 kg N ha^{-1}.), *Paspalum notatum* (27.7 kg N ha^{-1}.) and *Andropogon lateralis* (1.6 kg N ha^{-1}), estimating that on average, the percentage of N from BNF is 33 %, 22 %, and 25 % respectively[2].

Soil effect on forage BNF

Soil characteristics also influence BNF[22], a greater diversity of diazotrophic populations in soils with high organic matter is highlighted. The persistence of these microorganisms is modulated by the type and quality of nutrients in the soil[22], it is explained that diazotrophs increase their activity with the presence of iron (Fe), molybdenum (Mo), and vanadium (V) because these elements can be exchanged to be part of the nitrogenase structure. This enzyme, when inactivated by oxygen, requires anaerobic microsites to catabolize nitrogen fixation, which is why it seems that in clay soils there is greater chemical and mineral mobilization, and eventually greater BNF[21].

Effect of anthropogenic activities on forage BNF

Soil is a system that is naturally self-regulating, but abrupt changes in its characteristics due to anthropogenic management activities (tillage, fertilization) and use (permanent pastures with and without intervention, livestock) cause imbalance in bacterial communities since they alter the structure of the pores, the availability of elements, the content of organic carbon and the pH, factors that determine the richness, uniformity, and diversity of microorganisms[2,22].

Excessive application of Ca, nitrate and N during fertilization has a negative effect on diazotrophic populations[17]. The main cause is related to soil pH[12,22]; variations of 1.5 in the soil pH value can reduce the growth of microorganisms by up to 50 % in soils with a pH between 5 and 7[12,22]. There are reports of the inhibition of the growth of some microbial populations, such as *Azotobacter*, *Azospirillum*, *Herbaspirillum* and *Gluconacetobacter diazotrophicus*, with high fertilization doses of N[2,10,26], for example, with the application of 430 kg N ha^{-1} in *B. brizantha* and *B. ruziziensis*[27]. Nevertheless, the type and amount of fertilizer applied influences the abundance and diversity of microbial populations; an increase in methanotrophs with inputs greater than 200 μg N g^{-1} of ammonia has been observed when the active site of ammonia monooxygenase is exceeded[28]. In general, the structural modification of the bacterial community is a natural mechanism for controlling the nitrogen status in the soil[2].

Effect of the plant factor on forage BNF

The morphophysiological characteristics of grasses generate dissimilar microenvironments in leaves, stems, and roots, which promote the selective growth of members of the bacterial population during the growth phase[4]. In the early phase, the activity of rhizospheric diazotrophic populations is greater due to an increase in rhizo-depositions as a mechanism for plant recovery after grazing[13]. The interaction between diazotrophic bacteria and plants occurs through rhizo-depositions that include several molecules such as sugars, polysaccharides, inorganic organic acids, amino acids, vitamins, flavonoids, siderophores, peptides, proteins, and fatty acids[29]. These chemical signals control the interactions that take place in the soil and are responsible for promoting the selective growth of members of the rhizospheric community and allow the movement of bacteria to the plant root and root hairs[2,4]. The diverse functional capacity of diazotrophic bacteria allows them to modulate the growth response of forage and generate positive, negative, or neutral interactions. The main findings in relation to forage response with diazotroph inoculation are discussed below.

Biofertilizers made up of diazotrophs that have been used in grasses

From 1985 onwards, the first scientific studies in the area of biofertilizers applied to forage were reported, although historically it is a practice that dates back to 500 B.C., originating in India, a country that continues to lead scientific advances with a 30 % global share, followed by Brazil (10 %) and China (8.8 %). In the area of biofertilizers applied to forages, authors such as Gupta *et al*[4], Li H *et al*[15] and De Sousa *et al*[30] stand out. Rapid growth is estimated in the area with an inflection point by 2034 (Table 2), a projection that shows the existence of study opportunities that are linked to the phenomenon of climate change and the challenge of using sustainable fertilization strategies that reduce the application of chemicals obtained by burning fossil fuels such as urea.

Table 2: Nonlinear regression models obtained for the searches "Biofertilizer" and "Biofertilizer and grass"

Boolean code	Model	Inflection year	Durbin Watson	a	b	R^2	*P*-value
Biofertilizer	Sigmoidal 3 Parameter	2034	1.07	10988	5.7	0.99	0.01
	Sigmoidal, 3 Parameter	2029	1.87	21.42	4.34	0.96	0.01
	Sigmoidal, 4 Parameter	2018	2.89	26.96	5.4	0.90	0.01
Biofertilizer and grass	Gompertz, 3 Parameter	2018	2.93	33.68	10.42	0.90	0.01
	Hill, 3 Parameter	2016	0.82	21.34	92.85	0.58	0.01

Source: Authors' own preparation.

The trend of biofertilizer use in forages is sigmoidal with an inflection point towards the year 2029, as observed in the logistic model with the highest fit that obtained a Durbin Watson value close to 2[(7)], although the prediction by the Gompertz and Hill models is earlier, they have a lower fit (R^2), therefore, they do not predict reliable behavior (Table 2). The inflection point is associated with the rapid growth phase of the technology and corresponds to the maximum value of the curve from which biofertilizer-related publications are expected to begin to decline. These predictions with high variation are related to areas of application in increasing development, and organic fertilization is beginning to gain importance in the livestock sector due to the rise in the cost of chemical fertilizers.

From the review analysis, it was found that biofertilizers used in pastures have been applied by seed inoculation in the product for 30 min to 24 h, followed by a drying time prior to sowing[(2,31)] or by spraying in dosages ranging from 200 – 500 ml of inoculant ha^{-1} diluted in water at 0.1 - 1.3 % in a minimum concentration of 10^6 CFU ml^{-1} or 10^6 CFU g^{-1}[(32-36)].

The inoculation of microorganisms can modify the development of forage with high variability between genera and strains applied or even cause no effect or generate a negative response[(35)] (Table 3). When biofertilizers have been applied together with a synthetic N source, responses greater than or equivalent to the application of 100 % of the N requirement have been achieved due to more efficient absorption, reducing N losses caused by leaching by up to 95 %[(36)]. The best results in terms of production and economy have been observed with the combined application of the inoculant and N[(36-40)].

Table 3: Some studies of the effect of diazotroph application on forage grasses

Forage	Inoculant	Percentage increase in biological parameters compared to non-inoculated plants	Source
Brachiaria decumbens	*Herbaspirillum rubrisubalbicans* and *H. seropedicae*	12 % in crude protein	(1)
Megathyrsus maximus	*Bacillus* sp. and *Bacillus megaterium*	7.32 %, 25.3 % , 3.32 %, 20.3 %, 2.43 % in height, root biomass, digestibility, protein and neutral detergent fiber, respectively	(15)
Avena saliva L.	*Klebsiella* sp.	20 % in biomass	(16)
Panicum virgatum L.	*Burkholderia phytofirmans*	27 % in height	(19)
Brachiaria ruziziensis	*A. brasilense*	31.49 % in the relative content of water in leaves	(27)
Lolium multiflorum	*Pseudomonas fluorescens* and *Bacillus subtilis*	63 and 51 % in the production of dry mass of plants and biomass, respectively	(32)
Brachiaria brizantha	*Burkholderia pyrrocinia* and *Pseudomonas fluorescens*	770 %, 300 %, 17 % in root biomass, dry matter and chlorophyll, respectively	(33)
Panicum virgatum L.	*Azospirillum brasilense*	23 % in biomass	(34)
Avena saliva L.	*Sinorhizobium meliloti*, *Bacillus megaterium*, *Enterobacter* sp., *A. chroococcum*, *Pseudomonas* sp.	10.34 and 28.92 % in height and root length (28.92 %)	(37)
Pennisetum clandestinum	*Klebsiella* sp., *Beijerinckia* sp., *Achromobacter* sp.	52 %, 170 %, 134 % in shoot length, shoot dry weight and root length, respectively	(41)
Megathyrsus maximus	*Bacillus* sp.	30.8 % and 12.7 % in biomass production and height, respectively	(42)
Avena saliva L.	*Providencia rettgeri*, *Advenella incenata*, *Acinetobacter calcoaceticus*, *Serratia plymuthica*,	81.19 %, 26.89 %, 10.94 % in height, root length and chlorophyll, respectively.	(43)

	Acinetobacter calcoaceticus		
Avena saliva L.	*Bacillus thuringiensis* and *B. thuringiensis*	92 % in germinated seeds	(44)
Phleum pratense L.	*Bacillus subtilis*	26.6 % and 63.8 % in shoots and roots, respectively	(45)
Pennisetum purpureum Schumach	*Sphingomonas, Pantoea, Bacillus* and *Enterobacter*	116.01 % increase in shoot dry weight	(46)
Sorghum bicolor L.	*Azotobacter* sp. and *Burkholderia* sp.	21.5 % and 16.8 % in crude protein and dry matter digestibility, respectively	(47)

Source: prepared based on the indicated quotations.

The positive response of the plant with the inoculation of diazotrophs is mainly due to two main conditions; first, because it favors the availability of nitrogen in the soil, which is an element that is part of proteins, amino acids, DNA, RNA, cytochromes, nucleic acids, and chlorophyll[2,21]; and second, because of the production of secondary metabolites of bacterial origin such as: a) auxins that are involved in cell growth, differentiation, and division[16], b) gibberellins, which are hormones involved in the regulation of cell division and elongation, seed germination, bud appearance and stem growth[48], c) cytokines, which are related to the regulation of cell growth[48], d) siderophores, which are compounds that can bind to iron, making it available for use in metabolic processes[26] and e) biosurfactants, which are chemical agents that form micelles and allow better interaction between the membrane of microorganisms and nutrients dissolved in the soil and in rhizo-depositions[49].

Of these biomolecules, auxins are the most studied; indole-acetic acid stands out, which is synthesized from tryptophan, which can be derived from the following pathways: indole-3-acetonitrile, indole-3-acetamide, indole-3-pyruvic acid or tryptamine[48,50]. This hormone is produced by some diazotrophs, for example: *Stenotrophomonas* spp., *Pseudomonas* spp.[49], *Azospirillum* spp.[51], *Azotobacter* spp., and *Pseudomonas* spp.[26]. Its main effect is related to the modification of the structure, elongation and increase of forage root biomass[37], which favors the absorption of nutrients.

The hormonal stimulus that can be indirectly caused by the application of diazotrophs to the plant can favor its phenotypic plasticity in shady environments[23], in drought conditions[15] or saline soils[46]. Physiologically, tolerance to stress conditions is related to an increase in the activity of the superoxide dismutase and catalase enzymes that eliminate H from free radicals generated under stressful conditions[32]. An increase in the contents of proline, glutathione reductase[42], and ACC-deaminase[46] has also been reported.

On the other hand, greater availability of N in the soil due to bacterial effect allows the plant to increase the production of chlorophyll as it is part of its chemical structure, which leads to an increase in the photosynthetic rate of the plant and consequently in the production of biomass[32]. Compositionally, it can promote the crude protein content of forage[1] and the production of unsaturated fatty acids[14].

Despite the aforementioned synergisms, antagonistic responses are reported with the inoculation of diazotrophs[2], due to the effect of nitrogenase inactivation due to exposure to high doses of N. Nevertheless, the lack of response may also be due to a low dose of inoculant applied[23], which can be inhibited by allelopathic control of the plant, which generates low survival, adaptation, and persistence of the inoculated microorganisms. In fact, the variability among the ecosystem can limit the response of bacteria because the BNF process occurs only in favorable environments that allow the persistence of the alpha-proteobacterial taxonomic group[9,51].

Conclusions

BNF is the main source of N in perennial meadows where synthetic N is not applied and in areas of severe drought where the plant manages to maintain its growth thanks to structural adaptations such as the reduction of aerial material to increase root length. The specific signaling mechanisms that allow the expression of proteins for the production of hormones and enzymes that make these modifications possible and potentiate microbial communities specialized in BNF to favor plant survival under extreme conditions are unknown. However, it has been identified that the species of *Brachiaria* spp. and *Pennisetum* spp. have high potential to contribute to the BNF process due to the persistence of alpha proteobacteria in the rhizosphere and in the tissue of roots, stems, and leaves.

Azospirillum spp. and *Azotobacter* spp. are highlighted, but of these, *Azospirillum brasilense* has the greatest potential to fix N due to the ability to infect forage tissue, which eventually facilitates its survival. Nonetheless, it is unknown whether the colonization of this isolate along with other endophytic microorganisms resists the plant's defense system during prolonged exposure times, and perhaps this is related to the lack of productive response with the application of some inoculants. This is why the biotechnological development of these products aims at the study of native microorganisms to avoid a negative allelopathic response by the plant.

Increased dry matter with the application of biofertilizers is the main response observed according to the review analysis, this effect may eventually allow shorter grazing intervals and the intensification of rotations in livestock systems. It has also been observed that the application of diazotrophs can stimulate the phenotypic plasticity of the plant in shaded

conditions, which is why the use of biofertilizers can be a cost-effective option in silvopastoral systems.

There are still challenges such as ensuring positive interactions between applied microorganisms and native strains, developing biofertilizers combined with chemical fertilizers and biostimulants, reducing the technical costs of isolation, massification and obtaining the final product, formulating products by crop and according to the stage of growth, using monitoring methods for the detection and quantification of persistent bacterial populations that allow adjusting the dosage and frequency of use of biofertilizers according to management, crop, environmental conditions and soil type, and encouraging their application in farm systems as an ecosystem service.

Acknowledgements and conflict of interest

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Technical note

Frequency of seropositivity against porcine circovirus type 2 (PCV2) in the metropolitan area of Monterrey, Nuevo León, and its peripheral area

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Abstract:

Porcine circovirus type 2 (PCV2) is a DNA-type virus that has an affinity for cells of the immune system and generates lymphocyte depletion so it favors the development of diseases caused by other opportunistic agents, it is also related to the generation of different syndromes. Therefore, this virus causes major illnesses on the pig industry; nevertheless, PCV2-associated syndromes can easily be prevented through the proper application of biosecurity and vaccination measures. On the other hand, small-scale production units (SPUs) often lack this type of preventive management, as well as routine surveillance by a veterinarian. Although PCV2 is considered a widely distributed virus, there are no reports of its presence in SPUs in Nuevo León. The presence of antibodies against PCV2 was determined using a commercial kit and complete blood count was performed on the animals. A total of 48 SPUs were found, with 91.67 % positivity and 89.7 % seropositivity in the animals. In the complete blood count, it was found that HGB and HCT were decreased in individuals who were positive for antibodies compared to negative ones (P=0.03 and P=0.01, respectively); on the contrary, the value of total white blood cells was found to be decreased in individuals who were negative for the presence of antibodies against PCV2 (P=0.01).

Keywords: Backyard, PCV2, Porcine circovirus type 2, ELISA, Seroprevalence, Pigs.

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Porcine circovirus type 2 (PCV2) is a single-stranded DNA virus that only infects pigs, so it has no zoonotic significance. PCV2 is a pathogen that is implicated in the development of different syndromes, such as post-weaning wasting syndrome, dermatitis and nephropathy syndrome, as well as reproductive failure[1]. It is recognized that infection with this virus is a predisposing factor for syndromes, however, it requires co-infection with another pathogen to trigger the disease process. Among the risk factors that increase the possibility of introducing the agent into the pig population and its dissemination are: low birth weight, low weaning weight, as well as those related to facilities and management practices, such as housing a large number of animals in a small space, contact between pigs and hygiene[2].

PCV2 has an affinity for the cells of the immune system, especially macrophages and lymphocytes[3]; in fact, one of the expected clinical findings during infection by this virus is the reduction of total leukocyte counts, as well as lymphopenia[1,4,5]. Among the diagnostic methods for this virus are PCR and immunohistochemistry (IHC)[6]; for the latter, lymphatic tissue is used to detect antigens of the virus, which indicates the presence of the virus in the target cells. It has been confirmed that lymphocyte depletion in lymphatic tissue is related to the activation of apoptosis through the pathways of caspases 3 and 8 within lymphocytes[3], although it is not ruled out that there are other mechanisms involved. Lymphocyte depletion induces a state of immunosuppression that is also aggravated by co-infection with other pathogens, such as porcine parvovirus, PRRS virus, and others[7], which allows the appearance of the aforementioned syndromes.

In Mexico, small-scale production units (SPUs) are still present in some areas. The characteristics of these systems present in the metropolitan area of Monterrey, Nuevo León, have previously been researched, and among them, the lack of biosecurity, medication and routine surveillance by a veterinarian has been confirmed. The aforementioned conditions favor the entry of pathogens into production units, as well as their subsequent perpetuation in the environment; nevertheless, PCV2 is especially important since being immunosuppressive, it allows the appearance of clinical manifestations of secondary infections in some cases[8-10]. Therefore, this study aimed to determine the presence of antibodies against PCV2 in backyard pigs that did not have previous vaccination against this pathogen, so a cross-sectional study was carried out, which included pigs in SPUs from 9 municipalities corresponding to the metropolitan area of Monterrey, Nuevo León.

The small-scale production system (also known as backyard or artisanal) was defined as one in which pig rearing activities will be carried out in the home of the owners of the animals, or as a complementary economic activity, that is, one that will not represent the main family income. The minimum number of samples was calculated with the WinEpi software under the following considerations: an expected prevalence of 92 % was considered, which was taken from a previous report in Mexico[11], a margin of error of 5 %, and a confidence level of 95 % for an unknown population, yielding a minimum number of 114 samples. Once the SPUs were identified, permission was requested from the owners to take samples from the animals and a questionnaire was applied at the end. Samples were collected from May 2019 to March 2020. Pigs of different ages were included; however, sampling in pregnant females was avoided to prevent the risk of abortion, as well as in suckling piglets to avoid detection of maternal antibodies.

For sampling, the pigs were physically restrained. During immobilization, the body condition of each individual was rated on a scale of 1 to 5. Two blood samples were taken from the jugular vein, one in a collection tube with EDTA and the other in a collection tube with serum separator. The samples with EDTA were processed in the clinical laboratory of the Veterinary Hospital of Small Species (HVPE, for its acronym in Spanish) of the Autonomous University of Nuevo León under the standard procedure on the KONTROLab 5R+Vet equipment.

The serum was separated from the clot at 1,000 rpm for 10 min at 4 °C and then fractionated into 500 µL aliquots and stored at -80 °C until later use. For the detection of antibodies against PCV2, a commercial kit (Bio Check®) was used according to the manufacturer's instructions. This kit has a sensitivity of 92.1 % and a specificity of 95.6 %. The absorbance of the samples was read at 405 nm on the Awareness technology Chromate® equipment (Awareness technology Inc.).

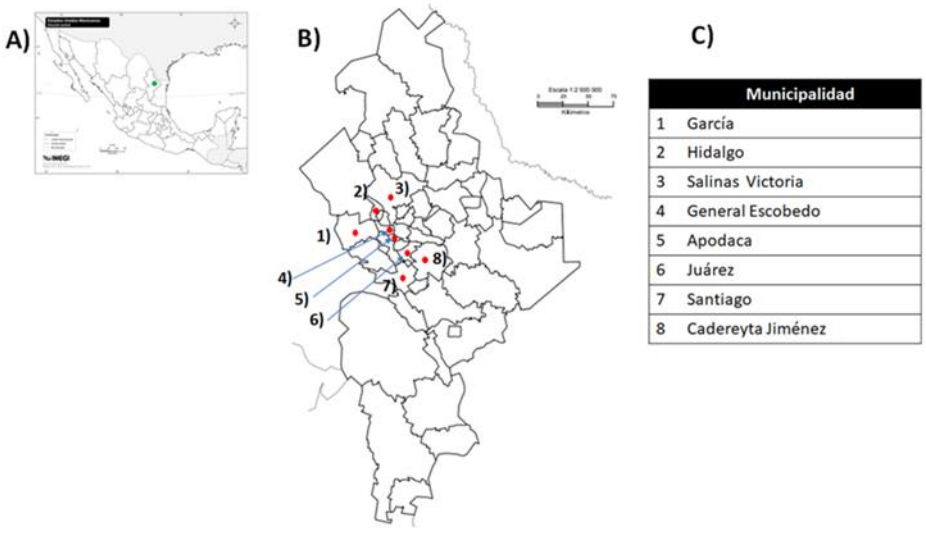
Data were captured in a spreadsheet to determine the percentage of seropositivity. A Student's T-test was performed to determine the difference between the means of the hematological parameters between the antibody-positive and antibody-negative groups, as well as of the frequency of the animals' body condition. Statistical analysis was performed using the GraphPad Prism 6 software (San Diego, CA). A P-value ≤ 0.05 was considered significant.

There were localized 48 SPUs, in which access to sample was allowed, which were in the municipalities of Apodaca, Cadereyta Jiménez, García, General Escobedo, Hidalgo, Juárez, Santiago and Salinas Victoria. It was found that, in 44 of the sites sampled, at least one animal tested positive for antibodies, so the percentage of positivity was 91.67 %. The rest of the production units corresponded to four sites where no animal was detected as positive for

antibodies against PCV2. In all municipalities, it was possible to detect positive production units.

A total of 204 animals were sampled, of which the presence of antibodies against PCV2 was confirmed in 183, resulting in 89.7 % seropositivity. Some studies have previously been carried out in Mexico, where 92.29 % positivity for antibodies against PCV2 was determined among animals and 98.14 % positivity among production units with at least one positive animal[11], so, in congruence with findings of other authors, seropositivity against this virus was found ubiquitously in the SPUs in the metropolitan area of Monterrey, Nuevo León, and its peripheral area.

Figure 1: A) Map of Mexico. B) Map of Nuevo León. Each red dot represents a sampled municipality. C) Identification of sampled municipalities



	Municipalidad
1	García
2	Hidalgo
3	Salinas Victoria
4	General Escobedo
5	Apodaca
6	Juárez
7	Santiago
8	Cadereyta Jiménez

On the other hand, different research groups have explored the presence of PCV2 through the use of real-time PCR; an example is in Brazil where they have found the presence of the virus genome in 15.6 %[8] of the lung samples studied. On the other hand, by using quantitative PCR, a 90 % prevalence has been detected in Colombia by using white blood cell samples[9]. In Spain, another group of researchers have taken on the task of identifying the presence of the virus in technified production units in different areas using quantitative PCR in environmental samples, which included swabs from the surfaces of pens, workers' boots and even inside the offices of five production units[12], finding a 42.9 % positivity rate, thus reiterating the easy spread of this virus and its wide dissemination in the environment.

The seroprevalence results obtained were entered into the WinEpi platform, and the sensitivity and specificity specifications provided by the kit manufacturer were also entered

in order to estimate the positive and negative predictive values. The platform yielded a positive predictive value of 99.9 % and a negative predictive value of 25.4 %, as well as an actual prevalence of 97.3 %.

In this study, pregnant females were not sampled due to the risk of inducing abortions or, where applicable, preterm births. Nonetheless, there are studies in the context of other infectious agents, such as influenza A, in which it has been shown that females with a higher number of births have a greater immune experience due to their age, as well as a higher concentration of specific antibodies[13]. Therefore, it is to be assumed that, if positivity was found in animals of other ages, there is also seropositivity among females. On the other hand, pigs are animals that are born agammaglobulinemic unless they are exposed to an agent *in utero*, therefore, the intake of colostrum is important for their survival, since in this way they acquire IgG and IgA from the mother[14]; in this study, piglets that had not been weaned were not included because the presence of maternal antibodies can be detected through the ELISA method without representing seroconversion due to exposure to the agent.

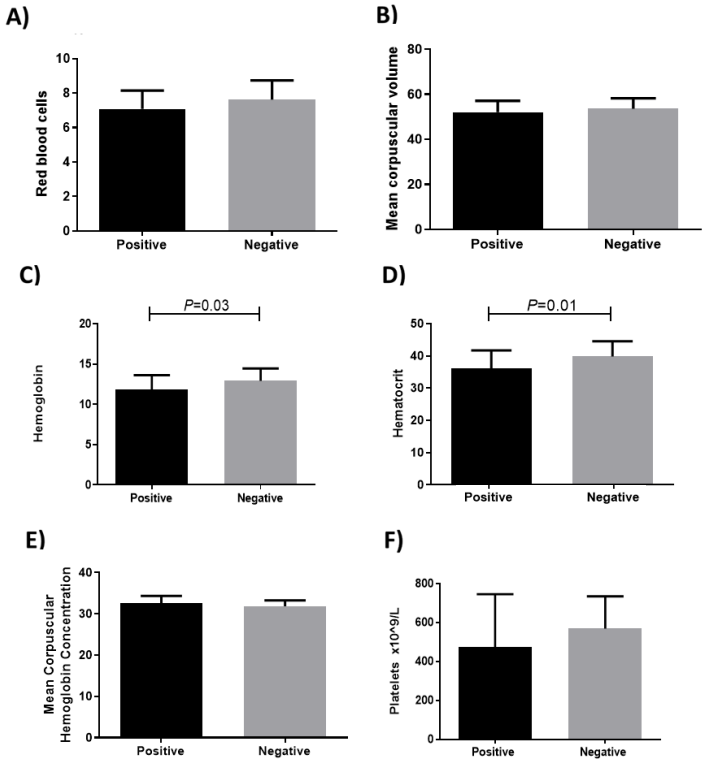
Regarding the body condition of the animals, only one animal with condition of 1 (0.41 %), 49 animals with condition of 2 (20.16 %), 86 with condition of 3 (35.39 %) and 107 animals with condition of 4 (44.03 %) were observed. No animals with a condition of 5 were observed. Mean body condition in the antibody-positive group was compared with the negative group (n= 139 animals), but no difference was found ($P>0.05$). Since vaccination against PCV2 was not reported for any of the animals, it is presumed that the presence of antibodies is due to seroconversion due to previous immune experience against the field virus, however, these antibodies could be fulfilling a protective role against the development of PCV2-associated syndromes. It has been shown that vaccination does not always prevent viremia, but it does reduce systemic viral load in vaccinated individuals[15]. In addition to the above, a group of researchers demonstrated that vaccination has a positive effect on the cellular and humoral immune response even in animals that previously had viremia[16], that is, that had been infected before being vaccinated.

Although a high frequency of antibody positivity was found in this study, no animals with apparent clinical symptoms were found. Most of the animals had medium to good body condition; it was not possible to differentiate antibody-negative animals from positive ones by body condition either. Other researchers have found that the presence of the virus on farm is not necessarily compatible with the presence of PCV2-associated syndromes[12], and although this virus is recognized as necessary to trigger associated syndromes, its presence alone is not sufficient to produce the disease[17].

On the other hand, the means of the hematological parameters in the antibody-positive group were compared with the negative group, and in terms of the red line, a significant difference was found for hemoglobin (HGB) ($P=0.03$) and hematocrit (HCT) ($P=0.01$), which were

found to be decreased in the positive group compared to the antibody-negative group (Figure 2).

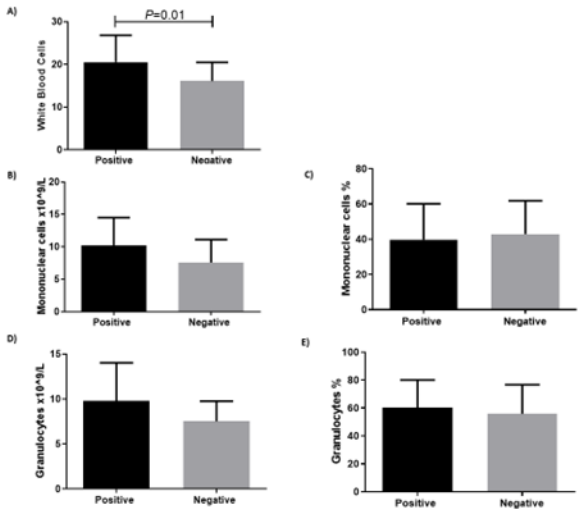
Figure 2: Comparison of hematological parameters in the red line



The number of individuals included in each analysis were: A= 124, B= 124, C= 141, D= 141, E= 141, F= 141.

For the white line (Figure 3), a decrease in total white blood cells was found in the group negative for antibodies against PCV2 compared to those who were seropositive (*P*=0.01). Although there were found differences in the decreased parameters of HGB and HCT in the positive individuals and total white blood cells in greater amounts in the antibody-positive compared to the negative ones, all three parameters were within the expected normal ranges in both groups. Interestingly, it was found a decrease in the total leukocyte count in the antibody-negative group; nevertheless, the finding suggests that these pigs could be in a state of infection and even viremia in which they have yet to develop antibodies; likewise, it must be taken into consideration that most of the individuals who remained negative for the presence of antibodies were in places where at least one positive animal was found, so it is very likely that they will have contact with the virus at some point in their lives.

Figure 3: Comparison of hematological parameters in the white line



The number of individuals included in each analysis were: A= 147, B=81, C=134, D= 81, E= 134.

In conclusion, there is presence of antibodies against PCV2 in a large proportion of the SPUs and in the pigs within them, which are located in the metropolitan area of Monterrey, Nuevo León, and its peripheral area; however, this does not imply the presence of clinical symptoms.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Technical note

Prevalence and infection intensity of honey bee (*Apis mellifera*) viral diseases in six regions of the state of Jalisco, Mexico

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Abstract:

Jalisco is one of the foremost honey-producing states in Mexico. However, there is no information on viral diseases that affect honey bees (*Apis mellifera*) in the different beekeeping regions of the state. The objective of this study was to determine the prevalence and intensity of four viral diseases of *Apis mellifera* during the spring, in six regions of Jalisco. Bee samples from 79 colonies were analyzed, of which, 66 % and 38 % were positive for black queen cell virus (BQCV) and deformed wing virus (DWV), respectively. Two viral diseases were not detected, those caused by the Israeli acute paralysis virus (IAPV) and the chronic bee paralysis virus (CBPV). The infection levels of BQCV were relatively low but elevated for DWV, with infection intensities 8,000 higher than those of BQCV. The prevalence of DWV was significantly higher in the regions of the Highlands, Center, and South, while for BQCV there were no differences between regions. For infection intensity, there were no differences between regions for DWV, but there were for BQCV. The regions with the highest infection levels were the South and Center. Surveys during other seasons of the year are recommended to identify possible seasonal viral effects on the bees and to design control strategies.

Keywords: *Apis mellifera*, Deformed wing virus, Black queen cell virus.

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Viral diseases of honey bees (*Apis mellifera*) are increasingly being associated with colony losses[1], so it is important to know their prevalence and distribution to be able to control them. More than 20 viruses are known to infect honey bees, but few of them appear to have a serious impact on their health. Among them, we can mention deformed wing virus (DWV), black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), and chronic bee paralysis virus (CBPV)[2,3]. In Mexico, the presence of DWV, IAPV and BQCV[4,5] has been reported in the high plateau region. Moreover, DWV and IAPV were identified in *Varroa destructor* mites, with DWV being the most prevalent in both, bee and mite samples[4]. Not much is known about honey bee viral diseases in Mexico, and the information about them, is limited to a few regions of a few states. For Jalisco's case, there are still no official reports about the distribution and levels of viruses in honey bee colonies of the state's different beekeeping regions. It would be important to know this information because Jalisco is one of the foremost honey producing states in Mexico, ranking third in 2021 with 6,073 t[6].

For beekeeping purposes, Jalisco has been divided into six different regions that vary in topography and climate, including the regions of the Highlands, Center, North, Sierra Amula,

South and Southeast. More than half of the state's producers and hives are located in the South and Southeast regions[7]. Because there is no information on the prevalence and infection intensity of honey bee viral diseases for the different regions of Jalisco, it seems relevant to conduct surveys to identify and quantify viruses in honey bee colonies from those regions, as well as to find out if there is a relationship between viruses and regions. Therefore, the objective of this study was to determine the prevalence and infection levels of the main viral diseases that affect adult honey bees, including DWV, BQCV, IAPV and CBPV, in samples of bees from colonies of six regions from Jalisco, Mexico.

Adult bee samples were collected during early spring, in the months of March, April and May 2018, from 12 to 16 colonies of each of the six beekeeping regions of Jalisco. In each apiary, three colonies were randomly selected and sampled. A total of 81 colonies from 27 apiaries were sampled, although data were obtained from only 79 colonies. Two samples of three bees each were collected from the entrance of each hive. The bees were introduced into 2 mL microfuge tubes containing RNAlater® (Thermo Scientific; Mississauga, ON, Canada) to preserve viral RNA. The samples were transported in coolers with freezing packs and were stored at -70° C until processed.

The molecular analyses to diagnose and quantify viral infections were conducted at the Honey Bee Research Centre, School of Environmental Sciences, University of Guelph, in Guelph, Ontario, Canada. First, the presence of DWV, BQCV, IAPV and CBPV, was determined by RT-PCR. RNA was extracted from three bees per sample with TRIzol (Fisher Scientific; Mississauga, ON, Canada), as per the manufacturer's instructions. cDNA was synthesized with the RevertAidTM H Minus First Strand kit (Fermentas; Burlington, ON, Canada), following the manufacturer's instructions.

The PCR reactions were carried out using a Master thermocycler (Eppendorf; Mississauga, ON, Canada). Each reaction contained 1.5 µL of 10x pH buffer for PCR (New England BioLabs; Pickering, ON, Canada), 1 µL of both primers (10 mM), 0.2 µL 5U/µL of Taq polymerase (New England BioLabs; Pickering, ON, Canada), 2 µL of cDNA and 8.8 µL of dH_2O. The primer sequences and amplification cycles used were those described in previous studies for DWV[4,8], BQCV[9], IAPV[10] and CBPV[11]. The PCR products were separated by electrophoresis on agarose gels and the amplified bands were photographed with a digital camera under UV light. Additionally, viral copies of DWV and BQCV were quantified with real time PCR (qRT-PCR). The other two viruses were not detected and therefore, not quantified. The calibration standard curve for DWV and BQCV was created using a 300 bp synthetic gene fragment or gBlock® (Integrated DNA technologies; Coralville, IO, USA) for each virus. The lyophilized of the synthetic genes (500 ng) were diluted with 50 µL of nuclease free dH_2O to obtain an initial concentration of 10 ng/µL that was used for serial dilutions from 10^9 to 10^2 viral copy numbers.

The qRT-PCR reactions were done using a BioRad CFX96™ thermocycler (Bio-Rad Laboratories; Mississauga, ON, Canada) with PowerUp™ SYBRgreen™ (Supermix 2X) (Applied Biosystems; Foster City, CA, USA) on 96-well PCR plates (Hard-Shell®). The reactions had a final volume of 20 µL that contained the following. For DWV, 10 µL of Supermix 2X (Applied Biosystems; Foster City, CA, USA), 0.4 µL of both primers (200nM), 7.2 µL of nuclease free dH_2O (Invitrogen; Burlington, ON, Canada) and 2 µL of cDNA or the synthetic gene dilutions. For BQCV, 10 µL of Supermix 2X, 0.8 µL of primers (400nM), 6.4 µL of nuclease free dH_2O and 2 µL of cDNA or the synthetic gene dilutions. The primer sequences and amplification cycles were those described in previous studies for DWV[12] and BQCV[13].

The thermocycler software calculated the efficiency, determination coefficient (R^2), and the slope of the viral RNA standard curve. To calculate the amount of viral RNA in the serial dilutions the following equation was used:

Number of viral RNA copies = (ng of synthetic gene) ($6.022x10^{23}$) /(length of synthetic gene) ($1x10^9$) (650 D). Where: 650 D is the average weight of a base pair and $6.022x10^{23}$ is the Avogadro number[14]. A graph using Ct values with the initial number of RNA viral copies and the number of DWV and BQCV copies of the samples was calculated using a regression equation.

To determine if there were differences between regions for viral prevalence, the data were analyzed with comparative tests for equality of proportions, using the Benjamini-Hochberg correction. Also, the data on infection intensity were subjected to Shapiro-Wilk and Bartlett tests to analyze the assumptions of normality and homoscedasticity, respectively. The data did not comply with the assumptions and thus, were log transformed and subjected to analyses of variance. When significance was detected, the regional means were compared with t tests using the Benjamini-Hochberg correction. All the statistical analyses were performed with the R 3.3.1 program (Foundation for Statistical Computing, Vienna, Austria).

Two viruses were detected in the honey bee samples from all regions of Jalisco, BQCV (Figure 1) and DWV (Figure 2). The other two viruses, IAPV and CBPV, were not detected. Of the detected viruses, the prevalence of BQCV at the state level was 66 % and that of DWV was 38 %. The prevalence and intensity of the viral infections identified are shown in Table 1.

Figure 1: Photograph of an agarose gel that shows bands of 698 bp of black queen cell virus (BQCV) in columns 1, 2, 5, 6, 7 and 8. A honey bee gene (RpS5) is used as a control in the RT-PCR reaction

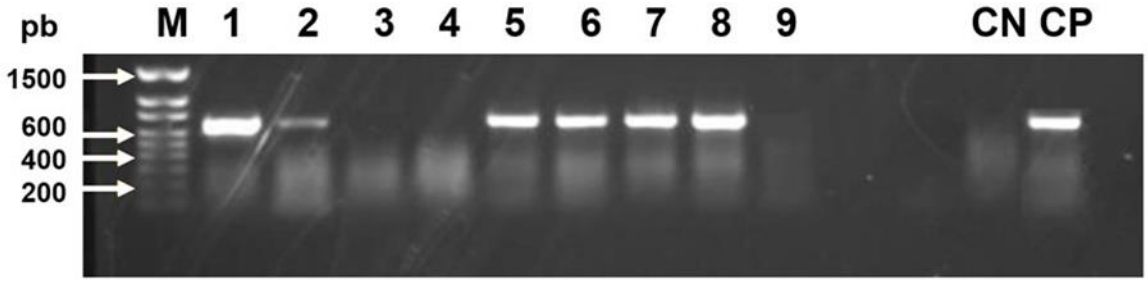

Figure 2: Photograph of an agarose gel that shows bands of 642 bp of deformed wing virus (DWV) in columns 2, 4 and 6. A honey bee gene (RpS5) is used as a control in the RT-PCR reaction

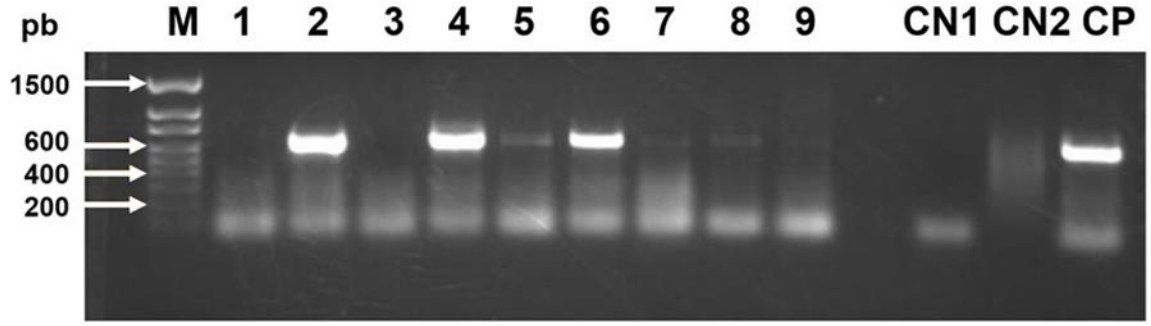

Table 1: Prevalence and mean intensity of viral infections that affect honey bee colonies in the state of Jalisco, Mexico

Pathogen	N	Prevalence (%)	Intensity ± E.E.[1]
Deformed wing virus	79	38.0	4,083.40 ± 2,676.05[1]
Back queen cell virus	79	65.8	0.49 ± 0.23[3]

[1] Number of viral copies per µg of RNA x 10^6

At the regional level, DWV prevalence in colonies from the Southeast and North regions was only 8 %, which was significantly lower than those of colonies from the South, Sierra Amula, and Center regions (P<0.05, Table 2). For DWV infection intensity, there were no significant differences between colonies of different regions ($F_{5,73}$= 0.64, P=0.67).

Table 2: Prevalence and mean infection intensity of deformed wing virus (DWV) in adult workers of honey bee colonies in different regions of the state of Jalisco, Mexico

Region	N	Prevalence (%)	Intensity ± E.E.[1]
Highlands	12	33.3 [a,b]	368.81 ± 239.91
Center	12	66.7 [a]	955.33 ± 611.20
Sierra Amula	15	60.0 [a]	14,652.31 ± 13,740.36
North	12	8.3 [b]	176.67 ± 85.12
South	16	50.0 [a]	955.19 ± 476.21
Southeast	12	8.3 [b]	5,778.83 ± 3,860.24

[1] Number of viral copies per µg of RNA x 10^6.

[ab] Different literals indicate significant differences based on tests for equality of proportions using the Benjamini-Hochberg correction (P<0.05).

BQCV was detected in 42 to 81 % of the colonies from the different studied regions, but there was no significant difference between regions for BQCV prevalence (P>0.05, Table 3). However, for BQCV infection intensity, regions varied significantly ($F_{5,73}$= 7.14, p< 0.01). For example, the South region had colonies with infection intensities significantly higher than those of colonies from the rest of the regions, except the Center region that was second for BQCV levels. The North region had the colonies with the lowest titers of BQCV infections.

Table 3: Prevalence and mean infection intensity of black queen cell virus (BQCV) in adult workers of honey bee colonies in different regions of the state of Jalisco, Mexico

Region	N	Prevalence (%)	Intensity ± E.E.[1]
Highlands	12	41.7	0.12 ± 0.07 [b,c]
Center	12	66.7	0.37 ± 0.21 [a,b]
Sierra Amula	15	66.7	0.06 ± 0.05 [c,d]
North	12	58.3	0.02 ± 0.01 [d]
South	16	81.2	1.94 ± 1.08 [a]
Southeast	12	75.0	0.03 ± 0.01 [d]

[1] Number of viral copies per µg of RNA x 10^6.

[abcd] Different literals indicate significant differences based on ANOVA and t tests using the Benjamini-Hochberg correction (P<0.05) of log transformed data.

In Mexico, there is little information about the presence of honey bee viral diseases, since viruses like DWV, IAPV, and BQCV were molecularly diagnosed for the first time just a decade ago in the Mexican high plateau[4,5], but nothing is known about the prevalence or infection intensity of these viruses in almost all states of the country. In northern Mexico, IAPV, DWV, sac brood bee virus (SBV), Kashmir bee virus (KBV), and filamentous virus (FV) were reported in colonies from the state of Chihuahua, but its prevalence and infection intensity were not determined[15,16]. Therefore, the results of this study are a reference point for future research in Mexico's regions of beekeeping importance.

In other countries of the Americas, several prevalence rates of honey bee viruses have been reported. However, most colonies of other countries have in common the prevalence of DWV and BQCV. For example, in Uruguay, 100% of the analyzed colonies were infected with DWV and BQCV[17,18]. In Argentina and Chile, the most prevalent honey bee virus was DWV, which was detected in 35 y 37 % of the colonies sampled, respectively[19,20]. In Cuba, DWV was the most prevalent virus, which was detected in 91 % of the colonies surveyed, but BQCV was not detected[21]. In Colombia, both viruses were detected at a prevalence of 19.9 and 10.6 %, respectively[22]. In North America, the prevalence of eight honey bee viruses was determined during six years in the USA, and in every single year, DWV was the most common of all viruses at a prevalence that ranged between 65 and 92 %, closely followed by BQCV with a prevalence range of 60 to 92 %[23].

Regarding viral infection intensities, with the exemption of reports from the USA and Canada, no study so far conducted in Central America, the Caribbean, or Mexico, has reported infection levels of honey bee viruses in different regions of a state, like this study does. Therefore, to the best of our knowledge, this is the first study to report infection levels of honey bee viruses at a regional level.

The prevalence of viruses varied between regions. DWV prevalence was significantly lower (8 %) in colonies of the Southeast and North regions than in other regions. Conversely, the prevalence of DWV in the regions South, Sierra Amula, and Center, was over 50 %. However, there were no differences for the intensity of infections of DWV between regions because viral infection levels were high in all regions. No differences in the prevalence of BQCV were found between colonies of different regions, however, their infection levels varied between colonies from one region to another. The colonies of the South region had higher BQCV infection levels than the colonies of the rest of the regions, except for the Center region. Regarding infection intensity, mean DWV infection levels were very high, with 4083.4 X 10^6 viral copies per µg of RNA, whereas for BQCV infection levels were relatively low, with 0.49 X 10^6 viral copies per µg of RNA. Thus, the intensity of DWV infection in honey bees from Jalisco was approximately 8,000 times higher than that of BQCV infection.

Some of the factors that could have influenced the differences in viral prevalence and infection intensity in honey bee colonies between Jalisco's regions include environmental effects, bee genotype, and possibly different viral strains. Regarding climatic effects, it is known that DWV infections are more prevalent and intense in colonies located in temperate climates than in colonies established in tropical climates[24]. The authors of the cited study proposed that this occurs because colder climates favor the transmission and replication of DWV and could reduce the immune responses of bees, making them more susceptible to the virus. The authors also found an effect of the interaction between climate and parasitism by *V. destructor*, a mite that is strongly related to the prevalence and infection intensity of DWV,

since it not only serves as a vector of the virus, but the virus multiplies in the mite's tissues[25,26]. Therefore, colonies with greater *V. destructor* infestation rates tend to have higher DWV prevalence and infection intensity than colonies with low mite infestation levels[27]. Furthermore, the genotype of bees varies with their degree of Africanization. It has been shown that the intensity of infection caused by DWV and BQCV is higher in colonies with bees of European mitotype or morphotype than in colonies with bees of African mitotype or morphotype[28]. It is possible that the colonies that were less infected with viruses in this study, had a greater degree of Africanization than the more infected ones. However, this hypothesis would have to be investigated in future studies.

The high levels of DWV infection found in this study are concerning because if beekeepers neglect their *V. destructor* control measures, the prevalence and intensity of DWV infections could increase. It is known that together with *Varroa* parasitism, this virus can weaken colonies until they collapse[1]. Therefore, it is essential to emphasize the importance of implementing an adequate control strategy for *V. destructor* infestations to keep DWV infections as low as possible in honey bee colonies. Regarding BQCV, although it had a high prevalence in colonies of most regions, its infection levels were low. However, this study was seasonal, and thus, studies would have to be carried out throughout an entire year and for several years, to confirm if it is a virus that could represent potential damage to the beekeeping industry in Jalisco.

In conclusion, the most prevalent honey bee virus in the state of Jalisco was BQCV, which was detected in 66 % of the colonies, while DWV was detected in 38 % of them. Infection levels of DWV were high (8,000 times higher than those of BQCV). The regions with the highest DWV prevalence were Center, South, Highlands, and Sierra Amula. Regarding the intensity of DWV infections, there were no significant differences between regions. There were also no significant differences between regions for BQCV prevalence, but there were for infection intensity of this virus. The regions with the highest infection levels were the South and Center regions. Additional studies are recommended with surveys conducted during different seasons of the year and for several years, to find out under what conditions and seasons, viruses could be harmful to the beekeeping industry, and to design control strategies.

Acknowledgments and conflict of interest

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