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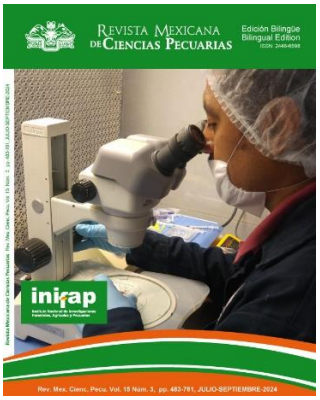

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NOTAS AL AUTOR

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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   Página del título
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   Resumen en inglés
   Texto
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Introducción Material
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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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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.

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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.

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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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17. Los trabajos no aceptados para su publicación se regresarán al autor, con un anexo en el que se explicarán los motivos por los que se rechaza o las modificaciones que deberán hacerse para ser reevaluados.

18.

Abreviaturas de uso frecuente:

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

Cualquier otra abreviatura se pondrá entre paréntesis inmediatamente después de la(s) palabra(s) completa(s).

19. Los nombres científicos y otras locuciones latinas se deben escribir en cursivas.

Updated: October, 2023

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Revista Mexicana de Ciencias Pecuarias is a scientific journal published in a bilingual format (Spanish and English) which carries three types of papers: Research Articles, Technical Notes, and Reviews**.** Authors interested in publishing in this journal, should follow the below-mentioned directives which are based on those set down by the International Committee of Medical Journal Editors (ICMJE) Bol Oficina Sanit Panam 1989;107:422-437.

1. Only original unpublished works will be accepted. Manuscripts based on routine tests, will not be accepted. All experimental data must be subjected to statistical analysis. Papers previously published condensed or *in extenso* in a Congress or any other type of Meeting will not be accepted (except for Abstracts).

2. All contributions will be peer reviewed by a scientific editorial committee, composed of experts who ignore the name of the authors. The Editor will notify the author the date of manuscript receipt.

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4. To facilitate peer review all pages should be numbered consecutively, including tables, illustrations and graphics, and the lines of each page should be numbered as well.

5. Research articles will not exceed 20 double spaced pages, without including Title page and Tables and Figures (8 maximum and be included in the text). Technical notes will have a maximum extension of 15 pages and 6 Tables and Figures. Reviews should not exceed 30 pages and 5 Tables and Figures.

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Title page
Abstract
Text
Acknowledgments and conflict of interest
Literature cited

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   Materials and Methods
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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.

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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

Modeling lactation curves for milk production, fat and protein, and evaluation of factors that affect them in Holstein cattle in Mexico

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Abstract:

The knowledge and modeling of lactation curves make it possible to identify factors that help explain environmental and genetic variations that allow the implementation of a selection program. This work aimed to evaluate different models for milk production, fat, and protein curves in Holstein cattle in Mexico and some factors that affect them. The information used was from 125,982 lactations belonging to 68,804 animals born from 2000 to 2020. The effect of calving number, season of the year, and herd was evaluated. R's Lactcurves package was employed to fit the 38 models included in the package, of which the best four (Wood, Wilmink, Ali & Schaeffer, and modified Pollot) were chosen and then used to model the individual curves through a nonlinear regression model. The parameters

calculated for each model were statistically different among the number of lactations ($P<0.05$), as well as the number of calving, calving season, and herd ($P<0.01$). The modeled curves have similar shapes to those reported in other studies, except those obtained for protein in the third and fourth or more calvings with the modified Pollot model. The equation proposed by Wilmink was the one that presented the best fit for the study population according to the different evaluation criteria. Using the model that best suits the data will give a closer predictions to reality, and it can be applied to different areas, such as genetic improvement.

Keywords: Lactation curves, Milk production, Fat, Protein, Holstein.

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Introduction

The lactation curve, defined as the graphical representation of milk production during the production cycle, can be described through mathematical functions explaining a biological production process subject to genetic and environmental influences[1,2]. Proper modeling of lactation curves allows for a good forecast of total production from partial samples, herd planning based on reliable production prediction, and animal selection through knowledge of the different parts of the curve. Therefore, it is essential to find the mathematical function that best describes the lactation curve of animals in each system of production[2,3].

The lactation curve is usually analyzed through four consecutive sections: a) Initial production, estimated by the average production during days 4 to 6 after the colostrum period, b) Ascending or increasing production phase, which is the rate of ascent, until reaching the maximum level of production, c) Maximum point or peak of production, determined by the highest level of production that the cow reaches within the first 90 days of lactation, and d) Decline or reduction in production, also called persistence, which refers to the decrease in milk secretion from peak production[4].

The use of mathematical models has made it possible to know the lactation curves in different dairy production systems. However, not all populations and production systems adjust to a typical lactation curve, with its different parameters and phases, such as start, ascending phase, peak, and decrease. The parameters of a model that fit the lactation curve must reflect various factors, such as genetic, physiological, productive, and environmental

factors, and the interactions between them[2,3]. Therefore, it is possible to generate as many curves as there are lactations and sources of variation. Hence, it is essential to know the standard levels of milk production by groups of animals with similar characteristics, such as the same lactation stage, calving season, production level, or lactation number[5].

Nonlinear models to represent lactation curves were initially proposed by Wood and have been used in cattle, sheep, goats, buffaloes, and South American camelids[2]. The different mathematical models proposed have presented advantages in the specific modeling of sections of the lactation curve, or they fit correctly to various production systems. For example, Wood's model fits milk production data well, better predicts actual data during early and late lactation, and less accurately predicts data during middle lactation than other nonlinear models[2,6]. Wilmink's model is also widely used to describe lactation curves in dairy cattle, mainly used to detect environmental effects; however, it has been reported that in some populations, this model tends to underestimate the middle part of the curve and overestimate the final part. The Ali-Schaeffer model fits well for lactations that start with low production and peak earlier than usual[7].

One of the main problems with empirical models is that it has been difficult to give physiological meaning to the parameters derived from them. Several modifications have been made to some models in order to have an interpretation closer to the physiological aspects of the lactation curve, such as those proposed by Pollot[8], where the resulting parameters have a biological interpretation, based on changes in the number of cells in the mammary gland during gestation, lactation, and involution, and their effects on milk production[9].

This work aimed to evaluate different mathematical models and some factors that affect the lactation curves of milk production and its components (fat and protein) in a population of Holstein cattle in Mexico.

Material and methods

Data editing and description

The study included information on milk production in kilograms and fat and protein percentages from 68,804 Holstein cows born from 2000 to 2020, belonging to 198 herds of the intensive production system. The data comes from 17 states of the country: Aguascalientes, Baja California, Coahuila, Chihuahua, Durango, Guanajuato, Hidalgo,

Jalisco, State of Mexico, Michoacán, Nayarit, Puebla, Querétaro, San Luis Potosí, Tlaxcala, Veracruz, and Zacatecas, where temperate (central zone) and semi-desert climates (northern zone) usually predominate. Querétaro, Guanajuato, Chihuahua, and the State of Mexico concentrate most information. The Holstein Association of Mexico provided the data. The database excluded lactations that did not have production weighing in the first 30 d, those greater than 500 d, and those that had double or triple peak production since it does not correspond to a standard production curve. Each lactation had information from 4 to 12 weightings, and lactations that had fewer than four useful weightings were eliminated.

The milk days of each weighing were adjusted to minimum and maximum values from 5 to 305 d. When the record was outside this range, it was not included in the analysis. Milk production in kilograms, and fat and protein in percentage were adjusted to the mean ±3 standard deviations. When no fat or protein information was available, information on both components was removed. To define the calving season variable, the animals were grouped into three categories according to the month in which they calved, which correspond to cold, hot, and rainy seasons, respectively. The first group covers from November to February, the second from March to June, and the third from July to October.

After the edition, the study included information from 68,804 Holstein cows, with information from 125,982 lactations (72,979 belonging to the first lactation, 31,371 to the second, 11,922 to the third, and 9,710 to 4 or more lactations), and there were 1,319,810 weightings in total.

RStudio[10] was used to evaluate different mathematical models to describe the representation of lactation curves. A total of 38 models included in the R[11] Lactcurves package were fitted, and the four best models were chosen according to the following selection criteria (Table 1): residual standard error (RSE), coefficient of determination (R^2), adjusted coefficient of determination (R^2 adjus), log-likelihood (LogL), Akaike information criterion (AIC), corrected Akaike information criterion (CAIC), Bayesian information criterion (BIC), and Durbin-Watson coefficient (DW).

The best models were adjusted to lactations per animal by means of a nonlinear (NLIN) regression model using the Statistic Analysis System[12] program. The parameters that describe the curve, persistence, days to the peak, and peak yield were obtained from each curve.

In addition, through the process of generalized linear models (PROC GLM) in SAS[12], it was evaluated whether, in each model, the number of calving, the herd, and the calving season were statistically important in milk production, with the intention of evaluating parameters that could be incorporated into the prediction model. The ggplot package of R was used to plot the curves by lactation.

The Wood[2] model used was as follows:

$y_t = at^b \exp(-ct)$

Where: y_t= milk yield at *t* days in kg, *a*= initial yield, *b*= phase of increase in the curve, *c*= phase of decline in the curve, and *t*= days.

From the calculated parameters, it is possible to estimate the days to the peak ($\frac{b}{c}$), maximum yield at the peak ($a(b\ /\ c)^b\ exp(-b)$), and persistence ($((1\ /\ c)^{b+1}$).

Wilmink's [13] model is described as:

$y_1 = a + be^{-kt} + ct$

Where: y_t= milk yield at *t* days in kg, *a*= initial yield, *b*= phase of increase in the curve, *c*= phase of decline in the curve, *k*=parameter associated with the days to the peak, and *t*= days in production.

The calculated parameters are used to estimate the persistence ($\frac{c*305}{a\times100}$), days to the peak ($\frac{1}{k}log\left(\frac{c}{kb}\right)$), and peak yield ($(a + ck\ (1 + log(bkc))$)[14].

The Ali-Schaeffer model[15] is:

$$y_t = a + b\left(\frac{t}{340}\right) + c\left(\frac{t}{340}\right)^2 + d\ log\ \left(\frac{t}{340}\right) + f\ \left(\frac{t}{340}\right)^2$$

Where: *t*= days in milk, *a*= related to peak production, *b* and *c*= related to decreased production, *d* and *f*= related to increased production.

The modified Pollot model[8] is described as:

$$y_t = \left(a\ /\ 1 + b * e(-c * t)\right)^* (2 - e^{(-d*t)})$$

Where: y_t= milk production at day *t*, *t*= days in milk, *a*= maximum lactation secretion potential, *b*= related to milk production potential, *c*= relative proliferation rate of secretory cell number during early lactation, and *d*= relative decrease in cell number as lactation progresses.

Results

According to the selection criteria, the best-evaluated models were Wood, Wilmink, Ali-Schaeffer, and modified Pollot. Table 1 shows the results of the four models and the values

of the selection criteria for estimating the milk production curves for Holstein cattle in Mexico in the intensive production system. In most of the criteria, Wilmink's model is the one with the best results.

Table 1: Selection parameters of Wood, Wilmink, Ali-Schaeffer, and modified Pollot models in Holstein cattle in Mexico

Models	R^2	R^2 adj	RSE	LogL	AIC	CAIC	BIC	DW
Wood	0.1378	0.138	8.911	-4998010	9996029	9996025	9996078	0.555
Wilmink	0.1381	0.138	8.910	-4997858	9995726	9995721	9995786	0.555
Ali-Schaeffer	0.1380	0.138	8.911	-4997941	9995894	9995888	9995967	0.555
Pollot modified	0.1381	0.138	8.910	-4997874	9995758	9995753	9995819	0.555

R^2= coefficient of determination, R^2adj= adjusted coefficient of determination, RSE= residual standard error, LogL= log-likelihood, AIC= Akaike information criterion, CAIC = corrected Akaike information criterion, BIC= Bayesian information criterion, DW= Durbin-Watson coefficient.

Table 2 shows the results of the ANOVA and Tukey tests for the parameters of the four selected models, differentiated by the number of lactations, and the mean of all the animals. In Wood's model, it is observed that estimators *a*, *b*, and *c* are statistically different between the different lactation numbers, except for the estimator *c* for lactations 3 and 4 or more. The values of persistence, peak production, and days to the peak for each lactation are also presented.

Regarding Wilmink's model, parameter *a* of lactation 1 differed from those of lactations 2 and 3, which in turn differed from that obtained for 4 or more lactations. Regarding parameters *b* and *k*, there were no significant differences between the groups; in contrast, in parameter *c*, lactations 2 and 4 are the same but differ from the rest. Ali & Schaeffer's model shows that parameters *a*, *b*, *c*, *d*, and *f* in lactations 1 and 4 are statistically different from those in lactations 2 and 3.

For the modified Pollot model, it is observed that parameter *a* is different between the first, fourth, and second-third lactations; as for parameter *c*, that of the first lactation differs from that of all the others; as for parameters *b* and *d*, they are different between lactations.

Table 2 also shows the estimated values of persistence, peak production in kilograms, and days to the peak by lactation for Wood, Wilmink, Ali-Schaeffer, and modified Pollot by calving number, as well as the mean for all animals. Figure 1 shows the lactation curves for each of the models.

In the evaluation of factors that affect the lactation curve presented in Table 2, it was found that the number of calving, calving season, and herd are significant ($P<0.05$) in the models used, except for the herd in the Ali-Schaeffer model. Table 3 shows the parameters for fat

and protein with the different models used, where it can be seen that all the parameters are different between the number of calvings ($P<0.05$). Table 4 shows the estimates for the components of the curve with the different models, while Figures 2 and 3 show the curves calculated for fat and protein, respectively. In the modified Pollot model for protein, the parameters did not model a curve in lactations 3 and 4 or more, so it was not possible to obtain the days to the peak, peak production, and persistence.

Figure 1: Lactation curves for milk production by lactation number with Wood, Wilmink, Ali & Schaeffer and modified Pollot models

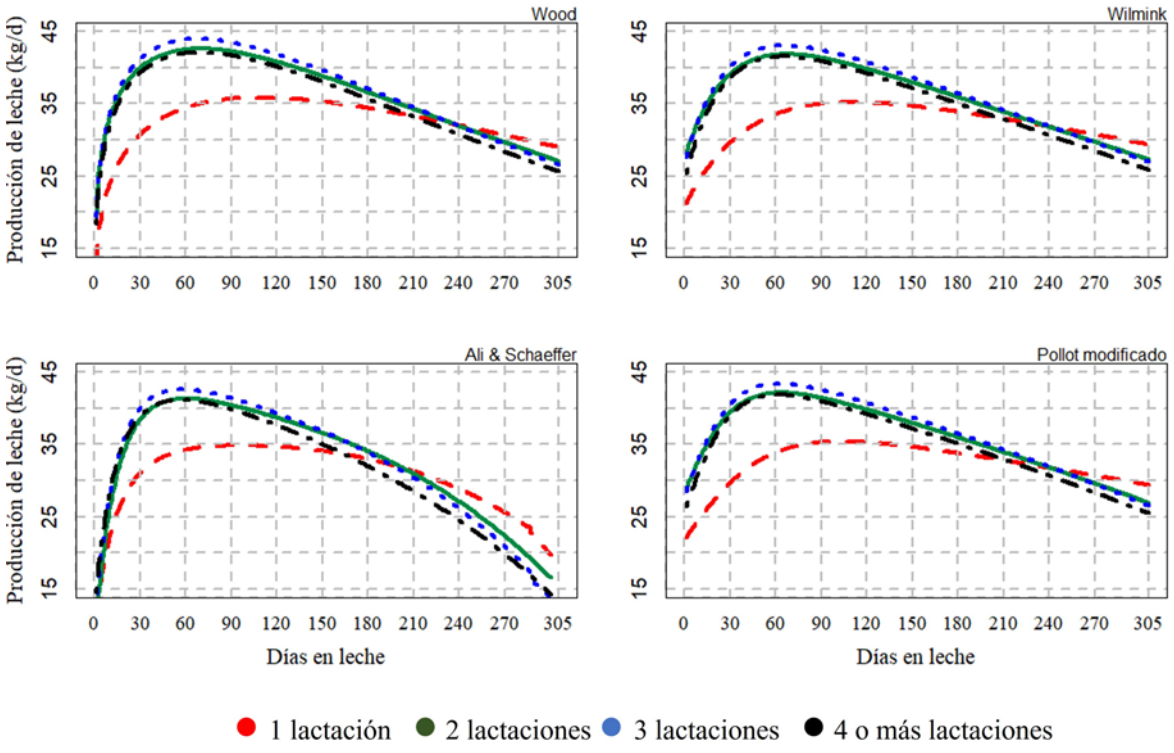


Figure 2: Lactation curves for milk fat percentage by lactation number using Wood, Wilmink, Ali & Schaeffer and modified Pollot models

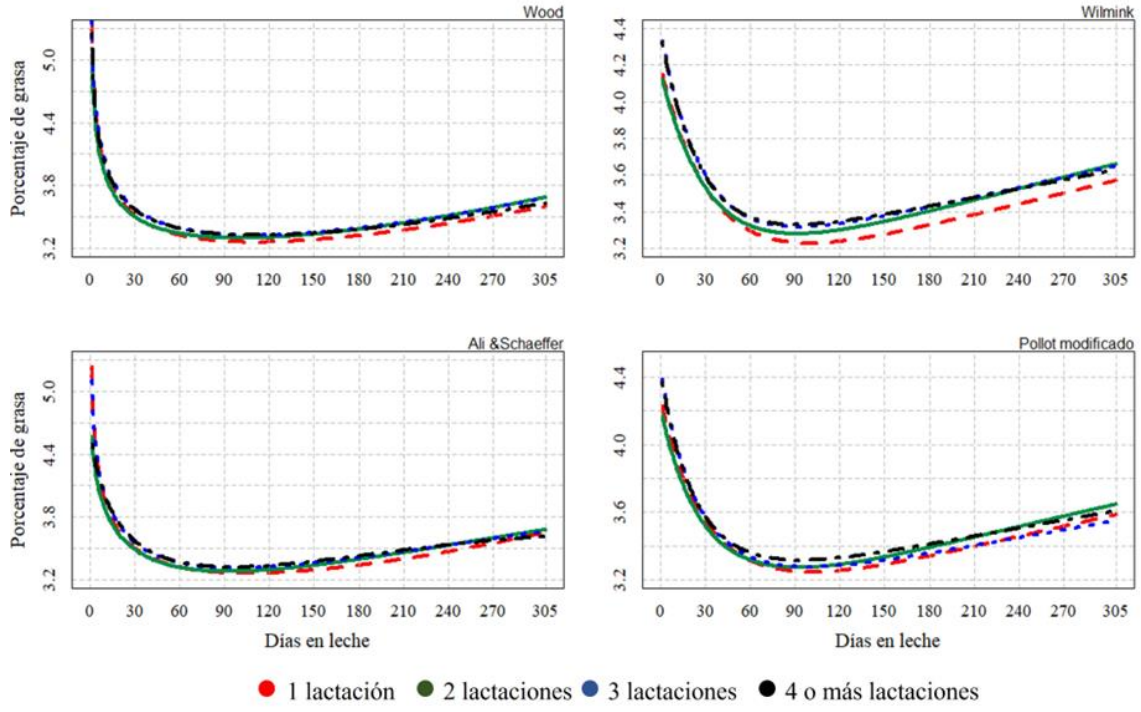
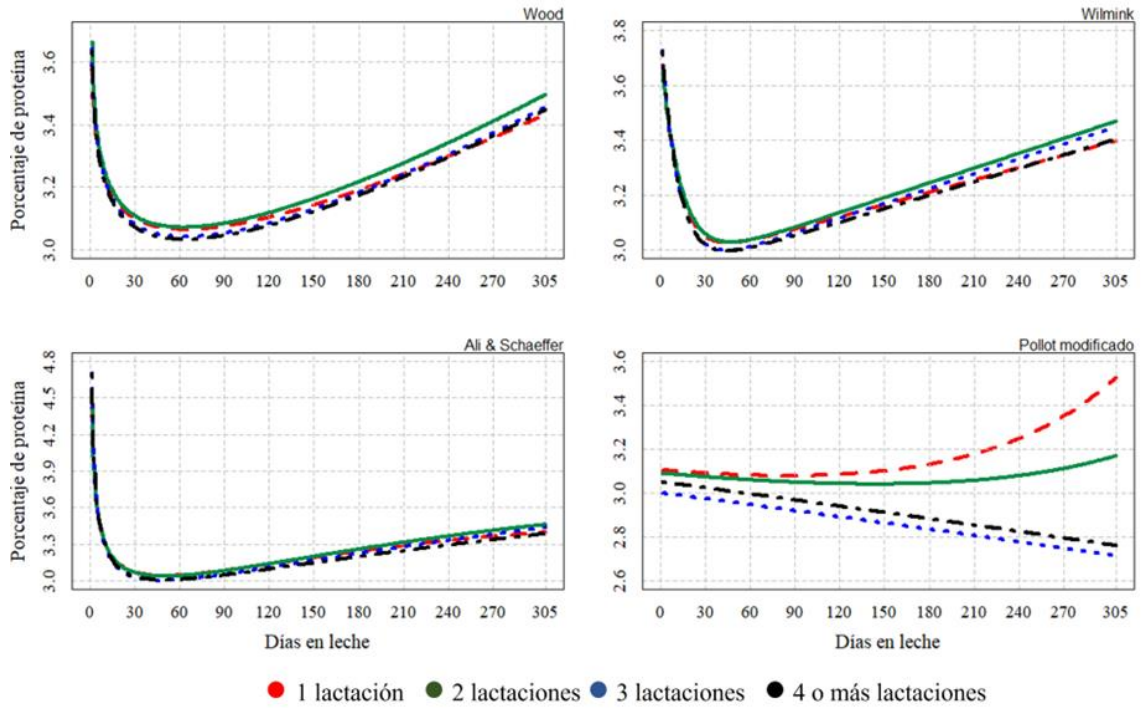


Figure 3: Lactation curves for protein percentage by lactation number with Wood, Wilmink, Ali & Schaeffer and modified Pollot models



Discussion

The mean square error was similar among the different models, being slightly lower in the first lactation. The same is true for the other model selection criteria, with Wilmink's model being slightly better.

The parameters obtained by the model proposed by Wood are different in lactation numbers, results that are far from those found by Duque *et al*[16] with Wood's model in the Colombian tropics with grazing Holstein cattle. Duque *et al* (2018) estimated a mean of parameter *b* (0.12) and peak production (26.5 kg) lower than what was found in the present study; the same happens with the days to reach maximum production among the different numbers of lactations (between 28 to 32 d). It is known that milk production in the tropics is usually lower due to various factors that limit production, such as temperature, where Holstein cows do not adapt adequately to hot climates; in addition to this, being in an extensive system, grazing feed tends to vary at different times of the year. In terms of persistence, they had higher values (66 to 82 %) than reported in this study, which ranges from 15 to 19 %. This may be because they are subjected to less production stress in addition to the variation in diet depending on the time of year and heat stress.

In a study conducted by Vázquez *et al*[17], where they evaluated cows mostly of the Holstein breed under an intensive system in Lima, Peru, the values of parameters *a* and *c* of Wood's model among the different lactations (16.41 to 18.11, and 0.0023 to 0.004, respectively) and the peak production (31.13 to 43.91 kg) are similar to those found in the Holstein population of Mexico. In both studies, the animals were subjected to intensive production systems, and the climatic conditions were similar, corresponding to a subtropical desert climate. For parameter *b* and days to peak production, Vázquez *et al*[17] show lower values (0.1880 to 0.3043 and 66 to 82 d, respectively). This difference could be attributed to the fact that the cows in the Peruvian study were milked 3 times a day, so the amount of milk produced in the first stage of the curve increases compared to cows that are milked 2 times a day, as is the case of the majority of the Mexican population.

In a study by Boujenane & Btissam[18] in semi-intensive production herds in Morocco with Holstein animals, the results show some differences compared to this study. The values by lactation reported in Morocco for parameter *a* in the first three calvings (15.9, 16.9, and 17.2, respectively) present a higher value, especially for first-calving animals. The mean of parameter *b* (0.1039) is the one with the biggest difference, which is reflected in the results of each lactation, where they are also higher (0.073, 0.091, and 0.096 for the first, second, and third lactations). In the same study, the parameter *c* is slightly higher in each lactation.

In terms of lactation components, there are differences in both studies. The three components shown by Boujenane & Btissam[18] (41.4 for days to the peak, 23.6 for peak production, and 6.56 for persistence) are lower than those found in the present study (Table 2), especially in days to the peak and peak yield. This is possibly caused by the semi-intensive production system and the high temperatures of the African country. In general, production in the animals in the Moroccan study is lower.

Regarding fat percentage, Gołębiewski *et al*[19] conducted a study on Holstein cattle in Poland, reporting values of 3.05, -0.07, and 0.04 for parameters *a*, *b*, and *c*, respectively, with Wood's model; and for protein percentage, they report values of 4.59, -0.19, and 0.04, for parameters *a*, *b*, and *c*, values similar to those found in the Holstein population of Mexico (Table 3). Both the Polish and Mexican Holstein animals in both studies were under an intensive system, so the environmental conditions are similar.

Regarding Wilmink's model, the results presented by Bouallega *et al*[20] in Holstein cows in Tunisia, the value of the parameters differs from that obtained in the Mexican Holstein population. The authors show values close to 28 and -7 for parameters *a* and *b*. The parameter *c* was similar to what was obtained (-0.3), while the value of *k* was set to 0.05. The values calculated in the present study for peak production and days to the peak (Table 4) were higher than those presented by the authors (26 kg and 48 d). In terms of persistence, they report values around 94 %. The number of animals used in the study was small (5,649), where the authors mention that a larger amount of data is suggested; the main difference between the cows in the Tunisian research and the Mexican population was temperature. The former were subjected to heat stress due to the climate in Tunisia, which can reduce production in Holstein cattle since animals of this breed do not usually adapt well to this type of climatic conditions.

Regarding parturition, the results found in this study are similar to those reported by Bouallega *et al*[20], which reiterates that the number of lactations is a significant source of variation, showing differences in animals with 1, 2, and 3 or more births, because first-calving animals have not completed the mammary gland maturation process; therefore, their production is usually lower than in subsequent lactations. In addition, Bouallega *et al*[20] recommend using the age at calving as a source of variation. Regarding herd as a factor affecting lactation curves, the aforementioned authors found that it is significant, attributing 30 % of the variation in milk production to it. This highlights the importance of environmental conditions in modeling the production curve, which are different between herds.

The protein percentage parameters show little similarity compared to what was reported in the present study (Table 4). An example is the peak and days-to-peak results shown for Tunisian animals (2.84 % and 53.4 d, respectively), which are lower than those of the

Mexican population. In terms of fat percentage, the biggest differences in comparison to this study are observed in parameter *b* (1.19), where the authors present slightly higher values, while the days to the peak (50.63) they show are much lower than those found in this study (Table 4). The differences in some results for protein and fat may be due to the high Mediterranean temperatures to which the animals were exposed; however, the percentage of fat tends to vary less due to environmental conditions and during lactation than the percentage of protein.

Torshizi *et al*[7] found that, in first-calving Holstein cows under intensive production systems in Iran, herd and calving season are sources of significant variation using Wilmink's model to model lactation curves, similar to what was found in the Mexican population. In addition, they used 4 fixed values for parameter *k* in their analyses (0.05, 0.065, 0.61, 0.10), the first being the one that yielded the highest correlation between the observed and predicted production values.

Regarding the other parameters, they are also very different, being most evident in parameters *b* and *c* (-20.227 and -0.036). Peak day and peak production (66 d and 32 kg, respectively) are lower in Iranian cows. These differences may be due to the length of lactations since Torshizi *et al*[7] study included animals with production cycles adjusted to 200 d, contrary to the usual in Mexico, which is to adjust to 305 d, and minimum productions of 3 kg of milk. This is due to the variation in production due to climatic issues. The authors mention that the best model for first-calving animals is Wood's.

Results presented by Gök *et al*[21], with first-calving Holstein cows in the Turkish province of Konya, where they used the Ali-Schaeffer model, show values similar to those found in the present study for parameter *b* (138). Regarding parameters *a* and *f*, these authors show higher values (-51.92 and -3.62); in contrast, the estimates for parameters *c* and *d* (-648.66 and 32.68) are lower compared to those found in the Mexican Holstein population (Table 4). In the same way, days to the peak and peak production are lower in animals from Konya. The main difference with this study was the production system. Turkish animals were under a grazing system, and where the climate is usually extreme in the different seasons of the year.

In their study of Holstein cattle from Turkey, where they classified the animals by lactation number (from 1 to 3), Koçak & Ekiz[15], by using the Ali-Schaeffer model, reported values for parameter *b* (165.3, 259.3, and 280.9) and *c* (-101.3, -121.1, and -127.0, respectively) similar to those found in this study (Table 2). In contrast, the rest of the parameters were higher in the research carried out in Turkey (-49.0, -55.7, and -50.7 for *a*; 103.36, 126.97, and 41.58 for *c*; -0.10, -6.91, and -14.71 for *f*). These differences in parameters are reflected in the days to the peak, where Turkish cows have their maximum production (74.94, 47.62, and 39.62 for the first, second, and third lactations, respectively) earlier than Mexican

cows, despite the fact that productions at this stage are similar. The animals in Koçak & Ekiz's[15] study belonged to semi-intensive production systems and were milked three times a day; in said study, despite the high environmental temperatures, the houses had temperature regulation systems, unlike what happened in Mexico, where the environmental conditions of the animals are not usually controlled.

On the other hand, Nanda *et al*[22] carried out a study on a housed herd in Indonesia, where they modeled the curves using Ali & Schaeffer's model by calving numbers (from 1 to 4). The parameters *a*, *c*, and *f* in each of the lactations of the cows in Java were higher than the parameters calculated in the present study (-40.79, -16.19, -20.86, and -26.89 for *a*; -16.50, -7.06, -14.74, and -25.00 for *c*; -6.83, -4.59, -4.52, and -4.59 for *f*); with respect to parameters *b* and *d*, they report lower values (68.32, 32.87, 44.25, and 59.15 for *b*; 38.85, 24.43, 25.52, and 26.83 for *d*). These values may indicate that the curves of the animals in the Indonesian study show peak production in a shorter time and yield lower than in the Mexican population. The hot and humid climate of the island of Java may be an important factor explaining the differences in the curves, as these animals were not housed in places where temperature was controlled.

In Holstein cows in Australia under a grazing system in a warm climate, Adediran *et al*[23] used the modified Pollot model and found that parameter *a* (13.36) was lower than that obtained in the present work, while parameter *b* was higher (1.23). Parameters *c* and *d* (2.80 and 0.0012) were similar in both studies. The main difference with the Mexican population is the type of production system. In the study in Australia, as the animals are grazing, there is less control of the environmental conditions, coupled with the intense heat reported by the authors, which may limit milk production, unlike the population in this study, which was in a housed system.

Information on the parameters of the fat and protein production curves with the different models is scarce, especially for Ali-Schaeffer and Pollot modified, so the results were compared with other models; however, it should be noted that the results presented in this study will serve as a reference for these characteristics in intensive production systems.

Conclusions and implications

Of the models evaluated in this study, the one proposed by Wilmink was the one that best fit the data of the Holstein population of Mexico. The importance of choosing a model that best suits the information lies in obtaining more accurate predictions, which translates into values that are closer to reality. In addition, the study evaluated environmental factors such

as calving number, calving season, and herd; they were significant for the modeling of lactation curves, so it is essential to consider them as a source of variation in the predictions made with the different models. Future research could investigate other environmental factors that may affect the curves. The practical application of lactation curve modeling is extensive, including genetic improvement, so having identified environmental sources of variation and choosing the most appropriate model will allow the selection of animals with the highest genetic value.

Table 2: Lactation curves parameters, days to the peak (Dpeak), peak production (peak), and persistence of the curve (persistence) for Wood, Wilmink, Ali-Schaeffer, and modified Pollot by lactation number.

	Lactation	Parameters						Lactation curve components			MSE (kg)
		a	***b***	***c***	***d***	***k***	***f***	**Dpeak**	**Peak**	**Persistence**	
Wood	Mean	14.600	0.380	0.004	-	-	-	85.020	41.140	16.990	
	1	11.920[a]	0.370[a]	0.003[a]	-	-	-	102.970	36.780	15.970	7.27
	2	16.120[b]	0.380[b]	0.004[b]	-	-	-	76.620	43.400	17.730	8.27
	3	15.770[c]	0.400[c]	0.005[c]	-	-	-	74.870	44.370	19.260	9.29
	4 or more	16.290[d]	0.370[d]	0.005[c]	-	-	-	72.760	42.990	15.560	9.64
Wilmink	Mean	258.040	-316.000	-0.337	-	0.027	-	85.940	42.100	-47.440	
	1	282.120[a]	-192.180[a]	-0.320[a]	-	0.017[a]	-	107.340	37.340	-36.0050	7.27
	2	250.040[b]	-432.310[a]	-0.340[b]	-	0.033[a]	-	75.010	44.560	-54.240	8.87
	3	243.320[b]	-453.410[a]	-0.350[c]	-	0.038[a]	-	72.250	46.110	-53.510	9.29
	4 or more	227.920[c]	-317.580[a]	-0.330[b]	-	0.041[a]	-	69.480	44.260	-55.730	9.64
Ali & Schaeffer	Mean	-91.640	184.090	-77.400	93.570	-	-20.680	87.250	39.990	9.270	
	1	-69.020[a]	159.520[a]	-72.310[a]	70.810[a]	-	-15.530[a]	116	34.870	5.930	7.27
	2	-127.120[b]	233.080[b]	-93.630[b]	120.330[b]	-	-26.300[b]	79	41.330	9.690	8.87
	3	-138.560[b]	247.320[b]	-98.880[b]	130.450[b]	-	-28.620[b]	79	42.610	10.930	9.29
	4 or more	-74.110[a]	151.350[a]	-67.760[a]	86.210[a]		-19.600[a]	75	41.160	10.540	9.64
Pollot modified	Mean	99.250	-31.930	2.95’	-0.001	-	-	72.250	40.710	5.720	
	1	105.300[a]	-37.620[a]	1.850[a]	-0.000[a]	-	-	103	35.420	2.980	7.27
	2	97.800[b]	-50.650[b]	3.740[b]	-0.001[b]	-	-	63	42.150	6.280	8.87
	3	94.170[b]	-64.980[c]	3.940[b]	-0.001[c]	-	-	62	43.420	6.940	9.29
	4 or more	89.350[c]	-35.810[d]	3.370[b]	-0.001[d]	-	-	61	41.860	6.700	9.64

Dpeak= days to the peak, Peak= peak production, MSE= mean square error.

[abcd] Significant differences at $P<0.05$.

Table 3: Parameters of lactation models for protein and fat percentages with Wood, Wilmink, Ali-Schaeffer, and modified Pollot models by lactation for the models with better goodness of fit

		Protein							Fat						
	Lactations	Parameters						MSE (kg)	Parameters						MSE (kg)
		a	*b*	*c*	*d*	*k*	*f*		*a*	*b*	*c*	*d*	*k*	*f*	
Wood	Mean	3.630	-0.055	0.0009	-	-	-		5.250	-0.126	0.001	-	-	-	
	1	3.600[a]	-0.051[a]	0.0008[a]	-	-	-	0.24	5.310[a]	-0.132[a]	0.001[a]	-	-	-	0.62
	2	3.660[b]	-0.056[b]	0.0009[b]	-	-	-	0.26	5.080[b]	-0.120[b]	0.001[b]	-	-	-	0.65
	3	3.640[c]	-0.057[c]	0.0009[c]	-	-	-	0.26	5.370[c]	-0.130[c]	0.001[c]	-	-	-	0.66
	4 or more	3.630[d]	-0.057[d]	0.0009[d]	-	-	-	0.27	5.250[d]	-0.123[d]	0.001[d]	-	-	-	0.67
Wilmink	Mean	2.920	0.832	0.001	-	0.082	-		3.040	1.201	0.001	-	0.032	-	
	1	2.940[a]	0.798[a]	0.001[a]	-	0.084[a]	-	0.24	2.960[a]	1.221[a]	0.002[a]	-	0.029[a]	-	0.62
	2	2.920[b]	0.799[b]	0.001[b]	-	0.075[b]	-	0.26	3.020[b]	1.138[b]	0.002[b]	-	0.031[b]	-	0.65
	3	2.900[c]	0.90[c]	0.001[c]	-	0.087[c]	-	0.26	3.100[c]	1.276[c]	0.001[c]	-	0.035[c]	-	0.66
	4 or more	2.900[d]	0.89[d]	0.001[d]	-	0.087[d]	-	0.27	3.140[d]	1.230[d]	0.001[d]	-	0.037[d]	-	0.67
Ali & Schaeffer	Mean	3.090	0.657	-0.284	-0.230	-	0.083		1.980	2.151	-0.400	0.59	-	-0.01	
	1	2.950[a]	0.913[a]	-0.441[a]	-0.150[a]	-	0.06[a]	0.24	2.650[a]	0.864[a]	0.249[a]	0.23[a]	-	0.03[a]	0.62
	2	2.940[b]	0.917[b]	-0.355[b]	-0.150[b]	-	0.07[b]	0.26	1.700[b]	2.648[b]	-0.594[b]	0.75[b]	-	-0.04[b]	0.65
	3	3.380[c]	0.148[c]	-0.037[c]	-0.420[c]	-	0.11[c]	0.26	1.980[c]	2.288[c]	-0.537[c]	0.59[c]	-	-0.09[c]	0.66
	4 or more	3.360[d]	0.117[d]	-0.045[d]	-0.400[d]		0.10[d]	0.27	0.930[d]	4.138[d]	-1.439[d]	1.18[d]		-0.09[d]	0.67
Pollot modified	Mean	2.940	-0.044	-0.004	-0.0004	-	-		2.960	-0.312	0.025	0.0008	-	-	
	1	2.860[a]	-0.079[a]	-0.004[a]	-	-	-	0.25	2.890[a]	-0.323[a]	0.022[a]	0.0009[a]	-	-	0.63
	2	2.970[b]	-0.039[b]	-0.005[b]	0.0006[a]	-	-	0.27	2.940[b]	-0.302[b]	0.025[b]	0.0009[b]	-	-	0.66
	3	3.000[c]	-0.019[c]	-0.003[c]	-	-	-	0.45	3.040[c]	-0.316[c]	0.028[c]	0.0007[c]	-	-	0.68
	4 or more	3.050[d]	-0.000[d]	-0.002[d]	0.0002[b] - 0.0004[c] - 0.0003[d]	-	-	0.44	3.090[d]	-0.302[d]	0.030[d]	0.0006[d]	-	-	0.67

MSE= mean square error. [abcd] Significant differences at $P<0.05$.

Table 4: Days to the peak, peak yield, and persistence for milk protein and fat percentages with Wood, Wilmink, Ali-Schaeffer, and modified Pollot models.

Model	Lactation number	Lactation curve components			Lactation curve components		
		Protein			Fat		
		Days to the peak	Peak yield (%)	Persistence	Days to the peak	Peak yield (%)	Persistence
Wood	Mean	60.890	3.050	1.670	103.130	3.050	1.420
	1	61.020	3.030	1.690	105.290	3.030	1.410
	2	59.950	3.080	1.670	97.370	3.080	1.440
	3	60.040	3.090	1.670	105.650	3.090	1.410
	4 or more	63.340	3.050	1.660	106.120	3.050	1.430
Wilmink	Mean	44.650	3.020	17.820	92.650	3.280	19.850
	1	44.370	3.030	16.480	97.270	3.250	21.110
	2	46.030	3.030	19.140	89.430	3.270	21.510
	3	43.190	3.000	19.020	91.050	3.310	17.770
	4 or more	44.320	2.990	17.180	85.5500	3.330	15.990
Ali & Schaeffer	Mean	49.250	3.010	0.150	49.250	3.010	0.150
	1	50	3.040	0.140	105	3.260	0.190
	2	50	3.030	0.160	93	3.270	0.180
	3	47	3.000	0.160	97	3.310	0.160
	4 or more	50	3.000	0.140	90	3.320	0.130
Pollot modified	Mean				96.50	3.270	0.140
	1	85	3.07	0.20	101	3.24	0.16
	2	144	3.04	0.08	92	3.27	0.17
	3	-	-	-	99	3.27	0.13
	4 or more	-	-	-	93	3.31	0.13

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Artícle

Corn oil in Pelibuey ewes embryo transfer

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Abstract:

The effect of including corn oil (CO) in the diet of Pelibuey ewes in an embryo transfer protocol was evaluated. Twenty-four (24) donor ewes were randomly assigned to each of two groups (G), G1: twelve ewes fed a base diet (BD) with CO, and G2: twelve ewes fed BD without CO. Embryos were obtained 6.5 d after estrus and transferred to recipient ewes, forming four treatments: T1: recipient ewes fed a base diet with CO (BD+CO), transferred with embryos from a G1 donor (n=23), T2: recipient ewes fed BD without CO (BD-CO), transferred with embryos from a G1 donor (n=18), T3: recipient ewes fed BD+CO, transferred with embryos from a G2 donor (n=9), and T4: recipient ewes fed BD-CO, transferred with embryos from a G2 donor (n=11). The inclusion of CO in the diet increased (P<0.05) the ovulatory rate (10.5 ± 2.07 *vs*. 6.3 ± 2.07 corpora lutea), the number of transferable embryos (5.5 ± 1.4 *vs* 2.8 ± 1.4), quality 1 embryos (4.41 ± 1.1 *vs* 2.08 ± 1.1), and the number of total structures (5.9 ± 1.5 *vs* 3.1 ± 2.7) per donor ewe, and no differences (P>0.05) were found in the percentage of gestation (43.4, 55.5, 55.5, and 36.3 %) in the

recipient ewes for any of the treatments. Including CO in the diet of Pelibuey ewes increases the superovulatory response in an embryo transfer protocol.

Keywords: Corn oil, Pelibuey ewes, Embryos.

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Introduction

Reproductive technologies are valuable tools that help increase sheep production units' productive and economic efficiency. For example, embryo transfer (ET) has been successfully used to reproduce genetically superior animals[1]. Nevertheless, even though this technology has advanced considerably in recent decades, the response to superstimulation protocols and the percentage of fertility have been inconsistent, so research has focused on two fundamental aspects: Improving the response in follicular superstimulation of embryo donor females to obtain a bigger number of transferable embryos and increasing the percentage of gestation in recipient females.

Some specific diet components, such as energy, can positively influence certain reproductive aspects of the female[2]. Supplementation with corn oil (CO) with a high content of polyunsaturated fatty acids (PUFAs; oleic, linoleic, and linolenic) can be used to modify some processes of ovarian physiology in ewes[3] because these processes are related to energy availability[4]. It has been shown that dietary CO supplementation can improve the population of large follicles[5] and promote the number of corpora lutea[6] and prolificacy[7]. PUFAs have a more substantial effect in the early stages of folliculogenesis and, therefore, can increase the number of embryos obtained by donor females and promote the conception rate in dairy cows[8]. Based on this background, this research aimed to determine if the inclusion of CO in the diet of Pelibuey ewes improves the superstimulatory response, increases the number of transferable embryos in donor ewes and improves the pregnancy rate of recipient ewes in an ET program in Pelibuey ewes.

Material and methods

The study was carried out from July to December 2017 at the Sheep and Goat Reproduction Laboratory (LaROCa, for its acronym in Spanish) of the College of Postgraduates, Montecillo Campus, located at 19° 29' N, 98° 53' W, and 2,240 m asl[9].

Twenty-four (24) Pelibuey donor ewes aged 3.5 ± 0.3 yr with an average weight of 51.9 ± 3.2 kg were used. Of this group, 12 ewes received a base diet with corn oil (G1) at a rate of 2.0 kg ewe^{-1} d^{-1} consisting of 60 % oat hay, 25 % achicalada (sun-dried) alfalfa, 6 % corn oil (Mazola®), and 9 % commercial concentrate (Borrega plus: Alimentos Unión Tepexpan®); the rest of the ewes (12) received the following diet without corn oil (G2): 63 % oat hay and 26 % achicalada alfalfa, and 11 % commercial concentrate (Borrega plus: Alimentos Unión Tepexpan®; 12 % CP, 40 % ADF, 51 % NDF, 2.3 % EE, and 9 % ash); the experimental diets were isoenergetic and isoproteic, containing 12 % CP and 3.2 Mcal ME, and were offered for 16 d from day three of the estrus synchronization protocol until the day before transfer (the time of CIDR insertion was considered day 0).

As recipient ewes, 61 Pelibuey ewes aged 3.4 ± 0.3 yr with an average weight of 54.6 ± 1.2 kg were used. Thirty-two (32) ewes received the same diet as G1 at a rate of 2.0 kg ewe^{-1} d^{-1}; the rest (29) of the ewes received the same diet as G2. The experimental diets were offered for 16 d from day three of the estrus synchronization protocol until the day before the transfer (the time of CIDR insertion was considered as day 0). The treatments were assigned as follows (Table 1):

Table 1: Allocation of experimental treatments in recipients

Treatments	Description	N
T1	Recipients fed BD+CO, transferred with embryos from G1 donors.	23
T2	Recipients fed BD-CO, transferred with embryos from G1 donors.	18
T3	Recipients fed BD+CO, transferred with embryos from G2 donors.	9
T4	Recipients fed BD-CO, transferred with embryos from G2 donors.	11

*BD+CO= Base diet with corn oil, BD-CO=Base diet without corn oil.

The number of recipient females in the treatments was subject to the availability of the transferable embryos at the time of donor flushing.

All the ewes were handled according to the standards established by the "Regulation for the use and care of animals intended for research in the College of Postgraduates" and the Official Mexican Standard NOM-062-ZOO-1999 in order to avoid unnecessary stress and suffering of the animals.

All recipient ewes were subjected to an estrus synchronization protocol by insertion of an intravaginal device (CIDR-Pfizer®) impregnated with progesterone (0.3 g of P_4) for 9 d; on day seven, 300 IU of equine chorionic gonadotropin (eCG) (Folligon-Intervet ®) and a dose of 5 mg of prostaglandin F2α (Lutalyse®, Pharmacia Animal Health Laboratories) were applied via IM. The timing in the donors was similar, only the IM application of 200 mg in decreasing doses (40-40, 30-30, 20-20, 10-10 mg) of follicle-stimulating hormone (FOLLTROPIN®, Vetoquinol Laboratories) at 12-h intervals between each application (am-pm) was included on d 6 to 9 of CIDR insertion for the superstimulation process.

On d 9 of the synchronization protocol, the CIDR was removed, and the detection of females in estrus began 4 h after its removal with the help of a ram provided with an apron in order to avoid copulation. Donor ewes that presented estrus behavior were inseminated intrauterinely (laparoscopy) from 12 to 18 h after the onset of estrus and remained without feed for 24 h, a procedure to reduce rumen content and avoid bronchoaspiration[10]. For insemination, two straws of 0.25 ml of fresh semen containing 80 x 10^6 sperm per straw were used.

Embryo collection and transfer

Ewes that presented a superstimulatory response (>2 corpora lutea[11]) underwent embryo collection 6.5 d after estrus by means of ventral midline laparotomy[12]. To do this, the ewes were exposed to a 24-h fast before the procedure, and the number of CL present in each ovary was recorded. An anesthesia protocol with a combination of xylazine (Procin®, Pisa Laboratories) and ketamine (Anesket®, Pisa Laboratories) in a 0.8:0.2 dilution was applied intravenously in the jugular vein. Subsequently, the ewe was prepared on the stretcher placed on a plane at 45°, and 1 ml of lidocaine (Pisacaina®, Pisa Laboratories) was applied. The surgical drapes were placed, a 7 cm incision was made on the ventral midline, and the reproductive system was exposed with Babcock forceps. With the help of an intravenous catheter (Punzocat®, 32 mm), a puncture was made in the uterus-tubal junction to enter the lumen of the uterine horn; a second puncture was then performed at the level of the

intercornual ligament to introduce a Foley No. 10 catheter. A syringe with 60 ml of a flushing medium (Vigro® Complete Flush Solution, Bioniche Laboratories) at 37 °C was connected to the intravenous catheter so that the embryos were collected by dragging them into gridded Petri dishes with the solution that came out of the front end of the Foley catheter. The Petri dishes were transferred to the laboratory to begin the search and evaluation of the embryos.|

The embryo search was carried out meticulously with a stereoscopic microscope (Barnstead, USA) at 40X magnification, observing each quadrant of the Petri dish. The structures found were placed in a four-well Petri dish containing 200 µL of holding medium (Holding®, Bioniche Laboratories) in each well, placed in a thermal stage at 37 °C. The embryos were evaluated and classified under the stereoscopic microscope at 100X magnification according to their morphology and based on the criteria of the International Embryo Transfer Society (IETS)[13].

The embryos were transferred fresh six and a half days after the onset of estrus (trying to reduce the asynchrony time between the donor and the recipient), selecting females from both the BD+CO group and the BD-CO group, making sure that all the females in the four treatments had the same number and quality of embryos transferred. With a previous period of 24 h of fasting, the recipient was placed on a stretcher, and the same protocol as for embryo collection was carried out. The uterine horns were visualized laparoscopically, and by means of a ventral midline laparotomy, a 2 cm incision was made in the abdominal midline, and with a Babcock forceps, the ipsilateral uterine horn of the ovary with one or more normal corpora lutea was externalized, permanently hydrating it with physiological saline solution (CS® Solution, Pisa Laboratories). With the help of an intravenous catheter (Punzocat®, 32 mm), a puncture was made in the uterus-tubal junction, and an embryo of quality 1 or 2 (depending on availability) was placed in a catheter (TOMCAT®) under the following order: a column of medium, air, medium with two embryos, air and finally medium. The embryos placed in the catheter were propelled into the lumen of the uterine horn using an insulin syringe. The surgical planes were sutured with zero-gauge chromic Catgut® for peritoneum and muscle, and the skin was sutured with zero-gauge nylon. To prevent postoperative infections and once the suture was finished, 100 mg of oxytetracycline (Emicina LA®, Pfizer Laboratories) was applied via IM.

The variables evaluated were the following:

Estrus manifestation (%): percentage of ewes that showed signs of estrus within each treatment.
Time to estrus manifestation (h): hours elapsed from removal of the device to the occurrence of estrus.

Ovulatory rate (OVR): number of corpora lutea present at the time of embryo extraction (6.5 days after estrus).
Quality 1 embryos (EQ1): number of quality 1 morulae or blastocysts.
Quality 2 embryos (EQ2): number of quality 2 morulae or blastocysts.
Transferable embryos (TE): number of quality 1 and 2 morulae or blastocysts.
Oocytes (OO): number of oocytes that were not fertilized.
Total structures (TS): number of total collected structures.
Percentage of gestation: number of pregnant recipient ewes at 40 d post-transfer divided by the number of ewes in each treatment multiplied by 100.
Lambing rate: number of recipient ewes that lambed in relation to the number of ewes transferred in each treatment.

The response in donor ewes was analyzed using a Poisson regression using the Generalized Linear Model (PROC GENMOD). To analyze the percentage of pregnancy in the recipients, the analysis of contingency tables for two factors (DAM and RAM) was employed, using the Log-linear model through the CADMOD procedure of the SAS statistical package[14]. The manifestation of estrus was analyzed through a Chi-square independence test using the PROC FREC procedure, and the time to estrus manifestation was analyzed with the Kaplan Meier survival curves method using the LIFETEST procedure, and differences were established between treatments with the LOG RANK test.

Results

The proportion of donor ewes with signs of estrus and the time to the estrus were similar between treatments ($P>0.05$); however, the inclusion of CO in the diet of donor ewes increased ($P<0.05$) OVR, TE, EQ1, OO, and the number of TS per ewe (Table 2).

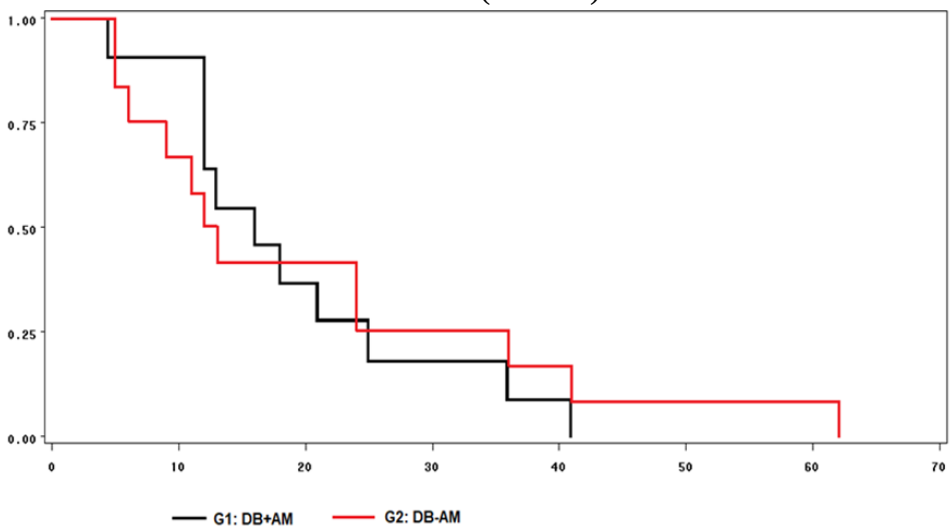
Table 2: Response of Pelibuey donor ewes fed a base diet with corn oil (BD+CO) or with a base diet without corn oil (BD-CO) (Means ± SE)

Variable	G1 BD+CO (n=12)	G2 BD-CO (n=12)	*P*-value
Ewes in estrus, %	91.6[a]	100[a]	
Time to the estrus, h	19.13±3.04[a]	20.6±3.1[a]	
Ovulatory rate, n	10.5±2.07[a]	6.3±2.07[b]	<0.0001
Transferable embryos, n	5.5±1.4[a]	2.8±1.4[b]	<0.0001
Quality 1 embryos, n	4.41±1.1[a]	2.08±1.1[b]	0.0002
Quality 2 embryos, n	1.08±0.52[a]	0.83±0.52[a]	
Oocytes, n	0.41±0.25[a]	0.33±0.25[b]	0.0163
Total structures, n	5.9±1.5[a]	3.1±2.7[b]	<0.0001

[ab] Values with different literal within rows show differences ($P<0.05$).

No differences were observed in the probability of estrus manifestation between groups, obtained by means of survival curves (Figure 1). Thus, 100 % of the ewes with BD+CO manifested estrus 42 h after CIDR removal, while the BD-CO ewes did so until 62 h.

Figure 1: Survival curves for the time between CIDR removal and estrus manifestation in Pelibuey donor ewes fed a base diet with corn oil (BD+CO) or with a base diet without corn oil (BD-CO)



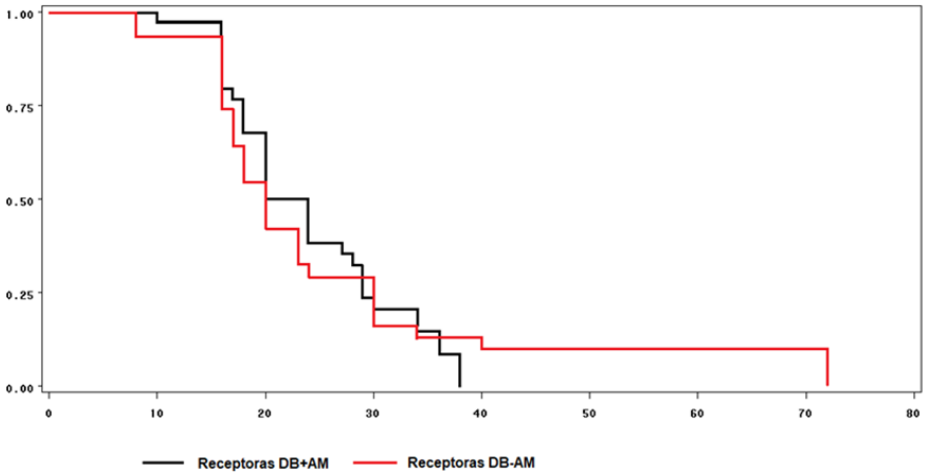
Response in recipients

The inclusion of CO in the diet did not alter the time or number of recipient ewes in estrus

with a base diet with corn oil (BD+CO, 24.08 ± 1.03) or with a base diet without corn oil (BD-CO, 25.8±3.0).

Survival curves for estrus manifestation in recipients showed no differences between treatments (Figure 2). One hundred percent of the ewes with CO manifested estrus at 38 h after CIDR removal, while the control ewes did so at 72 h.

Figure 2: Survival curves for the time between CIDR removal and estrus manifestation in Pelibuey recipient ewes fed a base diet with corn oil (BD+CO) or a base diet without corn oil (BD-CO)



No differences were found (P>0.05) in the percentages of pregnancy and lambing for any of the recipients' treatments (Table 3).

Table 3: Response in Pelibuey recipient ewes fed a base diet with corn oil (BD+CO) or with a base diet without corn oil (BD-CO)

Treatments	Recipient ewes		
	Transferred (n)	% Gestation 40 days	% Lambing
T1	23	43.4 (10)	39.1 (9)
T2	18	55.5 (10)	50.0 (9)
T3	9	55.5 (5)	22.2 (2)
T4	11	36.3 (4)	9.0 (1)

(P>0.05).

Discussion

The response to manifestation and time to estrus in donor and recipient Pelibuey ewes did not show differences between treatments (P>0.05). Similarly, in a study with Corriedale ewes, 100 % were reported to have superovulated in estrus with eCG and FSH during the breeding season[15]. In the present study, 25 % of donor ewes did not respond to the superovulation protocol, which coincides with other studies in which the proportion of females that did not respond to superovulation was between 20[16] and 30 %[17]. Supplementing omega-6 (n-6) PUFAs increases energy consumption as they are essential for feeding ruminants in addition to participating in the processes of folliculogenesis, ovulation, and estrous behavior[18]. The population of medium and large follicles increased when corn oil was supplemented as a source of PUFAs in ewes, which may result in increased estrogen production by those follicles, exerting an effect similar to eCG[19]. In the present study, the inclusion of PUFAs in the diet of ewes did not modify their reproductive behavior, possibly due to the short time of exposure to PUFAs in the diet since it has been mentioned that, in order to observe a response in the reproductive behavior of females, exposure of at least 20 d before the day of mating and continuing during mating is required[20].

The time to the manifestation of estrus between recipients (24.08 *vs* 25.8 h) and donors (19.13 *vs* 20.6 h) was similar between treatments (P>0.05). Results from other authors showed an estrus onset of 34.5.0 ± 2.6 h[21] or 32 ± 5.6 h[22] when 500 IU of eCG was applied, differing from what was found in this study. It has been mentioned that the time to the beginning of estrus is determined by the breed and type of FSH used in superovulation protocols[23]. The time to the beginning of estrus was 24 h, shorter when ewes were subjected to an intravenous infusion of olive oil compared to soybean oil. It has been mentioned that olive oil stimulates plasma concentrations of prostaglandin F2 alpha metabolite (PGFM) and prostaglandin E2 (PGE2)[24], providing evidence of the possible relationship between fatty acids, prostaglandins, and the onset of estrus[25]. In the present research, it was not possible to observe such an effect, probably due to the hormones used for synchronization.

The inclusion of CO in the diet of donor ewes improved the response to superstimulation in G1, results that are similar to those found by Herrera-Camacho[6] when evaluating superovulated Pelibuey ewes supplemented with CO (4 % DM), observing an increase in the number of corpora lutea, embryos collected and morulae; on the other hand, Zeron[26] reported a higher quality in oocytes and an increase in the proportion of PUFAs in plasma, follicular fluid, and *cumulus oophorus* cells when supplementing ewes with calcium soaps of fish oil. Nutrition during oocyte maturation has important effects on embryo viability in superovulated ewes[27]. FAs can directly affect oocyte maturation through the composition of its membrane[28] or indirectly by affecting the concentration of metabolites in follicular

fluid, impacting its subsequent development and viability[29]. Therefore, it is suggested that the inclusion of CO in this research could have influenced the quality of oocytes and embryos during the early stages of development since more transferable embryos were obtained when PUFAs were added to the diet; in addition, the increase in the number of corpora lutea may have modified the secretion of progesterone as there was a greater availability of cholesterol for the luteal tissue[6], optimizing the conditions for better embryonic development.

No differences in pregnancy percentage were observed between the four treatments ($P>0.05$). Similar results were found in other studies where, when adding CO to the diet of Pelibuey ewes, they did not observe differences in the percentage of gestation[7,19]. In this regard, it has been mentioned that both undernutrition and overfeeding can cause alterations in the intrauterine environment that prevent adequate development of the embryo, affecting its viability[30].

Diets containing high concentrations of PUFAs, such as linoleic acid in corn oil, can regulate prostaglandin synthesis and its subsequent effects on corpus luteum persistence, E_2 synthesis[4,8,25], and maternal recognition of pregnancy[31]. In addition, PUFAs can increase circulating concentrations of progesterone due to an increase in the availability of cholesterol[32], the main precursor for the synthesis of progesterone (P_4) in the corpus luteum[33].

The response to superovulation found in the present study was similar to that reported in other studies, obtaining an increase in the number of transferable embryos in the donors who received the PUFAs; nevertheless, the addition of PUFAs in the diet of recipient ewes did not improve the percentage of gestation, so it is necessary to carry out more studies on the time of exposure of females to the diet with PUFAs and the quantity and quality of PUFAs offered since, in ewes, there are very few published studies that explain the direct effects of polyunsaturated fatty acids on fertility[25].

Conclusions and implications

In conclusion, donor ewes respond well to including corn oil in an embryo transfer program. The use of corn oil in the diet of Pelibuey ewes increases the ovulatory rate, the number of total structures, and the number of transferable embryos, but it has no effect on the pregnancy percentage of the embryo recipient females.

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Artícle

Alternative model to measure the adoption of innovations: application in the Puebla beekeeping system

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Abstract:

This work aimed to adapt a methodological tool capable of improving the way to obtain the Innovation Adoption Index in the Puebla beekeeping system. A questionnaire was designed and applied to a sample of 62 beekeepers, from which information was obtained on the use of management, genetics, food, and health innovations, from which 32 original variables and seven innovation categories were defined. The Innovation Adoption Index (InAI) was redesigned and adapted using the analytic hierarchy process (AHP), which made it easier to identify social aspects of beekeepers and innovations that contribute to improving honey productivity. The results show that the innovations that contribute the most to production are

those contained in the categories of apiary location and health. On the other hand, the $InAI_{alt}$ evaluation proved to be a pertinent alternative for the explanation of the behavior of the yield per hive in addition to showing the specific contribution percentages on the innovations assessed, which can be used to calculate estimates that are more precise and consistent with the expected yields of the region.

Keywords: Beekeeping, Analytic Hierarchy Process, Agricultural innovation, Adoption of innovations.

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Introduction

The current economic environment has placed unprecedented demands for competitiveness on all companies, underlining the importance of innovation, research, and development activities. Innovation, being a dynamic process, not only drives business growth and economic development but also becomes a strategy for social and cultural change within the organization. In addition, it promotes the creation of new technologies that replace old ones[1], thus driving continuous evolution.

It is true that not all innovations have the same impact or value; innovation processes vary significantly from one sector to another, influenced by their conditions and adoption rates[2,3]. In addition, what may be considered new for one person in one region may not be new for others within the same geographical area; even some innovations, once they are no longer novel, become common practices, forming part of a technological set regularly applied by some, while for others who are learning and using them, they are still considered innovations, if not good production practices[4].

Currently, there is a consensus on a number of ideas to characterize innovation in agriculture. It is recognized that innovation requires knowledge from various sources, including the users of those innovations. In addition, different sources of expertise interact, sharing and combining ideas in processes usually specific to a given context. Each context has its own orders, reflecting its historical origins determined by cultural, political, and social factors[5]. Therefore, having methodologies that allow measuring innovations is a fundamental link for understanding them.

A methodology is defined as a set of principles, procedures, and practices aimed at achieving a specific goal[6]. These methodologies are relevant in product development, as they not only ensure that the final product is suitable and adaptable to the user's needs but also contribute to structuring and improving the development process itself. In this sense, understanding the characteristics of innovation and having appropriate methodologies is essential to promote development and continuous improvement in the agricultural sector[7].

The first methodology used to measure agricultural innovation was developed by Fliegel[8], who proposed an indicator of adoption of agricultural practices based on the percentage of practices that producers adopt compared to the total practices available. Later, Muñoz *et al*[9] proposed an innovation adoption index (hereinafter referred to as InAI) to assess a producer's innovative capacity. This index is similar to the one proposed by Fliegel; however, the second authors categorize innovations according to technological packages and calculate a specific InAI for each category, dividing the number of innovations made by the producer by the total number of innovations recorded in that category. Then, they average the InAIs of each category to get the overall InAI of each producer.

Nonetheless, in both methodological proposals, a clear differentiation is not established between the innovations assessed, which means that all innovations have the same weight. On the other hand, Pérez *et al*[10] argue that in order to measure a producer's innovation level, it is necessary to consider both the quantity and the type of innovations implemented. They propose implementing an alternative model that combines elements of traditional approaches with new perspectives with the aim of striking a balance between complexity and dynamism in measurement. To do this, it is necessary to determine the strength of the interrelations between the elements of a hierarchy.

One method used to hierarchize and weight criteria is the Analytic Hierarchy Process (AHP), proposed by Thomas Saaty[11] in the 80s. This multicriteria tool is based on pairwise comparisons of criteria or alternatives using a defined scale, allowing one to prioritize solving various complex multicriteria problems. The process involves obtaining subjective opinions and evaluations. In the AHP, items are compared to each other using a square matrix defined by a set of criteria, which involves weighting the number of rows and columns and assigning each item a relative importance based on expert judgment.

In this context, this research aimed to adapt a methodology to obtain an Alternative Innovation Adoption Index ($InAI_{alt}$) through the AHP in order to obtain an indicator that more accurately reflects the measurement of the innovation adoption process and that, in turn, allows understanding this process of vital importance for the agri-food sector, with a particular emphasis on the beekeeping sector, which plays a fundamental role in the

pollination of crops and the production of various products that contribute significantly to food security, biodiversity, and the development of rural communities.

Material and methods

The research was exploratory, descriptive, and cross-sectional, supported by primary and secondary sources with technicians, suppliers of inputs in the beekeeping sector, and honey producers. To ensure the integrity of the sample, the credibility of the sources was verified by reviewing their field experience, their reputation in the beekeeping community, and the consistency of their data with the existing literature. The surveys were carried out from July 2021 to March 2022 in the municipalities of Acatlán de Osorio, Guadalupe, San Pablo Anicano, and San Pedro Yeloixtlahuaca in the Mixteca region of the state of Puebla. From a list provided by technicians and suppliers in the region, 48 beekeepers were interviewed, and those who were not registered (14) were identified using the linear snowball technique during the same period. This research stands out for the relevance it acquires by focusing on the state of Puebla, which ranks eighth in honey production in Mexico[12]. This information underlines the significant presence of beekeeping in the region, which deserves a detailed analysis of its impact on the local and national economy. Specifically, the Mixteca region of Puebla emerges as an area conducive to developing beekeeping and producing high-quality honey. This phenomenon is attributed to a combination of factors, among which the following stand out: favorable climatic conditions, the richness of the local flora, the deep-rooted beekeeping tradition, as well as the economic and social impact generated by this activity in the region.

Variables and their analysis

A total of 32 items were analyzed, which were grouped into seven categories (Table 1) based on the manual of good livestock practices in primary honey production of the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA, for its acronym in Spanish) as a basis for analysis.

Table 1: Good honey production practice items

Category	Technological innovation in honey production
Health	**TI01.** Inspection frequency; **TI02.** Logging; **TI03.** Hive tool flaming; **TI04.** Varroosis control; **TI05.** Thymol removal 15 days before harvest; **TI06.** Change of the queen every year; **TI07.** Change of brood chamber frames 2/year.
Artificial feeding	**TI08.** Input for support or maintenance feeding; **TI09.** Input for stimulus feeding; **TI10.** Input for supplementary feeding. **TI 11.** Feeding is suspended at the beginning of flowering.
Location	**TI12.** Distance to the nearest source of water; **TI13.** Location of the apiary human settlements; **TI14.** Distance to the flowering area; **TI15.** Apiary clean of weeds; **TI16.** Hives on a base at 20 cm; **TI17.** Distance to inhabited areas; **TI18.** Knowledge of chemical application; **TI19.** Distance between hives.
Material of protection for the hive	**TI20.** Current condition of the hive equipment; **TI21.** Correct material for coating the hive (resins, waxes); **TI22.** Use of smoker with plant-based combustible material.
Harvest	**TI23.** Correct percentage of capping; **TI24.** Material used to dislodge bees during harvest.
Staff	**TI25.** Staff knows GHPP; **TI26.** There is a hygiene log; **TI27.** Clean clothing; **TI28.** Clothing for exclusive use.
Cleaning and hygiene	**TI29.** Program of procedures for hygiene and personal cleanliness; **TI30.** Procedures for hygiene and cleaning of protective equipment; **TI31.** Procedures for cleaning utensils and containers; **TI32.** Attendance at training workshops.

TI= Technological innovation.

Source: Prepared by the authors based on what was proposed by SAGARPA[13]

The calculation of the InAI or good production practices is carried out for each beekeeping production unit, which allows the evaluation of the degree of innovation. The InAI is a measure that varies between zero and one, where zero indicates a zero level of innovation, and one represents the maximum level of innovation a producer achieves. This index reflects the average percentage of practices implemented by the producer. To calculate the InAI of each producer, the values of the innovation adoption index in each category are averaged using Equation 1[9].

$$InAI_i = \frac{\sum_{j=1}^{n} IAIC_k}{K} \text{ (Equation 1)}$$

Where $InAI_i$= innovation adoption index of the i-th producer; IAICik = adoption index of the i-th producer in the k-th category; K= total number of categories.

To be able to compare the $InAI_i$ *vs* the $InAI_{alt}$, judgments had to be made about the importance of innovation in honey production per hive. To exemplify an application of the AHP, the

$InAI_{alt}$ was created. The hierarchical model established in this factor is illustrated in Figure 1, where it is observed that the $InAI_{alt}$ is explained by seven criteria (health, artificial feeding, location, protective material for the hive, harvest, staff, and cleaning and hygiene) and, in turn, each of them is the result of the variables that constitute the subcriteria and their alternatives.

To determine the relative importance of each innovation, an evaluation process was carried out by a group of five experts in the field of beekeeping and innovation in this sector. These experts were selected due to their experience and practical knowledge in beekeeping, as well as their technical mastery of innovations and good production practices in the sector. The profile sought was one that encompassed several aspects fundamental to the study, including a solid understanding of the needs and challenges faced by the beekeeping production units. During the evaluation, the experts assigned a weight to each innovation, considering two main criteria: first, they weighted the contribution or importance of each innovation within the corresponding categories, and second, they used a scale of 1 to 9 to indicate the relative priority of an alternative over the options compared. On this scale, the value 1 indicates that both options are equally important for the object of study; in contrast, the value 9 indicates the higher priority of an alternative over the options compared. The details of the numerical criterion are specified in Table 2.

Table 2: Preference comparison scale

Value	Definition	Explanation
1	Equally important	Innovations contribute equally to productivity
3	Moderately important	Innovation contributes moderately to productivity
5	Strongly important	Innovation makes a strong contribution to productivity
7	Very strongly important	Innovation is more favored than the other; its predominance was demonstrated in productivity
9	Extremely important	Evidence unquestionably favors innovation over the other
2, 4, 6, 8	Intermediate values	They are used when an intermediate value cannot be defined between adjacent innovations

Source: Prepared by the authors, adapted from Saaty[11].

Once the comparison matrices were filled, a consistency analysis of the judgments issued by the experts was carried out following the procedure described by Zamudio Sánchez and Núñez Vera[14]; the matrices that were not consistent were reevaluated until consistency was achieved with a level of sample significance less than 0.05. Once the consistency of the assignment of values in the matrices was tested, the weights of each attribute or alternative were calculated considering the eigenvalue associated with the maximum eigenvalue of each comparison matrix following the procedure described by Saaty[11].

The two previous tasks were automated using the SAS® V9 tool to program the matrix calculations in dynamic and interactive conditions with the IML (Interactive Matrix Language) procedure, where auxiliary routines were programmed to generate the outputs that included the specific weights of each innovation or category.

In order to assess the congruence of the values obtained with the $InAI_{alt}$ with respect to variables of productive importance, a multiple linear regression model (Equation 2) was used to explain the yield recorded per hive as a function of the $InAI_{alt}$ in interaction with the size of the apiary. The number of hives that each interviewed beekeeper has was used for the grouping into clusters, dividing them into three groups (Cluster 1, 1-10 hives; Cluster 2, 11-20 hives; and Cluster 3, 21-32 hives). This exercise was contrasted with the one used by $InAI_i$. It is essential to mention that the decision to use the number of hives as a grouping criterion was based on its relevance within the regional beekeeping context, as well as its ease of measurement and management in the study. This choice allowed for a balanced and representative distribution of beekeeping production units, thus facilitating analytical comparison between different productivity levels and the adoption of innovations.

$$E(Y_i/X = x_i) = \beta_1\chi_{1i} * Z_{1i} + \beta_2\chi_{1i} * Z_{2i} + \beta_3\chi_{1i} * Z_{3i} \text{ (Equation 2)}$$

Where Yi: Yield per hive of the i-th producer; X1= $InAI_i$ or $InAI_{alt}$ of the i-th producer; Zji= 1 if the ith-producer belongs to the cluster j=1,2,3, and 0 otherwise.

The purpose of the regression model was fundamental for the evaluation of both the predictive and explanatory accuracy of the relationships between the variables, considering the $InAI_{alt}$ as an additional technique to more effectively capture the complexity of the data and the interactions between the variables. In addition, the $InAI_i$ values were graphically represented against the $InAI_{alt}$ values obtained in each category in order to show the differences between these indices.

Results and discussion

The average age of beekeepers was 39 yr; more than 20 % were under 26 yr of age, and the rest were under 55 yr of age, which allows to assume that beekeeping activity is in the hands of adults; although it is true that the average age coincides with what has been reported in other studies in Mexico[15-18], beekeeping in the study region shows a generational change where young people begin to resume this activity as an alternative source of income.

As for the average years of schooling, these were 8.8, a higher figure than that reported by Güemes *et al*[19] and Magaña *et al*[20] for other states of the Mexican Republic, who indicated an incomplete level of primary education.
The region has an average of 8 yr engaged in beekeeping. Nevertheless, other studies[21,22,23] reported an average of 16, 21, and 22 yr of beekeeping, respectively, higher than what was found in this region, which indicates that the activity is relatively young compared to the states of Jalisco, Yucatán, and Veracruz.

As for the weekly time spent on the activity, the average was 1.65 h, but the range varies from 1 to 3.5 h, depending on the number of hives the beekeeper has. This flexibility in working hours is because beekeeping does not require long work hours to obtain good results, making it an excellent complementary activity according to the beekeepers' perception.

Beekeeping in the region is closely linked to staple crops and wild vegetation areas. A total of 62 apiaries were identified, which together housed 757 hives. The municipalities that stand out for having the highest number of apiaries are Guadalupe and Acatlán de Osorio, with 21 and 16 apiaries, respectively, followed by San Pablo Anicano with 15 and San Pedro Yeloixtlahuaca with 10 apiaries.

Regarding the size of the beekeeping production units, it is observed that the average is 12.21 hives; however, there are variations, from a minimum of three to a maximum of 32. This range of sizes reflects the diversity in the scale of beekeeping production in the region, which may be influenced by factors such as resource availability, beekeepers' experience, and local market demand.

The primary source of income for beekeepers comes from agricultural activities, representing 57.17 % of the total, according to data that coincide with those reported in other works[19,22]. This data suggests a strong economic dependence on agriculture in the study region. In second place are remittances from the United States, a phenomenon that has also been documented[24], where it is mentioned that 80 % of the remittances that arrive in the state of Puebla benefit the inhabitants of the Mixteca region. Beekeeping ranks third as a source of income, being considered a complementary activity due to the seasonal nature of its production process. This characteristic can limit its economic contribution compared to agricultural activities and remittances. Nonetheless, some producers, such as technical advisors and veterinarians, find beekeeping a second source of income.

According to the World Bank[25], the diversification of income sources not only reduces vulnerability to possible fluctuations in a single source of income but also strengthens the capacity for financial resilience in the face of unexpected events, proving to be a key factor in promoting local economic development.

Table 3 shows the specific weights by category according to the type of InAI; as mentioned above, in the traditional $InAI_i$, both the value of each innovation and that of each category will always be the same. The overall $InAI_i$ was 54.14 %; that is, beekeepers are applying 17 of 32 technological innovations assessed for honey production, which means that few innovations have been adopted, leaving a margin to continue advancing in this regard. According to this methodology, the harvest category is the one that contributes the most to honey production (13.71 %).

Table 3: Specific weights and number of innovations (INOV) by category

Category	INOV (n)	%					
		$InAI_i$	**Weight**	**$InAI_i$ weighted**	**$InAI_{alt}$**	**Weight**	**$InAI_{alt}$ weighted**
Location	8	65.52	14.29	9.36	81.82	31.74	25.97
Health	7	36.87	14.29	5.27	52.57	29.15	15.32
Feeding	4	47.98	14.29	6.85	31.12	19.21	5.98
Materials	3	91.94	14.29	13.13	79.98	7.78	6.22
Harvest	2	95.97	14.29	13.71	76.86	6.87	5.28
Staff	4	27.42	14.29	3.92	51.55	2.77	1.43
Cleaning and hygiene	4	13.31	14.29	1.90	17.96	2.48	0.45
Total	32		100.00	54.14		100.00	60.65

$InAI_i$= Innovation Adoption Index; $InAI_{alt}$= Alternative Innovation Adoption Index.

On the other hand, according to the weights obtained through the AHP, the categories of location and health of the apiary contribute almost 61 % of the innovations for honey production. The relevance of these categories in beekeeping production entails several important implications. Firstly, it points out that the correct selection of the location of the apiary must consider variables such as the availability of flower sources, climatic conditions, and the presence of pesticide agents, as these can considerably influence honey production volumes. In addition, adopting health management innovations, including disease control and pest prevention, is crucial to safeguard the health and well-being of beekeeping colonies.

In this sense, several authors state that the productivity of honey is the result of a combination of several factors, including the density and quality of flowering, the natural physical environment, and health[26,27]. Thanks to the richness of the natural resources available in the study region, the practice of artificially feeding (mainly sugar syrup) hives in times of scarcity is low (2.71 times/year). Nonetheless, Tucuch-Haas *et al*[28] mention that supplementary feeding increases the number of bees and the number of cells with capped brood, nectar-honey, and pollen.

Table 4 exemplifies the construction of the categories and how each innovation contributes a percentage to each of them. As stated, the location of the apiary is a category that accounts for 27.21 % of honey production, but within this category, distances of less than 1 km to the water source and the flowering area contribute 68 % to this category, which is why they are priority activities for the installation or management of an apiary; similar data were reported by other researchers[29].

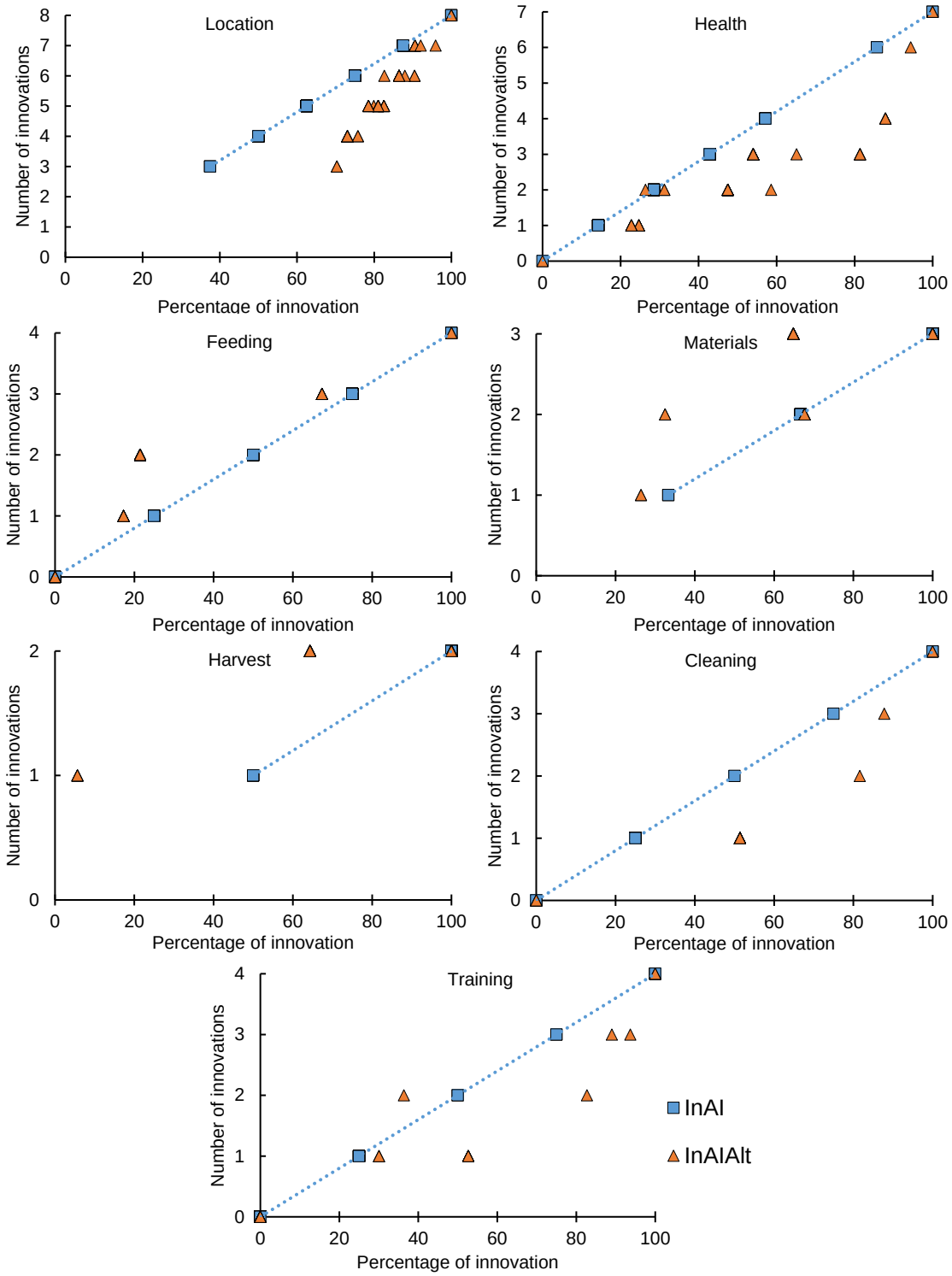
Table 4: Weight of the main innovations in honey production

Apiary location Innovation	%	Health Innovation	%
Distance < 1,000 m to the nearest source of water	48.35	Inspection frequency ≤ 15 d	33.89
Distance < 1,000 m to the flowering area	19.69	Hive tool flaming before inspecting a hive	24.71
Minimum distance between hives ≥ 2 m	9.44	Mite control (*Varroa destructor*) (thymol)	22.78
Hives on a base ≥ 20 cm	7.95	Change of brood chamber frames 2/year	6.52
Apiary clean of weeds	5.44	Change of the queen every year	5.61
Knowledge of chemical application dates	4.10	Thymol removal 15 d before flowering	3.60
Distance to inhabited areas > 200 m	2.74	Logging	2.90
Distance > 400 m to human settlements	2.30		
Total percentage	100.0		100.00

Health is another fundamental pillar because poor sanitary management increases production costs and the mortality of bee colonies; keeping a hive strong translates into greater productive efficiency. Even though there are seven innovations within health, inspection frequency, hive tool flaming, and varroosis control contribute more than 80 % of this category, making them high-impact activities.

The $InAI_{alt}$ presented variations for different innovations (Figure 2); it can be observed that, for the same number of innovations, a different percentage of innovation can be obtained within each category (higher or lower) according to the weight obtained through the AHP, except for the extreme values that always maintain the same percentage (0 or 100) regardless of the number of innovations or the weight assigned by any methodology. This situation favors both researchers and producers in knowing the current level between one producer and another, even having the same number of innovations, because each one has different objectives and priorities in terms of innovation.

Figure 2: Comparison of the main categories of innovation that contribute to honey production



One of the advantages of using the AHP is that values can be assigned to innovations that, although they do not comply with what is set out in the manual of good practices, could have a value other than zero or be with a higher value without fully complying with what is required, as is traditionally done with the InAI methodology. To exemplify this situation, the graph of materials (Figure 2) shows that, despite meeting all three characteristics, the percentage of innovation is less than 100; this situation could be explained by the fact that, despite having the necessary materials to cover this category, their current condition may not be optimal for proper management in the apiary, but it cannot be completely ruled out because it is better to have this innovation in not so favorable conditions than not to have it.

About the $InAI_{alt}$ evaluation, Table 5 shows the general results. Both regressions (InAI and $InAI_{alt}$) show a significant overall test (<.0001) and the RMSE is around 3.4, meaning that both models are statistically relevant in explaining the behavior of yield per hive. Judging by the R^2 statistic, both models explain 69 % of the variability inherent in yield per hive.

Table 5: Result of the multiple regression model

		InAI				
	Parameter	**Coefficient**	**S.D.**	**t-value**	**Significance**	**VIF**
InAI	Yield/hive*Cluster 1 (1-10 hives) n=30	15.80	1.37	11.55	<.0001	1.75
$InAI_{alt}$		14.33	1.22	11.79	<.0001	2.07
InAI	Yield/hive*Cluster 2 (11-20 hives) n=19	18.69	1.44	12.94	<.0001	1.75
$InAI_{alt}$		16.68	1.25	13.37	<.0001	2.07
InAI	Yield/hive*Cluster 3 (21-32 hives) n=13	26.09	1.39	18.75	<.0001	1.75
$InAI_{alt}$		22.95	1.19	19.24	<.0001	2.07

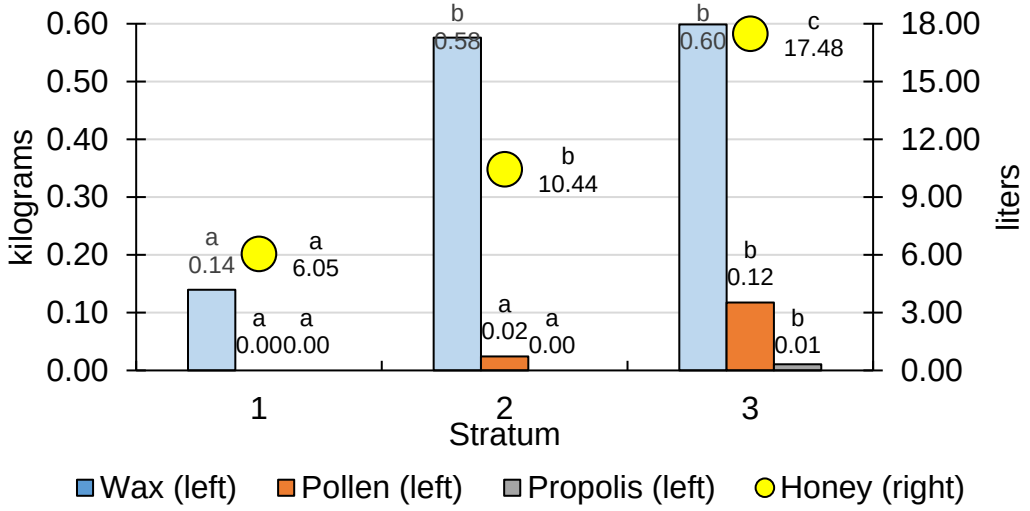
VIF= Variance inflation factor.

InAI general regression rest: F-value 217.48, *P*-value <.0001, R^2 0.652, RMSE 3.544.

$InAI_{alt}$ general regression test: F-value 229.29, *P*-value <.0001, R^2 0.668, RMSE 3.459.

It is also observed that the coefficients associated with InAI and $InAI_{alt}$ in each cluster are statistically significant (<.0001); that is, they are all non-zero; however, the standard errors associated with InAI are greater than $InAI_{alt}$, between 8 and 10 %. The estimated coefficients reveal a significant difference between the proposed and traditional models. This finding suggests that the expected yields in each cluster (small, medium, and large) are overestimated when the traditional method is used; in contrast, the proposed alternative produces estimate that are more adjusted to the analyzed reality. This discrepancy can be attributed to the proposed model minimizing errors, resulting in more accurate estimates (Figure 3). In addition, the new $InAI_{alt}$ could help to understand why stratum 3 producers can obtain more byproducts from the hive.

Figure 3: Average yield per hive in the Mixteca region of Puebla, Mexico



[abc] Means with different letters by column for the respective variable indicate significant differences ($P<0.05$).

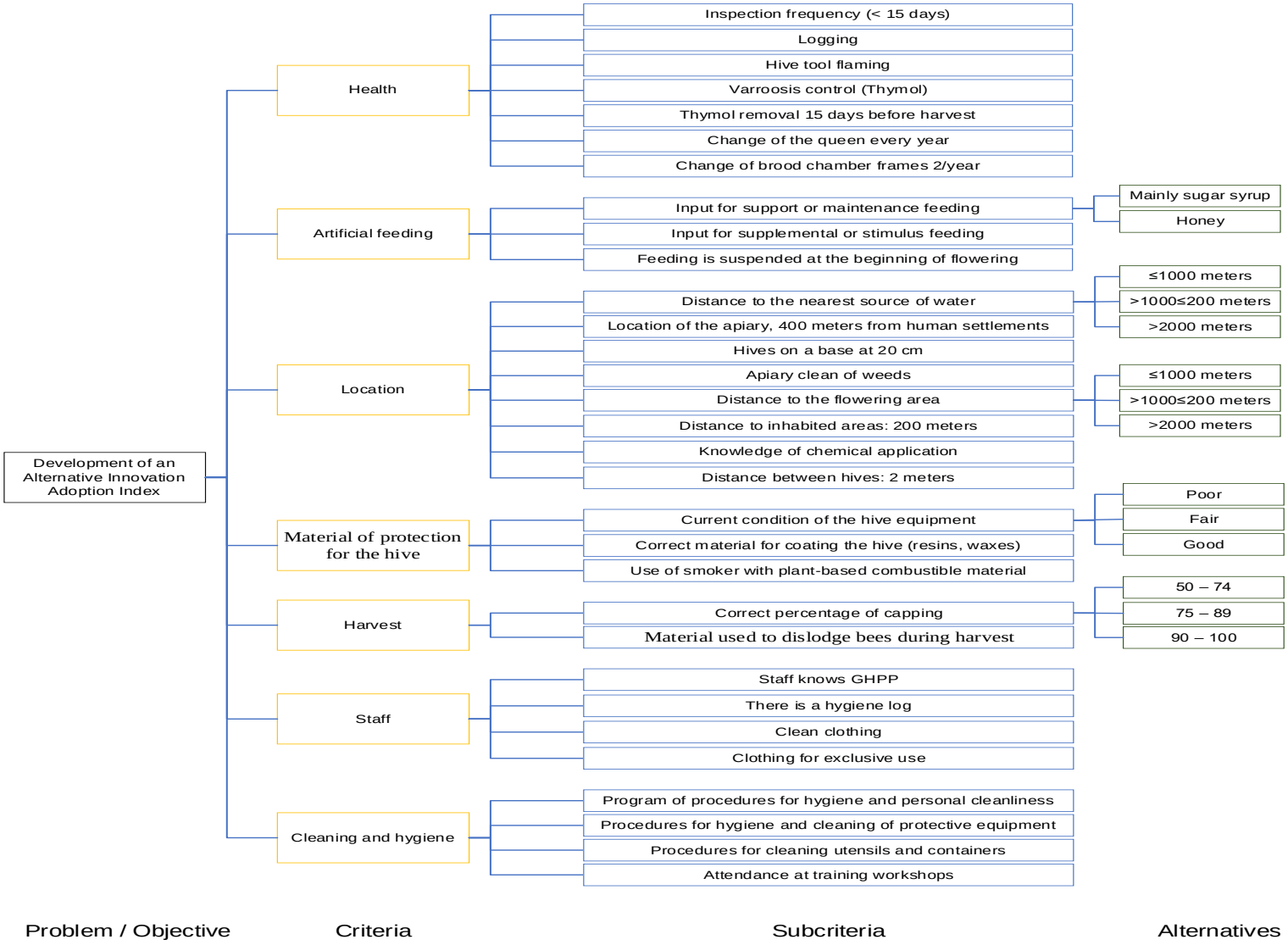
Using $InAI_{alt}$ made it possible to identify the most relevant categories and practices in the field of beekeeping, which suggests a new way of planning and executing training and extension programs in this activity. By defining priority areas, beekeeper training can focus on crucial aspects such as hive health, feed management, and selecting the best site for the apiary. This approach allows beekeepers to concentrate on improving practices that have the greatest impact on bee health and productivity rather than trying to innovate in all aspects simultaneously. This strategy not only optimizes the available resources but also promotes a gradual and successful transition to more efficient and sustainable beekeeping methods.

Conclusions and implications

In the Mixteca region of Puebla, beekeeping is practiced mainly in small-scale units and usually as a complementary activity. Nevertheless, on the other hand, there is a productive potential due to its agroecological conditions, in addition to being a source of income with reduced working hours. The proposed $InAI_{alt}$ made it possible to identify the categories and innovations with the greatest contributions to honey production in the region of study based on the weights granted through the AHP, which allows adapting this methodology to different regions and production systems thanks to its adaptability, which only requires a database with the innovations that are to be evaluated in a production system and a panel of experts from the area to reach a result that is more conclusive with the reality of the region. It has been shown that the measurement of innovation includes not only technical and productive aspects but also environmental aspects; this holistic view allows for a more accurate

assessment of the overall impact of innovation and its contribution to beekeeping development. The evaluation of the suitability of the $InAI_{alt}$ carried out through the regression models indicates that the results are consistent with those obtained in the InAI (fit of the models); however, the proposal produces estimates that are more accurate and consistent with the expected yields of the area. Therefore, it is proposed that for future research, regressions be carried out considering each of the byproducts of beekeeping to corroborate the model's scope. Finally, the proposed methodology exhibits sectoral and territorial flexibility, characterized by its versatility and adaptability, distinguishing it as an ideal instrument for implementation in multiple production scenarios. Consequently, its adoption would contribute to generating tangible improvements in terms of efficiency and quality in the operational sphere in order to have a better measurement of the process of adoption of innovations in the agricultural sector.

Figure 1: Hierarchical Model for obtaining the beekeeping InAI with the AHP



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Article

Characterization of sheep slaughterhouses for barbacoa production in a municipality in the Central Mexican Plateau

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Abstract:

Ensuring the quality and safety of meat from slaughter animals is a matter of global concern. Among the factors that must be taken care of are the activities that generate stress to the animal during *antemortem* handling (transport, rest, and stunning), *postmortem* carcass handling (aging and storage), and hygiene practices in facilities and staff. This work aimed to characterize sheep slaughter units within the municipality of Capulhuac de Mirafuentes, State of Mexico, based on current Mexican regulations. For this, a principal component (PC) analysis was carried out, highlighting that those that represented the highest variability in the slaughter centers were the price of the carcasses and their products, place of marketing, slaughter volume, sex of the animal, and safety of the carcasses, which represented 50.4 % of the explained variance. A cluster analysis was also carried out, which represented the integration of four groups of slaughter descriptors ($P<0.05$). As a result, it was found that

65 % of animals are slaughtered in commercial premises and houses that do not comply with the technification described in the regulations; they also present deficient *antemortem* and *postmortem* handling of animals; it was also observed that 98.3 % of the establishments use a slaughter method called “descabellado” (pithing), not reported in NOM-033-SAG/ZOO/2014, coupled with the lack of knowledge of the staff on animal welfare issues. This affects the quality and safety of meat and puts consumers’ health at risk.

Keywords: Slaughterhouses, Safety, Animal welfare, Meat quality, Cluster.

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Introduction

Slaughtering an animal constitutes the physicochemical change from muscle to meat[1]; this practice must ensure the humane, professional, and painless death of the animal, in addition to taking care that the animal is exposed to a low level of stress, guaranteeing animal welfare and the quality of the final product called meat[2].

The most important characteristics of fresh meat that determine quality, safety, and consumer acceptance are the physicochemical properties (pH, water retention capacity, color, and texture), organoleptic properties (softness, consistency, smell, taste, and color), and microbiological properties (absence of enteropathogenic bacteria and fungi)[3].

These properties are influenced by factors such as the production system (type of feeding, animal handling, and health, reproductive and genetic care), *antemortem* factors (transport, rest, fasting, and handling of the animal)[4], and *postmortem* factors (aging time and storage temperature)[2,3]. The operators’ handling of animals during slaughter also has an impact[5].

Sheep meat is considered one of the most complete foods from a nutritional point of view in the human diet[6] because it provides essential fatty acids, proteins, and fats of high biological value[7] in addition to being rich in vitamins and minerals[8].

In Mexico, 95 % of the consumption of this meat is in the form of the typical dish called barbacoa, a product obtained from the steaming of sheep meat in an underground hole

covered with bricks, wrapped in leaves of pulquero maguey (*Agave salmiana* Otto), added with seasonings and spices; the remaining 5 % is consumed as fine cuts[9,10].

In the municipality of Capulhuac de Mirafuentes, State of Mexico, around 400,000 head of sheep are slaughtered annually to supply the demand of the country's central area[11]. In this municipality, around 8 thousand sheep carcasses are marketed weekly, which is why it is considered the number one producer and marketer of fresh sheep meat nationwide. This municipality, although it currently has a municipal slaughterhouse with an installed capacity to house 67 % of the slaughters, is exceeded, which has led producers to generate their own slaughter units, not knowing if they comply with current regulations, which puts animal welfare, meat quality, and the health of consumers at risk. For this reason, this work aimed to characterize sheep slaughter units within the municipality of Capulhuac de Mirafuentes, State of Mexico, based on current Mexican regulations.

Material and methods

This study was defined as qualitative and descriptive research and was carried out in July 2022 in the municipality of Capulhuac de Mirafuentes (19°12'N, 99°28'W; 2700 m asl) in the State of Mexico (Central Mexican Plateau).

Preparation of the survey

To create the survey, the following standards were consulted: NOM-008-ZOO-1994 (Animal health specifications for the construction and equipment of establishments for the slaughter of animals and those engaged in the industrialization of meat products, in those points that were appropriate)[12], NOM-033-SAG/ZOO/2014 (Methods for killing domestic and wild animals)[13], NOM-213-SSA1-2018 (Products and services. Processed meat products and the establishments engaged in their processing. Sanitary provisions and specifications)[14], NOM 194-SSA1-2004 (Sanitary specifications in establishments engaged in killing and slaughtering animals for human consumption, storage, transport, and sale)[15], NOM-120-SSA1-1994 (Hygiene and sanitary practices for the processing of food, and non-alcoholic and alcoholic beverages)[16], NOM-051-ZOO-1995 (Humane treatment in the movement of animals)[17]. Primary and secondary information was also obtained through field visits and unstructured interviews with owners and employees of slaughterhouses and municipal slaughterhouse staff.

Firstly, the survey was validated by academic experts and zootechnical veterinarians who carry out the sanitary inspection on behalf of the Institute of Health of the State of Mexico (ISEM, for its acronym in Spanish), and it was used to carry out a pilot test, which was applied to 10 producers, which were not included in the results of the research.

Secondly, the data collected was used to generate a final survey structured with open-ended, closed, and multiple-choice questions in order to facilitate its application; it integrated 74 questions according to the most important specifications mentioned by university experts and sheep producers, as shown in Table 1.

Sample size

The number of establishments evaluated was calculated using a simple random sampling, considering a finite population. The components of the formula were a confidence value of 95 % (Z=1.96), a precision of 5 %, an estimator of variance equal to 0.25 [$\sigma 2 = \pi(1-\pi)$], and a value of N=65, based on the database of the establishments registered in the operating register of the Municipal Council of Capulhuac. The sample size obtained was n=57.

Study description

The surveys were conducted using a purposive random probabilistic sampling method due to the high number of sheep slaughters that are carried out. To minimize the error, it was mentioned that participation would be voluntary, and it was ensured that the owners and managers of the establishments did not know the day of sampling in addition to not offering any economic remuneration to the participating establishments and indicating that all the information would be confidential and only for research purposes.

Statistical analysis

Two multivariate statistical techniques were used: principal component analysis and cluster analysis. The information from the survey, which was applied to 57 sheep slaughter units (SSUs), was first used to carry out a discriminant analysis in order to eliminate those variables that did not allow the differentiation of sheep slaughter units. Subsequently, the variables that permitted differentiation were used to perform the principal component method

for factor extraction, the Kaiser-Mayer-Olkin (KMO) index, and Bartlett's test of sphericity to measure the correlation between variables. Those variables with a communality ($h<0.9$) were not included in the factor analysis because it indicated that these variables were not correlated with the new factors. The factors selected were those with eigenvalues ≥1. To better understand the factors obtained, an orthogonal rotation method (Varimax) was carried out; consequently, the scores of the factors in the analysis were estimated using the regression method and saved as new variables. Subsequently, a hierarchical analysis of clusters was carried out to identify similarities and differences in the slaughter rooms. The distance used was the squared Euclidean distance as a measure of similarity and clustering, performed by Ward's method. To select the most significant variables that would allow differentiation between the groups obtained, the non-parametric tests of Kruskal-Wallis and Mann-Whitney were performed, considering the characteristics of the study and the variables.

Results

Discriminant analysis

The discriminant analysis results allowed to rule out 28 variables that did not present a significant difference ($P>0.05$). Therefore, only 46 variables were finally considered for subsequent analyses, which allowed the explanation of the variability of the sheep slaughter descriptors (Table 2).

Table 2: Discriminant analysis results

Slaughter variables or descriptors selected	**Slaughter variables or descriptors discriminated**
1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, 20, 24, 26, 27, 28, 29, 30, 32, 33, 34, 36, 37, 38, 39, 43, 44, 45, 46, 47, 48, 50, 54, 55, 57, 58, 59, 65, 67, 69	2, 16, 21, 22, 23, 25, 31, 35, 40, 41, 42, 49, 51, 52, 53, 56, 60, 61, 62, 63, 64, 66, 68, 70, 71, 72, 73, 74

Thirteen (13) principal components (PCs) were obtained, which explained 78.64 % variability of the data (Table 3), which were renamed according to the variables that were correlated. Three groups were formed, where it can be observed that the study variables, price of carcasses and byproducts, which represented 22.58 %, and place of commercialization of products, with 9.96 %, were the ones that generated the highest values. Subsequently, the second component was integrated by the following variables: volume of slaughter, sex of the

animal, factors affecting the safety of the carcasses, generation of waste, social impacts, and hygiene practices of the staff; as a third component of importance, it was only the variable of training of staff; these three principal components together represented 46.14 % of the variability.

Table 3: Principal components of sheep slaughter in the municipality of Capulhuac de Mirafuentes

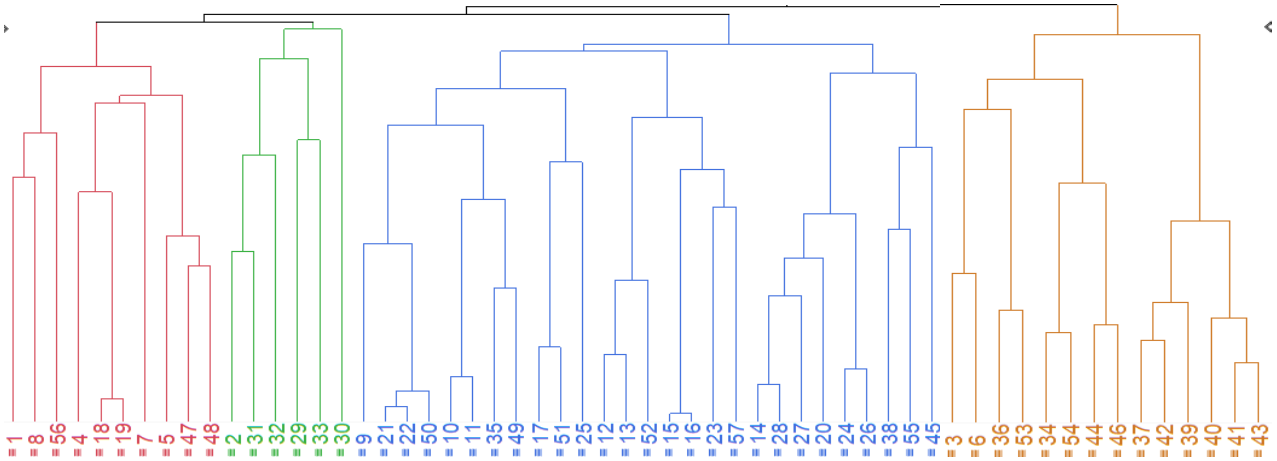
PC	Name	CV	Eigenvalue	Percentage 1	Percentage 2
PC1	Price of carcasses and byproducts	7, 8, 9, 10, 11, 12	9.71	22.58	22.58
PC2	Place of marketing	5, 6	4.28	9.96	32.54
PC3	Volume of slaughter	3, 4	2.81	6.54	39.09
PC4	Sex of the animal	13, 14	2.53	5.89	44.99
PC5	Factors affecting carcass safety	32, 34, 50, 55, 69	2.32	5.39	50.38
PC6	Generation of waste and social impacts	20, 57, 59	2.16	5.03	55.42
PC7	Staff hygiene practices	45, 46, 47, 48, 54, 58	1.94	4.51	59.92
PC8	Staff training	54	1.61	3.75	63.67
PC9	Slaughterhouse infrastructure	15, 17, 26, 27, 29, 30, 65	1.61	3.63	67.30
PC10	Factors affecting meat quality	33, 37	1.41	3.29	70.59
PC11	Type of slaughterhouse	1	1.28	2.98	73.58
PC12	Stunning method	55	1.14	2.65	76.24
PC13	Rest period before slaughtering	19	1.05	2.40	78.64

PC= principal component; CV= correlated variables; Percentage 1=% of the total variance explained; Percentage 2= cumulative % of the explained variance.

Cluster analysis

Figure 1 shows the dendrogram of the clusters formed from the slaughter environments.

Figure 1: Hierarchical clusters (dendrogram) of clustering analysis from slaughter descriptors (N=46)



Description of clusters by similarities in slaughter environments

Cluster 1

Made up of 10 SSUs (sheep slaughter units), it is characterized by being composed of only private establishments that slaughter an average of 31 animals per week (male sheep in 84 %) for the sale of meat in the municipality of Capulhuac and the commercialization of barbacoa in the metropolitan area of Mexico. As for carcasses, two types are marketed: tough carcass (adult animals) at a price of $91.00 and tender carcass (animals under 9 mo of age) at a price of $97.00; they also market byproducts such as viscera ($163.00), legs ($34.00), head ($53.00), and the dish called barbacoa and sheep belly at a price of $391.00 per kilo; regarding the infrastructure conditions of the establishments that comply with current regulations, they have an area for unloading animals and a loading area for carcasses and viscera, with rest pens where the animals are given a time of 12 to 24 h, and the joints of the floors and walls are easy to clean.

The slaughter area has sanitary mats with disinfectant solution. All areas of the slaughter unit are kept free of pests, and domestic animals are prevented from entering; all employees wear face masks and are prohibited from entering the slaughter area with any type of accessory. Regarding *postmortem* handling, the establishments have freezers, giving them an aging time of 1 to 6 h, separating and identifying the viscera by an animal. Nonetheless, they do not have pens to identify sick animals. They do not have a pest control plan or protections in windows and vents that help reduce the entry of dust, rain, and insects, and in general, the blood that

is discarded is composted; as for the liters of water used per animal, it ranges from 7 to 12 L (Table 4).

Cluster 2

Made up of 6 SSUs, it comprises three types of slaughterhouses: the municipal slaughterhouse, slaughterhouse facilities with private staff, and private slaughter units; in general, they are units with large slaughter volumes (average of 86 animals per week). Their primary purpose is the sale of meat, byproducts, and barbacoa in the municipality of Capulhuac and, mainly, for resale. They market two types of carcasses: tender, for $99.00, and tough, at $89.00; they are also characterized by the marketing of byproducts such as viscera: $151.00, legs: $35.00, head: $53.00, and the marketing of a dish called barbacoa and sheep belly for $360.00/kg. As for the preference for slaughter by sex of the animals, they do not give it importance. The infrastructure complies with the disembarkation area and loading area of carcasses and viscera; they also have pens for sick animals and rest pens, giving a period of between 13 and 24 h. They comply with materials in floor and wall joints that facilitate cleaning. The cleaning of pens, ramps, tunnels, *antemortem* baths, and drying and draining areas is carried out every day due to the high volumes of slaughter, complying with the identification of viscera by animal. There is no compliance with pest control and sanitary mats. The protections in windows and vents are not in good condition to reduce the entry of dust, rain, and harmful fauna. There are no signs that tell staff to wash their hands after using the restrooms. There are no measures to prevent the entry of domestic animals into the slaughter, carcasses, and viscera areas. Nor is it ensured that all plant areas are kept free of insects, birds, or rodents. The staff complies very little with the existence of clothing or personal belongings in the slaughter area. There is no prohibition on employees entering the slaughter or carcass processing areas with jewelry, clips, earrings, rings, watches, or bracelets. The blood is discharged into the public drainage. The water expenditure for processing the animal ranges from 25 to 48 L.

Cluster 3

This group comprises 26 SSUs; it includes private slaughterhouses and slaughterhouse facilities with private staff, which have an average slaughter volume of 60 animals per week (65 % male sheep). They are sheep from different states of the republic, which are slaughtered, and their meat and byproducts are marketed only in the municipality of Capulhuac, with two types of carcasses: tough, at an average price of $88.00, and tender, at $97.00. They also sell byproducts such as viscera: $159.00, legs: $36.00, and head $53.00.

The regulations they comply with include the existence of a pest control plan and easy-to-clean floor and wall joints, prevention of entry of domestic animals into the slaughter area, and a carcass aging time (7 to 12 h). All areas of the plant are kept free of insects, birds, and rodents; the viscera of each carcass are also identified to be inspected and they have freezers. As for the employees, all wear masks and partially comply with the non-existence of clothing or personal objects in the slaughter area. The regulations that are not complied with include the lack of sanitary mats at the entrances of the establishments. The blood is marketed within the municipality for preparing a moronga-type dish (blood sausage). The water they use in processing is 13 to 24 L per animal.

Cluster 4

Made up of 14 SSUs, only slaughters in slaughterhouse facilities with private staff that kill and process the least number of animals (27/wk). They use all the animals to prepare barbacoa and belly ($379.00/kg), which are only marketed in the metropolitan area of Mexico City; they process 76 % of male sheep to prepare barbacoa. The regulations they comply with are that the establishment has an area for unloading animals and a loading area for carcasses and viscera. They have pens for sick or suspicious animals, give a rest time before slaughter of between 13 and 24 h, have a pest control plan, have easy-to-clean floor and wall joints, prevent domestic animals from entering the slaughter area, allow an aging time of carcasses between 7 and 12 h, all have freezers, all employees wear face masks and comply with the non-existence of clothing or personal belongings in the slaughter area; the regulations they do not comply with are that there are no sanitary mats and a pest control plan, they do not have easy-to-clean joints between floors and walls either, they do not prevent the entry of domestic animals in the slaughter, carcass, and viscera areas, they give a deficient aging time of between 7 and 12 h, they do not keep the areas of the company free of insects, birds, and rodents; employees are not prohibited from entering the slaughter and carcass processing areas with jewelry, clips, earrings, rings, watches, or bracelets; the viscera of each animal are not identified, and they spend an average of 7 to 12 L (Table 5).

Table 5: Main differences in strengths and weaknesses between clusters

	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Strengths in sheep slaughter	They give an appropriate rest period before slaughter of 13 to 24 h[(4)]	The establishment has pens for sick or suspicious animals[(4)]	They control pests[(4)]	They give an appropriate rest period before slaughter 13 to 24 h[(4)]
	They comply with the existence of sanitary mats with disinfecting	They give an appropriate rest period before	They comply with preventing domestic animals from	They allow an aging period, as indicated by the

solution, as indicated by the standard[4]	slaughter of 13 to 24 h[4]	entering the slaughter, carcass, and viscera areas[4]	standard NOM-194-SSA1-2004, which is 7 to 12 h[4]
They comply with easy-to-clean floor and wall joints[1]	They comply with daily washing of pens, ramps, tunnels, *antemortem* baths, and drying and draining areas[4]	They allow an adequate aging time (7 to 12 h)[4]	
They comply with preventing the entry of domestic animals into slaughter, carcass, and viscera areas[4]	They comply with having easy-to-clean floor and wall joints, as indicated by the norm	They fully comply with the use of face masks by production staff[3]	They fully comply with the use of face masks by production staff[3]
They are very compliant in the use of face masks in production staff[3]	The comply with preventing the entry of domestic animals into slaughter, carcass, and viscera areas, as indicated[4]	They handle blood properly (sale)[4]	They use between 7 to 12 L of water per animal slaughter
They handle the blood properly (compost), as indicated[4]	They fully comply with the use of face masks by production staff, as indicated in the standard[3]	They give a very long rest period before slaughter of 24 to 48 h[4]	They wash ramps, tunnels, *antemortem* baths, and drying and draining areas weekly; for this reason, they do not comply[4]
They fully comply with the identification of viscera of each carcass, as indicated[4]	The establishments do not have a pest control plan, as indicated by the norm	They wash ramps, tunnels, *antemortem* baths, and drying and draining areas weekly; for this reason, they do not comply[4]	The establishments do not have a pest control plan[4]
They use between 7 to 12 L of water per animal slaughter	They do not comply with the existence of a sanitary mat with a disinfectant solution at the entrance to the slaughter area[4]	They do not comply with the existence of a sanitary mat with a disinfectant solution at the entrance to the slaughter area[4]	They do not comply with the existence of a sanitary mat with a disinfectant solution at the entrance to the slaughter area[4]

Weaknesses in sheep slaughter	They wash ramps, tunnels, *antemortem* baths, and drying and draining areas weekly; for this reason, they do not comply with the standard[4]	They do not comply with preventing the entry of domestic animals into slaughter, carcass, and viscera areas[4]	They do not comply with the identification of viscera of each carcass[4]	Floor and wall joints are not easy to clean[4]
	The establishment has a pest control plan[4]	They allow a very short carcass aging time of 1 to 6 h[4]	They spend between 13-24 L per animal	They do not comply with preventing the entry of domestic animals into slaughter, carcass, and viscera areas[4]
	They allow a very short carcass aging time of 1 to 6 h	They have no freezers[4]		They have no freezers[4]
		They do not give an adequate destination for the blood (drainage)[4]		They do not give an adequate destination for the blood[4]
		The viscera of each carcass are not identified[4]		The viscera of each carcass are not identified[4]
		They spend between 25-48 L per animal		

* Qualitative variable, ** Quantitative variable, Likert scale (not compliant, very little compliant, partially compliant, substantially compliant, fully compliant), [1](NOM-008-ZOO-1994), [2](NOM-033-SAG/ZOO/2014, [3](NOM-213-SSA-1 2018), [4](NOM-194-SSA1-2004), [5](NOM-120-SSA1-1994), [6](NOM-051-ZOO-1995).

Discussion

In Mexico, there are few studies that have documented the conditions in which sheep are slaughtered in different areas of the country and their effect on the health of consumers. The results of this study describe the conditions of the slaughter of more than 400 thousand sheep per year in Capulhuac, which are destined for human consumption through the sale of meat as fine cuts and barbacoa, a very popular dish to consume especially on Saturdays and Sundays in different areas of the metropolitan area of Mexico City, in addition to their use in social events[18]. Three types of sheep slaughter establishments were characterized: the first corresponds to all the animals slaughtered in the municipal slaughterhouse of Capulhuac with hired staff. The second corresponds to all animals slaughtered in an alternate outdoor area with pens, pools, and concrete tables, which the slaughterhouse rents to the general public to

carry out the slaughter of sheep, and the third corresponds to slaughterhouses with private establishments, of which 35 % have the adequate infrastructure and facilities to carry out the slaughter of sheep and 65 % correspond to premises and houses conditioned to carry out these activities.

It was also found that the three types of SSU have pens for the *antemortem* rest period of the animals. Nevertheless, they have poor management regarding rest time and prolonged fasting, factors related to the generation of periods of stress to the animal; this can be explained by the long distances that animals travel. Capulhuac is characterized by being an introducer of animals, which come mainly from the states of Coahuila, Zacatecas, and Jalisco and have even been imported from other countries such as New Zealand[19,20]. However, meat producers give the same rest times without considering truck infrastructure, distances, or transportation times, crucial factors that can trigger the formation of dark, firm, and dry (DFD) meats and thus affect carcass yield and consumer preference[21-25]. They have between one and five employees, their usual clothing being street clothes covered with an apron and plastic boots, not complying with the regulations.

The hygiene habits they comply with in full are the washing and disinfection of hands, forearms, and nails before entering the slaughter areas and the prohibition of employees from smoking, drinking, eating, and spitting in areas of slaughter and processing of carcasses. Regarding the desensitization method, less than 2 % use a method approved by the NOM-033-ZOO/SAG-2014 standard, such as the use of a penetrating captive bolt gun and electro-desensitization, methods that guarantee the unconsciousness of the animal and the null generation of suffering, while the rest (98 %) use a method that they locally call "descabellado", which refers to a method of killing reported by SADER and known as "puntilla" (pithing), which consists of a process of destruction of nervous tissue in the brainstem region to ensure the death of the animal; it is performed by inserting a "puntilla" (knife) that injures the medulla oblongata when it enters the atlanto-occipital joint, causing motor paralysis but there is no immediate loss of consciousness, leaving the cerebral faculties intact[26]. This method, despite being recommended in health emergencies, could violate the standard for methods to kill domestic and wild animals (NOM-033-SAG/ZOO-2014) as it is unknown if it nullifies the generation of stress and pain to the animal. The above are determining factors, as reported by some researchers[6] who observed that a deficient slaughter method could result in poor-quality meats with a shorter shelf life.

Regarding *postmortem* handling, it can be observed that none of the slaughter establishments has cooling chambers for the correct aging of meat, and only 12 % give it a time of between 12 and 48 h, while the rest are characterized by marketing hot carcasses, a detrimental factor for the tenderness of the meat, as mentioned in a study[27] that evaluated different aging times of sheep meat, concluding that the tenderness of the meat increases as the aging time of the carcasses increases. On the other hand[28], it is pointed out that pre-slaughter handling and

aging time, as well as meat storage conditions, play a determining role in the quality of the final product, which is consistent with what has been reported[29], which indicates that the stress generated by the poor handling of animals together with the deficient conditions of aging and storage of carcasses affects the loss of carcass weight, tenderness and generates cuts with dark colorations, directly affecting the sensory characteristics of the meat and thus the purchase decision or conditioning its sale to lower prices[30].

As for employees, no establishment provides adequate work clothes, nor is it required to disinfect footwear before entering the slaughter area. In 50 % of slaughterhouses, there were problems with pests, such as rodents, birds, insects, or domestic animals in the slaughter areas, coinciding with what was observed by others[31], who mention that the presence of pests reflects the poor cleaning conditions in worktables, vehicles, utensils, and work clothing. On the other hand, the staff lacks training as it was found that more than 90 % are unaware of good practices in animal slaughter and welfare, elements of utmost importance[32] according to the author of a study that evaluated the effectiveness of training staff in the handling and killing of animals and its effect on the quality of meat, concluding that appropriate equipment and staff training significantly improve the efficiency of the process, ensuring animal welfare and meat quality.

In 93 % of the handling of the carcasses of the establishments, *antemortem* examinations are not performed, in addition to not bathing animals, which has the purpose of reducing the microbiological load that the animal brings, such as remains of excrement, urine, or soil[33], results that coincide with a reported study[34] that found irregularities in veterinary inspection, compromising the safe reception of animals and increasing the risks of introduction of foodborne disease (FBD) causative agents from farms to the slaughterhouse.

Conclusions and implications

The three types of establishments formed do not have basic knowledge about animal welfare standards, and adequate staff training is lacking. The conditions of infrastructure, staff, and waste handling are not acceptable to ensure the safety and quality of the slaughter in accordance with current regulations. Particularly, of the three types of establishments to carry out the slaughter, the municipal slaughterhouse is the one that, to a certain extent, adheres to a higher level of compliance with current regulations. Nevertheless, the facilities are already old and lack the necessary technology for the number of animals slaughtered, and there is limited staff, thus causing long periods in the slaughter process. It is suggested that training programs be implemented by pertinent official authorities in order to improve the conditions in the slaughter process following the current regulations. It is also recommended to

condition and technify the facilities of the municipal slaughterhouse of Capulhuac de Mirafuentes to guarantee Good Slaughter Practices and the safety of the marketed meat, as well as make the installed capacity efficient to the current demand in the slaughter processing of this municipality.

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Table 1: Survey questions

(1) Type of slaughterhouse?*	(26) Are the floors of the slaughter facilities waterproof, homogeneous, and of characteristics that allow them to be easily cleaned and disinfected? (Yes, No)*(5)	(51) Is the *antemortem* inspection performed? (Yes, No)*(4)
(2) Origin of animals?*	(27) Is there a sanitary mat with a disinfectant solution at the entrance to the slaughter area? (Yes, No)*(4)	(52) Who performs the *antemortem* sanitary inspection?*(4)
(3) How many animals do you slaughter a week? (N)**	(28) Are the floor and wall joints easy to clean? (Yes, No)*(1)	(53) Do you perform *antemortem* bathing? (Yes, No)*(4)
(4) How often are sheep slaughtered? (N)**	(29) Are windows and vents provided with well-maintained protections to reduce the entry of dust, rain, and harmful fauna? (Yes, No)*(1)	(54) Are staff trained to do their jobs? (Likert Scale)*(5)
(5) Destination for the carcasses?*	(30) Are there signs instructing staff to wash their hands after using restrooms? (Yes, No)*(4)	(55) Stunning method?*(2)
(6) Place of marketing?*	(31) Does the establishment have an exclusive area for the temporary deposit of waste and garbage, delimited and outside the production area? (Yes, No)*(4)	(56) Are there rails or hooks for handling the carcasses? (Yes, No)*(4)
(7) Tender carcass price? ($/kg)**	(32) Are domestic animals prevented from entering slaughter, carcass, and viscera areas? (Yes, No)*(4)	(57) Destination for blood?*(4)
(8) Tough carcass price? ($/kg)**	(33) Carcass aging time? (hours)*(4)	(58) Do you have containers for disinfecting knives? (Yes, No)*(1)
(9) Viscera price? ($/kg)**	(34) Are all plant areas kept free of insects, rodents, birds, or other animals? (Yes, No)*(4)	(59) Are the viscera of each carcass identified? (Likert scale)*(4)
(10) Leg price? ($/kg)**	(35) Is the water used to wash equipment and utensils drinkable? (Yes, No)*(4)	(60) What are the viscera deposited in?*(1)
(11) Head price? ($/kg?) **	(36) Do you have a cooling chamber? (Yes, No)*(4)	(61) Are there separate rooms for handling green and red viscera? (Yes, No)*(1)
(12) Barbacoa price? ($/kg)**	(37) Do you have freezers? (Yes, No)*(4)	(62) Is *postmortem* inspection performed? (Yes, No)*(4)
(13) Percentage of male sheep sold (%)**	(38) How many employees work in the establishment? (N)**(5)	(63) Who performs the *postmortem* sanitary inspection?**(4)
(14) Percentage of ewes sold (%)**	(39) Is there no presence of clothing or personal belongings in the slaughter area? (Likert Scale)*(5)	(64) Are there incinerators? (Yes, No)*(1)(4)
(15) Does the establishment have an animal unloading area and a loading area for carcasses and viscera? (Yes, No)*(4)	(40) Are there lockers where employees can store their belongings? (Yes, No)*(3)	(65) What is the destination for confiscated viscera and carcasses*(4)

(16) Does the establishment have an identified area with water intake and drainage for washing and disinfecting transport? (Yes, No)*[4]	(41) Do employees show up to work neat? (Yes, No)*[5]	(66) Are carcasses washed after skin removal? (Yes, No)*[4]
(17) Does the establishment have pens for sick or suspicious animals? (Yes, No)*[4]	(42) Do they wear a cap? (Likert Scale)*[5]	(67) How many liters of water are used per animal? (L)**
(18) Does the establishment have pens for the rest period before the slaughter? (Yes, No)*[4]	(43) Do they wear face masks? (Likert Scale)*[3]	(68) Where is the wastewater discharged?*[4]
(19) Rest period before the slaughter? (hours)**[4]	(44) Is footwear disinfected before entering the slaughtering area? (Likert Scale)*[3]	(69) Is there signage for dangerous areas? (Likert Scale)*[5]
(20) How often are pens, ramps, tunnels, *antemortem* baths, and drying and draining areas washed?**([4]	(45) Does the establishment provide appropriate clothing for work? (Likert Scale)*[3]	(70) Do you have any health promotion programs?* (Yes, No)*[5]
(21) Does the establishment have drainage? (Yes, No)*[4]	(46) Are employees prohibited from entering the slaughter or carcass processing areas with jewelry, clips, earrings, rings, watches, or bracelets? (Likert Scale)*[5]	(71) Do you know what good slaughter practices are? (Yes, No)**[5]
(22) Does the establishment have restrooms? (Yes, No)*[4]	(47) Are employees prohibited from smoking, drinking, eating, and spitting in slaughtering and carcass processing areas? (Likert Scale)*[5]	(72) Are staff trained in GSP? (Yes, No)*[5]
(23) Are the restrooms located outside the slaughter and carcass processing facilities? (Yes, No)*[4]	(48) What type of clothing do employees wear to work?*([5]	(73) Do you know what animal welfare is? (Yes, No)*[6]
(24) Does the establishment have a pest control plan? (Yes, No)*[4]	(49) Is access to the slaughter rooms restricted to sick staff? (Likert Scale)*[5]	(74) Do you carry out animal welfare practices? (Yes, No)*[6]
(25) Floor and wall building material?*[1]	(50) Are staff required to wash and sanitize their hands and forearms and brush their nails before entering processing areas? (Likert Scale)*[3][5]	

N (number), * Qualitative variable, ** Quantitative variable, Likert scale (not compliant, very little compliant, partially compliant, substantially compliant, fully compliant), [1](NOM-008-ZOO-1994), [2](NOM-033-SAG/ZOO/2014, [3](NOM-213-SSA1-2018), [4](NOM-194-SSA1-2004), [5](NOM-120-SSA1-1994), [6](NOM-051-ZOO-1995).

Table 4: Relevant and important characteristics of the four clusters formed in the sheep slaughterhouses in the municipality of Capulhuac de Mirafuentes

No	Slaughter variable or descriptor	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Value of (*P*)
1	SSU number	10	6	26	14	
2	Type of slaughterhouse	Private slaughterhouses (100%)	Private slaughterhouses (33.4%), the Municipal Slaughterhouse (16.6%), Slaughterhouse facilities with private staff (50%)	Private slaughterhouses (84.6%), Slaughterhouse facilities with private staff (15.4%)	Slaughterhouse facilities with private staff (100%)	0.0001
3	How many animals are slaughtered per week	31±26.8	86±114.0	60±58.0	27±30.0	0.1078
4	How often sheep are slaughtered	Weekly	Weekly	Weekly	Weekly	0.2285
5	Destination for the carcasses	Meat and barbacoa sale	Meat and barbacoa sale	Sale of meat	Barbacoa	0.0001
6	Place of marketing	Capulhuac and Mexico City Metropolitan Area	Capulhuac	Capulhuac	Mexico City Metropolitan Area	0.0001
7	Tender carcass price/kg	96.9±4.03	99.4±8.00	97.0±6.25	N/C	0.0001
8	Tough carcass price/kg	91.50±4.03	89±8.00	88±6.50	N/C	0.0001
9	Viscera price/kg	163±10.59	151±18.60	159±13.20	N/C	0.0001
10	Leg price/pcs	34±5.27	35±5.00	36.30±4.05	N/C	0.0001
11	Head price/pc	53±4.40	50±0.00	53.04±5.50	N/C	0.0001
12	Barbacoa price/kg	391±16.93	360±28.28	N/C	379±24.66	0.0001
13	% of sheep sold	84.44±7.26	50±20.54	65.83±20.14	76.5±22.11	0.0001
14	% of ewes sold	16.67±5.47	50±20.54	34.2±16.32	23.5±14.12	0.0001
15	The establishment has an area for unloading animals and a loading area for carcasses and viscera	Yes (100%)	Yes (100%)	No (100%)	Yes (100%)	0.0001
16	The establishment has pens for sick or suspicious animals	No	Yes	No	Yes	0.0001
17	The establishment has pens for the rest period before the slaughter	Yes	Yes	Yes	Yes	0.3930
18	Rest period before the slaughter	13- 24 h	13-24 h	24-48 h	13-24 h	0.0490
19	How often are pens, ramps, tunnels, *antemortem* baths, and drying and draining areas washed?	Weekly	Daily	Weekly	Weekly	0.0172
20	The establishment has a pest control plan	No	No	Yes	No	0.0053
21	There is a sanitary mat with a disinfectant solution at the entrance to the slaughter area	Yes	No	No	No	0.0012
22	The floor and wall joints are easy to clean	Yes	Yes	Yes	No	0.0001

23	The windows and vents are provided with well-preserved protections to reduce the entry of dust, rain, and harmful fauna	No	No	No	No	0.0580
24	There are signs instructing staff to wash their hands after using the restrooms	No	No	No	No	0.8340
25	Domestic animals are prevented from entering slaughter, carcass, and viscera areas	Yes	No	Yes	No	0.0001
26	Carcass aging time	1-6 h	1-6 h	7-12 h	7-12 h	0.0470
27	There is a cooling chamber	No	No	No	No	0.3643
28	There are freezers	Yes	Yes	Yes	Yes	0.0253
29	How many employees work in the establishment	1-5	1-5	1-5	1-5	0.9080
30	All areas of the plant are kept free of insects, birds, and rodents	Yes	No	Yes	No	0.0001
31	There is no presence of clothing or personal belongings in the slaughter area	Fully compliant	Very little compliant	Partially compliant	Fully compliant	0.0164
32	Meat product establishment managers provide clean work clothes to workers	Not compliant	Not compliant	Not compliant	Not compliant	0.7601
33	They wear face masks	Substantially compliant	Fully compliant	Fully compliant	Fully compliant	0.0035
34	Footwear is disinfected before entering the establishment	Not compliant	Not compliant	Not compliant	Not compliant	0.0980
35	Employees are prohibited from entering the slaughter or carcass processing areas with jewelry, clips, earrings, rings, watches, or bracelets	Not compliant	Not compliant	Not compliant	Not compliant	0.0481
36	Employees are prohibited from smoking, drinking, eating, and spitting in the slaughter and carcass processing areas	Fully compliant	Fully compliant	Fully compliant	Fully compliant	0.4727
37	What type of clothing employees show up to work in	Plastic apron and rubber boots	Plastic apron and rubber boots	Plastic apron and rubber boots	Plastic apron and rubber boots	0.0708
38	Staff must wash and sanitize their hands and forearms and brush their nails before entering the processing areas	Fully compliant	Fully compliant	Fully compliant	Fully compliant	0.0766
49	Staff are trained to do their jobs	Not compliant	Not compliant	Not compliant	Not compliant	0.0609
40	Stunning method	Pithing	Pithing	Pithing	Pithing	0.0609
41	Destination for the blood	Compost	Drainage	For sale	Drainage	0.0193
42	There are containers for disinfecting knives	Fully compliant	Not compliant	Fully compliant	Not compliant	0.1743
43	The viscera of each carcass are identified	Fully compliant	Fully compliant	Fully compliant	Not compliant	0.0041
44	What is the destination for the confiscated viscera and carcasses	Incinerated	Incinerated	Incinerated	Incinerated	0.0697
45	How many liters of water are used per animal	7-12 L	25-48 L	13-24 L	7-12 L	0.0238
46	There is signage for dangerous areas	Not compliant	Not compliant	Not compliant	Not compliant	0.1245

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Article

Producer typology and indirect effects of climate change on cattle ranching in Sinaloa

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Abstract:

The objective of the work was to typify dual-purpose production units and characterize the resources for fodder production and the issues affecting livestock production in Sinaloa, Mexico. Through non-probabilistic sampling, 61 ranches were selected from eight municipalities in the state of Sinaloa, and four groups of producers were identified through factor analysis and cluster analysis: E1, E2, E3, and E4. Producers have diverse land uses for fodder production: planting of annual crops, pastures, grazing on fallow land, and use of pasture lands. Drought is the main issue for 52.5 % of the producers. Producers with larger herd sizes (E3 and E4) have more agricultural and grazing land; however, their production

systems are more vulnerable and, therefore, they have to resort to the purchase of forage. 86.7 % of the producers pointed out that the herd has decreased due to the problem of drought, which requires the development of technological strategies and policies to improve forage production within the context of climate change, and thus reduce the pressure and potential deterioration of agricultural and pasture land in the study region.

Keywords: Pasture land, Pastures and forage, Cattle, Drought, Tropic.

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Introduction

The main threats to the production sector relate not only to climate change trends, but also, and more importantly, to climate variability and extreme weather events such as heat waves, droughts, floods, cyclones, and forest fires[1]. These weather events affect livestock health through heat stress, metabolic disturbance, oxidative stress, and immune suppression, resulting in increased susceptibility to disease incidence and death[2]. In general, it has been identified that a drought event reduces the average agricultural gross domestic product by 0.8 % worldwide[3]. Direct effects of climate change on livestock include affecting livestock growth rates, milk and egg production, reproductive performance, as well as morbidity and mortality, along with feed supply[4], while indirect effects relate to the impact of climate change on pastures, forage crops, and feed productivity[5].

In Mexico, there are recent studies on the management, recovery, conservation of vegetation cover, and sustainable use of pasture land in livestock farming[6,7,8]. However, they do not refer to the relationship between these and the level of agricultural resources for forage production available to producers in a drought context. At the producer level, the main perceived climatic changes include erratic and reduced rainfall, increased temperature, and prolonged and frequent periods of drought, which have had negative impacts on livestock production, namely forage and water shortages, leading to starvation, malnutrition, and mortality of livestock, reduced productivity, and low market prices[9].

At the producer level, the main perceived climatic changes include erratic and reduced rainfall, increased temperature and prolonged and frequent periods of drought, which have had negative impacts on livestock production; forage and water shortages, leading to

starvation, malnutrition and mortality of livestock, decreased productivity and low market prices. At the national level, livestock production is associated with an area with natural vegetation of 26.4 million hectares in forests (28.3 %), of which 12.2 % correspond to the humid tropics and 16.1 % to the dry tropics, respectively[10]. Livestock production in Sinaloa is mainly located in the dry tropics, where a diversity of land and pasture uses converge in the region, with specific problems and management from the producer's perspective. In addition, under the current context, there is very little information on the direct and indirect effects of climate change on livestock production.

This study describes the agricultural and pasture land utilized for forage production, pinpoints the main issues in livestock production, and identifies drought as a consequence of climate change from the perspective and opinion of different groups of producers. The objective of the work was to typify dual-purpose production units and characterize the resources for forage production and the problems affecting livestock production in Sinaloa, Mexico. The hypothesis is that environmental vulnerability in the livestock production system has a direct relationship with the level of productive resources that the producer has; thus, the larger the herd size, the greater the purchase of forage and pasture land and the greater the perception of drought as a serious problem that affects the production system.

Material and methods

Location of the study area

The study area is located in the northwest of the country, in the state of Sinaloa, at the following extreme coordinates: 27°02'32" N to the north, 22°28'02" N to the south; east 105°23'32" W to the east, and 109°26'52" W to the west. The state represents 2.9 % of the country's surface and is bordered to the north by the state of Sonora and Chihuahua; to the east, by Durango and Nayarit; to the south, by Nayarit and the Pacific Ocean, and to the west, by the Gulf of California[11]. Sinaloa is made up of 18 municipalities; this study was carried out in eight municipalities, which represent 44.44 % and are located in three geographical regions: Southern area (Rosario, Mazatlán, Concordia, San Ignacio); Central area (Elota), and Northern area (Guasave, Mocorito, El Fuerte). These municipalities were selected in order to have information from the three geographic zones of the state.

Climate conditions in Sinaloa are very dry; in general, it has a warm sub-humid, dry, and semi-dry climate, and only 2 % of the state has a temperate sub-humid climate in the highlands[12]. Precipitation occurs irregularly, with average precipitation values increasing

from north to south and as one moves up from the coast to the high mountains. In the coastal plain, they range from 200 to 700 mm, and in the southeastern portion, they exceed 1,000 mm. In the northwest, rainfall is 600 mm, and in the southeast, it varies from 800 to more than 1,500 mm[13].

Vegetation types and livestock management

A total of 45.1 % of Sinaloa's surface area is covered by natural vegetation (jungle, forest, hydrophilic vegetation, scrubland, other types of vegetation, and pastureland), i.e., it has not been altered by man or natural events. While 54.9 % corresponds to agricultural land, cultivated pastures, urban areas, areas with no apparent vegetation, water bodies, and secondary vegetation[10]. The natural vegetation existing in the pasturelands of Sinaloa corresponds mainly to the so-called "tropical deciduous forest"[14], also known as "dry forests"[15]. Livestock management in Sinaloa uses pasture land; this resource is fundamental for the provision of forage for livestock feeding during the rainy season, in addition to the use of grazing annual crops (sorghum, corn) in the traditional way[16], and the rainy and dry season use of perennial grasslands established as a result of technology transfer by local research centers.

Sample selection and applied instrument

The study used information obtained through producer surveys. The sample was obtained through the use of non-probabilistic purposive sampling[17]. Purposive sampling prioritizes the selection of cases that provide quality information on a specific topic for in-depth analysis and is carried out through the definition of criteria defined by the researcher[18,19]. The survey was conducted by six livestock extensionists located in the study area and hired by the Directorate of Livestock of the Sinaloa State Government; they selected the municipalities and producers to be interviewed based on ease of access and security; the interviewees must: 1) be dual-purpose cattle producers (representative system of Sinaloa), and 2) agree to answer the survey.

A total of 61 surveys were conducted in three different areas: North (10), Central (7), and South (44). This survey was conducted in the first quarter of 2022. It was designed to obtain information related to the age of the producer, the total area used for livestock production, sowing areas, grazing areas, including information on whether or not they have pasture, months of use and total pasture area, the livestock inventory of each production unit, the

perception of the dates related to the beginning and end of the rainy season (when did the rainy season begin and when did it end?), the behavior of the herd size in the last ten years (Do you consider that the number of cattle had increased, decreased or remained the same in the last ten years? What was the reason for the decrease?). In order to identify the issues, the farmer was asked to select, in order of importance from most to least important, the problems that, in his perception, most affected livestock production. The issues raised were: high forage costs, high fuel costs, low milk prices, low price per kilo of calves, lack of government support, and drought.

Information analysis

Factor analysis (FA) was used to reduce the dimension of the data and explain a phenomenon from a smaller number of variables called factors[20]. The main purpose of a FA is "to try to establish an underlying structure between the variables of the analysis, based on the correlation structures between them, i.e., it seeks to define groups of variables (better known as factors) that are highly correlated with each other"[21]. In order to determine the number of factors to be extracted, the criterion of the percentage of explained variance was considered, which for social sciences can be set at a minimum of 60 %[22]. The factor matrix was estimated using the Varimax rotation method with Kaiser normalization; the rotated solution stops when the weights at the factor level are maximized. In other words, each item or variable is expected to be representative in only one of them, to minimize the number of variables within each factor as much as possible; the factor matrix was thus obtained, which contains the weights (loadings or weights) of each variable, so that a variable is contained in a factor when its contribution is above 0.5[23].

The FA used 10 quantitative variables, which have been used in other studies for producer typologies[24,25,26]: number of animal units and herd size, planted area, pasture area, number of offspring working on the ranch, total number of offspring, producer's age, pasture area, fallow area, and number of months with forage shortage. To verify the usefulness of factor analysis, the Kaiser-Meyer-Olkin (KMO) sample adequacy measure was obtained: values of this statistic below 0.5 would indicate that FA would not be a useful technique, and values between 0.5 and 0.6, that the degree of intercorrelation is medium, but applicable, while a KMO with values above 0.7 would indicate a high intercorrelation between the variables[27]. In addition, Bartlett's test of sphericity was utilized to test the null hypothesis that the variables are intercorrelated, that is, to evaluate whether the correlation matrix is not an identity matrix, that is, one in which there is no relationship between the variables; this test is accepted as valid if the significance level is less than 5%[28].

In order to identify the different groups of producers, a cluster analysis (CA) was performed, which allowed clustering producers with similar characteristics within the group and with a wide variability among them. According to Rao and Srinivas[29] in CA the groups are formed in such a way that each object is similar to those within the cluster. Hierarchical cluster analysis with Ward's method and the squared Euclidean distance were utilized to identify the groups[30]. An analysis between groups was performed using the Kruskal-Wallis test and Chi-square tests for qualitative variables to determine differences ($P<0.05$) between groups. A Spearman correlation analysis was performed to verify whether there is a relationship between pasture area, number of months of purchased fodder, and number of heads in the herd, given that the normality of the data was not fulfilled. Statistical analyses were carried out with SPSS software[31].

Results and discussion

Factor analysis

The FA identified four factors that explain 68.79 % of the variance of the data (Table 1). The components obtained were denominated as follows: agricultural resources (C1), forage resources (C2), family resources (C3), and additional forage resources (C4); the variables were positive in each component. The sample adequacy measure KMO presented a value of 0.61 and Bartlett's test of sphericity showed a Chi-square (X^2) value of 444.73 and a significance of $P<0.0001$, so it can be affirmed that the PA was a suitable and appropriate model for the reduction of variables. The cluster analysis identified four groups: group 1 (G1) represented 27.80 % of the sample, G2 represented 49.20 % and had the highest percentage of producers interviewed, G3 represented 9.80 % and finally, G4 represented 13.10 % of the total producers.

Table 1: Matrix of rotated components and percentage of explained variance

Variable	C1	C2	C3	C4	Communality
Herd size	.964	.053	-.068	-.089	.945
Animal units	.964	.053	-.065	-.093	.945
Planted surface area, ha	.754	.261	-.008	.233	.691
Surface area of pasture lands, ha	.529	-.400	.114	-.177	.484
No. of children working on the ranch, #	-.011	-.062	.873	-.082	.774
Total number of children	-.052	.344	.783	.177	.766
Producer's age	-.068	.559	.220	.109	.378
Surface area with meadows, ha	.207	.621	.181	-.047	.464
Fallow surface area, ha	-.040	-.034	.039	.958	.922
Months with fodder shortage	.062	.694	-.130	-.090	.511
Inherent value	2.813	1.861	1.185	1.021	
% of the variance	28.132	18.606	11.845	10.214	
% cummulative	28.132	46.738	58.583	68.797	

Family resources

The age of the producers was similar among the four groups ($P>0.05$), ranging between 50 and 57 years; G4 producers were the youngest with a median age of 50 yr. The four groups have 2 or 3 children on average. In general, there is very little participation by the offspring in the productive activities of the ranches (Table 2). These results coincide with Cuevas *et al*[32] who point out that the socioeconomic characteristics of the producer in Sinaloa are homogeneous.

Table 2: Family resources of producer groups (median±IQR*)

Variable	G1	G2	G3	G4	*P***
Age	56.00±26.00	57.50±21.25	56.00±23.25	50.00±17.00	0.338
Total number of children, #	3.00±3.50	2.00±3.20	2.00±3.20	3.00±3.50	0.544
Number of working children, #	0±1.00	0±1.00	0±1.00	0.50±1.00	0.657

*IQR= interquartile range, ** Kruskal-Wallis test.

Farming resources

Herd size was similar between G1and G2 (36 and 42.5 head of cattle per group), but different (P<0.05) between the rest of the groups (180 for G3 and 110.5 in G4); this behavior was similar for the animal unit (AU) variable. There were no differences (*P*>0.05) between groups G1, G2, and G4 in the planted area (*P*>0.05), unlike in the area of pasture land owned by farmers, which exhibited differences (*P*<0.05) between groups G1, G2, and G3 (Table 3).

Table 3: Agricultural resources of the producer groups (median±IQR*)

Variable	G1	G2	G3	G4	*P***
Herd, No. of heads	36.00±28.50[a]	42.50±27.25[a]	180.00±69.50[b]	110.50±21.25[c]	0.001
AU	32.75±26.00[a]	37.25±25.61[a]	154.50±61.70[b]	95.20±13.42[c]	0.001
Planted area, ha	20.00±21.50[a]	12.00±12.18[a]	50.00±62.50[b]	13.00±15.25[a]	0.027
Pasture land, ha	38.00±40.50[a]	3.50±90.00[b]	65.00±126.00[c]	15.00±80.80[a]	0.001

*IQR=interquartile range, ***P* is the probability obtained by the Kruskal-Wallis test.

[abc] Values with distinct literal are different (*P*<0.05).

The use of agricultural resources (sown area and pasture) for forage production depends on the rainy season. Producers reported a three-month rainy season (63.90 % mentioned that the rainy season starts in July, while 41% said it ends in September). Thus, the rainy season would correspond to a period of three months, July through September, while the rainy season could be up to nine months a year: October to June.

The pasture land ("agostadero") is used during the rainy season when the tropical deciduous forest is renewed; previous studies indicate that, during the rainy season, unproductive cattle, calves, and weaned calves are sent to the "pasture land" to graze grasses and trees[33], these same authors describe the main species that exist in the pasture land; the vertical structure is made up of dominant trees with heights of 10 to 15 m, the upper floor is made up of species such as *Lysilpma divaricata, Caesalpinia sclerocarpa, Pithecellobium mangense*, and *Conzattia serícea*. During the summer, the undergrowth is covered by a dense carpet of herbaceous species, which are highly preferred by cattle: *Carlowrightia costarina, Henrya imbricans, Henrya scorpioides, Ruellia donnell-smithii*, and *Siphonoglossa sessilis*. This resource is used by producers and is one of the most threatened plant resources in Mexico; a study conducted on this type of vegetation found an annual deforestation rate of 1.4 %, as well as fragmented and disturbed areas[34].

Finally, during the "dry season, the land planted with annual crops is used as "paddocks", that is, after harvesting the corn or sorghum, the rest of the plant (stubble) serves as feed for

livestock. At this time, all cattle are concentrated in these paddocks, which are fenced with barbed wire and regional wood posts obtained from the pasture, and feeding is complemented with the purchase of forage and the use of the state's irrigated areas. These results are consistent with a study of the dual-purpose bovine system (DPBS) carried out in northern Sinaloa[35] which indicates that the DPBS is based on the grazing of different forage resources: grazing on residues in cultivated areas (corn and sorghum crops), on established pastures, and on the grazing of areas of common use called *agostadero*, combined with feed supplementation.

Livestock forage resources

The use of grasslands and "savannas" was similar in the four producer groups (P>0.05). There is a small amount of grasslands and fallow land: only 45.90 % of the producers reported the use of grassland, and 21.30 % allowed land to lie fallow. However, all groups have purchased fodder, but those with the largest number of animals (G3 and G4) do so for a larger number of months, namely, 5 to 6.6 mo per year (Table 4).

Table 4: Fodder resources of the producer groups (median±IQR*)

Variable (ha)	G1	G2	G3	G4	*P***
Meadows	0±12.50	0.50±3.00	0±16.00	0±12.75	0.927
Purchase of forage, months	3.00±2.50	3.00±3.00	5.00±4.50	6.50±90	0.057
Fallow surface area, ha	0±10.00	0±0	0±2.00	0±0	0.107

*IQR=interquartile range, **Kruskal-Wallis test.

Cattle management in regard to this type of resource is as follows: at the beginning of the rainy season, lactating cows remain in the fallow areas or "savannas" (agricultural areas open to cultivation that are not sown and, therefore, allow this type of cattle to continue grazing on natural vegetation or native grasses). The use of savannas is a necessity for maintaining livestock, even though crop residues are often low quality.

Producers who have pastures use forage during the dry season, as, during the wet season, the savannas provide enough forage for the cows. In this regard, a study on small producers conducted in Sinaloa[36] shows that "producers who have perennial pastures use them as reserve lots in the dry months i.e., January through June; the animals graze continuously until they totally consume the pastures, which then are allowed to lie fallow and recover during the wet period (July to December), a situation that goes against pasture management, but the

producer's decisions in this regard are conditioned by the rainy period during which the pastures are utilized as a source of food".

The results of the correlation between herd size (HS) and the purchase of fodder was significant (P<0.05), with a value of rho59=.255, P=.047, and the correlation between HS and the number of hectares of pasture was moderate (P<0.05), with a value of rho59=.305, P=.017. This seems to indicate that, for the sample analyzed, producers with a larger HS have a larger surface area of pastureland and a greater need to purchase fodder, which may lead to a loss of productivity of this resource. As Enríquez *et al*[37] point out, in at least 24 states of the country, the number of head of cattle exceeds the carrying capacity based on forage production. This situation results in the gradual degradation of grasslands and, consequently, in a reduction of their productivity.

Issues in the livestock system

The first and second issues for livestock production in the study region were drought and the high cost of fodder; there were no differences (P>0.05) between the four groups analyzed; the only problem that differed among the groups was the low price of the calves (P<0.05), between G1 and G4 (Table 5). These results agree with Habte *et al*[9] in the sense that drought is one of the most important indirect effects of climate change on livestock production, given that 52.50% of the interviewed producers indicated that the main issue has to do rather with the intense droughts that limit the production of fodder for livestock feed.

Table 5: Main cattle raising problems in the study region (%)

Issue	*G1 (17)	G2 (30)	G3 (6)	G4 (4)	Average	X^2
Droughts	64.70	43.30	50.00	62.50	52.50	0.691
High cost of fodder	29.40	26.70	33.30	12.50	26.20	0.687
Low price per kilo of calves	35.30[a]	13.30[b]	0.0	37.50a	21.30	0.005
Lack of government support	17.60	16.70	16.70	12.50	16.40	0.173
Low milk prices	23.50	13.30	0.0	12.50	14.80	0.188
High fuel costs	0	6.7	0.0	0.0	3.30	0.748

X^2= Xi-square test, * The total number of producers in the group is shown in parentheses.
[ab] Values with distinct literal are different (P<0.05).

Through the drought monitoring carried out by the National Water Commission[38] at the national level and in Sinaloa, this institution has identified several years with critical drought periods; in its report for the year 2021, it identified in the study region five municipalities (Concordia, Elota, Mazatlán, Mocorito and San Ignacio) with extreme drought conditions,

while the other three municipalities (El Fuerte, Guasave, and Rosario) exhibited severe drought in the year 2021.

86.70 % of the producers pointed out that the livestock inventory has decreased in the last ten years, and 67.30 % mentioned frequent periods of drought as the main reason. Given that periods of intense drought reduce the availability of forage, extreme events such as hot spells, intense droughts, and floods will also have adverse effects on the agricultural sector and livestock productivity, as well as affecting the producer inventory[8,9]. It is worth mentioning that the months and mechanisms to provide water to the animals were not directly researched; however, water management for the animals is provided by wells, streams near the corrals, and dams. Producers in the north of the state (El Fuerte, Guasave) have their land close to irrigation canals and also "haul" water in pickup trucks. Drought and water management for livestock is a topic that should be further explored in future studies on livestock production in the tropics.

Conclusions and implications

The drought period in the analyzed sample was nine months; the shortage of forage during this period forces producers to buy pasture and other feed for up to six months of the year. In this sense, the hypothesis was corroborated by the fact that producers with larger herds are more vulnerable in the production of fodder for livestock feed, so they have to resort to the purchase of fodder and the use of a larger agricultural and pasture area. As for vulnerability to drought as a climate change issue, producers with larger herd sizes indicated drought as the main problem; however, the percentage of producers who pointed at drought as the main problem was higher among producers with small herds. These results apply to the interviewed producers; however, they could be used for regions with similar geographic conditions. Technological strategies and policies differentiated by types of producers according to their resources must be developed to improve fodder production within the context of drought and thereby reduce the pressure on and potential deterioration of agricultural and pasture lands in the state of Sinaloa.

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suelos en el trópico seco de México" ("Sustainable forage production under a context of climate change and soil degradation in the dry tropics of Mexico").

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Article

Effect of sex on meat quality traits and sensory properties in Argentine crossbred pigs

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Abstract:

The objective of this study was to evaluate the effect of sex on final live weight, carcass characteristics meat quality traits and sensory properties of a specific cross breed pig line (Landrace 75% x Yorkshire 25% "Degesa"). Eight randomly selected barrows (CM) and eight gilts (F) were used in the present study. No differences ($P>0.05$) between sexes for carcass characteristics, shear-force value or sarcomere length were observed. However, back fat thickness, pH@45, pH@24, water-holding capacity, marbling score and intramuscular fat content were higher ($P>0.05$) in CM than in F. Meat from CM had lower ($P=0.04$) lightness than F but similar ($P\geq0.34$) redness and yellowness. Total saturated fatty acids (SFA) proportion as well as individual SFAs (C16:0 and C18:0) were greater in CM than in F, but n-6:n-3 ratio was lower in males than females. In general, meat from males were better scored than meat from females by the trained panel in flavor attributes but the result was opposed when textural properties were evaluated. In addition, greater overall color score as well as flavor attributes were positively associated with intramuscular fat content and rate of monounsaturated FA but negatively associated with rate polyunsaturated FA proportion. In conclusion, results suggest that pork quality from Degesa crossbred pigs showed marked sex-related differences and therefore, it could be deferentially commercially by sex in the meat market.

Keywords: Fatty acids, Intramuscular fat, Meat color, Sarcomere length, Sensory panel, Shear force.

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Introduction

Argentina has been traditionally recognized as an important producer and consumer of meat from beef cattle. However, in the last past years the pork industry has been growing, leading to a greater local *per capita* consumption of meat (from 8.5 kg in 2011 to approximately 16 kg in 2020[1]. Although meat quality is a critical issue for the meat industry, Argentinian pork classification system is based only on the proportion (%) of lean tissue and on carcass yield (kg) (Resolution S.A.G. and P. No. 57/95).

The main sensory attributes defining pork quality are color, tenderness, juiciness, odor and flavor[2]. Productive managements such as diet and feeding practices[3] can affect these attributes. In addition, intrinsic aspects such as, breed, weight and sex are important[4,5,6]. Several studies[7,8,9] suggested that carcass and pork quality traits could be highly dependent on animal sex including type of castration. However, these studies obtained inconsistent results since different swine genetics lines were evaluated. This suggest that differences in meat pork quality traits related to sex are highly dependent on the breed and/or genetic hybrid considered[6].

Despite pig sex plays a key role in meat quality aspects, Argentine pig carcasses are currently commercialized as a single category "*capon*" including whole entire or castrated males and females whereas there are not researches to the current knowledge evaluating meat quality aspect of swine hybrid line (Landrace 75% x Yorkshire 25%). Therefore, the present study represents a novel approach to evaluate the effect of sex of females and castrated males' pigs on carcass and meat quality traits of swine hybrid line (Landrace 75% x Yorkshire 25% "Degesa")

Material and methods

Animal management, carcass measurements and sample collection

The trial was carried out in La Isla, Cerrillos, province of Salta (24°52'46"S, 65°24'20"W, 1,217 m altitude) Argentina, under good manufacturing practices management and welfare standards in accordance with Argentine national recommendations for animal handling. The procedure was approved by the institutional ethical and technical committee of the Catholic University of Salta (RR N° 1294/15).

Sixteen (16) crossbred pigs Degesa (Yorkshire 25% x Landrace 75%) were randomly selected from the same herd: eight females and eight males. Males were surgically castrated (CM) and females (F) remained entire. Each group was assigned to separate pens, with an area of 1.2 m^2 per animal. Animals were fed *ad libitum* with the same commercial feed and water using a hopper system. All animals were slaughtered on the same day in a commercial slaughterhouse, located 30 km from the experimental farm. At slaughter, animals were 25 wk of age and their average live weight was 125 ± 5 kg.

Individual pre-slaughter weight (PSW) and hot carcass weight (HCW) were recorded in the slaughterhouse. Muscle pH was determined 45 min (pH@45) and 24 h post-slaughter

(pH@24) on the *Longissimus lumborum* (LL) muscle, between the 12th and 13th ribs of each right half carcass. Back fat thickness was measured with a manual caliper (Starrett ®, Athol, Massachussetts, USA) and the Loin eye area (LEA) was traced and determined with ImageJ® software at the level of the 11th rib (BFT; cm) on the left carcass side. Marbling score was determined on the same rib through the Official Marbling Quality Standard score cards (Official Color and Marbling Quality Standards, Pork checkoff, USA). *Longissimus lumborum* (LL) sections between 9th and 13th ribs from each left and right carcass were cut into steaks, perpendicular to the longitudinal axis of the LL muscle. A 2.5-cm thick steak of the LL muscle was obtained from the 12–13th rib section (from cranial to caudal), of the left carcasses for proximate analysis. For this analysis, all external fat and connective tissues were previously removed. An additional 0.5-cm thick steak was obtained from the 12–13th ribs section and stored for further determination of sarcomere length. The LL muscle from the 9–11th rib section was cut into 2.5-cm thick steaks to evaluate color, warner–bratzler shear force (WBSF) and cooking loss. After 24 h of slaughter, meat samples were vacuum-packed and stored at -20 °C, until further analysis at the meat quality laboratory of EEA INTA Balcarce, Argentina. Sensory analyses were performed at the Laboratory of Sensory Analysis of the Faculty of Agronomy, National University of Buenos Aires, Argentina.

Meat quality measurements

Proximate analysis

Dry matter content was calculated as the difference between initial (fresh meat) and final weight after drying the meat for 48 h at 60 °C, in duplicate. Total lipid content was determined using an automatic extraction system (Ankom xt10, Ankon, Macedon NY, USA).

Meat color evaluation

Instrumental color was recorded using a Minolta chromameter (CR-310; Minolta Inc., Osaka, Japan) with a 50-mm-diameter measurement area using a D65 illuminant, calibrated against a white ceramic disk provided by the manufacturer. Color readings were determined 24 h *post mortem* on the exposed cross-sections of the 12th rib of the LL muscle from the left carcass. The meat sample was bloomed at room temperature for 30 min before color measurement. Each sample was measured six times and the value is expressed as an average. The system used was the CIE Lab, which provides three-color components: L* (lightness,

0 = black, 100= white), a* (red index, -a*= green, +a*= red) and b* (yellow index, -b= blue, +b= yellow).

Warner-Bratzler shear force and cooking loss

WBSF procedure was conducted according to AMSA (1995)[10] guidelines. Frozen samples (steaks of 2.5 cm of thickness) were thawed at 4 °C for 12 h, weighed and cooked on open heart electric grill (Farberware, Bronks NY). During cooking, steaks were flipped at 35.5 °C at the geometric center and grilled until temperature reached 71 °C. Internal temperature was controlled using a multi-scan digital thermometer (Scanning Thermometer, Digi-Sense, Cole Palmer). The cooked samples were chilled at 4 °C for 20 min and weighed again. Cooking loss was calculated as follows: cooking loss (%)= (weight of uncooked sample – weight of cooked sample)/(weight of uncooked sample) × 100. Chops were cooled at room temperature; six 1.27-cm diameter cores were removed parallel to the muscle fiber, and cores were sheared perpendicular to the fiber longitudinal axis. Peak shear force was measured using a digital force gauge (BFG500N, Quantro l TM, Dillon/ Quality Plus, Inc., Kansas City, MO, USA), equipped with a WBSF attachment at a cross head speed of 200 mm/min (Warner–Bratzler meat shear, G-R Manufacturing CO., Manhattan, KS, US).

Sarcomere length

Sarcomere length (SL) was determined in LL muscle samples, using a helium-neon laser diffraction method (CVI Melles Gliot. Series 7822 FH-1)[11]. Twenty (20) myofibril fragments of each sample were measured to determine the average sarcomere length.

Fatty acid profile

Fatty acid methyl esters in lyophilized LL muscle samples were obtained by direct transmethylation[12]. Fatty acid methyl esters were analyzed with a Clarus 500 (Perking Elmer) gas chromatograph provided with a capillary column CP-Select CB for FAME fused silica WCOT 100 m_0.25 mm (Cat.no.CP7420; Varian Inc.). Individual fatty acids were identified by comparing retention times with standards (Sigma, St. Louis, MO; Supelco, Bellefonte, PA; Matreya, Pleasant Gap, PA). Fatty acids were quantified by incorporating methyl tricosanoic acid (C23:0) as an internal standard, in each sample during methylation.

Sensory analysis

Twenty-four (24) hours before the sensory analysis, samples were thawed at 2.5 ± 0.5 °C at the Laboratory of Sensory Analysis of the School of Agronomy of the National University of Buenos Aires, Argentina. Loin samples (2.5 cm thick) were cooked in a double contact grill until the internal temperature reached 71 ± 1 ºC. Samples were analyzed by an analytical panel of six trained members according to international standards and meat[13-16] experience in sensory analysis. Each panelist received the samples (cubes: 1x1x2.54 cm) in Petri dishes with a three-digit randomized code. Steak samples were evaluated for the following sensory attributes: overall color (OC); odor intensity (OI); flavor persistence (FP), flavor characteristic (FC); firmness (F) and hardness (H). Panelists scored the samples using an unstructured linear 10-cm scale, where each end point corresponded to low or high score of each attribute, i.e.: OC: light pink to dark red, OI: not intense to extremely strong, FP: not persistent to extremely persistent, FC: none to strong off-flavor, F: extremely soft to hard, H: very tender to very hard (lower limit: 0 to upper limit: 10)[10].

Statistical analysis

The analysis was performed using a completely randomized design. The effect of sex on meat quality parameters was analyzed using a T test. Each animal was considered an experimental unit. The differences were considered significant at $P \leq 0.05$ and trends were considered when $P \leq 0.10$. The degree of association between physicochemical and sensory data was assessed using Pearson's correlations (significant at $P \leq 0.05$; trends $P \leq 0.10$). The statistical analysis was performed using the rcmdr package of the R core team statistical program (2013).

Results

Carcass characteristics and meat quality traits

Sex did not affect to PSW, HCW, carcass yield or LEA (P=0.48, P=0.20, P=0.22 and P=0.61, respectively; Table 1). Back fat thickness was 19 % higher (P<0.001) in CM than in F. Meat from CM tended (P=0.07) to have higher pH@45 than meat from F and, at 24 h *post mortem*, muscle pH was higher (P=0.03) in CM than in F.

No differences (P≥0.34) were observed for redness (a*) or yellowness (b*) parameters in loin samples, except for L* in F, which was 7 % higher (*P*=0.04) than in CM. In addition, shear force and sarcomere length did not differ (*P*>0.05) between meat from F and CM. No differences (*P*=0.55) in cooking loss were observed between sexes. Meat from CM had higher (*P*=0.03) marbling score and intramuscular fat content than meat of F.

Table 1: Effect of sex on live weight, carcass characteristics and meat quality

	CM	**F**	**SEM**	***P*-value**
Pre-slaughter weight, kg	124.50	121.50	7.52	0.48
Hot carcass weight, kg	101.60	98.00	5.78	0.22
Carcass yield, %	81.80	80.70	1.82	0.20
Backfat thickness, mm	25.90	21.06	3.14	<0.001
Loin eye area, cm^2	36.16	36.98	3.11	0.61
Warner bratzler shear force, N	36.00	31.00	0.86	0.26
Sarcomere length, µm	2.04	2.01	0.05	0.22
Marbling	2.60	1.70	0.83	0.03
Intramuscular fat, %	3.48	2.60	0.79	0.02
Cooking loss, %	25.52	26.74	0.96	0.55
pH@45	5.67	5.43	0.06	0.03
pH@24	5.41	5.23	0.05	0.07
	Color			
L*	52.44	56.13	3.60	0.04
a*	5.10	4.57	1.49	0.34
b*	14.56	15.16	2.16	0.94

CM= castrated male; F= female; SEM= standard error of the mean; PSE= pre-slaughter weight; HCW= hot carcass weight; CY= carcass yield; BFT= back fat thickness; LEA= loin eye area; WBSF= warner bratzler shear force; SL= sarcomere length ; MAR= marbling; CL= cooking loss; IMF= intramuscular fat; pH@45= pH of the *Longissimus lumborum* muscle at 45 min *post mortem*; pH@24= pH of the *Longissimus lumborum* muscle at 24 h *post mortem;* L* (lightness), a* (red index) and b* (yellow index).

Fatty acid profile

Total saturated fatty acid (SFA) proportion was higher (*P*<0.01) in CM than in F (Table 2); individual SFA was also higher in CM than F, with C16:0 and C18:0 ratios higher in CM than F, respectively (*P*≤0.04). The C22:5 ratio was higher (*P*<0.05) in F than in CM. No differences (*P*>0.10) were found between sexes for the remaining measurements.

Table 2: Effect of sex on fatty acids composition from the *Longissimus lumborum* (%)

Fatty acids	**CM**	**F**	**SEM**	***P*-value**
SFA	37.90	36.46	0.27	<0.01
C12:0	0.10	0.09	0.01	0.19
C14:0	1.34	1.31	0.01	0.29
C16:0	23.20	22.44	0.17	0.02
C18:0	11.91	11.19	0.17	0.04
MUFA	40.03	39.97	0.47	0.95
C16:1 cis 9	2.65	2.70	0.08	0.79
C18:1 *cis*-9	33.94	33.63	0.37	0.69
C18:1 *cis*-11	2.87	3.03	0.06	0.22
PUFA	18.99	20.15	0.54	0.29
C18:2 n-6	15.01	15.77	0.43	0.39
C18:3 n-3	1.24	1.21	0.03	0.76
C20:4 n-6	2.06	2.35	0.08	0.08
C20:4 n-3	0.03	0.04	0.01	0.21
C20:5 n-3	0.10	0.11	0.01	0.37
C22:5 n-3	0.34	0.39	0.01	0.03
PUFA n-6	17.07	18.12	0.49	0.29
PUFA n-3	1.82	1.88	0.04	0.57
		Ratios		
n-6:n-3	9.33	9.64	0.06	0.12
PUFA:SFA	0.50	0.55	0.09	0.38
MUFA:SFA	1.04	1.09	0.07	0.19

CM= castrated male; F= female; SEM= standard error of the mean; SFA (saturated fatty acids)= C12:0+ C14:0+ C16:0+ C18:0; MUFA (monounsaturated fatty acids)= C14:1 cis-9 + C16:1 cis-9 + C18:1 cis-9 + C18:1 cis-11 ; PUFA (polyunsaturated fatty acids)= C18:2 n-6 + C18:3 n-3 + C18:4 n-3 + C20:4 n-6 + C20:4 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3; PUFA n-6: C18:2 n-6 + C20:4 n-6; PUFA n-3: C18:3 n-3 + C18:4 n-3 + C20:4 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3.

Sensory characteristics

Sensory attributes in meat were influenced by the sex of animals. Meat from CM had higher score of flavor persistence ($P<0.01$) and tended to have higher overall color (OC, $P<0.09$) and flavor characteristic (FC; $P=0.06$) than meat from F. Hardness (H) and firmness (FI) were higher in F ($P< 0.05$). There were no significant differences in the remaining attributes ($P>0.10$; Table 3).

Table 3: Effect of sex on variability of visual, olfatory-gustatory, textural sensory variables in trained sensory panel

Attributes	Descriptors	CM	F	SEM	*P*-value
Visual	OC	6.01	5.53	0.15	0.09
Olfatory-gustatory	FC	6.39	5.84	0.11	0.06
	FP	6.70	5.63	0.13	0.001
Textural	H	4.19	4.89	0.14	0.01
	FI	3.92	4.72	0.16	0.01

CM= castrated male; F= female; SEM= standard error of the mean; OC= overall color; FC= flavor characteristic; FP= flavor persistence; H= hardness; FI= firmness.

Association between variables

Table 4 shows the correlation between physicochemical and sensory variables. Overall color grade (OC) of steaks was positively correlated with marbling score, intramuscular fat content and total MUFA proportion (r= 0.61, $P<0.01$; r= 0.52, $P<0.05$; r= 0.84, $P< 0.001$), but negatively associated with total PUFA proportion and PUFA: SFA ratio ($r\geq 0.83$; $P<0.001$). Meat hardness (H) was negatively correlated with pH24 (r= -0.46, $P<0.05$) and with marbling score (r= -0.63, $P<0.001$), but positively correlated with PUFA: SFA (r= 0.43, $P<0.05$). Overall firmness score (F) was negatively correlated with pH45 (r= -0.54, $P<0.05$), pH24 (r= -0.42, $P<0.1$), marbling score (r= -0.49, $P<0.05$), and cooking loss (r= -0.35, $P<0.05$). FC was positively correlated with intramuscular fat content (r= 0.45; $P<0.10$) and total MUFA proportion (r= 0.54; $P<0.05$), but negatively associated with total PUFA proportion (r= -0.45; $P<0.10$). Persistence (FP) showed a positive correlation with BFT, intramuscular fat content ($P<0.01$) and MUFA proportion ($P<0.05$), and a weak negative correlation with PUFA proportion and PUFA: SFA ratio ($P<0.1$). The remaining associations were not significant ($P>0.05$).

Table 4: Pearson correlation coefficient between physicochemical and sensory variables

	OC	H	FI	FC	FP
pH@45	0.03	-0.26	-0.54*	-0.27	0.06
pH@24	0.03	-0.46*	-0.42^{t}	-0.14	0.09
BFT	0.23	-0.37	-0.24	0.08	0.41^{t}
MAR	0.61**	-0.63***	-0.49*	0.25	0.29
WBSF	0.02	0.12	-0.10	0.32	0.12
SL	0.30	-0.13	-0.16	0.09	0.52*
CL (%)	-0.04	0.38	-0.35*	0.11	-0.26
IMF	0.52*	-0.46^{t}	-0.28	0.45^{t}	0.57**
MUFA	0.84***	-0.41^{t}	0.20	0.54*	0.48*
PUFA	-0.83***	0.45^{t}	-0.18	-0.45^{t}	-0.45^{t}
PUFA:SFA	-0.80***	0.43*	0.18	-0.01	-0.46^{t}

OC= overall color; H= hardness; FI= firmness; FC= flavor characteristic; FP= flavor persistence; pH@45= pH of the *longissimus lumborum* muscle at 45 min *post mortem*; pH@24= pH of the *longissimus lumborum* muscle at 24 h *post mortem*; BFT= back fat thickness; MAR= marbling; WBSF= warner bratzler shear force; SL= sarcomere length; CL= cooking loss; IMF= intramuscular fat; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; PUFA:SFA= polyunsaturated-monounsaturated fatty acids ratio.
^{t} $P<0.1$; *$P<0.05$; **$P<0.01$; *** $P<0.001$.

Discussion

Productivity and meat quality play a key role in the meat industry, since they have a direct effect on profitability. In line with previous reports[17], no differences in HCW were observed between F and CM. The results of this study showed that CM had higher BFT and intramuscular fat content percentage than F, in agreement with values reported by other authors[18,19,20], regardless of the genetic line. The lower concentration of sexual hormones present in CM may have promoted fat deposition instead of muscle[4,5].

Loin eye area (cm^2) in samples from CM and entire F were similar with other authors[8,17]. Meat from F had higher L values than meat from CM, in line with the lower final muscle pH and higher pH rate decline from F (lower pH@45). This result may be attributed to the fact that females are more susceptible to pre-slaughter stress[21], which results in pale pork cuts.

The lack of sex effect on shear force values measured by Warner Bratzler procedure was in line with the lack of sex effect on sarcomere length. It has been suggested that sarcomere length greater than 2 µm in muscle from pigs, as in the present study, would be enough to ensure tender meats[22]. However, these results were not in agreement with the difference in rate decline observed between sexes, probably because these differences were small.

A negative correlation was observed between marbling and tenderness (r= -0.49 $P<0.05$)[23]. However, the difference in the percentage of intramuscular fat between CM and F observed in the current study does not seem to be enough to produce a significant effect on objective tenderness. This result is in agreement with the lower fibrousness values in CM that F, as indicated by visual and textural attributes and Pearson's coefficient.

Meat flavor and palatability are highly dependent on the total amount of fat and on the fatty acid profile[24]. Therefore, the level of IMF found in CM with respect to F as well as some differences in the fatty acid profile would be responsible for differences in pork flavor and odor characteristics observed in the current study. Similarly, other authors found that meat from CM had greater flavor score than meat from F samples[7,25]. The significant correlation between intramuscular fat content and FP or FC observed in the present study supports the hypothesis that intramuscular fat composition and flavor attributes could be linked.

The fatty acid profile of pork is an important factor for several sensory properties, such as flavor and firmness of tissue[26]. The flavor of pork is directly associated with the lipid oxidation that occurs during the cooking procedure[26], generating a characteristic profile of volatile compounds. The differences in some individual polyunsaturated fatty acids observed in the present study could lead to differences in the perception of flavor compounds by the sensory panel. However, such differences were very small and should be confirmed in further studies. The proportion of mono-unsaturated fatty acids was positively correlated with the characteristic flavor and its persistence[27]. In addition, as expected, a higher proportion of some individual or total polyunsaturated fatty acids seems to contribute negatively to odor and flavor attributes but positively to textural attributes in female meat[27].

Conclusions and implications

Meat from surgically CM and entire F of Argentine crossbred pigs “Degesa” (Yorkshire 25% x Landrace 75%) presented some differential quality traits in the *longissimus lumborum* muscle. CM seem to have better colorimetric and sensorial characteristics than F. The main differences observed between sexes were related to a greater amount of intramuscular fat content in meat from CM. This result implies that the sex of animals needs to be considered when producing cuts or meat products with certain quality characteristics. This means that the Argentine pork category could be differentiated by meat quality according to the sex. Further studies with a higher number of animals would be necessary to corroborate these findings.

Acknowledgments and conflict of interest

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Artícle

Ivermectin resistance in *Rhipicephalus microplus* (Acari: Ixodidae) in northeastern Mexico and associated risk factors

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Abstract:

Rhipicephalus microplus is the parasitic species that causes the most damage to Mexican and global livestock due to direct and indirect losses, such as the increase in multidrug resistance and cross-resistance. Currently, there are few studies on resistance to macrocyclic lactones in Mexico, most of them in the south. This study aimed to evaluate the status of ivermectin resistance in *R. microplus* in northeastern Mexico and its associated risk factors. A total of 20 populations of *Rhipicephalus microplus* were collected in the states of Veracruz, Nuevo León, Tamaulipas, and San Luis Potosí, and they were analyzed with the larval immersion test. Mortality data were subjected to a Probit analysis, estimating lethal concentrations (LC) of 50 % and 99 % and their respective 95 % confidence intervals (95 % CI), and to determine possible risk factors, a multivariate analysis and 2 x 2 contingency tables were performed for

the exposure variables, with a 95 % confidence interval, and a binomial logistic regression model for those variables with a $P \leq 0.05$. Eighty (80) percent of the analyzed populations showed resistance with ranges of RR50= 2.07-11.14 and RR99= 3.03-47.93 ($P \leq 0.05$), and through the binomial logistic regression, it was observed that the variable of frequency of treatments obtained a $P \leq 0.0134$, a result that proved to be significant.

Keywords: Cattle tick, Veterinary epidemiology, Dose-response, Acaricides.

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Introduction

Ticks are hematophagous ectoparasites that are important in human and animal health due to the damage they cause by transmitting pathogens and feeding[1]. *Rhipicephalus microplus* is the most important species in cattle farming because it is the main vector of hemoparasites such as *Babesia* spp. and *Anaplasma* spp., in addition to this, the economic losses it causes at the productive level in Mexico amount to more than 573.6 million dollars per year[2]. This species is dispersed in the tropical, subtropical, and semiarid regions of all continents, except Europe[3]. The geographical distribution of *R. microplus* in the country is recorded by SENASICA, which states that 30.60 % of the country is free of ticks, 3.44 % is under eradication, and 65.96 % are natural free zones and control zones[4].

Ixodicides have been used for years for the control of *R. microplus*, such as: organophosphates, amidines, synthetic pyrethroids, growth regulators, phenylpyrazolones, and macrocyclic lactones (MLs); the latter are a broad-spectrum family (endectocide) and act by binding to the transmembrane (TM) domains of Cys-loop receptors, such as the glutamate-gated chloride channel (GluCl), which are expressed in the motor and sensory systems of arthropods and nematodes, causing hyperpolarization and ultimately death[5,6,7].

What all these drugs have in common is that they have generated resistance due to operational factors such as inappropriate and continuous use[8]. In Mexico, in 2010, resistance to ivermectin was reported for the first time in *R. microplus* populations[9], and it has been used since the beginning of the 21st century and currently there are few studies on resistance to MLs in Mexico, which are scarce in the northeast of the country. Therefore, the objective of

this research was to determine the status of ivermectin resistance in *R. microplus* in cattle ranches in northeastern Mexico, as well as the possible risk factors associated with such resistance.

Material and methods

Area and place of study

The study was carried out in the Bacteriology Laboratory and the Multidisciplinary Research Laboratory (LMI, for its acronym in Spanish) of the Faculty of Veterinary Medicine and Zootechnics (FMVZ, for its acronym in Spanish) of the Universidad Autónoma de Nuevo León (UANL, for its acronym in Spanish).

From September 2021 to October 2022, 20 populations of ticks belonging to the *R. microplus* species were collected, which were located in 20 different cattle ranches belonging to the four states of the northeastern region of Mexico: Veracruz (Ver.), Nuevo León (N.L.), Tamaulipas (Tamps.), and San Luis Potosí (S.L.P.). To determine the sample size, a simple randomized model was used, which was based on data from SIAP-SADER[10].

Tick collection and identification

In the morning, 20 to 30 engorged (teleogynous) females belonging to the species *R. microplus*, which were located in the body areas of the bovine, were collected by hand following the recommendations of FAO[11]. The identification of the specimens was carried out by means of an observational morphological analysis, with the use of dichotomous keys[12] and a Carl Zeiss™ Stemi™ DV4 stereoscopic microscope (Göttingen, Germany), in order to distinguish between other tick species that also parasitize cattle.

Production of infesting larvae

To carry out oviposition, the teleogynes were washed with distilled water and dried with paper towels; they were placed in groups of 10 in a Petri dish (100 x 15 mm) dorsoventrally

and incubated in an ECOSHEL BOD-250 incubator at a temperature of 27 ± 2 °C and a relative humidity between 80 and 90 %. After oviposition (14 to 18 d), the eggs were collected and transferred to 10 mL glass tubes sealed with a cloth and a rubber band to await the hatching of the larvae; after another 14 d, we waited for the larvae to mature, and once the negative geotropism characteristic was observed, the larval immersion test modified for ivermectin was then carried out[8,9,13].

Larval immersion test modified for ivermectin (LIT)

A stock solution of 1 % IVM (Sigma-Aldrich, USA) was prepared in absolute ethanol and 2 % of Triton X-100 (Sigma-Aldrich, USA). From this solution, the maximum dose of IVM was prepared at 0.01 % (100 ppm). Subsequently, 11 serial dilutions were prepared at 30 %: 0.01 %, 0.007 %, 0.0049 %, 0.00343 %, 0.0024 %, 0.00168 %, 0.00117 %, 0.00082 %, 0.00057 %, 0.0004 %, and 0.00028 %. A solution of 1 % ethanol and 0.02 % Triton X-100 in distilled water was used as a diluent. In 2.0 mL Eppendorf tubes, 500 µL of each dilution was added in triplicate and a quantity of between 100 and 150 infesting larvae was placed; they were immersed for 10 min and then transferred to 8.5 x 7.5 cm Walkman papers closed with foldback clips. After 24 h, live larvae and the initial number of pack larvae were counted[8,13,14].

Statistical analysis

A PROBIT dose-response analysis was performed; lethal concentrations (LC) of 50 % and 99 %, with their respective 95 % confidence intervals (95 % CI) were calculated using the SPSS V.24 software. The hypothesis of normality and equality of variance was tested with a Chi-square test ($P \leq 0.05$).

The resistance ratio (RR) of each population was determined and compared with data previously obtained from the susceptible reference strain Deutch (USDA, Cattle Fever Tick Research Laboratory, Edinburg, TX, USA)[13]. To determine susceptibility and resistance, the following classification was followed: RR50 ≤ 1: susceptible; RR50 > 1 < 2 incipient resistance, and RR50 ≥ 2 resistant[12]. The formula for calculating the RR was:

$$RR50 = \frac{\text{LC50 population}}{\text{LC50 reference strain}}$$

Epidemiological questionnaire

An epidemiological questionnaire was applied to each of the owners or managers of the cattle ranches studied in order to determine the practices in the use and management of MLs, as well as the control of *R. microplus*. Information related the following aspects was included: production, facilities, breeds, presence of ticks and other parasites, history of the use of macrocyclic lactones (MLs) and ixodicides, frequency of applications, rotation of ixodicides and pastures, among others.

The group with incipient resistance (RR50 > 1 < 2) was considered susceptible and a descriptive analysis was performed to calculate the frequencies of the variables found, as well as a multivariate analysis using 2 x 2 contingency tables to evaluate the interaction between the exposure variables, with a 95 % confidence interval using the Epi Info V.7.2 software. Fisher's exact test was used to determine the level of significance of each association and associations with a value of $P \leq 0.20$ were included in the binomial logistic regression model. A value of $P \leq 0.05$ was considered statistically significant in the binomial regression analysis[8,9,15].

Results

Place of collection of the populations

The data collected from the populations of *R. microplus* belonging to the northeastern region of Mexico are shown in Table 1, which were distributed as follows: four from Tamps., seven from Ver., five from N.L., and four from S.L.P.

Table 1: Geographic location of each *R. microplus* population collected in the northeastern region of Mexico

Population	Location	Geographic coordinates
ETHM	Tantoyuquita, Tamps.	22°31'05.5"N 98°31'26.5"W
JCG4	Ciudad del Maíz, S.L.P.	22°25'01.6"N 99°35'20.7"W
JAM5	Tantoyuca, Ver.	21°12'38.7"N 98°08'33.5"W
DALC	Cadereyta, N.L.	25°33'43.4"N 99°49'11.4"W
RAMT	Soto la Marina, Tamps.	23°48'30.3"N 98°08'24.9"W
JNSE	Santa Engracia, Tamps.	24°04'05.5"N 99°14'07.7"W
JVML	Los Ramones, N.L.	25°42'24.6"N 99°37'27.9"W
SNTM	General Bravo, N.L.	25°50'17.0"N 99°15'56.4"W
VMA1	General Terán, N.L.	25°10'06.4"N 99°32'55.3"W
PRVA	Aramberri, N.L.	24°06'19.8"N 99°55'20.1"W
MRNA	Hidalgo, Tamps.	24°04'41.0"N 99°14'28.8"W
ANGS	Tantoyuca, Ver.	21°23'42.1"N 98°08'32.3"W
LEX15	Tantoyuca, Ver.	21°18'06.0"N 98°15'42.4"W
ESHP	Tantoyuca, Ver.	21°19'42.7"N 98°20'44.0"W
JHE2	Tantoyuca, Ver.	21°24'05.1"N 98°11'15.5"W
JPN1	Tantoyuca, Ver.	21°17'15.3"N 98°15'57.3"W
VIHM	Tantoyuca, Ver.	21°27'41.4"N 98°18'30.5"W
KML1	Ciudad Valles, S.L.P.	22°01'19.9"N 99°04'23.5"W
EBEV	Casas Viejas, S.L.P.	22°11'22.2"N 99°05'53.2"W
ISALI	El Naranjo, S.L.P.	22°30'58.1"N 99°21'05.0"W

Cattle ranches with ivermectin-resistant *R. microplus* populations and the resistance ratio

Using the mortality rate and the PROBIT methodology, the lethal concentration in % (LC50 and LC99) and the resistance ratio (RR50 and RR99) were calculated (Table 2). The VMA1 population was susceptible to IVM (RR50= 0.73; RR99= 3.94) and the JCG4, JAM5 and JNSE populations showed incipient resistance (RR50 of 1.20, 1.55, and 1.61 respectively). On the other hand, the remaining 16 populations showed resistance to IVM (RR50= 2.07-11.14; RR99= 3.03-47.93) and of these, the JVML and LEX15 populations were highly resistant to ixodicide (RR50= 6.98; RR99= 11.11; RR50= 11.14; RR99= 47.93).

Table 2: Analysis of dose-response to IVM in *R. microplus* populations, lethal concentrations at 50 % and 99 % and resistance ratios 50 and 99 (RR_{50} and RR_{99})

Population	Slope	LC_{50}	95 % CI	RR_{50}	LC_{99}	95 % CI	RR_{99}
JCG4	4.77	0.00067	0.00028-0.00123	1.20	0.00203	0.00114-0.17975	1.20
ETHM	3.82	0.00154	0.00135-0.00174	2.75	0.00626	0.00491-0.00874	3.68
JAM5	3.10	0.00087	0.00074-0.00102	1.55	0.00490	0.00362-0.00758	2.88
DALC	3.46	0.00230	0.00200-0.00264	4.11	0.01083	0.00820-0.01616	6.37
RAMT	4.29	0.00148	0.00133-0.00164	2.64	0.00515	0.00418-0.00684	3.03
JNSE	2.82	0.00090	0.00072-0.00110	1.61	0.00602	0.00410-0.01090	3.54
JVML	3.40	0.00391	0.00313-0.00513	6.98	0.01889	0.01173-0.04563	11.11
SNTM	3.83	0.00226	0.00191-0.00269	4.03	0.00913	0.00695-0.01362	5.37
VMA1	1.91	0.00041	0.00027-0.00053	0.73	0.00669	0.00403-0.01585	3.94
PRVA	3.26	0.00206	0.00188-0.00225	3.68	0.01067	0.00883-0.01346	6.28
MRNA	3.62	0.00303	0.00265-0.00346	5.40	0.01326	0.01014-0.01927	7.80
ANGS	3.32	0.00213	0.00202-0.00224	3.80	0.01068	0.00948-0.01220	6.28
LEX15	2.09	0.00624	0.00547-0.00727	11.14	0.08148	0.05465-0.13760	47.93
ESHP	3.32	0.00177	0.00163-0.00192	3.16	0.00889	0.00741-0.01110	5.23
JHE2	2.70	0.00156	0.00138-0.00174	2.78	0.01136	0.0088-0.01572	6.68
JPN1	2.38	0.00225	0.00198-0.00256	4.02	0.02138	0.01526-0.03357	12.58
VIHM	3.10	0.00255	0.00222-0.00293	4.56	0.01435	0.01036-0.02122	8.44
KML1	2.53	0.00149	0.00134-0.00166	2.66	0.01242	0.00947-0.01750	7.31

ISALI	3.03	0.00116	0.00101-0.00132	2.07	0.00679	0.00513-0.00995	4.00
EBEV	2.87	0.00116	0.00095-0.00139	2.07	0.00750	0.00522-0.0129	4.41
DEUTCH[a]	4.72	0.00056	0.00052-0.00060	NA	0.0017	0.00150-0.00210	NA

a USDA susceptible reference strain, Cattle Fever Tick Research Laboratory, Edinburg, TX, USA.
LC= lethal concentration; CI= confidence interval; RR= resistance ratio; NA= not applicable.

Separating the populations by state, resistance to IVM was found to exceed 70 % in each of these. In the state of San Luis Potosí, there were three resistant populations (75 %) and one population showed incipient resistance (25 %); in Tamaulipas, values similar to those found in the state of San Luis Potosí were obtained: 75 % resistant, 25 % with incipient resistance. On the other hand, in Nuevo León it was found that 80 % of the population present resistance, while one population (20 %) showed susceptibility, it is highlighted that it was the only one in the present study. Finally, 86 % of the populations analyzed in Veracruz showed resistance, while 14 % showed incipient resistance.

Risk factors associated with resistant populations

A total of 14 independent variables were analyzed as possible risk factors associated with resistance to IVM (Table 3). On the one hand, the main farming system is the rangeland; just over half of the ranches have semi-technified facilities and landrace breeds between zebu and European. The density of animals per ranch is less than 50 head per herd, with a proximity of less than 5 km between ranches. Half of the ranches sampled have ticks year-round.

Regarding the management history of ixodicides and MLs, it was observed that all ranches implement ixodicide rotation by using various product families, such as organophosphates, amidines, synthetic pyrethroids, phenylpyrazolones, and developmental inhibitors. In addition, all ranches apply IVM and other MLs, such as doramectin, half of which are used for the treatment of ectoparasites. More than 50 % of the farmers surveyed mentioned using IVM formulations with concentrations greater than 1 %, applying them more than five times a year and adjusting the dose according to the weight of the bovine. In addition, most ranches have veterinary assistance and carry out pasture rotation.

The exposure variables “frequency of treatments” (P=0.026) and “formulation administered” (P=0.1531) showed statistical significance according to Fisher’s exact test (Table 4). Therefore, both variables were included in the binomial logistic regression model (Table 3),

where regression estimates, 95 % confidence intervals (95 % CI), odds ratios (OR), *P*-values, and standard error of the regression coefficient were obtained. A value of $P \leq 0.05$ was considered significant, indicating a positive statistical association between the variables.

Table 3: Frequency analysis of exposure-independent variables as possible risk factors associated with *R. microplus* resistance to IVM

Variable	Analysis	Frequency (%)	*P* (Fisher's exact test)
Farming system	Housed	6/20= 30	
	Rangeland	14/20= 70	0.6573[a]
Type of facilities	Semi-technified	11/20= 55	
	Familiar	9/20= 45	0.6253[a]
Breeds	Pure	2/20= 10	
	Landrace	18/20= 90	0.3684[a]
Animal density (number of heads)	> 50	8/20= 40	
	< 50	12/20= 60	0.5345[a]
Proximity to another ranch	> 10 km	5/20= 25	
	< 10 km	15/20= 75	0.2487[a]
Season with ticks	Seasonality	10/20= 50	
	All year	10/20= 50	0.7089[a]
Target parasite (s)	Ectoparasites	10/20= 50	
	Endo- and ectoparasites	10/20= 50	0.7089[a]
Frequency of treatments (year)	1- 3	9/20= 45	
	4- > 5	11/20= 55	0.026[b]*
Application of treatments	Prevention	7/20= 35	
	Presence	13/20= 65	0.5607[a]
Formulation administered	1	8/20= 40	
	3.15 - 4 %	12/20= 60	0.1531[b]*
Application according to the weight	Yes	17/20= 85	0.5087[a]
	No	3/20= 15	
Veterinary assistance	Yes	15/20= 75	0.2817[a]
	No	5/20= 25	
Ixodicide rotation	Yes	20/20= 100	0.4738[a]
	No	0/20	
Pasture rotation	Yes	14/20= 70	
	No	6/20= 30	0.3426[a]

a= not significant; b*= significant ($P \leq 0.20$).

Table 4: Binomial logistic regression analysis in significant variables as possible risk factors associated with *R. microplus* resistance to IVM

Variable	OR	95 % CI	SE (β)	*P*≤0.05
Frequency of treatments	Not defined	0.0	291.26	0.0134
Formulation administered	6.59	0.5428	1.27	0.1101

OR= odds ratio; CI= confidence interval; SE (β) = standard error.

Discussion

Chemical control of ticks in Mexico and the world has become ineffective, given the emergence of populations resistant and multi-resistant to ixodicides[16,17,18]. Since its introduction in the 1980s, IVM has been the most important animal health product worldwide[19]. There have been few studies on the status of resistance to IVM in *R. microplus* in Mexico[8,9,15]. This highlights the importance of conducting studies on the evaluation and diagnosis of resistance to this drug in the northeast of the country.

Applying the LIT and following the Probit methodology, the LC50 and LC99 of the study populations were determined. In the results obtained, a significant difference was found with the reference strain Deutch, with a susceptible population (5 %) (RR50= 0.73), three populations with incipient resistance (15 %) (RR50= 1.20-1.61), and the rest (80 %) with resistance (RR50= 2.07-11.14). These results coincide with those reported for the first time in Mexico[9], where 100 % of the populations analyzed showed resistance to IVM, with RR50= 2.04-8.59 and RR99= 2.67-87.86, in addition to exponential growth in different sampling periods. The importance of using a susceptible reference strain lies in the fact that it is a reference parameter for biochemical and molecular resistance studies[20]. In addition, they are regulated by international organizations. In the study carried out in 2006[9], a comparison was made between the results obtained in their research using the Deutch strain and another study[15], which used the Porto Alegre strain. This study[9] highlights that the result obtained by this team is superior to those of the second, even so, slightly higher or equal RR50 values were obtained. In the present research, similar results were found when analyzing the Porto alegre, Mozo and Deutch strains[9,13,21] as possible candidates for the reference strain, so it was decided to select the Deutch strain because, when analyzing the results of the three, there was no significance at the time of determining the already stipulated classification, and it was more in line with what was desired. On the other hand, the Mexican

strain Media Joya is only susceptible to organophosphates, synthetic pyrethroids and amidines, and there is no toxicological characterization of susceptibility to ivermectin[22].

Authors[23] mention that resistance is given by biochemical/genetic factors, operational factors, and ecological factors; the latter include intrinsic traits and interactions of populations with their surroundings and environment. In addition, the development of resistant individuals is dependent on the frequency of occurrence and the selection pressure[9,24,25]. In addition, in different studies of Latin American countries, resistant populations of between 40 and 100 % of the populations analyzed were obtained[26,27,28].

The response of populations to dose increase (slope) is an important indicator of resistance. A low slope ≤ 2 and a high LC (higher than the reference strain) are common in resistant populations, while a high slope ≥ 2 and low LC are common in susceptible populations with heterogeneous response[13,29]. In the present study, populations that respect this statement were found: JCG4 (S.L.P.), JAM5 (Ver), JNSE (Tamps), VMA1 (N.L.), and the JPN1 population (Ver), while, surprisingly, three populations from Tamaulipas (ETHM, RAMT, and MRNA), four from Nuevo León (JVML, SNTM, PRVA, and DALC), five from Veracruz (ANGS, LEX15, ESHP, VIHM, and JHE2) and three from San Luis Potosí (KML1, EBEV, and ISALI) showed high LCs and slopes. To date, there are no reports that determine a strain of *R. microplus* that is highly resistant to IVM[28]; according to these statements, the populations described have suffered a loss of heterogeneity and susceptible genes, demonstrating for the first time in the present research that resistant alleles are fixed in the population and they present a homogeneous resistance response. Other studies mention that the heterogeneity of resistant alleles would lead to the loss of susceptible populations and the emergence of resistant populations with homogeneous alleles[9,30,31].

Of the resistant populations obtained in this study, two were classified as highly resistant (RR50= 6.98 and RR50= 11.14), results that are similar to those that showed the highest values of resistance (RR50= 6.84, 7.37 and 10.23) and RR50= 5.89, 6.25 and 8.21[8,9,15]. Even so, molecular studies are needed to analyze all frequencies of resistant alleles in populations.

On the other hand, frequencies were analyzed based on the responses obtained in the epidemiological questionnaire (Table 3). The municipalities included in this study are located between parallels 26° N to 21° N, relative humidity between 65 and 79 %, average temperatures of 21° C, and an average water evaporation between 1,200-1,400 mm, optimal conditions for the development, distribution, and survival of the tick, as well as for the increase of generations per year[32,33,34]. Some authors mention that geographic location and abiotic niche are factors that promote the greater development of ticks[3,35].

Of the 14 variables studied, two showed significances of $P \leq 0.20$: frequency of treatments ($P=0.026$) and formulation administered ($P=0.1531$), which were included in the binomial logistic regression model.

Animal management systems, as well as the number of annual treatments, are considered factors that influence the efficacy of drugs, playing an important role in the development of resistance[4]. In 55 % of the ranches, IVM treatment is applied 4 to more than 5 times per year, similar to that obtained by Fernández-Salas *et al*[36], where cattle ranches that apply MLs 4 or more than 5 times a year are up to 13 times more likely to develop resistance[8]. IVM has a period of decrease in concentration after application, but due to its high affinity to fat and its persistence in tissues, it is not completely eliminated, so prolonged exposure to therapeutic doses favors the emergence of resistant organisms[9,15,36]. This assumption is known as the "tail effect"; if organisms are present during this period, the selection of IVM-resistant organisms is possible[37,38]. *R. microplus* reacts quickly to selection pressure and higher concentrations of ixodicides[39], therefore, the application of the chemical should be carried out less frequently at 30-d intervals with the intention of reducing this pressure, not only for the tick, but also for non-target organisms such as helminths[40,41].

By applying the binomial logistic regression, it was observed that a $P \leq 0.0134$ was obtained for the variable of frequency of treatments, a result that proved to be significant, but with an undefined OR due to the fact that in one of the groups of the 2 x 2 contingency table, there was a box in which there was no susceptible population and that the IVM was applied 4 or more than 5 times a year, which had to be computed as a zero; since the OR is the quotient of two ratios[42]. Including a zero in the division generates an incalculable result. It was determined that the administration of the treatment 4 or more than 5 times a year may be a risk factor since, on the one hand, the calculated frequency measures resulted in values greater than 1; the relative risk obtained was 1.8 and the ORs are in a range from 1.27 to infinity. Therefore, the increase in frequency in the exposed group can be considered to be due to the effect of the independent variable. One way to solve the fact that the OR is incalculable is to proportionally increase the values of each box[43], so when doing so, a value of OR= 11.14 and $P=0.032$ was obtained; although this result cannot be taken as reliable, it leaves open the possibility that, in future studies, including a larger number of farms studied, the increase in ORs for farms that apply treatments 4 or more times a year can be verified.

Regarding the independent variable of formulation administered, it was observed that more than half of the farmers use IVM-LA formulations of 3.15 % to 4 % due to the lack of efficacy of the 1 % formulation. IVM-LA formulations have a higher risk of generating resistant populations when applied with high frequency compared to 1 % short-acting formulations[1]. This is due to several factors, such as a higher concentration of the active ingredient in IVM-LA formulations, an applied dose that is three times higher (630 μg/kg), a prolonged

withdrawal period, a decrease in natural immunity and a faster selection of resistant parasites[41,44,45]. The binomial logistic regression analysis showed that for the variable of formulation administered, a $P \leq 0.1101$ (OR= 6.59, 95 % CI= 0.5428 and SE= 1.27) was obtained, which was not significant as a possible risk factor, but with a positive association. With these data, the only susceptible population (VMA1) was related to the possible associated risk factors due to the fact that, in this population, a lower frequency of treatments was found: 1-3 per year and a lower formulation administered: IVM at 1 %.

Conclusions and implications

Based on the results obtained, it was shown that, in the states of Veracruz, San Luis Potosí and Tamaulipas, there are no populations susceptible to IVM and 14 to 25 % of these have incipient resistance. On the other hand, in the state of Nuevo León, only one susceptible population was found. *R. microplus* is resistant to IVM in northeastern Mexico (80 %). Currently, the frequency of applications of 4 or more than 5 times a year is the only risk factor that could be associated with the presence of resistant populations. Therefore, it is necessary to migrate to new control methods, such as including several families of ixodicides, carrying out integrated control, responsible management, and a culture of diagnosis in order to reduce the selection pressure to which populations are exposed.

Acknowledgments and conflicts of interest

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Article

Effect of grazing, cutting, and irrigation on the production and nutritional value of Buffelgrass

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Abstract:

This study aimed to determine the effect of the type and intensity of utilization of buffelgrass grown under natural rainfall or irrigation conditions on the production and nutritional value of the dry matter. Sixteen plots (64 m^2 each) were grazed by Charolais cattle to obtain a utilization intensity of 50 % (GR 50) or 75 % (GR 75). Eight plots (40 m^2 each) were hand-cut up to 50 % (CU 50). The annual forage harvest was higher ($P \leq 0.05$) for GR 50 than for CU 50 (1,491 *vs* 954 kg DM/ha). No differences ($P \geq 0.05$) were found in dry matter production per hectare between GR 50 and GR 75 (1,707 *vs* 1,491 kg DM/ha). Irrigation increased dry matter production by 22 % ($P \leq 0.05$) compared to rainfed conditions (1,524 *vs* 1,245 kg DM/ha). There were no differences ($P > 0.05$) due to the type and intensity of utilization in the content of CP, NDF, and ADF; however, ADF increased ($P \leq 0.05$) in irrigated plots. In the same way, the *in vitro* digestibility of

DM was higher ($P \leq 0.05$) in CU 50 than in grazing plots GR 50 and G75 (55.7, 53.0, and 52.7 %). Finally, it can be conclude that buffelgrass production increased with grazing, but the IVDDM was better in hand-cut forage.

Keywords: Grazing, Irrigation, Rainfed conditions, Buffel.

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Introduction

For livestock farmers, the rational use of forage resources is of great importance; one of the grass species that prevails is buffelgrass (*Cenchrus ciliaris* L); this grass is widely grown in tropical and subtropical areas around the world due to its high tolerance to drought and the ability to withstand intensive grazing[(1)]. Its development in the semi-arid Northeast of Mexico intensified and reached, after its introduction in the fifties of the twentieth century, at least 500,000 ha in the state of Nuevo León[(2)].

During grazing, forage is not uniformly removed from all stems, as is the case with that harvested by mechanical cutting[(3)]. In addition, animals produce indirect effects such as soil compaction and recycling of nutrients from manure and urine[(4)]. On the other hand, the cutting intensity can generate differences in photosynthetic activity, influencing biomass production[(5)]. The determination of the optimal amount of residual forage is of fundamental importance to establish the limits of grazing, taking care that the plant retains enough forage for adequate production and storage of reserves for the next regrowth; the production of forages also depends largely on the water that is stored in the soil and reaches it through rain or irrigation. In the same way, by intensifying grazing, stem reforestation is promoted, and the highest values can occur with a medium and high grazing intensity[(6)]. This work aimed to evaluate the effect of different grazing, cutting, and irrigation conditions on the production and nutritional value of buffelgrass.

Material and methods

The work was carried out in the Experimental Field of the Faculty of Agronomy of the Autonomous University of Nuevo León (FAUNL, for its acronym in Spanish), located at 25° 52' N and 100° 03' W, and with an altitude above sea level of 393 m. The reports of the last 10 yr from the FAUANL meteorological station indicate that the average

temperature for September (the date of the start of the experiment) is slightly higher than that of the present work (26.3 °C). The average for January, in which the lowest temperatures were recorded, was 14.1 °C, and the maximum monthly average corresponds to June with 29.4 °C.

The total rainfall during this work was 386 mm. For the last 10 yr, the station recorded an average rainfall of 355 mm, 8 % lower than that recorded in this work. The soils are calcareous, and the texture is sandy loam to clay loam.

The experiment was conducted over 10 months distributed over two calendar years (September to July); by virtue of the above, there are three records: the first in the autumn of the first year (A1Y) and the second and third in the summer (S2Y) and autumn of the second year (A2Y). Out of 24 plots, half were irrigated (I), and the other half were used under rainfed conditions (R). Of the 12 plots used under rainfed conditions, four received moderate grazing at 50 % utilization (GR 50), another four plots received intense grazing at 75 % utilization (GR 75), and the remaining four received moderate cutting at 50 % utilization (CU 50); in all cases the grazing was continuous. The grazing plots had 8 x 8 m (64 m^2) dimensions, while the cutting plots measured 8 x 5 m (40 m^2). The 12 irrigated plots were assigned to the previous treatments but with the application of 70 mm of irrigation water per m^2 on two dates: first at the beginning of autumn and second at the beginning of spring.

Grazing intensity at 50 % utilization of available forage was achieved using two animals of the Charolais breed; the animals were two-year-old males weighing approximately 400 kg. Three animals with similar characteristics were used for grazing at 75 % utilization of the available dry matter. The cutting at 50 % utilization was done manually at the same time as the grazing of the plots. A first cut was made to standardize the plots (FC), and the treatments were applied two months later (autumn cut of the first year; A1Y), subsequently (5 months later) a cut was made in summer (S2Y), and finally, another in autumn of the second year (5 months later, A2Y).

To determine the dry matter per hectare (DM/ha) of forage available before each use (Pre), the amount of forage was recorded in each plot in two areas of one square meter taken randomly by cutting the grass at ground level to weigh it immediately. After cutting or grazing, the data corresponding to after cutting or grazing (Post) were recorded.

The dried samples were ground in a Willey mill with a 2 mm sieve and stored at room temperature for chemical analysis. The amount of dry matter of forage in each experimental plot before (Pre) and after (Post) use (cutting or grazing) was determined by weighing a representative sample of the cut forage and drying it in an oven at 62 °C for 48 h. Forage production was calculated as the difference between the quantity recorded after each use (post) and before (pre) the next. The utilization intensity was calculated by dividing the amount of forage recorded after (post) each use and before (pre) it.

The grass samples from the cutting and grazing plots were analyzed to determine their contents of dry matter, ash[7], and crude protein (CP) by the Kjeldahl method[8]. The contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) and *in vitro* digestibility of dry matter (IVDDM) were also analyzed[9,10].

During the 10 months of the experiment, soil moisture content was determined biweekly. To do this, a site from each plot was randomly selected, and a soil sample was extracted at a depth of 30 cm with the help of an auger. The samples obtained were placed in glass jars, weighed on a scale, and taken to an oven at 100 °C for 48 h; then, they were weighed to calculate the content and gravimetric moisture[11].

$$Gravimetric\ moisture\ (\%) = \frac{Wetsoilmass - drysoilmass}{drysoilmass} x100$$

The results obtained were analyzed under a split-block design; this arrangement is used when evaluating two factors, and both can be assessed more easily in large plots. The SPSS program was used[12]. The effect of the type of utilization (cutting and grazing), the intensities of utilization (50 and 75 % utilization), and moisture levels (irrigation and rainfed) on the production of dry matter and nutritional value of buffelgrass was evaluated. For each treatment, four replications were performed. The statistical model used was:

Yijk= μ + βi + Lj t Eij(a) + Hk + Eik(b) + (LH)jk + Eijk(c)

Yijk is the observation of the type or intensity j at the level k of moisture in block i;
μ is the overall true mean;
βi is the effect of block i, i= 1,2 r;
Lj is the effect of the level j of type or intensity, j= 1,2 a;
Eij(a) is the experimental error of the ij-th plot for the types or intensities;
Hk is the effect of the moisture level k, k= 1,2 b;
Eik(b) is the experimental error of the ik-th plot for moisture levels;
LHjk is the effect of the interaction of the type or intensity j and the moisture k;
Eijk(c) is the experimental error of the ijk-th subplot.

Results

Table 1 presents the data before the assignment of each treatment, the amount of forage used in the first treatment (FC), and its residue. What was initially planned as GR 50, CU 50, and GR 75 resulted in the use of FC in actual utilization rates of 57 % for moderate

grazing, 54 % for moderate cutting, and 71 % (69 % under rainfed conditions and 73 % under irrigation) for intense grazing ($P \leq 0.05$).

Table 1: Available, residual, and used forage, and intensity of utilizations of the plots assigned to each treatment prior to the start of the experiment (kg DM/ha)

Factor	Available forage	Residual forage	Used forage	% Utilization
GR 50	4,167[a]	1,805[a]	2,362[a]	57[b]
CU 50	3,892[a]	1,792[a]	2,100[a]	54[b]
GR 75	3,974[a]	1,172[a]	2,802[a]	71[a]

GR 50= moderate grazing at 50 % utilization; CU 50= moderate cutting at 50 % utilization; GR 75= intensive grazing at 75 % utilization.

[ab] Different letters in the same column indicate significant differences ($P<0.05$).

Table 2 shows the data on available, residual, and used forage for the three periods into which the experiment was divided: autumn of the first year, summer of the second year, and autumn of the second year. The forage available for use in A1Y was similar ($P>0.05$) for the plots assigned to the different treatments; the amount of residual forage was different ($P \leq 0.05$) for GR 50, CU 50, and GR 75.

Table 2: Available, residual, and used forage, and intensity of utilization according to type and intensity of utilization (kg DM/ha)

Factor	Available forage	Residual forage	Used forage	% Utilization
		Autumn of the first year=A1Y		
GR 50	2365[a]	822[a]	1543[a]	64[a]
CU 50	1809[a]	509[b]	1300[a]	72[a]
GR 75	1842[a]	557[b]	1285[a]	70[a]
		Second and third cut in summer= S2Y		
GR 50	3147[a]	1397.5[a]	1749.5[a]	56[b]
CU 50	2425[a]	1077[a]	1348[a]	56[b]
GR 75	2871[a]	737.5[a]	2134.5[a]	74[a]
		Autumn of the second year= A2Y		
GR 50	3581[a]	1663[a]	1919[a]	54[a]
CU 50	2895[a]	1476[a]	1419[a]	49[a]
GR 75	3636[a]	1294[a]	2343[a]	65[a]

GR 50= moderate grazing at 50 % utilization; CU 50= moderate cutting at 50 % utilization; GR 75= intensive grazing at 75 % utilization.

[ab] Different letters in the same column indicate significant differences ($P \leq 0.05$).

For the use in S2Y, what was initially planned as GR 50, CU 50, and GR 75 resulted in actual utilization rates of 56 % for moderate grazing, 56 % for moderate cutting, and 74 % for intense grazing ($P \leq 0.05$). For A2Y utilization, the amounts of forage available before (Pre) utilization were similar ($P>0.05$) for GR 50 and GR 75, and CU 50. For that same season, what was initially planned as GR 50, CU 50, and GR 75 resulted in actual

utilization rates of 54 % for moderate grazing, 49 % for moderate cutting, and 65 % for intense grazing ($P \geq 0.05$).

On average, for the 10 months of the experiment, what was initially planned as GR 50, CU 50, and GR 75 resulted in actual utilization rates of 57 % for moderate grazing, 58 % for moderate cutting, and 70 % for intense grazing.

Forage production in the period between the first cut (FC) and the autumn of the first year (A1Y), between A1Y and the summer of the second year (S2Y), and finally, between S2Y and the autumn of the second year (A2Y), for each of the six treatments established is shown in Table 3.

Table 3: Buffelgrass production by type and intensity of utilization (kg DM/ha)

Factor	FC-A1Y	A1Y-S2Y	S2Y-A2Y	Total
GR 50	559[a]	1,604[a]	2,184[a]	4347[a]
CU 50	18[b]	1,280[a]	1,818[a]	3,115[b]
GR 75	670[a]	1,587[a]	2,899[a]	5,155[a]

GR 50= moderate grazing at 50 % utilization; CU 50= moderate cutting at 50 % utilization; GR 75= intensive grazing at 75 % utilization.

[ab] Different letters in the same column indicate significant differences ($P \leq 0.05$).

The forage production recorded according to the type of utilization (moderate cutting or grazing) between FC and A1Y (2 mo) was minimal, as there was a decrease in soil temperature and moisture due to an absence of precipitation. In the period between A1Y and S2Y and between S2Y and A2Y, forage production under the two types and intensities of grazing was similar ($P \geq 0.05$).

For the three seasons of utilization, plots used in moderate grazing (GR 50) produced, on average, 26 % more forage ($P < 0.05$) than CU 50. Likewise, the total forage produced in more intensive grazing (75 %) was 16 % higher ($P > 0.05$) than that produced in moderate grazing (50 %).

Table 4 shows the values of forage production under irrigation and rainfed conditions. In the FC-A1Y period (2 months), there was a higher forage production ($P \leq 0.05$) in the irrigated plots compared to the rainfed plots (890 *vs* -59 kg DM/ha). This can be explained by the fact that even with a drop in temperature in the autumn months (average values of 13 °C were recorded for November), soil moisture increased considerably in irrigated plots (soil moisture values were 23 % in irrigated plots, compared to values of 13 % in rainfed plots).

Table 4: Buffelgrass production according to moisture level (kg DM/ha)

Factor	FC-A1Y	A1Y-S2Y	S2Y-A2Y	Total
Irrigation	890[a]	1514[a]	2272[a]	4676[a]
Rainfed	-59[b]	1466[a]	2328[a]	3735[b]

[ab] Different letters in the same column indicate significant differences ($P \leq 0.05$).

The effect of irrigation was mainly seen in the period from FC (first cut to standardize the plots) to A1Y and the total for the entire period (Table 4). For AIY-S2Y and S2Y-A2Y, dry matter production both under irrigation and rainfed conditions was similar. The total rainfall for the 10 mo was 386 mm; the highest levels occurred in week 4 of October of A1Y with 55 mm and between May and June of the second year, just before the cut of the S2Y, with rainfall that varied between 16 and 116 mm. The presence of rainfall at that year's season equaled these two markers. In total, for the three periods in the study years, 21 % more forage ($P<0.05$) was produced due to irrigation than in the rainfed plots.

The interaction of the factors indicated that the highest total forage production (10 mo duration in two calendar years) corresponded to the most intense grazing that received irrigation, with 5,585 kg DM/ha; 50 % grazing with irrigation produced 4,896 kg, and intense grazing under rainfed conditions produced 4,622 kg. The lowest forage productions were recorded in rainfed and irrigated moderate cutting, with 2,788 and 3,444 kg, respectively. There were no statistical differences for the interaction of the factors.

Table 5 presents the average nutritional values of buffelgrass before and after grazing in each of the established treatments. It includes both rainfed and irrigated treatments.

Table 5: Average values for the entire experiment of crude protein CP, neutral NDF and acid detergent fiber ADF, and *in vitro* digestibility of dry matter IVDDM of buffelgrass according to the type and intensity of utilization and moisture level

Factor	Moisture level	CP		NDF		ADF		IVDDM	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
GR 50	Rainfed	6.7	6.6	74.3	79.6	43.8	46.0	53.4	49.9
GR 50	Irrigation	6.7	6.4	77.7	78.5	46.2	47.7	52.6	48.5
CU 50	Rainfed	6.8	5.9	75.6	77.1	43.9	45.3	56.0	52.2
CU 50	Irrigation	6.7	6.5	76.9	76.0	45.3	47.5	54.9	49.1
GR 75	Rainfed	7.4	5.4	73.9	79.2	43.7	46.1	53.0	50.6
GR 75	Irrigation	6.8	6.5	73.7	78.5	45.3	47.1	52.5	46.6

GR 50= moderate grazing at 50 % utilization; CU 50= moderate cutting at 50 % utilization; GR 75= intensive grazing at 75 % utilization
($P>0.05$).

No significant differences were recorded for CP either before or after grazing at any season of the year. The difference in CP in irrigated plots compared to rainfed meadows was insignificant. The NDF content of forage produced in rainfed and irrigated plots was

higher after use than before use for all periods of the experiment (Table 5). On the other hand, the NDF content for forage in GR 50, CU 50, and GR 75 was higher after each use (post) compared to the values found before (pre) use in all study periods. After use, the grass NDF content in the cut plots was lower than in the grazed plots. The highest value for NDF was recorded in GR 50 after use in rainfed meadows in S1Y, with 80.9 %. The lowest NDF values were recorded in GR 75 before use in A1Y and A2Y, with 73.7 %.

For the three seasons of the year and on average for the entire study period, the content of NDF before use was lower than that recorded after use for the types and intensities of utilization. The proportion of stems is higher than that of leaves after the plants are grazed or cut.

For NDF, it is highlighted that, in the autumn of the second year of study (data not shown), the NDF content in the grass before its use was similar ($P>0.05$) for GR 50, CU 50, and GR 75, with 75.6, 75, and 74.9 %, respectively. After use, CU 50 recorded lower values of NDF ($P<0.05$) than those of GR 50 and GR 75 (76.5, 79.1, and 78.8 %, respectively).

The grass ADF content in GR 50, CU 50, and GR 75 plots was lower before than after use, both for irrigation and rainfed conditions, at all seasons of the two years of study; however, there were no statistical differences. In general, before use, the average ADF content of rainfed plots was 43.8 %, and that of irrigated plots was 45.6 % ($P<0.05$). After use, the average ADF content under rainfed conditions was 45.8, and under irrigation, it was 47.4 ($P<0.05$). The highest value for ADF was recorded in CU 50 plots subjected to irrigation after use in A1Y, with 49.5; on the other hand, the minimum value was recorded in GR 75 in rainfed plots in A2Y, with 42.2.

The buffelgrass ash content before and after each use, in each of the three periods of the experiment and on average, was similar ($P\geq0.05$) for type and intensity of utilization and moisture level.

In general, the IVDDM recorded lower values before than after use. Before the autumn use, there were higher values ($P<0.05$) of IVDDM for GR 50 (59.3 %) and CU 50 (60.3 %) compared to GR 75 (56.4 %). Before use in the autumn of 2000, IVDDM values of CU 50 were higher ($P<0.05$) than those recorded for GR 50 and GR 75 (53, 50.2, and 50.2 %, respectively). After use, the values were higher ($P<0.05$) for GR 50 (48.8 %) and for GR 75 (47.4 %) compared to CU 50 (45.9 %). Before use, the average IVDDM of buffelgrass was higher ($P<0.05$) in CU 50 (55.7 %) than the values of GR 50 (53 %) and GR 75 (52.7 %). After use, the average IVDDM was 50.6 % for CU 50, 49.2 % for GR 50, and 48.6 % for GR 75 ($P\geq0.05$).

In A1Y, 56.7 % of IVDDM was recorded in rainfed plots after moderate cutting, while irrigated plots registered 52.0 % ($P<0.05$). At the same intensity of utilization and after it, in S2Y, the rainfed plots recorded 52.4, while irrigated plots recorded 42.4 ($P<0.05$). Before use, the average IVDDM was higher ($P<0.05$) in CU 50 (55.5 %) compared to GR

50 (53 %). After use, there was no statistical difference in rainfed plots compared to irrigated plots. In A1Y, the highest value for IVDDM was recorded before use in CU 50 and in rainfed plots, with 61.0; in contrast, in S2Y, the lowest value was recorded after use in GR 75 and in irrigated plots, with 42.4 %.

Discussion

In the present study, plots subjected to moderate grazing produced 35 % more dry matter than plots subjected to cutting. When grazing, cattle tend to be more selective in choosing the consumed parts of the plant, improving the renewal of the grasses and their palatability; on the other hand, the forage harvested by mechanical cutting is more uniform[4]. Animals trample, move seeds and minerals, and select when and how they eat; likewise, the populations of nitrogen-fixing bacteria may be in greater quantity in grazed meadows than in cut meadows[13].

The selective consumption of certain plants depends on external and intrinsic factors of the animal that modulate the consumption behavior. The factors affecting consumption behavior and selectivity are those of the animal, social factors, and environmental factors[14]. Compared to cut plants, higher production of grazed plants may be due to greater photosynthetic activity caused by a higher incidence of light and microclimate changes resulting from different cutting heights in grazed plants. In cutting, its uniformity means that lower parts of the plant are left without photosynthesis, as light does not penetrate[3].

Intensive cutting or grazing affects the production of new shoots either by the elimination of organic reserves or non-structural carbohydrates located in the stems and crowns or by lack of leaf area for the resumption of photosynthesis[15]. In the same way, a higher forage production in grazed plots can also be explained by a greater exchange of CO_2 as a result of greater light penetration and a warmer microclimate near the soil surface[16]. Increasing grazing intensity promotes stem reforestation, and the highest values were recorded at medium and high grazing intensity[6].

Large herbivores affect plants by removing biomass, but also due to indirect effects on soil microorganism communities; grazing causes a decrease in vegetation cover, a reduction in organic matter, and with it, changes in the soil microbiome; this produces a reduction of nutrients, mainly phosphorus and nitrogen[17]. One of the alternatives to increase the concentration of nitrogen is the application of manure; in a study that evaluated the effect of pig manure on the yield of *Cenchrus americanus*, they reported no differences in growth between fertilized and unfertilized forage, but they did report a protein increase in the fertilized forage in addition to higher concentrations of nitrogen in the soil[18].

In the case of cattle, the amount of manure excreted per animal unit can be 5 to 6 t of fresh matter per hectare when using rotational grazing. However, trampling influences the soil, which could increase its bulk density (compacting it), decreasing its aeration and, therefore, decreasing soil moisture retention[19]. In the present study, buffelgrass produced 62 % more forage when used under grazing than cutting (2,750 *vs* 1,700 kg DM/ha). A little further north, in Pennsylvania, the Trailblazer species produced only 8 % more when grazing it compared to two cuts per year; however, the Cave-in-Rock and Shawnee species produced more forage by cutting them two and three times per year compared to grazing[20].

On the other hand, when studying the influence of grazing on soil characteristics, it was found that rotational grazing positively influenced physical characteristics by not increasing bulk density values, keeping penetration resistance values low, increasing porosity, and producing a lower average pore radius size compared to continuous grazing. These characteristics would also be positively affected in mechanical cutting[21]. In this study, more intense grazing (GR 75) recorded a 16 % increase in dry matter compared to less intense grazing. When cutting buffelgrass in a greenhouse at 4, 8, 12, and 16 cm, respectively, it was found that it produces the highest forage yield when cut twice a week at 8 cm. Plants harvested at 12 and 16 cm caused a greater increase in the accumulation of dead material[22].

In *Cenchrus ciliaris* and *Chloris gayana*, cutting significantly increased the crude protein content and the digestibility of the organic matter; in contrast, the ash and lignin contents decreased by increasing the cutting frequencies[23]. In the present study, due to irrigation, 31 % more forage ($P \leq 0.05$) was produced than in rainfed plots (1,558 *vs* 1,245 kg DM/ha). By using a sprinkler irrigation system at different percentages of evo transpiration, a maximum of 28 t/ha of dry matter were reported in 12 cuts per year[24]. The results suggest that forage quality depends on various factors such as species, soil, season of the year, temperature, water availability, and solar radiation, among others. In livestock production, low forage quality may be associated with low forage consumption and low livestock behavior. Ideal pasture management is achieved when the quality and quantity available to the animals is maximized.

As for nutritional quality, the first aspect to determine is the effect of grazing intensity. In the present study, there was no statistical difference in CP, NDF, ADF, and IVDDM; however, more intense grazing was recorded in rainfed pastures, 10 % more CP (7.4 *vs* 6.7 %). In *Dactylis glomerata* L., under two grazing intensities (severe: 3 to 5 cm and light: 6 to 8 cm residual forage height), there were similar values of protein and digestibility. A significant effect was only observed during autumn ($P \leq 0.05$), with severe grazing showing the highest IVDDM (64 *vs* 56 %)[25].

Minerals are a key element for plant growth in addition to being essential for animal feeding; in *Cenchrus purpureus*, it was reported that the total contents of ash, magnesium, and phosphorus were variable, contrary to nitrogen, which decreased with regrowth;

however, the magnesium and phosphorus contents were below what was required for plant growth[26]. On the other hand, in *Trifolium repens* under intense grazing, there was an increase in protein in forage (17.4 %) compared to that produced in plots subjected to moderate grazing (14.9 %); in contrast, no differences were found in ADF content when it was subjected to moderate or intense grazing (26.2 and 25.6 %, respectively)[27].

In the present study, there was no difference in nutritional quality when the type of utilization (cutting or grazing) was compared. There was an increase in CP of plots already grazed compared to those already cut and when these were not irrigated (6.6 *vs* 5.9 %). Similar values of CP and NDF digestibility of trailblazer grass subjected to cutting or grazing were reported. The authors only reported differences in both cutting and grazing for NDF. In this regard, the biggest changes in both yield and nutritional quality are due to the climate and crop management[20].

An increase in soil moisture due to rain or irrigation directly impacts the fiber content and, therefore, the digestibility of forages. In *Stipa grandis* P. Smirn. and *Leymus chinensis* (Trin.) Tzvel., from Mongolia, an increase of 0.1 g kg^{-1} in the digestibility of cellulose from organic matter was reported for every 50 mm increase in precipitation and a decrease of 0.1 g kg^{-1} of NDF[28]. In the present work, the NDF content was higher (P>0.05) in rainfed plots compared to irrigated plots.

Regarding grazing intensity, they only observed a significant effect during autumn (P≤0.05), with severe grazing recording the highest IVDDM (64 *vs* 56 %). This can be attributed to the higher proportion of green leaves and a lower percentage of dead material present in the most severe grazing[25]. In the present work, the digestibility values were practically the same in the two grazing intensities. In a study conducted by Ordaz-Contreras *et al*[26] with King grass (*Pennisetum purpureum* Schumach), a decrease in protein was reported as the cutting interval increased. Finally, the height of the cut did not affect the percentages of ash, NDF, and ADF in Guinea [*Megathyrsus maximus* (Jaqc.)], Tanzania, and Mombasa pastures[29].

Conclusions and implications

It can be concluded that a grazing intensity of 70 % exercised for two years did not affect the productivity of buffelgrass compared to that recorded with an intensity of 57 %. The nutritional values of buffelgrass subjected to these two grazing intensities were similar. There was a higher forage production when buffelgrass was used for moderate grazing compared to moderate cutting. Plots subjected to moderate cutting registered higher values for IVDDM than those obtained with moderate grazing. In the cumulative for the two years of study, irrigation produced more forage (22 %) than non-irrigated meadows. When grazing was compared at different intensities of utilization, more intense grazing

produced 14 % more forage than moderate grazing, with no significant differences between the two.

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Review

Genomic regions, genes, and single nucleotide polymorphisms in resistance to gastrointestinal nematodes in sheep. Review

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Abstract:

Several factors can modify productivity in sheep flocks; one of them is gastrointestinal (GI) parasitosis by nematodes, which can cause weight loss, growth retardation, and, in extreme situations, death. Parasite infections involve the immune system for resistance or susceptibility; therefore, strategies are currently being sought that will currently be efficient in the long term to reduce this affectation. One of these strategies is precision livestock breeding, which consists in the identification and selection of genetically resistant animals, using molecular markers. The objective of this review is to gather novel information on quantitative traits (IQT) and genome-wide association studies (GWAS), which confirm the relevance of certain regions or genes in resistance to ovine gastrointestinal parasitosis. Likewise, the potential relevance of new regions was analyzed to perform finer mappings

and find sets of polymorphisms that may allow a more efficient selection, while also considering the particular conditions of the sheep herds.

Keywords: Polymorphisms, Resistance, Gastrointestinal parasitosis, Sheep.

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Introduction

One of the factors that can modify sheep productivity is gastrointestinal (GI) parasitosis; among its adverse effects are weight loss[1] growth retardation and, in extreme situations, death[1,2], aspects that directly affect the economy of sheep producers. Strategies are continually being developed to lessen the effects of infection, either by testing new drugs or by searching for animals that are genetically more resistant in order to reproduce them. Some authors mention that these strategies tend to be more efficient, in the long run, when they are based on multiple approaches[3,4].

Genetic variation between or within breeds allows the detection and genetic selection of individuals with a greater capacity to resist the consequences of helminth infections. Selection of sheep or goats for improved parasite resistance is considered a valuable option to complement other control measures[5]. The term disease resistance is commonly used generically to refer to resistance to infection or resistance to the consequences of disease, i.e. disease tolerance. Strictly speaking, however, disease resistance describes the host's ability to interact with and control the parasite's life cycle. Within the context of GI parasitosis, this may include the probability of establishment of ingested larvae, the rate and degree of parasite development within the host, mortality and fecundity of the parasites, and thus the fecal egg count. On the other hand, disease tolerance is used to describe the ability of the host to resist the pathogenic effects of infection[1].

Genetic improvement for resistance is possible due to the existence of a wide genetic variation in animals. When looking for genetic associations, traits of resistance or susceptibility (fecal egg count (FEC), parasite load, worm size and fecundity): immune response (major histocompatibility complex-MHC, concentration of antibodies such as IgA, IgG and IgM); impact of infection (anemia, presence of pepsinogen, or fructosamine concentrations), or resilience (growth rate and frequency of treatment required) are regularly

studied[3,6]. There are a number of important scientific contributions linking genes of the ovine major histocompatibility complex (Ovar-MHC) to the ability of sheep to resist infection by gastrointestinal parasites[7–16]; however, the effect of the MHC is reportedly small and accounts for approximately 11 % of the total phenotypic variation[7]. Class 1 genes are among the most polymorphic genes; this diversity, together with the lack of clarity of genomic organization, makes it difficult to identify new alleles of interest in sheep, which results in an evident scarcity of information[17]. The products of Class I and II genes are glycoproteins that present antigenic peptides to the T-cell receptor (TCR) of cytotoxic CD8+ and helper CD4+ lymphocytes, respectively[18,19]. Class II DRB genes have been more extensively studied[18,20] and have shown consistent associations with the phenotype of resistance to GI nematodes[7]. Current approaches may neglect this important molecule because statistical analyses to detect association between MHC alleles and disease depend partly on haplotype frequencies[21], whereas the ability to discriminate with causal point mutations depends on the degree of linkage disequilibrium (LD), as, when LD is high, alleles at different loci are often inherited together by the offspring, and the effects of the various loci cannot be easily disentangled[22]. Due to the high polymorphic variation in MHC, it is necessary to construct haplotype combinations to associate it with resistance/susceptibility traits[21,23], since most MHC genes are inherited in bloc as a haplotype with rare recombination events[24]. Use of MHC as a generalized marker requires deepening of knowledge to the point of sequencing already associated haplotypes and knowing in which race and for which parasite they can be validly utilized; the association of haplotypes may then become stronger than the allelic association with single nucleotide polymorphisms (SNPs).

New strategies such as the use of arrays or chips with thousands of polymorphisms for simultaneous genotyping may predict the genetic merit of an individual through clusters of single nucleotide polymorphisms[25]. Genotyping arrays have been developed by such companies as Illumina and Affymetrix, together with the Sheep Genomics Consortium, at different densities and with different coverage within the genome; currently the most commonly used in genome-wide association studies or determination of genetic merit are high and medium density, which can detect approximately 606,000 and 50,000 (50 K) evenly spaced SNPs. It has been observed that the estimated level of development for markers separated by less than 1Mb in arrays of up to 12 K can be a suitable tool to identify genomic regions associated with traits related to resistance to GI parasites[26]. In addition to this, there are intergenic regions, called "genetic deserts"[27], which are regions with non-coding sequences but also with unannotated regulatory elements that offer a promising potential for future research.

While resistance or susceptibility to gastrointestinal parasitosis may be controlled by multiple loci with small effects, epistasis relationships could be evaluated as part of the resistance architecture. In addition, epistatic relationships allow the regulation of the expression of

neighboring genes, which in turn make the expression of other genes possible. To date, no studies have identified major genes as the only genes involved in resistance in gastrointestinal parasitic infections. Therefore, the objective of this review is to present genomic information that confirms the relevance of certain regions or genes, and to give new relevance to others in GI parasitosis by nematodes in sheep.

Recent findings of genes or genomic regions involved in resistance/susceptibility to gastrointestinal nematodes

Nematodes can be located in different regions of the gastrointestinal tract; for example, in the abomasum the most frequent are *Haemonchus contortus*, *Trichostrongylus axei*, *Mecistocirrus digitatus*, and *Telodorsagia circumcincta*, while *Trichostrongylus colubriformis*, *Cooperia* spp., and *Nematodirus* spp. are prevalent in the small intestine, and *Oesophagostumum* spp., *Chabertia ovina,* and *Trichuris ovis*[28–30] predominate in the large intestine. In general, it can be said that the nematode usually found worldwide, especially in tropical and subtropical regions or climates, is *H. contortus*[31], whereas *Telodorsagia circumcincta* is one of the most prevalent in cold regions[32]. It has been hypothesized that the inherent GI resistance to parasites is given by several genes (polygenic), and that it is related to the immune system[33–35].

Four main mechanisms can be identified that determine the host's response to GI parasite infection: 1) the mechanism of the innate immune response, 2) protection of gastric mucous membranes, 3) hemostasis pathways, and 4) acquired immunity[36]. On the other hand, among the mechanisms that allow the expulsion of parasites are the following: hypermotility, gastric hypersecretion and goblet cell hyperplasia with subsequent increased mucus production. *In vitro* and *in vivo* studies have shown that the immediate expulsion of parasites is associated with the presence of histamine and leukotrienes in the mucus of the abomasum that inhibit parasite motility[37,38]. High concentrations of histamine in the abomasal mucosa of hemoncosis-resistant sheep may allow parasite expulsion, promoting abomasal hypersecretion that decreases worm fecundity and motility[39,40].

With respect to the host immune response, it has been observed that there is a clear difference in the immune response of lambs that have been challenged once or twice in their lives, and adult ewes that have been challenged several times during their productive lives with different larval or worm stages[31,37,40]. Lambs demonstrate competent immunity by the age of 2 to 3 mo[41], and if larval exposure challenge is constant, immunity develops with a significant protective response by the age of 10 to 12 mo[42,43]. In adult sheep this immunity tends to remain, rendering them relatively resistant to infection, and low-level exposures

make them retain immunity[44]. In some studies, protection against GI parasitosis has been associated with the Th2 helper immune response[45,46], characterized by the production of interleukin (IL) 4 which is an important cytokine in the immune control of parasitic GI diseases[47,48], essential in the maturation of virgin CD4+ T cells through the STAT6 pathway[49,50]; it also promotes the differentiation of high synthesis rate B cells (changing the heavy chain from IgM to IgE and IgA)[51–53], as well as the recruitment of eosinophils, basophils and mast cells to control infection and participate in the expulsion of helminths[54–56]. IL-13 acts in concert with IL-4 to stimulate IgE class switching, promote healing by tissue fibrosis, and enhance larval expulsion by increasing mucosal permeability, mucus production and muscle contraction[57–59]; IL-5 too stimulates eosinophil maturation. The positive upregulation of these two cytokines after infection with *T. columbriformis*[58,60], coincides with the increase in IgE and IgA production[61]. The development of the Th1 cellular response prevents the expression of proinflammatory cytokines such as interferon gamma (IFN-γ), and, therefore, the Th2 response[55,62]. There is a link between IFN-γ and susceptibility, as it negatively regulates IL-4 and, consequently, the differentiation towards the Th2 response[63,64].

During parasite infection, the concentration of IgA is more important in the abomasum than in serum, and a negative correlation has been observed between the amount of specific IgA in abomasal mucus and the parasite load in infections by *H. contortus*[65]. This situation has also been evident with the nematode *T. circumcinta* found in sheep abomasum, where high levels of specific IgA in the abomasal mucus have decreased the fertility and length of the nematode[66]. A typical feature of helminth infections is the production of specific IgE as a result of a Th2-type response. IgE is able to induce antibody-dependent cytotoxicity of eosinophils, mast cells and macrophages. Increased local IgE levels have been associated with resistance to GI parasitosis in sheep and goats[67–69].

There are reports of resistance/susceptibility in such breeds as Churra, Red Maasai, Merino[70], Dorper x Red Maasai crosses[71], Scottish Blackface[72], St. Agnes[26], Soay feral sheep, Djallonké[73], Border Leicester x Merino, Poll Dorset x Suffolk or white Dorper crosses, Kathadin[74], Tunisian Native sheep[75], Corriedale, Pampinta[76], Sardas[77], and Florida Native sheep among others[78]. In addition, other studies in hair breeds such as the Red Maasai[79], Florida, Santa Cruz, Barbados Black Belly, and Navajo[80] have shown that they are more resistant to nematode infection and its consequences than European breeds. However, there is variation between hair breeds, as shown in a study comparing Pelibuey versus Kathadin lambs; Pelibuey lambs showed greater resistance to natural GI nematode infection compared to Kathadin lambs, sharing the same climatic and grazing conditions, associated with the phenotype of egg counts per gram of feces and peripheral eosinophil counts[81].

Traditionally the association is made with such traits as fecal egg count, because it is a direct manifestation of the host's inability to control parasite reproduction[37,82]. Another is the FAMACHA index, which is an indirect measure of the presence of parasites in the abomasum and the severity of the anemia they can cause to the host, and is related to the reduction of the agglomerated cell volume (measured in percentage), sequelae of infection with parasites such as *H. contortus*, which highlights the host's inability to replenish red blood cell levels and, in an extreme situation, could lead to death; but if the individual tolerates the acute infection and does not die, maintaining its zootechnical activity may be a resilience trait[76]. Animals infected with *H. contortus* show more severe anemias[82,83]. In initial studies, these traits have been associated with regions on chromosome 20 (OAR20; OARn = *Ovis aries* chromosome number "n"), which contain MHC II alleles, and OAR3 on the gamma interferon gene (IFN-ɣ) or genes close to this region[84–86]. Another systematic review, mentions that there is sufficient evidence regarding the association of the IFN-ɣ gene and resistance to *T. circumcincta*, and it has been suggested that this region and its neighboring genes are of interest in host resistance[36,84–88].

Other studies in which no association has been found with the abovementioned regions, point to this difference as being attributable to the characteristics of the study subjects, as associations have been found only in lambs, not in adult sheep[87,89].

Other data provided for Red Maasai x Dorper sheep suggest that variation in SNP markers located in immune cell signaling genes such as suppressor of cytokine signaling (SOCS2), ubiquitin E2-conjugating enzyme (UBE2N) and protein tyrosine kinase substrate 15 (EPS15), could favor Th2 cytokine production to enhance the biological function of eosinophilia, mastocytosis and humoral response (high IgE levels) at the site of infection. Mucus production by the action of genes such as MUC15 or GALANT4 and hemostasis pathways (ATP2B1) may be important mechanisms contributing to the phenotype or in the differences in parasite resistance in the Red Maasai x Dorper population[72], and describes two regions that had not been associated until then: OAR2 (162-163Mpb) and OAR3 (44Mpb). The data also show that the OAR6_81718546 polymorphism (close to the platelet-derived growth factor receptor-α PDGFRA) is associated with effects on the aggregate cell volume, as has been previously reported in sheep of the Brazilian Morada Nova, Spanish Churra [34], and Soay Feral breeds[78] and in the Red Maasai x Dorper cross. Markers affecting fecal egg count (OAR5_111342555, OAR15_35337227, OAR5_100699982.1, DU183841_402.1, OAR15_40719719.1, OAR15_40926306.1, OAR7_4206430, and OAR17_42673146) do not affect agglomerated cell volume or live weight according to this study[72].

On the other hand, twelve SNP's listed in Table 1 were analyzed in the Soay Feral breed, of which, RORC2 p.A404T (100,653,186 bp) is associated with IgA; in addition it was concluded that the IL23R p.V32M polymorphism (42,512,431 bp) is related to the IL-23

receptor, an inflammatory cytokine that exhibited association with body weight at 20 days in blackface lambs[78].

In 2016, associated genes were found in Spanish Churra breed for the fecal egg count trait in OAR6 (with peak at 88. 1 cM) as AFP, ALB, AMBN, AMTN, AREG, BTC, CXCL1, CXCL10, CXCL11, CXCL9, EREG, GC, IGJ, IL8, MUC7, PF4, PPBP, RASSF6, SCARB2, TMPRSS11D; in OAR8 (peak at 2 cM) for the same trait, as CD109, COL12A1, MYO6, and in OAR 22 (peak at 3.4 cM), for the IgA trait of gene PCDH15. Among the nematode species found most frequently in this study were *Trichostrongylus* spp. and *Teladorsagia* spp. In addition, other genes encoding for chemokines were found, including IL-8, CXCL1, CXCL10, CXCL11, CXCL9, PF4, PPBP —molecules that are of great importance in the immune system, since they are involved from leukocyte recruitment to cell communication and activation during infection; particularly IL-8, CXCL8, and CXCL1 are involved in the recruitment and activation of neutrophils. Notably, this author found no clear correspondence with previously described classical regions related to IFN-ɣ or those involving MHC class II genes[34].

On the other hand, when evaluating Santa Inés sheep using a SNP chip with 12 785 single nucleotide polymorphic markers, an association was found between regions in OAR1, OAR2, OAR3, OAR5, OAR8, and OAR15[78] (Table 1). Several candidate chromosomal regions described by the authors are related to the development of the immune system, its activation, inflammatory response, lymphocyte regulation, and leukocyte proliferation (B2M, SFXN1, IL25, BMP4, TSHR, CCL28, PIK3R1, FGF10, IL15, TP-1, BPMG, BCL10, HSPD1, MALT1), highlighting genes such as CD109, which is a surface antigen expressed by CD34 or IL-25 cells; coincidentally some of these were reported by another study[34] as potential genes in resistance to GI nematodes in sheep.

As of 2018, GWAS studies with high or medium density chips have elucidated more candidate genes that could be relevant in resistance/susceptibility to nematodes and other parasites. This is indicated by the findings of several authors[75,77,90], one of them in Djallonké lambs from West Africa, where five genes (TRIB3, CDK4, CSNK2A1, MARK1, and SPATA5) are associated with resistance traits related to immunity and cell proliferation. It is also suggested that the MBL2 gene (as the basis of a QTL) in OAR22 is related to IgA levels[27,82]. Also, it has been hypothesized that genes involved in lamb growth and size (such as the ADAMTS17 gene in OAR18) may be pleiotropic with certain genes that determine resistance traits to GI parasite infection; however, the association between these genes has not yet been clearly determined[34,73,91].

OAR2 is also highlighted in a study in Australian sheep of breeds such as Merino or Border Leicester x Merino, Poll Dorset/Suffolk/Suffolk/White Suffolk/White Dorper/Border Leicester crosses, where, in a first analysis, the authors provide an outline of three SNP's in

OAR2 with a strong association to the trait of fecal egg count (rs421630816, rs424521894, rs413835864): the SNP rs421630816 (position in OAR2: 110.8 Mbp) in the PALLD gene, while rs424521894 and rs413835864 (position in OAR2:107.3 and 107.4 Mbp, respectively) in the GALNTL6 gene related to the synthesis of mucin-like glycans, which influence host-pathogen interaction. Likewise, these authors point out a region in OAR6 that includes six SNP's, where rs416517011 stands out for its level of significance in the association; they also found other associated genes in OAR18 and OAR24, hypothesizing that these genes share certain mechanisms with the immune system, suggesting potential interaction effects between genes[70]. Another contribution found significant associations in OAR2, 3, 16, 23, and 24 in Kathadin sheep[74]. A relevant finding is a locus located in OAR3, close to the C3 complement pathway receptor 1 gene (C3AR1). C3AR1 has been reported to be differentially expressed in susceptible versus resistant sheep[92] and has been associated with the Th1 response[93], also located in OAR16, 87 kb towards 5' of the *ITGA2* gene (α-2 integrin) that mediates adhesion of platelets and other cell types to the extracellular matrix. One region that stands out in OAR2 —the DIS3L2 gene (rs406850490 and rs422243920), an exoribonuclease involved in regulating the relative expression of Toll receptor type 4— was significantly associated and suggests a potential role in mediating resistance. The DIS3L2-associated SNP had a minor allele frequency (MAF) overrepresented in resistant sheep (0.479) compared to susceptible sheep (0.094); this exoribonuclease may affect IL-10 expression by repression of let-7, a miRNA. Other findings of importance in the study refer to OAR3 ALK-tyrosine kinase receptor (rs437558829 and rs407346502) and C3AR1, OAR19 (rs406978752) GRM7-(metabotropic glutamate receptor 7), OAR23 (rs399876637) SLC14A2 (urea transporter 2) and OAR24 ZP3 glycoprotein (rs423186265); however, it has been suggest that these findings need to be validated[74].

In order to give prominence to the effects of the immune system on the response to parasitosis, another group studied indigenous Tunisian sheep under traditional grazing management and they highlighted RUFy4 and VIL1, two IL-8 receptors (CXCR1 and CXCR2) as candidate genes involved in the immune response in the GI tract, hypothesizing that they may be involved in repairing damaged tissue in the intestine and enhancing neutrophil recruitment and inflammation. They also found two cation transporter genes such as SLC22A4 (OCTN1) and SLC22A5 (OCTN2) involved in the transport of oxourea. The authors stress that the traditional management of these sheep allows them to develop multiple adaptive strategies that make them resistant to parasitosis, and the information gathered from this type of native livestock is very valuable in understanding the architecture of resistance[75]. In Mexico there are herds with Creole characteristics and extensive management; therefore, it would be interesting to determine if the adaptive strategies of the immune system coincide with those of other herds, or other breeds, managed under similar conditions, and thus be able to identify coinciding mechanisms for use as markers for resistance/susceptibility to nematodes or other parasitosis.

Notably, a fine mapping carried out by Argentine researchers in Pampinta and Corriedale lambs under natural challenge found that certain regions that had been previously associated[36], in OAR3 and OAR6, and OAR20, contain genes involved in MHC-mediated antigen processing and lymphocyte signaling pathways. The OLA-DRA1_479 SNP was the only SNP that showed a significant association for the traits under study in Corriedale lambs; it also associated polymorphism of C-type lectin receptors that mediate functions such as cell signaling transduction processes, pathogen recognition, and innate immunity, although CLEC12A acts by inhibiting the production of IL-12, TLR4-dependent TLR4 and IL-12[94]. It also marked three significant *de novo* SNP´s, FOS_109, IL20RA_422 and TIMP3_716 —the first, located in FBJ murine viral osteosarcoma homologous gene; the next, in IL-20 receptor gene, and the last, located in TIMP a metalloproteinase inhibitor in OAR 3, 7 and 8, respectively—; FOS_109 belongs to a group of proteins that regulate cell proliferation, differentiation and transformation. The duplicated expression of this gene in abomasal tissue was found to be associated with resistance in Merino sheep and it is hypothesized to be a relevant gene in primary infections by *H. contortus*. In some cases, FOS gene expression has also been associated with cell death by apoptosis. TIMP3_716 showed evidence that suggests association when using fecal egg count as a breeding value estimated as an association phenotype, and may be involved in the remodeling of damaged tissue in response to parasitic infections. The results obtained confirm genomic regions previously reported to be associated with nematode resistance in other sheep breeds, both for innate immunity (MASP, CLR, NLR, TLR, IL20R, FOS, TIMP) and adaptive immunity (CLR, IL2, OLA-DRA, TIMP) reinforcing the role of the host immune response against parasites[76].

In Sarda ewes and crosses of this line with Lacune, 10 regions with significant association to the trait of fecal egg count were mapped, pointing to 3,538 polymorphisms causing high-impact effects that can generate termination codons (nonsense mutations) in genes coding for 530 proteins. The authors of this study hypothesize that QTLs located in OAR 1, 12, 19, and 20 are strongly implicated in a complex mechanism of resistance in sheep to GI parasitosis; some of the polymorphisms they report can be seen in Table 1(77). In OAR12, the missense mutation c.103G>A in exon 2, position 39, 567,687 bp, in the TNFRSF1B gene (member of the TNF1B receptor superfamily), which is also close to the *SELE* gene (selectin E gene, four relevant nonsense mutations), encodes a protein in endothelial cells and is responsible for the accumulation of leukocytes at sites of inflammation mediated by vascular lining cells. Another authors[95] mentions that the *SELE* gene is negatively expressed in abomasal lymph nodes of lambs recently infected with *T. circumcincta*, suggesting it as a component of the resistance response to infection in GI parasitosis. In OAR19 the most significant association was in the MGR gene (metabotropic glutamate receptor, associated with nervous mechanisms in humans), in addition to 13 nonsense variants in the IL5RA gene (α-subunit of rIL-5). This protein has been found to be expressed in animals resistant to *T. circumcincta* (Scottish blackface lambs, churras ewes, and merino lambs)[95–97]. In the OAR20 region, a large region encompassing MHC class II was found, although these are reportedly located at

a distance of 4 to 6 Mb from the most significant location, highlighting that, due to the polymorphic nature of the gene, it is difficult to identify causal mutations or SNP's that are useful in resistance selection[98]. Also reported were mutations in IL17A, IL17F, TRIM26, TRIM38, TNFRSF21, LOC1011118999, VEGFA and TNF. A significant SNP (rs404860664) was reported in the LOC101111058 gene (butyrophilin-like protein); however, butyrophilin-like proteins suggest that it plays a role in the regulation of local intestinal inflammation in other species[99], with nine mutations in TRIM 26; these proteins play roles in the regulation of pathogenesis in autoimmune diseases and pathogen defense in particular against viruses[100], and they also may be involved in the down-regulation of several immune response genes[77].

In a first study detecting repeat variants by GWAS in native sheep in Florida, 8124 copy number variations (CNV) were identified, although only 14 were significantly associated with the traits under study, such as fecal egg count and aggregate cell volume. The genes that stand out in this study in relation to the immune response are CCL1, CCL2, CCL8, CCL11, NOS2, TNF, CSF3, and STAT34, which may play an important role in the resistance to *H. contortus*. These genes could be used as potential markers of resistance in this breed; it is also possible that genes close to repeat regions such as LOC101110424, DOCK9, ITGBL1, BIVM, TNFSF13B, ING1, F7, F10, PCID2, and GAS6 may have important effects on the immune response against the parasite[90]. For example, ITGBL1 gene expression is associated with immune cell infiltration[101], while genes F7 and F10 play a relevant role in the initiation of coagulation and defense against pathogens[102]. The CCL1 gene is part of an eotaxin chemokine and promotes the migration of activated eosinophils[103], eosinophilia is a common event in sheep infected with *H. contortus*[104], and this gene is commonly used as a marker of resistance[92,105]. In addition, three galectin genes (LOC101117947, LOC101118202 and LOC101102156) near a repeat region were associated with the egg count trait at day 28. Galectins are proteins involved in the immune response to parasitic infections of the gastrointestinal tract in sheep and are upregulated during infection with *H. contortus*[106]. Some of these galectins, like No. 11, can regulate larval growth and development by binding to *H. contortus* larvae No. 4 and adults[107]. The associated repeats for the cell package at day 0 and 28 (LOC101108321) are contained in genes related to multidrug resistance proteins (MRP), expressed at the same level in CD3+/CD4+ T cells according to a study performed in the peripheral blood of normal and refractory lymphoma patients[108]; they can also regulate the inflammation of intestinal mucous epithelia[109]. There is a possibility that all the repeated sequences found in this study may be segregated among the population, but as in other studies, they must be validated in other populations. These findings may contribute to the development of new strategies to improve parasite resistance in sheep and promote selective breeding through marker-assisted selection[90].

Conclusions

Parasitosis and parasitic resistance are an issue that affects sheep production systems, especially grazing sheep. Knowledge about the architecture of resistance/susceptibility in sheep contributes to genetic improvement at a faster rate, resulting in higher productivity in flocks that contribute to precision livestock breeding. Although effective drug treatments to fight parasitosis can be found in parallel, when new formulations become available, they can potentially be more expensive. There is also a growing interest in reducing the use of anthelmintics to contribute to the environment by reducing their excretion into the environment. The information from QTLs has been refined by GWAS analysis with high-density chips, creating the need for further fine mapping of candidate genes, so that the information may be utilized to screen sheep for resistance to GI parasites or to elucidate epistatic relationships between immune response genes in order to generate areas of research for functional or expression studies, providing greater clarity on the function of the immune system. Dissection of the architecture of resistance and susceptibility to gastrointestinal parasitosis, as well as the validation of associated loci in different herds, create the challenge of generating a marker test with the best possible combination of SNPs to allow characterizing individuals resistant to GI parasitosis among certain populations as a strategy to address parasitic resistance and implement more effective and direct selection programs.

Table 1: Findings of genomic regions, SNP's and genes linked to association variables in resistance to gastrointestinal parasitosis in sheep

Author	**Parasite**	**Association variables or traits**	**QTL**	**SNP**	**Genes**
Benavides, 2015	*Haemonchus contortus*	AVCA*, LW**	OAR2 (15 Mbp), OAR11 (58 Mbp) OAR15 (54 Mbp). New OAR2 (162-163Mpb) and OAR3 (44Mpb).	OAR6_81718546, OAR5_111342555, OAR15_35337227, OAR5_100699982.1 DU183841_402.1, y OAR15_40719719.1 OAR15_40926306.1 OAR7_4206430 y OAR17_42673146,	*SOCS2, UBE2N y EPS15 ATP2B1 y LRP8 MUC15 y GALNT4*
Atlija, 2016	*Trichostrongylus* spp *y Teladorsagia* spp		OAR6 (peak in 88.1 cM), OAR8 (peak in 2cM) y OAR22 (peak in o 3.4 cM)		*AFP, ALB, AMBN, AMTN, AREG, BTC, CXCL1, CXCL10, CXCL11, CXCL9, EREG, GC, IGJ, IL8, MUC7, PF4, PPBP, RASSF6, SCARB2, TMPRSS11D, CD109, COL12A1, MYO6 PCDH15, IL8, CXCL1, CXCL10, CXCL11, CXCL9, PF4, PPBP, CxCL8 y CXCL1*
Berton, 2017	*Haemonchus contortus*	FEC***, FAMACHA index, AVCA	OAR 2:91681809-9470993 2:140765269-143337545 OAR 3:195904655-195904655		*LPAR1; TXN; ALDOB; PLPPR1; CTSV; PTCH1; AGTPBP1; AQP3; ADRA1A; LOXL2; SFTPC; HR; LPL; LOC101123612; TGFBR1; GNA14; PCSK5; RORB; ALDH1A1; TYRP1; FREM1; PSIP1; CCDC171; BNC2; CNTLN; ADAMTSL1; RPS6; TP-1 XIRP2; LOC101109253; SCN7A; SCN9A; SCN1A; TTC21B; GALNT3; CSRNP3; LOC101110039; SCN2A;*

			OAR 1:56799547-56799547 OAR 16 :41876371-41876371 OAR 18:68738392-68738392		*SCN3A, DHX57; GEMIN6; RSF7; GALM; HNRNPLL; LOC101119897; LOC101120157; ATL2; LOC101120655; LOC101120913; LOC101119706; RMDN2; CDC42EP3; TRNAC-GCA; TRNAS-GGA; QPCT; PRKD3; NDUFAF7; CEBPZ; SULT6B1; EIF2AK2; GPATCH11; HEATR5B; STRN; VIT; FEZ2; LOC101122183; LOC101122430; LOC101122685; LOC101123283* *GALNT2; TRNAE-UUC; PGBD5; LOC101103868; LOC101104120; LOC101104369; LOC101104630; LOC101104883; LOC101105131; LOC101105384; LOC101105628; LOC101105878; LOC101106137; LOC101106392; LOC101106652; LOC101106903; LOC101107159; LOC101107409; LOC101107663; LOC101107927; LOC101108188; LOC101108450; LOC101108625; LOC101108717; LOC101108881; LOC101109143; LOC101108983; LOC101109240; LOC101109508; LOC101109767* *PDZD2; LOC101119673; C16H5orf22; DROSHA; CDH6* *INF2; ADSSL1; SIVA1; AKT1; TMEM179; PLD4; LOC101104938; C18H14orf79; LOC101105444; GPR132; LOC101105953; BTBD6;*

					BRF1; LOC101106466; LOC101106718; C18H14orf80; TMEM121; LOC101107475; LOC101107738; LOC101107998; LOC101108260; LOC101108522; LOC101108781
Wilkie, 2017	****NE	FEC, IgA, LW		RORC2 c*25T>C and RORC2 c.*109[a]>g E294Q y A404T) IL23R p.V324M y RORC2 p. A404T	*TBX21, RORC2 e IL23R*
Álvarez, 2019	NE	AVCA, FEC_{log}, AVCA, FAMACHA		OAR1_55820164.1 OAR2_117867801.1 OAR8_16568165.1 OAR15_88875909.1 OAR18_43101149.1 OAR2_140684314.1 S16493.1 (OAR16) S43307.1 (OAR7) OAR8_8982479.1 OAR15_2525103.1 OAR17_3451123_X.1 S43852.1 (OAR19) OAR2_64824262.1 OAR3_77774489.1 OAR3_161498140.1 OAR12_22189408.1 S32476.1 S09612.1 (OAR13) OAR18_5508052_X.1 OAR22_6293170.1 OARX_107840506.1	*TMOD1; TDRD7, MFSD6, INPPI, HIBCH, C2H2orf88, SV2C, IQGAP2, NUDT6 TRIB3, CDK4, CSNK2A1, MARK1 y SPATA5, MBL2, ATP6V1E2, TMEM247, EPAS1, ATP23, CTDSP2, AVIL, TSFM, METTL21B, METTL1, LOC101116039, MARCH9, CDK4, TSPAN31, MARK1*

Kaladeh, 2019	*H. contotus, T. colubriformis, T. circumcincta*	FEC		rs421630816, rs424521894, rs413835864, rs421630816, rs424521894 y rs413835864, rs413835864, rs424521894 y rs421630816, rs416517011	*PALLD, GALNTL6*
Becker, 2020	*Haemonchus contorutus*	Estimated genetic values and FEC, and FAMACHA index		rs406850490 y rs422243920, rs437558829 y rs407346502, (rs406978752, rs399876637, rs423186265	*C3AR1, DIS3L2*
Ahbara, 2021	NS		QTL FECGEN		*SLC22A4, SLC22A5, IL-4, IL-13, IL-4, VIL1, CXCR1, CXCR2, IL-4, IL-13, FECGEN, TFEC_1, HFEC, NFEC, LATRICH_2, IGA, OSAS, WORMCT, PEPSL y CEOSIN QTL, RUFy4 y VIL1, ITLN*

* AVCA= average volume of the cell agglomerate; ** LW= live weight; *** FEC= fecal egg count; ****NS= not specified.

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Review

Use and evolution of sperm sexing in cattle. Review

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Abstract:

Since the commercial beginning of sperm sexing in artificial insemination, the adoption of this technology by the livestock industry (producers, veterinarians, and genetics companies) has been a reality in cattle production, mainly in dairy cattle. This review describes the beginnings of sperm sexing, its development, commercial application, and evolution to the present. The most significant events were undoubtedly the determination of the difference in DNA content between spermatozoa carrying the “Y” or “X” chromosome, the flow of these in the cytometer, and their separation into the so-called “Y” and “X” spermatozoa. The subsequent achievements that favored the application of this technology commercially were the determination of the optimal concentration and the successful cryopreservation of sexed semen; since then, research to try to reduce the deleterious effects of the sexing process has

not stopped, leading to the emergence of new sperm sexing technologies where this effect is minimal. The most widely used technique commercially is the ultrasexing of 4 million spermatozoa (SexedULTRA-4M™), in which the method, media, and cytometers were completely modified so that this technology has results very similar to those obtained with unsexed semen (conventional semen). There is another sperm sexing technology called Sexcel™ that is offered commercially, in which they have obtained similar results to those obtained with conventional semen, but only in heifers. With these advances, sperm sexing is shown to be a technology in constant development and of high impact on cattle farming.

Keywords: DNA, Sperm sexing, Sex chromosomes, Flow cytometry.

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Introduction

As far as reproductive biotechnologies in cattle are concerned, sex preselection has a long history, in which attempts have been made to separate spermatozoa with "X" and "Y" chromosomes by various techniques based on principles of difference in mass and motility, sperm kinetics, changes in sperm surface, and differences in volume; however, none of these methods (sedimentation, centrifugation, and antiserum Y) was able to produce an effective separation of fertile sperm populations[(1)].

Sex predetermination was achieved thanks to advances in computation, biophysics, cell biology, and applied reproductive physiology, among others. From 1980 onwards, a technique called flow cytometry began to be applied, which allowed spermatozoa to be separated according to their sex chromosomes. It took 20 yr for this technology to be commercialized for use in artificial insemination (AI) in cattle. This technique is based on differentiating "X" and "Y" spermatozoa in terms of their DNA content. In the case of cattle, the "X" spermatozoa that produce females contain an average of 3.8 % more DNA than the "Y" spermatozoa that produce males[(2)]. Sperm sexing by flow cytometry is a valuable tool that undoubtedly had a beneficial impact on the genetic improvement of the livestock industry. This technology had an efficiency of 85 to 95 % regarding the birth of offspring with the preselected sex; nevertheless, it was not completely perfected[(3)].

The first commercial production of sexed semen was carried out by the Cogent company in the United Kingdom[4]. Although it had a relatively slow start, the production of bovine-sexed semen increased exponentially, with an estimated 4 million doses in 2008[4]. The sexed semen was marketed in 0.25 ml straws at a concentration of 2.1 million spermatozoa[5]. A minimum efficient concentration was used because there were losses of approximately 80 % of the ejaculate between the spermatozoa of the unwanted sex and the spermatozoa that could not be differentiated[6]. This semen was less fertile and more delicate than conventional semen since the spermatozoa were subjected to several processes for the separation of spermatozoa with an "X" chromosome and those with a "Y" chromosome, in addition to the freezing and thawing process[7]. Despite the limitations of sexed semen, there was clearly good acceptance[4]. Acceptable gestation percentages were achieved with the reduced dose (2.1 x 10^6 spermatozoa) of sexed semen in heifers, but few studies were carried out with lactating cows[2]. Nowadays, sperm sexing technology has evolved, modifying techniques, increasing the speed of sexing, reducing stress, increasing sperm concentration, and therefore improving sperm viability parameters. At the moment, three sexing techniques are applied commercially, all through flow cytometry: SexedULTRA™[8], Sexcel™[9], and Lumisort™[10]; however, there are other promising techniques other than flow cytometry: sperm sexing using gold nanoparticles[10] and sperm sexing using magnetic nanoparticles[11], but which are not yet used commercially.

Historical overview of sperm sexing

Quantification of sperm DNA

Undoubtedly, the determination of the DNA content in the sperm opened the doors to sperm sexing technology. In 1976[12], the sperm DNA content in different animal species (hamster, mouse, rabbit, bull, pig, horse, oysters, abalone, and octopus) was evaluated by flow cytometry. In this work, they found that the distribution of the populations depended on the shape of the sperm head and how they were oriented. In the case of abalone spermatozoa (cylindrical heads), they presented a symmetrical fluorescence pattern; nevertheless, in the case of eutherian spermatozoa (elongated heads), the fluorescence pattern was asymmetrical, which presented a problem for reproductive biology applications; however, the authors concluded that this problem could be solved using a flat flow given the shape of the sperm head (Figure 1).

Figure 1: Morphology and morphometry of the sperm head in different species[13]

Dimensions and profiles of sperm heads and flow cytometric sorting indices for some domestic mammals and man

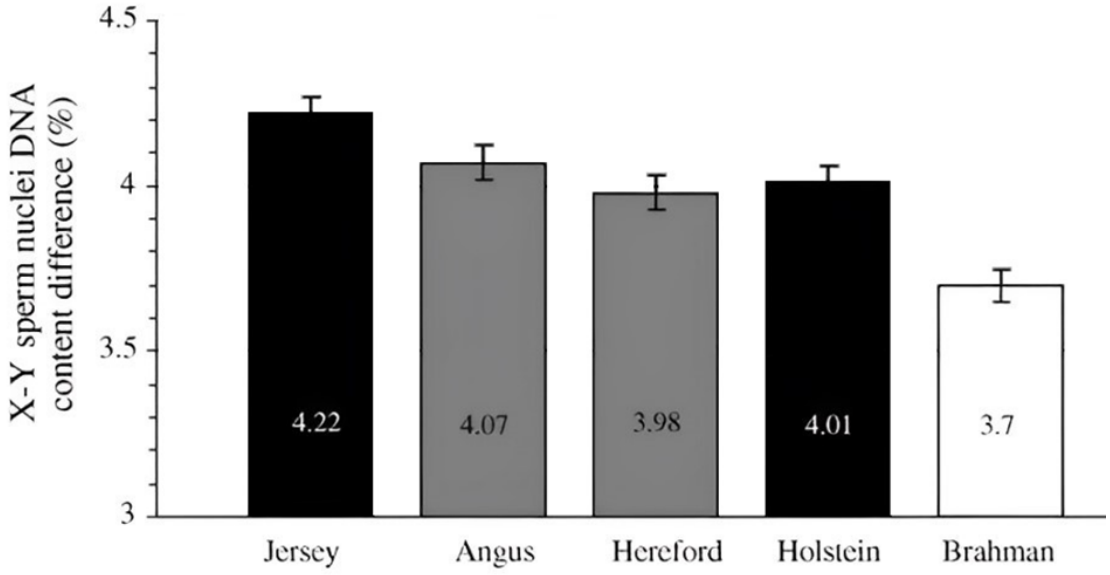
Dimension	Bull	Boar	Ram	Rabbit	Cat	Dog	Horse	Man
Length (µm)	9.1	9.0	8.1	7.7	7.7	7.0	6.5	4.6
Head sagital section								
Width (µm)	4.7	5.0	4.0	4.5	3.2	3.5	3.4	3.2
Head profile								
Area (µm^2)	34.5	37.5	26.6	28.0	19.0	20.9	15.2	10.8
X-Y difference (%)	3.8	3.6	4.2	3.0	4.2	3.9	3.9	2.8
Sorting index [a]	131	115	112	84	80	82	59	31

[a] An approximation of the ability to flow cytometric sort sperm consisting on the head profile area (µm^2) x X-Y Sperm DNA difference (%).

"X" and "Y" chromosomes in productive species

Since flow cytometry opened up the possibility of separating spermatozoa based on their DNA content, the next step in the development of sperm sexing technology was the quantification of DNA from "X" and "Y" spermatozoa from domestic species. In 1983[14], the difference in DNA content between spermatozoa with "X" and "Y" chromosomes from domestic animals was evaluated, where a difference of 3.9 % was found in the case of bulls, 3.7 % in pigs, 4.1 % in sheep, and 3.9 % in rabbits. In the case of bulls, they used 25 bulls representing five breeds (Jersey, Holstein, Hereford, Angus, and Brahman), and they observed that the average among the population of spermatozoa with the "Y" chromosome was in the range of 49.5 to 50.5 % for all breeds. The differences between spermatozoa with "X" and "Y" chromosomes did not vary within each breed but significantly differed when compared between breeds. The Jersey breed had the largest difference between "X" and "Y" chromosomes, and the Brahman breed had the smallest difference (Figure 2); this indicates that the Jersey breed is easier to sex than the Brahman breed.

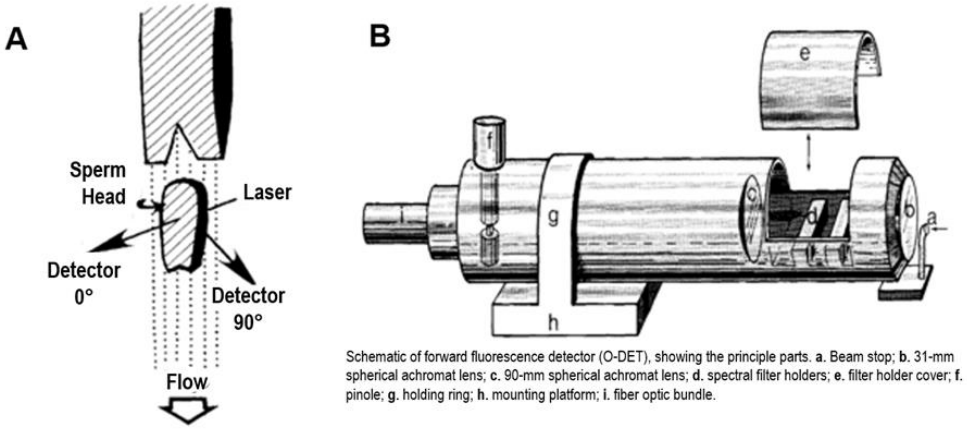
Figure 2: Difference between the DNA content of "X" and "Y" spermatozoa between different cattle breeds[13]



First modifications to flow cytometers for sperm sexing

Once it was possible to differentiate the "X" and "Y" spermatozoa based on their DNA content, work began on the flow cytometer to be able to make more efficient readings. In 1986[15], the first modifications to the flow cytometer were made to separate sperm populations. In this work, they used an EPICS V cytometer (Coulter Corporation, FL, USA), which they adapted to improve orientation. These adjustments consisted of modifying the bevel of the sample injection tube and the addition of a second fluorescence detector at 90° (Figure 3A) along the axis of the laser beam, directing the fluorescence collected by a beam of optical fibers toward the photomultiplier tubes (Figure 3B).

Figure 3: Bevel and injection tube used in the EPICS V cytometer. **A.** Beveled tip and fluorescence detectors. **B.** Photomultiplier tubes[15]



Subsequently, the sample injection tube modifications were continued, making a beveled tip. This beveled tip (25°) caused a sample flow of a flat shape, so the hydrodynamic force in the sperm nuclei caused them to be preferentially oriented in the plane of the flow. The fluorochrome-stained nuclei were excited by a laser beam perpendicular to the sample flow plane. The laser hit the flat side of the oriented sperm nuclei, and the fluorescence was simultaneously detected from the flat side by a fluorescence detector at 0°; in addition, a standard detector at 90° was added. To generate the fluorescence, a Coherent Innova 90-5 Argon-ion laser (Coherent Inc, CA, USA) was used, operating in ultraviolet light (351, 364 nm) at 150-200 mW of power. The fluorescence emitted individually by each sperm nucleus was collected by both detectors (0° and 90°) and stored as frequency distributions (histograms) in a multiparameter data acquisition display system. The separation process was carried out by droplet formation using a drop flow through ultrasonic vibration. Each droplet contained a sperm nucleus that emitted fluorescence, which was detected and electrostatically charged in one of two containers for each population, "X" or "Y". With these modifications and working with chinchilla spermatozoa, it was possible for the nuclei of the spermatozoa to be separated into "X" and "Y" at a rate of 55 nuclei/sec for each population, with a purity of 95 %[16].

Progeny obtained from spermatozoa with "X" and "Y" chromosomes

Until now, invasive techniques have only been used for both staining and selecting spermatozoa with "X" and "Y" chromosomes; therefore, the next step would be selecting viable spermatozoa with which AI could be performed. In 1989[17], the first AI test was carried out using sexed sperm in rabbits. For this test, fresh semen from two rabbits was used,

which was sexed in an EPICS V flow cytometer with previous modifications[15,16]. This test resulted in sperm populations with an "X" chromosome with a purity of 86 % and 81 % for spermatozoa with a "Y" chromosome. With the semen obtained, surgical inseminations were performed on previously synchronized females with unsexed semen, with semen with an "X" chromosome, and with semen with a "Y" chromosome. Of the females inseminated with semen with an "X" chromosome, 94 % of the offspring were female; in the case of females inseminated with semen with a "Y" chromosome, 81 % of the offspring were males. This work demonstrated the accuracy of the sperm sexing technique employing flow cytometry.

Subsequently, there were reports of the first bovine offspring born from embryos produced *in vitro* with sexed semen, which had a purity of 79 % for spermatozoa with the "X" chromosome and 70 % for the "Y" chromosome. The embryos produced *in vitro* were sexed by PCR; the analysis indicated that 73 % were female and 69 % were male, with no statistically significant difference compared to what was obtained in the flow cytometry analysis. In this research, it was observed that sexed semen continued to have its fertilizing capacity and that it had an acceptable purity; however, the number of selected spermatozoa was too low to be used in AI, but it was feasible to be used in the case of *in vitro* embryo production (IVP)[18].

In 1996[19], a field trial was carried out in which Holstein heifers were inseminated (deep insemination, ipsilateral to the ovary with a larger follicle) with sexed semen (90 % purity, 1 x 10^5 spermatozoa) refrigerated at 5 °C. In this work, approximately 18 h passed from the time the semen was collected until the heifers were inseminated. Twenty-two inseminations were performed, of which 11 females were diagnosed pregnant at 60 d; of these pregnancies, the sex was determined by ultrasonography (between 60 and 70 d of gestation); one of the 11 fetuses was not of the predicted sex.

Creation of the company XY Inc.

The results obtained in the AI test in cattle[19] encouraged the USDA (United States Department of Agriculture) to license the Colorado State University Research Foundation (CSURF), Fort Collins, CO, USA, to proceed with the commercialization of the Beltsville sperm sexing technology for sex selection in non-human mammalian spermatozoa. With the issuance of this license in 1996, the company XY Inc. was formed, which was a collaboration between CSURF, Cytomation Inc. (CO, USA) and private investors. This company acquired the rights to high-speed flow cytometry and marketed it as the MoFlow™ cytometer (CO, USA). This cytometer included the modifications made to the injection needle[15,16] and it was improved by adding a selection nozzle that oriented 70 % of the spermatozoa through

the pressure of the hydrostatic fluid system. With this improvement, around 20,000 spermatozoa/sec could be analyzed, and up to 6,000 or more spermatozoa/sec from each of the "X" or "Y" populations could be sorted with 90 % accuracy. In 2003, Cytomation Inc. was bought by the Danish biotechnology company Dako, and it became Dako A/S; the company continued to produce the cytometer for sperm sexing, which they renamed MoFlow SX™ (CO, USA). Subsequently, the flow cytometry instrumentation division was acquired by Beckman Coulter, located in Fullerton, CA, USA[5].

Artificial insemination with low doses of sexed semen

In 1997, a study was carried out with two objectives: 1) to evaluate pregnancy rates of heifers that were synchronized and inseminated (in the uterine horn, ipsilateral to the ovary with a larger follicle) with very low doses of semen (1 x 10^5; 2.5 x 10^5; 2.5 x 10^6 spermatozoa/0.21 ml) refrigerated at 5° C under ideal conditions at the field level; 2) to evaluate the pregnancy rates of heifers that were synchronized and inseminated (in the uterine horn, ipsilateral to the ovary with a larger follicle) with low doses of sexed semen (1-2 x10^5 spermatozoa/0.1 ml) refrigerated at 5 °C. In the first experiment, the pregnancy rates at 40 d were 41 %, 50 %, and 61 % for 1 x 10^5, 2.5 x 10^5, and 2.5 x 10^6 spermatozoa/insemination, respectively. Regarding the second experiment, 22 % of 67 inseminated heifers were pregnant, and 82 % of the offspring were of the selected sex[20].

Successful cryopreservation of sexed semen

Later, in 1999, another research was carried out with the aim of evaluating the process of freezing the sexed semen; this could be done because the semen was processed in a MoFlow SX™ flow cytometer, with which it was possible to have a sufficient number of spermatozoa unlike when working with the EPICS V flow cytometer. In this work, it was determined that the use of the laser at a power of 100 mW had a lower impact on the progressive motility of the post-thawed semen than when it was used at 150 mW. It was also observed that the post-thawed progressive motility was higher when using a TRIS-based diluent than when using citrate-egg yolk or TEST. Regarding the equilibrium time at 5 °C before freezing, it was concluded that the progressive motility after thawing from 3 to 6 h was better than when it lasted 18 h. On the other hand, it was determined that it was better to keep raw semen (freshly collected, undiluted semen) at 22 °C than to dilute it with TALP medium added with Hoechst 33342 fluorochrome (ICN Biomedicals Inc., OH USA). With these new procedures for sperm sexing, slightly lower results were achieved than with conventional semen in terms of

motility and acrosomal integrity, and it was considered that the use of sexed semen for artificial insemination on a commercial basis would be available in approximately 2 yr[21].

Beginnings of the commercialization of sexed semen

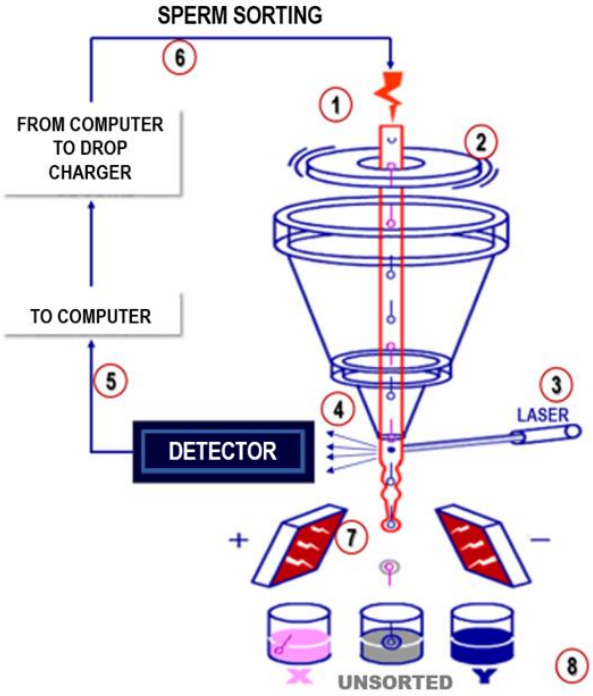
The Monsanto company, located in St. Louis, Mo, USA, developed a one-of-a-kind sperm selection system, which used 16 selection nozzles instead of just one, as in the case of the MoFlow SX™ cytometers. This equipment was intended to be commercialized, but apparently, due to problems with low conception percentages that were detected in its first tests, the company gave up[5]. In 2003, Genetic Resources International / Sexing Technologies in Navasota, TX, USA, purchased the intellectual property and sperm sexing equipment developed by Monsanto and the entire infrastructure of XY Inc[5]. The company has now changed its name to STgenetics®[22].

Sperm sexing with the conventional technique (Legacy or XY)

Legacy sperm sexing overview

The Legacy sperm sexing system used a MoFlow SX™ flow cytometer, which consisted of a closed-loop high-speed fluid flow that allowed spermatozoa to be aligned and read individually in microdroplets. The fluorescence produced by each stained sperm was processed by software that allowed the operator to select the sperm population with minimum and maximum luminosity according to the sex to be separated. The chosen spermatozoa were electrically charged, diverted from the original flow in a magnetic field, and finally collected[6] (Figure 4). They were then concentrated by centrifugation and frozen, leaving only half of the total alive[4].

Figure 4: Flow cytometry sperm classification system

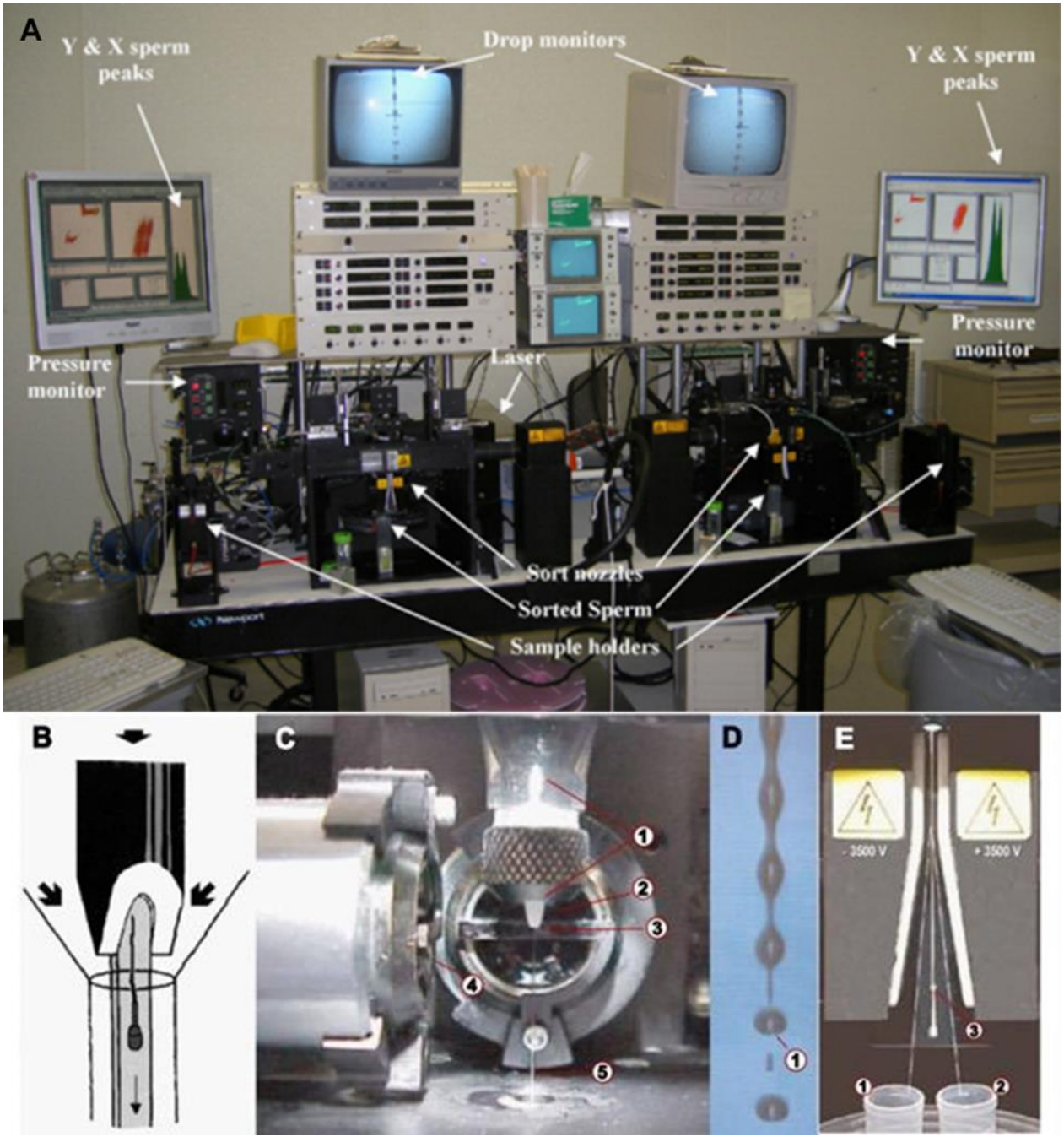


1) Spermatozoa are injected through the system after being stained with a DNA-binding fluorochrome, 2) A vibrating ring of piezoelectric crystal causes 90,000 droplets per second to form as the stream exits the system, 3) A UV laser illuminates the spermatozoa as they flow through the beam, 4) X spermatozoa fluoresce with 4 % more intensity than Y spermatozoa, 5) The signal detected with a photomultiplier tube is sent to a computer that processes the detected fluorescence and categorizes whether the sperm is X, Y, or unoriented, 6) Negative, positive or uncharged, it is applied to the droplets that emerge from the flow, 7) As the charged droplets pass between continuously charged plates, they are diverted, 8) The spermatozoa are collected in three containers: X, Y, unoriented or sperm-free[2].

The sperm quality and concentration of the ejaculates were perhaps the most important factors in obtaining a good separation of the two populations since a high correlation between motility, concentration, and separation of the populations was demonstrated with high-speed flow cytometers. Therefore, the separation of "X" and "Y" spermatozoa was usually carried out in ejaculates with more than 50 % progressive motility and 75 % normal spermatozoa[6].

With the high-speed cytometers, the MoFlow SX™ (Figure 5), spermatozoa passed through the cytometer at a speed of 80 km/h, approximately 20,000 total spermatozoa/second[3] and it took 9 min to sex a straw of 2 x 10^6 spermatozoa, approximately seven straws per hour[5].

Figure 5: MoFlow SX™ cytometer for XY sperm sexing



A. Sperm sorter and computer. **B.** Beveled tip. **C.** Optics and hydrodynamics, 1) Nozzle with X Y orienting tip, 2) Side fluorescence objective: cell orientation, 3) Locking bar, 4) Front fluorescence objective: cellular DNA quantifier, 5) Flow output. **D.** Formation of microdroplets, 1) Last drop united. **E.** Deflection plates, 1) Spermatozoa with Y chromosome, 2) Spermatozoa with X chromosome, 3) Residual stream[1,2,5].

In the sexing process, of 100 % of the spermatozoa, approximately 20 % ended up collected in the "X" fraction and 20 % in the "Y" fraction; the remaining 60 % consisted of

spermatozoa that could not be detected by the cytometer, dead spermatozoa, and droplets without spermatozoa[3,6].

The sperm characteristics and survival of sexed sperm were poor compared to unsexed spermatozoa (the increase in dead spermatozoa reached 18.6 %); this was attributed to the sexing process[7] that began with many hours of maintenance from semen collection until the semen was sexed[1].

Factors affecting Legacy sexed semen outcomes in AI

Viability

The damage due to the sexing process with the Legacy technique had a direct impact on the pregnancy percentages. The lower fertility of sexed semen was mainly due to exposure to mechanical forces during the sexing process and, to a lesser extent, due to staining and exposure to lasers[3]. After the sexing process, the spermatozoa were partially capacitated, reducing the lifespan and consequently reducing fertility[23]. For all of the above, the straws had a minimum of 35 % spermatozoa with progressive motility and a minimum of 85 % sex certainty to reach the approval standards[6].

Concentration

In addition to the damage caused by the sexing process, another cause of the decrease in fertility of Legacy sexed semen was due to the low number of spermatozoa contained in the dose[24,6]. A dose of 2.1 million spermatozoa is a low dose for AI[25]; however, it was observed that for most bulls, the concentration of spermatozoa to obtain a percentage of 80 % of normal conception is approximately 2 million spermatozoa per dose[23]. In cows inseminated (12 h after natural heat) with doses of 2 million spermatozoa of sexed and conventional semen, the pregnancy rates were less than 30 % and did not differ between sexed and conventional semen, indicating that the total number of inseminated spermatozoa seems to have a more significant impact on conception than the use of sexed or conventional semen[23]. On the other hand, no difference (P=0.64) was found when inseminating (12 h after natural heat) Holstein cows with 2.1 and 3.5 million sexed spermatozoa, obtaining percentages of 23 % and 25 %, respectively. Nonetheless, under ideal insemination conditions and with doses of 3 million sexed spermatozoa in lactating beef cows, the pregnancy rates were similar to those of

heifers[27]. Another study found that pregnancy rates were virtually identical with 1, 1.5, and 3 million spermatozoa per dose (54 %, 56 %, and 51 %, respectively)[28]. Based on the work carried out, it can be seen that the low sperm concentration of the sexed semen doses was sufficient to obtain adequate pregnancy rates.

Differences between bulls

Differences have been reported between bulls regarding sperm tolerance to the sexing process[3,27]. In addition, a difference of up to 18 % of gestation was found according to the bull used[23,27], which indicates that the fertility of the sexed semen seems to differ between bulls. This implies that field tests cannot accurately predict the fertility of sexed semen as with conventional semen[24]. Therefore, care should be taken when interpreting the results obtained with sexed semen since there is a strong influence of the bull used with the gestation percentages[23]. Thus, monitoring the results of sexed semen and keeping bulls (Holstein) with the highest fertility for sexing is the best way to increase its fertility[24].

Other applications of Legacy sexed semen

Reverse-sorted semen

Reverse-sorted semen (RSS), also known as reverse semen, is a technique that allows spermatozoa with “X” and “Y” chromosomes to be obtained from conventionally frozen semen. An advantage of this technology is that it is possible to obtain sexed semen from bulls of high genetic merit that have died[29]. RSS has been associated with other biotechnologies, such as AI[30] and IVP[31]. In tests carried out with AI, pregnancy rates were low, from 4 to 10 %[30], with 14.2 % of offspring born[32]. Therefore, this technology is primarily used with IVP[29].

In vivo production of embryos with Legacy sexed semen

The use of Legacy sexed semen for multiple ovulation of donors has had very variable results, generally poor or very low compared to conventional semen, where between 1.4[33] and 2.3 transferable embryos[34] per collection are reported. Some promising results using heifers

report that there is no significant difference between sexed and conventional semen[35]. For all of the above, the use of Legacy sexed semen in multiple ovulation programs has been limited.

In vitro production of embryos with Legacy sexed semen

Historically, it has always been considered that the most economical method of using sexed semen in breeding programs in cattle is through IVP since, with this reproductive biotechnology, a very small number of spermatozoa is required. Combined with ultrasound-guided follicular aspiration, large quantities of embryos generated from both "X" and "Y" spermatozoa are obtained. Many studies have been carried out using Legacy sexed semen to produce embryos *in vitro*, and many aspects related to the *in vitro* production of bovine embryos with this semen have been described; among these are the low rates of fertilization, divisions, blastocyst production, gestation, and variation between bulls[36]. When evaluating fresh sexed semen compared to fresh conventional semen and frozen sexed semen compared to frozen conventional semen for IVP, it was found that, in the case of fresh semen, the results seemed to be similar in terms of motility parameters; nevertheless, in the percentage of divisions, they were lower (P<0.001) for fresh sexed semen compared to fresh conventional semen (66 *vs* 76 %, respectively). When using frozen sexed semen and frozen conventional semen, they had no differences in the percentage of divisions. Another aspect observed with the sexed semen was that there was a delay of half to one day in development to the blastocyst stage. These authors found that blastocyst production with sexed semen was ~30 % lower compared to conventional semen[37]. In another study, it was reported that the percentage of blastocyst production obtained from oocytes collected by ultrasound-guided follicular aspiration was lower (P<0.05) when sexed semen was used compared to conventional semen[38]. In general, blastocyst production with conventional semen is around 30 to 40 % and 10 to 20 % with sexed semen[36].

On the other hand, in the case of IVP with RSS, no significant difference (P>0.1) was found between the percentage of blastocysts obtained using sexed semen and RSS[31,39]. Commercially, in *Bos taurus* and *Bos indicus* breeds, the use of RSS for IVP had an average percentage of 30 % blastocyst production[29]. A relevant aspect is that it has been found that offspring produced from IVP with RSS have significantly higher birth weights (P=0.028), higher postnatal growth (P=0.001), higher mortality percentages (in the first 6 mo of age; P=0.008), and reduction in milk (P=0.001), fat (P=0.007), and protein (P=0.031) production, compared to offspring born from AI[40].

SexedULTRA™ sexed semen

Technique overview

The causes of lower fertility of sexed semen have been attributed to the various biochemical changes to which spermatozoa are subjected during the sexing process. There are about 20 different subprocesses involved in sperm sexing; among the most critical and important are the maintenance time before staining, exposure to the laser to generate fluorescence, and achieving separation between spermatozoa (with "X" and "Y" chromosomes), and finally, exposure to an electric field for the separation of relatively pure populations in a container[1,3,13]. According to the above, the challenge was to find new ways to control these events using new hardware, software, and new processing techniques during, before, and after the sperm separation stages[13].

The Legacy or XY technology described in previous publications[41,21] has been modified and has now changed to an entirely new sexing system called ultrasexing or after its brand, SexedULTRA™ (Navasota, TX, USA). Ultrasexing technology has been designed to be less aggressive for the sperm during the most critical points of the process, particularly improving changes in pH (buffer system) and oxidative stress[42].

Modifications to the technique

Although there is currently very little data about this new technology (due to intellectual property issues), it has been reported that, in this new technique, the sperm physiology was altered to facilitate the entry of the Hoechst 33342 fluorochrome and to retain it within the cell, which allows for greater fluorescence and thus better discrimination between the "X" and "Y" populations. On the other hand, the cryopreservation process is another very stressful step for the sperm cell, which is why the SexedULTRA™ technology was designed to simplify and optimize the media and control these stressors for the sperm. The protocol was modified with a treatment before the staining process and the use of a new staining medium that maintains the pH stable for a more extended period of time. The freezing medium was also modified, considering the dose of sexed semen[42].

The success of the ultrasexing process was mainly influenced by two factors: modifications in the media and equipment for sexing. MoFlow SX™ cytometers (Cytomation Inc, Fort Collins, CO, USA) were very expensive, bulky, had low performance, and required highly

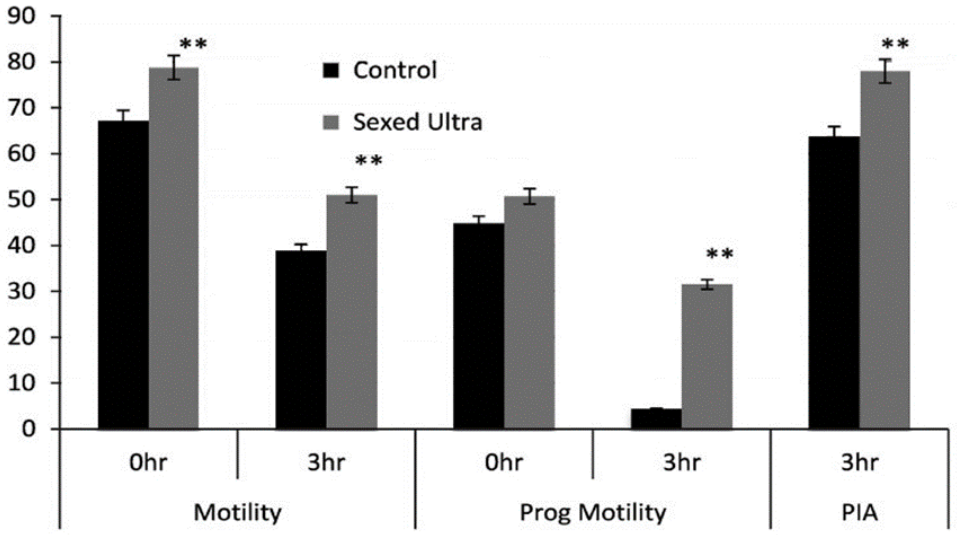
trained personnel to operate them (Figure 4). The modern Genesis cytometers developed by Cytonome ST™ (Boston, MA, USA) have advanced and automated electronic features with multiple heads in one machine for parallel separation. The Genesis III™ cytometer (Figure 6) uses a solid-state laser, two orthogonal detectors (0° and 90° to the laser), an orientation nozzle, and a subpopulation separation of ~8000 spermatozoa/second with ~90 % purity, reaching a maximum separation of 500 million spermatozoa/hour[42].

Figure 6: Genesis III™ cytometer for sperm sexing[42]

Laboratory tests of SexedULTRA™ technology

With these changes, in laboratory tests, sperm motility and the integrity of the acrosome were increased compared to XY Legacy technology (conventional sexing), considering the same sperm concentrations (Figure 7)[8].

Figure 7: Comparison of SexedULTRA™ and XY Legacy (Control) sexing methods on *in vitro* semen quality assessment



Sperm motility and progressive motility were assessed using computer-aided semen evaluation, and the percentage of intact acrosomes was determined by differential interference contrast microscopy (n=12 bulls). Bars with two asterisks differ significantly (P<0.001)[8].

In addition, in *in vitro* fertilization tests, ultrasexed semen had a higher number of freezable embryos compared to Legacy sexed semen, with 13.2 % and 9 %, respectively[8].

On the other hand, in 2018[43], the considered sperm quality, plasma membrane integrity, percentage of intact acrosomes, and DNA fragmentation index (DFI) of SexedULTRA™ semen compared to conventional semen were evaluated. In SexedULTRA™ semen at 3 h post-thawed, the percentage of intact acrosomes was significantly higher than in conventional semen (Table 1). In terms of DFI, SexedULTRA™ semen had a significantly lower DFI at all evaluation points compared to conventional semen. The authors conclude that the SexedULTRA™ technology maintains semen quality and, in many cases, has greater longevity *in vitro* compared to conventional semen.

Table 1: Comparison of characteristics of SexedULTRA™ semen and frozen-thawed conventional semen

Value	Time	Least square means		Tukey		
		Conventional	SexedULTRA™	Mean difference	SE	*P*
Visual motility	0 h	61.0	63.8	2.8	2.4	0.250
	3 h	50.1	51.0	0.9	2.4	0.709
Total motility	0 h	60.6	63.8	3.2	2.2	0.157
	3 h	49.6	50.0	0.4	2.2	0.862
Progressive motility	0 h	49.8	53.0	3.3	2.5	0.198
	3 h	28.5	29.4	1.0	2.5	0.698
Intact plasma membrane	0 h	55.6	56.7	1.1	1.6	0.502
	3 h	40.7	43.4	2.6	1.6	0.121
Intact acrosomes (%)	0 h	72.6	76.0	3.3	2.1	0.126
	3 h	55.6	62.3	6.7	2.1	**0.004**

SE= standard error. The differences were considered significant with a value of $P<0.05$ (underlined and bold values), n=10[43].

Evaluation and standardization of SexedULTRA™ technology in the field

In the first field-level evaluation using SexedULTRA™ technology for AI (Table 2)[44,45], there was a 7.4 % increase in heifer conception rates compared to XY Legacy technology. The second test was carried out in collaboration with the commercial company Select Sires (OH, USA); in this test, eight Holstein bulls were used, from which semen was collected and processed using both SexedULTRA™ technology and XY Legacy technology, with which 6,930 heifers were inseminated. The results showed that SexedULTRA™ semen increased conception rate by 4.5 % ($P<0.001$) compared to XY Legacy semen (46.1 and 41.6 %, respectively[44,45].

Table 2: Results of field fertility tests of heifers inseminated with SexedULTRA™ semen[44,45]

	Number of inseminations	Conception rates
Sexing Technologies test		
XY Legacy	1,166	47.3 [a]
SexedULTRA™	957	54.7 [b]
Mean difference		7.4
Select Sires test		
XY Legacy	3,384	41.6 [a]
SexedULTRA™	3,546	46.1 [b]
Mean difference		4.5

[ab] Within the test, rows with different superscripts differ ($P<0.01$).

With these tests, it was observed that the deleterious effects of the XY Legacy technology were partially lessened with the new SexedULTRA™ technology, so the next logical step was to increase the sperm concentration per dose; however, in the past, the increase in sperm concentration did not improve fertility. The following test was carried out in collaboration with the company German Genetics International, for which they used five Holstein bulls, from which semen was collected, and each ejaculate was divided into four parts to be processed with XY Legacy technology of 2.1 million spermatozoa, SexedULTRA™ of 2.1, 3 and 4 million spermatozoa per dose; in addition, semen from these same bulls of contemporary ejaculate frozen conventionally was used, with a concentration of 15 million spermatozoa per dose. The rates of non-return to estrus at 65 d were calculated from 7,855 inseminations with sexed semen and 62,398 inseminations with conventional semen. Overall, XY Legacy semen of 2.1 million spermatozoa per dose resulted in lower rates of non-return to estrus compared to all SexedULTRA™ and conventional semen treatments. SexedULTRA™ treatments of 2.1 and 3 million spermatozoa per dose were similar to but lower than conventional semen; nevertheless, the SexedULTRA™ treatment of 4 million spermatozoa per dose had non-return rates to estrus similar to conventional semen of 15 million spermatozoa per dose (Table 3)[45]. With the data obtained, the effect of the dose-response using sexed semen was demonstrated for the first time and the SexedULTRA-4M™ technology ($4x10^6$ spermatozoa/straw) emerged.

Table 3: Effect of increasing sperm dose with SexedULTRA™ semen on the rates of non-return to estrus at 56 days[45]

Treatment	Number of inseminations	Rate of non-return to estrus at 56 days (%)
Legacy 2.1 millions	1,953	55.9 [a]
SexedULTRA™ 2.1 millions	1,999	59.9 [b]
SexedULTRA™ 3.0 millions	2,013	60.0 [b]
SexedULTRA™ 4.0 millions	1,890	66.7 [c]
Conventional 15.0 millions|	62,298	66.5 [c]

[abc] Different literals in the same column differ ($P<0.001$).

In the case of multiple ovulation, the use of SexedULTRA™ semen was evaluated in lactating Holstein embryo donors. In this study, they used three doses of FSH for multiple ovulation and inseminated with SexedULTRA™ semen. With the highest doses of FSH, four point five embryos were obtained, with no difference between the qualities (Table 4)[46].

Table 4: Percentages of all recovered structures, transferable and non-transferable embryos of lactating dairy cows superovulated with three protocols[46]

	F700	F1000	F700 P300
Total structures	4.7 ± 3.0 [a]	8.1 ± 3.8 [b]	8.5 ± 6.4 [b]
Transferable embryos (%)	1.9 ± 1.7 [a] (41.2)	4.4 ± 2.6 [b] (54.7)	4.5 ± 3.3 [b] (52.9)
Non-transferable embryos (%)	2.8 ± 3.2 (58.8)	3.6 ± 2.9 (45.3)	4.0 ± 5.4 (47.1)
Grade 1* (%)	19/33 (57.6)	96/150 (64.0)	66/117 (56.4)
Grade 2* (%)	13/33 (39.4)	46/150 (30.7)	43/117 (36.8)
Grade 3* (%)	1/33 (3.0)	8/150 (5.3)	8/117 (6.8)
Grade mean*	1.45 ± 0.5	1.41 ± 0.6	1.50 ± 0.6

F700= Folltropin 700 IU, F1000= Folltropin 1000 IU, F700P300= Folltropin 700 IU+Pluset 300 IU.

* Quality grades (IETS 1-3) of transferable bovine embryos recovered from lactating dairy cows superovulated with three protocols.

[ab] Different literals in the same column differ ($P<0.05$).

SexedULTRA-4M™ technology and its application in the field compared to conventional semen

In the case of the SexedULTRA-4M™ technology ($4x10^6$ spermatozoa/straw)[47], the use of SexedULTRA-4M™ semen in fixed-time artificial insemination was evaluated using cows and heifers. Its results show that there was no significant difference (P=0.61) in terms of the pregnancy rates between conventional semen (61.9 %) and SexedULTRA-4M™ semen (63.8 %) when females were in heat before fixed-time artificial insemination.

Another experiment[48] compared the use of conventional semen and SexedULTRA-4M™ semen in AI using three different bulls (Angus) and beef cows. In this study, it was found that fertility is influenced by the bull since only one out of three bulls had no differences in terms of the percentage of pregnancies when comparing conventional semen and SexedULTRA-4M™ semen, which shows that there is a difference between bulls, as is the case with Legacy sexed semen.

In the case of dairy cattle, through AI of grazing Holstein cows, they evaluated conventional semen and SexedUltra-4M™ semen from 10 bulls and concluded that SexedULTRA-4M™ semen has a lower conception rate compared to conventional semen; however, this depends on the bull, the fertility of the cow, and the herd[49].

In vitro production of embryos with SexedULTRA-4M™ semen

To date, there is very little information about the use of SexedULTRA-4M™ sexed semen in IVP. In one study, this semen was evaluated in IVP, and it was found that the SexedULTRA-4M™ semen generated a bigger number of freezable embryos compared to the Legacy sexed semen (13.2 and 9.2 %, respectively; P>0.05)[8]. In two other studies, IVP was evaluated using conventional semen and SexedULTRA-4M™ semen from the same bull, using oocytes from adult animals[50] and using oocytes from 6-mo-old prepubertal females[51] and no significant differences (P>0.05) were found between blastocysts produced with conventional semen and those produced with SexedULTRA-4M™ semen in both studies; nonetheless however, in the case of adult animals, there was a higher number of blastocysts with SexedULTRA-4M™ semen (43.6 and 37.8 %, respectively; P>0.05). In another study, IVP was evaluated using conventional semen and SexedULTRA-4M™ semen from four bulls of the Angus breed; in this study, it was found that two bulls were significantly superior for blastocyst production with SexedULTRA-4M semen compared to conventional semen [24.2 and 20.4 %; 14.2 and 10.4 %, respectively (P<0.05)]. In this study,

it was also concluded that the results of IVP with SexedULTRA-4M semen were similar to those obtained with conventional semen[52].

Other sperm sexing techniques

Lumi sort™

Lumisort™ (Microbix Biosystems Inc., ON, Canada) is a next-generation sperm sexing technology for the livestock industry. The Lumisort method combines an optical system for detecting the sex of spermatozoa with a fast and effective laser that destroys spermatozoa that are not of the desired sex. The spermatozoa do not suffer damage due to hydrostatic pressure; it does not use droplets, so it does not require vibrations to align the sperm, it does not require electrical charges, and the selected spermatozoa are gently separated. It was first started in 2005 and later introduced in the dairy industry in 2013[10]; nevertheless, there are no studies published in scientific journals evaluating this technology.

SexCell™ (Gender ablation)

This technology is very recent, like Lumisort technology; sexing is performed by flow cytometry, and the spermatozoa of the unwanted sex are destroyed[9]; nonetheless, the sexing process is not described in detail. This technology has been developed by the company Genus-IntelliGen Technology[53] and is marketed by the company ABS (WI, USA)[54]. There is only one publication in which they evaluated the conception rate in beef cows and heifers inseminated with conventional semen and semen sexed by gender ablation. Conventional semen had statistically superior results compared to sexed semen in cows; however, in heifers, there was no significant difference between conventional semen and sexed semen[9].

Techniques in development

Sperm sexing using gold nanoparticles

This technique uses functionalized gold nanoparticles (AuNPs) to detect specific sequences of the "Y" chromosome in morphologically and functionally intact spermatozoa. The first step consists of the entry of AuNPs through the sperm membrane. Subsequently, there is a non-invasive coupling of a specific DNA sequence with the double strand of sperm DNA. Once mated, the specific signal pattern of the "Y" chromosome is recognized to identify the sperm population[10].

Sperm sexing using magnetic nanoparticles

This technique has only been reported in donkeys; however, it could later be used in other species. Magnetic nanoparticles (MNPs) have a diameter of 50 nm, are composed of an iron magnetite core covered with silica, and are negatively charged. MNPs are mixed with semen and exposed to a magnet for 20 min. The interaction between the negative charge of MNPs and the electrical potential of spermatozoa is different for spermatozoa with an "X" chromosome (20 mV) and those with a "Y" chromosome (16 mV). In this way, spermatozoa with a "Y" chromosome will be kept closer to the MNPs and will form an accumulation of spermatozoa, and in this way, the populations can be separated[(11)].

Prospects

The advancement of the different technologies involved in sperm sexing is remarkable. This shows that sperm sexing is in continuous evolution and with increasingly better results, both for artificial insemination and for other biotechnologies, such as the *in vivo* and *in vitro* production of embryos in cattle, which could be applied to other species, such as sheep, goats, horses, and pigs. For this reason, it is envisaged that in the not-too-distant future, this technology will displace conventional semen, or even that expensive and sophisticated equipment will not be required to carry it out.

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Review

Winemaking by-products and grape polyphenols extracts as phytogenic feed additives in the pork production. Review

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Abstract:

Winemaking by-products (WBP) such as grape pomace (GP), seed, and skin represent a rich source of phenolic compounds (PCs), fiber, fatty acids, and essential nutrients. Due to their profile and content of PCs, WBP can exert multiple bioactive properties on animal health, nutrition, and production. Specifically, beneficial effects have been observed in pigs. Therefore, these by-products and wine polyphenols extracts have been considered as valuable ingredients and as promising alternative to replace conventional resources of monogastric diets and thus minimize feeding cost. Nevertheless, these by-products are discarded and improperly disposed. Indeed, only 3% of the recovered by-products are used in animal nutrition without prior treatment. Emphasis has been placed on generating added value to obtain more significant economic and technological benefits and greater efficiency in animal production. This review discusses the most relevant and recent studies on the inclusion of WBP and their PCs during different stages of the pork production system (gestation-lactation, weaning, growth, and finishing) and their effects

on the final quality of pork products. Additionally, strategies and treatments applied for the use of pomace in pig diets are described.

Keywords: Bioactivity, Phenolic compounds, Grape pomace, Monogastric, Phytochemicals.

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Introduction

In view of the continuous intensification of pig production systems to meet world demand for animal products, the cultivation of grains (corn, soybeans, sorghum) has been frequently used as energy and protein sources for monogastric feed[(1)]. In this context, it is estimated that 60 % of the biomass generated for feed production is used by the livestock sector and this item represents up to 70 % of the total costs of a swine farm[(2)]. This dependence on agricultural crops has promoted food-feed competition between human consumption, the livestock sector and biodiesel production[(1-3)]. Therefore, to face this problem, various economic, viable and promising alternatives have been sought, through which the use of conventional dietary ingredients can be minimized without affecting their nutritional contribution, and at the same time improve productivity, animal health and the final quality of animal foods[(1,3)]. In this sense, an attractive option could be the inclusion of agro-industrial by-products and their extracts as phytogenic feed additive in monogastric nutrition[(3–5)]. These wastes are generated in large amounts each year (1.3 billion tons) and represent a rich source of bioactive compounds (BCs). However, their use has been inefficient due to their low economic value that lacks alternative uses, and therefore most of these residues tend to be wasted and disposed of inadequately, generating a negative environmental impact[(6)]. A significant amount of these by-products come from the winemaking process for which 75 % of the grapes harvested are used[(7,8)] while the remaining (25 %) represents the by-products (residues of skin, pulp, stems, and seeds) obtained during the pressing process (called pomace) and are discarded by the wine industry (13.1 million tons)[(7–11)]. Inclusive, it is estimated that approximately 70 % of the phenolic content is retained in the pomace after processing. In this context, pomace and seeds are considered a rich source of PCs (tannins, anthocyanins, phenolic acids, and resveratrol); and possesses multiple bioactive properties that contribute to health and animal production[(12,13)]. Although approximately 30 to 40 % of WBP have been valued worldwide (5.24 million tons) for use in the agricultural sector[(13)], it is estimated that only 3 % of the pomace produced is reused for animal feeds (0.39

million tons) without prior treatment[8,14]. Therefore, in recent years, there has been an emphasis on generating added value to WBP to obtain more significant economic and technological benefits and greater efficiency in animal production. Likewise, the use of agro-industrial by-products could represent a non-conventional feeding alternative for monogastric animals and as a circular economy strategy that promotes the sustainability of pork production systems[15]. Thereby, WBP represent an excellent opportunity to recover multiple phytochemical substances as PCs[8,14,16] which present great potential as phytogenic additives in pork production considering the importance of this young class of feed additives for pig farmers[5,17,18]. Interest in these natural additives has increased in recent decades in response to the ban on the use of antibiotics and beta-adrenergic compounds[19].

Scientific reports show some beneficial effects in pigs on a diet including different dosages of grape by-products and an inclusion limit of 9 % has been reported[3,20]. However, there is variability and inconsistency in the results obtained. The studies about WBP and grape polyphenols extracts in production cycle of swine are limited, and it is necessary to highlight what advances have been achieved in each stage of the pork production cycle, to determine future areas of opportunity in animal nutrition research, due to the importance of each of these phases for the success of the pork production. Therefore, the purpose of this review is to discuss the most relevant and recent studies on the inclusion of WBP and their PCs during different stages of the pork production system (gestation-lactation, weaning, growth, and finishing) and their effects on the final quality of pork products. Emphasis has been placed on the strategies and treatments applied to GP for inclusion in monogastric diet. Additionally, it is essential to evaluate the beneficial effects of including pomace phytochemicals or the raw matrix on productive efficiency.

Importance of winemaking by-products as alternative resources and feed additive in animal feeding

Grape is one of the most important fruit crops worldwide (7.3 million hectares), and about 36 % of total production (72.9 million tons) is concentrated in China, United States, and Italy[7,8,21]. It is estimated that 52 % of this grape volume is pressed and destined for wine production (34.1 million tons)[22]. During the final fermentation process, valuable by-products are obtained with multiple technological and health benefits, representing approximately 25 % of the total volume of grapes required by the wine industry[10]. Vinification residues are classified into two categories: solids (stems, pomace, and seeds) and liquids; the former is generated during the harvesting and pressing of the grapes, whereas the latter is obtained during winemaking[23]. The solid residues consist of 45 % pomace, 7.5 % stems, 6 % seeds, and other residues[24]. The pomace represents the leading organic waste or by-product generated during the process of separating the solid waste

(skins, seeds, and stems) from the liquid product or juice and constitutes 20 to 30 % of the processed grape[10]. These residues are a rich source of various nutrients (protein, fatty acids, fiber) and PCs. However, these by-products are discarded, and their proper disposal represents one of the most critical challenges facing the wine industry.

Physicochemical composition of grape pomace

The composition of GP is determined by intrinsic factors such as grape maturity, variety, and sanitary conditions. Additionally, factors such as viticultural practices, edaphoclimatic conditions, harvest practices, and winemaking processes are determinants of composition. Fresh GP represents a rich source of dietary fiber (DF), that is composed of non-starch polysaccharides, tannins, and lignin[25,26]. The fiber proportion in the pomace ranges from 43 to 7 5%. There are significant differences in soluble fractions, the insoluble fraction represents 61.3 to 73.5 %, while the soluble fraction compromises 10 and 3.7 % respectively. It is concentrated in the seeds (Table 1) and is higher in red varieties (51 %) than in white varieties (28 %)[13]. Due to its high fiber content, pomace has been used as a feed additive in the diets of sows and the finishing stage; however, low inclusion rates have been reported in monogastrics (3 %), although other authors have obtained satisfactory results with rates of 9 %[3,20,27]. Satisfactory results have been obtained regarding the production of volatile fatty acids (VFA) and beneficial bacteria[27]. This food matrix also contains phenolic fractions that give it antioxidant properties and greater complexity[21]. It is estimated that the content of PCs oscillates around 0.9 %, and the proportion of condensed tannins is 17.43 % and 7.29 % for red and white GP, respectively. However, the fiber and condensed tannin contents in pomaces could limit the bioavailability of dietary nutrients, causing a reduction in diet quality and therefore in the productive performance of animals[21,28,29]. Bioavailability is determined by the inclusion of this by-product in the diet and the previous treatment[9]. The moisture content of this by-product can range from 55 to 75 %, depending on processing conditions and is considered an indicator of the microbial and enzymatic degradation of fresh GP[30]; this is one of the limitations of its application. Although low counts of aerobic mesophiles (3-6 Log CFU/g) and molds and yeasts (3 to 6 log CFU/g) have been reported, few studies have been conducted on the microbiological quality of this by-product[31,32]. The protein content varies from 6 to 15 % of dry matter and is slightly higher in the skin than in the seeds[33]. GP is rich in aspartic and glutamic acid, whereas it is deficient in sulfur amino acids and tryptophan. Additionally, it has been reported that unlike the seeds, the skin is a rich source of lysine and alanine. The lipid content of pomace derives from the seeds and ranges from 14 to 17 %. The lipid fraction is rich in unsaturated fatty acids and poor in saturated fatty acids. The fatty acids that predominate in grape seeds are linoleic (70 %), oleic (15 %), and palmitic (7 %). Likewise, it is necessary to highlight that its nutritional value is close to that of other foods such as corn and it has been reported that its crude protein content can be like that of conventional flours, which emphasizes its potential as

a functional ingredient in animal feed[3].

Table 1: Chemical composition (g/100 g D.M) and phenolics compounds content of grape pomace, skin, and seeds (g /kg as D.M.)

Component		**Grape pomace**	**Skin**	**Seeds**
Dry matter		90-93	81-93	91-93
Protein		11.2-13.8	11.0-13.8	9.3-14.6
Fat		5.6-11.7	3.2-6.3	9.5-11.1
Ash		2.4-5.8	6.2-7.5	2.9
Fiber		32.5-56.3	30.6	41.4
Acid detergent fiber		48-70.4	19.3-49	45.4-57.0
Neutral detergent fiber		54.2-70.8	24.3-70.4	50.3-67.0
Acid detergent lignin		30.7-47.5	28.3-43.7	21.4-43.7
Condensed tannins	Free	1.6-3.8	------	------
	Fiber bound	1.9-3.4	------	------
	Protein bound	5.6-13.1	------	------
	Total	9.1-20.3	------	------
	Phenolic compounds (g/kg DM)			
Total phenol content		19-40.5	20.2-52.3	36.6-88.7
Total tannins		39.1-105.8	44.9-73.0	62.3-167.8
Phenolic acids		0.03-8.31	0.17-8.23	0.10-0.11
Catechins		0.03	0-0.3	2.14-2.42
Epicatechin		0-0.2	0-0.13	0.88-1.60
Epigallocatechin		0-0.05	ND	0.05
Epicagallocatechin gallate		0-0.007	ND	0.06-0.07
Epicatechin gallate		0.003	0.04	0.25-0.31
Procyanidin B1		0.11-0.60	0.18-0.6	0.14-0.17
Procyanidin B2		0.01-0.84	0.01-0.84	0.04-0.18
Anthocyanins		11.47-29.82	11.47-29.82	ND
Total flavanols		0.03-0.63	ND	0.02-0.05

Phenolic composition of grape pomace

WBP such as GP are considered a rich source of PCs, and these have been shown to possess multiple bioactive properties on animal health and production**:** antioxidant, antimicrobial, immunomodulator, anti-inflammatory[34]. These phytochemicals are secondary metabolites of plants that act as a defense mechanism against pests, pathogens, herbivores, environmental factors, and stressful situations[35,36]. Their chemical structure

determines their biological properties and, therefore, their beneficial effects on animal health. A PC has one or two aromatic rings with one or more hydroxyl group substituents; this conformation determines its ability to capture free radicals[9]. These compounds are classified into four categories based on their chemical structure and molecular weight: hydroxycinnamic acids (HA), stilbenes, lignans, and flavonoids[33]. The latter is the broadest and most diverse group of polyphenols and has been studied most frequently in animal nutrition[37,38].

The main compounds found in GP are flavanols (catechin, epicatechin, and epigallocatechin), anthocyanins (cyanidin, pelargonidin, and delphinidin), and condensed tannins (Table 1). The former predominates in white grapes, whereas the latter is only present in red grapes[39]. Grape skins and seeds are rich in epigallocatechin and gallocatechin, whereas anthocyanins and stilbenes such as resveratrol are found in the skin. Hydroxycinnamic acids predominate in the form of tartaric esters (caftaric, and courtaric) both in the skin and in the pulp of the grape, whereas the seeds are rich in gallic acid and protocatechuic acid. However, the phenolic composition varies depending on climatic conditions, growth, and fermentation time[9].

The beneficial effects attributed to these PCs include antimicrobial, antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, cardioprotective, antidiabetic, anthelmintic, and intestinal microbiota modulation properties[36]. The antioxidant activity exerted by PCs, mainly resveratrol (RES), in production animals has been emphasized. In this context, it has been suggested that the antioxidant capacity of RES is more significant than that provided by vitamins C and E; i.e., it is more effective in capturing and preventing free radicals[40,41]. In turn, the PCs present in GP are readily accepted by consumers and producers, as pomace has been considered an alternative to traditional medicine for decades[41].

Potential use of winemaking by-products (grape pomace, seeds, skin) and its extracts as phytogenic feed additive in swine nutrition

In the last decade, agro-industrial by-products such as GP have received significant attention as alternative sources of unconventional animal feed[18]. This by-product has a unique polyphenol profile[42]. The content and diversity of PCs have stimulated in this food matrix as a supplement or additive to monogastric diets. Recently, these phytochemicals have been used as additives in feeds for pigs and poultry through various inclusion strategies (whole portions of the plant resource, by-products, extracts, isolated BCs, and complex blends of compounds). It is estimated that approximated 70 to 80 % of the industry that manufactures feed for monogastric uses phytochemicals as additives[36]. The use of heterogeneous blends of these secondary metabolites with different plant

origins (phytogenic) has been emphasized, whereas studies on the isolated forms of the compounds have been limited.

Phytogenic additives refer to compounds of plant origin that have been used in monogastric feed to improve animal productivity, health, and feed quality. These food additives have been classified into four categories according to their functionality in feed or/and animals: 1) Sensory (modifying the palatability and organoleptic properties of the feed); 2) Technological (acting as mycotoxin sequestrants and antioxidants); 3) Zootechnical (acting as immunomodulators, growth promoters of nonmicrobial origin, intestinal function modulators, digestive stimulants, or enhancers of quality in animal products, or productive and reproductive performance); and 4) Nutritional (acting as vitamins, minerals, or enzymes). Within these categories, they have been used as technological and zootechnical additives in pork production, particularly during the gestation-lactation and weaning stages, respectively[40,41] .

The dietary inclusion of phytochemicals and phytogenics in monogastric has shown that these compounds exert multiple beneficial effects on the animal due to their bioactive properties[43]. The benefits including, improvements in palatability, productive performance (feed intake, average daily gain, and feed conversion), carcass quality, blood profile, animal welfare, health (immunity, antioxidant, antimicrobial), intestinal function (gastrointestinal morphology and nutrient digestibility) and as growth promoter[36,40]. However, the variability among reports regarding the efficacy of some BCs subjected to specialized treatments and the partial understanding of their possible mechanisms of action have limited their use as feed additives. Therefore, future applications will be determined by the characteristics of the vegetal resource (primary and secondary compounds), complete knowledge of the mechanism of action, and safety of both the animals and the products generated[17].

Potential use of winemaking by-products in feeding pigs at different production stages

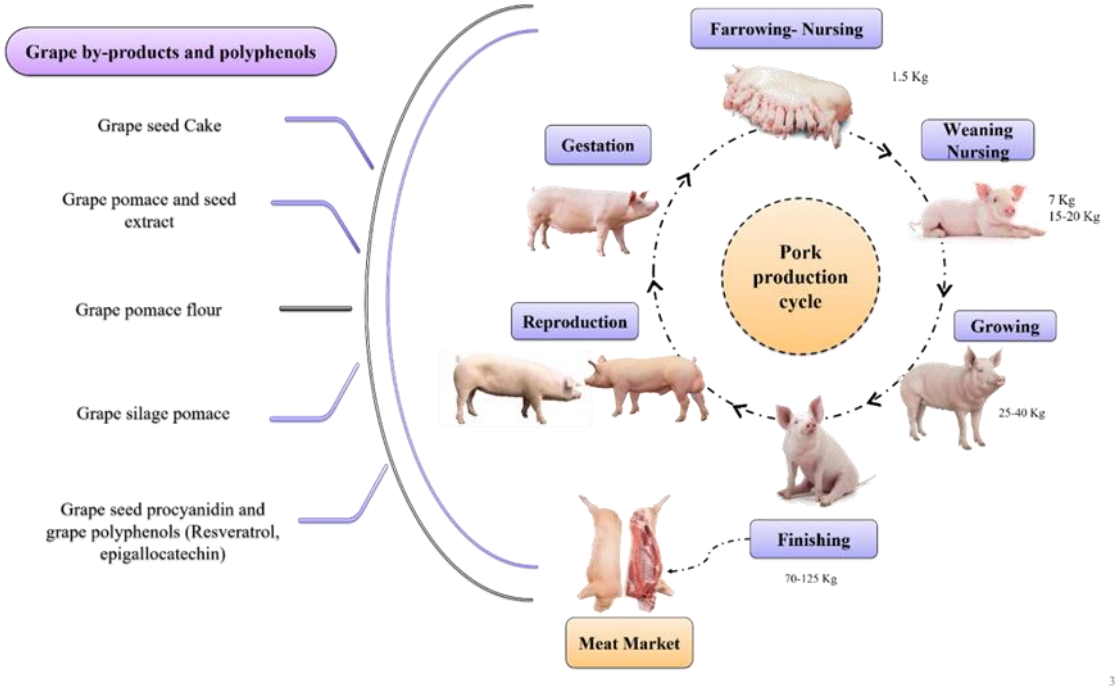
Impact on the function and reproductive organs of boars and sows

Reproduction represents a fundamental and integral component of sustainable swine production systems[42,44]. It is also a complex process that greatly influences nutritional and feeding factors. The intensive inclusion of polyphenols in the diets of breeding

animals has been evaluated[42]. Typically, these compounds are obtained from alternative food sources such as agro-industrial by-products and natural feeding systems and have been used as additives to enrich animal diets[45]. Previous research has evaluated the bioavailability of PCs in reproductive organs (ovary, uterus, placenta, fetus, and testis), the pituitary gland, and the hypothalamus and has shown that these PCs can cross diverse blood barriers and exert their physiological effects in the animal[46]. However, the type of polyphenols, the selectivity of the tissues, and the physiological status of the animal determine the bioavailability of PCs in reproductive organs and, therefore, their effect on different reproductive events.

In this context, including these compounds in the diet can generate positive or negative changes in events such as fetal programming, pregnancy, gametogenesis, sexual behavior, reproductive function, and hormonal secretion in boars and sows. These effects are attributed to similarities in the chemical structure of PCs and estrogens; as a result, they can activate estrogen receptors (ERs) to exert a hormone-like effect that can modify the hormonal balance and reproductive events mentioned previously[42]. Additionally, these compounds can control the expression of genes and the activity of sexual enzymes involved in regulating certain reproductive phases[47]. Recently, the effects of PCs such as resveratrol and epigallocatechin have been evaluated in various assisted reproductive techniques (ARTs) in males and females (Figure 1). Thus far, promising results have been obtained during the thawing and cryopreservation of boar semen. It has been reported that the inclusion of epigallocatechin 3-gallato (EGCG) (25, and 50 µM) increases the fertilization rate of ovules, while the inclusion of resveratrol (0, 0.5, 1, and 2 mM/mL) and GP (2 and 4 %) maintains the integrity of the acrosome, sperm viability and reduces lipid peroxidation of fresh and cooled semen[47,48]. These results have been attributed to the antioxidant activity of the PCs. In contrast, other studies conducted on *in vitro* fertilization have shown that compounds such as EGCG (0, 5, and 50 g/mL) can inhibit steroidogenesis and proliferation of granulosa cells in sows. Similarly, a decrease in progesterone synthesis and the percentage of fertilized oocytes was observed when a dose of 25 µg/mL of EGCG was included[49]. The effect of these phytochemicals on the reproductive cycle of farm animals is still controversial and inconsistent[42].

Figure 1: Grape by-products as phytogenic additives in pork production cycle



Gestation phase: Effects in pregnant sows, development of fetal piglets and farrowing

Oxidative stress represents one of the main critical points that swine production systems must face during the gestation (early, middle, and late) and lactation phases, in which systemic damage occurs in response to the high level of reactive oxygen species (ROS)[41,50]. Gestation reflects a state of high oxidative stress in which various tissues and organs that actively metabolize oxygen (O_2), such as the placenta, can present dynamic changes that modify the metabolic state of both the sow and the fetus. This state responds to an increase in O_2 consumption and energy levels required to support the metabolic load involved in placental, embryonic development and fetal growth, which implies tissue mobilization. However, an excess of ROS impairs the physiological function of the placenta, triggering uterine restrictions, that increase embryonic mortality rates and impair the development of the progeny[50].

Therefore, to mitigate the impact of oxidative stress, different antioxidant sources have been included in the diets of gestating sows. In this context, previous studies[50] have determined that the inclusion of 200 mg/kg and 300 mg/kg of grape seed polyphenols (GSP) in multiparous sows during late gestation (d 80) reduced stillbirth (0.63) and increased farrowing survival (89.32 %). These results reflected higher circulating levels of progesterone and estrogen in serum of pregnant sows (d 110), which are associated with pregnancy maintenance and maternal recognition[50]. Likewise, this supplementation showed a significant improvement in antioxidant status of pregnant sows by increasing the enzymatic activity of superoxide dismutase (SOD) and glutathione peroxidase

(GSHpx)[41,50]. Similarly, supplementing of resveratrol (300 mg/kg) y catechins (200 and 300 mg/kg) from early (d 20) and mid gestation (d 40) until farrowing improved the antioxidant status (SOD, GSHpx, and catalase [CAT]) of sows and, their progeny during lactation phase[51,52]. These results were attributed to the Keap 1-Nrf2 and Sirt 1 pathways regulating placental antioxidant genes[41]. This same behavior has also been reported with the inclusion of other plant extracts, which reaffirms the importance of adequate maternal supplementation from any third of gestation to ensure adequate performance at parturition and during lactation[53].

Lactation phase: Lactating sows and suckling piglets

Like late gestation and parturition, lactation also leads to an overproduction of ROS, triggering lipid peroxidation. In this case, oxidative stress is attributed to the heightened metabolic energy demand required for mammary gland development and milk production. Moreover, the severity of oxidative damage affects the reproductive performance of the sow and hampers the adequate development of the offspring during the early stages of life. Therefore, maternal supplementation with phenolic compounds from gestation through lactation has been evaluated as a strategy to enhance the antioxidant status in lactating sows, which in turn may lead to improved development, pre-weaning performance, and overall health of their progeny (suckling piglets)[53].

In this context, it has been determined that the inclusion of GSP (200 and 300 mg/kg), resveratrol (200 and 300 mg/kg), and standardized blend of polyphenols-Proviox (catechin, procyanidins, and anthocyanins) increases the content of immunoglobulins (IgG and IgM) in the colostrum of supplemented sows[50], thereby improving the antioxidant status of suckling piglets by enhancing the enzymatic activity of CAT, SOD, and GPx[41,54,55]. In addition, preweaning survival rate improved (96.9 %) with GSP supplementation (200 and 300 mg/kg) while the inclusion of resveratrol in the sows' diets increased litter weight (57.26 kg *vs* 48.98 kg) as did the weight of the piglets at weaning (5.84 kg *vs* 5.24 kg), in comparison to unsupplemented females[41,50]. Similarly, with the inclusion Proviox, a significant effect was observed in litter weight of sucking piglets and colostrum[54]. Therefore, the nutritional strategies that have been evaluated at this stage are focused on improving the antioxidant status of pregnant sows and thus attenuating the impacts of oxidative stress, which, can be associated with an improvement in immune status and this information suggests that is possible to obtain higher number of piglets for the growing and finishing phases.

Weaning and postweaning phases

The weaning stage represents a change in piglet diet (from liquid to solid) and separation from the dam. Moreover, the establishment of social hierarchies, triggers a series of physiological responses that compromise immune function, intestinal metabolism, and antioxidant capacity, favoring the appearance of oxidative stress and an increase in the rates of morbidity and mortality[56]. At the same time, feed intake decreases, causing growth retardation. The addition of WBP has been evaluated as a mitigating factor for this situation and as an alternative to replace the use of antibiotics as growth promoters in this phase[19]. In this context, it has been demonstrated that the addition of PCs such as tannic acid (500 mg/kg and 1000 mg/kg) in the diets of weaned piglets (3-wk-old for 14 d) has beneficial effects on the transport of nutrients at the intestinal level along with the intestinal microbiota and morphology[19,57]. This reflects the potential of GP as a source of various PCs. A previous study determined that the inclusion of 5 % dried GP improved the antioxidant status of the liver, kidney, and spleen in weaned piglets. Reduction in lipid peroxidation (MDA) and triglycerides was observed on d 36, whereas CAT, GSHpx, and SOD activities increased[58]. Similar effects on enzyme activity and total antioxidant capacity (T-AOC) have been reported by other authors as presented in Table 2.

A recent study[59] reported that the inclusion of grape seed flour (8 %) in starter diets attenuates the oxidative stress induced by aflatoxin B1 (AFB1) and decreases inflammation markers (Table 2). In this context, a protective effect against aflatoxicosis has been shown, and the antioxidant status of birds improved with resveratrol supplementation (0.5 and 1 %)[58]. Other studies have reported that agroindustrial by-products such as white GP (Malvasia) and red GP (Primitivo) have an excellent ability to adsorb ochratoxin (OTA), zearalenone (ZEN), fumonisin (FB1), and AFB1from aqueous solutions at pH 3 and 7[60]. These results show that these compounds have considerable potential to sequester mycotoxins in weaned piglet diets. The study showed that the inclusion of white pomace (Malvasia 2.8%) in the diet of weaned pigs reduces gastrointestinal absorption of the mycotoxins ZEN and OTA by 67 and 69 %, respectively. Although the mechanism of action as a mycotoxin sequestrant has not yet been elucidated, it is suggested that the cellulose content in the pomace has a considerable potential to absorb AFB1 through electrostatic attractions; polyphenols may also could form complexes with mycotoxins[61].

Other biological activities, such as immunomodulatory, anti-inflammatory, and intestinal modulation have been evaluated more frequently in weaned piglets, given their immature digestive organs and immune systems. Evaluation has been emphasized in the first 3 wk after weaning because the immune system can only generate an effective response at 5 or 6 wk of age[62,63]. In this context, it has been shown that supplementation with 50,100, and 150 mg/kg grape seed procyanidins[46]; 1 % grape seed and grape marc extract (GSGME) with a phenol content of 8.5 % (piglets 7 kg BW for 4 wk)[49,64]; and 5 % dried GP (20.41 mg/g dry matter, 5-wk-old for 28 d) decreases the incidence of diarrhea[58,62]

increases the height of intestinal villus: depth ratio of crypts in the duodenum (VCR)[62] and jejunum[65]; increases the proportion of *Olsenella umbonata*, *Lactobacillus delbrueckii,* and *Selenomonas bovis* in the cecum[65]; decreases the populations of *Streptococcus* and *Clostridium*; and decreases VFA levels in the fecal microbiota of weaned piglets (5-wk-old for 25 d)[58] .

These results were attributed to the potential of PCs to improve antioxidant status, reduce intestinal permeability, increase surface area, improve nutrient absorption, and modulate intestinal populations by increasing butyrate-producing species that enhance colon health. These beneficial effects could be related to microbial metabolites such as 4-hydroxyphenylvaleric, 3-hydroibenzoic, caffeic, syringic, and protocatechuic acid[66]. Similarly, other studies have verified that the PCs of WBP reduce the expression of proinflammatory genes in the intestine (cecum, ileum, and colon)[67]. Supplementation with GSF (grape seed flour) in weaned piglets in an induced colitis model (iron dextran sulfate) attenuated the effect of this inducer, modulated the colonic microbiota, and reduced the impact of intestinal dysbiosis[25,27]. Therefore, the PCs of the WBP represents a strategy to inhibit inflammation and modulate intestinal health during the postweaning stage. Among the productive parameters, a significant improvement of 4 to 7 % was observed in the weight gain:feed ratio (G:F)[67], and it has been suggested that this is due to an improvement in nutrient digestibility[25]. However, other authors did not obtain a significant improvement in this indicator and stated that there was no consistent effect on intestinal morphology[68]. Therefore, the improvement in G:F has been associated with reduced expression of proinflammatory genes and changes in microbial composition.

Growth and finishing stages

Previous studies have shown that grape seed pomace cakes (GSC 5% of the basal diet), as a rich source of proanthocyanidins, stilbenes, and flavanols (catechins, epicatechins, gallocatechins, epigallocatechins, and procyanidins), increased IgA levels (49.9 %) in animal plasma[41]. Additionally, pomace decreased the inflammatory response in the liver and spleen, together with the production of hepatic cytokines (IFN-γ, IL-1, IL-8, and IL-6), and the gene expression and concentration of proinflammatory markers (IL-1β and, IFN-γ) in the spleen[63]. Similarly, with the same dose, it was reported that cholesterol levels were reduced, which is attributed to the ability of resveratrol and epigallocatechin to bind to key regulators of liver lipid metabolism[69]. Although malondialdehyde (MDA) levels decreased in the liver (13 %), the results were inconsistent in terms of enzyme gene expression; CAT, SOD, and GPx activities, T-AOC; which indicates that the antioxidant status of the pigs did not improve during supplementation and can be attributed to the low CF content in the matrix. In contrast, other authors[63,68] reported a significant increase in the expression of enzymatic genes (CAT, GPx); a similar trend in the activity of CAT, SOD, GPx, and T-AOC (49 %); and decreased MDA levels in the spleen (20 %). Similarly, with the addition of 9 % GP silage during the growth stage, the decomposition of H_2O_2 increased[20].

It has been indicated that the inclusion of 1 % GSGME with a polyphenol content of 8.5 % and the addition of 3 % fermented GP is effective in improving the productive performance (body weight, average daily gain [ADG], intake feed, and feed conversion) in growing pigs[70]. However, it has also been shown that the inclusion of GSC (5 %) in finisher pigs does not influence the productive performance[64]. Similar results were obtained by other authors in all finishing stage[62]. Although GP has been reported to improve sensory abilities and metabolism in pigs[58], few specific studies for the finishing stage (> 70 kg) have evaluated any by-product of vinification, unlike the first two production phases[71]. In general, the investigations integrate the initiation phase with growth[27]. Even the results of GP supplementation on productive performance (ADG, feed conversion, and feed intake) are inconsistent[17] and it has been suggested that this nutritional matrix may suppress growth performance. The concentration and profile of PCs will determine this impact together with the binding to digestive enzymes and proteins[72].

A study on pigs reported that the inclusion of 300 and 600 mg/kg of resveratrol in finishing diets for 49 d induced a transition in the type of muscle fibers and a change in energy metabolism[73] that favored the expression of oxidative fibers (IIA) over glycolytic fibers (IIB). These results are consistent with those reported by other authors, who observed a lower proportion of IIB fibers when animals were supplemented with 400 ppm and 600 ppm of resveratrol for 42 d, which could indicate a growth-promoting effect[74]. However, the authors did not observe a significant effect on production efficiency. Likewise, it has been shown that with the inclusion of dry pomace (5 %) in finisher pigs, the profile of polyphenols in plasma (273 nm and 279 nm) is modified and these changes could be reflected in the muscle[75].

Effects of winemaking by-products and wine polyphenols extracts in pork meat quality, nutritional value and meat products

Grape pomace supplementation of animal feed or its direct inclusion in meat or meat products exerts an antioxidant and antimicrobial effect that determines the final quality of the product. Likewise, it has been reported that the oxidative stability of meat products is determined by the composition of unsaturated fatty acids[76,77]. Although a higher PUFA content in meat has been associated with greater susceptibility to lipid oxidation, it has also been shown that the inclusion of sources rich in PCs gives it greater stability, which is attributed to an increase in antioxidant enzyme activity and a decrease in MDA and TBARS in the muscle of monogastric[1,3]. The antioxidant effects of pomace by-products such as grape seeds and skins, which have been seen in animal production, have been associated with end products of the degradation of low molecular weight PCs, which can be transferred to tissues and responsible compounds such as epicatechin, among others,

have been detected[51,76].

Therefore, it has received significant attention as a natural preservative during meat storage to extend shelf life. Indeed, it has been shown that oral supplementation of flavonoids (quercetin, epicatechin, and catechin) in the diet increases the *in vivo* concentrations of vitamin E[78], which could explain the antioxidant effect of PCs on animal tissues. Additionally, the storage and deposition dynamics of vitamin E differ in monogastric animals. In pigs, the accumulation of this vitamin in response to supplementation with grape pomace is significant in fat, liver, and muscle, whereas, in birds there is more deposition in the liver and less deposition in the fat[79]. In addition, it has been reported that the inclusion of 30 g of fermented pomace/kg in the diets of pigs improved the percentage of marbling (3.5 %), increased the a* (25 %) and b* (45 %) values of the meat, and reduced MDA levels by 53 %[27]. Similarly, pomace modifies the polyunsaturated fatty acids (PUFA) content and the SFA/PUFA ratio, which has been attributed to the moderating effect of grape pomace on vitamin E in the intestine[78]. Likewise, it has also been shown that the supply of 5 % grape pomace from the feed of finisher pigs increases the levels of omega 3 fatty acids (1.27 times), alpha-linoleic (1.35 times) and eicosapentaenoic acid (1.30 times), while it tends to decrease the n-6:n-3 ratio with respect to the control[75].

Furthermore, it has been shown that the b* value is mainly affected by the type of myoglobin in the muscle[27]. However, the supplementation of 3 %, 5 %, 6 %, and 10 % grape pomace with a mixture of oils from the finishing stage did not affect the production of thiobarbituric acid reactive substances (TBARS) in the pork loin samples[79] but increased the a* value together with the color saturation index, which suggests a possible antioxidant effect.

For texture of the meat, satisfactory results have been obtained in shear force, which have been associated with an increase in pH values in response to supplementation with grape pomace extracts. In addition, it has been proposed that grape proanthocyanin may decrease collagen deposition by suppressing the expression of protein promoters involved in collagen synthesis (TGF-β)[80].

On the other hand, in the maturation of meat products, it has been determined that for every 100 g of raw meat, a powdered grape pomace (1L) mixture should be used at 4 °C for 72 h. Marinating the pork tenderloin with this mixture, the crude protein, crude fat, ash, pH, a* value, and b* value in the meat decreased, and its shear force and moisture content increased. During meat storage, the concentration of TBARS, volatile basic nitrogen, and the total microbial count decreased[80,81]. These results could be attributed to the organic acids and PCs present in the WBP, which could inhibit the growth of microorganisms by reducing the activity of some enzymes and inhibiting the production of basic substances[82]. Therefore, marinating with grape pomace powder improves some meat quality characteristics and stabilizes the pork loin during storage. Similar results were observed when adding two different grape pomace extracts to pork burgers[83],

resulting in an inhibition in lipid oxidation, greater color stability, and improved acceptability with the HLIP extraction method (Table 3).

Table 3: Addition of winemaking by-products on pork meat and pork products

Grape by-product	Muscle or meat product	Dose	Storage	Results	Reference
GSE		0.005%, 0.01%, 0.02% (49 d)		↑ in pH24h (3%) ↑ Redness (15%) ↑PUFA (20%), n-3 PUFA content (13%) ↑PUFA/SFA ratio (26%) ↓ Shear force ↓Drip loss (39%)	[94]
GSE	*L. thoracis Semimembranosus*	3-5 % (21 d) 6-10% (17 d)	----------	↑value and color saturation ↑ omega 3 and PUFA content in backfat	[79]
GP powder	Marinated tenderloin	20%;40% 0.5%;1% 2%	4°C for 10 d	↓ pH and color; ↑ Shear force Inhibits lipid peroxidation, volatile nitrogen, and microorganism growth	[82]
GSE	Pork burgers	0.06 g/100 g	4°C for 6 d	Color stability Inhibition of lipid oxidation Improves overall acceptability after 6 d of storage	[83]
GSE	Pork patties	1 g/kg	2 °C for 1, 6, 13 and 20 d	Reduces lipid oxidation ↓ Total bacteria (BAL, pseudomonas, and psychotropic bacteria)	[81]
GSE	Pork patties	0.2 g/kg	18°C for 6 months	↓ TBARS values ↑ Antioxidant activity than rosemary oleoresin,	[80]

				oregano extract, BHA and BHT	
Fermented GP	*Longissimus*	3% (105 d)*	---------- --------	↑ Value a* (20%) and b (31%) ↑ Marbling and modify fatty acid pattern (PUFA and SFA / PUFA ratio); ↓TBARS (47%)	[27]
GP silage	*L. thoracis*	3.5%* 7.0%* (86 d)	---------- ---------	↑PUFA/SFA (38%) ↑PUFA and n-3 PUFA ↓SFA (8%) and n-6/ n-3 ratio (13%) ↑TBARS (85%) No effect on marbling, color, drip loss	[92]
RES	*L. thoracis*	300 mg/kg *		↓ Drip losses; ↑ pH 24h ↑ Intramuscular fat	[55]

* Supplementation from animal feed

Technological considerations for the inclusion of winemaking by-products in monogastric diets

WBP such as grape pomace, have been subjected to various pre-treatments (endogen enzymes, cellulolytic enzymes, polyethylene glycol, and fermentation) to increase the bioavailability of their BCs especially of PCs and improve its nutritional value to favor their inclusion in animal feed. In this context, it has been demonstrated that the inclusion of enzyme complexes (tannases) and polyethylene glycol in monogastric diets can partially inactivate condensed grape pomace tannins, minimize the content of antinutritional factors and increase antioxidant and antimicrobial activity[28,84]. Among these treatments, fermentation processes with yeasts, bacteria, and fungi have been emphasized[27]. In this context, a systematic biotechnological approach was developed, through which the metabolic capacities of several species of fungi (*Aspergillus, Rhizopus*, and *Trichoderma ssp*.) were evaluated about the bioconversion of grape pomace and lees to feed for animals[85]. In this study, the authors obtained an improvement in the protein content (5 to 26 %) and digestibility of the feed (25 to 50 %).

Similarly, the supplementation of 3 % fermented grape pomace with *Saccharomyces boulardii* in pigs during their growth-finishing stage increased the apparent digestibility of dry matter (79 %) and nitrogen (82.5 %) after 11 wk[27]. These results suggest that the PCs of this matrix can stimulate intestinal fermentation and influence the production of specific microbial metabolites, which explains the improvement in growth. This strategy makes it feasible to provide rich protein sources and an optimal nutritional status to the animals[66]. However, it has also been reported that with the inclusion of 20 % fermented grape pomace in finisher pigs, the digestibility of dry matter, crude protein, energy, and essential and nonessential amino acids was reduced with respect to the control[25]. These results were attributed to the formation of complexes between some PCs and the food protein, which limited the action of digestive enzymes; thus, it is necessary to consider the inclusion levels for each production stage.

Other technological processes such as the elaboration of flours, powders, and cakes from WBP (seeds, pomace, skins) are indispensable in the formulation of diets for monogastric animals. Therefore, the optimum conditions of temperature, time and particle size have been evaluated and standardized to obtain better results in animal feed with respect to the raw by-product. GP has been subjected to a drying process with hot air flow at 65 °C for 4 d, 60 °C for 20 h[16,86], 50 °C for 12 h[87] and 60 °C until reaching a constant weight. Also, after grinding, particle sizes from 1 mm to 6 mm have been defined for poultry and swine respectively[67,70,86]. Likewise, it is also important to define and specify the temperature/time conditions for the GP intended for pig feeding and to standardize the particle sizes depending on the production stage, which has made it possible to obtain better results in animal feeding concerning the crude by-products[8,20,70].

Conclusions and implications

A large number of completed studies have evaluated the antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory properties of wine industry by-products, emphasizing weaned piglets and early growth stages. However, the effect on muscle fibers with the raw matrix has not been determined. In the final stage of production, it is still necessary to continue evaluating different presentations of this matrix and new inclusion levels and consider mortality rates as another indirect measure of productive performance. The inclusion of grape pomace in extract or flour form and its BCs in the pig diets at each productive phase is considered a low-cost antioxidant and antimicrobial source that exerts multiple benefits on the productive performance of pigs as well as the meat quality. Therefore, it represents a promising alternative for the animal nutrition sector that could minimize the use of synthetic antioxidant compounds, growth promoters such as antibiotics, and beta-adrenergic agents in the pork production system.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 2: Effects of phenolic compounds from winemaking by-products in pork production systems

Productive phase	Animal weight (kg)	Grape by-product	Duration (days)	Dosage	Additive type[b]	Results[c]	Reference
Female pigs	130-150	GSE (grape seed extract)	6	1% p/p	Z MIF	↑*Lachnospiraceae,* ↑*Clostridales,* ↑*Lactobacillus* ↑*Ruminococcacceae*	[88]
Lactation	NR	GSP	35	200 and 300	T Antioxidant	↓ Stillborn (0.63)	[50]
Nursing		(grape seed polyphenols)	21	mg/kg	Z PP; RP and nutritional composition and Ig content in colostrum	↑ survival to delivery (89.33%) ↑ pre-weaning survival (95.23) ↑ P4 and E2;↑ SOD and GSH-Px	
Gestation	NR	RES	94	300 mg/kg	T Antioxidant		[41,55]
Lactation		(resveratrol)	21		Z PP	↓ MyHC IIb expression	
Weaning			68		Growth promoter	↓ MDA; ↑ SOD	
Finishing			108				
Gestation	NR	RES	94	300 mg/kg	T Antioxidant	↑ Weaning weight per litter and piglet	[41]
Weaning			21		Z DP	↑ AOS in milk, placenta and plasma of females and piglets; ↑ gene expression (CAT, GPx, SOD). ↑ SIRT1 expression in placenta	

							↓ IL-8 expression in the placenta	
Lactation	180.53	Spent grapes	63	Replacement of 25% maize	Z	PP	Tendency to decrease FCR; No effect on ADC of NFE, ADG, ADFI, final body weight	[89]
Weaning	8.4	GSP	28	40,70, 100 mg/Kg	T Z	Antioxidant MIF PP	40 mg/ Kg ↑ ADG and ↓ FRC ↓ Diarrhea incidence (40-70 mg/Kg), ↓MDA ↑Amylase and lipase activity ↑Antioxidant enzymes (GSH-Px, SOD and T-AOC)	[90]
Weaning	6.9	GSE	56	0.015%	Z	PP Digestibility	Improve the ADC in tract, No effects on microbial metabolites	[91]
Weaning (day 28)	NR	GSF (grape seed flour)	30	8% p/p	T Z	Micotoxin binder Antioxidant Inmunomodulator	↑ SOD (119.3%) ↑ GPx (105.9%) ↑ T-AOC (112%) ↓ Lipid peroxidation (12.3%) ↓ Inflammatory markers AFB1 (IL6 IL1-β)	[63]
Weaning	9.13	GSF	30	8%p/p	Z	Colonic microbiota modulation	↑ Butyrate, isobutyrate ↑ *Prevotella* and *Megasphaera*; ↓ *Roseburia*	[69]
Weaning (d 20) Growing	4.8	GP silage	30	9%	T Z	Antioxidant MIF PP	↑ T-AOC and H_2O_2 decomposition activity ↑ GDP (23.5%); ↓ MDA	[20]

							↑ Facultative probiotic bacteria growth and BAL Inhibits the growth of pathogenic bacteria	
Weaning	10.70	Dried GP	36	5%	T Z	Antioxidant PP	↑ CAT, SOD, GPx; lipid peroxidation ↑ food intake	[20]
Weaning-Growing (d 21)	6.99	GSP	28	50, 100 150 mg/kg	T Z	Antioxidant Inmunomodulator PP; MIF	100 and 150 mg / Kg ↑ Ig GEe Ig M and IL-2 ↓ Incidence of diarrhea ↓ serum MDA; ↑TAOC, GSH-Px; SOD	[64]
Weaning-Growing (d 42)	NR	GP and GSE	28	1%	T Z	Antioxidant Inmunomodulator PP; MIF	↓ oxidative stress; ↓ NFKb and Nrf2 ↓ Inflammation; ↑ Gain: feed ratio ↑ Height / depth ratio of crypts (2.11 ± 0.11)	[62]
Weaning-Growing (d 35)	10	Grape Polyphenols	28	1%	Z	Inmunomodulator PP; MIF	↓ Expression of pro-inflammatory genes in duodenum, ileum, and colon; ↓ Streptococcus and clostridium ↑ Villus height ratio↓ VFA in feces	[58]

Post-weaning (31 days)	7.5	Red GP	19	3.5%	Z	Inmunomodulator MIF; Blood parameters	↑ Total number of colonic bacteria (Steptococci / Enterococci), Lactobacilli	[71]
Growing	48.6	GP silage	90	3.5%;7.0%	Z	PP	It did not affect daily weight gain	[92]
Initiation Growing Finishing	23 36 64	GP fermented by *S. boulardii*	105	30 g/kg	Z S	PP; Meat quality Digestibility	Improved DP (↑ GDP); ↑ apparent DM digestibility (79%) and (82.5%)	[27]
In vitro	----	GSE	----	250 µg/ mL	Z	MIF	Modifies ileal and fecal fermentation patterns	[72]
Finishing	75.53	GSC	24	5%	Z	Antioxidant Inmunomodulator PP	↑ T-AOC (spleen); ↓ MDA ↑ Antioxidant gene expression (CAT, SOD, GPx) ↓ Gene expression of pro-inflammatory markers ↓ IL-1β (52.66%); IFN-γ (42.13%) ↓ IL-6 (13.25%); TNFα (9.6%); IL-8 (11.08%) ↓ Cholesterol; ↑ IgA (49.9%) in plasma ↓ Pro-inflammatory response	[67,93]
				1				[25]

Finishing	63.42	GP fermented	7	0 %; 20%	Z	MIF; Amino acid digestibility	20%: ↑ Stool excretion ↑ Yeast; ↓ amino acid digestibility and VFA

a) Type of additive: Z= zootechnical; T= technological; S= Sensory; PP= productive performance; DR= reproductive performance; T-AOC (Total antioxidant capacity).
b) Results: MDA (malondialdehyde); AOS (Antioxidant status); NR= not reported, MIF= modulation of intestinal function; ADC= apparent digestibility coefficient.

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Review

Re-seed or not re-seed? Factors affecting rangeland grass-seedling establishment. Review

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Abstract:

Although seedling has a significant role in the maintenance of plant diversity, productivity, and biochemical cycles in the rangeland. However, little is known about the influence of environmental factors in the seedling establishment, as well as the differences in the morphological development among species. To understand of seedlings establishment becomes of crucial importance to improve the success of reseeding of natural ecosystems. This literature review investigated which factors are addressed with failures in the seedling establishment of native grasses in rangeland conditions. Germinating seed grass is not big a problem if there are optimum environmental conditions. The heart of the matter is to ensure the survival and growth of these seedlings until the complete establishment as plant. The moisture and temperature of soil are the main environmental factors associated with failures in seedling establishment. The studies reviewed showed that annual plants have higher seedling growth rates, however lower allocation to reproductive structures when compared to mid-seral and late successional plants. These differences also promote different rates of seedling survival rate, with early seral grass showing higher rates than late seral. Apparently, the main cause of seedlings failures in the establishment is correlated with the development and extension of the adventitious roots. Where the reports describe that seedling emerge quickly and abundantly in most grasses, but the seedlings died between six and ten weeks of

age. It was addressed that a plant can germinate and sprout the primary roots, however, for an unknown reason the plant does not sprout the adventitious root.

Keywords: Rangelands, Adventitious root, Rangeland restoration, Seedling establishment, Rangeland reseeding.

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Introduction

Rangelands are widely diverse, including grasslands, savannas, shrublands, deserts, tundras, marshes, and meadows. Rangelands cover about 50 % of land area in the world. Only in north America stats indicates that 1.3 billion of hectares can be classified as rangelands[(1)]. During the last century, the man has increased the ways to use rangelands due to expansion of croplands, urbanization, and mainly the expansion of livestock production. Overuse of these ecosystems promotes a non-natural phenomenon denominated "Rangeland degradation." There are two factors that might cause rangeland degradation: natural cycles lead by droughts, and the second one, related to human factors addressing the overuse of natural resources.

The effects of climate catastrophes and human land use promote the depletion of soil nutrients, and decline in water retention, causing a breakdown in soil structure, and therefore the changes in vegetation composition, increasing the percentage of less desirable plants for the livestock, such as thorny and low palatable plants. Also, intensification of land use without proper rangeland management reduces biomass productivity, which may lead to further agricultural expansion in even more marginal areas.

D'Odorico, *et al*[(2)] mentioned that among the human activities that degrade rangelands, overgrazing as the main factor for the losses of forage species diversity on rangeland. The fast increase of the world's livestock population has been causing overuse of rangeland. Overgrazing of rangelands initially reduces biomass productivity, breaking all natural bio-cycles in the ecosystems. Overall, overgrazing converts the rangeland into a desert place (desertification). Currently worldwide more than 680 million hectares of rangelands are in some degradation stage, which is five times more than the U.S. cropland area, or 100 times

the agricultural land area of México[3], Gaitán *et al*[4] affirm that continuous grazing with excessive stocking rates has been occurring for many decades over all rangelands of the America.

The previous situation has encouraged several projects aiming to mitigate the effects of rangeland degradation, including the reseeding of the rangelands with native and exotic plant species. It is important to mention that currently, most of the projects recommend reseeding exotic species, believing in an increasing stocking rate. However, in some initiatives, for example, in México and Argentina, reseeding attempts to restore the ecosystem to a condition near the original, thus, to recover the natural landscape conditions, the Plan Maestro de la Alianza Regional para la Conservación de los Pastizales del Desierto Chihuahuense in México and the Buenas Practicas Ganaderas in Pastizales of the Argentina government, have become as mandatory in reseeding programs the use of only native species.

Johnson *et al*[5] affirms that reseeding native forage grasses increase the percentage of desirable grass species in the rangeland, increasing the forage available to livestock and wildlife, and consequently a higher stocking rate. Nonetheless, some reseeding projects in several parts of the world (such as Mexico, USA, Canada, and Argentina) are trying to compensate for the loss of plant diversity using native grass species. However, the high cost and risk involved in the process of reseeding and the lack of knowledge of the physiological mechanisms in seedlings seem to be a big obstacle to the success of this practice. In addition, Rector[6] mentioned that the high risk of reseeding rangelands is associated with the temporal and spatial unpredictability of rainfall pattern in many rangelands during the seedling establishment period.

The chance of success decreases dramatically in semiarid zones due to the short rainfall season. Rector[6] also states that the rate of success in reseeding programs is more effective in areas with annual rainfall superior to 800 mm/yr. Conversely, in areas with precipitation inferior to 350 mm per year the probability of risk of failure in seeding establishment is more than 80 %. According to the Plan Maestro de la Alianza Regional[7] rangeland reseeding promotes multiple benefits, such as 1) Increasing plant diversity; improving forage quantity and quality for livestock; 2) Producing large or abundant seeds for wildlife; 3) Promoting a beautiful spring view, and 4) Facilitating soil stabilization in disturbed areas. If all environmental conditions (soil temperature and moisture) are met, germinating of a seed grass will be completed. The problem is to ensure the survival and growth of these seedlings until the entire establishment as plants; in México the the occurrence of the intra-seasonal drought and early frosts in the rangelands affect the seedling estableshment. After a considerable number of papers reviewed, it was regard to seedling establishment failure and addressing by not ideal conditions to germinate seedling grass.

Orloff *et al*[8] point out the three major factors associated with seedling failures on rangeland grasses during the establishment: inadequate environmental conditions, seed size, and genetic factors. About the genetic factor Esau[9] and Tischler *et al*[10] reported that seedling establishment is correlated with the ability to initiate growth of adventitious roots and the subsequent elongation of these roots in the seedling stage. Regarding the environmental factors associated with the seedling establishment in perennial grasses, most of the published literature mentioned four factors as the most important for seedlings establishment: 1) Inadequate soil moisture, especially in the surface[11,12] 2) Inadequate environmental temperature, especially the soil temperature[13] 3) Competition for sunlight and nutrients among species[14] and 4) planting depth[15]. Concerning to environmental factors, Briske and Wilson[13] studied the optimum temperature and moisture in blue grama seedlings, and they conclude that even with optimum environmental conditions, some other unknown factors also affect seedling establishment.

Although this review aims to discern seedling challenges during stable development, it is essential to mention that some seed/germination limitations should also be overcome. Size and weight of seeds are also reported as a factor that affects the seedling development[15]. Maron *et al* [16] concluded that not only the size but also the weight of seeds influences the seedling survival of rangeland plants. Hyder *et al*[17] affirm that unknown genetic factors could also promote failures in the seedling establishment. In addition, other works[18] suggest that photomorphogenic factors associated with the seed and crown could cause failures. Several approaches have shown which mechanisms affect seedling establishment; however, all conclude that development and extension of adventitious roots is the most important process associated with seedling establishment. Therefore, in this review, it was discussed the main factors that cause failures in the seedling establishment of native grasses in rangeland conditions.

Environmental factors that affect the seedlings establishment and survival

There is no single environmental attribute that could entirely explain seedling vigor in grasses. Thus, environmental influences on seedling establishment in grasses should be analyzed together. Most literature indicates that interaction among moisture, temperature, and light are the main environmental factors that affect the success of seed germination and seedling survival.

Sluijs & Hyder[19] affirm that in blue grama, adventitious root grows out of tillering crowns and becomes successfully established when damp and cloudy weather persists for 2 or 3 d after germination. But if the roots are exposed to harsh environmental conditions the chances of survival are poor[20]. It is important to mention that each site has an optimum environmental condition for seedling establishment. The effect of environmental factors on seed germination of rangeland grasses has been broadly studied[21,22]. However, few studies tried to understand the impact of environment after germination until entire establishment (juvenile phase). In this segment of this review, the effect of environmental factors on seedling establishment will be discussing it.

Temperature

Temperature seems to have more influence on seed germination than seedling establishment. Overall, environmental temperature determines the rate of development in all organisms. Snyman [23] affirms that soil temperature is the major factor associated with seedling emergence because it controls evaporation and transpiration rate in ecosystems. It is affirmed that the ideal temperature of seedling growth for warm-season grasses is between 25 and 30 ºC[24]. While, for cool-season grasses, McGinnies[25] stated that a temperature around 20 ºC promotes the best root development in these species groups. By studying the effect of moisture and temperature in two grass and four forbs natives from north American grasslands, it was concluded that a temperature lower than 15 ºC resulted in delays in the seedling establishment caused by an inefficient root distribution in the soil[26].

Soil temperature seems to be more important than air temperature. Hsu *et al*[24] suggested that high soil temperature in the first 30 cm of soil is a major factor associated with failures in root emergence. Therefore, the faster the root reaches the deepest soil layers, the greater the chance of the plant surviving and establishing in the rangeland.

Hsu *et al*[24] affirm that the optimum soil temperature for root grows for warm-season grasses ranges between 9.4 and 11.4 ºC. However, this temperature is usually higher during the summer in all rangelands located between the parallels 30º N and 30º S[27], which probably is one of many factors associated with failures in the reseeding on drylands. Briske and Wilson[13] stated that for a successful seedling establishment in drylands the seedling roots of grass need to develop quickly in order to avoid the excessive exposure to high temperatures. The optimum temperature for shoot and root shows to be different. The optimum temperature for shoot growth for dryland grasses is around 5 ºC lower than the temperature for optimum root growth, causing a disbalance in terms of environmental condition[28].

Moisture

There is no doubt that moisture has an essential role for plants in the seedling stage. Moisture seems not to be limited in pasturelands, which can receive an additional water by irrigation. In contrast, rangelands, rainfall is the only source of water. The moisture in the soil is not a limiting factor to grass growth in tropical lands because the moisture in the soil of these regions is abundantly available for a large part of the year[29]. However, this is a limiting factor for seedling establishment in semiarid and arid environments.

Concerning the U.S. rainfall pattern, Rajagopan and Lall[30] affirm that U.S rangelands western of the 100º of meridian longitude exhibit considerable complexity distribution temporal and spatial , in comparison to rainfall patterns in the eastern part of the country. Thus, an analysis based on annual or monthly precipitation for drylands does not allow conclusive results about water availability for the native plants.

It was mentioned that it is necessary to appreciate that rain precipitation does not imply that rainfall water will be available 100 % to the plants[31]. Therefore, it is essential to understand the hydrology patterns and its implication in the ecosystem. Thus, this could be the initial step to comprehend the influence of environmental factors on seedling establishment. Some researchers[32] addressed the response of plants with pulses of precipitation. The pulses of precipitation theory suggest that frequency of precipitation has the same importance as the volume precipitated in some area.

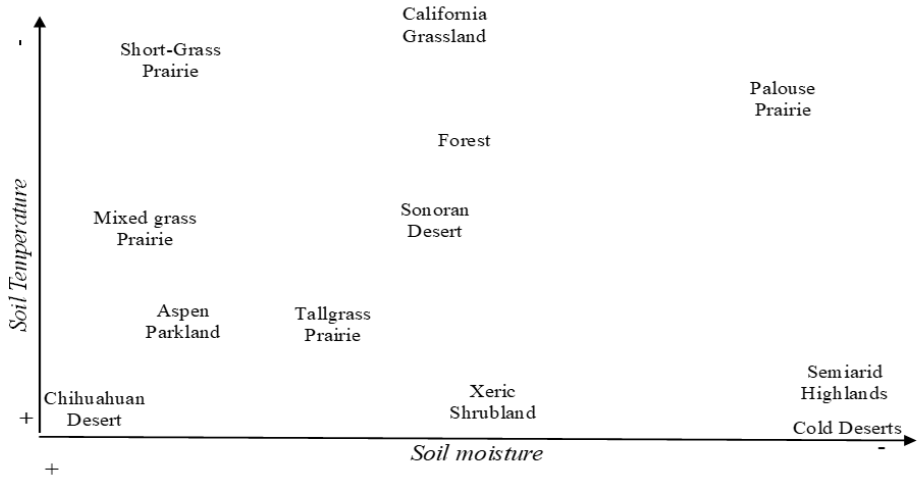
In one work[33] states that it is necessary to have two wet days for a grass seed to germinate and five wet days for the seedling to establish in rangeland conditions. After this time, the seedling can resist up to seven dry days consecutively. In arid lands, the growth of plants is primarily controlled by soil water availability than any other factor. Water has an intrinsic ratio with all aspects of grasses growth on rangeland species including anatomy, morphology, physiology, and biochemistry[34–36].

The key for seedling establishment in rangelands seems to be a positive balance in soil water available. In other words, there should be more rainfall than evaporation in that area. Frasier *et al*[33] studied the effect of drought on sideoats grama seedlings and concluded that five consecutive dry days promoted mortality over 50 % of the seedlings. Drought and soil desiccation are the main factors for the limits of seedling establishment in many environments[37]. It was suggested that differences exist in seedling survival among grass species during drought. The development and extension of adventitious roots play an important role in the plant establishment and soil exploration[38].

Studying blue grama, it was concluded that optimum moisture for maximum development of adventitious root is 90 % of soil saturation[13]. However, the adventitious root could grow slowly in low soil water potential conditions. Harrington[39] studied the effect of soil moisture on shrub seedling survival in a semi-arid grassland in Australia and concluded that to obtain success in the seedling establishment, it was necessary to apply at least 100 mm of supplementary irrigation during summer on three occasions after the pre-sowing irrigation in early spring and twice after late-spring irrigation. It is important to highlight that the author reached seedling survivorship above 80 % in irrigated plots when compared with zero survivorship on the unirrigated plots. Davis[40] affirms that most native species of the California chaparral have seedlings adapted to drought showing shallow-roots and high efficiency of water available. It was concluded that failures in the root establishment and development of 12 grass species of prevalent occurrence in the California chaparral occur during droughts in the summer[41].

According to some information[1] moisture and temperature in the soil are the most important factors associated with failures in the seedling establishment of grasses of North American rangelands. Thus, using this affirmation, we reviewed several articles which studied the influence of these two environmental factors on the seven main rangelands ecosystems of the United States. Figure 1 represents graphically the level of importance of moisture and temperature in the soil reported in the scientific literature associated with failures in the seedling establishment of grasses in the rangeland of North America.

Figure 1: Importance of soil temperature and moisture associated with risk of failures in the grasses seedling establishment of grass seedlings in the rangelands of North America *†



† The figure is based on results of 18 studies in the 12 rangeland ecosystems of North America. *The level of importance had been set based on several scientific reports which associate failures in the seedling establishment with soil temperature and moisture. Thus, the closeness of ecosystems (name) with signal (+ and –) represents graphically the level of importance of these variables in the ecosystem.

In the Chihuahua Desert the combination between soil moisture and soil temperature has a similar level of importance in the seedling establishment of rangeland grasses. On the other side, cold deserts and semiarid highlands we found more reports demonstrating a strong association between failures in the seedling establishment and soil temperature. In the xeric shrublands (e.g., Matorral xerofilo and Mesquitales of Mexico) the temperature of soil was reported more than moisture as cause of seedlings failures in the establishment. Similar pattern to tallgrass prairie and the Aspen parklands of Canada. For the rest of group, could also be noted that in the tall grass and mid-grass prairies, seems to exist a combination between these two factors that assure a successful development of rangeland grass-seedlings.

Others (light, soil, and planting depth)

The light can also control the seedling establishment because a low light intensity reduces the leaf and root size. Pang *et al*[42] affirm that shade could reduce the soil temperature, but it did not increase survival of grass seedlings. By studying the effect of shade in growth response of four perennial southwestern grasses it was concluded that morphological, physiological, and yield responses were high in plants in full sunlight condition than plants under different levels of shade[43]. Although the light affected the size of leaves and roots, this variable did not affect the seedling establishment directly.

Concerning soil properties that affect seedling establishment, the hydraulic conductivity seems to be the most important, in other words, the capacity of moisture retention and water availability to the plants. Okami[44] stated that hydraulic conductivity is the most important soil variable related with grass seedling development, once that 75 % of the time during the seedling establishment, the seedling is not dependent on nutrient content in the soil. The physical soil features such as texture, structure, density, and capillarity determine the water retention in the soil and the contact surface between soil moisture and the seed. Berti and Johnson[45] studied the seedling establishment of switchgrass in different soil types, and they concluded that seedling emergence is 25 % faster in sandy soil than in clay soils. Similar results stated that soil texture affected seedling emergence in some tropical grass[46].

Planting depth also has been reported as a factor that influences the development of seedlings in grasses. One report[11] affirm that planting depth affects the seedling establishment of rangeland grasses, especially during the emergence stage. Analyzing the influence of planting depth in the seedling emergence of native grasses it was stated that Bromegrass (*Bromus inermis*) planted deeper than 1.3 cm in a silty clay loam soil decreased the emergence, and consequently seedling survival[47]. While other authors[11] studied the influence of planting depth on the emergence, morphology, and establishment of big

bluestem (*Andropogon gerardii*), indiangrass (*Sorghastrum nutans*), and switchgrass (*Panicum virgatum*). They concluded that results obtained from these experiments were not convincing to affirm that planting depth affects the seedling survivorship. In arid condition the switchgrass, had the higher emergence when planted under pre-sowing irrigation and shallow seeding[48]. Anderson[49] affirms that optimum planting depths from native grasses of northern US and southern Mexico are between 6 to 12 mm, the difference inside this range is according to grass species and soil type.

Differences in the rate of utilization of seed reserves may explain why certain species emerge at greater planting depths than others. Others authors suggested that optimum planting depth is correlated with the amount of carbohydrate reserves content in the seed[50-52].

Non-environmental factors that affect the seedling establishment

Plants have several anatomorphological adaptations to be more effectively to compete with each other plants for resources (light, water, nutrients, soil, and air). Harris[53] stated that in a general concept the perennial grasses have a natural competitive advantage over annual grasses, so that, it is not necessary for them to seed following each dormant period. However, annual plants have a fast-seedling establishment.

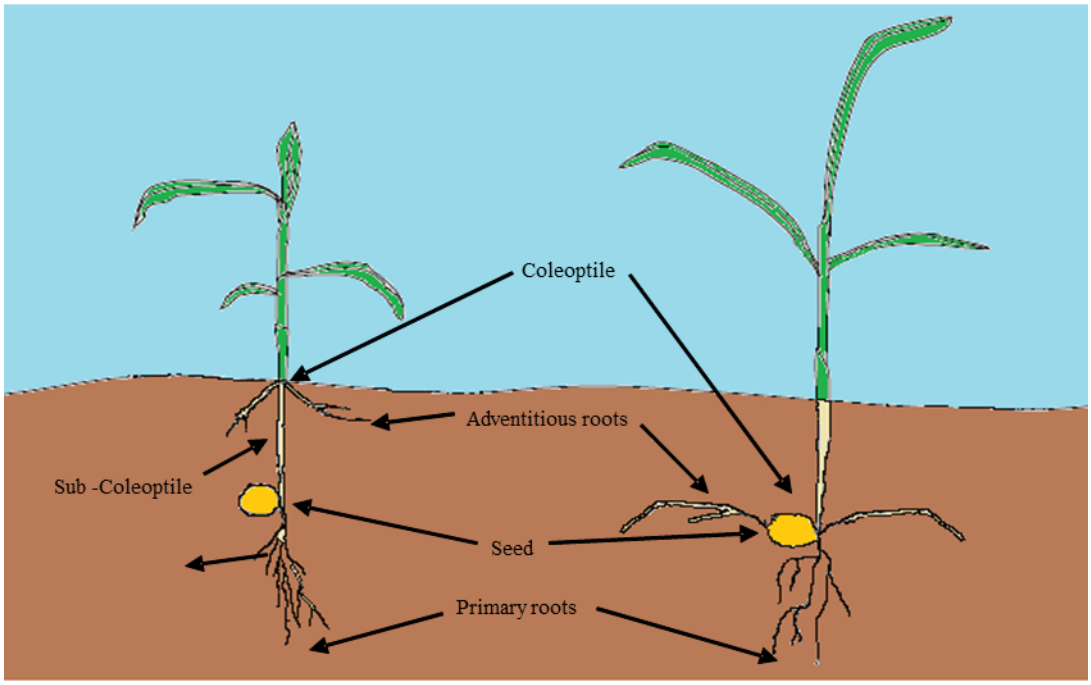
Thus, by studying the influence of growth form and plant morphology in the seedling establishment of dryland grasses, researchers. concluded that neither variably affected emergence, survival, nor relative growth rates in all growth forms studied[54]. Corroborating with authors mentioned above Larson *et al*[12] affirm that variation in anatomy, morphology and physiology among seeds and seedlings explained over 90 % of the variation in cumulative survival of rangeland grasses, regardless of seedling survival probabilities or precipitation pattern.

There are three major non-environmental factors related to seedling establishment: morphology, physiological and genetic characteristics intrinsic for each species (seral stage) and competition among species[21]. Below was done a brief review of how these factors affect the seedling establishment in rangeland grass species.

Morphology

Grass seedlings are hypogeal, which means that the cotyledon in most cases remains below ground during the germination. The elongation of the coleoptile is different between cool and warm season grasses. In the cool season grasses, the coleoptile is long with short sub-coleoptile; contrarily the warm season grasses have a short coleoptile and short sub-coleoptile (Figure 2).

Figure 2: Seedling morphology (right festudicoid plant); left (panicoid plant)



(Adapted from Tischler *et al* 1989).

The extension of coleoptiles and sub-coleoptiles have an important role in the emergence of seedlings because near the top of these structures are located the meristematic points where the first leave will grow[55]. The roots are also important in the seedling establishment; it was reported[56] that the root system in seedling grasses consists of seminal and adventitious roots. The seminal roots start to grow immediately after germination; they arise directly from a structure in the seed called scutellar note. The seminal roots are divided into primary roots and lateral roots. Seminal roots are entirely dependent on adequate levels of water content in the young plant, as well, moisture in the soil[57].

After the entire development of seminal roots, the seedling starts to release the adventitious root from the coleptilar node. Tischler and Voight[9] affirm that adventitious are considered the mature root system. Some authors suggest that establishment of seedlings is associated with the development of adventitious roots[56].

The seminal roots start growth after 1 or 2 consecutive wet days; once the seminal roots are developed, plants begin to sprout the adventitious root. This phase is called the transitional stage. In other words, the transitional stage consists of development and extension of the adventitious root, and a weakening and death of the seminal root[58]. Hyder *et al*[17] affirm that in field conditions where blue grama seedlings fail in the extension of adventitious roots, the seedlings die between 6 to 10 wk of age. It was stated that for a successful seedling establishment, the rate of root elongation of adventitious roots should be sufficiently fast to keep a portion of the root in moist soil ahead of the drying soil[13].

Newman and Moser[11] advocate that adventitious root development undoubtedly controls the seedling establishment. However, little is known about how many, and extensions of adventitious roots will be enough to affirm when a plant is established. Even though some authors associate the establishment of seedlings with the transitional stage, it was mentioned that the development and activities of roots of cultivated grasses and concluded that the seminal roots remain alive and active until the time of harvest in crop plant species[57].

After examining reports about root systems of 14 perennial grasses, conclude that in these species the seminal root grew deep and spread widely, and they remained alive and active as absorbing organs during four months of experimental analysis[13]. Most of the articles reviewed affirm that adventitious root development in the transitional stage determines the seedling establishment, and the death of seminal roots is required for the development of adventitious roots. However, there is not a consensus about this hypothesis, since some researchers indicate that seminal roots remain alive for a long time after the plant reaches the mature phase.

Competition between species

The competition among plants occurs when the demands of neighboring plants exceed the resource supply, inducing the stress and then the death of the plants. Plant completion could occur in two levels, among individuals in the same species (inter-species) or among individuals of different species (intra-species). Whatever the level, competition affects the availability of environmental resources for plants. It was reported[59] that initial densities and timing of establishment promote changes in the dynamics of plant competition because they

lead to asymmetries in plant size and resource capture. Range plants have many adaptations (morphological, anatomical, physiological, and phenological) suiting them to a place in the ecosystem. Therefore, understanding the effect of competition among species is a basic requirement to increase the chances of success of rangeland reseeding.

Stands of perennial grasses have a natural competitive advantage over annual grasses. Since it is not necessary for them to begin from seed following each dormant period[24]. Ries and Svejcar[60] reported that seedlings of annual plants readily invade and become established on disturbed sites. Favorable root phenology is one of the adaptive strategies allowing this superior competitive ability.

Some authors[61] defines five most important phenological characteristics in seedlings concerning competitive relationships in juvenile plants. The features are (1) easy germination, (2) precocious initial root growth, (3) rapid extension of root-soil contact, (4) easy dormancy break, and (5) survival of drought.

Plant competition also could be divided according to the zone where it occurs. In this classification, it can be divide the competition into two levels: above and belowground. Several studies have provided evidence that in arid environments the belowground competition is more important than aboveground[62].

Harris and Wilson[61] suggested that in areas where the season of favorable moisture coincides with the season of low temperature, the ability of seedlings to continue root growth at low temperatures can be a deciding factor in the outcome of competition between species. They also studied the effect of soil moisture during the seedling establishment of forage cool-season grasses at low temperatures, concluding the existence of differences in root adventitious growth where the *Bromus tectorum* and *Taeniatherum asperwerum* were more successful than seedlings of *Agropyron spicatum*.

Invasive species have shown be more efficient in the seedling establishment than native species. It was reported[63] that invasive species germinates faster than native species, getting an advantage in the competition for light, space, and moisture. As mentioned, more studies are necessary to know how to occur the interaction between plants in the seedling stage in native grasses. It is important to mention that the first step in land reclamation is to eliminate the seed bank.

Seral stage

Several studies have indeed shown that annual plants have higher seedling growth rate[55,64] and higher allocation to reproductive structures[65,66] when compared to mid-seral and late successional plants. Newman and Moser[11] compared the seedling development among 12 grass species. They conclude that annual plants sprouted the first leaf faster than late seral species. Early seral species seem to allocate more energy to the shoot development than to the root system. Contrarily, mid and late seral seem to allocate more energy to root development. They conclude[56] that if the proportion of shoots is more than the roots in the seedling, higher are the risks of failure in the establishment caused by seedling acidification or an inefficient control in the water uptake.

Importance of the seminal and adventitious root pattern in the plant establishment (implications in the seedling survival)

As previous mentioned the establishment and survival of a plant are intrinsically correlated with emergence and extension of seminal and adventitious roots[56]. Other work[67] supports the idea that a plant cannot be considered established until it shows a plausive development of adventitious roots, which will allow extending down, catching moisture in deep levels of soil. To facilitate understanding the chronological sequence of morphological events that occur in a plant until the development of adventitious root the description mentioned by Whalley *et al*[68] was used. The author divides the growth of seedlings into three stages: the heterotrophic stage, a transitional stage, and the autotrophic stage.

The heterotrophic stage begins when the seed has contact with water being in this stage independent of other environmental factors, which means that the plant uses in its metabolism the energy reserves (starches) stored in the seed. After that phase, the plant still does not have a photosynthetic tissue. Hyder *et al*[17] affirm that plants easily surpass this stage in field conditions. Most of the researchers mistakenly consider the percent of seeds germinated as an indicator of seedling establishment. Hyder *et al*[17] affirm that less than eight percent of seeds germinated will reach the adult phase. The next transitional stage as the name suggests is a transition evolution to a photosynthetic phase. In this stage, the plants begin photosynthesis but still use energy reserves from the seeds for the expansion of root systems and the formation of new leaves. This phase can be divided into three sub-stages based on the development of the root system.

In the first step, the seedling shows only seminal roots. The seminal root system consists of one to five roots that developed from radicle and two pairs of lateral roots. Some investigators[21] affirm that seminal roots only are able to absorb water; while all nutrients necessary for the seedling come from the seeds reserves. The thick seminal roots has a limited capacity to absorb and translocate water in the plant. In the second step of this stage, the plants start to sprout the adventitious roots from the nodes in the crown. Some authors report this type of roots as true roots because they can absorb moisture and nutrients for the plant. In this step the seedlings show these two types of roots. Little is known about the interaction between these types of roots. The third stage is often marked by the weakening and death of seminal roots and the strength, extension and consolidation of adventitious roots as real roots. Haling *et al*[69] states that seminal roots persist only a short time after germination, their place being taken by adventitious roots. Some authors assert that the third seedling stage as the most important time in seedling establishment[40,70]. If the plant starts to lose its seminal roots before the adventitious roots reach a reasonable deep penetration in the soil profile, there is a high probability of failure in the establishment.

Due to a misunderstanding of the physiological bases of seedlings, most range managers associate the seedling establishment with the development of shoots. In American native grasses it was concluded that the stage of root development did not coincide with the stage of shoot development among species, which means that only a simple visual analysis cannot be considered a good indicator of success in the seedling emergence of American grasses[11]. Thus, it was affirmed[9] that to evaluate the success of the seedling establishment, variables such as size and age in the Klein grass seedling are also important and should be measured additionally the visual analysis.

In selected cultivars of big bluestem based on shoot weight and tiller number and they conclude that seedling tiller number and weight are not good indicators of seedling establishment success[71]. The development and extension of adventitious roots seem also to be associated with genetic factors. It was mentioned[72] that genetic variability appeared to correlate with adventitious root elongation in the blue grama, and that, in plants under the same edaphoclimatic conditions showed a broad diversity of development that could not be explained by environmental factors.

Conversely Chen *et al*[57] after investigated the root systems of several crops, conclude that the seminal root remains alive and active until the time of harvest, may is the reason of the success in the establishment of most cosmopolite crops. However some grass species seems to move in the same direction, Sánchez-Valdés *et al*[73] states that the seminal root of a ryegrass plants remains functional throughout the entire life of the plant. Same results were reported for other species[74].

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Technical note

Estimation of genetic parameters for milk flow rate and conductivity traits in a robotic milking system

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Abstract:

This work aimed to estimate the variance components and genetic correlations for milk yield (MiY), mean flow rate (MnF), maximum flow rate (MxF), and electrical conductivity (EC) of milk, in a robotic milking system. IT was analyzed a total of 137 lactations from 110 primiparous and multiparous Holstein cows, with 42,009 observations, from 2018 to 2020 in a dairy herd in the state of Querétaro. Genetic evaluation was performed using a mixed regression animal model. To estimate heritability (h^2), the restricted maximum likelihood algorithm was used to calculate the variance components, the BLUE estimator and the BLIP predictor, for each of the variables subject to the research. The estimated h^2 for MiY (0.62) was the highest of those calculated, and h^2 was also estimated for MnF (0.44), MxF (0.33),

and EC (0.28); it is considered that one of the aspects that influenced the values was the variability of each daily observation. Genetic correlations for MiY were negative for MnF (-0.6117) and MxF (-0.7666); in contrast, for the trait of EC (-0.1669), the correlation was low. The estimated genetic correlations for MxF were positive for MnF (0.7422) and EC (0.5351); finally, a positive genetic correlation was estimated for MnF and EC (0.3546). The results presented allow to understand the relationships between flow rate, conductivity, and yield, and they indicate the importance of these characteristics for a genetic selection program.

Keywords: Heritability, Milk yield, Electrical conductivity, Milk flow rate.

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In order to improve the production indicators of dairy cattle and modify the desirable frequency of genes in a population, genetic evaluation and selection programs are employed. These programs, based on knowledge of genetic parameters, have frequently been used for the selection of traits such as milk yield and composition, udder conformation, and animal longevity. Thanks to technological advances in milking equipment, it is easier to measure yield, milk flow rate, and electrical conductivity, characteristics that can be included in a selection scheme[1,2].

In order to increase animal production, it is important to know the genetic and environmental factors, and to work on those that can be improved[3]. The morphology of the animals is usually the first direct indicator of milk production and of the ease of correct and fast milking. Nonetheless, it should be borne in mind that environmental factors must be considered in order to express genetic potential[4].

The milk flow rate trait can be considered of great importance because it is associated with milking efficiency and udder health. The higher the milk flow rate, the shorter the time spent using milking labor and machinery, which significantly influences the economy of the establishment[5,6]; however, a higher milk flow rate decreases the tension of the teat sphincter, which increases the risk of mastitis and is associated with a greater number of somatic cells (SCC). On the other hand, slow flow rate is associated with incomplete milk extraction, which causes increased intramammary tension. Although increasing the milking speed per cow reduces costs, when planning to make selection based on milk flow rate, it is advisable to keep the flow rate at a medium level[7,8]. The speed of milk ejection depends on the pressure accumulated within the mammary gland. Thus, a greater amount of milk stored in the udder

increases the intramammary pressure, with the consequent increase in the speed of milk let down. The release of oxytocin into the bloodstream is essential to trigger the let-down and ejection of milk[9].

The milking routine, the machine, and the animal itself are factors directly related to milk flow rate. There is a great influence of the milking technique and the vacuum level of the machine; for example, a vacuum higher than specified results in an increase in milk flow rate but irritates the nipple lining. At the same time, flow rate measurement allows the identification of animals with longer milk ejection times, which are negatively associated with production per milking[10,11]. One of the main problems is how to measure flow rate; Tancin *et al*[12] concluded that the maximum flow rate is a biologically significant measure since as the maximum milk flow rate increases, total milking time and the duration of plateau phase decrease. In addition, flow rate measurement has been used to monitor the efficiency of the milking equipment and estimate the production per milking[13], as well as to establish the most appropriate flow rate that determines the end of milking and does not affect cow comfort[14].

Electrical conductivity (EC) is the ability of a solution to conduct electric current; it is related to the concentration and mobility of milk ions, 60 % depending on its dissolved salt content[15]. EC has been considered as a trait that indicates udder health, being used for the prediction of mastitis in goats and cows[15,16,17]; the information is easy to record in automated systems. Therefore, EC could be useful not only for cow management but also as a selection trait. It has been mentioned that the high correlation between SCC and EC values holds promise for improving mastitis resistance and functional capacity of dairy cows. In automated systems, EC records are available within a few seconds after milking, making EC information useful for early detection of mastitis.

EC may fluctuate; it may vary between quarters, between milking phases, and due to the presence of mastitis. The components of the milk can also play a role as any change in the ion concentration will be reflected in the EC[16]. Other studies[18] have reported that milk production and EC change significantly at least one day before the onset of clinical mastitis.

Robotic milking systems (RMSs) record milking parameters related to yield, EC, and quarter and total flow rate for each event[19,20].

Genetic improvement programs are the cornerstone of increasing the efficiency of livestock production units and are based on increasing the frequency of desirable genes in a population of dairy cows[21,22]. The effectiveness of an improvement program will depend on the genetic variability of the population and, therefore, on the heritability of the traits to be improved. Carrying out genetic evaluations allows the identification of those animals with the greatest

genetic potential for the traits of productive interest. In genetic programs, the parameters of heritability, repeatability, and genetic correlations are estimated.

By means of heritability, the extent to which the phenotypic variance corresponds to the variance due to the genes is estimated. These components determine the response to selection, establish the strategy to be used in the improvement of traits of interest, and are essential for the successful construction of decisions in selection and genetic improvement programs[23].

Although electrical conductivity and flow rate are important for increasing milk yield and are closely related to a decrease in production costs, there is not enough information on their genetic components or the influence this information would have on milk production.

The work was carried out in a cowshed that has a DeLaval VMS^{TM} robotic milking system, located in the Municipality of El Marqués, Querétaro. This system permanently records the individualized information of each cow each time it enters the milking module. At each milking event, the system records the amount of milk produced per quarter and the total yield (kg), milking time (min), milk flow rate (kg/min), and conductivity (mS/cm).

The cows were integrated into the trial at the beginning of their lactation period; individual information was collected throughout the period. Information from cows that did not complete lactation for reasons beyond the control of the project was discarded.

The management of the animals was based on one-way traffic; that is, the animals could be in the trough area, in the stall area, or in the milking module area, and circulate in that order, but they could not return to previous areas. The cows came to the milking module voluntarily and attracted by the offer of concentrated feed in the trough located in the module. Feeding consisted of the permanent offer of a partially mixed ration and a limited supply of concentrate in the milking module; the diet was formulated to meet the nutritional requirements of the animals. The cows were permanently monitored to carry out the necessary activities related to reproductive and health maintenance aspects; all in accordance with the practices established by the Veterinarian responsible for the establishment.

Information on milk yield in kg/day (MiY), electrical conductivity in mS/cm (EC), mean milk flow rate (MnF) in kg/min, and maximum milk flow rate in kg/min (MxF) was collected daily from 110 cows, of which 47 were in first lactation (FL), 45 in second lactation (SL), 28 in third lactation (TL), and 17 in fourth or more lactations (FoL), giving a total of 137 lactations. Table 1 shows the values recorded by calving year and Table 2 by calving season for the above-mentioned characteristics. The seasons were defined as follows: Season 1, the first 3 mo of the year; Season 2, mo 4 to 6; Season 3, mo 7 to 9; Season 4, mo 10 to 12. The yields reported by the cowshed are within the usual parameters for a high producing herd in Mexico.

Table 1: Average, minimum, and maximum values of the traits of milk yield (MiY), electrical conductivity (EC), mean milk flow rate (MnF), and maximum milk flow rate (MxF) per calving year

Variable	N	Minimum	Maximum	Mean	Std Error
			Calving year 2018		
MiY, kg/day	9313	0.41	114.54	38.61	0.140
EC, mS/cm	9313	1.97	6.65	4.57	0.004
MnF, kg/min	9313	0.25	2.70	1.21	0.004
MxF, kg/min	9313	0.52	3.62	1.68	0.005
			Calving year 2019		
MiY, kg/day	21205	0.95	134.70	39.86	0.087
EC, mS/cm	21205	0.99	7.84	4.59	0.003
MnF, kg/min	21205	0.24	5.15	1.23	0.002
MxF, kg/min	21205	0.37	13.49	1.72	0.003
			Calving year 2020		
MiY, kg/day	4965	5.83	85.18	41.92	0.180
EC, mS/cm	4965	1.57	6.85	4.77	0.008
MnF, kg/min	4965	0.35	2.21	1.18	0.004
MxF, kg/min	4965	0.54	3.00	1.66	0.005

Table 2: Average, minimum, and maximum values of the traits of milk yield (MiY), electrical conductivity (EC), mean milk flow rate (MnF), and maximum milk flow rate (MxF) by calving season

Variable	N	Minimum	Maximum	Mean	Std Error
			Calving season 1		
MiY, kg/day	8576	2.66	91.21	39.97	0.130
EC, mS/cm	8576	1.57	6.85	4.64	0.005
MnF, kg/min	8576	0.30	2.24	1.13	0.003
MxF, kg/min	8576	0.55	3.00	1.59	0.003
			Calving season 2		
MiY, kg/day	5408	6.44	84.85	39.20	0.153
EC, mS/cm	5408	0.99	6.38	4.54	0.006
MnF, kg/min	5408	0.24	2.19	1.24	0.004
MxF, kg/min	5408	0.37	2.96	1.72	0.004
			Calving season 3		
MiY, kg/day	6697	4.76	88.01	40.67	0.137
EC, mS/cm	6697	1.75	7.84	4.58	0.006
MnF, kg/min	6697	0.30	2.35	1.29	0.004
MxF, kg/min	6697	0.52	3.14	1.72	0.004
			Calving season 4		
MiY, kg/day	14802	0.41	134.70	39.58	0.120
EC, mS/cm	14802	1.34	7.50	4.64	0.004
MnF, kg/min	14802	0.25	5.14	1.24	0.003
MxF, kg/min	14802	0.52	13.49	1.75	0.004

The information was recorded daily, obtaining 1 to 4 records per day. For MiY, the total yield of each milking was summed per day, while for EC, MnF, and MxF, the values were averaged per day (total and per quarter). To make the lactation curve, the daily MiY was added, and then per week. To calculate the genetic parameters, a total of 137 observations were obtained for MiY, EC, MnF, and MxF (Table 3).

Table 3: Average, minimum, and maximum overall values of the traits of milk yield (MiY), electrical conductivity (EC), mean milk flow rate (MnF), and maximum milk flow rate (MxF).

	MiY, kg/day	EC, mS/cm	MnF kg/min	MxF kg/min
Average	39.82	4.61	1.22	1.70
Minimum	0.41	0.99	0.23	0.37
Maximum	134.7	7.84	5.14	13.49
Std Error	0.069	0.002	0.001	0.002

In order to identify the genetic effects, it was necessary to consider and correct for the environmental effects that could have an effect on the variables studied. Therefore, environmental effects were represented in the model, including the year and season of calving and the age of the animal at calving; in addition, the possibility of having permanent environmental effects (common to the same animal, but not genetic) was also considered as there was more than one record per animal.

The variance components for MiY, MnF, MxF, and EC were estimated per lactation with a repeatability animal model, eliminating atypical and extreme data. A mixed linear model was used, which included as fixed effects: the number of calvings/year/calving season (four seasons depending on the month of calving: January-March, April-June, July-September, and October-December). The animal and the permanent environment were included as random effects.

The estimators of the variance and covariance components were performed by means of restricted maximum likelihood and the heritabilities, repeatability, and genetic correlations were calculated from the variance components, using the BLUPF90 suite programs[24].

To estimate variance components, the model used was:

$$y_{ijklmn} = \mu + year_i + season_j + numc_k + animal_l + perenv_m + e_{n(ijklm)}$$

Where:

y_{ijklmn}= vector of observations of interest (MiY, EC, MnF, and MxF) corresponding to observation n in calving year i, calving season j, calving number k, animal l, permanent environment m;

year$_i$= effect of the calving year i;

season$_j$= effect of calving season j (from 1 to 4);

numc$_k$= effect of calving number k (from 1 to 4);

animal$_l$= genetic random effect of the animal l,

perenv$_m$= random effect of the permanent environment m;

***e*$_{n(ijklm)}$**= vector of the error or residual effects of observation n within animal l, calving year i, calving season j, and calving number k.

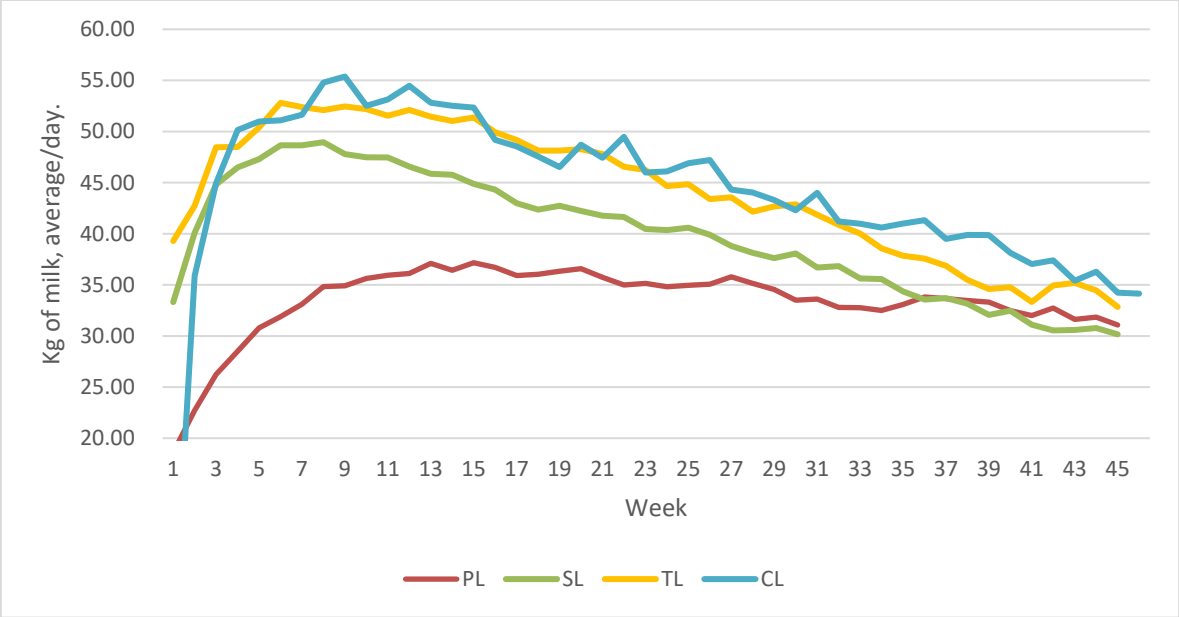
To estimate the components of covariance, bivariate analyses were performed using the following matrix model:

$$\begin{bmatrix} y_1 \\ y_2 \end{bmatrix} = \begin{bmatrix} X_1 & 0 \\ 0 & X_2 \end{bmatrix} \begin{bmatrix} b_1 \\ b_2 \end{bmatrix} + \begin{bmatrix} Z_1 & 0 \\ 0 & Z_2 \end{bmatrix} \begin{bmatrix} u_1 \\ u_2 \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \end{bmatrix}$$

Where subscripts 1 and 2 identify the pair of traits to be evaluated, ***y*** (MiY, EC, MnF, and MxF), b= fixed-effect vector (calving/year/calving season), u= random-effects vector (animal and permanent environment), X and Z are incidence matrices for vectors b and u, respectively; *e*= vector of error or residual effects.

Figure 1 shows the milk yield of cows by lactation number. First calving cows had lower yield and yield peak and greater persistence than cows with more lactations.

Figure 1: Daily milk yield over 45 weeks in cows in first lactation (FL), second lactation (SL), third lactation (TL), and fourth or more lactations (FoL)



The variance components calculated for milk yield, electrical conductivity, mean flow rate, and maximum flow rate, as well as heritability and repeatability are shown in Table 4.

Table 4: Variance components for milk yield (MiY), electrical conductivity (EC), mean milk flow rate (MnF), and maximum milk flow rate (MxF)

	MiY	EC	MnF	MxF
σ^2_A	183.60	0.110	0.055	0.065
σ^2_{PE}	23.74	0.099	0.082	0.109
σ^2_e	87.78	0.129	0.025	0.052
h^2	0.62	0.44	0.33	0.28
r^2	0.70	0.48	0.84	0.77

σ^2_A= additive genetic variance; σ^2_{PE}= variance of the permanent environment; σ^2_e= residual variance; h^2= heritability; r^2= repeatability.

The heritability of milk yield (0.62) was higher than the estimated values in Holstein cattle in Mexico; it has been reported between 0.17 and 0.49 for the first lactation and between 0.16 and 0.41 for the first five lactations[23]. A medium-high heritability value (0.44) was estimated for EC, similar to that estimated by other authors[25,26]; under automated milking conditions, as in this study, heritability was reported to fluctuate between 0.38 and 0.49[27]. It has been argued that the heritability of EC is important because the genetic correlations

between EC and mastitis have been estimated to range from 0.65 to 0.8; therefore, obtaining the genetic response for mastitis should be possible by using EC information in genetic evaluation[17].

The estimated heritabilities for MnF and MxF were medium (0.33 and 0.28); in cows managed in automated milking systems, values of 0.47 to 0.58 were reported for MnF[27]; similarly, in Italian Holstein-Friesian cows[28] under traditional milking, high heritability (0.50) was obtained for the initial milk flow rate and high heritability (0.54) for MxF.

Table 5 presents the genetic correlations for the traits studied. A negative correlation was estimated for MiY and EC (-0.167); other authors[29] also reported a negative correlation (-0.12), which suggests that selection made to increase milk production decreases EC.

Negative correlations (-0.612) were estimated for MiY and MnF, as well as for MiY and MxF (-0.767); in contrast, other authors[30], who worked with Jersey cows in tropical climates, estimated positive genetic correlations for these traits (0.46 to 0.89). The above should be reviewed since if the genetic correlations were negative as reported here, the increase in milking time would not be proportional to the increase in yield as the flow rate of milk would decrease, with significant decreases in milking efficiency.

Table 5: Genetic correlations between milk yield (MiY), electrical conductivity (EC), mean milk flow rate (MnF), and maximum milk flow rate (MxF)

	MiY	**EC**	**MnF**	**MxF**
MiY	1	-0.167	-0.612	-0.767
EC		1	0.3546	0.5351
MnF			1	0.7422
MxF				1

The relationships of EC with MnF and MxF were 0.35 and 0.53, respectively, values that contrast to those reported by some authors who worked with dairy goats and showed a negative relationship for MxF and EC (-0.003)[31].

The positive correlations found between the flow rate and conductivity traits allow to infer that the selection programs may be based on one of the three traits and show progress. Nevertheless, the negative correlations between milk yield and these characteristics, especially with flow rate traits, are contrary to what has been reported by other authors[30] and present a challenge for producers since milk yield is the most economically important characteristic in the production system and its improvement implies deterioration in the other traits.

The correlations between milk flow rate traits were high and positive (0.74), so it is not necessary to select for both variables when increasing milk flow rate is desired.

The present results allow a better understanding of the relationships between flow rates (average and maximum), conductivity, and milk yield, and indicate that the selection made to increase milk production has decreased EC, which implies that the average levels of EC will have to be recalculated periodically in order to interpret this parameter correctly. However, this improvement in MiY is associated with decreases in milk flow rates, so there is the potential to improve the efficiency of milk production by shortening the duration of milkings through increased flow rate, with consequent savings in milking costs. Negative genetic correlation will make the individual selection of these traits difficult, and their improvement will require the development of selection indicators that allow both traits to be improved at the same time.

Acknowledgments and conflict of interest

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The authors state that there is no conflict of interest.

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Technical note

Fatty acids and terpenes from the methanolic extract of *Artemisia cina* as possible compounds responsible for the ovicidal effect on *Haemonchus contortus*

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Abstract:

Haemonchus contortus is a hematophagous nematode with a high reproduction rate, considered to be the main problem in grazing small ruminants. Therefore, treatment

alternatives based on the use of plant extracts are sought. This study aimed to evaluate the ovicidal activity of *Artemisia cina* against the parasite *Haemonchus contortus* and to chemically characterize the extract with the highest biological activity through gas chromatography coupled with mass spectrometry (GC-MS). The extracts to be evaluated were obtained through the maceration technique using methanol, ethyl acetate, and n-hexane. The extracts were taken to total dryness and challenged against *H. contortus* eggs using the egg-hatching inhibition technique described by the World Association for the Advancement of Veterinary Parasitology (WAAVP). The methanolic extract (ME) showed 100 % ovicidal activity at a concentration of 4.25 mg/ml, being the most active at a low concentration; therefore, it was characterized by GC-MS. ME mainly contains fatty acids and terpenes; among them are hexadecanoic acid and 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl][cyclohex]-1-en-carboxyaldehyde. The characterized compounds have shown previously reported anthelmintic activity so that ovicidal activity may be associated with them. In conclusion, the methanolic extract of *A. cina* had a higher ovicidal activity at low concentrations; this is probably due to the presence of fatty acids and terpenes.

Keywords: *Artemisia cina*, *Haemonchus contortus*, Egg hatching, Anthelmintic.

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Haemonchus contortus infection is one of the biggest challenges faced by sheep and goat production worldwide. This nematode parasite is highly virulent and has a great economic impact due to the loss of production and the need to control the infection[(1)].

Resistance to commonly used anthelmintics has been a growing problem in the fight against *H. contortus*[(2)]. Although options for sustainable management, such as those contemplated by integrated parasite control, are a strong alternative, more studies are still required to validate the methods and contribute to reducing resistance or protecting molecules present on the market[(3)].

The host's immune response to the presence of *H. contortus* is a crucial factor in counteracting the infection so that individuals in the herd can be classified as resistant, resilient, or susceptible animals. Resistance is associated with a zero parasite load and a low impact on the productive parameters of the herd[(4)]. However, susceptible individuals, who are sometimes fewer in number but who are severely affected by nematodiasis damage, require frequent treatments, inducing problems due to anthelmintic resistance. Therefore, it is necessary to identify new molecules with antiparasitic potential[(4)].

The identification of new molecules and compounds that have activity against parasites is an area in constant evolution. Some plants have been shown to have anthelmintic properties, including effects against *H. contortus*[5]. Currently, antiparasitic control must focus on sustainable strategies that have the least possible impact on the environment, in addition to reducing pressure on nematode strains in constant mutation and selection to genes associated with resistance. Efforts should be directed towards searching for compounds that can cause damage to the parasite, reducing its populations, or even reversing the overexpression of genes responsible for anthelmintic resistance[6].

The genus *Artemisia* contains different species with proven anthelmintic activity, including *A. cina*, which has been used in traditional medicine as an antiparasitic along with plants of the same species, and they have excellent effects on intracellular parasites, nematodes, or even cestodes[7].

The genus *Artemisia* biosynthesizes different secondary metabolites such as sesquiterpenes, diterpenes, sterols, phenoxychromes, phenylpropanes, flavonoids, coumarins, isoprenylcoumarin, caffeoylquinic acid, acetylenes, and lignans that are responsible for anthelmintic activity[8]. Among the molecules with reported antiparasitic activity are artemisinin, santonin, norisoguaicin, and 3'-demethoxy-6-O-demethylisoguaiacin[9-11]. Other authors, such as Sakipova *et al*[10], have reported the presence of artemisinin and santonin[10]. *Artemisia cina* has been shown to be a plant with a high anthelmintic potential for nematodes and cestodes of veterinary importance[8,12]. This study aimed to evaluate the ovicidal activity of *Artemisia cina* against the parasite *Haemonchus contortus* and to propose the structures of the major volatile molecules of the extract with the highest anthelmintic activity through gas chromatography coupled with mass spectrometry.

Plant material: The previously dried and ground aerial parts of *Artemisia cina* in the pre-flowering state (40 to 60 cm in height) were provided by Hunab® laboratories, Mexico, who produce the plant commercially under the following conditions: humidity of 24.6 %, pH 8.7, and salinity of 1.6 %. The plant with voucher number No. 11967 was identified as *Artemisia cina* by Dr. Alejandro Torres-Montúfar from the herbarium of FES Cuautitlán, Cuautitlán, State of Mexico.

Obtaining the plant extract: Samples of approximately 1 kg of plant material were used to perform the solvent extraction by maceration for 72 h at room temperature, using methanol, ethyl acetate, and n-hexane to obtain extracts of high, medium, and low polarity, respectively. After the maceration time, it was filtered using gauze, cotton, and filter paper (Whatman® #4). The resulting filtrate was concentrated at reduced pressure at 40 °C and 100 rpm in a DLAB RE-100 Pro® rotary evaporator. The resulting extract was vacuum-dried and stored in a desiccator at reduced pressure until use.

Thin layer chromatographic (TLC) analysis: Merck® aluminum TLC plates were used with the following conditions: silica gel 60 F254. The mobile phase used to perform the elution of the extracts was 5:5 *n*-hexane:ethyl acetate. For each lane, 15 µl of a solution of 16 mg/ml of each extract and the reference was applied; therefore, a higher intensity of the bands corresponds to a higher concentration. The reference was the *n*-hexane extract reported by Higuera Piedrahita *et al*[21], of which the anthelmintic activity of *A. cina* on *Haemonchus contortus* eggs is reported. The chromatography plates were checked at two wavelengths (254 and 365 nm) before being developed with ceric sulfate. The retention factor (R_F) was calculated with the following equation:

$$Rf = \frac{\text{Solute distance traveled}}{\text{Solvent distance traveled}} \qquad (1)$$

Egg hatching inhibition (EHI): The eggs of *Haemonchus contortus* were obtained from the strain isolated and kept in the FES Cuautitlán. The EHI was performed in 96-well ELISA plates; the protocol used was the one reported by Coles *et al*[13], where 100 eggs were used per well with four replications; the eggs exposed to the treatments were incubated in a wet chamber for 48 h before reading. The EHI reading was performed using an iodine-lugol solution, which was added to each well after incubation. The number of unhatched eggs (dead and larval) and larvae 1 were counted to determine the percentage of egg hatching inhibition using a microscope with 10 X magnification (Olympus, model CK-2, Japan®). Ivermectin (5 mg/ml) was used as a positive control, and water as a negative control. The photographs were taken on the 40X lens using an HK-10 CMOS camera and the ISCapture V3.6.6 software.

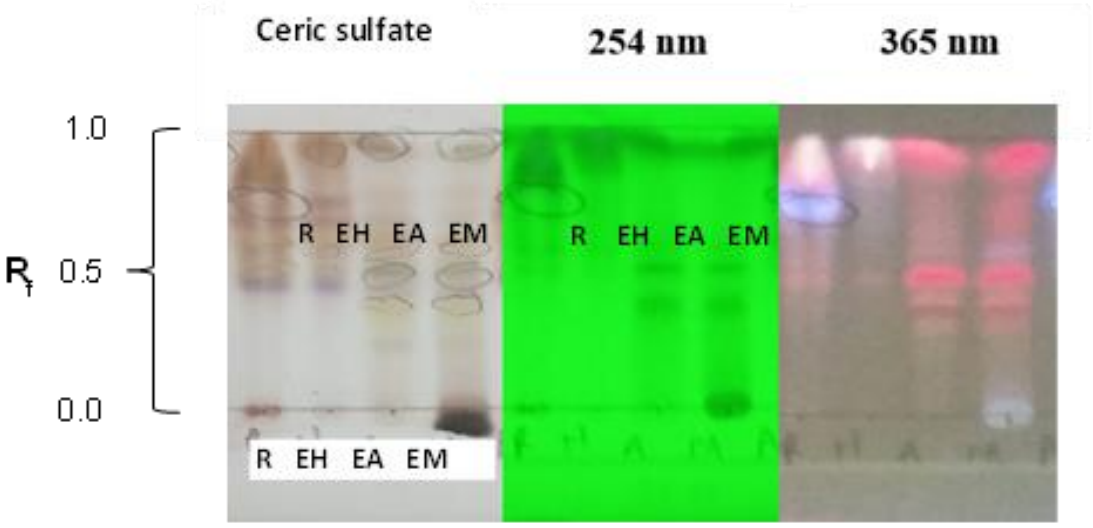
Gas chromatography coupled with mass spectrometry (GC-MS): The volatile components present in the crude extract with the highest activity in inhibiting the hatching of *H. contortus* of eggs were analyzed by GC-MS using an Agilent Technologies HP 6890 gas chromatograph coupled with an MSD 5973 quadrupole mass detector (HP Agilent) and an HP-5MS capillary column (length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 µM). A constant flow of helium as a carrier gas was adjusted to the column at 1 mL/min. The inlet temperature was set at 250 °C, while the furnace temperature was initially kept at 40 °C for 1 min and increased to 280 °C at intervals of 10 °C/min. The mass spectrometer was used in positive electron impact mode with an ionization energy of 70 eV. The detection was performed in selective ion monitoring mode. The signals were identified and quantified using target ions. The compounds were identified by comparing their mass spectra with the NIST library version 1.7a. The relative percentages were determined by integrating the signals using the GC Chem Station software, version C.00.01. The composition was reported as a percentage of the total signal area.

Statistical analysis: Three replications were performed in duplicate for each extract. The LC_{50} and LC_{90} were calculated through a PROBIT analysis using the SAS 9.0 software. The mean and its standard error were obtained for each extract; a Tukey's multiple comparison of means was performed at 95 % confidence using the Statgraphics program.

Extraction by polarity of *Artemisia cina* extracts allowed the following yield percentages to be obtained: methanol extract (ME) had a yield percentage of 4.1 %, ethyl acetate (EA) 3.86 %, and *n*-hexane (EH) extract 1.09 %. The ME was the one that presented the highest yield, followed by EA and EH.

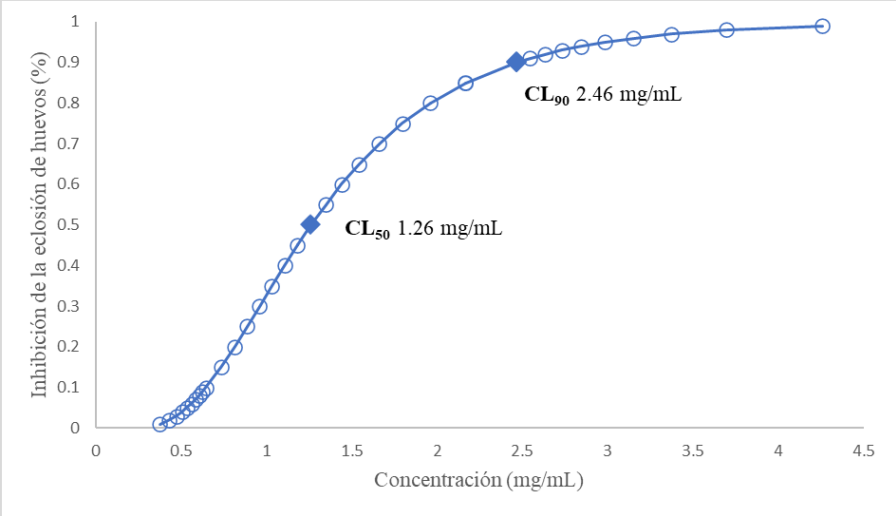
In the comparison of the chemical profile of the different extracts through thin layer chromatography (TLC), an *n*-hexane:ethyl acetate (5:5) system was used, which allowed the separation of a bigger number of bands than other systems. In this system, it was possible to observe the difference in the chemical profile of each of the extracts, where EH presents the highest concentration of compounds between the retention factors (R_F) 0.5 and 1.0, EA between 0.4 and 0.7, and ME in 0.0. According to the R_F and intensity of the bands, the compounds present in EH are mainly of low polarity, those of the EA are of medium polarity, and those of the ME are of higher polarity compared to the other extracts (Figure 1). The resulting extracts were obtained employing a simple maceration using a different plant material for each solvent, avoiding exhaustive extractions.

Figure 1: Reference thin-layer chromatography (R), extracts of *n*-hexane (EH), ethyl acetate (EA), and methanol (ME) and Mobile phase:*n*-hexane: 5:5 ethyl acetate, developer: ceric sulfate



Once the difference in the chemical composition of the three extracts was observed, the inhibition of *H. contortus* eggs hatching was evaluated. A dose-response relationship was observed (Figure 2) in the EHI, which allowed the use of the Probit analysis to calculate the LC_{50} and LC_{90} of the three extracts.

Figure 2: Lethal concentrations LC_{50} and LC_{90} required to inhibit hatching of *H. contortus* eggs after 48 h incubation with an *Artemisia cina* methanolic extract determined by PROBIT analysis



The ME had the highest EHI (LC_{50} 1.26 mg/ml and LC_{90} 2.46 mg/ml), > followed by EA (LC_{50} 2.42 mg/ml and LC_{90} 3.80 mg/ml) and > EH (LC_{50} 3.08 mg/ml and LC_{90} 3.84 mg/ml). In other words, a greater effect of EHI was observed as the polarity of the extracts increased (Table 1).

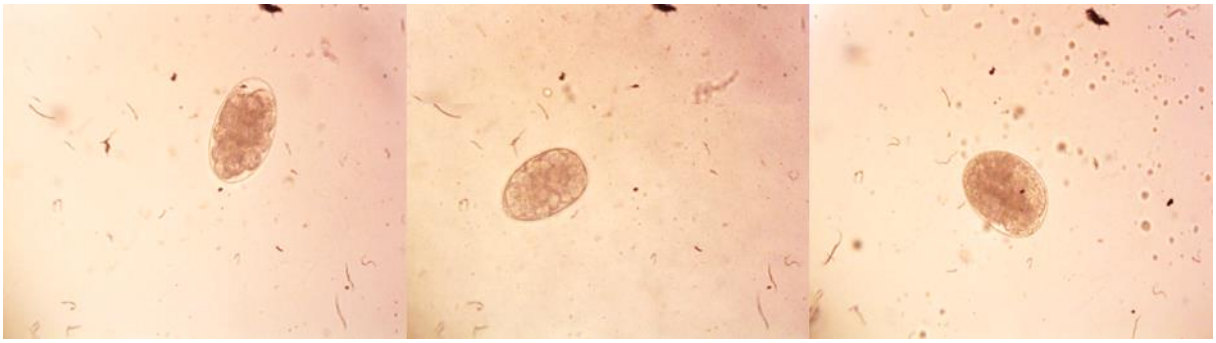
Table 1: Percentage of inhibition of hatching of *Haemonchus contortus* eggs exposed to *n*-hexane, ethyl acetate, and methanolic extracts of *Artemisia cina*

Treatment	LC_{50} (mg/ml)	LC_{90} (mg/ml)
n- hexane	3.08 (2.96 – 3.18)^{a}	3.84 (3.70 – 4.07)^{a}
Ethyl acetate	2.42 (2.27 – 2.55)^{b}	3.80 (3.64 – 4.10)^{a}
Methanol	1.26 (1.18 – 1.34)^{c}	2.46 (2.32 – 2.66)^{b}

^{ab} Equal letters indicate no significant difference between groups. Duncan $\alpha<0.05$.

Photographs were taken of the eggs observed under the microscope at 40X subjected to ME, and hatching inhibition was observed in the treatment with ivermectin and larval eggs in the treatment with methanolic extract (Figure 3). Figure a shows a morulated egg exposed to distilled water without damage before 48 h of exposure to the treatments. It should be noted that at 48 h, eggs exposed to distilled water developed into larvae 1.

Figure 3: *Haemonchus contortus* eggs observed at 40X under different conditions: a) negative control with water; b) positive control of ivermectin (5 mg/ml); c) methanolic extract of *Artemisia cina* at 2.46 mg/ml after 48 h of exposure



a) **b)** **c)**

The ME showed the highest inhibition of egg hatching at lower concentrations compared to the other extracts. Therefore, the main volatile compounds of the ME were determined through GC-MS, and the structure of the primary compounds was proposed according to the fragmentation pattern, which were compared with the NIST library. Considering the above, about 15 different volatile compounds are proposed, of which three are fatty acids, and 12 are terpenes (Table 2).

Table 2: Volatile compounds present in the methanolic extract of *Artemisia cina*

Compound	Retention time (min)	Name	Molecular weight (m/z)	% of area	Type of compound
(1)	9.20	4H-Pyran-4-one, 2,3-dihydro- 3,5 dihydroxy-6-methyl.	144	8.376	Hemiterpene
(2)	11.85	Dihydro aromadendrene	202	1.519	Bicyclic sesquiterpene

(3)	12.85	Caryophyllene	204	0.891	Bicyclic sesquiterpene
(4)	14.91	Caryophyllene oxide	220	9.601	Bicyclic sesquiterpene
(5)	16.80	Spathulenol	220	5.256	Bicyclic sesquiterpene
(6)	17.54	(-) Spathulenol	220	8.552	Bicyclic sesquiterpene
(7)	10.80	Platambin	238	2.794	Bicyclic sesquiterpene
(8)	18.94	Hexadecanoic acid	256	19.185	Saturated fatty acid
(9)	20.241	Phytol	296	3.691	Linear diterpene
(10)	20.55	9-12 Octadecanoic acid (Z,Z)	290	8.463	Unsaturated fatty acid
(11)	20.63	9-12-15 Octadecatrienoic acid methyl ester (Z,Z,Z)	292	9.736	Unsaturated fatty acid
(12)	21.390	Azulene [6,5-b] furan -2,5-dione, decahydro-4a,8 dimethyl-3-methylene, [3aR-(3aα, 4aβ, 7aα, 8β, 9aα)]	248	1.497	Sesquiterpene lactone
(13)	23.07	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl) hexa-1,3,5-trienyl][cyclohex-1-en-carboxyaldehyde]	280	13.677	Bicyclic diterpene

(14)	23.42	Spiro [7H-cyclohepta[b]furan 7,2'(5H')-furan]-2,5'(3H)-dione, octahydro-8-hydroxy-6,8-dimethyl-3-methylene, [3aS-(3aα, 6β, 7α, 8α,8aα)]	280	2.772	Sesquiterpene lactone
(15)	25.77	Azulene [6,5-b] furan -2,5-dione, decahydro-4a,8 dimethyl-3-methylene, [3aR-(3aα, 4aβ, 7aα, 8β, 9aα)]	248	3.989	Sesquiterpene lactone

In general, the volatile compounds of ME are mainly terpenes and some fatty acids, with sesquiterpenes being the most chemically diverse. Figure 4 shows seven compounds that, according to the percentage of the area under the curve of the total compounds (≥ 8 %), could be considered as the main. According to the fragmentation pattern of the seven major volatile compounds of the *Artemisia cina* ME, the proposed structures are shown in Table 3.

Figure 4: GC-MS chromatogram of the chemical compounds present in the methanolic extract of *Artemisia cina*

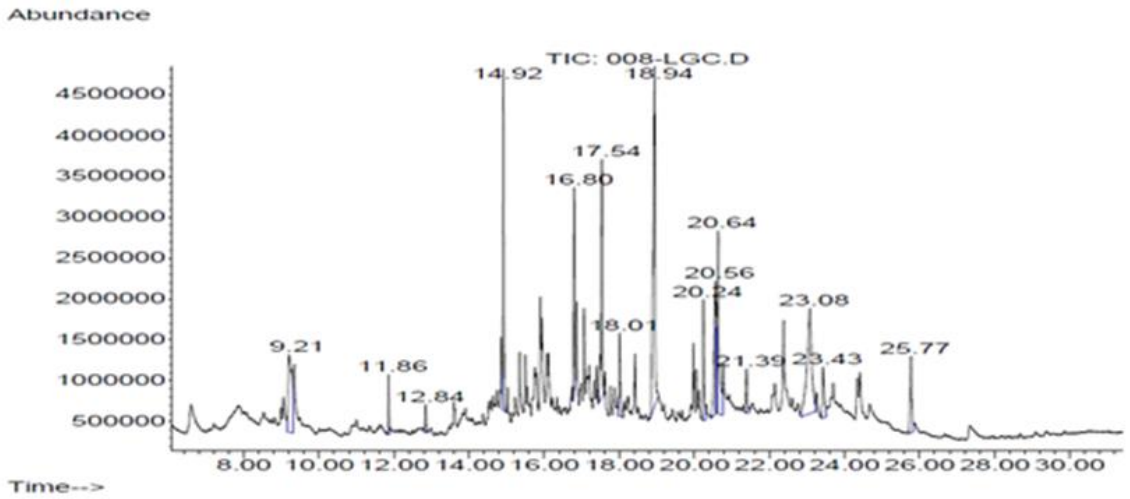
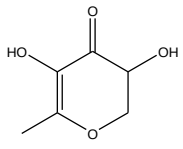
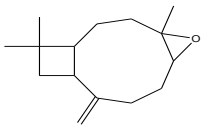
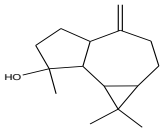
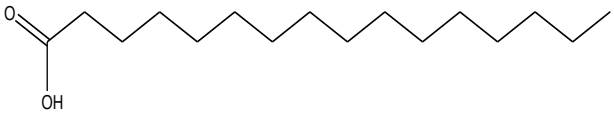
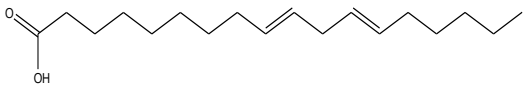
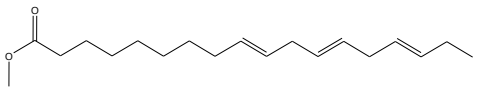
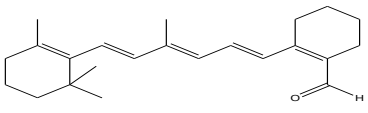


Table 3: Major volatile compounds of the methanolic extract of *Artemisia cina* determined through GC-MS

Compound	RT	Proposed structure
(1)	9.20	
(4)	14.92	
(6)	17.54	
(8)	18.94	
(10)	20.55	
(11)	20.63	
(13)	23.07	

RT= retention time (min).

According to Table 2, the possible major volatile compounds are terpenes and fatty acids. Of the terpenes, the following are present: compound (**1**) a hemiterpene, (**4**) a bicyclic sesquiterpene, (**6**) a tricyclic sesquiterpene, and (**13**) a bicyclic diterpene. Of the fatty acids: (**8**) a saturated fatty acid, (**10**) an unsaturated fatty acid, and (**11**) an unsaturated and esterified fatty acid. Hexadecanoic acid (**8**) is the most abundant in ME, followed by 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl][cyclohex-1-en-carboxyaldehyde (**13**).

The egg-hatching inhibition (EHI) of ME can be attributed to the presence of saturated and unsaturated fatty acids, such as hexadecanoic acid, which is the most abundant in the *Artemisia cina* ME. Pineda-Alegría *et al*[14] evaluated pentadecanoic acid $CH_3(CH_2)_{13}COOH$), hexadecanoic acid $CH_3(CH_2)_{14}COOH$) (**8**), and stearic acid ($CH_3(CH_2)_{16}COOH$) and found an increase in the EHI by increasing the number of carbons of unsaturated fatty acids, with the most active being palmitic and stearic acids at a dose of 20 mg/ml, where they obtained 100 % EHI of *H. contortus*. Considering that the ME presented LC_{100} in the EHI of 4.25 mg/ml and that one of its primary compounds is hexadecanoic acid, it could be thought that there is a synergism with the other chemical compounds present in ME. Due to the nature of these fatty acids, they could be the potential compounds with ovicidal activity of ME.

The presence of secondary metabolites in plants is a consequence of their interaction with the surrounding environment. These interactions are typical of the biotic and abiotic factors of the place where the plant is located. Regarding the phytochemical profile of the ME of *A. cina* used in this study and the concentration, it is a response to the controlled conditions of the crop since the plant material was obtained from a greenhouse[15].

The ME of *A. cina* has a high content of terpenes, which have been reported with a high ovicidal activity against gastroenteric nematodes of ruminants. These terpenes are caryophyllene oxide (**3**) and spathulenol (**5**), which are part of the main compounds (15.4 % and 5.1 %, respectively) of the essential oil of *Achyrocline satureioides*[16], which have an EC_{50} of 10.42 mg/ml in the EHI on *H. contortus*, compared to the EC_{50} of 1.42 mg/ml in the EHI of the *Artemisia cina* ME, in which (**3**) and (**5**) are present in 9.60 % and 5.25 %.

Considering the above, it is hypothesized that fatty acids and terpenes have different mechanisms of action and could be working together, thus generating a pharmacodynamic interaction[15], in this case, a synergism. Synergism occurs when the effect or response of the mixture is greater than the sum of the combination of the drugs alone[15]. Although it is not common to find drug interactions between chemical compounds, it is desirable to find synergisms between them, as they could be the basis for implementing a drug combination therapy, which could reduce the side effects that usually occur in drug monotherapy[17], which could be an excellent alternative to the use of anthelmintics due to the resistance that currently

exists. A particularity of secondary metabolites is that they are multitarget due to the presence of different functional groups[18]. This synergistic effect should be tested in future studies.

Although terpenes and fatty acids are typical for the genus *Artemisia*, only the presence of santonin, pectolinarigenin[10], 3'-demethoxy-6-O-demethylisoguaiacin, norisoguaicin[19], artemisinin and derivatives[20] has been reported in the *A. cina* plant. Therefore, this work reports the presence of three fatty acids and twelve terpenes other than artemisinin in *A. cina*, of which there is no report. The anthelmintic activity of *A. cina* has been attributed mainly to the *n*-hexane extract[19-21]; for the specific case of the EHI, it was found that the activity increases as the polarity of the extracts increases, thus opening a new perspective to design a phytomedicine with anthelmintic effect.

All the evaluated extracts of *Artemisia cina* showed inhibitory activity of *Haemonchus contortus* egg hatching, with the methanolic extract (ME) being the one that presented the highest activity. ME contains 15 different volatile compounds, of which three are fatty acids and 12 terpenes. Hexadecanoic acid and 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl][cyclohex-1-en-carboxyaldehydehyde] are the major compounds, which are presumed to be responsible for the ovicidal activity.

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Conflict of interest

The authors declare no conflict of interest.

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Technical note

Frequency and factors associated with the diagnosis of *Ehrlichia canis* and *Anaplasma* spp. in dogs

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Abstract:

This study assesses the number of reported cases of canine anaplasmosis and ehrlichiosis in the district of Rímac, Lima, Peru, as well as their association with factors involved in the occurrence of these diseases. In these cases, the presence of anemia and thrombocytopenia is common, which affect normal hematological parameters. All the medical records of the 2018-2021 period of canine patients of the Municipal Veterinary Clinic of Rímac located in the district of Rímac, Lima – Peru, were sampled. The Chi-square statistical test and the contingency coefficient were used to determine the association. All variables were also analyzed using logistic binomial regression. A significance level of 0.05 was used. *Ehrlichia canis* and *Anaplasma* spp. were diagnosed in 4.308 % (224/5,200) of medical records. The Chi-square test was used to evaluate the association with the factors of sex, race, age, and season of the year, concluding that there was an association of the diseases with the age group; at a 95 % confidence interval, it was observed that the frequency of cases of *E. canis* and *Anaplasma* spp. was 95.98 % and 1.79 %, respectively, and the co-infection of both pathogens was 2.23 %. The logistic regression model included the effects of live weight and sex on the diagnosis of ehrlichiosis and anaplasmosis, which were significant. There was a significant association between the diagnosis of canine ehrlichiosis and anaplasmosis with age and weight, but there was no effect of breed and season of the year.

Keywords: Anaplasmosis, Anemia, Ehrlichiosis, Medical records, Thrombocytopenia.

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Canine ehrlichiosis, considered one of the most important diseases affecting dogs, has a wide worldwide distribution and is caused by the infectious agent *Ehrlichia canis*, with co-infection with *Anaplasma* spp. (*A. phagocytophilum* and *A. platys*) being common, also transmitted by *Rhipicephalus sanguineus* ticks, which enhances its clinical signs[1], being mostly identified in areas where *E. canis* is endemic[2,3]. Infection may be suspected when the dog lives in or travels to an endemic region or has had previous exposure to ticks, with the common diagnostic techniques being hematology, cytology, serology, and isolation, but the definitive diagnosis requires molecular techniques[4]. In addition, it is common in warm and temperate climates, such as the summer season, where the vector is active[5]. Being on the street, sex, age, German shepherd breed, tick infestation, and not using ectoparasiticides have been mentioned as factors associated with the diagnosis of diseases[6].

Canine ehrlichiosis was first reported in 1982 in Peru, and since then, cases have increased[7]. Metropolitan Lima reported a prevalence of canine ehrlichiosis of 4.05 % in the north, 11.5 % in the center, 33.78 % in the south, 43.24 % in the east, and 7.43 % in the west[8]. In the districts of Chorrillos, La Molina, and San Juan de Miraflores, 16.5 % of positive cases were reported in 2001[9], another study reported 31.1 % of cases of *E. canis* in Chorrillos in 2019[10], and in 2020 an increase in positive cases of ehrlichiosis was reported, with 59.4 % in the northern zone[11]. Current studies conducted in metropolitan Lima reported a total of 29.2 % of positive cases for *Anaplasma platys*[12]. Due to the aforementioned information, the objective was to determine the frequency of canine ehrlichiosis and anaplasmosis and the degree of association of the factors of sex, the season of the year, breed, age, and live weight in the Municipal Veterinary Clinic of Rímac (MVCR) in the 2018-2021 period.

To achieve the proposed objectives, a basic, cross-sectional, retrospective, and descriptive study was developed. It has approval from the institutional ethics committee in research on animals and biodiversity of the Scientific University of the South (Code: 399-2021-PRE16). Information from medical records of the MVCR located in the district of Rímac, province of Lima, subregion of Lima-Centro, Peru, was used to carry it out. The climate is subtropical temperate desert with an average annual temperature of 19 °C, ranging between 14 and 30 °C. The average yearly rainfall is less than 15 mm, being more accentuated between July and August. The approximate casuistry per year is 150. The medical records collected were those of canines treated in the period between 2018 and 2021 in the MVCR that have been diagnosed as positive for *E. canis* or *Anaplasma* spp., using the Anigen CaniV-4 kit (BioNote Inc., South Korea), which has a sensitivity and specificity of 97.6 % and 99 % for *E. canis*, while for *Anaplasma* spp., it is 88.5 % and 97.1 %, respectively.

The reading of the medical records was considered to establish factors associated with the infections mentioned above, achieving the following study variables: number of diagnosed clinical cases (Table 1) and age at diagnosis, categorized into three groups (Table 2). For this grouping, it was considered that dogs at an early age present greater risks of being exposed to the vector than dogs considered elderly because when they complete the vaccination schedule, they begin to have regular walks outside[13,14]. Season of the year (Table 3) and breed (Table 4) were also considered.

The information collected was tabulated in the Microsoft Excel 2016 program. The Chi-square statistical test and the contingency coefficient were used to determine a preliminary association between the diagnosis and the associated factors. All variables were analyzed through a logistic binomial regression model (multivariate analysis) using the SPSS v.25 program for Windows, through which regression estimates, odds ratio (OR) 95 % confidence intervals, and significance values were obtained. The dependent variable was the evaluation diagnosis of each animal, and the independent variables were sex, breed, age group, and weight. A significance level of 0.05 was used for all calculations.

After processing and analyzing the information, the cases of *E. canis* and *Anaplasma* spp. represented 4.308 % (224/5,200) of the population, being 95.98 % (215/224) for *E. canis*, 1.79 % (4/224) for *Anaplasma* spp., and 2.23 % (5/224) for the co-infection of both pathogens. The significance of the association between the three groups of cases and sex, together with the contingency coefficient, is shown in Table 1. The ages of the dogs positive for both diseases ranged from 1 mo to 14 yr, with most of them being younger than 2 yr, with 47.76 % (107/224), followed by older than 3 yr, with 32.58 % (73/224) (Table 2). There were more cases of canine ehrlichiosis and anaplasmosis in the autumn season, with 40.18 % (90/224), followed by summer with 33.48 % (75/224) (Table 3). For the breed variable, the majority were crossbred, with 58.04 % (130/224). In crossbred and purebred dogs, canine ehrlichiosis accounted for the majority of cases, with 56.25 % (126/224) and 39.73 % (89/224), respectively, with the Shih Tzu breed standing out (Tables 4 and 5).

Table 1: Frequency of positive cases of canine ehrlichiosis and anaplasmosis associated with sex, with Chi-square *P*-value and contingency coefficient in parentheses

	E. canis	***Anaplasma* spp.**	***Co-infection* of *E. canis* and *Anaplasma* spp.**	**Medical records**	**Percentage of the total**	***P*-value**
F	80	4	0	84	37.5	0.072
M	135	0	5	140	62.5	(0.174)
T	215	4	5	224	100	

F= females; M= males; T= total.

Table 2: Diagnosis of canine ehrlichiosis and anaplasmosis associated with the age group, with Chi-square *P*-value and contingency coefficient in parentheses

	E. canis	***Anaplasma* spp.**	**Co-infection**	**Medical records**	**Percentage of the total**	***P*-value**
< 2 years	101	2	4	107	46.98	0.003 (0.283)
2-3 years	44	0	0	44	20.47	
>3 years	70	2	1	73	32.56	
Total	215	4	5	224	100	

Table 3: Diagnosis of canine ehrlichiosis and anaplasmosis associated with season of the year, with Chi-square *P*-value and contingency coefficient in parentheses

	E. canis	***Anaplasma* spp.**	**Co-infection**	**Medical records**	**Percentage of the total**	***P*-value**
Spring	22	1	1	24	10.71	
Summer	72	2	1	75	33.48	0.051
Autumn	89	1	0	90	40.18	(0.264)
Winter	32	0	3	35	15.63	
Total	215	4	5	224	100	

Table 4: Diagnosis of canine ehrlichiosis and anaplasmosis associated with breed, with Chi-square and contingency coefficient in parentheses

	E. canis	***Anaplasma* spp.**	**Co-infection**	**Total cases**	**Percentage of the total**	***P*-value**
Crossbred	126	3	1	130	58.04	0.774
Purebred	89	1	4	94	41.96	(0.074)
Total	215	4	5	224	100	

Table 5: Diagnosis of positive cases of canine ehrlichiosis and anaplasmosis according to breed at the Municipal Veterinary Clinic of Rímac

Breed	***E. canis***	***Anaplasma* spp.**	***E. canis* and *Anaplasma* spp.**	**Total cases**
American bully	2	0	0	2
Bichon	3	0	0	3
Bull terrier	3	0	0	3
English bulldog	3	0	0	3
Chihuahua	2	0	0	2
Chow chow	2	0	0	2
Cocker	8	0	0	8
Dobermann	1	0	0	1
Argentine Dogo	1	0	0	1
Golden	7	0	0	7
Labrador	4	0	1	5
Maltese	2	0	0	2
Shepherd	1	0	0	1
German Shepherd	2	0	0	2
Pekingese	1	0	0	1
Peruvian hairless dog	1	0	1	2
Pit Bull	8	0	0	8
Poodle	7	0	0	7
Pug	1	0	0	1
Rottweiler	1	1	0	2
Samoyed	3	0	0	3
Schnauzer	8	0	0	8
Shar-pei	1	0	0	1
Shih Tzu	12	0	0	12
Siberian	3	0	2	5
Dachshund	1	0	0	1
Yorkshire terrier	1	0	0	1
Crossbred	126	3	1	130

Regarding the results of the complete blood count recorded in the medical records and the result of multinomial regression: regression coefficient (β), odds ratio at a 95 % confidence interval, it was found that the risk of thrombocytopenia and anemia with thrombocytopenia in females is 0.28 and 0.41 times less likely than males, respectively. On the other hand, the risk of thrombocytopenia for each kilogram gained in weight is 1.172 times more likely (Table 6).

Table 6: Multinomial regression result: regression coefficient (β), odds ratio, and 95 % confidence interval

			OR 95% confidence interval	
	β (SE)	**Odds ratio**	**Lower**	**Upper**
Normal *vs* Anemia				
Intercept	0.402 (0.746)			
Female	-0.401 (0.453)	0.67	0.275	1.629
Male		Reference		
Crossbred	0.306 (0.465)	1.359	0.546	3.377
Purebred		Reference		
Age group G1	0.640 (0.856)	1.896	0.354	10.157
Age group G2	-2.168 (1.459)	0.114	0.007	1.998
Age group G3		Reference		
Weight	0.03 (0.049)	1.03	0.936	1.134
Age group G1 x Weight	-0.122 (0.074)	0.885	0.766	1.023
Age group G2 x Weight	0.206 (0.140)	1.228	0.934	1.616
Age group G3 x Weight		Reference		
Normal *vs* Thrombocytopenia				
Intercept	-1.183 (1.830)			
Female	-1.274 (0.532)*	0.28	0.099	0.794
Male		Reference		
Crossbred	-0.148 (0.517)	0.863	0.313	2.378
Purebred		Reference		
Age group G1	1.932 (1.154)	6.9	0.719	66.204
Age group G2	1.401 (1.571)	4.06	0.187	88.199
Age group G3		Reference		
Weight	0.158 (0.056)**	1.172	1.051	1.307
Age group G1 x Weight	-0.138 (0.073)	0.871	0.755	1.005
Age group G2 x Weight	0.047 (1.141)	1.048	0.796	1.381
Age group G3 x Weight		Reference		
Normal *vs* A AND T				
Intercept	0.254 (0.762)			

Female	-0.892 (0.427)*	0.41	0.178	0.946
Male		Reference		
Crossbred	0.406 (0.433)	1.502	0.642	3.51
Purebred		Reference		
Age group G1	1.011 (0.843)	2.748	0.527	14.340
Age group G2	-1.022 (1.414)	0.360	0.023	5.752
Age group G3		Reference		
Weight	0.040 (0.050)	1.041	0.943	1.149
Age group G1 x Weight	-0.016 (0.062)	0.984	0.872	1.111
Age group G2 x Weight	0.155 (0.139)	1.167	0.889	1.533
Age group G3 x Weight		Reference		

Note: R^2= 0.219 (Cox and Snell), 0.237 (Nagelkerke); Final model= β + Sex + Breed + Age Group + Weight + Age Group * Weight; Ji^2= 55.508; * *P*<0.05; ** *P*< 0.01; Normal: it considers canines that have no alterations in the complete blood count. G1= under 2 yr of age; G2= 2 to 3 yr; G3= over 3 yr old.

In 2009, cases of canine ehrlichiosis with a history of origin were reported in multiple districts of Lima, obtaining 4.05 % in the north, 11.5 % in the center, 33.78 % in the south, 43.24 % in the east, and 7.43 % in the west[8]; in the districts of northern Lima, there was a frequency of 36.7 % for *E. canis* in 2017[15], increasing to 59.4 % in 2020[11]; in Callao, the overall seroprevalence for canine ehrlichiosis was 57.5 % in Ventanilla[16]; for the districts of Chorrillos, La Molina, and San Juan de Miraflores, 16.5 % of antibodies against *E. canis* were reported for the first time with the ELISA technique[9]. In Chorrillos, the prevalence of *E. canis* was 31.1 % in 2019[10]; in San Juan de Lurigancho, it was 46.44 % in 2016[17], increasing to 47.5 % in 2017[14]; in Lima, at the Cayetano Heredia University, 45.5 % of canine ehrlichiosis and 10.6 % of canine anaplasmosis were obtained. In 2015, positive cases of canine anaplasmosis were 29.2 % in Lima[12]. Having these reference data to compare the frequency of 4.308 % of cases of canine ehrlichiosis and anaplasmosis in the district of Rímac, it is suggested that they are less frequent than in other districts, probably due to different inclusion criteria or the use of other variables included in the analysis or even the use of more precise molecular techniques[12]. Nonetheless, there may also be a low prevalence, as found in the districts of the northern zone or the central zone mentioned above[8], considering that the district of Rímac is close or even adjacent to these areas.

Paiva and Giset[18] mention that for the vector to complete its biological cycle, it must have optimal conditions of climate and humidity, ideally high temperatures of 30 °C and humidity of 20 % to 93 %, otherwise the cycle can extend for several months, which is why the tick-borne disease is considered to be present in tropical and subtropical regions[3], such as Lima, which has an arid subtropical climate with annual temperatures ranging from 19.5 °C to 20.3[19], with the highest temperature recorded in February with an average of 26.5 °C[7]. This study recorded a higher frequency of cases of canine

ehrlichiosis and anaplasmosis in autumn, with 40.18 % (Table 3); this does not necessarily indicate that there is a greater probability of contagion in autumn, as another study indicates higher cases in summer, with 64.6 % of canine ehrlichiosis[14]; it is known that canine ehrlichiosis can be present throughout the year, probably due to climate change and temperature variation in Peru, or the incubation period to present clinical signs according to the canine's immune system[20]; on the other hand, because the vector is sensitive to cold, its presence decreases in winter[21], but after winter rest, some of the different stages of its biological cycle survive and simultaneously infect the susceptible animal, mainly in spring and autumn, reaching its maximum multiplication in summer[14].

It is known that the infection of both diseases does not distinguish the host by sex, age, or breed[22,23]; nevertheless, another author considers females more susceptible to contracting the disease during the estrus season due to exposure to males that do not always have control against ectoparasites[9]. Previous studies by Rodríguez *et al*[24] and Zambrano[25] found more cases of anaplasmosis and ehrlichiosis in males. Infection may be related to the degree of immune response and the presence of the vector[18,26]. These studies coincide with those of the present study since 62.5 % of the dogs positive for *E. canis* and/or *Anaplasma* spp. were males (Table 1); these results may probably be influenced by the number of male, crossbred, and medium-sized dogs, 56.6 %, 54.1 %, and 42.2 %, respectively, according to the study by Arauco *et al*[27].

It has been reported that one of the risk factors associated with canine ehrlichiosis disease is early age, indicating more cases in dogs under 1 yr of age[16,28], over 1 yr of age[29], followed by 6 to 11 mo of age[30]; in addition, it has been reported that most dogs affected with *E. canis* and *A. platys* are between 13 and 24 mo old[31]; between 2 and 4 yr old[8,11,32], older than 4 yr[33], 2 to 6, and 6 yr old or older[10]; on the other hand, Villaverde[13] mentions that the median age of dogs with antibodies positive for *Ehrlichia* spp. is 24 mo, coinciding with the results of this study since the age group that had the highest number of cases was that of less than 2 yr, with 47.76 % (Table 2). These results suggest that, at the end of the vaccination schedule, canines at an early age are more exposed to the vector since owners consider that they are fully protected against pathogens[7,14].

It is known that all breeds have the same probability of infection[22]; however, the German Shepherd breed seems to have a greater predisposition to develop the clinical form[8], as does the Springer Spaniel[22]; in contrast, in this study, the Shih Tzu breed stood out among breeds (Table 5). In this study, the majority of cases of canine anaplasmosis and/or ehrlichiosis were obtained in crossbred dogs, with 58.04 % (Table 4), coinciding with Coello *et al*[34], who indicate that cases of anaplasmosis predominate in crossbred dogs, and what was reported by Lorsirigool and Pumipuntu[35], Villaverde[13], and Cusicanqui and Zuñiga[11], where dogs infected with *E. canis* are mostly crossbred, being common in dogs that have never used an ectoparasiticide or have used it intermittently. The number of dogs positive for these diseases did not allow to identify significant differences, or there were no differences, as has already been shown in other studies[6].

These diseases often alter hematological values, and a complete blood count is essential for diagnosis since thrombocytopenia is considered a factor associated with the disease during all phases[5,36], appearing in 80 % of cases and may be accompanied by regenerative or non-regenerative anemia[20]. These results were found in most of the medical records of dogs affected by *E. canis* and/or *Anaplasma* spp., obtaining, according to the odds ratio, a lower probability of presenting thrombocytopenia, and anemia with thrombocytopenia in females compared to males (Table 6); this may be associated with the fact that some canines may be undergoing the subclinical stage of the disease or may be incubating the agent without presenting relevant symptomatology or hematological findings. On the other hand, the variation in the kilograms of weight of each canine described in the medical records corresponds to different sizes, breeds, and ages, obtaining a greater probability of presenting thrombocytopenia for each kilogram increase (Table 6); in addition, considering that a different physiological behavior has been described between young and adult dogs for erythrocyte and leukocyte values[37], it has been mentioned that adult dogs positive for the disease have lower values of the red, white and platelet series, and puppies show a lower mean of hemoglobin and red blood cells[11], suggesting that the findings of this study are likely influenced by age between puppy or adult. Finally, this work allows us to conclude that the frequency of cases of *E. canis* and *Anaplasma* spp. was 4.308 %. Of this sample, the canines diagnosed with *E. canis* were 95.98 %, with *Anaplasma* spp., they were 1.79 %, and the co-infection of both was 2.23 %. There was a significant association between the diagnosis of canine ehrlichiosis and anaplasmosis with age, sex (OR), and weight (OR), but there was no association with the factors of breed and season of the year.

Conflict of interest

The authors declare that they have no conflict of interest.

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