Universidad Austral de Chile

Facultad de Ciencias Veterinarias

Austral Journal of Veterinary Sciences

ISSN 0719-8000 / ISSN 0719-8132

VOLUME 55 / VALDIVIA - CHILE / 2023 / Nº 2

This journal is subsidised by
Dirección de Investigación y Desarrollo de la Universidad Austral de Chile (DID-UACh)

Periodicity: Triannual (January-May-September). Funded in 1969.
Indexed in:

– Current Contents Agriculture, Biology and Environmental Sciences (CC/AB and ES).
– Commonwealth Agricultural Bureau, International (C.A.B.I.).
– Dairy Science Abstracts.
– Veterinary Bulletin.
– Animal Breeding Abstracts.
– Helminthological Abstracts S.A.
– Agrindex.
– Biological Abstracts.
– Periódica.
– Focus on: Veterinary Sciences and Medicine.
– Science Citation Index Expanded.
– Scielo - Chile.
– Google Scholar.
– Ebsco.

ISSN 0719-8000
ISSN 0719-8132

Austral Journal of Veterinary Sciences

VOL. 55, Nº 2, 2023

Universidad Austral de Chile
Facultad de Ciencias Veterinarias
Casilla 567 - Valdivia - Chile

VOLUME 55, Nº 2, 2023

ORIGINAL ARTICLES

SHORT COMMUNICATION

Austral J Vet Sci 55, 81-86 (2023)

ORIGINAL ARTICLE

Evolution of IFN-γ response against mycobacterial antigens used for the diagnosis of bovine tuberculosis in BCG vaccinated cattle under a natural transmission setting in central Chile

María B. Benavides[a], Pedro E. Ábalos[a], Nicolás Valdivieso[b], Patricio Retamal[a*]

ABSTRACT. Bovine tuberculosis (bTB) is a chronic disease of animals mainly caused by *Mycobacterium bovis*, a zoonotic pathogen that generates economic losses in the milk and meat industry. In central Chile, the Metropolitan Region concentrates dairy herds with the highest bTB prevalence of the country and the official veterinary service has supported the evaluation of the *M. bovis Bacillus* Calmette-Guerin (BCG) vaccine in this area with the replacement of tuberculin purified protein derivative (PPDs) by the DIVA (Differentiating Infected from Vaccinated Animals) peptides for the bTB diagnosis in the herds. This study aimed to describe the IFN-γ response against PPDs (bovine and avian PPD) and DIVA antigenic cocktails (ESAT-6/CFP-10 and Rv3615c) in BCG vaccinated 11-month-old heifers under a natural transmission scenario. Sixty-two animals were vaccinated via subcutaneous route with a 2-8 x 10^5 colony forming units of BCG Russia strain and 60 control animals received sterile saline. Blood sampling was performed at time 0, previous to vaccination, and then at 3, 6, 9, 12, 15 and 18 months post-inoculation. The follow up of the IFN-γ response in animals determined that the BCG vaccination interferes with the diagnosis of bTB using the traditional bovine PPD between 9 and 12 months post-inoculation. Furthermore, the sensitization with non-tuberculous mycobacteria (NTM) was also interfering the diagnosis relying in PPDs, suggesting the need of using DIVA antigens under this epidemiological condition, whether or not the BCG vaccine is administered in cattle, in order to improve the accuracy of bTB diagnosis in central Chile.

Keywords: IFN-γ response, BCG, cattle, Chile.

INTRODUCTION

Bovine tuberculosis (bTB) is a zoonotic infectious disease mainly caused by *Mycobacterium bovis* (Olea-Popelka *et al*., 2017), a pathogen distributed across the world which causes significant economic losses to the dairy industry, especially in low- and middle-income countries. Due to the mandatory milk pasteurization programs in many countries, the zoonotic infection currently becomes more important in occupational settings where the frequent contact with diseased animals in closed enclosures is the highest risk activity (Torres-Gonzalez *et al*., 2013; Vayr *et al*., 2018).

The clinical disease in cattle generally appears along their productive cycle, although asymptomatic and infectious animals contribute significantly to bacterial transmission. Thus, an effective control scheme of bTB requires diagnostic tests to identify infected animals and their prompt removal from herds. However, without compensations for these animal losses, this sanitary management is generally not affordable by farmers in high prevalence areas (Buddle *et al*., 2018). This epidemiological and economic scenario encourages the study of the vaccination as an additional strategy for supporting the control of the disease.

Received: 19.05.2022

Accepted: 07.09.2022

[a]Laboratorio de Enfermedades Infecciosas, Departamento de Medicina Preventiva Animal, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile.

[b]Servicio Agrícola y Ganadero, Coyhaique, Chile.

*Corresponding author: pretamal@uchile.cl

In animals, vaccination strategies have been proposed and tested in many studies, evaluating attenuated, subunit and recombinant products (Vordermeier *et al*., 2016). However, the *M. bovis* BCG strain constitutes a major vaccine candidate for use in cattle due to its approved use in humans, its availability and safety record (Buddle *et al*., 2018). What has prevented its official implementation is the interference with the standard tuberculin skin test, on which control and eradication programs rely for the *in vivo* diagnosis of animals. To overcome this limitation, novel DIVA (Differentiating Infected from Vaccinated Animals) antigens are now available among which ESAT-6, CFP-10 and Rv3615c have shown comparable sensitivity and specificity levels with the tuberculin test (Vordermeier *et al*., 2016; Srinivasan *et al*., 2019). Both ESAT-6 and CFP-10 are proteins encoded by genes located within the RD1 region of *M. bovis* and *M. tuberculosis* genome, which was deleted from the genome of *M. bovis* BCG vaccine strains (Vordermeier *et al*., 2016). Due to this, the IFN-γ response against these antigens discriminates between *M. bovis*-infected and BCG-vaccinated cattle (Vordermeier *et al*., 2016). The Rv3615c antigen is encoded in the BCG genome, but its secretion is dependent on a secretion system encoded in the RD1 region (Millington *et al*., 2011). In BCG-vaccinated populations, the use of Rv3615c peptide cocktail in combination with ESAT-6/CFP-10 peptide cocktail in DIVA tests has the potential to significantly increase diagnostic sensitivity without reducing specificity (Sidders *et al*., 2008; Vordermeier *et al*., 2016).

The tuberculin skin test consists in the intradermal inoculation of purified protein derivative (PPD) prepared from a culture of *M. bovis* (PPDB) or *M. avium* (PPDA)

(Vordermeier *et al*., 2016). In the last years, the interferon-γ (IFN-γ) release assay (IGRA) has been implemented as an ancillary test for the diagnosis of bTB, using PPD and DIVA antigens (Vordermeier *et al*., 2011; Buddle *et al*., 2018).

In Chile, the distribution of bTB is characterized by low prevalence areas in northern and southern regions and a high prevalence area in the central zone (Rivera & Vega, 2014) where farmers have been less receptive to cull reactor animals due to the lack of compensation (Max *et al*., 2011). On the other hand, the prevalence of non-tuberculous mycobacteria (NTM) in dairy cattle has been scarcely reported with some evidence in southern Chile about *M. avium* subsp. *paratuberculosis* infection in dairy herds, with a within-herd prevalence ranging between 3.2% and 38% (Verdugo *et al*., 2018) and a herd-level prevalence between 28% and 100%, depending on the herd size (Kruze *et al*., 2013).

In some dairy herds from the central zone, the Agricultural and Livestock Service of Chile (SAG) allowed a field trial for the study of BCG vaccination in cattle, obtaining a significant efficacy which encourages its implementation as an alternative strategy for the prevention of the disease in high prevalence farms (Retamal *et al*., 2022). The aim of this work is to describe the dynamics of IFN-γ responses against tuberculin PPD (PPDB and PPDA) and DIVA (ESAT-6/CFP-10 and Rv3615c) antigens in BCG vaccinated and unvaccinated cohorts of heifers raised under a natural transmission setting in central Chile.

MATERIALS AND METHODS

The dairy herd, with 294 Holstein-Friesian breeding cows in milk production, was located in the Metropolitan Region. It had a Brucellosis-free status and a bTB incidence of 24% at the beginning of the BCG vaccination in 2017. The presence of other infectious diseases was unknown. For controlling bTB, the owner was periodically using the single intradermal tuberculin test for detecting reactor animals, some of which were segregated and others culled. For the study in this farm, SAG allowed the use of the IGRA with traditional and DIVA antigens (described below) in replacement of the tuberculin test as an official diagnostic technique.

ANIMAL VACCINATION SCHEDULE

Between May 2017 and July 2018, 11-month-old Holstein-Friesian female heifers were included in a double-blinded cohort study, in which 62 animals were vaccinated by the subcutaneous route with 2-8 x 10^5 colony forming units (CFU) (0.1 mL) of the BCG Russia strain (Serum Institute of India, Pune, India). The control group was constituted by 60 animals that received 0.1 mL of NaCl 0.9% (vaccine diluent).

This study was originally designed to evaluate the BCG efficacy in heifers, in which a protection of 66.5% was determined (Retamal *et al*., 2022). Hence, vaccinated and unvaccinated animals that were reactors to DIVA IGRA were assumed as infected with *M. bovis* and were excluded from subsequent sampling activities. In addition, a group of animals gradually disappeared from the study due to herd management practices, which were locally prioritized by health or production issues. For these reasons, 41 and 27 animals were included in the last sampling activity in the vaccinated and control groups, respectively.

BLOOD SAMPLING

Animals were sampled the day of inoculation (time 0) and at 3, 6, 9, 12, 15 and 18-months post vaccination. For this, 5 mL of blood was collected in heparinized tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) and maintained at environmental temperature until arriving at the laboratory.

IFN-γ RELEASE ASSAY (IGRA)

The stimulation of whole blood was performed on the day of sampling with 250 IU/mL of the traditional avian (PPDA) and 300 IU/mL of bovine (PPDB) Purified Protein Derivatives (Applied Biosystems® Bovigam®) and DIVA antigens, represented by the ESAT-6 and CFP-10 combined peptides (21 peptides in total) and the Rv3615c peptides (11 peptides in total) (Pepceuticals Ltd., United Kingdom) (Sidders *et al*., 2008; Vordermeier *et al*., 2001). These DIVA peptides were diluted in RPMI 1640 medium with Glutamax® supplement (Gibco, Grand Island, NY, USA) at a final concentration of 55 µg/each peptide/mL. Then, 25 µL of this solution was used to stimulate 250 µL of blood. In addition, a PBS control solution and the Pokeweed mitogen (Applied Biosystems® Bovigam®) were also utilized for blood stimulation. After an incubation period at 37°C for 18 h, plasma supernatants were harvested and stored at -20°C. For the IFN-γ detection, plasmas were thawed and processed with the Bovigam 2G® Test Kit for cattle (Prionics AG, Tullamarine, Australia), according to manufacturer recommendations.

The cut-off value for both bovine and avian PPD was a ≥ 0.05 difference (ΔOD_{450}) between them (PPD B-A for PPDB reactors and PPD A-B for PPDA reactors) and a ≥ 0.05 difference with the phosphate-buffered saline (PBS) control solution, according to the national control and eradication program definition. The cut-off value for DIVA antigens was OD_{450} ≥0.1 difference with the PBS control in either cocktail. The criterion for diagnosing DIVA reactors was testing positive to at least one peptide cocktail.

ANALYSIS OF RESULTS

The IGRA results (ΔOD_{450}) were compared between vaccinated and unvaccinated groups of animals at each sampling time with the non-parametric Mann-Whitney

Table 1. Comparisons of interferon gamma release assay (IGRA) reactors using PPD and DIVA antigens for the diagnosis of bovine tuberculosis in BCG vaccinated and control groups of animals.

	BCG				Control			
Month	PPD N° (%)	DIVA N° (%)	p	k	PPD N° (%)	DIVA N° (%)	p	k
3	15 (24.2)	2 (3.2)	0.001	0.06	6 (10)	8 (13.3)	0.79	-0.13
6	16 (28.1)	2 (3.5)	<0.001	0.05	7 (14.6)	2 (4.2)	0.12	0.17
9	8 (15.7)	1 (2.0)	0.04	-0.04	2 (5.3)	0 (0)	<0.001	0
12	6 (12.5)	0 (0)	<0.001	0	3 (7.7)	3 (7.7)	0.63	0.28
15	5 (11.4)	2 (4.6)	0.45	-0.07	5 (14.3)	2 (5.7)	0.38	0.22
18	8 (19.5)	0 (0)	<0.001	0	2 (7.4)	2 (7.4)	0.5	0.46

PPD, purified protein derivative; DIVA, differentiating infected from vaccinated animals; BCG, *Mycobacterium bovis* BCG strain.
p, McNemar test; k, Kappa test.

test. The comparison within groups between sampling times was performed with the Friedman test. The bTB diagnosis attained with PPD and DIVA antigens was contrasted with the McNemar and Kappa tests, to identify changes in proportions and interrater reliability of results, respectively.

These analyses were performed using Infostat® software (Di Rienzo *et al*., 2016).

RESULTS

The IGRA results with PPD and DIVA antigens can be seen in Figure 1 and Table 1S. The unique stimulus in which IFN-γ responses were significantly different between vaccinated and control groups was observed with the bovine PPD at 3, 6 and 18 months post-vaccination ($p<0.05$, Figure 1B).

Comparisons performed with the Friedman test showed that the IFN-γ responses to the bovine PPD in vaccinated animals increased significantly at 3, 6, 9, 12 and 15 months post-vaccination ($p<0.0001$), in contrast to time 0. Within the unvaccinated group, differences were only observed between times 0 and 3 months ($p<0.05$). In the analysis of PPD B-A, the vaccinated group showed differences between times 0 and 6, 9, 15 and 18 months post-vaccination ($p<0.01$). The unvaccinated group did not fluctuate significantly alongside the study ($p>0.05$). With DIVA antigens, the unique difference was observed with ESAT-6/CFP-10 cocktail in the vaccinated group, between time 0 and 6 months ($p<0.05$).

The diagnosis of bTB through IGRA using cut-off values, showed variable proportions of reactor animals and interrater agreements when IFN-γ responses to PPD and DIVA antigens were compared, especially in the BCG vaccinated group (Table 1).

Within the group of DIVA positive animals, 58% of them (14/24) were also identified as *M. avium* reactors (PPD A-B ≥0.05), 29% (7/24) were PPDB reactors (PPD B-A ≥0.05) and 12.5% (3/24) were classified as negative by PPD antigens.

Table 2. Interferon gamma release assay (IGRA) reactors using PPD antigens (PPD A-B ≥0.05 and PPD A-PBS ≥0.05) in BCG vaccinated and control groups of animals.

Month	PPDA reactors N° (%)	
	BCG	Control
0	19 (30.6)	22 (36.6)
3	27 (43.5)	29 (49.2)
6	17 (29.8)	12 (25)
9	18 (35.3)	9 (23.7)
12	14 (29.2)	13 (33.3)
15	9 (20.5)	10 (28.6)
18	10 (24.4)	5 (18.5)

PPD, purified protein derivative; PPDA, PPD avium; PPDB, PPD bovis; PBS, phosphate-buffered saline; BCG, *Mycobacterium bovis* BCG strain.

The sensitization with non-tuberculous mycobacteria (NTM) was similar in both groups of animals during all sampling times ($p>0.05$), with the highest values of avium PPD reactors at 3 months post-vaccination (Table 2).

The strength of IFN-γ responses of DIVA positive animals was also compared between groups without significant differences ($p>0.05$) (Table 3).

DISCUSSION

The application of the *M. bovis* BCG vaccine in cattle has been evaluated in experimental and natural transmission settings, at different doses, strains, ages and breeds, suggesting an average efficacy around 25% of vaccinated animals (Srinivasan *et al*., 2021) and some potential non-specific benefits (Retamal *et al*., 2022). Despite of this level of protection, which would significantly contribute to bTB

Table 3. Interferon gamma release assay (IGRA) results of DIVA reactor animals using PPD (PPDB, PPDA) and DIVA (ESAT-6/CFP-10, Rv3615c) antigens.

Group	DIVA reactors N°(%)	IFN-γ responses (ΔOD_{450} nm) (mean ± SEM)			
		PPD B-A	PPDB	ESAT-6/CFP-10	Rv3615c
BCG	7 (11.3)	-0.01 ± 0.15	0.92 ± 0.44	0.21 ± 0.05	0.08 ± 0.06
Control	17 (28.3)	-0.03 ± 0.21	0.75 ± 0.20	0.40 ± 0.16	0.17 ± 0.07

PPD, purified protein derivative; PPDA, PPD avium; PPDB, PPD bovis; DIVA, differentiating infected from vaccinated animals; BCG, *Mycobacterium bovis* BCG strain.

control and prevention (Conlan *et al*., 2015), the official veterinary services have not allowed its implementation, due to the interference that the vaccine elicits with the World Organization for Animal Health (WOAH)-recommended tuberculin skin test (Bayissa *et al*., 2021). However, the required diagnostic assays that discriminate infected from vaccinated animals (DIVA) have already been developed, for both skin and IGRA procedures (Srinivasan *et al*., 2019; Vordermeier *et al*., 2016).

In this study, the BCG vaccination elicited a raise in the IFN-γ response against the bovine PPD (Figure 1), which determined significant differences ($p<0.05$) with the diagnosis based on DIVA cocktails, especially during the first 12 months post-vaccination (Table 1), as has been observed previously (Whelan *et al*., 2011). Accordingly, the agreement between these PPD and DIVA peptides in vaccinated animals was negligible in all sampling times, contrasting with the control group, in which this indicator was progressively improving along the study (Table 1). This result accounts for the interference that the *M. bovis* BCG strain generates with the tuberculin-based diagnosis and justifies its replacement by a DIVA assay when the vaccination is applied in cattle (Vordermeier *et al*., 2001).

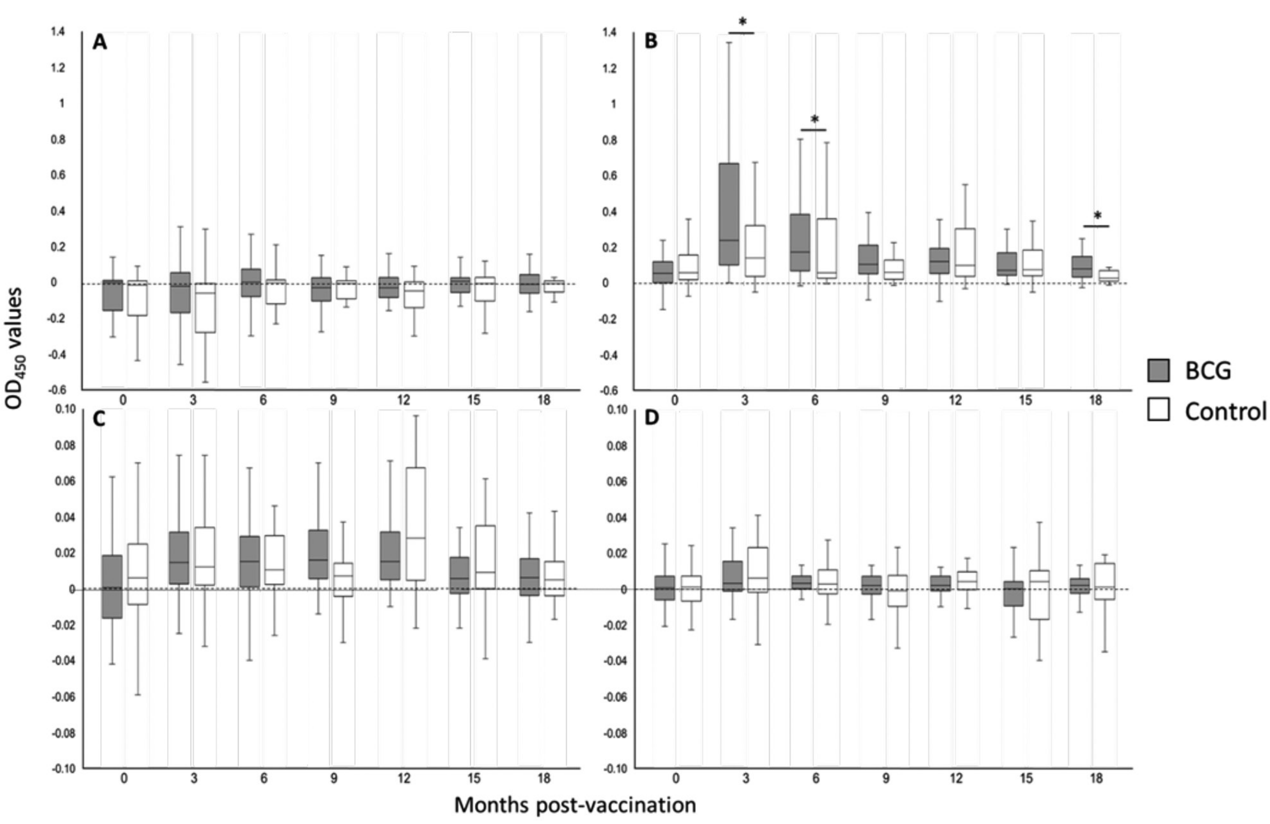

Figure 1. Box-plot diagrams showing IFN-γ responses at 0, 3, 6, 9, 12, 15 and 18 months post-vaccination in BCG and control groups. The figure shows the IFN-γ release assay (IGRA) results obtained with **A)** PPD B-A (bovis minus avium), **B)** PPDB minus saline, **C)** CFP-10/ESAT-6 and **D)** Rv3615c. Within box-plot diagrams, the median is represented with a line, the interquartile range with a box and the minimum and maximum with the whiskers. Outliers were not depicted (* $p<0.05$).

Furthermore, differences between PPD and DIVA peptides were also observed in the group of non-vaccinated animals, represented with low agreement rates between them during the first 9 months of the study (Table 1). In this regard, the higher IFN-γ response of animals to NTM during the first three months, assumed by the PPDA reactors (Table 2), determined that 9 out of 10 DIVA reactors were also identified as reactors to the avium PPD (Table 1S). This implies that when PPD antigens are occupied for bTB diagnosis in the context of a high NTM exposition, a proportion of truly *M. bovis* infected animals will be identified as PPDA reactors, decreasing the sensitivity of the test and the effectiveness of diagnosis for controlling the disease. In the whole study, a higher proportion of DIVA and PPDA reactors were observed in the control group (65%, 11/17) than the vaccinated group (43%, 3/7), probably due to BCG increased the IFN-γ response against bovine PPD, partially compensating the mentioned NTM effect in vaccinated animals. Therefore, the highest disagreement for *M. bovis* diagnosis between PPD and DIVA antigens were observed at 3 months in the control group (Table 1).

This NTM exposition was not explained by any known risk or management factor in animals. The NTM corresponds to a diverse group of microorganisms, widely distributed in nature, some of which can produce disease in animals (Pereira *et al*., 2020). In Chile, a high prevalence of *M. avium* subsp *paratuberculosis* has been reported in dairy cattle (Kruze *et al*., 2013), affecting the accuracy of PPD based tests for the diagnosis of bTB (Raffo *et al.*, 2017, 2020). Under this epidemiological context of high exposition to NTM, the use of DIVA reagents would also support diagnostic efforts of bTB.

Previous reports have shown that BCG vaccination also increases IFN-γ responses against avian PPD (Buddle *et al*., 2013; Vordermeier *et al*., 2001). However, in this work we did not observe differences between vaccinated and unvaccinated animals (data not shown), suggesting that the BCG strain, the age of inoculated animals or their previous exposition to NTM might be influencing the cross reaction between BCG and the avian PPD.

In *M. bovis* infected animals, the amount of secreted IFN-γ in response to DIVA peptides has been documented variable between vaccinated and unvaccinated animals, suggesting a negative correlation with protection and a positive correlation with bTB-associated pathology (Parlane *et al*., 2014; Vordermeier *et al*., 2002; Bayissa *et al*., 2021). Despite of this work determined a lower mean IFN-γ release in BCG vaccinated than control DIVA-reactors, such differences were not significant ($p>0.05$) (Table 3). To clarify if such prognostic association can also be observed in the Chilean field conditions, a higher number of reactor animals and the inclusion of *post-mortem* analyses are needed.

A major drawback to be addressed with BCG vaccination in cattle is the additional cost associated with the application of the DIVA test. Because of this, better cost-effective scenarios need to be explored, such as its application to confirm reactor animals to the traditional tuberculin skin test (Parlane & Buddle, 2015; Buddle *et al*., 2013; Jones *et al*., 2017).

It was determined that the implementation of DIVA-IGRA for the diagnosis of bTB was essential in BCG vaccinated dairy cattle, under a natural transmission setting in central Chile. In addition, this assay appeared useful in a context of high exposition of NTM, avoiding the interference that these mycobacteria could have with classical diagnosis based on bovine and avium PPD antigens. These results and the significant protection conferred by the vaccine (Retamal *et al*., 2022) suggest the need for a more committed effort of official veterinary services around the globe for the inclusion of BCG vaccine and DIVA diagnosis in bTB control programs, especially in those more prevalent areas.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ETHICS STATEMENT

The study was performed after an informed consent was signed by the farm owner. The University of Chile committee on the care and use of animals' guidelines were followed (Permit N°11021 VET-UCH).

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation and analysis were performed by MBB. The organization and coordination of the field work was performed by PA and NV. Analyses of results was performed by MBB and PR. The manuscript was written by PR and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

FUNDING

This research received financial support from the SAG-FAVET collaboration and transfer agreement for the BCG vaccination of dairy cattle.

ACKNOWLEDGEMENTS

We would like to thank Dr. Martin Vordermeier and Dr. Gareth Jones by their support with DIVA antigens and protocols. In addition, we thank Karina Saadi, Jaime Figueroa, Carolina Perez-Watt, Romina Orellana, Natalia Muñoz, Constanza Salinas, Constanza Ávila, Valentina Padilla, Mathias Wistuba, Nicole Sánchez, Martín Vásquez, Camila Ortega and Ayalen Mella for their help in the fieldwork.

REFERENCES

Bayissa, B., Sirak, A., Worku, A., Zewude, A., Zeleke, Y., Chanyalew, M., Gumi, B., Berg, S., Conlan, A., Hewinson, R., ETHICOBOTS, Wood, J., Vordermeier, H., & Ameni, G. (2021). Evaluation of the Efficacy of BCG in Protecting Against Contact Challenge With Bovine Tuberculosis in Holstein-Friesian and Zebu Crossbred Calves in Ethiopia. *Frontiers in Veterinary Science, 8*, 702402. https://doi.org/10.3389/fvets.2021.702402

Buddle, B., Hewinson, R., Vordermeier, H., & Wedlock, D. (2013). Subcutaneous Administration of a 10-fold-lower Dose of a Commercial Human Tuberculosis Vaccine, Mycobacterium Bovis Bacillus Calmette-Guerin Danish, Induced Levels of Protection Against Bovine Tuberculosis and Responses in the Tuberculin Intradermal Test Similar to Those Induced by a Standard Cattle Dose. *Clinical and Vaccine Immunology*, *20*(10), 1559-1562. https://doi.org/10.1128/CVI.00435-13.

Buddle, B., Vordermeier, H., Chambers, M., & de Klerk-Lorist, L. (2018). Efficacy and Safety of BCG Vaccine for Control of Tuberculosis in Domestic Livestock and Wildlife. *Frontiers in Veterinary Science*, *5*, 259. https://doi.org/10.3389/fvets.2018.00259

Conlan, A. J., Brooks Pollock, E., McKinley, T. J., Mitchell, A. P., Jones, G. J., Vordermeier, M., & Wood, J.L. (2015). Potential benefits of cattle vaccination as a supplementary control for bovine tuberculosis. *PLoS Computational Biology*, e1004038. https://doi.org/10.1371/journal.pcbi.1004038

InfoStat. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

Jones, G. J., Coad, M., Khatri, B., Bezos, J., Parlane, N.A., Buddle, B.M., Villarreal-Ramos, B., Hewinson, R.G., & Vordermeier, H.M. (2017). Tuberculin Skin Testing Boosts Interferon Gamma Responses to DIVA Reagents in Mycobacterium bovis-Infected Cattle. *Clinical and Vaccine Immunology*, *24*(5), e00551-16. https://doi.org/10.1128/CVI.00551-16

Kruze, J., Monti, G., Schulze, F., Mella, A., & Leiva, S. (2013). Herd-level prevalence of Map infection in dairy herds of southern Chile determined by culture of environmental fecal samples and bulk-tank milk qPCR. *Preventive Veterinary Medicine*, *111*, 319-324. https://doi.org/10.1016/j.prevetmed.2013.05.011.

Max, V., Paredes, L., Rivera, A., & Ternicier, C. (2011). National control and eradication program of bovine tuberculosis in Chile. *Veterinary Microbiology*, *151*, 188-91. https://doi.org/10.1016/j.vetmic.2011.02.043

Millington, K., Fortune, S., Low, J., Garces, A., Hingley-Wilson, S., Wickremasinghe, M., Kon, O., & Lalvani, A. (2011). Rv3615c is a highly immunodominant RD1 (Region of Difference 1)-dependent secreted antigen specific for Mycobacterium tuberculosis infection. *Proceedings of the National Academy of Sciences USA*, *108*, 5730-5735. https://doi.org/10.1073/pnas.1015153108

Olea-Popelka, F., Muwonge, A., Perera, A., Dean, A.S., Mumford, E. Erlacher-Vindel, E., Forcella, S., Silk, B.J., Ditiu, L., El Idrissi, A., Raviglione, M., Cosivi, O., LoBue, P., & Fujiwara, P.I. (2017). Zoonotic tuberculosis in human beings caused by Mycobacterium bovis-a call for action. *Lancet Infectious Diseases*, *17*, e21-e25. https://doi.org/10.1016/S1473-3099(16)30139-6.

Parlane, N. & Buddle, B. (2015). Immunity and vaccination against Tuberculosis in cattle. *Current Clinical Microbiology Reports, 2*, 44-53. https://doi.org/10.1007/s40588-014-0009-4

Pereira, A., Ramos, B., Reis, A., & Cunha, M. (2020). Non-Tuberculous Mycobacteria: Molecular and Physiological Bases of Virulence and Adaptation to Ecological Niches. *Microorganisms*, *8*, 1380. https://doi.org/10.3390/microorganisms8091380

Raffo, E., Steuer, P., Monti, G., & Salgado, M. (2017). Effect of Mycobacterium avium subsp. paratuberculosis (MAP) infection on the diagnostic accuracy for Mycobacterium bovis (M. bovis) infection under field conditions in cattle belonging to low M. bovis prevalence herds. *Tropical Animal Health and Production*, *49*, 771-775. https://doi.org/10.1007/s11250-017-1259-y.

Raffo, E., Steuer, P., Tomckowiack, C., Tejeda, C., Collado, B., & Salgado, M. (2020). More insights about the interfering effect of Mycobacterium avium subsp. paratuberculosis (MAP) infection on Mycobacterium bovis (M. bovis) detection in dairy cattle. *Tropical Animal Health and Production*, *52*, 1479-1485. https://doi.org/10.1007/s11250-019-02151-2.

Retamal, P., Ábalos, P., Alegría-Morán, R., Valdivieso, N., Vordermeier, M., Jones, G., Saadi, K., Perez-Watt, C., Salinas, C., Ávila, C., Padilla, V., Benavides, B., & Orellana, R. (2022). Vaccination of Holstein heifers with Mycobacterium bovis BCG strain induces protection against bovine tuberculosis and higher milk production yields in a natural transmission setting. *Transboundary and Emerging Diseases*, *69*(3), 1419-1425. https://doi.org/10.1111/tbed.14108.

Rivera, A. & Vega, M. (2014). Ocurrencia de la Tuberculosis bovina en Chile. Web site: http://www.sag.cl/sites/default/files/ocurrencia_tb_2000_2014_ar-mv.pdf: Servicio Agrícola y Ganadero de Chile.

Sidders, B., Pirson, C., Hogarth, P.J., Hewinson, R.G., Stoker, N.G., Vordermeier, H.M., & Ewer, K. (2008). Screening of highly expressed mycobacterial genes identifies Rv3615c as a useful differential diagnostic antigen for the Mycobacterium tuberculosis complex. *Infection and Immunology*, *76*, 3932-3939. https://doi.org/10.1128/IAI.00150-08

Srinivasan, S., Conlan, A., Easterling, L., Herrera, C., Dandapat, P., Veerasami, M., Ameni, G., Jindal, N., Raj, G., Wood, J., Juleff, N., Bakker, D., Vordermeier, M., & Kapur, V. (2021). A Meta-Analysis of the Effect of Bacillus Calmette-Guérin Vaccination Against Bovine Tuberculosis: Is Perfect the Enemy of Good? *Frontiers in Veterinary Science*, *8*, 637580. https://doi.org/10.3389/fvets.2021.637580.

Srinivasan, S., Jones, G., Veerasami, M., Steinbach, S., Holder, T., Zewude, A., Fromsa, A., Ameni, G., Easterling, L., Bakker, D., Juleff, N., Gifford, G., Hewinson, R., Vordermeier, H., & Kapur, V. (2019). A defined antigen skin test for the diagnosis of bovine tuberculosis. *Science Advances*, *5*. https://doi.org/10.1126/sciadv.aax4899

Torres-Gonzalez, P., Soberanis-Ramos, O., Martinez-Gamboa, A., Chavez-Mazari, B., Barrios-Herrera, M.T., Torres-Rojas, M., Cruz-Hervert, L.P., Garcia-Garcia, L., Singh, M., Gonzalez-Aguirre, A., Ponce de Leon-Garduno, A., Sifuentes-Osornio, J., & Bobadilla-Del-Valle, M. (2013). Prevalence of latent and active tuberculosis among dairy farm workers exposed to cattle infected by Mycobacterium bovis. *PLoS Neglected Tropical Diseases*, *7*, e2177. https://doi.org/10.1371/journal.pntd.0002177

Vayr, F., Martin-Blondel, G., Savall, F., Soulat, J.M., Deffontaines, G., & Herin, F. (2018). Occupational exposure to human Mycobacterium bovis infection: A systematic review. *PLoS Neglected Tropical Diseases*, *12*, e0006208. https://doi.org/10.1371/journal.pntd.0006208

Verdugo, C., Valdés, M.F., & Salgado, M. (2018). Within-herd prevalence and clinical incidence distributions of Mycobacterium avium subspecies paratuberculosis infection on dairy herds in Chile. *Preventive Veterinary Medicine*, *154*, 113-118. https://doi.org/10.1016/j.prevetmed.2018.03.022

Vordermeier, H. M., Jones, G.J., Buddle, B.M., Hewinson, R.G., & Villarreal-Ramos, B. (2016). Bovine Tuberculosis in Cattle: Vaccines, DIVA Tests, and Host Biomarker Discovery. *Annual Review of Animal Biosciences*, *4*, 87-109. https://doi.org/10.1146/annurev-animal-021815-111311

Vordermeier, H. M., Whelan, A., Cockle, P.J., Farrant, L., Palmer, N., & Hewinson, R.G. (2001). Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. *Clinical Diagnostic Laboratory Immunology*, *8*, 571-578. https://doi.org/10.1128/CDLI.8.3.571-578.2001

Vordermeier, M., Gordon S. V., & Hewinson, R. G. (2011). Mycobacterium bovis antigens for the differential diagnosis of vaccinated and infected cattle. *Veterinary Microbiology*, *151*, 8-13. https://doi.org/10.1016/j.vetmic.2011.02.020

Whelan, A.O., Coad, M., Upadhyay, B.L., Clifford, D.J., Hewinson, R.G., & Vordermeier, H.M. (2011). Lack of correlation between BCG-induced tuberculin skin test sensitisation and protective immunity in cattle. *Vaccine*, *29*, 5453-8. https://doi.org/10.1016/j.vaccine.2011.05.057

Austral J Vet Sci 55, 87-94 (2023)

ORIGINAL ARTICLE

Physical quality of different industrial versus non-industrial eggs obtained from groceries and markets in southern Chile

Mónica Gandarillas[a*], Valeria Olmos[a], Bárbara Piña[b], Juan Pablo Keim[a], Einar Vargas-Bello-Pérez[b]

ABSTRACT. The aim of this study was to determine external and internal quality parameters of industrial (cages and cage-free) and family farms eggs that are normally available at groceries in developing countries such as Chile. Two experiments were performed to evaluate 1) quality differences between family farms and industrial eggs and 2) to determine quality differences between brown shell eggs from different industrial cage and cage-free systems. Experiment 1 consisted of five groups where three of them were industrial eggs: i) cage white shell eggs, ii) cage brown shell eggs, iii) brown shell cage-free eggs; and two of them were non-industrial: iv) family farm brown shell eggs and v) family farm blue shell eggs. Experiment 2 had four groups, all brown-shell types of eggs were used: i) cage brown eggs, ii) cage-free from aviary eggs, iii) southern free-range eggs and iv) central free-range eggs. In both Experiments, egg weight, egg length, egg width, egg shape index, Haugh units, albumen ratio, egg yolk, yolk weight and albumen weight, blood and meat spots were determined. In Experiment 1, brown and blue-shelled family farm eggs were equal in terms of external and internal quality, except for blood spots, with brown eggs having more incidence. In Experiment 2, free-range eggs presented more intense yolk colors compared to those from battery and cages. In both experiments, free-range eggs presented the darker yolk color. It can be concluded that brown and blue-shelled family farm eggs are equal in terms of external and internal quality, except for blood spots, with brown eggs having more incidence. In addition, free-range eggs from the southern part of the country presented better shell quality, whereas free-range eggs presented more intense yolk colors, while those of battery.

Keywords: egg quality, industrialized eggs, cage-free eggs, free-range eggs, farm eggs.

INTRODUCTION

Egg production systems in the world are developed under conventional (cages) or alternative (cage-free) systems with different housing systems, productive parameters, egg quality, animal welfare and hen's health parameters (Wang *et al*., 2009; Sosnówka-Czajka *et al*., 2010; Rakonjac *et al*., 2014). Furthermore, the alternative systems can be further classified as indoor (i.e., floor, aviary and furnished cages) and outdoor (free-range and organic). Both free-range and organic outdoor systems have daytime access to pasture and some different conditions may exist across world regions. For example, in the European Union (EU) these systems must provide hens daytime access to open-air runs covered by vegetation keeping a density of 2,500 hens/hectare (or 1 hen/4 m2)(EC, 1999) whereas, in the United States, only outdoor access to pasture during the laying cycle is required (Ricke & Rothrock, 2020).

Alternative systems have become increasingly popular among consumers as they prefer animal welfare-friendly systems which also led to the ban on conventional cages in the EU legislation (Chielo *et al*., 2016). Consequently, the alternative egg sector in the world has been growing rapidly in the last 30 years (Hammeishøj, 2011). In Europe, 21.7% of layers are raised in free-range (16.4%) and organic (5.3%) systems, while in Australia 48% of eggs sold at the grocery are free-range (Ruhnke, 2015). It is estimated that globally, egg consumption will continue increasing due to a growing number of people who are adopting meat-free or vegetarian diets (Réhault-Godbert *et al*., 2019).

In Chile, 98.2% of the eggs sold at the market are produced in cages, while 1.2% are cage-free (Aguirre & Pizarro, 2018). Eggs sold at the supermarket are "industrial" since they are produced in cages and cage-free (aviary, floor, free-range and organic) systems. In most of those systems, hybrid hens (crossbred between breeds) have been selected for greater egg production and quality during the last 70 years (Thaxton *et al*., 2016). Thus, the egg industry is based on hybrids (commercial genetic lines) rather than pure breeds as the productivity of a pure breed is less efficient as compared to improved genetic lines (Besbes *et al*., 2007). In Chile, the most common genetic lines for industrial egg production are Hy-line and Lohmann which are reared in both cage and cage-free systems. In our country, there is a normative that regulates the egg labeling in many aspects such as the egg classification (Chilean normative 1372 Of. 78 (González, 2019)). Industrial eggs, most commonly sold at supermarkets, are classified the following categories: especial (> 68g), extra grande (61-68g), grande (54-61g).

Non-industrial eggs, such as farming eggs, belong to family farmers. They are defined as an organization for agricultural production that sustains the work of each family member, regardless of land possession, land surface and land destination (Elhawary *et al*., 2005). These systems

Received: 18.05.2022.

Accepted: 28.09.2022.

[a]Instituto de Producción Animal, Facultad de Ciencias Agrarias y Alimentarias, Universidad Austral de Chile, Valdivia, Chile.

[b]Department of Animal Sciences, School of Agriculture, Policy and Development, University of Reading, Reading, UK.

*Corresponding author: monica.gandarillas@uach.cl

are using local or native breeds which are less efficient and productive (Rizzi & Marangon, 2012; Lordelo *et al*., 2020). Normally, the eggs are for self-supply and sold at fairgrounds or small farmer markets. In general, these indigenous chickens are double purpose hens fed with pasture, kitchen leftovers, and mixed grains (Elhawary *et al*., 2005). Furthermore, in the specific case of Chile, indigenous hens have been introgressed with the oocyan gene from the Mapuche fowl which produces eggs with a blue/green shell color (Alcalde, 2015).

Berkhoff *et al*. (2020) reported that brown and blue-shell farm eggs are more favorably evaluated and preferred over industrial eggs from free-range and cage-free systems, by 30 untrained panelists in a sensory evaluation. The yolk color was the most important factor for the panelists when discriminating eggs from different production systems. Although, studies have reported how production systems affect egg quality (Stojanova *et al*., 2016; Gałązka-Czarnecka *et al*., 2019), to the best of our knowledge the comparison in terms of internal and external quality traits from industrial, cage-free and family farm eggs have not been evaluated in developing countries where these productions are more available. Such information is important due to the incipient internal market for these eggs (Patterson *et al*., 2001) and the expected increases in production (Aguirre & Pizarro, 2018).

Due to the aforementioned background, the main objective of this study was to determine external and internal quality parameters of industrial eggs (cage and cage-free) and family farms eggs that are normally available at groceries and fairs in developing countries such as Chile. To do that, two different experiments were carried out 1) to evaluate the quality differences between family- farms eggs and industrial eggs and 2) to elucidate quality differences between brown shell eggs from different industrial systems available at groceries.

MATERIAL AND METHODS

The study was carried out at the Animal Nutrition Laboratory of the Animal Production Institute, Universidad Austral de Chile. Two experiments were carried out to evaluate external and internal quality of eggs purchased from different supermarkets, grocery stores, mini markets, and producers. Experiment 1 was carried out between May and June 2018 while Experiment 2 was performed from October to December 2018.

EXPERIMENT 1: FAMILY-FARM VERSUS INDUSTRIAL EGGS

In Experiment 1, a family-farm focused on poultry production was defined as "owner of poultry for personal consumption or local sale with less than 500 birds" (SAG, 2016), thus they are considered non-industrial.

Five different groups were established, three of them with industrial eggs: i) cage white shell eggs (cage white); ii) cage brown shell eggs (cage brown); iii) brown shell cage-free eggs (cage-free); and two of them with non-industrial eggs: iv) family-farm brown shell eggs (farm brown); and v) family-farm blue shell eggs (farm blue). The industrial eggs were obtained from supermarkets (from the same brand and same expiration date) and farm brown and farm blue eggs from local farmer markets (produced in small-scale family farms). Collected farm brown and blue eggs were laid within a maximum period of 4 days and had no expiration date or brand. For each group, a dozen of eggs was purchased at five consecutive weeks, thus 60 eggs per group were subjected to egg quality measurements.

EXPERIMENT 2: BROWN SHELL EGGS FROM DIFFERENT INDUSTRIAL CAGE AND CAGE-FREE SYSTEMS

Four different groups were established, all brown-shell type of eggs were used: i) cage brown eggs (cage); ii) cage-free from aviary eggs (aviary); iii) southern free-range eggs; and iv) central free-range eggs. Free-range eggs differed in their geographical location (central and southern regions of Chile) as climate conditions affect pasture characteristics and therefore may affect egg quality. For each group, a dozen of eggs was purchased at five consecutive weeks, thus 60 eggs per group were subjected to egg quality measurements. All eggs were obtained from supermarkets (from the same brand for each group and similar expiration dates for all groups). The eggs of each group were subjected to the same measurements of external and internal quality.

For experiments 1 and 2, the following measurements were performed to establish external and internal egg quality three hours after purchase.

EXTERNAL QUALITY

Egg weight (EW) was measured using an electronic balance (Q-DG2000, Quimis®, Sao Paulo, Brazil) (+0.05). Egg length (EL) and egg width (EWi) were measured using a digital electronic caliper, and egg shape index (ESI) was computed as:

$$ESI = (EWi/EL) * 100$$

Eggshell weight (ESW) was determined by carefully separating the shell from internal membranes and oven-dried at 105°C for 24 hours. Afterward, eggshells were weighted, and the ESW/EW ratio was reported as a percentage.

$$\% \text{ shell} = (ESW/EW) * 100$$

INTERNAL QUALITY

After break, eggs were inspected for the incidence of blood and meat spots and those were counted. Albumen quality was assessed with Haugh units (HU) and the albumen ratio (AR). First, thick albumen height (AH) was measured with an electronic caliper, and further divided by EW. The HU is a logarithmic result between the EW and the thick albumen height using the following formula: First, thick albumen height (AH) was measured with an electronic caliper, and further divided by EW (Eisen *et al*., 1962). The formula for calculating the Haugh unit is:

$$HU = 100 * \log (h - 1.7w^{0.37} + 7.6)$$

Where:

HU = Haugh unit
h = observed height of the albumen in millimeters
w = weight of egg in grams

Egg yolk (EY) quality color was measured using the DSM YolkFan^{TM}, which is an industrial color scale varying from 1 (pale yellow) to 16 (dark orange) (DSM, 2021). The yolk was carefully separated from the albumen and weighted determining yolk weight (YW) and albumen weight (AW). Yolk height (YH) was determined using an electronic caliper and yolk ratio (YR, %) was calculated as YW/EW, whereas albumen ratio (AR, %) as AW/EW.

STATISTICAL DESIGN AND STATISTICAL ANALYSIS

Two different completely randomized block designs, with five (experiment 1) and four (experiment 2) groups were performed. Five dozen of eggs were analyzed for each group, considering the dozen as the experimental unit, and the egg was considered as the observational unit. Each dozen was analyzed on different sessions.

The data of the quality parameters obtained from the eggs were analyzed with the mixed procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NC), considering the fixed effect of the type of egg and the random effect of dozen and place where eggs were obtained. Statistically significant differences ($P \leq 0.05$) between least square means were tested using the PDIFF command, incorporating the Tukey test for pairwise comparison of group means. The statistical model used was: $Y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij}$. Where: μ = mean, τi = effect of group I, βj = block effect j, and εij = experimental error.

For the incidence of blood and meat spots, data were analyzed as binary and discrete dependent variables, where 0 represented the absence and 1 the presence of blood and meat spots, and results were expressed as logit values. Data were analyzed with the GENMOD procedure of SAS with the DIST = BIN and LINK = LOGIT defining a binomial distribution and a logit model:

ni = log[pi/(1-pi)] = m + τi, where p corresponds to the probability of success, m is the overall mean of the proportion on the logarithmic scale and τi the effect of group i

RESULTS

EXPERIMENT 1

The external and internal quality results of experiment 1 are shown in Table 1. The EW was heavier in the cage brown, cage-free, farm brown and farm blue compared with the cage white. Egg length of farm blue and farm brown eggs was longer than the cage-free, cage white and cage brown. However, the cage brown and cage-free eggs were significantly wider than the cage white (P = 0.046) and showed no differences with the farm blue and farm brown eggs. Regarding to ESI and shell percentages, they

Table 1. External and internal egg quality parameters from industrial white and brown shelled cage eggs, free range eggs versus blue and brown shell farm eggs with sixty eggs per group.

	Cage white	Cage brown	Cage- free	Farm brown	Farm blue	P-value	SEM
Egg weight, g	57.5^{b}	60.9^{a}	61.7^{a}	63.0^{a}	62.1^{a}	0.018	1.09
Egg length, cm	55.8^{b}	56.9^{b}	56.5^{b}	59.1^{a}	58.2^{a}	<0.001	0.43
Egg width, cm	42.8^{b}	43.7^{a}	44.2^{a}	43.6^{ab}	43.7^{ab}	0.046	0.31
Shape index	76.8^{a}	76.9^{a}	78.3^{a}	73.8^{b}	75.2^{b}	<0.001	0.54
% Shell	9.0^{a}	9.8^{a}	9.9^{a}	8.8^{b}	8.55^{b}	<0.001	0.15
Haugh units	87.1^{a}	80.3^{bc}	75.5^{c}	85.0^{ab}	81.9^{ab}	0.013	2.34
Albumen ratio	9.90^{a}	8.26^{ab}	7.06^{b}	9.7^{a}	8.94^{a}	0.017	0.60
Yolk color	7.8^{c}	8.4^{c}	12.8^{a}	10.6^{b}	11.0^{b}	<0.001	0.51
Yolk ratio	46.1	44.8	45.6	46.7	46.9	0.737	1.24
Blood spots	26.4(0/50)^{b}	1.87(8/59)^{a}	1.54(10/58)^{a}	1.01(16/60)^{a}	2.90(3/59)^{a}	<0.001	0.59
Meat spots	4.08(1/50)^{b}	1.29(13/59)^{a}	0.86(29/58)^{a}	0.48(23/60)^{a}	1.24(13/59)^{a}	<0.001	0.31

$^{a, b, c}$ Different letters indicate significant differences (P < 0.05); SEM = standard error of the mean. ( ) Numbers in brackets indicate the number of eggs with spouts from the total evaluated.

Table 2. External and internal egg quality parameters from brown shelled eggs from different housing origins using sixty eggs per group.

	Cage	Cage-free	Southern free-range	Central free-range	P-Value	SEM
Egg weight, g	59.9	61.0	59.7	61.5	0.664	1.23
Egg length, cm	56.7	56.8	55.8	57.2	0.473	0.62
Egg width, cm	43.6	44.0	43.5	43.9	0.454	0.28
Shape index, %	76.9	77.6	78.0	76.8	0.452	0.56
Shell percentage, %	10.0	10.1	10.2	10.3	0.823	0.19
Haugh units	78.9[a]	66.4[b]	82.3[a]	79.8[a]	<0.001	3.48
Albumen ratio	7.8[a]	5.30[b]	8.6[a]	8.19[a]	<0.001	0.61
Yolk color	9.9[c]	11.3[b]	14.0[a]	14.1[a]	<0.001	0.45
Yolk ratio	41.8	41.4	42.9	41.8	0.398	1.00
Blood spots	-1.07(15/59)	-1.26(13/59)	-1.79(7/49)	-2.2(5/52)	0.113	0.470
Meat spots	-1.08(15/59)[b]	-0.24(26/59)[a]	0.20(27/49)[a]	-1.70(8/52)[b]	<0.001	0.29

[a, b] Different letters indicate significant differences (P < 0.05); SEM = standard error of the mean. Numbers in brackets indicate the number of eggs with spouts from the total evaluated.

were lower for farm blue and farm brown when compared with cage white, cage brown and cage-free eggs.

Haugh units were greater for cage white eggs compared with cage-free and cage brown eggs (P = 0.013). This agrees with the results of the albumen ratio, where cage white along with farm blue and farm brown eggs also had a greater ratio compared with the cage-free eggs ($P = 0.017$). The YC differed among types of egg (P <0.001), where cage-free were more intense (redder) than those from the farm (blue and brown), and the YC of the farm eggs was more intense than the cage brown and white (more yellow) eggs. The YI was not affected by groups. No differences were found among groups in the yolk ratio ($P > 0.05$).

The appearance of meat and blood spots was lower in cage white eggs compared with all other eggs.

EXPERIMENT 2

The external and internal quality of brown eggs from different industrial systems are shown in Table 2. There were no differences among groups ($P > 0.05$) for EW, EL, EWi, ESI and % eggshell.

Results of internal quality are shown in Table 2. There was no difference in the YR among groups ($P > 0.05$). For HU and AR, aviary eggs showed the lowest values compared with eggs from free-range cage systems ($P < 0.001$). The YC was greater in eggs from both free-range systems, followed by aviary eggs and, cage eggs showing the lowest values ($P < 0.001$). The appearance of blood spots was not affected by housing system ($P = 0.113$). Meat spots were higher in aviary and southern free-range eggs compared to cage and central free-range ($P < 0.001$).

DISCUSSION

White-shelled eggs in experiment 1 had the lowest weight, which agrees with Curtis *et al*. (1985). Brown hens are larger than white hens, therefore they lay heavier eggs (Odabasi *et al*., 2007). Egg weight is related to hen's age and laying week, as the age of the hen flock increases, the weight of the eggs also increases (Fletcher *et al*., 1981). This explains the fact that white-shelled eggs from cage systems were lighter. Eggs from birds of greater live weight have a longer length and width, which allows us to support that there is a close relationship between the size of the bird and the dimensions of the laid eggs (Idahor, 2017). In Experiment 1, the greater weight and dimensions of family farm eggs may be explained since family farm egg systems in general cull their hens at irregular times, so, their flocks are age-mixed (Asencio, 2023).

In experiment 2, no differences were detected between egg weights under different production systems and the reason for this might be, in agreement with the aforementioned authors, that all the eggs used were brown in color and uniform in size and they all fall into the large classification (between 54 and 61 g), as proposed by the National Institute of Standardization (Chilean normative 1372 Of. 78 (González, 2019)). Also, no differences between egg length and width were detected under the different production systems, which may be because all the eggs used in this experiment came from specific genetics lines, so it is inferred that they are similar in live weight, laying week and therefore in the size of their eggs (Kingori, 2011; Idahor, 2017).

Although the shape index increases with the hen's age, the results found in Experiment 1 showed that family farm eggs have a more elongated shape, while eggs from intensive systems are rounder. As mentioned before, family farm hens have a higher average age than those from intensive systems, so it should be expected that their eggs have a higher morphological index (Lordelo *et al*., 2020). However, results from Experiment 1 agree with Rodríguez-Navarro *et al*., (2013) who indicated that the age of the hens has a significant influence on the shape index of the eggs, as flocks of 77-week-old produced more elongated eggs as compared to 21 to 44 weeks when the egg was more rounded. This could be explained by the fact that, at the beginning of the laying period, eggs have a round shape that gradually tends to lengthen which is due to a weakening of the muscle tone of the calcareous gland in older hens (Travel *et al*., 2010)

A strong eggshell is fundamental for consumers due to the egg's ability to resist shock (Rehault-Godbert *et al*., 2019). Eggshell quality is influenced by genetics, nutrition, environment and the flock age (Samiullah *et al*., 2017) as well as the sanitary condition (presence of infectious bronchitis virus, Influenza, and mycotoxins (Roberts *et al*., 2011). As the hen gets older, egg size and weight increase, and the eggshell does not increase in the same proportion, so it is highly expected that the percentage of eggshell over the egg weight decreases with age and laying week. The lower eggshell percentages in family farm eggs (either blue or brown) observed in Experiment 1 may be explained due to a calcium deficiency in the diet, as calcium is the most important nutritional factor for shell formation and must be supplied in the diet (Nys & Le Roy, 2018). Commercial layers are given a strictly formulated and balanced diet so that each nutrient, including calcium, is delivered as required, this may explain why eggs from intensive systems had a higher percentage of the shell. On the other hand, in the family farm system, birds are fed with a diet whose nutritional profile is often unknown, and where the main source of calcium is oyster shell, which is delivered *ad libitum* (Asencio, 2023). A study conducted with family farm hens pointed out that, in this system, hens receive a diet based on grains with limitations of calcium and phosphorus, for the same reason that there is a greater probability in these hens of having an unbalanced diet that originates eggs with a fragile shell (Juárez *et al*., 2010). It was determined that the greater the value of the morphological index, the greater force is required to break the eggs, so the resistance to fracture is highly dependent on the morphological index (Altuntas & Sekeroglu, 2008).

The eggshell percentage and its proportion related to the EW showed no differences among groups and all eggs were within the expected reference values for good quality, which indicates that the shell should be about 10% of the egg weight (Roberts, 2004). Layers' diets are balanced so the calcium administered is what they need to achieve nonfragile, good quality eggshells, which contributes to commercialization.

Cage white industrial eggs together with family farm eggs (either blue or brown shell) were superior to industrial cage brown and industrial cage-free eggs, whereas, in experiment 2, differences were found between cage eggs and southern and central free-range eggs which obtained a greater value for HU. The albumen ratio followed the same differences in experiment 2 and a little difference was observed for Experiment 1 where family farm eggs, and industrial white eggs were superior to industrial free-range eggs. In experiment 2, the aviary (non-cage) eggs obtained the smallest height and the largest diameter. Other factors influence albumen quality such as age, genetic line and storage conditions, and hen's nutrition (Scott & Silversides, 2000; Huang *et al*., 2012; Chang-Ho *et al*., 2014; Ramírez *et al*., 2016). As the hen gets older, the albumen quality decreases and days between oviposition and consumption or quality evaluation influence this quality parameters (Padhi *et al*., 2013). In both experiments we had no access to flock age records, however, the three industrial systems had the same oviposition-evaluation days that were longer concerning family farms eggs (Roberts, 2004; Chang-Ho *et al*., 2014). Also, it is known that older birds produce thinner eggshells and therefore may lose more CO_2 which causes an increase in the pH of the albumen (Alleoni & Antúnez, 2005). Since family farm eggs had less eggshell percentage, it is believed that greater UH was due to the days between oviposition and egg quality evaluation. The increase in pH in the albumen implies a degradation of the union of the ovomucin and lysozyme proteins, which makes the albumen more and more fluid. In this regard, the pH of the albumen of a freshly laid egg is between 7.6 and 8.5, which can increase to 9.7 after a storage time due to the loss of CO_2 through the pores (Coutts & Graham, 2007).

Also, a genetic effect on albumen quality was reported by Silversides & Scott (2000) who compared a white and brown genetic line, and found a higher albumen height, and therefore a higher UH value in the eggs from white lines. A third factor affecting the albumen quality is storage conditions such as duration and temperature, since an increase in storage temperature led to a significant decrease in HU (Chung & Lee, 2014). Moreover, as egg conservation time increases, the HU and albumen index decrease (Ramírez *et al*., 2016). The hen's nutritional status also affects albumen pH, as it implies a degradation of the union of the ovomucin and lysozyme proteins, which makes the albumen more fluid (Huang *et al*., 2012).

Yolk color is one of the most important parameters of egg preference (Skrivan *et al*., 2015; Berkhoff *et al*., 2020). The results of experiment 1 showed that free-range eggs obtained the highest value for color (the darkest) when compared to family farm eggs and these were darker than white cage and brown cage eggs. Moreover, free-range eggs in Experiment 2 showed the greatest value for yolk color as compared to aviary and cage eggs. These results are consistent with Van den Brand *et al*., (2004) who reported that yolk's color was considerably darker in free-range eggs rather than in cage eggs, since eggs produced under laying hen grazing systems may increase yolk redness (Skrivan *et al*., 2015). Variations in the yolk color are mainly due to the sources of pigmentation (natural or synthetic) (Titcomb *et al*., 2019). For example, Bovšková *et al*. (2014) observed a particularly high content of carotenoids in the eggs of hens raised at home, which is similar to family farm eggs, corroborating what was determined in this study. In the case of pasture systems, yolk egg redness from free-range hens is increased by natural pigment sources such as carotenoids (lutein, beta-carotene, zeaxanthin, among others) compared to a concentrate diet based on corn and soybean meal (Mugnai *et al*., 2014). Among cereals, corn is the only one with a considerable content of beta-carotene (Çalişlar, 2019), which is one of the best sources of energy for poultry producers due to its high metabolizable energy, palatability and digestibility (Rostagno *et al*., 2017).

Hens in an industrial cage and cage-free systems have corn as the basis of their diet, thus, it can be inferred that this cereal by itself does not generate such pigmented yolks (Seemann, 2000). For the yolk index, no differences between groups were observed in experiments 1 and 2. However, Khan *et al*. (2013) found that the yolk index values showed a significant decrease with increasing egg storage period. Opposite to albumen ratio, the decrease in yolk index occurs slower, showing changes in three weeks after the eggs were kept at 25°C (Romanoff & Romanoff, 1949; Elhawary *et al*., 2005; Khan *et al*., 2013). The fact that no differences were found in experiments 1 and 2 may be because all eggs were purchased within less than three weeks between oviposition and evaluation.

Meat spot incidence was greater than blood spots for all types of eggs, which coincides with Stadelman *et al*. (1952), who found that the percentage of meat spots was four times greater than that of blood spots. There was also an increased incidence of both kinds of spots as the hen gets older (Bustany & Elwinger, 1987; Ahmadi & Rahimi, 2011). These could be a valid explanation for family farm eggs, given that there is a great probability that the diet did not meet the nutritional requirements. However, according to the results of experiment 1, blood spots were greater in industrial cage-free, cage brown and family farm brown eggs with respect to industrial cage white and family farm blue eggs. In this regard, hen genetics has been shown to affect the presence of spots (Ahmadi & Rahimi, 2011). In addition, Jeffrey (1950) found that spots were very common in brown eggs of heavy breeds, much less common in lighter eggs, such as blue shell eggs, and rare in white eggs, which is in agreement with our study where white eggs showed a lower incidence of spots (Bustany & Elwinger, 1987). The aforementioned support results of experiment 1. In experiment 2, there were differences between the incidences of meat spots under the different systems, being higher in aviary and free-range eggs from the southern, and lower in eggs obtained under cages and central free-range system, while the blood spots were largely found in the cage eggs, then in aviary, and to a lesser extent in both the free-range systems.

Some aspects of this study need to be considered when interpreting and extrapolating our data. Although data obtained and discussed in the present study shows differences somehow attributed to the different retail points (i.e., supermarkets, grocery stores, and mini markets) where samples were obtained, further information will be needed to complement our findings. In this regard, data on age and hen's genetic line as well as ingredients and chemical composition of animal's diets, egg storing conditions and egg packing should be further analyzed. In this study, those details were not available as our approach was a retail survey.

In addition, further studies should increase the number of companies sampled as well as increase sampling time to reflect if there is a seasonal effect. Previous studies have done similar approaches sampling only 2 local groceries with 2 sampling times allowing us to reach far conclusions on the physical quality and composition of retail shell eggs (Jones *et al*., 2010). In a more recent study, Hisasaga *et al*. (2020) performed a survey of egg quality in commercially available table eggs evaluating 5 brands of brown eggs with 2 sampling periods spanning 7 months. Compared to the aforementioned studies, ours has more complexity as eggs were surveyed not only from industrial origin but also from informal markets where specific egg data is not available or even recorded by retailers. This should be taken into consideration and future efforts in Chile could focus on specific markets and retail types to obtain more data that could help to improve interpretation from laboratory analysis.

CONCLUSIONS

Brown and blue-shelled family farm eggs are equal in terms of external and internal quality, except for blood spots, with brown eggs having more incidence. Family farm eggs are different from those produced in cage and cage-free systems. However, it cannot be said that they are of higher quality, in general terms the main advantage of field eggs is that they are consumed fresh, with their quality attributes still intact. The family farm eggs were larger and wider, had fewer shells in relation to the total weight and had a yolk color of intermediate intensity between the intensive traditional systems. The factor that may have the greatest impact on the quality of these eggs is the less time spent during storage.

It can be concluded that free-range eggs from the southern part of our country presented better shell quality. The free-range eggs, regardless of the regional zone where they are produced, presented more intense yolk colors.

Results from this study could be used as an example of what could be found in terms of internal and external quality traits from eggs produced in developing countries where several systems are available but not much information is available for consumers.

AUTHOR CONTRIBUTIONS

Conceptualization, M.G.; methodology, M.G, and V.O.; software, J.P.K.; validation, M.G., V.O., and J.P.K.; formal analysis, V.O.; investigation, M.G., and V.O.; resources, M.G.; data curation, J.P.K.; writing —original draft preparation, V.O., and E.V.-B.-P. writing— review and editing, M.G., J.P.K and E.V.-B.-P.; visualization, E.V.-B.-P.; supervision, M.G. project administration, M.G.; funding acquisition, M.G. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was supported by the Instituto de Producción Animal of the Universidad Austral de Chile.

ACKNOWLEDGMENTS

We thank Sra. Ximena Molina and Srta. Jeniffer Berkhoff for their technical support at the Animal Nutrition Laboratory.

CONFLICTS OF INTEREST:

The authors declare no conflict of interest.

REFERENCES

Aguirre, R. & Pizarro M. J. (2018). *Panorama y mercado del huevo*. In ODEPA (ed.), Santiago, Chile.

Ahmadi, F., & Rahimi, F. (2011). Factors affecting quality and quantity of egg production in laying hens: a review. *World Applied Sciences Journal, 12*(3), 372-384.

Alcalde, J. A. (2015). Ethno-ornithology and history of the mapuche fowl. *Revista Chilena de Ornitología*, *1*(22), 126-132.

Alleoni, A. C., & Antúnez A. J. (2005). Perfil de textura e umidade espremível de géis do albume de ovos recobertos com soro de leite. *Food Science and Technology*, *25*(1), 153-157.

Altuntas, E., & Sekeroglu, A. (2008). Effect of egg shape index on mechanical properties of chicken eggs. *Journal of Food Engineering, 88*(1), 606-612. https://doi.org/10.1016/j. jfoodeng.2007.08.022

Asencio, K., Alcalde, J.A., Gandarillas, M., Keim, J.P., & Echeverría, R. (2023). Characterization of peasant family poultry farming in Southern Chile. *Poultry Science, 102*(2), 102339. https://doi.org/10.1016/j.psj.2022.102339

Berkhoff, J., Alvarado-Gilis, C., Keim, J. P., Alcalde, J. A., Vargas-Bello-Perez, E. & Gandarillas, M. (2020). Consumer preferences and sensory characteristics of eggs from family farms. *Poultry Science, 99*(11), 6239-6246. https://doi.org/10.1016/j.psj.2020.06.064

Besbes, B., Tixier-Boichard, M., Hoffman I, & Jain, G. L. (2007). *Future trends for poultry genetic resources*. In: Proceedings of the International Conference Poultry in the Twenty-first Century: avian influenza and beyond, Bangkok, Thailand.

Bovšková, H., Mikova, K. & Panovska, Z. (2014). Evaluation of Egg Yolk Colour. *Czech Journal of Food Sciences*, *32*, 213-217. https://doi.org/10.17221/47/2013-CJFS

Bustany, Z. & Elwinger, K. (1987). Shell and interior quality and chemical composition of eggs from hens of different strains and Ages Fed Different Dietary Lysine Levels. *Acta Agriculturae Scandinavica*, *37*, 213-217.

Çalişlar, S. (2019). The Important of Beta Carotene on Poultry Nutrition. S*elcuk Journal of Agriculture and Food Sciences, 33*(3), 256-263.

Chang-Ho, K., Jong-Ho, S., Jae-Cheong, L. & Kyong-Woo, L. (2014). Age-Related Changes in Egg Quality of Hy-Line Brown Hens. *International Journal of Poultry Science*, *13*(9), 510-514.

Chielo, L. I., Pike, T & Cooper, J. (2016). Ranging Behaviour of Commercial Free-Range Laying Hens. *Animals (Basel), 6*(5). https://doi.org/10.3390/ani6050028

Chung, S. H., & Lee. K. W. (2014). Effect of hen age, storage duration and temperature on egg quality in laying hens. *International Journal of Poultry Science*, *13*(11), 634-636

Coutts, J. A., & C. W. Graham. (2007). Optimum Egg Quality- A practical approach., 5M Publishing. Sheffield, GB.

Curtis, P. A., Gardner, F. A. & Mellor, D. B. (1985). A Comparison of Selected Quality and Compositional Characteristics of Brown and White Shell Eggs: I. Shell Quality. *Poultry Science, 64*(2), 297-301. https://doi.org/10.3382/ps.0640297

DSM. (2021). DSM egg yolk pigmentation guidelines. https://www.dsm.com/anh/news/feed-talks/articles/eggyolk-pigmentation-guidelines.html.

EC. (1999). Council Regulation (EC) No 1804/1999 and supplementing Regulation (EEC) Nº 2092/91 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs to include livestock production. *Official Journal of the European Communities* 24.98.1999: Brussels. L. 2221-2228.

Eisen, E. J., Bohren, B. B. & McKean, H. E. (1962). The Haugh unit as a measure of egg albumen quality. *Poultry Science, 41*(5), 1461-1468.

Elhawary, N. A., Shawky, R. M. & Nemat, H. (2005). Deletion Mechanisms in Egyptian Duchenne and Becker Muscular Dystrophy Families. *Molecules and Cells*, *19*(1), 155-155.

Fletcher, D. L., Britton, W. M. Rahn, A. P. & Savage, S. I. (1981). The Influence of Layer Flock Age on Egg Component Yields and Solids Content. *Poultry Science*, *60*(5), 983-987. https://doi.org/10.3382/ps.0600983

Gałązka-Czarnecka, I., Korzeniewska, E., Czarnecki, A., Sójka, M., Kiełbasa, P. & Dróżdź, T. (2019). Evaluation of Quality of Eggs from Hens Kept in Caged and Free-Range Systems Using Traditional Methods and Ultra-Weak Luminescence. *Applied Sciences*, *9*(12), 2430.

González, P. (2019). *Etiquetado de Huevos: Codex Alimentarius, Chile, Unión Europea y Estados Unidos de Norteamérica*. Biblioteca del Congreso Nacional de Chile/BCN.

Hammeishøj, M. (2011). Organic and free-range egg production. In Y. Nys, M. Bain, & P. Van Immerseel (Eds.). *Improving the Safety and Quality of Eggs and Egg Products*. Woodhead Publishing, 463-486.

Hisasaga, C., Griffin, S. E. & Tarrant, K. J. (2020). Survey of egg quality in commercially available table eggs. *Poultry Science*, *99*(12), 7202-7206. https://doi.org/10.1016/j.psj.2020.09.049

Huang, W., Eichenberger, A. P. & Van Gunsteren, W. F. (2012). Molecular dynamics simulation of thionated hen egg white lysozyme. *Protein Science*, *21*(8), 1153-1161. https://doi.org/10.1002/pro.2102

Idahor, K. O. (2017). Poultry Birds Egg: An Egg inside Egg whose Biological, Nutritional and Cultural Value Gives and Sustains Life. *International Journal of Research*, *3*, 1-11.

Jeffrey, F.P., & Walker, C.E. (1950). The Relationship between Egg Shell Color and Incidence of Colored Meat Spots. *Poultry Science, 29*(2), 244-247. https://doi.org/10.3382/ps.0290244

Jones, D. R., Musgrove, M. T., Anderson, K. E., & Thesmar, H. S. (2010). Physical quality and composition of retail shell eggs. *Poultry Science, 89*(3), 582-587. https://doi.org/10.3382/ps.2009-00315

Juárez, A., Guriérrez, E., Segura, J. & Santos, R. (2010). Calidad del huevo de gallinas criollas criadas en traspatio en Michoacán, México. *Tropical and Subtropical Agroecosystems,* (12), 109-115.

Khan, M. J. A., Khan, S. H., Bukhsh, A., Abbass, M. I., & Javed, M. (2013). Effect of Different Storage Period on Egg Weight, Internal Egg Quality and Hatchability Characteristics of Fayumi Eggs. *Italian Journal of Animal Science*, *12*(2), e51. https://doi.org/10.4081/ijas.2013.e51

Kingori, A. M. (2011). Review of the Factors That Influence Egg Fertility and Hatchabilty in Poultry. *International Journal of Poultry Science, 10*, 483-492.

Lordelo, M., Cid, J., Cordovil, C. M. D. S., Alves, S. P. Bessa, R. J. B., & Carolino, I. (2020). A comparison between the quality of eggs from indigenous chicken breeds and that from commercial layers. *Poultry Science, 99*(3), 1768-1776. https://doi.org/10.1016/j.psj.2019.11.023

Mugnai, C., Sossidou, E. N., Dal Bosco, A., Ruggeri, S., Mattioli, S. & Castellini C. (2014). The effects of husbandry system on the grass intake and egg nutritive characteristics of laying hens. *Journal of the Science of Food and Agriculture, 94*(3), 459-467. https://doi.org/10.1002/jsfa.6269

Nys, Y., & Le Roy, N. (2018). Calcium Homeostasis and Eggshell Biomineralization in Female Chicken. In D. Feldman (Ed), *Vitamin D* No. 1. Academic Press, London, UK. p. 361-382.

Odabasi, A. Z., Miles, R. D., Balaban, M. O., & Portier, K. M. (2007). Changes in brown eggshell color as the hen ages. *Poultry Sciences*, *86*(2), 356-363.

Padhi, M., Chatterjee, R. Haunshi, S., & Ullengala, R. (2013). Effect of age on egg quality in chicken. *Indian Journal of Poultry Science*, *48*, 122-125.

Patterson, P. H., Koelkebeck, K. W., Bell, D. D., Carey, J. B., Anderson, K. E., & Darre, M. J. (2001). Egg marketing in national supermarkets: Specialty eggs - Part 2. *Poultry Science*, *80*(4), 390-395. https://doi.org/10.1093/ps/80.4.390

Rakonjac, S., Bogosavljevic-Boskovic, S., Pavlovski, Z., Skrbic, Z., Doskovic, V., Petrovic, M. D. & Petricevic, V. (2014). Laying hen rearing systems: a review of major production results and egg quality traits. *World's Poultry Science Journal*, *70*(1), 93-104. https://doi.org/10.1017/S0043933914000087

Ramírez, A., González, J., Andrade, V., & Torres. V. (2016). Efecto de los tiempos de conservación a temperatura ambiente, en la calidad del huevo de gallinas camperas (*Gallus domesticus*) en la Amazonia Ecuatoriana. *REDVET. Revista Electrónica de Veterinaria*, *17*(12), 1-17.

Rehault-Godbert, S., Guyot, N., & Nys, Y. (2019). The Golden Egg: Nutritional Value, Bioactivities, and Emerging Benefits for Human Health. *Nutrients*, *11*(3), 684 https://doi.org/10.3390/nu11030684

Ricke, S. C., & Rothrock, M. J. (2020). Gastrointestinal microbiomes of broilers and layer hens in alternative production systems. *Poultry Science*, *99*(2), 660-669. https://doi.org/10.1016/j.psj.2019.12.017

Rizzi, C., & Marangon, A. (2012). Quality of organic eggs of hybrid and Italian breed hens. *Poultry Science*, *91*(9), 2330-2340. https://doi.org/10.3382/ps.2011-01966

Roberts, J. R. (2004). Factors Affecting Egg Internal Quality and Egg Shell Quality in Laying Hens. *The Journal of Poultry Science*, *3*(41), 161-177.

Roberts, J. R., Souillard, R., & Bertin, J. (2011). Avian diseases which affect egg production and quality. *Improving the Safety and Quality of Eggs and Egg Products*, 376-393. https://doi.org/10.1533/9780857093912.3.376

Rodriguez-Navarro, A. B., N. Dominguez-Gasca, A. Munoz, and M. Ortega-Huertas. (2013). Change in the chicken eggshell cuticle with hen age and egg freshness. *Poultry Science*, *92*(11), 3026-3035. https://doi.org/10.3382/ps.2013-03230

Romanoff, A. L., & Romanoff, A. S. (1949). *The Avian Egg*. John Wiley and Sons, New York.

Rostagno, H., L. F., Texeira, M. I., Hannas, D. J.L., Perazzo, F. G. Saraiva, A. Texeira de Abreu, M. L. Rodriguez, P. B. de Oliveira, R. F. de Toledo, S. L. & De Oliveira Brito, C. (2017). Tablas Brasileñas para Aves y Cerdos. H. S. Rostagno (Ed.). *Composición de Alimentos y Requerimientos Nutricionales*, Viçosa.

Ruhnke, I. (2015). Free-range egg production in Australia – industry trends and challenges. *Lohmann Information*, *50*, 24-31.

SAG. (2016). Bioseguridad en la avicultura familiar campesina. Programa de vigilancia epidemiológica, Gobierno de Chile. In M. d. Agricultura (Ed.), Santiago, Chile.

Samiullah, S., Omar, A. S. Roberts, J. & Chousalkar, K. (2017). Effect of production system and flock age on eggshell and egg internal quality measurements. *Poultry Science*, *96*(1), 246-258. https://doi.org/10.3382/ps/pew289

Scott, T. A., & Silversides, F. G. (2000). The effect of storage and strain of hen on egg quality. *Poultry Science*, *79*(12), 1725-1729. https://doi.org/10.1093/ps/79.12.1725

Seemann, M. (2000). *Factors which influence pigmentation Lohman Information*, No. 24, 20-26.

Skrivan, M., Englmaierova, M. Skrivanova, E., & Bubancova, I. (2015). Increase in lutein and zeaxanthin content in the eggs of hens fed marigold flower extract. *Czech Journal of Animal Science*, *60*(3), 89-96. https://doi.org/10.17221/8073-Cjas

Sosnówka-Czajka, E., Herbut, E. & Skomorucha, I. (2010). Effect of different housing systems on productivity and welfare of laying hens. *Annals of Animal Science*, *10*(4), 349-360.

Stadelman, L.W., Jensen, J.S., & Cyrus, M. (1952). The Relationship of Shell Color to Incidence of Blood and Meat Spots in Hens' Eggs. *Poultry Science, 31*(5), 855-856. https://doi.org/10.3382/ps.0310855

Stojanova, M., Kocevski, D., Vukovic, V., & Porcu, K. (2016). Comparison of qualitative properties of eggs from hens reared in battery cages and free-range system. *International Journal of Agronomy and Agricultural Research*, *9*(6), 77-83.

Thaxton, Y. V., Christensen, K. D., Mench, J. A., Rumley, E. R. Daugherty, C. Feinberg, B. Parker, M. Siegel, P., & Scanes, C. G. (2016). Symposium: Animal welfare challenges for today and tomorrow. *Poultry Science*, *95*(9), 2198-2207. https://doi.org/10.3382/ps/pew099

Titcomb, T. J., Kaeppler, M. S., Cook, M. E Simon, P. W. & Tanumihardjo, S. A. (2019). Carrot leaves improve color and xanthophyll content of egg yolk in laying hens but are not as effective as commercially available marigold fortificant1. *Poultry Science*, *98*(10), 5208-5213. https://doi.org/10.3382/ps/pez257

Travel, A., Nys, Y. & Lopes, E. (2010). Facteurs physiologiques et environnementaux influençant la production et la qualité de l'œuf. *INRAE Productions Animales*, *2*(23), 155-166.

Van den Brand, H., Parmentier, H. K., & Kemp, B. (2004). Effects of housing system (outdoor vs cages) and age of laying hens on egg characteristics. *British Poultry Science*, *45*(6), 745-752. https://doi.org/10.1080/00071660400014283

Wang, X. L., Zheng, J. X., Ning, Z. H., Qu, L. J., Xu, G. Y., & Yang, N. (2009). Laying performance and egg quality of blue-shelled layers as affected by different housing systems. *Poultry Science*, *88*(7), 1485-1492. https://doi.org/10.3382/ps.2008-00417

Austral J Vet Sci 55, 95-103 (2023)

ORIGINAL ARTICLE

Effects of the inclusion of brown seaweed (*Macrocystis pyrifera*) additive in the diet of grass-fed steers on carcass performance, meat quality, and nutrient composition

Nancy Jerez-Timaure[a*], Rubén Pulido[a], Florencia Heinsohn[b], Jonathan Mendoza[c], Josefa Fuentes[b], Marcelo Brintrup[c], Maureen Berkhoff[a]

ABSTRACT. The objective of this study was to evaluate the inclusion of a brown seaweed additive (SWA; *Macrocystis pyrifera*) in the diet of grass-fed steers on carcass performance, beef quality, and nutrient composition. A total of 20 Holstein-Friesian steers were randomly distributed into two groups: Control group (a basal diet without supplementation of SWA) and SWA group (2%-SWA) with basal diet + 30 g/day/animal of SWA during the breeding phase (11 months) and 48 g/day/animal of SWA during the fattening phase (4 months). Steers fed with 2%-SWA were not different (P>0.05) in final body weight, carcass weight, carcass dressing, fat thickness, ribeye area, and marbling score than those from the Control group. Likewise, no effects of 2%-SWA supplementation were detected (P>0.05) for beef quality traits, glycolytic potential, or their metabolites (muscular glycogen, glycose+glucose-6-phosphate, and lactate), evaluated in *longissimus lumborum* (LL) samples. Sensory evaluation showed a slight preference for Control group samples rather than those from the 2%-SWA group (58.93% and 41.07%; P=0.06). Regarding, proximal composition, the inclusion of SWA only affected the total lipids present in the LL samples, which decreased significantly (P=0.01) in LL samples of grass-fed steers fed with 2%-SWA. The composition of macro (Ca, Na, Mg, P, and K) and micro (Mn, Fe, Cu, and Zn) minerals in LL samples were not affected (P>0.05) by the inclusion of SWA in the diet. The inclusion of the additive based on brown seaweed had not a detrimental effect on carcass performance, beef quality, and mineral content, however, it reduced the total lipids content in the LL muscle.

Keywords: seaweed, carcass, lipids, proximal composition, mineral, beef.

INTRODUCTION

Beef is known as one of the main sources of protein with high biological value, bioavailable minerals (Fe, Zn, and P), vitamins of the B-complex (B1, B2, B3, B6, and B12), and other nutritional components like vitamins D, E, and β-carotenes (Williams, 2007; Klurfeld, 2018). Besides, it is considered that meat has been related to the evolution of humanity due to its important impact on human cognitive, morphophysiological, and social development (Psouni *et al.,* 2012).

At present, beef consumption in Chile is estimated around 24 kg/year per capita, foreseeing a slight upward trend in the next 10 years (OECD-FAO, 2019). In addition, in the last decades, there is an increasing preference for natural, organic, and antibiotic-free meat (Karmaus & Jones, 2021), creating a growing interest in the study of natural supplements in animal nutrition. Also, beef industries are facing constantly challenges, such as an enhanced request for certification on animal welfare (Rossi *et al.,* 2020), environmental emissions, and climate change (Halmemies *et al.,* 2018). These demands require animal productions to be more sustainable, in conjunction with the use of innovative resources (Halmemies *et al.,* 2018; Rossi *et al.,* 2020; Raja *et al*., 2022). In this context, marine algae could be able to become an economical and competitive option for animal production worldwide (Raja *et al.*, 2022; Madeira *et al.*, 2017) due to their nutritional value, and content of bioactive compounds which could contribute to enhance production and health in animals (Halmemies *et al.*, 2018). Also, some seaweed species have shown the potential to mitigate ruminal methane production *in vitro* (Maia *et al*., 2016).

Nutrition is one of the main factors that greatly influence growth and carcass performance (Guerrero *et al.,* 2013; Mwangi *et al.,* 2019), but it also produces important changes in the nutrient composition of meat, such as fatty acid composition, and mineral content (Rotta *et al.,* 2009).

Algae are autotrophic organisms with a simple structure, little or no cell differentiation and complex tissues, being considered talophytes. By taxonomy, they are classified into three groups: Chlorophyta or chlorophytes, Phaeophyta or pheophytes, and Rhodophyta or rhodophytes, which correspond to green algae, brown algae, and red algae, respectively (Quitral *et al.,* 2012).

Among the huge variety of marine algae, brown seaweed is widely found in large populations in North America (from Alaska to Baja California) and South America (from Perú to Tierra del Fuego), including the extensive Chilean coast (Baca *et al.,* 2008). Its large size and easily harvesting have allowed this type of algae to be studied and used in animal feeding (Makkar *et al.,* 2016). Within this group of algae, the best known in south America is *Macrocystis pyrifera* (popularly known as "huiro"), *Lessonia nigrescens*

Received: 09.09.2022

Accepted: 05.01.2023

[a]Instituto de Ciencia Animal, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile.

[b]Escuela de Medicina Veterinaria, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile.

[c]I+D Patagonia Biotecnología SA., Valdivia, Chile.

*Corresponding author: nancy.jerez@uach.cl

("huiro negro"), and *Durvillaea antarctica* ("cochayuyo") (Quitral *et al.,* 2012).

Brown seaweeds have a protein content of 5 to 15%, but very high levels of minerals, fatty acids, carbohydrates, and essential amino acids (Raja *et al.*, 2022; MacArtain *et al.,* 2007; Baca *et al.,* 2008; Makkar *et al.,* 2016). Particularly, *Macrocystis pyrifera* contains from 8.7 to 10.7% of crude protein, while the concentration of ashes ranged between 33.5 and 36.6% (Baca *et al.,* 2008). They are an excellent source of vitamins A, B1, B2, B3, B5, B12, C, D, E, and folic acid (Makkar *et al.,* 2016; Quitral *et al.,* 2012; Ortiz *et al.,* 2008). In addition, no anti-nutrients have been found in its composition (Casas *et al.,* 2005). In this context, brown seaweed could be considered a natural source of nutrients and bioactive compounds with great biological activity with potential benefits for animal health and growth, also as a choice to produce functional foods (Quitral *et al.,* 2012).

Extensive reviews about the inclusion of seaweeds in monogastric production have been published (Corino *et al.,* 2019; Angell *et al.,* 2016; Øverland *et al.,* 2019). However, fewer studies have investigated the effects of dietary seaweed on beef carcass traits, and its nutrient composition. The magnitude of the associate response to the inclusion of seaweed in the animals' diet on growth performance, carcass traits, quality and nutrient composition of meat depends on the type of seaweed used, the bioactive components present in the extract, and the proportion and frequency used in the diet (Makkar *et al.,* 2016). The objective of this study was to assess the inclusion of a seaweed additive (*Macrocystis pyrifera*) supplied during the backgrounding and fattening phases of grass-fed beef cattle on its productive performance, carcass parameters, beef quality, and nutrient composition.

MATERIALS AND METHODS

ANIMALS AND SAMPLING

A total of 20 Holstein-Friesian calves, after weaning were reared, at then finished at the Agricultural Austral Research Station of the Universidad Austral de Chile. The calves, prior to the start of the trial, were managed in artificial rearing receiving milk replacer (4 L/day), initial concentrate (increasingly until reaching 2 kg/day), and alfalfa cubes, up to 200 g daily. The control and treatment groups were weaned averaging 79.6 and 77.8 days, weighing 98.0 kg and 99.4 kg, respectively. The backgrounding period lasted 11 months and the fattening period 4 months. Animals were fed mainly with permanent pasture (*Lolium perenne* L. dominated sward (55% *L. perenne*, 33% *Bromus valdivianus* Phil., 5% *Trifolium repens* L. and 7% of other species) offered based on 3% body weight measured at ground level. Animals were also supplemented with a commercial concentrate (49.3 maize, 11.5 soybean meal, 30.0 beet pulp, 4.6 beet molasses), 4.5 of mineral mix and silage (approximately 17 kg/animal when needed) offered. The expected dry matter intake was 2.5% of body weight during the entire experiment. The pasture was managed on a rotational grazing with a resting period between 10 to 15 days. The concentrate and the silage were offered in feeding pens at 08:00 h and 15:00 h. Water was permanently offered *ad libitum* in the paddocks and enclosure pens. Samples of pasture, concentrate and SWA were collected on each period. All samples were immediately frozen at -20 °C and then freeze-dried for chemical analysis. Before chemical analysis, the samples were ground through a 1 mm screen (Willey Mill, 158 Arthur H, Thomas, Philadelphia, PA). The bromatological results of the analysis of forages, commercial concentrate, and SWA are described in Table 1.

Animals were assigned to one of the following treatments: Control group: basal diet with 0% of SWA; and 2%-SWA group: basal diet + 30 g/day/animal of SWA during the growing phase and 48 g/day/animal of SWA during the fattening phase. The levels of SWA used were determined in preliminary experiments. The additive was added to the concentrate and mixed manually and offered to animals in individual feeders. The SWA was fabricated by I+D Patagonia Biotecnología S.A., as an impalpable hydrolyzed seaweed powder produced by spray drying of *Macrocystis pyrifera*, that maintains its chemical-physical characteristics and bioactive compounds. Animals were sent to harvest when they reached between 16 to 18 months of age.

Table 1. Chemical composition of the experimental diet: forage, commercial concentrate, and seaweed additive (SWA).

Parameter	Forage	Commercial concentrate	SWA
DM, %	30.76	85.9	92.67
TA, %	7.15	5.81	25.60
CP, %	16.50	17.25	8.87
ADF, %	27.61	8.77	11.78
EE, %	0.29	3.41	0.29
CF, %	-	6.46	2.87
NDF, %	52.93	29.88	7.06
NFE, %	6.92	52.97	43.93
DV, %	73.31	77.83	70.91
ME, Mcal/kg DM	2.62	2.81	2.58

DM: Dry matter, TA: Total Ash, CP: Crude protein, ADF: Acid-detergent fiber Ether extract, and EE: Ether extract were determined by AOAC (1996). CF: Crude fiber determined by AOAC (1984). NDF: Neutral-detergent fiber obtained according to Van Soest *et al.* (1991). NFE: Nitrogen free extract. % NFE = 100 % – (% moisture + % EE + % CP + % TA + % CF). DV: Digestibility value according to Tilley & Terry (1963). ME: Metabolizable energy determined by Goering & Van Soest (1970).

CATTLE HARVESTING, CARCASS EVALUATION, AND SAMPLING

Steers were transported to a slaughterhouse plant facility located 15 km away from the farm and slaughtered after 12 h of lairage. Harvesting, dressing procedures, and *postmortem* inspection followed the standards of the Chilean regulation (INN, 1993). Final body (BW) and hot carcass weights were recorded to estimate carcass dressing yield. Ribeye area, fat thickness at the 10^{th} rib, and degree of marbling were evaluated according to the procedure stipulated by the USDA (2017). Carcasses were chilled for 24 h *postmortem* at 2 °C.

A core of *longissimus lumborum* (LL) samples from each carcass was taken at 24 h *postmortem*, immediately frozen in liquid nitrogen (-196 °C) and stored at -80 °C for muscular glycogen content (MGC), glucose+glucose-6 phosphate (G+G6P), and lactate concentration (LC) determination. After 48 h *postmortem*, the entire portion of the LL muscle was removed from each left side of the carcass, and samples of 2.5 cm thickness were obtained. Two samples were used immediately for pH and color evaluation, and four samples of each loin were individually packaged and frozen at -20 ºC during 30 days for the rest of the analysis.

MEAT QUALITY AND POSTMORTEM GLYCOLYTIC METABOLITES

A portable pH meter with a puncture electrode (Hanna, model HI 99163, Jud Cluj, Rumania) was previously calibrated with buffer pH 4 and 7 was used for pH measurement. A Hunter Lab Mini Scan XE Plus (Hunter Associates, Reston, VA, USA) was used with a 2.5-cm open port, Illuminant D65 and 10◦ standard observers to objectively evaluate color. Three readings were obtained from the muscle surface, and the mean was calculated. Readings were obtained after exposing the muscles to air for 30 min (bloom). The color scale used was Hunter L, a, b. The L value represents lightness; the a and b values represent redness, and yellowness, respectively.

The Warner-Bratzler Shear Force (WBSF) was estimated on samples cooked in a convection oven (Albin Trotter model E-EMB Digital) until reaching a final internal temperature of 70 °C following the guidelines of the American Meat Science Association (AMSA, 2016). The temperature was monitored using an oven thermometer ranged (-10 +110 °C; +/- 1°C) inserted into the geometric center of each steak. The cooked steaks were chilled for 2 h at 4 °C, and then eight cores (1.27 cm in diameter) were removed parallel to the muscle fiber orientation. Cores were sheared once each on the Warner- Bratzler Meat Shear apparatus (GR Manufacturing Co., Manhattan, KS, USA) to get WBSF values. The water holding capacity (WHC) was determined as cooking loss, which was determined by weight, expressed as a percentage compared to the original weight of the sample. A taste preference test was performed in two sessions (56 panelists). Two steaks of each treatment were used in each session. The tests were carried out in individual evaluation cabinets illuminated with red light. Each panelist, in each session, tasted two samples (one from each treatment) at random, and they were asked to select the best preference.

The MGC, G+G6P, and LC were determined as described in Apaoblaza *et al.* (2015). Briefly, muscle samples were homogenized in ice-cold phosphate buffer (pH 7). Ten µL of homogenate were hydrolyzed in 200 µL of 0.1M HCl at 100 °C for 2 h, after which pH will be adjusted to 6.5-7.5 and glucose determined via NADP reduction with a linked assay involving hexokinase and glucose-6-phosphate dehydrogenase (Glucose HK 16-50 Sigma). The LC was determined from the homogenate via NAD reduction with a linked assay involving lactate dehydrogenase and glutamate pyruvate transaminase (Boehringer Mannheim). Glycolytic potential (GPOT) was calculated with the following formula GPOT = (LC) + 2([MGC] + [G+G6P]) and was expressed as millimoles of lactate per kilogram of muscle (Monin & Sellier, 1985).

NUTRIENT COMPOSITION OF BEEF

Moisture, protein, and lipid content of meat samples were determined according to the AOAC (1990). Duplicates of 10 g of ground meat were calcined in a furnace at 550 °C for 6 h. After cooling, the residue (white ash) was subjected to an acid digestion process with 10 mL of a 20% *v*/*v* hydrochloric acid solution by heating on a hot plate for 10 min. Mineral analyses were conducted by atomic absorption and/or atomic emission (AOAC, 1990), following the analytical methods described by Perkin-Elmer (1994). Values were expressed as g/100 g or mg/g of dry matter (DM).

STATISTICAL ANALYSIS

The experimental design was a completely randomized design. A one-way ANOVA was performed using a mixed model with SWA treatment as the main factor and animal as the random effect. The value $P \leq 0.05$ was used to declare the significant difference between the average scores. Tukey's multiple comparison test was used for the comparison of means. The Bonferroni correction was also performed to adjust the probability of *P* values. χ^2 test was used for sensory preference data. Analysis was performed using the R Program (R Core Team, 2021).

RESULTS

CARCASS PERFORMANCE.

Both groups had similar BW ($P=0.69$) at the beginning of the study with 463.9 kg and 470.6 kg for treatment and control groups, respectively. Table 2 shows mean values for carcass traits. Carcass traits evaluated in this study were not affected (*P*>0.05) by the inclusion of 2%-SWA in the diet of steers. Most of the carcasses were described as practically devoid of marbling (scale 1; USDA, 2017), and all carcasses exhibited a similar fat thickness (P>0.05) and finish score or subcutaneous fat cover (1= Slight) according to INN (1993) and similar.

MEAT QUALITY TRAITS AND *POSTMORTEM* GLYCOLYTIC POTENTIAL AND THEIR METABOLITES

Table 3 shows the mean values for meat quality traits. There is no significant effect (*P*>0.05) of the inclusion of 2%-SWA in the diet of steers on muscular pH, cooking loss, and WBSF. Instrumental colour was determined in its three dimensions (a, b, and L values) and no significant differences were detected for any of them (*P*>0.05) when comparing both groups under study. The ANOVA detected the non-significant (*P*>0.05) effect of the inclusion of SWA on MGC, G+G6P, and LC and GPOT (Table 4) evaluated at 24 h *postmortem.*

Regarding sensory evaluation, in session 1, 18 panelists preferred samples from the control group equivalent to 64%. In session 2, it was counted 15 preferences for the control group (equivalent to 53%). Together, this represents 58.93% of taste preference for Control samples compared to 41.07% of preference for 2%-SWA group. Although there was no statistically significant difference in the preferences of the panelists, a tendency (*P*=0.06) to prefer the samples of the Control group compared to those of the SWA group was observed. In both sessions, the panelists considered that all samples had a normal taste, without the presence of a strange or unpleasant flavor. Some panelists even highlighted the juiciness and tenderness of the samples in their observations.

Table 2. Effects of the inclusion of seaweed additive (SWA) in the diet of grass-fed steers on growth and carcass traits.

Variable	Control	2%-SWA	SEM	*P*-value
Final body weight, kg	470.6	463.9	8.10	0.69
Hot carcass weight, kg	227.88	221.36	4.49	0.48
Hot carcass dressing, %	48.37	47.70	0.28	0.24
Fat thickness, mm	4.27	4.06	0.29	0.73
Ribaye area, cm^2	45.61	49.93	2.32	0.37
Marbling*	1.8	1.6	0.17	0.59

* 1= practically devoid; 2= scarce; 3= small amount of marbling (USDA, 2017). SEM: standard error of the mean.

Table 3. Effects of the inclusion of seaweed additive (SWA) in the diet of grass-fed steers on meat quality traits.

Variable	Control	2%-SWA	SEM	*P*-value
Muscular pH, 48 h	5.65	5.67	0.03	0.69
Redness (a value)	15.21	15.52	0.29	0.62
Yellowness (b value)	9.72	9.62	0.25	0.74
Lightness (L value)	27.51	26.51	0.96	0.24
Cooking loss, %	16.41	16.44	0.64	0.96
WBSF, kg	2.19	2.12	0.09	0.46

SEM: standard error of the mean. WBSF: Warner Bratzler shear force.

Table 4. Effects of the inclusion of seaweed additive (SWA) in the diet of grass-fed steers on *postmortem* glycolytic metabolites and glycolytic potential.

Variable*	Control	2% SWA	SEM	*P*-value
MGC	6.07	6.73	0.69	0.64
LC	35.18	40.26	2.10	0.23
G+G6P	10.81	10.20	0.36	0.42
GPOT	65.63	59.56	3.90	0.45

SEM: standard error of the mean. MGC: muscular glycogen content. LC: lactate content. G+G6P: Glucose + Glucose-6-phosphate. GPOT: Glycolytic potential. *Measured at 24 h *postmortem* (mmol/kg).

NUTRIENT COMPOSITION OF MEAT

Table 5 shows the mean and standard error of the mean of the proximal composition and mineral content of LL, according to the treatment groups. Proteins and total ash were not affected by the SWA additive (*P*>0.05). However, there was an effect (*P*<0.05) of the inclusion of 2% of SWA on the total lipid content in the bovine LL. Samples from animals that were fed with 2% SWA had a lower (*P*=0.01; Table 5) amount of total lipids (5.24 g/100 g dry matter) than those from the Control group (6.65 g/100 g dry matter).

The inclusion of SWA in the diet of fattening steers did not affect (*P*>0.05) the content of the macro (Ca, Na, Mg, P, and K) and micro minerals (Mn, Fe, Cu, and Zn) evaluated in this study. Steers that were fed with 2% of SWA exhibited less numerical values of Mn and Zn than the Control group (*P*>0.05; Table 5).

DISCUSSION

In recent decades, interest in the use of seaweeds as organic ingredients in farm animal has increased (Makkar *et al.,* 2016). The inclusion of seaweed has been investigated in the feeding of sheep (Marín *et al.,* 2003), pigs (Baca *et al.,* 2008), rabbits (Rossi *et al.,* 2020), and cattle (Morrill *et al.,* 2017a, b). Most of these researchers have been focused on growth parameters, like body weight, daily weight gain and feed conversion. However, few studies of this nature have been conducted on the evaluation of carcass performance, meat quality, and nutrient composition.

Table 5. Effects of the inclusion of seaweed additive (SWA) in the diet of grass-fed steers on proximal composition and mineral content of *longissimus lumborum.*

Variable	Control	2%-SWA	SEM	*P*-value
		Proximal composition[1]		
Ash	4.82	4.79	0.05	0.82
Crude protein	88.21	87.80	0.51	0.61
Total lipids	6.65	5.24	0.89	0.01
		Macrominerals[1]		
Ca	0.017	0.017	0.001	0.98
Na	0.164	0.166	0.003	0.75
Mg	0.075	0.074	0.001	0.72
P	0.723	0.719	0.006	0.76
K	1.39	1.41	0.03	0.81
		Microminerals[2]		
Mn	8.82	7.97	0.764	0.59
Fe	26.07	26.71	2.58	0.90
Cu	15.22	15.62	0.83	0.81
Zn	77.29	72.68	2.75	0.42

[1] g/100 g of dry matter (DM). [2] mg/g of DM. SEM: standard error of the mean.

In this study, the inclusion of 2% of SWA in the diet of grass-fed steers did not affect the final body weight. Several authors did not find significant variations in the growth of lambs (Al-Shorepy *et al.,* 2001) or steers (Anderson *et al.,* 2006) in response to the inclusion of supplements based on marine algae. On the other hand, Fike *et al.* (2001) reported an increase in the weight of lambs that were fed with seaweed extract during the summer grazing period. In Chile, only the studies of Mendoza (2017) and Nannig (2018) have evaluated the effect of brown algae on bovine, reporting similar average daily gains when comparing heifers treated with SWA *vs.* Control ones.

Carcass weight and dressing were not influenced by the inclusion of 2%-SWA in the diet of steers. Fat thickness, marbling, and ribeye area of the carcass from finishing steers fed with SWA were similar (P>0.05) to those from the Control group. Morrill *et al.* (2017a) also reported no differences in carcass weight, fat thickness, or *longissimus* muscle area in carcasses from steers consuming 9% of post-extraction algal residue (PEAR) compared to those that received glucose infusion (Control group). However, in this same study, the marbling score was 15% greater in PEAR-fed group compared to Control carcasses.

The inclusion of 2% of SWA did not affect the instrumental tenderness of meat. Control samples of LL had 2.19 ± 0.09 kg in WBSF and the mean ± SEM for the SWA group was 2.12 ± 0.12 kg. Jerez-Timaure *et al.* (2021) found similar results in pork LL samples. Morrill *et al.* (2017b) stated that feeding with PEAR resulted in a slight but no significant reduction of shear force, with values between 2.77 and 2.5 kg. Miller *et al.* (2001) developed a tenderness threshold based on consumer acceptability, stablishing that WBSF values < 3.0 kg can be classified as tender beef and WBSF values > 4.6 kg were tough beef. According to Miller *et al.* (2001), LL samples from this study could be categorized as tender (<3 kg), and those are very similar to the values reported by Morrill *et al.* (2017b).

The sensory evaluation showed that a slight majority (58.93%) of panelists had a tendency (P=0.06) to prefer samples of the Control group rather that those from the 2%-SWA. These results could be related to the increased content of total lipids detected in samples from de Control group (Table 5). Lipid compositions are related to flavor development, with a different range of flavor precursor being produced from saturated and polyunsaturated fatty acids (Wood & Enser, 1997). Braden *et al.* (2007) stated that the supplementation effect on sensory evaluation taste depends on the ingredient of the diet. Morrill *et al.* (2017b) found no significant differences, as in this study, in the tenderness and flavor of meat from bovines supplemented with an additive made of marine algae residues. Jerez-Timaure *et al.* (2021) reported similar results, they reported a greater taste preference (P<0.05) by consumers in samples coming from the Control group. However, in the case of studies in rabbits, it is reported that supplementation with brown marine algae improved the palatability of their meats (Rossi *et al.*, 2020). Morrill *et al.* (2017b) stated that cattle have limited ability to digest and absorb lipids to negatively affect meat flavor. Also, it is well known that changes in fatty acid composition are often associated with flavor differences (Arshad *et al.,* 2018). In this study, the fatty acid composition was not evaluated. Jerez-Timaure *et al.* (2021) did not detect differences in the fatty acid profile of loin samples from pigs that fed SWA at 2 and 4% compared to the Control ones (0%-SWA).

Meat color is considered one of the most valued and preferred attributes by the consumer at the time of purchase, preferring bright red meat and rejecting dull or brown meat (Holman *et al.,* 2017). It has been studied that supplementation with antioxidants, such as vitamin E, helps to extend the case and shelf life of fresh meat products, improving color stability, reducing lipid oxidation, and delaying the formation of metmyoglobin (Allen *et al.,* 2001; Braden *et al.,* 2007; Rossi *et al.,* 2020). It is important to emphasize that the additive based on brown marine algae *Macrocystis pyrifera* used in this study is rich in antioxidants (MacArtain *et al.,* 2007; Ortiz *et al.,* 2008), and an improvement in meat color was expected. However, instrumental color values of 2%-SWA samples were not statistically different (P>0.05) compared to the control ones. Jerez-Timaure *et al.* (2021) reported that the inclusion of 4%-SWA affected redness value of LL pork. The differences in the content of natural antioxidants such as polysaccharides and polyphenols present in the SWA, and some possible interactions between the polysaccharides present in the SWA, might affect the oxymyoglobin (Ortiz *et al.,* 2008) and therefore it influences meat color. Beef from pasture-raised animals tend to have higher levels of antioxidant compounds, like phenols, terpenoids, carotenoids and tocopherols (Van Vliet *et al*., 2021) which also affect color stability.

The pH represents one of the most important characteristics of meat because it is highly related to meat quality. In this study, muscle pH values were not affected by treatments. Some authors reported that feeding has little influence on water holding capacity, which is an important parameter to define the taste and technological quality of meat (Braden *et al.,* 2007). In this study, the water holding capacity expressed as total cooking losses were not affected by the inclusion of SWA in the diet of finishing steers. Previous studies carried out with the same additive, but in pigs (Jerez-Timaure *et al.,* 2021), also reported that the 2 and 4% inclusion of SWA does not affect the water holding capacity.

Postmortem pH directly affects quality characteristics, such as water holding capacity, color, and, to a lesser degree, tenderness (Jacob & Hopkins, 2014). In addition, the speed of the decrease in pH in the carcass is influenced by multiple factors or *antemortem* handling of the animal (Gallo *et al.,* 2013; Gallo & Huertas, 2016) or *postmortem* factors (Jacob & Hopkins, 2014; Ponnampalam *et al.,* 2017). For decades, GPOT has been used as a fair approximation for the total compounds transformable to lactic acid present in the muscle at slaughter (Monin & Sellier, 1985). It has been shown that anaerobic glycolytic processes occur early after exsanguination and these drastic biochemical changes are caused by the fast glycolytic activity that is triggered by the *postmortem* action of glycolytic enzymes. Muscular glycogen content was very low at 24 h *postmortem.* Steers used in this study were fed mainly with forage and were fasted for almost 12 h before slaughtering, which may explain the low levels of MGC. However, pH values were ranged in the normal pH threshold (<5.8). Values greater that 5.8 are associated with dark cutting beef (Ponnampalam *et al.,* 2017; Apaoblaza *et al.,* 2015). Previous studies performed in southern Chile also reported low levels of *postmortem* MGC. Amtmann *et al.* (2006) found that *longissimus* muscle in grass-fed carcasses with pH <5.8 had 35.5 ± 15.7 mmol/kg of MGC. Meanwhile, Apaoblaza *et al*. (2015) reported MGC at 24 h of 6.34 ± 0.05 in normal pH carcasses.

Our results show that muscle glycogen, lactate, and glucose content, evaluated at 24 h *postmortem*, were not affected by the effect of the inclusion of the seaweed additive in the diet (*P*>0.05). In addition, they indicate that the *postmortem* glycolytic metabolism was similar in both groups of carcasses. The glycolytic potential used to determine the ability to convert glycogen into lactate (Monin & Sellier, 1985) was similar in both groups (*P*>0.05), indicating that the seaweed inclusion treatment did not modify the *postmortem* glycolytic capacity, allowing a normal drop of muscle pH in early *postmortem*.

The lipid fraction and fatty acid composition are mainly influenced by three factors: the age of the animal, the composition of the animals' diet, and the breed. Mwangi *et al.* (2019) stated that notable modifications produced by the feeding systems in the chemical composition of the meat are mainly in the fat content. In this study, meat from steers that were fed with 2%-SWA decreased its amount of total lipids. Natural pigments of brown marine algae, like fucoxanthin is found, which could inhibit the differentiation of preadipocytes into adipocytes. This has also been demonstrated by other studies with other carotenoids that, like fucoxanthin, have an allenic group in their composition (Quitral *et al.,* 2012). Also, it is known that marine algae are rich in vitamin A (Quitral *et al.,* 2012), which have retinoic acid in their composition, which restricts hyperplasia and/or regulates the growth hormone gene, resulting in a decrease in the fat deposition (Mwangi *et al.,* 2019). Seaweeds also have high amounts of vitamin D, being its active form (1,25-dihydroxyvitamin D3), the cause of inhibiting the differentiation of adipocytes (Mwangi *et al.,* 2019). This occurs when its plasma levels increase, which occurs in situations of low dietary calcium intake (Mwangi *et al.,* 2019).

Given the growing demand for leaner meats or meats with lower fat content by consumers concerned about diet-health aspects, the use of the additive based on seaweed in cattle feed could become an alternative approach to achieve differentiated products with added value aimed at consumers who prefer lower lipid content in their protein foods of animal origin.

Morrill *et al.* (2017b) show that the meat of cattle fed with marine algae residues slightly modifies the fatty acid profile of the beef, so it is necessary to evaluate the composition of fatty acids in this study because it could result in meats with a higher concentration of polyunsaturated fatty acids, due to the contribution of these compounds offered by brown seaweed. On the other hand, Casas *et al.* (2005) mentioned that the presence of omega 3 fatty acids in seaweed is an aspect of interest, since it could be an alternative to produce meats with a higher content of polyunsaturated fatty acids, which are beneficial for human health.

CONCLUSIONS

In general, no harmful effects were found in this study by feeding grass-fed steers during fattening with brown sea algae extracts; however, a reduction of total lipids present in the LL samples of grass-fed steers supplemented with 2%-SWA was evidenced. Since seaweed represents a group of organisms with diverse types of bioactive compounds, further studies are needed to understand the biological effects of this SWA on adipogenesis or fatty acid oxidation, as an alternative to producing beef with low content of fat.

ETHICS STATEMENT

Animal handling for animal care and welfare were performed and revised according to the Institutional Committee for Animal care and use of the Universidad Austral de Chile. Resolution number 281/2021. (https://vidca.uach.cl/comite-bioetica-investigacion-uso-animales/)

AUTHOR CONTRIBUTIONS

N.J-T.: Conceptualization, data curation and analysis, original draft preparation, and edition.

R.P.: conceptualization, investigation, writing, and review.

F.H. & J.F.: data collection, experimental work, investigation, and writing,

J.M.: supervision of data collection, data curation, data analysis, and review.

M.Br.: funding acquisition, conceptualization, review, and edition.

M.B.: experimental work, laboratory analysis, and revision of the manuscript.

All authors searched and reviewed the literature, discussed the contents of the manuscript, and approved submission.

FUNDING

This research was funded by the Project CORFO INNOVA CHILE 171TE2-72098

ACKNOWLEDGMENTS

The authors are grateful to Agricultural Austral Research Station of the Universidad Austral de Chile. And I+D Patagonia Biotecnología S.A., for their valuable support during the experimental trial.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Allen, V., Pond, K., Saker, K., Fontenot, J., Bagley, C., Ivy, R., Evans, R., Schmidt, R., Fike, J., Zhang, X., Ayad, J., Brown, C., Miller, M., Montgomery, J., Mahan, J., Wester, D. & Melton, C., (2001). Tasco: Influence of brown seaweed on antioxidants in forages and livestock - A review. *Journal of Animal Science, 79*, E21-E3. https://doi.org/10.2527/jas2001.79E-SupplE21x

AMSA, American Meat Science Association. (2016). *Research Guidelines for Cookery, Sensory Evaluation, and Instrumental Tenderness Measurements of Meat*. 2nd ed. Illinois, USA.

Angell, A. R., Angell, S. F., de Nyr, R., & Paul, N. A. (2016). Seaweed as a protein source for monogastric livestock. *Trends in Food Science & Technology, 54*, 74-84. https://doi.org/10.1016/j.tifs.2016.05.014

Al-Shorepy, S., Alhadrami, G., & Jamali, I. (2001). Effect of feeding diets containing seaweeds on weight gain and carcass characteristics of indigenous lambs in the United Arab Emirates. *Small Ruminant Research, 41*, 283-287. https://doi.org/10.1016/S0921-4488(01)00204-8

Anderson, M., Blanton, J., Gleghorn, J., Kim, S., & Jhonson. W. (2006). Ascophyllum nodosum supplementation strategies that improve overall carcass merit of implanted English crossbreed cattle. *Asian-Australasian Journal of Animal Science, 19*(10), 1514-1518. https://doi.org/10.5713/AJAS.2006.1514

Amtmann, V. A., Gallo, C., Van Schaik, G., & Tadich N. (2006). Relaciones entre el manejo *antemortem*, variables sanguíneas indicadores de estrés y pH de la canal de novillos. *Archivos de Medicina Veterinaria, 38*(3), 259-264. https://doi.org/10.4067/S0301-732X2006000300010

AOAC, Association of Official Analytical Chemists. (1990). *Official Methods of Analysis*. 15th ed. Washington DC, USA, pp. 467-492.

AOAC, Association of Official Analytical Chemists. (1984) Supplement, Secs. 7.066-7.069, pp. 160-161. Washington DC, USA.

AOAC, Association of Official Analytical Chemists (1996). Official Methods of Analysis 16th edition. International, Gaithersburg, Maryland, USA.

Apaoblaza, A., Galaz, A., Strobel, P., Ramírez-Reveco, A., Jerez-Timaure, N., & Gallo, C. (2015). Glycolytic potential and activity of adenosine monophosphate kinase (AMPK), glycogen phosphorylase (GP) and glycogen debranching enzyme (GDE) in steer carcasses with normal (<5.8) or high (>5.9) 24h pH determined in M. *longissimus dorsi*. *Meat Science, 101*. https://doi.org/10.1016/j.meatsci.2014.11.008

Arshad, M. S., Sohaib, M., Ahmad, R. S., Nadeem, M. T., Imran, A., Arshad, M. U., Know, J. H., & Amjad, Z., (2018). Ruminant meat flavor influenced by different factors with special reference to fatty acids. *Lipids and Health Disease, 17*, 223. https://doi.org/10.1186/s12944-018-0860-z

Baca, S., Cervantes, M., Gómez, R., Espinoza, S., Sauer, W., Morales, A., Araiza, B., & Torrentera, N. (2008). Efectos de la adición de harina de algas marinas a dietas de trigo para cerdos en crecimiento y finalización. *Revista Computadorizada de Producción Porcina, 15*(4), 324-328.

Braden, K., Blanton, J., Jr, Montgomery, J., van Santen, E., Allen, V., & Miller, M. (2007). Tasco supplementation: Effects on carcass characteristics, sensory attributes, and retail display shelf-life. *Journal of Animal Science, 85*, 754-768.

Casas, M., Hernández, H., Marín, A., Águila, R., Hernández, C., Sánchez, I., & Carrillo, S. (2005). *El Alga Marina Sargassum (Sargassaceae): una alternativa tropical para la alimentación de ganado caprino*. [Tesis]. Departamento de Zootecnia, Universidad Autónoma de Baja California, Ejido de Nuevo León. Mexicali, México.

Corino, C., Modina, S.C., Di Giancamillo, A., Chiapparini, S., & Rossi, R. (2019). Seaweeds in pig nutrition. *Animals, 9*, 1126. https://doi.org/10.3390/ani9121126

Fike, J., Allen, V., Schmidt, R., Zhang, X., Fontenot, J. P., Bagley, C., Ivy, R., Evans, R., Coelho, R., & Wester, D. (2001). Tasco-forage: I. Influence of a seaweed extract on antioxidant activity in tall fescue and in ruminants. *Journal of Animal Science, 79*, 1011-1021. https://doi.org/10.2527/2001.7941011x

Gallo, C., Apaoblaza, A., Pulido, R., Jerez-Timaure, N. (2013). Efectos de una suplementación energética en base a maíz roleado sobre las características de calidad de la canal y la incidencia de corte oscuro en novillos. *Archivos de Medicina Veterinaria, 45*(3), 237-245. https://doi.org/10.4067/S0301-732X2013000300003

Gallo, C., & Huertas, S.M. (2016). Main animal welfare problems in ruminant livestock during preslaughter operations: A South American view. *Animal*, *10*(2), 357-364. https://doi.org/10.1017/s1751731115001597

Goering, H., & Van Soest, P.J. (1970). *Forage fiber analysis (apparatus, reagents, procedures, and some applications).* United States Department of Agriculture, Washington, D.C., USA.

Guerrero, A.; Valero, M. V.; Campo, M.M.; & Sañudo, C. (2013). Some factors that affect ruminant meat quality: From the farm to the fork. Review. *Acta Scientific of Animal. Science*, *35*, 335-347.

Halmemies, A., Rinne, M., Lamminen, M., Mapato, C., Ampapon, T., Wanapat, M., & Vanhatalo, A. (2018). Review: Alternative and novel feed for ruminants: nutritive value, product quality, and environmental aspects. *Animal, 12*(2), 295-309.

Holman, B. W. B., van de Ven, R. J., Mao, Y., Coombs, C. E. O., & Hopkins, D. L. (2017). Using instrumental (CIE and reflectance) measures to predict consumers' acceptance of beef colour. *Meat Science*, *127*, 57-62. https://doi.org/10.1016/j.meatsci.2017.01.005

INN. Instituto Nacional de Normalización (1993). *Canales de bovino – Definiciones y tipificación*. Norma Chilena Oficial NCh. 1306. Of. 93.

Jacob, R., & Hopkins, D. (2014). Techniques to reduce the temperature of beef muscle early in the *postmortem* period – a review. *Animal Production Science, 54*(4), 482-493. https://doi.org/10.1071/AN12338

Jerez-Timaure, N., Sánchez-Hidalgo, M., Pulido R., & Mendoza J. (2021). Effect of dietary brown seaweed (*Macrocystis pyrifera)* additive on meat quality and nutrient composition of fattening pigs. *Foods*, *10*(8), 1720. https://doi.org/10.3390/foods10081720

Karmaus, A. L., & Jones, W. (2021). Future foods symposium on alternative proteins: Workshop proceedings. *Trends Food Science and Technology, 107,* 12-129. https://doi.org/10.1016/j.tifs.2020.06.018

Klurfeld, M. D. (2018). What is the role of meat in a healthy diet? *Animal Frontiers*, *8*, 5-10, https://doi.org/10.1093/af/vfy009

MacArtain, P., Gill, C., Brooks, M., Campbell, R., & Rowland, I. (2007). Nutritional value of edible seaweeds. *Nutrition Reviews, 65*(12). https://doi.org/10.1301/nr.2007.dec.535-543.

Madeira, M.S., Cardoso, C., Lopez, P.A., Coelho, D., Afonso, C., Bandarra, N. M., & Prates, J.A.M. (2017). Microalgae as feed ingredients for livestock production and meat quality: A review. *Livestock science*, *205*, 111-121. https://doi.org/10.1016/j.livsci.2017.09.020

Maia, M.R.G., Fonseca, A.J.M., Oliveira, H.M., Mendonça, C., & Cabrita A.R.J. (2016). The potential role of seaweeds in the natural manipulation of rumen fermentation and methane production. *Scientific Report*, *6*, 32321. https://doi.org/10.1038/srep32321

Makkar, H., Tran, G., Heuzé, V., Giger-Reverding, S., Lessire, M., Lebas, F., & Ankers, P. (2016). Seaweeds for livestock diets: A review. *Animal Feed Science and Technology, 212*, 1-17. https://doi.org/10.1016/j.anifeedsci.2015.09.018

Marín, A., Casas, M., Carrillo, S., Hernández, H., & Monroy, A. (2003). Performance of sheep fed rations with *Sargassum* spp. Sea algae. *Cuban Journal of Agricultural Science, 37*(2), 119-123.

Mendoza, J. (2017). *Evaluación de un aditivo en base a algas marinas sobre crecimiento, comportamiento en pastoreo y características fermentativas de la dieta de vaquillas*. [Tesis de magister]. Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Chile.

Morrill, J. C., Sawyer, J. E., Smith, S. B., Miller, R. K., Baber, J. R., & Wickersham, T. A. (2017a). Post-extraction algal residue in beef steer finishing diets: I. Nutrient utilization and carcass characteristics. *Algal Research, 25*, 584-588. http://dx.doi.org/10.1016/j.algal.2017.05.026

Morrill, J. C., Sawyer, J. E., Smith, S. B., Miller, R. K., Johnson, M., & Wickersham, T. A. (2017b). Post-extraction algal residue in beef steer finishing diets: II. Beef flavor, fatty acid composition, and tenderness. *Algal Research, 25*, 578-583. https://doi.org/10.1016/j.algal.2017.07.007

Miller, M. F., Carr, M.A., Ramsey, C. B., Crockett K. L., & Hoover L. C. (2001). Consumer thresholds for establishing the value of beef tenderness. *Journal of Animal Science*, *79*, 3062-3068. https://doi.org/10.2527/2001.79123062x

Monin, G., & Sellier, P. (1985). Pork of low technological quality with a normal rate of muscle pH fall in the immediate post-mortem period: The case of the Hampshire breed. *Meat Science*, *13*(1), 49-63. https://doi.org/10.1016/S0309-1740(85)80004-8

Mwangi, F., Charmley, E., Gardiner, C., Malau-Aduli, B., Kinobe, R., & Malau-Aduli, A. (2019). Diet and genetics influence beef cattle performance and meat quality characteristics. *Foods,* 8, 648. https://doi.org/10.3390/foods8120648

Nannig, R. (2018). *Efecto de la suplementación con algas marinas, sobre la respuesta productiva y calidad de leche, en vacas lecheras en pastoreo restringido en primavera*. [Memoria de Título], Escuela de Medicina Veterinaria, Universidad Austral de Chile, Valdivia, Chile.

OCDE-FAO, Organización para la Cooperación y el Desarrollo Económico (OCDE) y la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO). (2019). Perspectivas agrícolas 2019-2028. Enfoque especial América Latina.

Ortiz, J., Uquiche, E., Robert, P., Romero, N., Quitral, V., & Lantén, C. (2008). Functional and nutritional value of the Chilean seaweeds *Codium fragile*, *Gracilaria chilensis* and *Macrocystis pyrifera*. *European Journal of Lipid Technology*, *110*. https://doi.org/10.1002/ejlt.200800140

Øverland, M., Mydland, L., & Skrede, A. (2019). Marine macroalgae as sources of protein and bioactive compounds in feed for monogastric animals. *Journal of Science Food and Agriculture*, *99*, 13-24**.** https://doi.org/10.1002/jsfa.9143

Perkin-Elmer. (1994). *Analytical methods for atomic absorption spectrophotometry*. Norwalk, Connecticut, USA.

Ponnampalam, E., Hopkins, D., Bruce H., Li, D., Baldi, G., & Bekhit, A. (2017). Causes and contributing factors to "dark cutting" meat: Current trends and future directions: A review. *Comprehensive Review in Food Science and Food Safety, 16*, 400-430. https://doi.org/10.1111/1541-4337.12258

Psouni E., Janke, A., Garwicz, M. (2012) Impact of Carnivory on Human Development and Evolution Revealed by a New Unifying Model of Weaning in Mammals. *PLoS ONE 7*(4), e32452. https://doi.org/10.1371/journal.pone.0032452

Quitral, V., Morales, C., Sepúlveda, M., & Schwartz, M. (2012). Propiedades nutritivas y saludables de algas marinas y su potencialidad como ingrediente funcional. *Revista Chilena de Nutrición*, *39*(4), 196-202. http://dx.doi.org/10.4067/S0717-75182012000400014

R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2021; Available online: https://www.r-project.org (accessed on 13 Mach, 2022).

Raja, K., Kadirvel, V., & Subramaniyan, T. (2022). Seaweeds, an aquatic plant-based protein for sustainable nutrition - A review. *Future Foods, 5*, 100142. https://doi.org/10.1016/j.fufo.2022.100142

Rossi, R., Vizzarri, F., Chiapparini, S., Rattim S., Casamassimam, D., Palazzo, M., & Corino, C. (2020). Effects of dietary level of brown seaweeds and plant polyphenols on growth and meat quality parameters of growing rabbits. *Meat Science, 161*, 1077987. https://doi.org/10.1016/j.meatsci.2019.107987

Rotta, P. P., Prado, R. M. do Prado, I. N. do Valero, M. V., Visentaine, J. V., & Silva, R. R. (2009). The Effects of genetic groups, nutrition, finishing systems and gender of Brazilian cattle on carcass characteristics and beef composition and appearance: A Review. *Asian-Australasian Journal of Animal Sciences*. https://doi.org/10.5713/ajas.2009.90071.

Tilley, J. M. & Terry, A. R. A. (1963). A two-stage technique for the in vitro digestion of forage crops. *Grass Forage Sciences*, *18*, 104-111.

USDA. United States Department of Agriculture (2017). Official United States Standards for Grades of Carcass Beef; Agricultural Marketing Service: Washington, DC, USA, Available online: https://www.ams.usda.gov/sites/default/files/media/CarcassBeefStandard.pdf (accessed on 30 May 2021).

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for Dietary Fiber, Neutral Detergent Fiber, and Non-starch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science*, *74*, 3583-3597.

Van Vliet, S., Provenza, F.D., & Kronberg, S.L. (2021). Health-promoting phytonutrients are higher in grass-fed meat and milk. Frontiers. *Frontiers in Sustainable Food Systems*, 4. https://doi.org/10.3389/fsufs.2020.555426.

Williams, P. Nutritional composition of red meat. (2007). *Nutrition and Dietetics*, 64, S113-S119. https://doi.org/10.1111/j.1747-0080.2007.00197.x

Wood, J.D., & Enser, M. (1997). Factors influencing fatty acids in meat and the role of antioxidants in improving meat quality. *British Journal of Nutrition*, 78, S49-S60. https://doi.org/10.1079/BJN1997013.4

Austral J Vet Sci **55, 104-112 (2023)**

ORIGINAL ARTICLE

Zoonoses and traumatic injuries among practicing veterinarians from Southern Chile

Luciano Herrera Rosales[a], Lucía Azócar-Aedo[a*], Javier Cabello Stom[b]

ABSTRACT. This research addresses the occurrence of clinical signs and related symptoms of zoonotic diseases, traumatic injuries, and the frequency of healthcare seeking among practicing veterinarians, whose job was performed in the Los Lagos Region, Southern Chile. An online standardized survey collected from 140 practicing veterinarians was conducted between March and July 2020. The surveyed participants reported the occurrence of brucellosis, ringworm, scabies, cat scratch disease, anthrax, toxocariasis, salmonellosis, pediculosis, and flea infestation among veterinarians. The clinical signs and symptoms related to these events include diarrhea, allergies, and muscle pain. Mild and severe traumatic injuries were also declared by the participants, in which the frequency of severe trauma increased as the time of professional practice increased. Nevertheless, the use of professional healthcare was low among the surveyed veterinarians. This study emphasizes the need to consider veterinarians' health-related occupational risks using the "One Health" approach.

*Keywords***:** veterinarians, zoonoses, traumatic injuries, healthcare seeking, Chile.

INTRODUCTION

Zoonoses are diseases transmitted from animals to humans (Hugh-Jones *et al*., 1995). Some of these pathogens have little or no chance for subsequent human-to-human transmission, as occurs with rabies (Karesh *et al*., 2012; Medline, 2021). The occurrence of zoonoses among veterinarians depends on their prevalence in the animal population, morbidity, use of personal protection equipment, and the quality of education obtained in veterinary schools (Baker & Gray, 2009). A study of Canadian veterinarians demonstrated a high potential for occupational risk in relation to events involving zoonotic diseases, exposure to rabies, traumatic injuries caused by bites or scratches from animals, and allergies (Epp & Waldner, 2012). These occupational events have been associated with a higher probability of zoonotic infection, along with the fact that veterinarians commonly do not use adequate protection equipment, such as gloves, masks, or protective clothing (Baker & Gray, 2009).

Veterinarians are often exposed to injuries while performing clinical practice on animals, the most common of which are bites by domestic dogs and cats, scratches, and blows or compressions in the body by large animals (Jeyaretnam & Jones, 2000). The odds of having severe injuries resulting from occupational accidents are nine times higher than those of other professions in the healthcare sector (Norwood *et al*., 2000). This risk increases among professionals who handle wild animals, especially those of the order Carnivora (Echarte & Vasallo, 2016). These types of injuries represent a considerable proportion of veterinarians' occupational risks. Multiple activities in the field and work environments lead to various potential risks because of trauma as well as biological, chemical, and physical elements (Samadi *et al*., 2013).

In the Los Lagos Region, Southern Chile, there are approximately 774.321 cattle, 227.798 sheep, 9.082 goats, 42.429 pigs, 9.749 horses, 390.138 hens/chickens, 12.893 turkeys, and 52.368 poultry, including ducks, geese, emus, quail, pheasants, partridges, and ostriches (INE, 2021). Regarding the number of pets, there are estimates of domestic dogs that amount to approximately 115.414 dogs with owners in Puerto Montt[1]. However, it is difficult to determine the exact number of veterinarians in the Los Lagos Region. A total of 198 professionals are registered in the "Servicio Agrícola y Ganadero" (SAG), of whom 189 are members of the "Colegio Médico Veterinario" (COLMEVET). However, these rosters were not freely available. Moreover, some veterinarians are not part of these associations. This study aimed to conduct a cross-sectional investigation of zoonoses and traumatic injuries among practicing veterinarians in the Los Lagos Region, Chile.

MATERIAL AND METHODS

STUDY DESIGN

The study design was observational, cross-sectional, quantitative, and descriptive (Hernández *et al*., 2010). An unknown population size was considered despite the SAG and COLMEVET registries, as there are veterinarians not included in these records, and some are even registered in both. A sample size of 139 people was selected following Fernández (1996) with a confidence level of 95%, a precision of 5%, and an expected proportion of 10% (Epp & Waldner, 2012). The questionnaire was created

Received: 30.09.2022.

Accepted: 11.01.2023.

[a]Facultad de Ciencias de la Naturaleza, Escuela de Medicina Veterinaria, Universidad San Sebastián, Sede de la Patagonia, Puerto Montt, Chile.

[b]Centro de Conservación de la Diversidad Chiloé Silvestre. Reserva Marina Pullinque, Nal Bajo, Ancud Chile.

*Corresponding author: lucia.azocara@uss.cl

on the Google Forms platform and shared via email with veterinarians with professional practice in the Los Lagos Region (these were veterinarians accredited by the SAG, whose data is freely available, and members of COLMEVET) between March and July 2020. The participants were contacted *via* email to inform them of the study objectives. Private veterinary clinics in the cities of Puerto Montt, Puerto Varas, and Osorno in the Los Lagos Region were contacted by phone. Emails were also sent to veterinarians graduated from the San Sebastián University, Patagonia campus, Puerto Montt, Los Lagos Region. In total, 140 individuals met the requirements to answer the survey. The inclusion criteria were 1) those practicing veterinary medicine and 2) those with professional work in the Los Lagos Region. The questionnaire is detailed and described in Supplementary Material 1.

DETERMINATION OF ZOONOSES

To better identify the respondents, their area (or areas) of activity or expertise was assessed using the following options: 1) companion animals, 2) farm animals, 3) wild animals, 4) equines, 5) public health, 6) research, and 7) laboratory. To determine exposure to zoonoses, veterinarians were asked about the diagnoses of these types of diseases by human health professionals, with medical certainty that such diseases were contracted within the region. This question was answered in writing by the respondent to avoid omitting any disease that could go unnoticed.

FREQUENCY OF INJURIES AND COMMON CLINICAL MANIFESTATION(S) RELATED TO A ZOONOTIC DISEASE AMONG VETERINARIANS

1) From a selection of injuries described in other studies (Epp & Waldner, 2012; Navarrete & Tarabla, 2018), exposure to traumatic injuries was determined as "minor" or "severe," considering the different nature of the animal species treated and the different areas of professional activity. Injuries were classified according to their severity as follow: a) "Minor injuries" included superficial cuts, superficial puncture wounds, abrasions, contusions, contractures, and sprains. The respondents indicated how often they suffered this type of injury in their professional practice, within the categories "never, rarely, sometimes, commonly, or always."
2) "Serious injuries" included all injuries caused during work that, for the respondent, required medical, outpatient, or emergency assistance.

The respondents freely indicated (in an open question) how many times they had required this type of assistance when injured, excluding vehicular accidents and medical consultations due to problems for ergonomic reasons. The proportion of the total number of veterinarians affected by one of the each categories was calculated. The average number of injuries was calculated based on the total number of respondents. Questions about the clinical manifestations caused by unknown agents that induced the respondents' personal suspicion of contracting a zoonosis were asked, including diarrhea; allergy; and ocular, respiratory, skin, and vector infections (biological or mechanical). These criteria were selected using a different survey (Garland-Lewis *et al*., 2017), naming other common symptoms and signs, such as vomiting, abdominal pain, joint pain, and muscle pain. The statistical tests used for the clinical manifestations was a chi-square test with a Yates correction using Epi Info™ version 6.0.4 (Centers for Disease Control and Prevention), considering a P value less than 0.05, as statistical significance. In addition, it was possible to evaluate the diseases that were more common according to the categories of responses "never", "rarely", "sometimes", "commonly", or "always" of a presumptive nature to a zoonotic disease. The existence of possible significant differences between the response categories mentioned for each clinical manifestation was determined using a chi-square test or Yates correction (P value less than 0.05).

FREQUENCY OF PRACTICING VETERINARIANS SEEKING MEDICAL ASSISTANCE

Respondents' frequency of seeking medical assistance after suspecting a zoonosis was calculated considering the number of veterinarians who sought medical attention after suspicion of disease in the following categories: "never", "rarely", "sometimes", "commonly", or "always". This was performed using the total number of respondents who answered the survey.

RESULTS

Approximately 250 questionnaires were sent and 140 responses were received. Of the 140 veterinarians surveyed, 27 reported being diagnosed with one or more zoonotic diseases. Out of 27, 24 specified which disease(s) they had suffered, registering a total of nine different reported zoonoses. The most frequent diagnoses were brucellosis and dermatophytosis, both having eight reports (30%), followed by scabies (22%). The area of activity or expertise with most reports was the category "farm animals" (67%), "companion animals" (44%), and finally equines (22%) (Table 1).

Regarding clinical manifestation and the frequency of the professionals suspecting a zoonosis, most of the answers were "never" and "rarely." Some responses were recorded in the "sometimes" category for allergy, skin infection, and infection by vector (Table 2). The results of the chi-squared test indicated that "diarrhea," "allergy," and "muscle pain" have statistically significant differences in their presentation frequencies between the categories "never," "rarely," "sometimes," "commonly," and "always".

Regarding the frequency of minor injuries, the category "sometimes" was the most frequent, followed by "rarely"

Table 1. Reports of suspected zoonotic diseases and professional specialty in veterinarians surveyed in Los Lagos Region, Southern Chile, 2020.

		N	Percentage among reports (n=27)	Percentage among the total (n=140)
Suspected Disease	Anthrax	1	3.70	0.71
	Bartonellosis	2	7.41	1.43
	Brucellosis	8	29.63	5.71
	Dermatophytosis	8	29.63	5.71
	Scabies	6	22.22	4.29
	Pediculosis	1	3.70	0.71
	Pulicosis	1	3.70	0.71
	Salmonellosis	1	3.70	0.71
	Toxocariasis	1	3.70	0.71
Professional Specialty	Companion animals	12	44.44	8.57
	Farm animals	18	66.67	12.86
	Wild animals	4	14.81	2.86
	Equines	6	22.22	4.29
	Public health	4	14.81	2.86
	Research	2	7.41	1.43
	Laboratory	2	7.41	1.43

Table 2. Survey answers (n=140) of veterinarians regarding a clinical manifestation and the frequency of a suspected zoonosis in Los Lagos Region, Southern Chile, 2020. The χ^2 and the P values are expressed.

Clinical manifestation	Never		Rarely		Sometimes		Commonly		Always		χ^2	*P value*
	N°	%	N°	%	N°	%	N°	%	N°	%		
Diarrhea	97	69.3	35	25.0	5	3.6	3	2.1	0	0	29.6	0.000002
Vomit	124	88.6	12	8.6	4	2.9	0	0.0	0	0	-	-
Allergy	73	52.1	45	32.1	15	11.0	7	5.0	0	0	15.0	0.001745
Ocular infection	105	75.0	30	21.4	4	2.9	1	0.7	0	0	-	-
Respiratory infection	116	82.9	16	11.4	6	4.3	2	1.4	0	0	-	-
Skin infection	70	50.0	46	32.9	19	14.0	3	2.1	2	1.4	-	-
Infection by vector	108	77.1	19	13.6	10	7.1	1	0.7	2	1.4	-	-
Abdominal pain	121	86.4	12	8.6	6	4.3	0	0.0	1	0.7	-	-
Join pain	121	86.4	10	7.1	6	4.3	3	2.1	0	0	-	-
Muscle pain	115	82.1	16	11.4	5	3.6	4	2.9	0	0	52.2	0.0

(Figure 1). For serious or severe injuries during professional practice, 139 respondents were considered because of the lack of clarity in the responses provided by one of them. The average score was calculated for each category of years of professional practice. In addition to the general average (n = 139) of 1.35 injuries, an increase in this average regarding years of professional practice was observed (Figure 2).

The frequency of the respondents visiting a healthcare center was calculated on a scale in which they considered that the clinical manifestations were attributable to the contagion of a zoonotic disease. A low frequency of seeking medical assistance was observed (in 66 veterinarians, the response was "never") (Figure 3).

DISCUSSION

This study demonstrated that veterinarians in the Los Lagos Region of Southern Chile are exposed to different zoonotic diseases, clinical manifestations related to zoonoses, and traumatic injuries of mild and severe degrees. This survey also showed the frequency of veterinarians seeking medical attention when suspecting a zoonosis. To the best

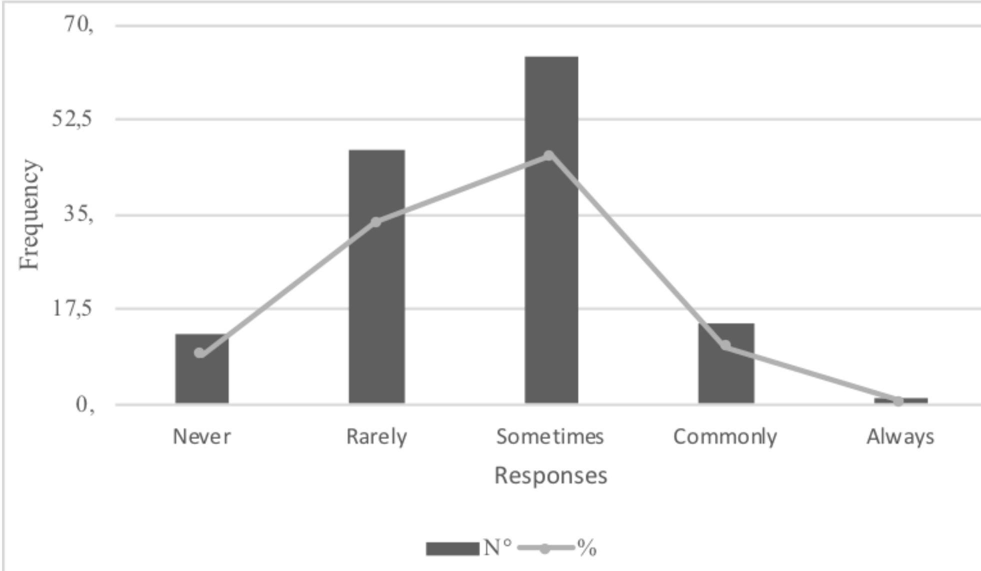

Figure 1. Frequency distribution of minor injuries during their professional practice among veterinarians surveyed in Los Lagos Region, Southern Chile, 2020.

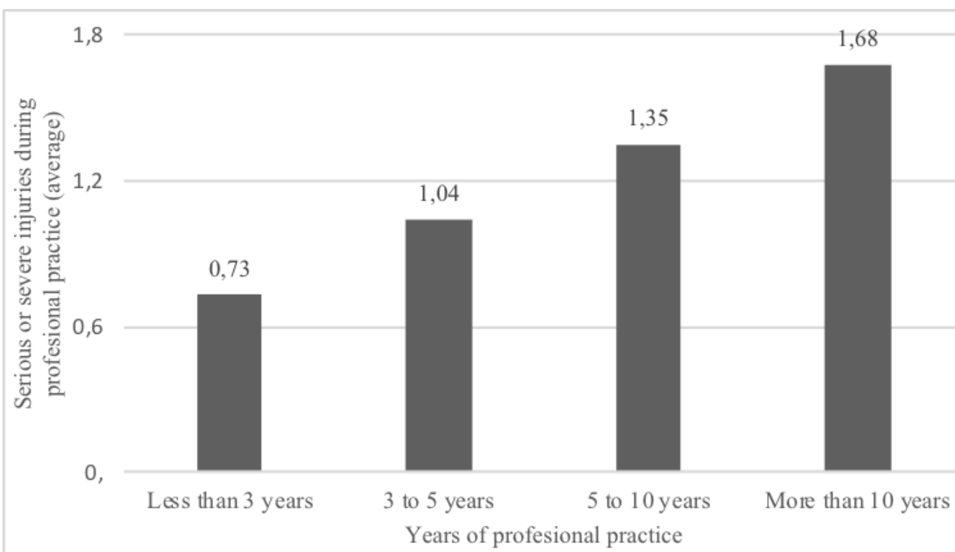

Figure 2. Average frequency of "serious" or "severe" injuries among veterinarians surveyed (n=139) along their professional practice in Los Lagos Region, Southern Chile, 2020.

of our knowledge, this is one of the first studies of this topic in Chile.

The most frequent zoonotic disease among the respondents was brucellosis, an infectious disease caused by bacteria of the genus *Brucella*. It affects a wide variety of mammals, including ruminants, swine, canids, rodents, and even marine mammals such as pinnipeds and cetaceans, causing acute or chronic symptoms (recurrent fever, chronic fatigue, orchitis, endocarditis, arthritis, and inflammation of the liver or spleen) (CDC, 2021). Owing to its prevalence, bovine brucellosis is of great importance in livestock, and efforts to eradicate it have been made throught an eradication program established in Chile in 1975 by SAG (MINSAL, 2015). The monitoring and control of this entity range from prevention and vaccination to the adoption of farming sanitary measures (SAG, 2022). Brucellosis transmission can occur through the consumption of unpasteurized dairy products and inhalation or contact with secretions from abortions through the mucous membranes or wounds. This transmission route is significant, as it presents a greater risk for veterinarians, laboratory technicians, and professionals working in the meat industry (CDC, 2021). The nonspecific

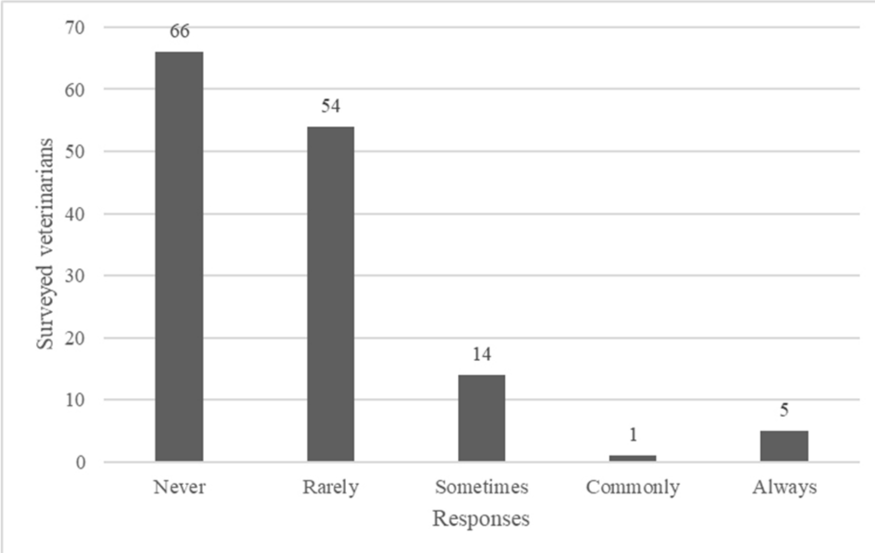

Figure 3. Responses of veterinarians regarding health care center assistance after a suspicion of a zoonotic disease in Los Lagos Region, Southern Chile, year 2020.

nature of the clinical signs of this disease and the lack of studies in humans in Chile have led to the suspicion of a high underdiagnosis (Olivares *et al*., 2017). However, 67% of the zoonosis cases in the present study were suspected of brucellosis. It is important that people working with "farm animals" are categorized as a group at a high risk of infection, as previously described in Turkey (Kutlu *et al*., 2014). Further, a higher probability of infection by *Brucella* was reported even among veterinary medicine students (Sánchez *et al*., 2017).

Other important diseases reported in this study were scabies and dermatophytosis, both of which affect the skin and cause irritation and itching. These diseases are transmitted by direct contact with infected animals or humans. Scabies is caused by the mite *Sarcoptes scabiei*, and dermatophytosis (also known as "ringworm") is caused by fungi of the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* (CDC, 2010; CDC, 2020a). Epidemiological studies have found that the main species causing dermatophytosis in pets is *Microsporum canis* (in 81.8%–97% of the cases), and their incidence is higher among dogs, cats, and humans (60.0%) than in other species (Paryuni *et al*., 2020). In Chile, scabies is more commonly present in canids than in felines (18.7% and 1.2% of cases, respectively), and dermatophytosis occurs more frequently in cats than in canines (10.6% and 6.4%, respectively); however, this frequency may vary due to the socioeconomic factors of pet owners (López *et al*., 2009). One study (Pereira Olivares, 2017) conducted between 2001 and 2013, based on the records of a Teaching Veterinary Hospital, estimated a prevalence of dermatophytosis of 2.2% (n = 5.293) in dogs. Although dogs and cats play key roles in the transmission of dermatophytosis, it is important to elucidate that these mycotic agents are ubiquitous, even in other domestic species (Cabañes *et al*., 2000). Infection among veterinarians is quite possible because of frequent animal handling during clinical examinations (a fundamental action in clinical practice). It is also worth noting that both diseases depend on the host immune status (CDC, 2010; CDC, 2020a).

Bartonella henselae causes various symptoms, such as fever, enlarged lymph nodes, papules or pustules at the wound site, myalgia, or encephalitis, mainly in individuals below 15 years of age. It is transmitted through scratches in cats infested with fleas (CDC, 2020b). Different prevalence studies have been conducted on animals in Chile. In Valdivia (Los Ríos region), a study was conducted using 370 blood samples from cats, in which *Bartonella sp.* DNA was detected in 18.1% of samples (Muller *et al*., 2016). In another study on stray dogs in Linares (El Maule Region), 72.7% (n = 66) of the canines were seropositive for the agent, whereas in Puerto Montt (Los Lagos Region), three dogs were positive for *Bartonella sp*. Although the disease is characterized in cats, these animals have a high ectoparasitic infection rate, which could facilitate the spread of the pathogen (Weinborn-Astudillo *et al*., 2020). Animals with a high ectoparasitic load can be a source of pulicosis or pediculosis, the reports of which were also presented in the aforementioned study. Scratches and bites by animals are common in veterinary practice (Jeyaretnam & Jones, 2000; Bonini *et al*., 2015; Gómez & Tarabla, 2015); therefore, bartonellosis is expected to be detected among veterinarians in the Los Lagos Region. In the Bíobío Region, a high presence of antibodies against *Bartonella* was detected by indirect immunofluorescence technique among veterinarians and individuals with occupational contact with cats, which was equivalent to 60.5% (n = 76) (Troncoso *et al*., 2016).

Anthrax is a bacterial disease caused by the agent *Bacillus anthracis*, the transmission of which is through spores that form upon contact with the air after infecting a wound (in herbivores) and remain suspended in the environment. Anthrax has different clinical manifestations, such as cutaneous, which occurs in 95% of cases, respiratory, and gastrointestinal, with the latter having high lethality (Acha & Szyfres, 2003). Control measures include vaccination and antibiotic treatment of animals, as well as environmental management (Laverde *et al*., 2008). The latest reported anthrax cases in humans in Chile occurred in a rural sector of the Ñuble Region, all in their cutaneous presentation, due to the slaughter and consumption of an infected equine (Arellano *et al*., 2018). Although this case occurred in individuals who were not veterinarians, the case shows that anthrax is present in the Chilean rural sectors and is closely related to herbivores. Therefore, it is not unusual for anthrax to be included in the survey reports. Among veterinarians, its presence could be associated with performance of necropsies on infected animals without the use of appropriate measures for personal protection. Carcasses suspected of anthrax should not be subjected to necropsy, but by mistake, this practice is not strictly followed, and veterinarians thus get infected (Laverde *et al*., 2008).

Pediculosis and pulicosis were also reported among the surveyed veterinarians but at low frequencies. Regarding pediculosis, like other blood-sucking insects, lice bite and cause itching on the macules, papules, vesicles, or pustules due to the irritant effect of their saliva by allergic reactions and local inflammation. Scratching results in excoriations and microhemorrhages that form scabs and facilitate secondary infections (López-Valencia *et al*., 2019). Fleas are important vectors of pathogens worldwide; they are rarely specific at the host species level, but some clades are associated with a particular host group. However, many fleas are associated with domesticated animals such as *Ctenocephalides felis* (cat flea) and *C. canis* (dog flea), which represent the majority of fleas in human homes and are vectors of the bacteria *Bartonella henselae*. (Desachy, 2018). In addition, *Xenopsylla cheopis* (Oriental rat flea) is common in many tropical and warm temperate environments worldwide and is the vector of *Yersinia pestis* involved in the transmission of murine (endemic) typhus, parasitic helminths, and *Bartonella* species (Bitam *et al*., 2010).

According to Costa *et al.* (2012) and Wiedemann *et al*. (2015) another zoonosis reported was salmonellosis. *Salmonella typhi* and *Salmonella paratyphi* A, B, and C are pathogenic to humans; however, animals are asymptomatic. *Salmonella choleraesuis* is carried mostly by pigs, but it also causes salmonellosis in humans. Common serotypes, such as *Salmonella enteritidis* and *Salmonella typhimurium,* cause infections in the gastrointestinal tract. Serotypes causing typhoid fever are transmitted between people with no mediation of an animal (McSorley, 2014), but salmonellosis non-typhoid is isolated from both humans and animals, including livestock. Serotypes Typhimurium, Enteritidis, Newport, and Heidelberg are most often responsible for food poisoning, but Choleraesuis and Dublin also cause diarrhea (Kurtz *et al*., 2017). Generally, salmonellosis can be more severe in immunocompromised individuals, resulting in bacteremia or systemic and localized infections such as meningitis and osteomyelitis. Although many pet species have been implicated in human salmonellosis, amphibians, reptiles, exotic animals, rodents, and young poultry pose the greatest risks (Stull *et al*., 2015).

Finally, toxocariasis was also reported affecting veterinarians. Although many *Toxocara canis* infections are subclinical, human toxocariasis can manifest as syndrome known as visceral *larva migrans*, ocular *larva migrans*, and neurotoxocariasis. People get infected through accidental ingestion of parasite eggs from contaminated soil, water, and food (raw vegetables and fruits), and contact with dogs and cats is described as an important risk factor (Ma *et al*., 2018). Toxocariasis is a neglected disease with high prevalence in humans, pets, and stray companion animals worldwide (Rostamia *et al*., 2019). In Slovakia, a cross-sectional study performed by Fecková *et al*. (2020) determined a low prevalence of anti-*Toxocara* antibodies among veterinarians, veterinary assistants, and veterinary medicine students (0.5%), however, a high seropositivity rate was observed in children and young people, as well as a high risk of professional exposure in hunters and farmers, highlighting the importance of taking preventive measures and enhancing knowledge of toxocariasis among both professionals and pet owners.

In this study, the relevant clinical manifestations among the surveyed veterinarians, supported by statistical significance, were "diarrhea," "allergy," and "muscle pain." Among these, diarrhea and allergies are commonly perceived to be associated with work involving animal handling (Garland-Lewis *et al*., 2017), although it is worth considering their presence in zoonotic diseases, such as bartonellosis (Vayssier-Taussat, 2016). However, it is noteworthy that the clinical manifestations of zoonoses vary, with septicemic, respiratory, digestive, nervous, and cutaneous signs with different evolution patterns and variable severity (Desachy, 2018). Studies conducted to understand the epidemiology of diarrhea have been limited to specific groups of individuals with certain comorbidities, such as children, the elderly, and people undergoing immunosuppression (Asenjo *et al*., 2008). However, among the causative agents of diarrhea in adults, pathogens such as *Escherichia coli*, *Salmonella sp*., *Campylobacter jejuni*, *Giardia sp.*, and *Cryptosporidium sp.* have been described (Lucero, 2014). These agents can be associated with a potential animal origin because are excreted in the fecal material, a common occupational exposure in veterinarians during clinical practice.

Regarding the "mild" or "severe" injuries reported here, the answers were not clear on how many injuries each participant experienced. The most common events were bites and scratches, which are not necessarily associated with clinical care. In addition to the difficulty of accurate quantification (Epp & Waldner, 2012), many veterinarians self-medicate these types of injuries (Jeyaretnam & Jones, 2000; Navarrete & Tarabla, 2022). However, this study demonstrated that mild injuries were present at different frequencies among the professionals. Serious injuries show a clear increase with more years of professional practice, as confirmed in some published studies (Mishra *et al*., 2020). This study did not evaluate the differences between the areas of professional performance among respondents. However, studies have noted that a greater proportion of occupational accidents in veterinarians working in animal production are concentrated in groups specializing in large animal species, such as ruminants and equines (López *et al*., 2014). This situation makes more sense considering the type of injuries that large animals can cause, for instance, physical trauma or cranial and facial trauma (Norwood *et al*., 2000), which would require medical assistance. Within the different categories, it is noteworthy that most respondents answered that "never" (n = 66) or "rarely" (n = 54) sought medical care in case of suspicion of a zoonotic disease (Figure 3). This is consistent with other study that demonstrated that most of respondents (56.4%, 31/55) did not contact a physician to diagnose or treat their zoonotic infection (Jackson & Villarroel, 2012) One reason for this phenomenon may be the prominent level of self-medication among veterinarians in cases of occupational accidents (Navarrete & Tarabla, 2018; Gardland-Lewis *et al*., 2017). One study showed that 77% of the participants resorted to self-treatment, including self-medication with antibiotics, wound suturing, and reduction of fractures or dislocations, which may explain the low attendance rate to healthcare centers (Landercasper *et al*., 1988).

One of the limitations of this study is the lack of knowledge of the number of veterinarians in the Los Lagos Region as there is no single entity that can group them all simultaneously in a mandatory manner. In addition, there was a great distribution between the performance areas; however, the difficulty was that many of the participants were dedicated to several areas simultaneously. Regarding the answers, only one question registered little clarity (presence of "serious" to "severe" traumatic injuries), with which, for this analysis, the sample size was reduced to 139 people, which may constitute a possible information bias. It is possible that some zoonoses go unnoticed because of the limited assistance of veterinarians to human healthcare centers, self-medication, and lack of consideration of these diseases in the human health system. Thus, the study of serum antibodies or the detection of zoonotic pathogens using direct diagnostic tests among veterinarians with a clinical suspicion of zoonoses as professional diseases is required in Southern Chile.

CONCLUSIONS

The most frequent zoonoses reported by veterinarians in the Los Lagos Region, Southern Chile, were brucellosis, dermatophytosis, and scabies, among other diseases such as bartonellosis, anthrax, external parasitosis, dermatophytosis, salmonellosis, and toxocariasis. Furthermore, reports of zoonotic diseases were higher among respondents working with farm animals. Based on the statistical results of association, the most common clinical manifestations of suspecting a zoonotic disease were diarrhea, allergy, and muscle pain. The frequency of minor injuries varied among the categories considered, and the average number of serious injuries increased according to the years of professional practice. Further, the veterinarian professionals do not frequently seek care from a medical professional after suspecting a zoonotic disease. It is important to recognize the occupational health problems of veterinarians and to increase their awareness of this health risk, as they are professionals with advanced knowledge about zoonotic diseases. Occupational safety must be improved and the risk factors that affect individuals, especially veterinarians, during diagnosis, which could be a zoonotic disease, must be publicized. This is a joint effort that must be carried out by Chile's Healthcare System, veterinarians' employers, and, of course, veterinarians themselves when considering and applying the "One Health" concept.

ETHICS STATEMENT

In this study, informed consent was not applied, as it was based on a questionnaire carried out online in those veterinarians who voluntarily participated, and no personal data were asked. The information collected was authorized for use for academic purposes and for the accomplishment of the statistical analyses (Dirección de Integridad, Seguridad y Ética de la investigación, Vicerectoría de Investigación y Doctorados, Universidad San Sebastián, Chile)

REFERENCES

Acha, P., & Szyfres, B. (2003). Zoonosis y enfermedades transmisibles comunes al hombre y a los animales. Publicaciones Científicas y Técnicas Organización Panamericana de la Salud.

Arellano, S., Soto, D., Reeves, M., Chávez, F., Loubies, R., & Ojeda, A. (2018). Ántrax cutáneo, último brote diagnosticado en Chile. *Revista Chilena de Infectología, 35*(2). https://dx.doi.org/10.4067/s0716-10182018000200195

Asenjo, A., Lledó, L., López, P., Barrena, P. (2008). Síndrome diarreico en el adulto. Tratamiento de la diarrea. *Medicine, 10*(4), 215-222.

Baker, W., & Gray, G. (2009). A review of published reports regarding zoonotic pathogen infection in veterinarians. *Journal of the American Veterinary Medical Association, 234*(10), 1271-1278. https://dx.doi.org/10.2460/javma.234.10.1271

Bitam, I., Dittmar, K., Parola, P., Whiting, M., & Raoult, D. (2010). Fleas and flea borme diseases. *International Journal of Infectious Diseases, 14*, 667-676. https://10.1016/j.ijid.2009.11.011.

Bonini, S., Buonacucina, A., Selis, L., Peli, A., Mutti, A., & Corradi, M. (2016). Occupational hazards in veterinarians: an updating. *Journal of Veterinary Science & Technology, 7*(3). https://dx.doi.org/10.4172/2157-7579.1000317

Cabañes, F. J. (2000). Dermatophytes in domestic animals. *Revista Iberoamericana de Micología, 17*, 104-108.

Centers for Disease Control and Prevention. (2010). *Scabies*. https://www.cdc.gov/parasites/scabies/index.html

Centers for Disease Control and Prevention. (2020)a. *Ringworm*. https://www.cdc.gov/fungal/diseases/ringworm/index.html

Centers for Disease Control and Prevention. (2020)b. *Bartonella Infection (Cat Scratch Disease, Trench Fever, and Carrión's Disease)*. https://www.cdc.gov/bartonella/index.html

Centers for Disease Control and Prevention. (2021). *Brucellosis*. https://www.cdc.gov/brucellosis/index.html

Costa, L., Paixão, T., Tsolis, R., Bäumler, A., Santos, R. (2012). Salmonellosis in cattle: advantages of being an experimental model. *Research in Veterinary Sciences*, *93*, 1-6. https://doi/10.1016/j.rvsc.2012.03.002

Desachy, F. (2018). *Las zoonosis, transmission de las enfermedades de los animales al ser humano*. De Vecchi SA.

Epp, T., & Waldner, C. (2012). Occupational health hazards in veterinary medicine: zoonoses and other biological hazards. *Canadian Veterinay Journal, 53*, 144-150.

Feckováa, M., Antolováa, D., Zalesny, G., Halánovác, M., Strkolcová, G., Goldová, M., Weissová, T., Lukác, B., & Nováková, M. (2020). Seroepidemiology of human toxocariasis in selected population groups in Slovakia: a cross-sectional study. *Journal of Infection and Public Health*, *13*, 1107-1111. http://doi/10.1016/j.jiph.2020.04.006

Fernández, P. (1996). Determinación del tamaño muestral. *Cuadernos de Atención Primaria*, *3*(138-14), 1-6.

Garland-Lewis, G., Whittier, C., Murray, S., Trufan, S., & Rabinowitz, P. (2017). Occupational risks and exposures among wildlife health professionals. *EcoHealth, 14*(1), 20-28. https://dx.doi.org/10.1007/s10393-017-1208-2

Gómez de la Torre, N., & Tarabla, H. (2015). Accidentes laborales, enfermedades profesionales y uso de elementos de protección personal en veterinarios de pequeños animales. *Investigación Veterinaria, 17*(2), 223-227.

Hernández, R., Fernández, C., & Baptista, M. del P. (2010). *Metodología de la Investigación*. Mc Graw Hill.

Hugh-Jones, M., Hubert, W., & Hagstad, H. (1995). *Zoonoses: recognition, control, and prevention.* Iowa State Press.

INE (Instituto Nacional de Estadísticas). (2021). Cuadros estadísticos 2021. Censo Nacional Agrícola y Pecuario. https://www.ine.gob.cl/estadisticas/economia/agricultura-agroindustria-y-pesca/censos-agropecuarios

Jackson, K., & Villarroel, A. (2012). A survey of the risk of zoonoses for veterinarians. *Zoonoses and Public Health*, 59, 193-201. https://doi.org/10.1111/j.1863-2378.2011.01432.x

Jeyaretnam, J., & Jones, H. (2000). Physical, chemical and biological hazards in veterinary practice. *Australian Veterinary Journal, 78*(11), 751-758. https://dx.doi.org/10.1111/j.1751-0813.2000.tb10446.x

Karesh, W., Dobson, A., LLoyd-Smith, J., Lubroth, J., Dixon, M., Bennett, M, Thomas, M. J. (2012). Ecology of zoonoses: natural and unnatural histories. *The Lancet,* 380(9857), 1936-1945. https://dx.doi.org/10.1016/S0140-6736(12)61678-X

Kurtz, J., Goggins, J., McLachlan, J. (2017). *Salmonella infection*: interplay between the bacteria and host immune system. *Immunology Letters*, *190*, 42-50. http://doi: 10.1016/j.imlet.2017.07.006

Kutlu, M., Ergonul, O., Sayin-Kutlu, S., Guven, T., Ustun, C., Alp-Cavus, & S, Kazak, E. (2014). Risk factors for occupational brucellosis among veterinary personnel in Turkey. *Preventive Veterinary Medicine, 117*(1), 52-58. https://dx.doi.org/10.1016/j.prevetmed.2014.07.010

Landercasper, J., Cogbill, T., Strutt, P., & Landercasper, B. (1988). Trauma and the veterinarian. *The Journal of Trauma, 28*(8), 1255-1259. https://dx.doi.org/10.1097/00005373-198808000-00020

Laverde, L., Moreno, F., & Pérez, J. (2008). Ántrax en bovinos, reporte de un caso. *Revista CES Medicina Veterinaria y Zootecnia, 3*(2), 78-83.

López, J., Abarca, K., Cerda, J., Valenzuela, B., Lorca, L., Olea, A., & Aguilera, X. (2009). Surveillance system for infectious diseases of pets, Santiago, Chile. *Emerging Infectious Diseases, 15*(10). https://dx.doi.org/10.3201/eid1510.081596

López, M., Andrade, R., Tarabla, H., Signorini, M., & Molineri, A. (2014). Factores asociados con la presentación de accidentes laborales en veterinarios zootecnistas del departamento de Boyacá (Colombia). *Salud Uninorte, 30*(1), 23-33.

López-Valencia, D., Medina-Ortega, A., Hoyos-Samboní, D., Salguero, C., Vásquez-Arteaga, L. 2019. Pediculosis capitis and potential transmission of re-emerging infectious diseases in Colombia. Literature review. *Revista Facultad de Medicina Veterinaria, 68*(2), 295-304. https://doi.org/10.15446/revfacmed.v68n2.76604

Lucero, Y (2014). Etiology and management of acute infectious gastroenteritis in children and adults. *Revista Medicina Clínica Las Condes*, *25*, 3, 463-472. http://doi.org/10.1016/S0716-8640(14)70063-X

Ma, G., Holland, C., Wang, T., Hofmann, A., Fan, C., Maizels, R., Hotez, P., Gasser, R. (2018). Human toxocariasis. *The Lancet*, *18*. http://doi.org/10.1016/S1473-3099(17)30331-6

McSorley, S. (2014). Immunity to intestinal pathogens: Lessons learned from *Salmonella*. *Immunology Reviews, 260*, 168-182. http://doi.org/10.1111/imr.12184

Medline (2022). *Enciclopedia médica.* https://medlineplus.gov/spanish/ency/article/002362.htm

Ministerio de Salud (MINSAL), Gobierno de Chile. (2015). *Brucelosis*. https://www.minsal.cl/brucelosis/

Mishra, S., & Palkhade, R. (2020). Risk factors and prevalence of work-related injuries and accidents among veterinarians in India. *Veterinary World, 13*(11), 2555-2564. https://dx.doi.org/10.14202/vetworld.2020.2555-2564

Müller, A., Walker, R., Bittencourt, P., Machado, R., Benevenute, J., & Do Amaral, R., André, M. (2016). Prevalence, hematological findings and genetic diversity of *Bartonella spp*. in domestic cats from Valdivia, Southern Chile. *Parasitology, 144*(6), 773-782.

Navarrete, M., & Tarabla, H. (2018). Factores asociados a los riesgos ocupacionales y el uso de elementos de protección personal en la práctica veterinaria con bovinos y equinos. *Investigación Veterinaria, 20*(2), 255-266.

Norwood, S., McAuley, C., Vallina, V. L., Fernandez, L. G., McLarty, J. W., & Goodfriend, G. (2000). Mechanisms and Patterns of Injuries Related to Large Animals. *The Journal of Trauma: Injury, Infection, and Critical Care, 48*(4), 740-744. https://dx.doi.org/10.1097/00005373-200004000-00025

Olivares, R., Vidal, P., Sotomayor, c., Norambuena, M., Luppi, M., Silva, F., & Cifuentes, M. (2017). Brucelosis en Chile: descripción de una serie de 13 casos. *Revista chilena de infectología, 34*(3), 243-247. https://dx.doi.org/10.4067/S0716-10182017000300006

Paryuni, A., Indarjulianto, S., Widyarini, S. (2020). Dermatophytosis in companion animals: a review. *Veterinary World, 13*, 1174-1181.

Pereira Olivares, D. (2017). *Estudio descriptivo retrospectivo de casos de perros con enfermedades dermatológicas parasitarias en el Hospital Clínico Veterinario de la Universidad de Chile, período 2001-2013.* Veterinary Medicine Thesis, Universidad de Chile. https://repositorio.uchile.cl/handle/2250/151126

Rostamia, A., Mab, G., Wangb, T., Koehlerb, A., Hofmannb, B., Changb, B., Macphersonc, C., & Gasser, R. (2019). Human toxocariasis - a look at a neglected disease through an epidemiological 'prism'. *Infection, Genetics and Evolution*. 74. http://doi.org/10.1016/j.meegid.2019.104002

Samadi, S., Wouters, I., & Heederik, D. (2013). A review of bio-aerosol exposures and associated health efects in veterinary practice. *Annals of Agricultural and Environmental Medicine, 20*(2), 206-221.

Sánchez, A., Prats-van, M., Tatay-Dualde, J., Paterna, A., de la Fe, C., Gómez-Martín, A., Contreras, A. (2017). Zoonoses in veterinary students: a systematic review of the literature. *PLoS ONE, 12*(1). https://dx.doi.org/10.1371/journal.pone.0169534

Servicio Agrícola y Ganadero (SAG). (2021). *Brucelosis bovina.* https://www.sag.gob.cl/ambitos-de-accion/brucelosis-bovina-bb

Stull, J., Brophy, J., & Weese, J. (2015). Reducing the risk of pet-associated zoonotic infections. *Canadian Medical Association Journal, 187*(10). https://doi.org/10.1503/cmaj.141020

Troncoso, I., Fischer, C., Arteaga, F., Espinoza, C., Azócar, T., & Abarca, K. (2016). Seroprevalencia de *Bartonella henselae* en personas con riesgo ocupacional. *Revista chilena de infectología, 33*(3). https://dx.doi.org/10.4067/S0716-10182016000300019

Vayssier-Taussat, M., Moutailler, S., Féménia, F., Raymond, P., Croce, O., La Scola, B., & Raoult, D. (2016). Identification of novel zoonotic activity of *Bartonella* spp., France. *Emerging Infectious Diseases, 22*(3), 457-462. https://dx.doi.org/0.3201/eid2203.150269

Villar, G., Ortiz, R. (2016). Accidentes ocupacionales en trabajadores relacionados con animales silvestres en el Parque Zoológico Nacional de Cuba. *Revista Cubana de Salud y Trabajo*, *17*(3), 15-20.

Weinborn-Astudillo, R., Pau, N., Tobar, B., Jaffe, D., Boulouis, H., Sepúlveda, P., & Chomel, B. (2020). *Bartonella* infection in stray dogs from central and southern Chile (Linares and Puerto Montt). *Vector-Borne and Zoonotic Diseases, 20*(3), 187-192. https://dx.doi.org/10.1089/vbz.2019.2505

Wiedemann, A., Virlogeux-Payant, I., Chaussé, A., Schikora, A., & Velge, P. (2015). Interactions of *Salmonella* with animals and plants. *Frontiers in Microbiology, 5*, 791. https://doi.org/10.3389/fmicb.201

Austral J Vet Sci 55, 113-123 (2023)

ORIGINAL ARTICLE

Effects of plant extracts on the growth of beneficial indigenous lactic acid bacteria (BLAB) for their potential use in preventing bovine reproductive tract infections

María Hortencia Miranda[a], María Elena Fátima Nader-Macías[a*]

ABSTRACT. There is a renewed interest in products based on phytocompounds, prebiotics and probiotics applied to different hosts to exert effects of immunomodulation, anti-inflammation and analgesia. The microbiome of the bovine reproductive tract can become unbalanced for many reasons, favoring the entry and proliferation of pathogenic microorganisms, currently treated with antibiotics that exert adverse effects and generate antimicrobial resistance. To deal with this situation, "phytobiotic" formulas are proposed that combine phytocompounds and probiotics. This work aims to study the effect of plant extracts, prebiotics and vitamins on the growth of native beneficial lactic acid bacteria (BLAB), to be further potentially applied in the design of phytobiotic formulas. Nine beneficial strains isolated from different bovine ecosystems were evaluated against nine phytocompounds, two prebiotics and five vitamins. Affinity was assayed using the diffusion technique on agar plates, and the effect of the phytocompounds on the growth of lactic acid bacteria by microplates. The growth of all the strains was affected by some plant extracts, showing a stimulating or inhibitory effect. Diffusion-agar plates show that only vitamin A affected the viability of *Lactobacillus johnsonii* CRL1702 at concentrations higher than 7.5 mg/ml. When studying the growth kinetics of the strains with the phytocompounds, the results show that the effect was different in each of one the associated strains + plant extracts, indicating a strain-specific effect of plant extracts on each BLAB strain. Lapacho and Malva stimulate the growth of most microorganisms, then were selected to be combined with BLAB to design a phytobiotic formula with potential therapeutic activity to treat bovine reproductive infections. Plant extracts at the evaluated concentrations did not inhibit the growth of most of the pathogens responsible for endometritis. On the other hand, the highest concentrations of phenolic compounds were detected in Echinacea, Lapacho and Llantén; and the best percentages of antioxidant activity were evidenced in Garlic, Blueberry and Chamomile (<60%).

Keywords: Probiotic lactic bacteria, *Handroanthus impetiginosus*, *Malva sylvestris*, phytobiotics, bovine reproductive health.

INTRODUCTION

The autochthonous microbiota of the different bovine tracts and mucosa are constituted by a wide variety of microorganisms in ecological balance, called "microbiome", which is currently considered one more organ since it provides metabolic activities, coding capacities and fulfills a fundamental role and variety of physiological functions including immunomodulation and prevention of infection in humans and animals (Li *et al*. 2013; Stumpf *et al*. 2013). Lactic acid bacteria (LAB) are one of the most significantly important groups of bacteria in the food industry. They have long been consumed in dairy products all around the world and most of them are classified as "Generally Recognized As Safe" (GRAS) and into Qualified Presumption of Safety (QPS) microorganisms, because they are non-pathogenic, suitable for technological and industrial processes, having the ability to produce many antimicrobial substances (Reuben *et al*., 2020; Shehata *et al*., 2016). In the last decade, LAB has received increased attention and are widely used as probiotics, defined as "live microorganisms that exert health benefits on the host when ingested in adequate amounts" (FAO/WHO, 2006). Furthermore, lactobacilli are also under development as delivery systems for vaccines and treatments (Sun *et al*., 2015).

On the other hand, there is a growing interest in natural ingredients, including plant sources and derivatives, both from consumers and producers in the food and pharmaceutical industries. People search into the market for products that are artificial and synthetic additives-free to promote their health and animal sanity (Marchesi *et al.*, 2020). In almost all countries, plants have been widely used throughout history for the treatment and prevention of different diseases and infections in humans and domestic animals. Today, these traditional treatments are recommended in human and veterinary medicine due to their promising therapeutic efficacy with minimal side effects and reduction of chemotherapeutic drug residues in animal products consumed by humans (Gurib-Fakim, 2006). Most of them are included in the Pharmacopoeia regulations, while the perspective and future approaches of ethnopharmacological research are developed in parallel with advances in clinical and laboratory sciences, mainly phytochemistry and pharmacology (González-Stuart *et al*., 2017). Medicinal plants with their well-established history are an excellent resource of natural products used as an alternative therapy (Mushtaq *et al*., 2018). The limitation of therapeutic options for emerging multi-resistant microorganisms and the urgent need for new (or old uses)

Received: 11.10.2022

Accepted: 20.03.2023

[a]Laboratorio Farmabióticos, Centro de Referencia para Lactobacilos, Consejo Nacional de Investigaciones Científicas y Técnicas. San Miguel de Tucumán, Tucumán, Argentina.

*Corresponding author: fnader@cerela.org.ar, fatynader@gmail.com

natural and safe combinations are emerging. Then, the concept of phytobiotics was conceived, to combine safe phytoderivatives and probiotic bacteria for the design of new formulations, to advance in the combination/synergy of the immunomodulatory, anti-inflammatory, anticancer, analgesic and antioxidant effects reported individually in both, LAB probiotics and plant extracts (Nader Macías *et al*., 2008; Ayrle *et al*., 2016; Martínez & Lujan, 2011). These bioactive ingredients must be included in formulas in such a way that they must be compatible, protect against harsh environmental and process conditions, and be safely delivered to cells and target organs. In the area of probiotics, our research group has a long history of experiments carried out *in vitro* or in animal models, complemented by studies on the design of different types of vaginal probiotic formulas (Ocaña *et al*., 1999; De Gregorio *et al*., 2016; Leccese Terraf *et al*., 2014).

Therefore, the objective of this work was to evaluate the effect of phytoextracts (powdered) on the growth of bovine homologous vaginal probiotic lactobacilli and their affinity/compatibility to select those to be combined for the further design of a phytobiotic formulas. Also, to determine the phytocompounds effect on bovine vaginal pathogens, and the phenolic content and antioxidant activity of the plant extracts, to define if are related with the beneficial effect. The best combinations were defined to advance in the design of an intravaginal formula to be further applied to the reproductive health of postpartum cows.

MATERIALS AND METHODS

BENEFICIAL AUTOCHTHONOUS LACTIC ACID BACTERIA (BLAB) AND GROWTH CONDITIONS

The bacterial strains used in this study were beneficial lactobacilli previously isolated from three bovine ecosystems: the vaginal tract and mammary gland of cows and the gastrointestinal tract of calves (Tucumán, Argentina). The strains used are included in Table 1, together with the previously determined beneficial properties (Otero *et al*., 2006; Espeche *et al*., 2012; Maldonado *et al*., 2012; Miranda *et al*., 2020). The microorganisms were preserved in yeast extract milk (13% skim milk, 0.5% yeast extract and 1% glucose) at -20 °C, subcultured 3 times in MRS broth (De Man, Rogosa and Sharpe, Biokar Diagnostics, Beauvais, France) and incubated for 12 hours at 37 °C. Protocols were performed with the third subculture at 2%. The vaginal tract strains were isolated from vaginal brushing samples (Otero *et al.,* 2006), from the mammary gland/milk (Espeche *et al*., 2012) adult cows. Calves LAB were obtained from saliva and gastrointestinal tract (faeces) (Maldonado *et al*., 2012).

PREPARATION OF PLANT EXTRACTS, VITAMINS AND PREBIOTICS

The solid vegetable compounds evaluated were obtained from SAPORITI (Buenos Aires, Argentina) and selected

Table 1. Beneficial characteristics of the lactic acid bacteria strains selected and evaluated.

Microorganism	Beneficial properties•	Isolation Origin
Lactobacillus gasseri CRL 1412	H_2O_2 producer, hydrophobic, biofilm former and EPS producer	Bovine vagine*
Lactobacillus gasseri CRL 1421	H_2O_2 and lactic acid producer, hydrophobic, biofilm-forming	
Lactobacillus gasseri CRL 1460	Hydrophobic	
Lactobacillus gasseri CRL 1461	Lactic acid producer. Hydrophobic, self-aggregating, high adhesion capacity	
Lactococcus lactis subsp. *lactis* CRL 1656	H_2O_2 and lactic acid producer. Inhibits *S. aureus*, *S. uberis*, *S.agalactiae* and SCN	Bovine mammary gland$^{\gamma}$
Pediococcus pentosaceus CRL 1831	H_2O_2 producer, biofilm former, EPS +	
Weisella cibaria CRL 1833	Hydrophobic, biofilm-forming, EPS +	
Lactobacillus johnsonii CRL 1693	Lactic acid producer. Self-aggregating, hydrophobic, inhibits *E.coli*, *S. aureus*, *S. dublin*.	Faecal matter calves+
Ligilactobacillus murinus CRL1695	H_2O_2 and lactic acid producer. Self-aggregating, biofilm-forming, inhibits *S. typhimurium*	
Limosilactobacillus mucosae CRL1696	H_2O_2 producer	
Ligilactobacillus salivarius CRL1702	Lactic acid producer. Hydrophobic, inhibits *S. typhimurium*	

CRL: Centro de Referencia para Lactobacillus
*Published previously by Otero *et al.* (2006).
γPublished previously by Espeche *et al.* (2012).
+Published previously by Maldonado *et al.* (2012).
•Published previously by Miranda *et al*. (2020).

from the list of natural extracts approved by different Pharmacopoeias (Argentinian Pharmacopoeia; European Pharmacopoeia) for human and veterinary applications, included in Table 2 with their ethnopharmacological properties: *Allium sativa* (Garlic), *Vaccinium myrtillus* (Blueberry), *Atropa belladonna* (Belladonna), *Echinacea angustifolia* (Echinaceae), *Matricaria recutita* (Chamomile), *Handroanthus impetiginosus* (Lapacho), *Malva sylvestris* (Malva), *Larrea divaricata* (Jarilla) and *Plantago major* (Llantén).

COMPATIBILITY OF BLAB WITH PLANT EXTRACTS BY THE AGAR PLATE DIFFUSION TECHNIQUE

The Minimal Inhibitory Concentration (MIC) of the plant extract was determined by the agar plate diffusion technique, by using a range between 1.8 and 30 mg/ml. They were prepared at 60 mg for the microplate assay, dissolved in 1 ml of 25% alcohol, and stored at 4 °C protected from light until use. The vitamins used were: A, B5, B7 and B12 (ICN Biomedicals Inc, Ohio, USA), while powdered corn syrup (Arcor, Lules, Tucuman, Argentina) and inulin (Sigma-Aldrich, USA) was used as prebiotic and dissolved in water (30 mg/ml).

MRS (De Man, Rogosa and Sharpe) agar plates (Biokar Diagnostics, Beauvais, France) (1% agar) were prepared by inoculating 100 µl of different BLAB ($1x10^6$ and $1x10^8$ CFU/ml) added to the melted and cooled agar. Once solidified, 25 µl (per spot) of the seriated dilutions of plant extracts, vitamins and prebiotics were inoculated**,** maintained quiescent until complete diffusion on the agar, and incubated at 37 °C for 48 hours. The inhibition zones (in millimeters) were determined by using a manual caliper after. Assays were performed in triplicate.

Table 2. Application/Effect of vegetal extracts (approved in pharmacopoeias) assayed

Scientific name	Vulgar name	Application*	References	Pharmacopoeia
Allium sativum	Garlic	Modulation of the immune system and inflammation. GI tract: antidiarrheal Antibacterial - Anti-inflammatory Insect Bites - Injuries and Wounds	Ayrle *et al.*, 2016; Mirabeau 2012; Harris, 2001; Pittler, 2007; Iciek, 2009; Oosthuizen, 2017; Anwar 2017; Cheng, 2018	Argentina 8th edition
Malva sylvestris	Malva	Modulation of the immune system and inflammation - Intestinal colic - Wounds and injuries. Mastitis - Intestinal colic - Eye lesions - Antioxidant	Ayrle *et al.*, 2016; Martínez & Lujan, 2011; Bensaad, 2016; Prudente, 2017; Martins, 2017; Lasparetto, 2011; Vahabi, 2019	Argentina 1er annex
Larrea divaricata	Jarilla	Bumps or swelling - Muscle pain - Placental retention	Martínez & Lujan, 2011; Canale, 2018; Davicino, 2007; Palacio, 2008, 2012; Davicino, 2008; Martino, 2010, 2011; Pedernera, 2006	Argentina 8th edition
Plantago major	Llanten	Shock/inflammation - Wounds and injuries - Antiulcer - Anti-inflammatory - Antidiarrheal - Antitumor.	Martínez & Lujan, 2011; Amos, 2017; Najafian, 2018; Chiang, 2003a, b; Huang, 2009	Argentina 8th edition
Matricaria recitita	Chamomile	Wounds and injuries - Treatment of castration - Antioxidant - Anticancer - Placental retention - Insect and animal bites	Martínez & Lujan, 2011; Miraj, 2016; McKay, 2006; Sharifi-rad, 2018; Park, 2017; Al-Dabbagh, 2019; Mohsenzadeh, 2011	Argentina 8th edition
Echinacea angustifolia	Echinacea	Immune system strengthens and enhancer	Martínez & Lujan, 2011; Barnes, 2005; Arland, 2016; Sharifi-rad, 2018; Sharma, 2009; Stevenson, 2005	European annex 2014
Vaccinium myrtillus	Blueberry	Antioxidants - Anti-inflammatory - Anti-hypertensive - Antimicrobial - Anticancer	Jakesevic, 2013; Korus, 2014; Mykkanen, 2014; Nohynek, 2006; Veljković, 2016	European 8th edition / Argentine Food Code
Atropa belladonna	Belladonna	Antipyretic - Modulates inflammation	Ahmad, 2018; Pedalino, 2004	Argentina 8th edition
Handroanthu simpetiginosus	Lapacho	Anti hyper uricemic - Anti-inflammatory - Antioxidant - Anti-tumor - Antiulcerogenic - Analgesic - Anti-allergy	Ferraz-Filha, 2016; Grazziotin, 1992; Kim, 2012; Suo, 2012	Argentina 3rd annex

*Ethnopharmacological effect of plant extractsΩ

GROWTH KINETICS OF BLAB WITH PHYTOCOMPOUNDS BY MICROPLATE TECHNIQUE

The behavior of 9 plant extracts (SAPORITI drugstore, Buenos Aires-Argentina) at a concentration of 60 mg/ml on 10 different BLAB strains was evaluated: *Lactobacillus gasseri* CRL1412, *Lactobacillus gasseri* CRL1421, *Lactobacillus gasseri* CRL1460, *Lactobacillus gasseri* CRL1461, *Lactococcus lactis* subsp. *lactis* CRL1656, *Pediococcus pentosaceus* CRL1831, *Weisella cibaria* CRL1833, *Lactobacillus johnsonii* CRL1693, *Ligilactobacillus murinus* CRL1695, *Limosilactobacillus mucosae* CRL1696 and *Ligilactobacillus salivarius* CRL1702. The solid plant extracts were weighed and resuspended in 1 ml of 25% ethanol and filtered through sterile 0.22µm membrane filters (Biofil-Syringe Filter). The BLAB inoculum was prepared from the third MRS broth subculture with O.D.560nm 0.9-1.0 (10^7-10^8 CFU / ml).

The effect of the plant extracts on the growth of the BLAB strains was evaluated in polystyrene microplates (Extragen-96-well ELISA plate), to which 150 µl of MRS broth (De Man, Rogosa and Sharpe) (Biokar Diagnostics, Beauvais, France) inoculated with 2% BLAB was added. Then 50 µl of the plant extracts were added, gently mixed and incubated at 37 °C for 48 hours. Bacterial growth was determined by optical density ($O.D._{560nm}$) (Spectronic 20, Bausch and Lomb, Rochester, NY) at 4, 6, 22, 24, 30 and 48 hours. Solvents, individual plant extracts and bacteria in MRS were included as a control. The values of O.D. of bacterial growth were obtained subtracting the O.D. value obtained for each plant extract. All experiments were performed in triplicate.

EFFECT OF PLANT EXTRACTS ON THE GROWTH OF BOVINE REPRODUCTIVE TRACT PATHOGENS

The effect of 9 plant derivatives against different pathogens isolated from the bovine reproductive tract was evaluated by using the modified agar plate diffusion technique (De Gregorio *et al*., 2019). *Escherichia coli* 99/14, *Pseudomonas aeruginosa*, *Streptococcus bovis*, *Enterococcus faecalis* and *Listeria monocytogenes* (Nader *et al*., 2008) were cultured in LAPTg broth (Biokar Diagnostics, Beauvais, France) for 12 hours at 37 °C under microaerophilic conditions, in an anaerobic jar (AnaeroGen Oxoid). Briefly, LAPTg agar plates (15 ml of LAPTg with 1% agar) were inoculated with 1 x 10^6 and 1 x 10^8 CFU/ml of each pathogen (Pasteris *et al*., 2011). Aliquots (25 µl) of the different solid plant extracts dissolved in 25% hydroalcoholic solution (30, 15, 7.5 and 3.25 mg/ml) were added to the holes (4mm in diameter) aseptically made in the agar plates (in triplicate), incubated later for 24 hours at 37°C. The inhibition halos were quantified in mm, determining the Minimum Inhibitory Concentration (MIC) of each of the plant extracts. The extracts were added also as spots on the surface of the pathogens-agar plates (De Gregorio *et al*., 2019).

DETERMINATION OF TOTAL PHENOLIC COMPOUNDS OF PLANT EXTRACTS

The total phenolic content of the phytocompounds was determined by the Zielinski & Kozlowska (2000) technique, with the following modifications: 100 µl of each plant extract (1mg/ml) and 900 µl bidistilled water were mixed with 100 µl Folin-Ciocalteu reagent (Sigma-Aldrich, Darmstadt, Germany) (diluted to 50% in bidistilled water). After 2 minutes at room temperature, 400µl of a Na_2CO_3 solution (Ciccarelli, Santa Fe, Argentina) (15.9%, in bidistilled water) was added and left to stand for 10 minutes in the dark. Then 200 µl were seeded in microplates (in triplicate). Absorbance was measured in a microplate reader at 765 nm in a UV-VIS spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY). The calibration curve was prepared with gallic acid (GA) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in ethanol as a standard, in a concentration range of 2.5 to 1000 µg. The regression equation used was $y = 0.0395x + 0.1633$, and the correlation coefficient R2 = 0.9907. The results were expressed in mg of GAE extract/ml.

ANTIOXIDANT ACTIVITY OF PLANT EXTRACTS

The antiradical activity was evaluated by the photometric method, according to Wu *et al*. (2003). The DPPHu radical (1,1-diphenyl-2-picryl hydrazyl, Sigma-Aldrich, Darmstadt, Germany) was dissolved in 80% methanol up to an absorbance equal to 1.0 at 517 nm. 100 µl aliquots of each phytocompounds (1mg/ml) were mixed with 200 µl of DPPHu. The decrease in absorbance was determined in a microplate spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY) at 517 nm, with readings every 30 seconds for 10 minutes. The percentage of inhibition of DPPHu radical was calculated through the equation described by Burda & Oleszek (2001).

$$AAR = 100 \times (1 - Am/A0)$$

Where Am is the absorbance of the mixture at an infinite time and A0 is the absorbance of the DPPH solution before the addition of the samples.

STATISTICAL ANALYSIS

The results were subjected to analysis of variance (ANOVA) and a significance level of 5% ($p \leq 0.05$) was applied, using the Minitab analytical program.

RESULTS

BLAB COMPATIBILITY WITH PLANT EXTRACTS

The diffusion method on MRS agar plates determined that all plant extracts assayed are compatible with BLAB, as

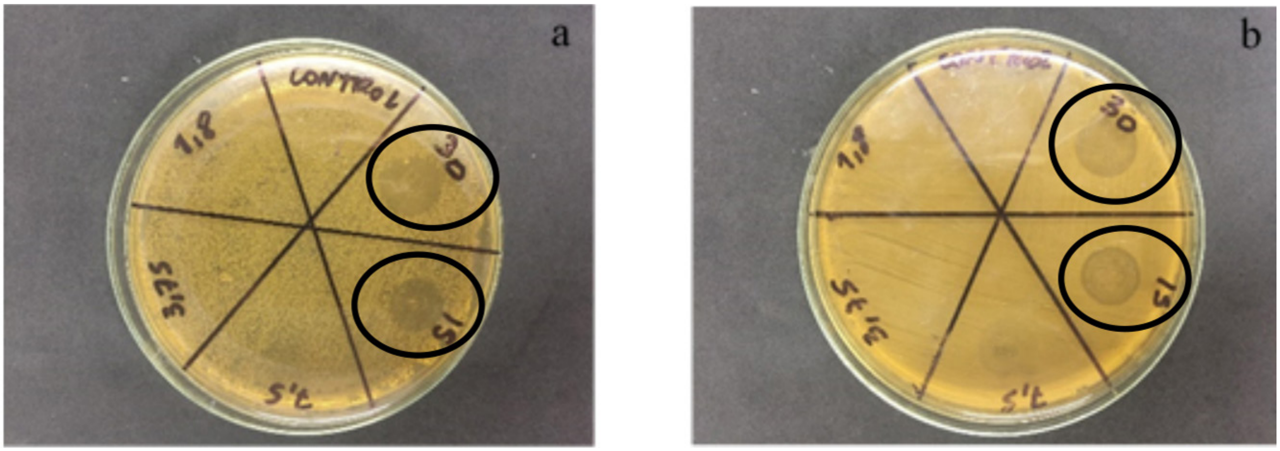

Figure 1. Inhibition of vitamin A on *Ligilactobacillus salivarius* CRL1702: 10^6 CFU/ml (a) and 10^8 CFU/ml (b). The black circles indicate the inhibition spots.

well as inulin and corn syrup used as prebiotics, except *L. salivarius* CRL1702 (10^6 and 10^8 CFU/ml) (Figure 1A-B) which was inhibited by vitamin A at 30 and 15 mg/mL concentrations (MIC = 7.5 mg/mL).

BLAB GROWTH IN THE PRESENCE OF DIFFERENT PLANT EXTRACTS

The plant extracts under study affected the growth of many BLAB strains since they were either stimulated or inhibited. Figure 2 shows the effect of different phytocompounds on BLAB growth, indicating that the effect is strain-specific. The claimed pharmacological effects of all the phytoderivatives assayed (Table 2), were not related to the degree of inhibition exerted on the growth of the strains; the growth of each BLAB with the added phytocompounds, evaluated by O.D.560nm, indicated that the effect is different in each one of the strains + plant extracts associations. Some examples are shown in Figure 2: a-g: represents obligate heterofermentative bacteria (OHe) and, h-i: obligate homofermentative lactobacilli (OHo). The growth in standard medium (MRS) of each strain is represented by a dotted line. Figure 2a shows the behavior of *L. gasseri* CRL1412 stimulated by Lapacho, Malva, Llanten and Manzanilla during the first 6 hours, but inhibited in a higher degree by Blueberry, Belladonna, Echinacea and Garlic. However, it is strongly stimulated by Jarilla starting at 12 hours. *L. gasseri* CRL1421 follows a growth pattern similar to CRL1412, showing good growth together with Lapacho and Malva, but inhibited by Belladonna, Llanten and Echinacea (Figure 2b). Figure 2c indicated the growth kinetics of *L. gasseri* CRL1460 strongly stimulated by Lapacho and Malva at 3 and 6 hours, respectively. After 6 hours, this strain was inhibited by most of the plant extracts, except Jarilla. *L. gasseri* CRL1461 (Figure 2d) which showed the highest growth between 3 and 6 hours. Malva, Manzanilla, Lapacho, Llanten and Equinacea produced even higher growth than in the standard broth (MRS broth). Lapacho, Malva and Llanten facilitated the growth of *L. lactis* subsp. *lactis* CRL1656 during the first 6 hours. Malva reaches a maximum O.D.540nm at 24 hours (1.43 ± 0.039), followed by Echinacea with an $O.D_{540\ nm}$ of 1.33 ± 0.10; while Belladonna suppressed the growth (Figure 2e). Figure 2f shows the growth of *P. pentosaceus* CRL1831 with plant extracts, indicating that Garlic and Malva inhibited this strain, while Blueberry, Lapacho and Manzanilla favor its growth at 24 hours. *L. salivarius* CRL1702 shows similar kinetics but only Llantén allowed higher growth rates at 24 h. Blueberry negatively affects both strains (Figure 2g).

The growth of *W. cibaria* CRL1833 is represented in Figure 2h, where Blueberry, Garlic, Jarilla, Belladonna and Echinacea were showed to inhibit it, while Lapacho and Malva strongly stimulated. *L. mucosae* CRL1696 evidenced an inhibitory pattern of all the plant extracts during the first 6 hours, however, Malva, Llantén, Lapacho and Echinacea produced a higher growth than in MRS at 24 hours (Figure 2i).

The degree of interaction between BLAB and plant extracts is summarized in Figure 4, showing the compatible or non-compatible combinations: stimulating (green) or inhibitory (red) effect of the phytocompounds on the growth of each strain. Gray boxes indicate "No effect" because there is no modification of the growth with the phytoderivatives when compared to the MRS control.

EFFECT OF PLANT EXTRACTS ON THE GROWTH OF UROGENITAL PATHOGENS

The plant extracts assayed at different concentrations was not able to inhibit the growth of most of the frequent pathogenic microorganisms that cause bovine metritis, although the antimicrobial effect of many of them was previously reported (Oosthuizen *et al*., 2017; Martínez & Lujan 2011; Nohynek *et al*., 2006; Sharifi-Rad *et al*., 2018; Canale *et al*., 2018).

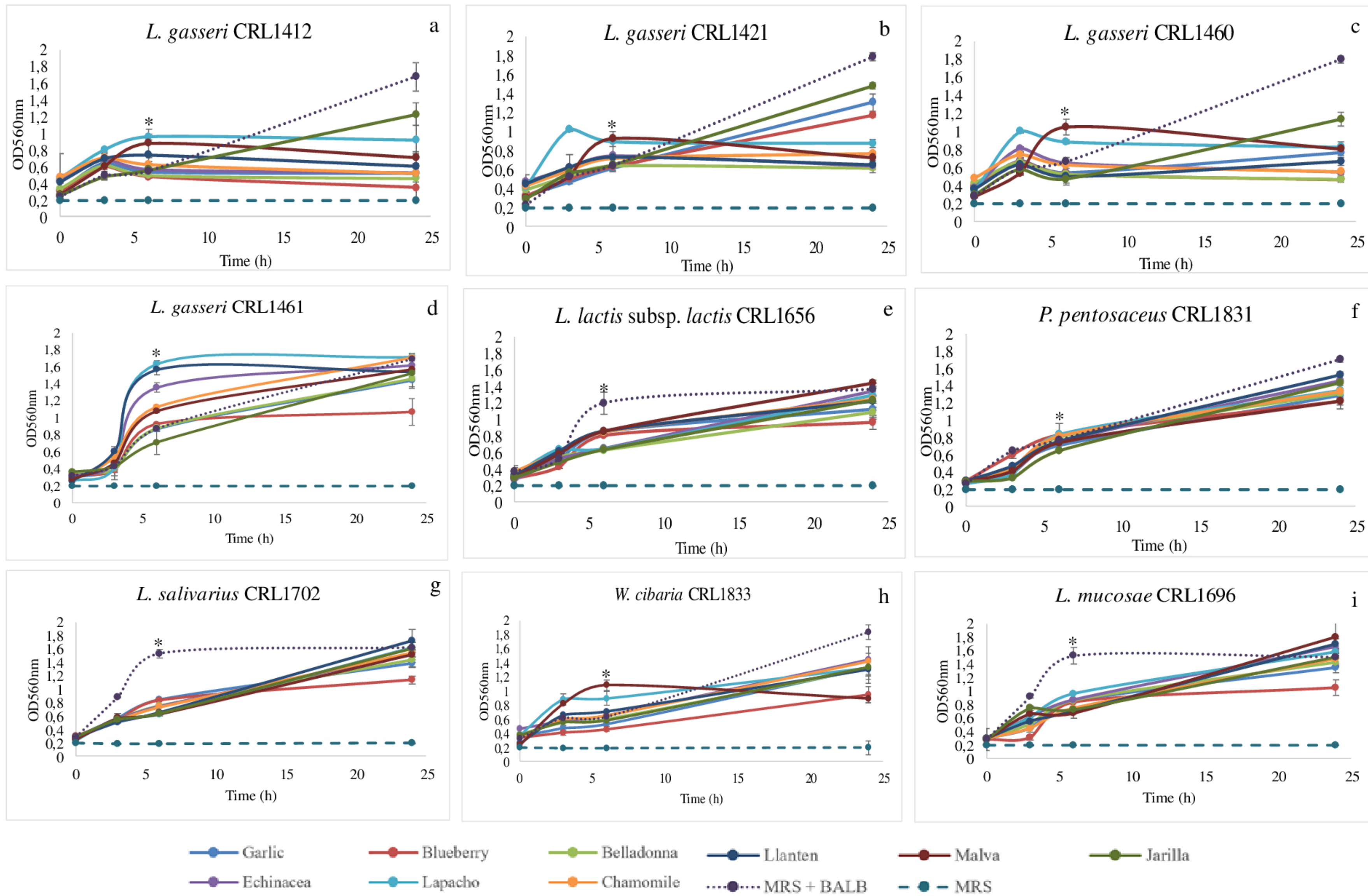

Figure 2. Effect of plant extracts on the growth of obligate heterofermentative (a-g) and obligate homofermentative (h-i) BALB. The asterisk (*) indicates significant differences (p<0.05) in the growth of the strain in different combinations of MRS + plant extract tested according to Tukey's test.

QUANTIFICATION OF TOTAL PHENOLIC COMPOUNDS (TPC) AND ANTIOXIDANT ACTIVITY IN PLANT EXTRACTS

The results obtained by evaluating the content of TPC and the antioxidant activity of the nine plant extracts were determined to select those with higher antioxidant activity, to promote the beneficial effect when combined with probiotics to the phytobiotic formula. Echinacea is the plant extract with the highest concentration of phenolic compounds. Lapacho and Jarilla did not show significant differences between them, or between Garlic and Belladonna. On the other hand, Blueberry and Malva did not present significant differences between them, showing the lowest TPC values (Figure 3a). Garlic, Blueberry, and Chamomile recorded the highest percentages of antioxidant activity, without significant differences between them, followed by Malva. Belladonna was the phytocompound with the lowest antioxidant activity (Figure 3b).

DISCUSSION

There is increasing interest in natural ingredients, including plant sources and their derivatives, and beneficial microorganisms from both consumers and producers in the food and pharmaceutical industries. People search the market for products and foods free of artificial additives that can promote their health, many of them with a very long application based on concepts or uses derived from custom. The same interest is shown in animal production systems.

On the other hand, the use of lactic acid bacteria as probiotics in different mucosal sites is widely recommended (ISSAP, 2016; McFarland *et al*., 2018). Specifically, in the bovine reproductive system, different scientists have shown that the application of an adequate combination of probiotics could effectively counteract an endometrial infection by *E. coli* (Genis *et al*., 2016, 2017) and improve productive parameters (Ametaj *et al*., 2014). Then, to increase/enhance beneficial effects (such as immunomodulation, and inhibition of pathogenic microorganisms), it is possible to combine them with extracts from plants currently or historically applied to the urogenital tract to design different formulas intended to exert or produce a synergistic effect on the host. Therefore, it is of the utmost importance to determine their compatibility and if the phytoderivatives affect the growth of BLAB to be used in a single beneficial formula.

In human medicine, some results have been published regarding the administration of a vaginal gel containing *Thymus vulgaris* and *Eugenia caryophilus* together with two *Lactobacillus* strains specifically formulated in slow-release capsules for the treatment of recurrent bacterial vaginosis or vulvovaginal candidiasis (Murina *et al*., 2018) however, there are no previous studies that report the compatibility between the two bioactive components of the commercial formula. It is very important, and as a first step for the design of the formula, to evaluate the effect

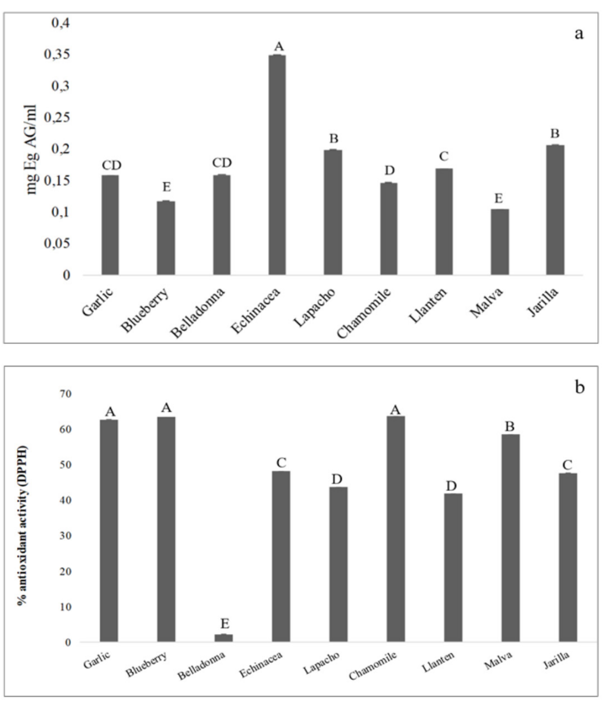

Figure 3. Quantification of phenolic compounds (a) and antioxidant activity (b) in plant extracts.

The different letters represent statistically significant differences (p<0.005) (Tukey's test).

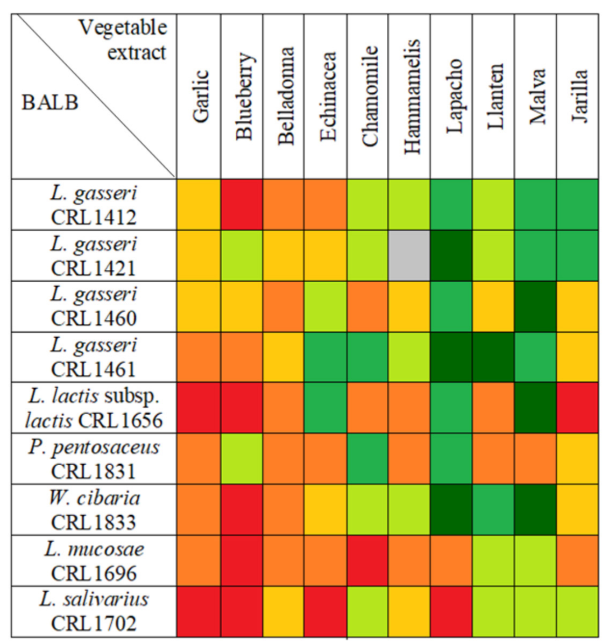

Figure 4. Compatibility between beneficial vaginal lactic acid bacteria in the presence of plant extracts. Red: "Inhibitory effect"; Green: "Stimulating effect"; Shades of color indicate greater or lesser inhibition ( ) or stimulation ( ) and "No effect" ( ).

of the phytoderivatives on the growth of lactobacilli. The results of this work show the interactions of a long list of phytoderivatives approved in the Pharmacopoeias to be used for both oral and local administration with bovine BLAB probiotic strains characterized in our laboratory. The compatibility of phytoderivatives and strains of bovine lactic acid bacteria in veterinary medicine has not been previously studied, therefore, the issue is of the utmost importance, and considered as a first step to design a novel formula, to evaluate the effect of phytoderivatives, whether liquid or extract, on the growth of BLAB. The results obtained in this work indicate that the effect of the phytoextracts is dependent on the strain since there are no general rules. Each strain and each extract must be tested to define their optimal or adequate combinations. In this work, the evaluation of their combinations was carried out through MIC tests and growth kinetics, which allowed defining which of them could be adequately combined. Different publications have reported the individual use of lactic acid bacteria and plant extracts as curative and/or preventive treatments in veterinary medicine (Pellegrino *et al*., 2017; Maldonado *et al*., 2017; Mansilla *et al*., 2020; Ayrle *et al*., 2016; Martínez & Lujan 2011; Lillehoj*et al*., 2018; Marume *et al*., 2017). Phytotherapy represents one of the most widely applied non-conventional medicines in both human and veterinary medicine (Hahn *et al*., 2005). According to Viegi *et al*. (2003), large animals (cattle, horses, sheep, goats and pigs) represent 70.5% of domestic animals treated with herbal remedies, because phytotherapy is mainly used on organic farms to further reduce the use of allopathic medicines. Although the use of phytotherapeutic products is increasing in animals, there are few studies and clinical trials reported in the literature on the therapeutic use of different plants for reproductive disorders in cows (El-Shanawani, 1996; Bruni *et al*., 1997; Lans *et al*., 2000; Uncini Manganelli *et al*., 2001; Alawa *et al*., 2002; Guarrera, 2005).

As previously indicated, there are no available studies on the combination of phytoderivatives and probiotic lactic acid bacteria and their application in the bovine reproductive system for the prevention of infections, reason by which there is a limitation to discuss/comparing the results obtained in this work with some other scientific reports. For this reason, the beneficial effects of the probiotic microorganisms and the plant extracts assayed in this work are included individually. Supported by the limited information available and the constant search for natural and innocuous products, the combination of probiotics and phytocompounds for veterinary use is an interesting and promising vacancy to study, even though there is a requirement to further assay their combination in the host animal.

The plant extracts evaluated in this work were selected for their variety of pharmacological effects and their use in veterinary medicine. Lapacho (*Handroanthus impetiginosus*) was the plant extract that stimulated the growth of the largest number of BLAB strains evaluated, with statistically significant differences (at 6 hours). Lapacho is traditionally used in folk medicine and mentioned for its therapeutic effects and included in pharmaceutical forms such as infusions and tinctures, mainly as an analgesic, anti-inflammatory and antitumor agent (Grazziotinv *et al*., 1992). Suo *et al*. (2012) demonstrated in an *in vitro* assay that compounds isolated from Lapacho bark significantly suppressed IL-1β and TNF-α cytokine production without significant cytotoxicity in LPS-stimulated cells, suggesting that Lapacho suppresses production the inflammatory cytokines. Therefore, the combination of Lapacho + BLAB strains is a promising strategy for cattle.

Another phytoderivative, Malva (*Malva sylvestris)*, shows a stimulating/promoting effect on *L. gasseri* CRL1412, *L. gasseri* CRL1421, *L. gasseri* CRL1460 and *W. cibaria* CRL1833. Several studies report the use of *Malva sylvestris* for veterinary purposes. The decoctions of whole plants are administered to cattle to treat colic, the leaves applied in compresses have shown high effectiveness in the treatment of mastitis in cattle, infusions and decoctions of aerial flowery parts were used against inflammation, wound infection, diarrhea in calves, its use as a curative for skin, reproductive and nervous disorders was also reported (Gasparetto *et al*., 2011; Viegi *et al*., 2003). Martins *et al*. (2017) evaluated the anti-inflammatory activity of the Malva extract and observed an inhibition of the release of proinflammatory mediators. Then the association of Malva with selected BLAB strains results in another interesting combination.

Plantain (*Plantago major*) was able to promote the growth of *L. gasseri CRL1461, P. pentosaceus CRL1831, L. mucosae CRL1696* and *L. salivarius CRL1702*. Velasco *et al*. (2006) found that the antibacterial activities of Plantago major leaves and seeds were shown to inhibit *Escherichia coli, Bacillus subtilis* and *Candida albicans* cultures. The rupture of the cell walls of Gram-positive bacteria and the formation of vesicles in Gram-negative bacteria (Metiner *et al*., 2012) were observed by electron microscope evaluations.

Jarilla (*Larrea divorcata*) shows a stimulating effect on the growth of vaginal strains, which is interesting, since different publications have shown that extracts of *L. divaricata* have immunomodulatory properties (Davicino *et al*., 2007, 2010). Jarilla also exhibited *in vitro* antimicrobial effects against pathogens (Quiroga *et al*., 2004; Stege *et al*., 2006). Then it is possible to indicate that the behavior of stimulation or inhibition of the extract is directly related and depends on each strain and species of microorganism. The Jarilla is an autochthonous phytoderivative widely used in northern Argentina.

Garlic and Belladonna extracts inhibited the growth, to different degrees, of all strains tested. Although their important therapeutic effects have been studied, it is not possible to combine them with probiotic microorganisms, possibly due to their antimicrobial properties.

Combinations of beneficial lactic acid bacteria and plant extracts for veterinary therapeutic purposes have not been previously reported. Phytobiotic formulations for female vaginal application were studied by combining probiotic lactobacilli with different active ingredients (Marchesi *et al*., 2020), but no products are marketed in our country. These authors advanced in the selection of viable beneficial lactobacilli for their inclusion in the design of phytobiotic formulas for the health of the female urogenital tract, based on their compatibility with natural compounds with ethnopharmacological properties. The phytocompounds studied in this work did not show to inhibit the growth of pathogenic microorganisms responsible for urogenital infections in cows, even though their antimicrobial effects were reported in the literature. Even though this is not an encouraging result, the definitive effect must be assayed in the host animal, because the *in vitro* experiments, predictive in some aspects, sometimes do not agree with the *in vivo* behavior or clinical/animal studies.

The concentration of phenolic compounds and the antioxidant activity in the extract are not directly correlated with the effect on the growth of BLAB nor with the compatibility between them. Further studies should be carried out to determine if any specific type of component is responsible for the stimulating or inhibitory effect. Lillehoj *et al*. (2018) reported that the beneficial effects of phytoextracts are attributed to their antimicrobial and antioxidant properties, which is not demonstrated in our results. The inclusion of phytoderivatives in the diets of animals should alter and stabilize the intestinal microbiota and thus reduce toxic microbial metabolites in the intestine, due to their direct antimicrobial properties on various pathogenic bacteria, resulting in the alleviation of intestinal challenges and immune stress, which would lead to improved performance (Kim *et al*., 2015). In ruminants, tannins, included in phenolic compounds, modulate the ruminal and intestinal microbiota and improve the growth of certain bacterial populations. The effects of tannins on the rumen microbiota may be different depending on the molecular nature of these polyphenols (Vasta *et al*., 2008; Min *et al*., 2015).

CONCLUSION

The BLAB strains that showed an affinity with the phytoextracts were: *L. gasseri* CRL1421, *L. gasseri* CRL1460, *L. gasseri* CRL1461 and *W. cibaria* CRL1833 with Lapacho, Malva, Llanten and Jarilla, which can be used to design phytobiotic formulas due to their pharmacological effects (antibacterial, anti-inflammatory, immunomodulatory and healing). These results are original, because the compatibility of natural extracts with probiotic lactic acid bacteria strains was studied, in a way to combine the beneficial effect of the two bioactive compounds in the host, which should be further assayed in *in vivo* protocols.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

No ethical approval was required in this work, as this is an original article with only bacterial isolates and data. No animal or human samples were employed.

FUNDING

This work was supported by PICT 2017-4324 and 1187 (FONCYT-ANPCYT-MINCYT) and PIP 744 and 545 from CONICET (Consejo Nacional de InvestigacionesCientíficas y Técnicas, Argentina).

REFERENCES

Alawa, J.P., Jokthan, G.E. & Akut, K. (2002). Ethnoveterinary medical practice for ruminants in the subhumid zone of northern Nigeria. *Preventive Veterinary Medicine*, 54(1), 79-90. https://doi.org/10.1016/s0167-5877(01)00273-2

Ametaj, B.N., Iqbal, S., Selami, F., Odhiambo, J.F., Wang, Y., Gänzle, M.G., Dunn, S.M. & Zebeli, Q. (2014). Intravaginal administration of lactic acid bacteria modulated the incidence of purulent vaginal discharges, plasma haptoglobin concentrations, and milk production in dairy cows. *Research in Veterinary Science*, *96*(2), 365-370. https://doi.org/10.1016/j.rvsc.2014.02.007

Ayrle, H., Mevissen, M., Kaske, M., Nathues, H., Gruetzner, N., Melzig, M. & Walkenhorst, M (2016). Medicinal plants – prophylactic and therapeutic options for gastrointestinal and respiratory diseases in calves and piglets? A systematic review. BMC *Veterinary Research*, *12*(89). https://doi.org/10.1186/s12917-016-0714-8

Bourda, E. & Oleszek, W (2001). Antioxidant and antiradical activities of flavonoids. *J. Agric. Food Chemistry*, *49*(6), 2774-2779. https://doi.org/10.1021/jf001413m

Bruni, A., Ballero, M., & Poli, F. (1997). Quantitative ethnopharmacological study of the Campidano Valley and Urzulei district, Sardinia, Italy. *Journal of Ethnopharmacology*, *57*(2), 97-124. https://doi.org/10.1016/S0378-8741(97)00055-X

Canale, F.P., Davila, SV., Sasso, C.V., Pellarin, N.W. & Mattar Dominguez, M.A. (2018). Immunization with *Larrea divaricata* Cav. proteins elicits opsonic antibodies against *Pseudomonas aeruginosa* and induces phagocytic activity of murine macrophages. *Microbial Pathogenesis*, *118*, 257-267. https://doi.org/10.1016/j.micpath.2018.03.029

Davicino, R., Mattar, A., Casali, Y., Porporatto, C., Correa, SG. & Micalizzi, B. (2007). *In Vivo* Immunomodulatory Effects of Aqueous Extracts of *Larrea divaricata Cav*. *Immunopharmacology and Immunotoxicology*, *29*(3-4), 351-366. https://doi.org/10.1080/08923970701619703

Davicino R., Manuele M.G., Turner S., Ferraro G. & Anesini C. (2010). Antiproliferative activity of *Larrea divaricata Cav*. on lymphoma cell line: participation of hydrogen peroxide in its action. *Cancer Investigation*, *28*(1), 13-22. https://doi.org/10.3109/07357900902849665

De Gregorio, P.R., Juárez Tomás, M.S. & Nader-Macías, M.E. (2016). Immunomodulation of *Lactobacillus reuteri* CRL1324 on Group B *Streptococcus Vaginal* Colonization in a Murine. Experimental Model. *American Journal of Reproductive Immunology*, *75*(1), 23-35. https://doi.org/10.1111/aji.12445

De Gregorio, P.R., Silva, J.A., Marchesi, A. & Nader-Macías, M.E.F. (2019). Anti-Candida activity of beneficial vaginal lactobacilli in in vitro assays and in a murine experimental model. *FEMS Yeast Research*, *19*(2). https://doi.org/10.1093/femsyr/foz008

Deng, Q., Odhiambo, J.F., Farooq, U., Lam, T., Dunn, S.M., Gänzle, M.G. & Ametaj, B.N. (2015). Intravaginally administered lactic acid bacteria expedited uterine involution and modulated hormonal profiles of transition dairy cows. *Journal of Dairy Science*, *98*(9), 6018-6028. https://doi.org/10.3168/jds.2014-8559

El-Shanawani, M.A.A. (1996). *Plants Used in Saudi Traditional Medicine KACST Riyadh*. King Saud University Press, Saudi Arabia.

Espeche, C., Pellegrino, M., Frola, I., Larriestrac, A. & Bogni, C. (2012). Lactic acid bacteria from raw milk as potentially beneficial strains to prevent bovine mastitis. *Anaerobe*, *18*, 103-109. https://doi.org/10.1016/j.anaerobe.2012.01.002

FAO/OMS. (2006). Probiotics in food, healthy and nutritional properties and guidelines for their evaluation. *FAO Food and Nutrition Paper*, (85). WHO/FAO, Rome, Italy.

Gasparettoa, J.C., Ferreira Martinsa, C.A., Hayashia, S.S., Otukyb, M.F. & Pontaroloa, R. (2012). Ethnobotanical and scientific aspects of *Malva sylvestris L.*: a millennial herbal medicine. *Journal of Pharmacy and Pharmacology*, *64*(2), 172-89. https://doi.org/10.1111/j.2042-7158.2011.01383.x

Genis, S., Bach, A. & Aris, A. (2017). Effects of intravaginal lactic acid bacteria on bovine endometrium: Implications in uterine health. *Veterinary microbiology*. https://doi.org/10.1016/j.vetmic.2017.04.025

Genís, S., Bach, À., Fàbregas, F. & Arís, A. (2016). Potential of lactic acid bacteria at regulating Escherichia coli infection and inflammation of bovine endometrium. *Theriogenology*, *85*, 625-637. https://doi.org/10.1016/j.theriogenology.2015.09.054

González-Stuart, A. & Rivera, J. (2017). Yellow Oleander Seed, or “Codo de Fraile” (*Thevetia spp.*): A Review of Its Potential Toxicity as a Purported Weight-Loss Supplement. *Journal of Dietary Supplements, 15*(3), 352-364. https://doi.org/10.1080/19390211.2017.1353565

Grazziotin, J.D., Schapoval, E.E.S., Chavesa, C.J., Gleyeb, J. & Henriques, A.T. (1992). Phytochemical and analgesic investigation of *Tabebuia chrysotricha*. *Journal of Ethnopharmacology*, *11*, 249-251 https://doi.org/10.1016/0378-8741(92)90051-r

Guarrera, P.M. (2005). Traditional phytotherapy in Central Italy (Marche, Abruzzo, and Latium). *Fitoterapia*, *76*(1), 1-25. https://doi.org/10.1016/j.fitote.2004.09.006

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*, *27*(1), 1-93. https://doi.org/10.1016/j.mam.2005.07.008

Hahn, I., Zitterl-Eglseer, K. & Franz, Ch. (2006). Phytomedicine in dogs and cats: web-based survey among veterinarians in Austria, Germany and Switzerland. *Schweiz Arch Tierheilkd*, *147*(3), 135-141. https://doi.org/10.1024/0036-7281.147.3.135

International Scientific Association for Probiotics and Prebiotics (ISAPP). (2016). International Scientific Association for Probiotics and Prebiotics. https://isappscience.org.

Kim, J.E., Lillehoj, H.S., Hong, Y.H., Kim, G.B., Lee, S.H., Lillehoj, E.P. & Bravo, D.M. (2015). Dietary *Capsicum* and *Curcuma longa* oleoresins increase intestinal microbiome and necrotic enteritis in three commercial broiler breeds. *Research in Veterinary Science, 102*, 150-158. https://doi.org/10.1016/j.rvsc.2015.07.022

Lans, C., Harper, T., Georges, K. & Bridgewater, E. (2000). Medicinal plants used for dogs in Trinidad and Tobago. *Preventive Veterinary Medicine*, *45*(3-4), 201-220. https://doi.org/10.1016/S0167-5877(00)00123-9

LecceseTerraf, M.C., Mendoza, L.M., Juarez Tomas, M.S., Silva, C. & Nader, M.E.F. (2014). Phenotypic Surface properties (aggregation, adhesion and biofilm formation) and presence of related genes in beneficial vaginal lactobacilli. *Journal of Applied Microbiology*, *117*(6), 1761-1772.https://doi.org/10.1111/jam.12642

Lillehoj, H., Liu, Y., Calsamiglia, S., Fernandez-Miyakawa M.E., Chi, F., Cravens, R., Oh, S. & Gay, C. (2018). Phytochemicals as antibiotic alternatives to promote growth and enhance host health. *Veterinary Research*, *49*(76). https://doi.org/10.1186/s13567-018-0562-6

Maldonado, N.C., Chiaraviglio, J., Bru, E., De Chazal, L. & Santos, V. (2017). Effect of Milk Fermented with Lactic Acid Bacteria on Diarrheal Incidence, Growth Performance and Microbiological and Blood Profiles of Newborn Dairy Calves. *Probiotics Antimicrob Proteins*, 10(4), 668-676. https://doi.org/10.1007/s12602-017-9308-4

Maldonado, N.C., Silva de Ruiz, C., Otero, M.C., Sesma, F. & Nader-Macías, M.E. (2012). Lactic acid bacteria isolated from young calves--characterization and potential as probiotics. *Research in Veterinary Science*, *92*(2), 342-349. https://doi.org/10.1016/j.rvsc.2011.03.017

Mansilla, F., Takagi, M., Garcia-Castillo, V., Aso, H., Nader-Macias, M.E.F., Vignolo, G.M., Kitazawa, H. & Villena, J. (2020). Modulation of Toll-like receptor-mediated innate immunity in bovine intestinal epithelial cells by lactic acid bacteria isolated from feedlot cattle. *Beneficial Microbes*, *11*(3), 269-282. https://doi.org/10.3920/BM2019.0189

Marchesi, A., Silva, J., AristimuñoFicoseco, C., Wiese, B. & Nader-Macías, M.E.F. (2020). Effect of phytoderivatives on the growth of homologous beneficial vaginal lactobacilli (BVL) strains and their compatibility for the design of phytobiotics for the vaginal tract health. *Research Square*. https://doi.org/10.21203/rs.2.22448/v1

Martinez, GJ, Lujan, MC (2011). Medicinal plants used for traditional veterinary medicine in the Sierras de Córdoba (Argentina): an ethnobotanical comparison with human medicinal uses. *Journal of Ethnobiology and Etnomedicine*, *7*(1), 23. https://doi.org/10.1186/1746-4269-7-23

Martins, C.A.F., Campos, M.L., Irioda, A.C., Pontarolo Stremel, D., Leal Badaró Trindade, A.C. & Pontarolo, R. (2017). Anti-Inflammatory Effect of *Malva sylvestris*, *Sidacordifolia*, and *Pelargonium graveolens* Is Related to Inhibition of Prostanoid Production. *Molecules*, *22*(11), 1883. https://doi.org/10.3390/molecules22111883

Marume, A., Matope, G., Katsande, S., Khoza, S., Mutingwende, I., Mduluza, T., Munodawafa-Taderera, T. & Ndhlala, A.R. (2017). Wound Healing Properties of Selected Plants Used in Ethnoveterinary Medicine. *Frontiers in. Pharmacology*. https://doi.org/10.3389/fphar.2017.00544

McFarland, L.V., Evans, C.T. & Goldstein, E.J.C. (2018). Strain-Specificity and Disease-Specificity of Probiotic Efficacy: A Systematic Review and Meta-Analysis. *Frontiers in Medicine (Lausanne)*, *5*, 124. https://doi.org/10.3389/fmed.2018.00124

Metiner, K., Ozkan, O.0 &, Ak, S. (2012). Antibacterial effects of ethanol and acetone Extract of *Plantago major L.* on gram positive & gram negative bacteria. *Kafkas Üniversitesi Veteriner Fakültesi*, *18*(3), 503-505. https://doi.org/10.9775/KVFD.2011.5824

Min, Y.W., & Rhee, P-L. (2015). The role of microbiota on the gut immunology. *Clinical Therapeutics*, *37*, 968-975. https://doi.org/10.1016/j.clinthera.2015.03.009

Miranda, M.H. & Nader-Macias, M.E.F. (2020). Advances in the Design of a Multi-Strain Homologous Probiotic Formula for Cattle. *Journal of Veterinary Medicine and Animal Sciences*, *3*(1), 1022.

Murina, F., Vicariotto, F. & Di Francesco, S. (2018). *Thymol*, *Eugenol* and lactobacilli in a medical devicefor the treatment of bacterial vaginosis and vulvovaginal candidiasis. *New Microbiologica*, *41*(3), 220-224.

Mushtaq, S., Shah, A.M., Shah, A., Lone, S.A., Hussain, A., Hassan, Q.P. & Ali, M.N. (2018). Bovine mastitis: An appraisal of its alternative herbal curen. *Microbial Pathogenesis.* https://doi.org/10.1016/j.micpath.2017.12.024

Nader, M.E.F., Otero, M.C., Espeche, M.C. & Maldonado, N.C. (2008). Advances in the desing of probitic products for the prevention of major diseases in dairy cattle. *Journal of Industrial Microbiology and Biotechnology*. https://doi.org/10.10007/s10295-008-0438-2

Nohynek, L., Alakomi, H.L., Kähkönen, M.P., Heinonen, M.., Helander, I.M., Oksman-Caldentey, K.M. & Puupponen-Pimiä, R.H. (2006): Berry Phenolics: Antimicrobial Properties and Mechanisms of Action Against Severe Human Pathogens. *Nutrition and Cancer*, *54*, 18-32. https://doi.org/10.1207/s15327914nc5401_4

Ocaña, V.S., Pesce De Ruiz Holgado, A.A. & Nader-Macías, M.E. (1999). Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain. *Applied and Environmental Microbiology*, *65*(12), 5631-5635. https://doi.org/10.1128%2Faem.65.12.5631-5635.1999

Oosthuizen, C., Arbach, M., Meyer, D., Hamilton, C. & Lall, N. (2017). Diallyl Polysulfides from *Allium sativum* as Immunomodulators, Hepatoprotectors, and Antimycobacterial Agents. *Journal of Medicinal Food*. https://doi.org/10.1089/jmf.2016.0137

Otero, M.C., Morelli, L. & Nader Macías, M.F. (2006). Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle. *Letters in Applied Microbiology*, *43*, 91-97. https://doi.org/10.1111/j.1472-765X.2006.01914.x

Pasteris, S.E., Guidoli, M.G., Otero, M.C., Bühler, M.I &, Nader-Macías, M.E. (2011). *In vitro* inhibition of *Citrobacter freundii*, a red-leg syndrome associated pathogen in raniculture, by indigenous *Lactococcus lactis* CRL 1584. *Veterinary Microbiology*. https://doi.org/10.1016/j.vetmic.2011.03.025

Pellegrino, M., Berardo, N., Giraudo, J., Nader-Macías, M.E.F. & Bogni, C. (2017). Bovine mastitis prevention: humoral and cellular response of dairy cows inoculated with lactic acid bacteria at the dry-off period. *Beneficial Microbes*. https://doi.org/10.3920/BM2016.0194

Quiroga, E.N., Sampietro, A.R. & Vattuone, M.A. (2004). *In vitro* fungitoxic activity of *Larrea divaricatacav*. extracts. *Letters in Applied Microbiology*, *39*(1), 7-12. https://doi.org/10.1111/j.1472-765X.2004.01521.x

Reuben, R.C., Roy, P.C., Sarkar, S.L., Rubayet, U.l., Alam, A.S.M. & Jahid, I.K. (2020). Characterization and evaluation of lactic acid bacteria from indigenous raw milk for potential probiotic properties. *Journal of Dairy Science*. https://doi.org/10.3168/jds.2019-17092

Sharifi-Rad, M., Mnayer, D. & Morais-Braga, M.F.B. (2018). Echinacea plants as antioxidant and antibacterial agents: From traditional medicine to biotechnological applications. *Phytotherapy Research*. https://doi.org/10.1002/ptr.6101

Shehata, M.G., El Sohaimy, S.A., El-Sahn, M.A. & Youssef, M.M. (2016). Detección de bacterias de ácido láctico probióticas potenciales aisladas para la propiedad de reducción del colesterol y la actividad hidrolasa de sales biliares. *Annals of Agricultural Sciences*, *61*, 65-75. https://doi.org/10.1016/j.aoas.2016.03.001

Stege, P.W., Davicino, R.C., Vega, A.E., Casali, Y.A., Correa, S. & Micalizzi, B. (2006). Antimicrobial activity of aqueous extracts of *Larrea divaricata Cav* (jarilla) against *Helicobacter pylori*. *Phytomedicine*, *13*(9-10), 724-727. https://doi.org/10.1016/j.phymed.2005.06.008

Sun, Z., Harris, H.M.B., McCann, A., Guo, C., Argimón, S., Zhang, W., Yang, X., Jeffer, I.B., Cooney, J.C., Kagawa, T.F., Liu, W., Song, Y., Salvetti, E., Wrobel, A., Rasinkangas, P., Parkhill, J., Rea, M.C., O'Sullivan, O., Ritari, J., Douillard, F..P, Ross, R.P., Yang, R., Briner, A.E., Felis, G.E., de Vos, W.M., Barrangou, R., Klaenhammer, T.R., Caufield, P.W., Cui, Y., Zhang, H., & O'Toole, P.W. (2015). Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nature Communications*. 6, 8322. https://doi.org/10.1038/ncomms9322

Suo, M., Isao, H., Kato, H., Takano, F. & Ohta, T. (2012). Anti-inflammatory constituents from *Tabebuia avellanedae*. *Fitoterapia*, *83*(8), 1484-1488. https://doi.org/10.1016/j.fitote.2012.08.014

Uncini Manganelli, R.E., Camangi, F. & Tomei, P.E. (2001). Curing animals with plants: traditional usage in Tuscany (Italy). *Journal of Ethnopharmacology*. https://doi.org/10.1016/S0378-8741(01)00341-5

Vasta, V., Makkar, H.P.S., Mele, M. & Priolo, A (2008). Ruminal biohydrogenation as afected by tannins *in vitro*. *British Journal of Nutrition, 102*, 82-92.

Velasco-Lezama, R., Tapia-Aguilar, R., Román-Ramos, R., Vega-Ávila, E. & Pérez-Gutiérrez, M.S. (2006). Effect of *Plantago major* on cell proliferation *in vitro*. *Journal of Ethnopharmacology, 103*(1), 36-42. https://doi.org/10.1016/j.jep.2005.05.050

Viegi, L., Pieroni, A., Guarrera, P.A. & Evangelista, R. (2003). A review of plants used in folk veterinary medicine in Italy as basis for a databank. *Journal of Ethnopharmacology*, *89*, 221-244. https://doi.org/10.1016/j.jep.2003.08.003

Wu, H.C., Shiau, C.Y., Chen, H.M. & Chiou, T.K. (2003). Antioxidant activities of carnosine, anserine, some free amino acids and their combination. *Journal of Food and Drug Analysis*, *11*(2), 148-153.

Zieliński, H. & Kozłowska, H., (2000). Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *Journal of Agricultural and Food Chemistry*, *48*(6), 2008-2016. https://doi.org/10.1021/jf990619o

Austral J Vet Sci **55, 124-130 (2023)**

ORIGINAL ARTICLE

Frequency distribution of polymorphisms on κ-casein and DGAT1 genes in dairy cattle used in Chilean milk production

Daniela Levicoy[a], Rodrigo de la Barra[a], Andrés M. Carvajal[a*]

ABSTRACT. Milk solids are very important to transform milk in dairy products like cheese. Several genes and polymorphic variants had been associated with this process, increasing the milk fat and/or protein content or regulating the coagulation milk properties. In the present study, we evaluate the frequency distribution of two major polymorphisms present in *DGAT1* and *CSN3* genes in 6 dairy biotypes commonly used in southern Chile: Holstein-Friesian (HF), Jersey (JE), Montbeliarde (MB), Overo Colorado (OC), Frisón Negro (FN) and hybrids (HYB). For *CSN3* the results revealed that the A variant was predominant (0.57 to 0.71) in all breeds except Jersey which showed a high frequency of the B variant (0.73), one that favors milk transformation, and in MB (0.58). For *DGAT1* a similar trend was seen. The A variant predominated (0.64 to 0.96) in all populations except Jersey, which displayed a high frequency (0.70) of the K variant that favors milk solids. The analysis of FIS for both markers gave negative and non-significant values in all populations some exception of OC and HYB, which are not in Hardy-Weinberg equilibrium, suggesting an excess of heterozygotes. By another hand, FST analysis suggest a high genetic structure for Jersey. The simultaneous analyses of both markers produced 16 combined genotypes with hybrid animals displaying the highest number (11) whereas Jersey showed a favorable combination (*DGAT1* KK and *CSN3* BB) renewing their productive orientation to transform milk to cheese. Also, some Holstein animals displayed a heterozygous combined genotype (*DGAT1* A/K–*CSN3* AB). For decades, Holstein-Friesian has been an important breed used for milk production in southern Chile and during the last time, several farmers have introduced Jersey in order to improve milk quality. In this sense, our results show the presence of potential favorable genotypes for the transformation of milk to dairy products.

Keywords: Dairy cattle, milk solids, molecular markers, kappa-casein, dgat1.

INTRODUCTION

Bovine milk is one of the major foods included at healthy recommendations for human consumers. With a wide composition, it contains macro and micronutrients that are fundamental for our health and development. Within these, milk protein and milk fat are key components due to contribution of essential aminoacidic and fatty acids (Gibson, 2011). Both of them make up the majority of the well-known milk solids and are important for the technological transformation of milk to cheese and other dairy products (Cipolat-Gotet *et al*., 2018).

The content of milk solids is determined by two major factors: nutrition and genetics (Osorio *et al*., 2016). Thus, currently one of the more important dairy breeding's objectives at world and national level is the increase in the milk solids content (Uribe *et al*., 2017).

From a genetic perspective, the presence of variants in several milk proteins and major enzymes regulating milk fat synthesis and secretion has generated several studies related to the identification of molecular markers, mainly SNPs but also candidate genes to modulate milk composition (Caroli *et al*., 2009). This relationship between markers and production traits has been widely strengthened with the arriving of genomics, which allows the association of thousands of markers along the cattle genome (Gebreyesus *et al*., 2019; Sanchez *et al*., 2019).

An important milk protein is kappa casein (κ-casein), which is related to milk production, composition, and coagulation properties, playing a direct role in the manufacturing process of milk products like cheese (Bonfatti *et al*., 2010; Vallas *et al*., 2012). This protein is encoded by *CSN3* gene, which is highly polymorphic and being the A and B variants the most important ones. Those animals carrying the BB genotype show a milk with increased levels of κ-casein and a lower micelles size, reducing the clotting time and promoting the firmness of the rennet (Cipolat-Gotet *et al*., 2018). On the other hand, animals with AA genotype are related to higher milk production but lower clotting time (Caroli *et al*., 2009).

Respect to milk fatty acids, *DGAT1* gene codes for diacyglicerol:acyl CoA acyltransferase 1 (EC 2.3.1.20; Cases *et al*., 1998), the limiting enzyme for triglycerides biosynthesis at mammary gland. This gene shows a polymorphism (K232A) with a major effect on milk composition (Grisart *et al*., 2004). The mutation corresponds to an aminoacidic change of lysine (K) to alanine (A) which can explain up to 50% of variation of milk fat content. It is widely described that those cattle carrying the K allele show a high yield and content of milk fat, whereas those with the A variant display a high milk yield (Grisart *et al*., 2004; Carvajal *et al*., 2016).

In southern Chile several cattle breeds are used for milk production including worldwide and local adapted breeds. In addition, today exists a growing interest to increase milk solids. This is given by a greater incentive from industry in the payment schedule. Therefore, the

Received: 25.07.2022.

Accepted: 21.03.2023.

[a]Instituto de Investigaciones Agropecuarias (INIA), Ruta 5 km 8 norte, Osorno, Región de Los Lagos, Chile.

*Corresponding author: andres.carvajal@inia.cl

objective of this study was to evaluate the distribution of allelic frequencies of C*SN3* and *DGAT1* polymorphisms in representative cattle breeds used for milk production.

MATERIAL AND METHODS

ANIMAL SAMPLES

The study included 263 dairy cattle distributed in six different herds located in Los Ríos and Los Lagos regions, Chile. These animals had 2-4 parturitions and belonged to five different dairy breeds: Holstein-Friesian (HF; n= 56), Jersey (JE; n= 41), Montebeliarde (MB; n= 39), Overo Colorado (OC; n= 44) and Frisón Negro (FN; n= 46) plus other group called hybrid (HYB; n=38) corresponding to crosses between HF and OC. Individual blood samples from each animal were obtained by caudal puncture using a venojet system including the anticoagulant EDTA (Vacutainer, USA) and transported to laboratory on ice for their analysis. All procedure with animals were performed in accordance with protocols approved by the Institutional committee for use and care of animals in research of INIA (CICUA 04/2022).

GENOTYPING

Genomic DNA was purified from 200 μL of blood using the GeneJet Whole blood Genomic DNA Purification Mini Kit (Thermofisher Scientific, USA) following the manufacturer protocol. Integrity of DNA was assessed by agarose gel electrophoresis and its quantification by measuring DNA absorbance at 260 and 280 nm and evaluating its ratio using a plate-reader (Infinity M200 Pro, TECAN, Switzerland). Genotyping of the K232A *DGAT1* marker was performed by PCR-RFLP according to Carvajal *et al*. (2016), using the primers F-TGCCGCTTGCTCGTAGCTTTGGCC and R-ACCTGGAGCTGGGTGAGGAACAGC where a 414 amplicon was digested with *Eae*I enzyme (Thermo Scientific, USA). For *CSN3* (GenBank Y380228.1) we designed primers using the Primer-BLAST tool (NCBI; F- GCGCTGTGAGAAAGATGAAAG and R-CCCATTTCGCCTTCTCTGTAA) to amplify a 631 bp product (T° annealing 59 °C) containing the polymorphisms rs43703015 and rs43703016 which determine the A, B, C and E variants. Then, amplicons were purified by column using the E.Z.N.A Pure cycle kit (Omega Bio-tek, USA) and sent to commercial sequencing (Macrogen, Korea) to reveal the variant.

DATA ANALYSIS

The analysis contemplated the estimation of allelic and genotypic frequencies for each of the markers by means of direct count. In addition, the Hardy-Weinberg equilibrium (HWE) was performed by Chi-test with a $P<0.05$ as significant (Falconer & MacKay, 1996).

Likewise, the genetic diversity of populations was determined using the Wright-F statistics Ho, He, FIS and FST. All calculations were performed using the GenePop 4.7.5 (Rousset, 2008).

RESULTS

Table 1 shows the allelic frequencies for *CSN3* marker. It showed that the A variant was predominant in four herds (HF, OC, FN e HYB) in a range between 0.569 to 0.709. At the same time, the results revealed that the B variant was best represented in JE (0.729) and MB (0.577) herds. The E variant, was poorly represented in HF, OC, FN, and HYB (between 0.022 and 0.071), and absent in JE and MB. Additionally, the C variant was only found in a few HYB animals with a frequency of 0.17. The analysis of these distributions showed that populations are in HWE, except for OC which displayed a χ^2 value of 6.812 (Table 1; $p<0.05$).

For K232A *DGAT1*, we found that frequency of the A allele was predominant (0.645 to 0.962) for all populations except JE (Table 1). The highest frequency was observed in MB and FN, and then OC, HF and HYB. Holstein-Friesian showed a higher frequency of AK genotype which was

Table 1. Allelic frequencies (%) and Hardy-Weinberg equilibrium for *DGAT1* and *CSN3* markers.

Breed	*DGAT1*				*CSN3*				
	n	A	K	χ^2	A	B	C	E	χ^2
HF	56	0.661	0.339	0.743	0.709	0.251	0	0.040	0.781
JE	41	0.305	0.695	1.781	0.271	0.729	0	0	0.650
MB	39	0.962	0.038	0.062	0.423	0.577	0	0	1.692
OC	44	0.750	0.250	1.980	0.569	0.360	0	0.071	6.812*
FN	46	0.967	0.033	0.049	0.658	0.320	0	0.022	3.171
HYB	38	0.645	0.355	5.149*	0.612	0.170	0.169	0.049	1.803

HF: Holstein-Friesian; JE: Jersey; MB: Montbeliarde; OC: Overo Colorado; FN: Frisón Negro; HYB: Hybrid; n, number of animals; χ^2: Chi-square test; *Indicates Hardy-Weinberg equilibrium deviation ($p<0.05$).

present in half of the animals (28 of 56 animals, data not showed). On the other hand, MB and FN did not show the KK genotype. Jersey, as expected, showed the highest frequency of the K variant (0.695; Komisarek *et al*., 2011; Anton *et al*., 2012). For this marker, all populations were in HWE with exception of hybrids animals (Table 1; $\chi^2 = 5.149$).

The analysis of FIS for *CSN3* (Table 2) gave negative and non-significant (p>0.05) values in almost all populations except for OC (-0.429; MB showed a positive value). Likewise, for *DGAT1* we observed the same tendency, except for HYB, which displayed a positive value (0.418). Regarding inbreeding, the FIT values for *DGAT1* and *CSN3* were non-significant (0.241 and -0.028, respectively). In relation to genetic differentiation, the paired FST values by breed were between 0.010 and 0.650 for *DGAT1,* and between 0.001 and 0.342 for *CSN3*, with significant differences in most of the populations (Table 3). For *CSN3* it was possible to observe high (FST 0.15-0.25) or very high values (FST>0.25) for JE/HF and JE/HYB pairs, and low values with a low differentiation for JE/MB. For *DGAT1*, the FST analysis yielded very high values (JE/FR and JE/MB), and low values as observed for HF/OC and HYB/OC pairs.

The simultaneous analysis of both markers (*DGAT1* and *CSN3*) gave 16 combined genotypes (Table 4) with hybrid animals displaying a high number of combinations (11). The Jersey breed showed five combinations with exactly the favorable (*DGAT1* AA/AA and *CSN3* BB) in the highest frequency (44%). The other populations including MB, OC and FN barely exhibited the favorable combination, but only Holstein-Friesian displayed a few animals with this combination and high frequency (38%) of heterozygous genotype *DGAT1* AK – *CSN3* (AB).

Table 2. Values of observed (Ho) and expected (He) heterozygosity and FIS index for *DGAT1* and *CSN3* markers.

Breed	*DGAT1*				*CSN3*			
	Ho	He	FIS	P-value	Ho	He	FIS	P-value
HF	0.281	0.253	-0.106	0.867	0.26	0.231	-0.129	0.909
JE	0.212	0.175	-0.197	0.955	0.14	0.165	-0.142	0.294
MB	0.030	0.029	-0.027	1.000	0.23	0.192	0.196	0.940
OC	0.203	0.166	-0.201	0.971	0.30	0.211	-0.429	0.005*
FR	0.034	0.029	-0.023	1.000	0.26	0.204	-0.276	0.990
HYB	0.102	0.171	0.418	0.014*	0.16	0.147	-0.083	0.822
Total			-0.030	0.967			-0.177	0.868

HF: Holstein-Friesian; JE: Jersey; MB: Montbeliarde; OC: Overo Colorado; FN: Frisón Negro; HYB: Hybrid; Ho: Observed heterozygosity; He: Expected heterozygosity; FIS: Inbreeding coefficient; (*) P<0.05.

Table 3. FST values for *DGAT1* and *CSN3* markers between different breeds pairs.

	Breed	HF	JE	OC	FR	MB
DGAT1	JE	0.216*				
	OC	0.010	0.326*			
	FR	0.248*	0.650*	0.172*		
	MB	0.227*	0.626*	0.152*	-0.011	
	HYB	-0.012	0.201*	0.011	0.287*	0.261*
	Total FST	0.263*				
CSN3	JE	0.324*				
	OC	0.015	0.208*			
	FR	-0.004	0.275*	0.001		
	MB	0.154*	0.040*	0.063*	0.112*	
	HYB	-0.010	0.342*	0.021	-0.003	0.166*
	Total FST	0.125*				

HF: Holstein-Friesian; JE: Jersey; MB: Montbeliarde; OC: Overo Colorado; FN: Frisón Negro; HYB: Hybrid; FST: Proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance; (*) P<0.05.

Table 4. Combined genotypes (%) for *DGAT1* and *CSN3* markers.

Combined genotypes			Frequency					
Nº	*DGAT1*	*CSN3*	HF	JE	MB	OC	FN	HYB
1	KK	AA	0	0	0	0	0	4
2	KK	AB	1	0	0	0	0	0
3	KK	ABC	0	0	0	0	0	3
4	KK	AE	1	0	0	1	0	1
5	KK	BB	3	18	0	0	0	0
6	AK	AA	4	2	0	12	0	4
7	AK	AB	21	14	0	1	0	1
8	AK	ABC	0	0	0	0	0	3
9	AK	AE	3	0	0	1	1	2
10	AK	BB	0	5	3	2	1	0
11	AK	BE	0	0	0	4	1	0
12	AA	AA	23	2	5	0	18	9
13	AA	AB	0	0	23	23	24	0
14	AA	ABC	0	0	0	0	0	8
15	AA	ABCE	0	0	0	0	0	1
16	AA	BB	0	0	8	0	1	1

HF: Holstein-Friesian; JE: Jersey; MB: Montbeliarde; OC: Overo Colorado; FN: Frisón Negro; HYB: Hybrid; Favorable genotypes are highlighted with underlining.

DISCUSSION

CSN3 and *DGAT1* markers are major polymorphisms related to cheese manufacturing and milk quality, respectively. They have been evaluated in several breeds in different production systems but there is little or no information available in our country. Thus, we evaluate the frequency distribution of these markers in five dairy breeds usually milky in southern Chile (three of them with worldwide presence and two local adapted breeds, Overo Colorado and Frisón Negro), and a hybrid population. For *CSN3* the most representative allele in almost all populations was A as have been reported in several studies including Holstein-Friesian and some crosses (Volkandari *et al*., 2017; Miluchová *et al*., 2018; Gurses *et al*., 2018; Adamov *et al*., 2020) and several local breeds worlwide (Akyüz & Çinar, 2014; Djedovic *et al*., 2015; Barbosa *et al*., 2019). For FN, a local crossed breed genetically related to Holstein we observed a high presence of the A variant but the percentage of animals with the B variant (0.32) was higher than that reported by Felmer & Butendick (0.18) (1998), suggesting a higher selection to milk quality during the last years. In turn, the B allele was more frequent in Jersey as expected. It is well known that Jersey cattle is highlighted because of their potential to transform milk to cheese, and it presents a higher frequency of B variant (Zepeda-Batista *et al*., 2015). Other alleles such as E and C were less represented according to reports in different breeds (Zepeda-Batista *et al*., 2015; Gurses *et al*., 2018). The analysis of heterozygosity showed that all populations were in HWE except for OC (Table 2; p<0.05). Although OC is well-known as dairy cattle, this biotype was in its origin a double purpose cattle (milk and meat) and today it is breeding with distinct schemes and the pursue of higher levels of milk solids could be affect their genetic distribution.

It was interesting that, for *DGAT1*, Holstein-Friesian, which is characterized by a high milk production, showed a higher frequency of AK genotype, which was present in half of the animals (data not showed). It is widely recognized that selection of milk production has a negative effect on milk fat content (Uribe *et al*., 2017), which is explained by a lower occurrence of the K variant. However, it has been described that the managing of animals can induce higher frequencies of K allele (Ardicli *et al*., 2018) and there is a margin to select animals for milk production and solids content. Jersey, as expected, showed the highest frequency of the K variant (0.70; Komisarek *et al*., 2011; Anton *et al*., 2012). This breed is characterized by a high milk solids content.

In relation to heterozygosity, the analysis of FIS estimator for *CSN3* gave negative and non-significant values in all populations except for OC. This indicates an excess of heterozygotes. Likewise, for *DGAT1* we observed the same tendency, except for HIB, which displayed a positive value suggesting a deficit of heterozygotes. These results are correlated with the absence of HWE in both populations (OC and HYB). Regarding genetic differentiation, the analyses of FST by breed for *CSN3* gave positive values with significant differences in most of the populations displaying high or very high values for JE/HF and JE/HYB pairs, suggesting a high differentiation according to Wright (1984), and also low values with a low differentiation for JE/MB.

These results must be considered cautiously because, though important, only two markers associated to milk composition yield were assessed. If a greater number of markers were included, the very high values of differentiation would be reduced to high values (in a range of FST between 0.15-0.25). However, these markers respond to an intraspecific diversity correlated to the differentiation of milk traits of every breed, determining a high genetic structure for Jersey biotype compared to the other breeds (Huson *et al*., 2020). Other studies using genomics tools, had described moderate values of FST (0.142) between Holstein and Jersey biotypes (Melka & Schenkel, 2012). On other hand, Brown Swiss cattle which display favorable traits for cheese manufacturing, shows a genetic differentiation trend, and recent studies confirm this with high values of FST (0.156) between Brown Swiss and Holstein (Signer *et al*., 2017).

The simultaneous analyses of both markers (*DGAT1* and *CSN3*) gave 16 combined genotypes. Hybrid animals displayed a high number of combinations (11) probably as a result of multiple crosses, according to breed records between HF and OC, but those didn't favor the milk solids or cheese manufacturing. The Jersey breed showed five combinations but with the favorable genotype (*DGAT1* KK and *CSN3* BB) in the highest frequency, renewing their productive orientation to transform milk to cheese or dairy products.

Since 2010 the national dairy chain set itself the goal of reaching a 7.6% of milk solids yield for 2020. Although progress has been made on this way, it has not been fully achieved, in part, by the utilization of genotypes mainly oriented to milk production but no milk solids. Holstein-Friesian and Overo Colorado are the main biotypes used in milk production in Chile, reaching together more than 60% of dairy cattle, whereas Jersey is less-represented reaching only a 5% (ODEPA, 2019). Holstein-Friesian and their crosses had been used worldwide including Chile as dairy cattle for milk production since the mid-twentieth century. Nevertheless, more recently milk solids have been incorporated to breeding through selection index (O'Sullivan *et al*., 2019). Although the results of previous (Carvajal *et al*., 2016) and current work show an important percentage of the *DGAT1* genotype associated to milk solids (K allele), their distribution in the total mass of dairy cattle is unknown. On the other hand, in Jersey cattle the presence of favorable genotypes for cheese manufacturing is widely described. Crosses between Jersey and New Zealand Holstein show a significant highly milk solids yield which could generate a high economic recovery (Delgadillo *et al*., 2016). This better performance results from heterosis and the favorable input from Jersey biotype (Buitenhuis *et al*., 2014).

In this sense, the dairy sector including the government should initiate a national or regional breeding program including a selection index considering dairy genotypes oriented to milk solids. However, it is important to consider that the association of genetic variants to milk production and milk solids is polygenic. This is, multiples genomic regions or QTLs are involved in these traits (Ibeagha-Awemu *et al*., 2016). For example, a genome-wide study by Buitenhuis *et al*. (2014) reveals that *DGAT1* can explain up to 8-23% of milk fat content in Holstein and Jersey breeds. Therefore, other molecular markers could be included in a prediction scheme of genetic merit, or better, use animals for breeding with validated genomic information for dairy production systems based in pastures.

CONCLUSIONS

This study shows that between dairy breeds used in southern Chile Jersey biotype display the highest frequency of *DGAT1*-KK and *CSN3*-B variants which favor a major content of milk solids and the potential to cheese manufacturing.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

The obtention of blood samples were according to the Institutional Committee of Animal Care and Use from the Instituto de Investigaciones Agropecuarias.

AUTHOR CONTRIBUTIONS

A.M.C. planned the research; D.L. and A.M.C performed the analysis and write the manuscript; A.M.C. and R.dlB. made the discussion of the data and its implications.

FUNDING

Part of these work was financed by the Fundación para la Innovación Agraria (FIA-2018-0274).

ACKNOWLEDGEMENTS

We are grateful to dairy farmers which facilitated the access to their herds and collaborated in the sample collection. Also, we thank to Dr. Nicolás Pizarro-Aránguiz for their intermediacy with farmers.

REFERENCES

Adamov, N., Atanasov, B., Ilievska, K., Nikolovski, M., Dovenska, M., Petkov, V., & Dovenski, T. (2020). Allele and genotype frequencies of the Kappa-Casein (CSN3) locus in Macedonian Holstein-Friesian cattle. *Macedonian Veterinary Review, 43*(1), 45-54. https://doi.org/10.2478/macvetrev-2020-0013

Akyüz, B., & Çinar, M.U. (2014). Analysis of prolactin and kappa-casein genes polymorphism in four cattle breeds in Turkey. *Annals of Animal Science, 14*(4), 799-806. https://doi.org/10.2478/aoas-2014-0036

Anton, I., Kovács, K., Holló, G., Farkas, V., Szabó, F., Egerszegi, I., Ratky, J., Zsolnai, A., & Brüssow, K P. (2012). Effect of DGAT1, leptin and TG gene polymorphisms on some milk production traits in different dairy cattle breeds in Hungary. *Archiv Tierzucht, 55*(4), 307-314.

Ardicli, S., Soyudal, B., Samli, H., Dincel, D., & Balci, F. (2018). Effect of STAT1, OLR1, CSN1S1, CSN1S2, and DGAT1 genes on milk yield and composition traits of Holstein breed. *Revista Brasileira de Zootecnia, 47*. https://doi.org/10.1590/rbz4720170247

Barbosa, S.B.P., Araújo, I.I.M., Martins, M.F., Silva, E.C., Jacopini, L.A., Batista, A.M.V., & Silva, M.V.B. (2019). Genetic association of variations in the kappa-casein and β-lactoglobulin genes with milk traits in Girolando cattle. *Revista Brasileira de Saúde e Produção Animal, 20*, 1-12. https://doi.org/10.1590/S1519-9940200312019

Bonfatti, V., Di Martino, G., Cecchinato, A., Degano, L., & Carnier, P. (2010). Effects of β-κ-casein (CSN2-CSN3) haplotypes, β-lactoglobulin (BLG) genotypes, and detailed protein composition on coagulation properties of individual milk of Simmental cows. *Journal of Dairy Science, 93*(8), 3809-3817. https://doi.org/10.3168/jds.2009-2779

Botaro, B.G., Lima, Y.V.R.D., Cortinhas, C.S., Rennó, F.P., & Santos, M.V.D. (2009). Effect of the kappa-casein gene polymorphism, breed and seasonality on physicochemical characteristics, composition, and stability of bovine milk. *Revista Brasileira de Zootecnia, 38*(12), 2447-2454. https://doi.org/10.1590/S1516-35982009001200022

Buitenhuis, B., Janss, L.L., Poulsen, N.A., Larsen, L.B., Larsen, M.K., & Sørensen, P. (2014). Genome-wide association and biological pathway analysis for milk-fat composition in Danish Holstein and Danish Jersey cattle. *BMC Genomics, 15*, 1112. http://www.biomedcentral.com/1471-2164-15-1112

Caroli, A. M., Chessa, S., & Erhardt, G. J. (2009). Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science, 92*(11), 5335-5352. https://doi.org/10.3168/jds.2009-2461

Carvajal, A.M., Huircan, P., Dezamour, J.M., Subiabre, I., Kerr, B., Morales, R., & Ungerfeld, E.M. (2016). Milk fatty acid profile is modulated by *DGAT1* and *SCD1* genotypes in dairy cattle on pasture and strategic supplementation. *Genetics and Molecular Research, 15*(2). https://doi.org/10.4238/gmr.15027057

Cases, S., Smith, S.J., Zheng, YW., Myers, H.M., Lear, S.R., Sande, E., Novak, S., Collins, C., Welch, CB., Lusis, AJ., Erickson, S.K. & Farese, RV. (1998). Identification of a gene encoding an acyl CoA: diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proc. Natl. Acad. Sci. USA, 95*(22), 13018-13023.

Cipolat-Gotet, C., Cecchinato, A., Malacarne, M., Bittante, G., & Summer, A. (2018). Variations in milk protein fractions affect the efficiency of the cheese-making process. *Journal of Dairy Science, 101*(10), 8788-8804. https://doi.org/10.3168/jds.2018-14503

Delgadillo, J., González, H., & Uribe, H. (2016). Genetic-economic comparison of New Zealand Holstein and Jersey-New Zealand Holstein cross under a pastoral scheme in southern Chile. *Archivos de Medicina Veterinaria*, *48*(3), 261-267. http://dx.doi.org/10.4067/S0301-732X2016000300003

Djedovic, R., Bogdanovic, V., Perisic, P., Stanojevic, J., Popovic, J., & Brka, M. (2015). Relationship between genetic polymorphism of κ-casein and quantitative milk yield traits in cattle breeds and crossbreds in Serbia. *Genetika, 47*(3), 23-32. https://doi.org/10.2298/GENSR1501023D

Falconer, D.S., & Mackay, T.F.C. (1996). *Introduction to quantitative genetics*. Longman. Essex, England.

Felmer, R., & Butendieck, N. (1998). Frecuencia alélica del gen de la k-caseína bovina en un rebaño Frisón Negro Chileno. *Archivos de Medicina Veterinaria, 30*(2), 145-150. http://dx.doi.org/10.4067/S0301-732X1998000200015

Gebreyesus, G., Buitenhuis, A.J., Poulsen, N.A., Visker, M.H.P.W., Zhang, Q., Van Valenberg, H.J. F., Sun, D., & Bovenhuis, H. (2019). Multi-population GWAS and enrichment analyses reveal novel genomic regions and promising candidate genes underlying bovine milk fatty acid composition. *BMC Genomics, 20*, 178. https://doi.org/10.1186/s12864-019-5573-9

Gibson, R.A. (2011). Milk fat and health consequences. Nestle Nutr Workshop Ser Pediatr Program 67, 197-207. https://doi.org/10.1159/000325585

Gurses, M., Yuce, H., Etem, E.O., & Patir, B. (2018). Polymorphisms of kappa-casein gene and their effects on milk production traits in Holstein, Jersey and Brown Swiss cattle. *Animal Production Science*, *58*(5), 778-784. https://doi.org/10.1071/AN15131

Grisart, B., Farnir, F., Karim, L., Cambisano, N., Kim, J.J., Kvasz, A., Mni, M., Simon, P., Frere, JM., Coppieters, W., & Georges, M. (2004). Genetic and functional confirmation of the causality of the DGAT1 K232A quantitative trait nucleotide in affecting milk yield and composition. *Proc. Natl. Acad. Sci. USA, 101*(8), 2398-2403. https://doi.org/10.1073/pnas.0308518100

Huson, H.J., Sonstegard, T.S., Godfrey, J., Hambrook, D., Wolfe, C., Wiggans, G., Blackburn, H. & VanTassell, C.P. (2020). A genetic investigation of Island Jersey cattle, the foundation of the Jersey breed: Comparing population structure and selection to Guernsey, Holstein, and United States Jersey cattle. *Frontiers in Genetics, 11*, 366. https://doi.org/10.3389/fgene.2020.00366

Ibeagha-Awemu, E.M., Peters, S.O., Akwanji, K.A., Imumorin, I.G., & Zhao, X. (2016). High density genome wide genotyping-by-sequencing and association identifies common and low frequency SNPs, and novel candidate genes influencing cow milk traits. *Scientific Reports, 6*, 31109. https://doi.org/10.1038/srep31109

Komisarek, J., Michalak, A., & Walendowska, A. (2011). The effects of polymorphism in DGAT1, GH, GHR genes on reproduction and production traits in Jersey cows. *Animal Science Papers and Reports, 29*(1), 29-36.

Melka, M.G., & Schenkel, F.S. (2012). Analysis of genetic diversity in Brown Swiss, Jersey and Holstein populations using genome-wide single nucleotide polymorphism markers. *BMC Research Notes, 5*, 161. http://www.biomedcentral.com/1756-0500/5/161

Miluchová, M., Gábor, M., Candrák, J., Trakovická, A., & Candráková, K. (2018). Association of HindIII-polymorphism in kappa-casein gene with milk, fat and protein yield in Holstein cattle. *Acta Biochimica Polonica, 65*(3), 403-407. https://doi.org/10.18388/abp.2017_2313

ODEPA. (2019). Oficina de Estudios y Políticas Agrarias Estadísticas productivas. Encuesta de ganado bovino. Chile.

Osorio, J.S., Lohakare, J., & Bionaz, M. (2016). Biosynthesis of milk fat, protein, and lactose: roles of transcriptional and posttranscriptional regulation. *Physiological Genomics, 48*(4), 231-256. https://doi.org/10.1152/physiolgenomics.00016.2015

O'Sullivan, M., Horan, B., Pierce, K.M., McParland, S., O'Sullivan, K., & Buckley, F. (2019). Milk production of Holstein-Friesian cows of divergent Economic Breeding Index evaluated under seasonal pasture-based management. *Journal of Dairy Science, 102*(3), 2560-2577. https://doi.org/10.3168/jds.2018-15559

Rousset, F. (2008). Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources, 8*(1), 103-106. https://doi.org/10.1111/j.1471-8286.2007.01931.x

Sanchez, M.P., Ramayo-Caldas, Y., Wolf, V., Laithier, C., El Jabri, M., Michenet, A., Boussaha, M., Taussat, S., Fritz, S., Delacroix-Bouchet, A., Brochard, M., & Boichard, D. (2019). Sequence-based GWAS, network and pathway analyses reveal genes co-associated with milk cheese-making properties and milk composition in Montbéliarde cows. *Genetics Selection Evolution, 51*(1), 34. https://doi.org/10.1186/s12711-019-0473-7

Signer-Hasler, H., Burren, A., Neuditschko, M., Frischknecht, M., Garrick, D., Stricker, C., Gredler, B., Bapst, B., & Flury, C. (2017). Population structure and genomic inbreeding in nine Swiss dairy cattle populations. *Genetics Selection Evolution, 49*(1), 83. https://doi.org/10.1186/s12711-017-0358-6

Uribe, H., González, H., & Gatica, C. (2017). Genetic parameter estimation to milk yield and fat and protein yield deviated from 3% of concentration in milk, in dairy herds of southern Chile. *Austral Journal of Veterinary Sciences, 49*(2), 71-76. https://dx.doi.org/10.4067/S0719-81322017000200071

Vallas, M., Kaart, T., Värv, S., Pärna, K., Jõudu, I., Viinalass, H., & Pärna, E. (2012). Composite β-κ-casein genotypes and their effect on composition and coagulation of milk from Estonian Holstein cows. *Journal of Dairy Science, 95*(11), 6760-6769. https://doi.org/10.3168/jds.2012-5495

Volkandari, S.D., Indriawati, I., & Margawati, E.T. (2017). Genetic polymorphism of kappa-casein gene in Friesian Holstein: a basic selection of dairy cattle superiority. *Journal of Indonesian Tropical Animal Agriculture, 42*(4), 213-219. https://doi.org/10.14710/jitaa.42.4.213-219

Wright, S. (1984). *Genetics and the Evolution of Populations*. *Volume 2: The Theory of Gene Frequencies*. The University of Chicago Press, USA.

Zepeda-Batista, J.L., Alarcón-Zúñiga, B., Ruíz-Flores, A., Núñez-Domínguez, R., & Ramírez-Valverde, R. (2015). Polymorphism of three milk protein genes in Mexican Jersey cattle. *Electronic Journal of Biotechnology, 18*(1), 1-4. https://doi.org/10.1016/j.ejbt.2014.10.002

Austral J Vet Sci 55, 131-136 (2023)

ORIGINAL ARTICLE

Frequency and risk factors of intestinal parasites in pet dogs from Mexicali, Mexico

Dhamaris Sharai Gonzalez-Saldívar[a], Enrique Trasviña-Muñoz[a*], Gilberto López-Valencia[a], Paulina Haro[a], Francisco Javier Monge-Navarro[a], José Carlomán Herrera-Ramírez[a], Nohemí Castro-del-Campo[b], Soila Gaxiola-Camacho[b]

ABSTRACT. Parasitic intestinal infections in dogs represent a problem for human health, because a wide variety of these parasites have zoonotic potential. Therefore, proximity to pets puts us at risk. The objective of this study was to determine the frequency and risk factors (age, sex, size, breed, presence of ectoparasites and gastrointestinal disorders) of intestinal parasites in the feces of dogs attending a Veterinary Hospital in the City of Mexicali, Baja California, Mexico. A total of 148 fecal samples were collected from canine patients and analyzed for parasite identification and parasite load. A 12.2% (18/148) of the samples were positive to parasitic intestinal infections. The frequency of specific infections was an 8.1% of *Cryptosporidium* sp., followed by a 2.7% of *Cystoisospora* sp., and 1.4% of *Toxascaris leonina.* A statistical significance was identified between the presence of intestinal parasites and mongrel breed. The predominance of protozoa shows the importance of diagnosis prior to treatment with anthelminthic drugs, since preventive antiparasitic protocols are commonly used, although these particular parasites are out of the spectrum of those drugs. *Cryptosporidium* spp. have zoonotic potential, particularly in immunocompromised patients, and there are few or no treatment options.

Keywords: zoonosis, *Cryptosporidium*, intestinal parasites, dogs.

INTRODUCTION

Animal ownership, particularly in dogs, has brought many physical and psychological health benefits to humans (Wells *et al*., 2022), such as increased physical activity, and social and emotional support (Martín, 2020). However, these companion animals can also be carriers of zoonotic diseases (Medina-Pinto *et al*., 2018). Among the wide variety of diseases, intestinal parasitic infections stand out, in which dogs act as reservoirs by dispersing them through feces containing eggs, cysts, and oocysts (Morales *et al*., 2016).

Previous studies in northwestern Mexico have reported the presence of dog parasite forms in public spaces (Ramírez-Rubio *et al*., 2019) and in stray dogs from urban and agricultural areas (Trasviña-Muñoz *et al.,* 2017, 2020), highlighting a large proportion of dogs infected with zoonotic parasites such as *Toxocara canis, Toxascaris leonina, Ancylostoma caninum, Dipylidium caninum,* and *Taenia* sp. Nevertheless, the greatest risk of exposure to humans is represented by domestic dogs owing to regular contact between owners and pets (El-Tras *et al*., 2011). In addition, due to COVID-19, a reduced number of veterinary visits has been reported, which could lead to an increase in the number of parasites in owned dogs (Yee *et al*., 2021). Moreover, it is important to identify the diversity and abundance of parasites carried by pet dogs to generate information that will enable clinicians and health personnel to establish appropriate preventive and control measures (Aguillon-Gutierrez *et al*., 2021). Therefore, the objective of the present study was to determine the frequency and risk factors for intestinal parasites in stool samples of dogs from a Veterinary Hospital in Mexicali, Baja California, Mexico.

MATERIALS AND METHODS

STUDY DESIGN

The study was conducted on dogs received at the Small Species Veterinary Hospital (IICV-UABC) between August 2021 and December 2021 in the district of Mexicali, Baja California (32° 39’ 48’’ north latitude). For this study, samples were collected from 148 owned dogs with the following inclusion criteria: older than 1-month, different sexes, breed, and body size. Sampling was performed when all owners stated their consent to participate. As a requirement to participate, the owners had to provide a fresh stool sample from the dog in a closed container (minimum of 3 g). The personal data, such as the address and phone number provided by the owner, were handled in accordance with Federal Law for the Protection of Personal Data in Possession of Individuals (DOF, 2010). The stool samples were refrigerated at 4ºC, placed in a cooler, and transported to the Parasitology Laboratory at the Institute of Research in Veterinary Science (UABC) in Mexicali, Baja California, Mexico for processing. Originally, the study design had considered a total of 280 dogs, a sample size calculated (Thrusfield, 2005) based on the prevalence reported in previous studies in stray

Received: 30.09.2022.

Accepted: 29.03.2023.

[a]Institute of Research in Veterinary Sciences, Autonomous University of Baja California.

[b]School of Veterinary Medicine and Animal Husbandry, Autonomous University of Sinaloa.

*Corresponding author: etrasvina@uabc.edu.mx

dogs (Trasviña-Muñoz *et al*., 2017), an error of 5%, a 95% confidence, and an expected proportion of 21.5%. However, due to COVID-19 restrictions, there was a low demand for hospital services; consequently, our sample size was reduced to 148.

EPIDEMIOLOGICAL QUESTIONNAIRE

A questionnaire was administered to all owners who participated in the study by hospital veterinarians and interns during sample reception. Age (>12 months or <12 months) and sex (male or female) were recorded, and breed was determined based on the American Kennel Club classification. If the dog did not present characteristics that determined a single breed, it was classified as "mongrel". Size was recorded as small, medium, and large, determined by the weight of the animal (<5 kg, 5-15 kg, and >15 kg, respectively). Individual data were omitted by the owner, and other risk factors were also recorded, such as the presence of ectoparasites and signs associated with gastrointestinal disorders (vomiting, diarrhea, and constipation).

COPROLOGIC ANALYSIS

Parasitic forms of intestinal parasites present in the feces were identified using the Hendrix & Robinson method (2012). The samples were examined macroscopically for helminths or proglottids. Subsequently, the flotation concentration technique described by Zajac & Conboy (2012) was performed on 2 g of stool with a slight modification because a saturated saline (NaCl) solution (specific gravity 1.20) was substituted for zinc sulfate. The quantitative analysis was carried out with Mc Master's technique described by Serrano *et al*. (2010) by mixing 2 g of stool with saturated NaCl solution to a total volume of 60 ml, filtered, and then filled both compartments of the McMaster´s counting chamber to obtain the estimation of the parasite load in 1 g of stool.

To detect *Cryptosporidium* sp. oocysts, the modified Ziehl-Neelsen staining method described by Serrano *et al*. (2010) was applied.

STATISTICAL ANALYSIS

Descriptive statistics were used to calculate the overall and specific frequencies of intestinal parasitic infections. Inferential analyses were performed using the Statistix 9® software.The Chi-square (χ^2) test was used to establish associations between parasitic infections and risk factors. Risk factors with a p-value < 0.10 was further analyzed using a logistic-binomial regression model, which provides exact regression estimates, 95% confidence intervals, odds ratios, and p-values.

RESULTS

None of the 148 samples analyzed showed evidenced presence of adult parasites, and 18 (18/148) were positive for a single parasite. No co-infections were recorded. The genera and species found, in order of frequency, were as follows: *Cryptosporidium* sp*., Cystoisospora* sp., and *Toxascaris leonina* (Table 1). There was a notable predominance of protozoa, 88.8% (16/18) in the total positive cases compared to nematodes 11.1% (2/18).

Dogs younger than 12 months presented the highest frequency of positive cases of intestinal parasitic infections (14.2%, 3/21) compared to dogs older than 12 months (12.8%, 15/117). Males presented a higher frequency of cases of infection (14.4%, 11/76) than females. The most infected breed in this study was the Mongrel (21.5%, 11/51), compared to 7.9% (7/88) of purebred animals. The most frequently infected purebreds were Husky (25%, 1/4), American Staffordshire Terrier (9.5%, 2/21), and Poodle (15.3%, 2/13). Small dogs were more frequently infected (13.8%, 5/36), followed by medium (13.7%, 8/58), and large (10.6%, 5/47) size dogs. Dogs did not present ectoparasites associated with the biological cycle of gastrointestinal parasites and only ticks were found. It should be noted that most animals with intestinal parasitic infections did not show gastrointestinal disorders (88.8%, 16/18) (Table 2). The only statistically significant risk factor was breed (p = 0.026) (Table 3); and an association was identified between the overall frequency of cases and mongrel dogs (OR= 3.1821; 95% CI = 1.15-8.83). Regarding

Table 1. Frequency, genera, and parasite loads calculated using the McMaster technique for parasites detected in the feces of dogs at the Small Species Veterinary Hospital of the UABC in Mexicali, Baja California, Mexico. *Missing value: Because of the small size of Cryptosporidium sp. oocysts, the parasitic load was not determined. Low: 50-100 EPG, OPG; Middle: 101-500 EPG, OPG; high: 500 EPG, OPG (Rodriguez-Vivas *et al.*, 2011).

Genera/species	Frequency (%)	Positives/total samples	Parasitic load per gram of feces
Cystoisospora sp.	2.7	4/148	(50 -150)
Cryptosporidium sp.*	8.1	12/148	-
Toxascaris leonina	1.3	2/148	(100 -200)

Table 2. Characteristics of the population of owned dogs sampled at the UABC Small Species Veterinary Hospital in Mexicali, Baja California, Mexico.

Risk factors			Number of individuals	Positive	Negative
Age		<12 months	21	3	18
		>12 months	117	15	112
Sex		Female	65	7	58
		Male	76	11	65
Breed	Breeds withs at least 1 positive individual	Schnauzer	12	1	11
		American Staffordshire Terrier	21	2	19
		Poodle	13	2	11
		Chihuahua	15	1	14
		Husky	4	1	3
	Other breeds		22	0	22
	Mongrel		51	11	40
Size		Small	36	5	31
		Medium	58	8	50
		Big	47	5	42
Ectoparasites		Present	7	2	5
		Absent	133	16	117
Gastrointestinal disorders		Present	13	2	11
		Absent	126	16	110

Table 3. Association between the risk factors and intestinal parasites in feces of dogs owned by the Small Species Veterinary Hospital of the UABC in Mexicali, Baja California, Mexico. *p-value <0.05.

Risk factors		Positive/total samples	OR	95% IC	p-value
Age	<12 months	14.2% (3/21)	1.13	0.30 – 4.31	0.85
	>12 months	12.8% (15/117)			
Sex	Female	10.7% (7/65)	0.71	0.26 – 1.95	0.51
	Male	14.4% (11/76)			
Breed	Pure	7.9% (7/88)	3.18	1.15 – 8.83	0.02*
	Mongrel	21.5% (11/51)			
Size	Small	13.8% (5/36)	1.17	0.61 – 2.22	0.64
	Medium	13.7% (8/58)			
	Big	10.6% (5/47)			
Ectoparasites	Present	28.5% (2/7)	2.92	0.52 – 16.32	0.22
	Absent	12% (16/133)			
Gastrointestinal disorders	Present	15.3% (2/13)	1.26	0.26 – 6.22	0.77
	Absent	12.6% (16/126)			

the association between cases of parasitic infections for each detected parasite, no association was found between cases of *Cryptosporidium* sp., *Cystoisospora* sp., and *T. leonina* and the risk factors.

DISCUSSION

Companion animals, owing to their proximity to humans, are a potential source of more than 70 zoonotic diseases (Stull *et al*., 2015). Intestinal parasites are a clear example of this, as they are considered a global health problem (Zanzani *et al*., 2014). These infections sometimes present with a long prepatent period, and their clinical manifestations may not be evident (Torrecillas *et al*., 2021). The absence of clinical signs in the patients whose samples were analyzed in this study and had a positive result suggests that although the disease is not perceptible, subclinical parasitized dogs play an important role in allowing the parasites to develop their life cycle (Maggi & Krämer, 2019). This finding has been demonstrated in cryptosporidiosis; despite not presenting any signs, dogs can continue to excrete oocysts, which can contaminate the environment and facilitate subsequent transmission (OIE, 2018; Murnik *et al*., 2022).

The higher frequency of positive cases in the mongrel breed agrees with that reported by Plúas & Sánchez (2021) in Ecuador. This may be due to the lower level of veterinary care that mongrel dogs receive, which is related to their low economic value compared to that of purebred dogs (Ojo *et al*., 2019). The data from the present study disagree with those reported by Tortolero *et al.* (2008) in Venezuela and Vega *et al*. (2014) in Peru, where there was a statistically significant relationship between intestinal parasites and purebred dogs. The authors attributed this to genetic manipulation of these animals, which can increase their susceptibility to infection.

Although there is no zoonotic risk, *Cystoisospora* sp. are important for pet health. In young animals, strong signs of acute diarrhea, either catarrhal or hemorrhagic, are observed (Bowman, 2014; Villeneuve *et al*., 2015). In the present study, the animals infected with *Cystoisospora* sp. were older than 12 months, and the owners did not report the presence of previous gastrointestinal signs. However, no statistically significant relationship was observed between the presentation of this parasite and age. These results differ from those reported by Smith *et al*. (2014) in Canada, who reported that adult animals were considerably less infected than young animals.

There are over 30 different species of *Cryptosporidium* sp. recognized; some are host-specific, and others infect a wider host range (Robertson *et al*., 2020). The most prevalent species reported in humans include *C. hominis, C. parvum,* and *C. canis* (Ryan *et al*., 2021). In the present study, a frequency of 8.10% was observed for *Cryptosporidium* spp. Thus, this study is the first to report cases of infection in dogs in Mexicali, Baja California, Mexico. Regarding cryptosporidiosis in dogs, there are discrepancies regarding its importance as a zoonosis, as some studies indicate that the risk is minimal (Lucio-Forster *et al.*, 2010). However, Mexicali borders the state of California in the United States, where historically, it has been indicated as the main risk of infection through contact with dogs at home (Acute Communicable Disease Control, 2014). In Mexico, there have been few studies on this protozoan in domestic dogs. In Mexico City, Martinez-Barbosa *et al*. (2015) reported a 11.5% prevalence of *Cryptosporidium* spp. in asymptomatic domestic dogs, which coincides with our findings, and the age of the dogs (younger than 12 months) was determined as a risk factor, which differs from our study. In Aguascalientes, Vitela-Mendoza *et al*. (2019) determined the frequency of *Cryptosporidium* sp. in dogs from dairy stables and an urban area captured by the Center for Control, Attention, and Animal Welfare (CCABA) and found that 20.5% of the canines were infected. The highest number of cases were observed in animals from dairy farms (30%), whereas the urban area showed a low level (9%). In Durango, Aguillón-Gutiérrez *et al*. (2021) reported a prevalence of 6% in pet dogs, which is the most frequent parasite in these canines. In Toluca, Lara-Reyes *et al*. (2019) reported that 4.7% of dogs submitted to different clinics in the area to be evaluated were positive for this parasite.

In humans, cryptosporidiosis can present with minimal to severe symptoms, such as watery and bulky diarrhea, abdominal pain, nausea, vomiting, anorexia, fatigue, and fever. These can last up to one month, with a risk of reinfection; in severe cases, it can compromise the human patient's life (Chalmers & Davies, 2010). Unfortunately, no drugs or vaccines are available to prevent this infection, and the treatment options for both animals and humans are extremely limited and may not be fully effective in immunocompromised patients (Innes *et al.*, 2020). According to the CDC (2021), no single cleaning measure is effective in preventing cryptosporidiosis and has even shown resistance to chlorine in water, but it also strongly suggests washing hands often with soap and water.

Toxascaris leonina has zoonotic potential, can occasionally infect humans as accidental hosts, and is commonly present in asymptomatic dogs (Rostami *et al.*, 2020). Canines usually cause mild gastrointestinal disturbances, and in most cases in humans, they do not cause any signs or symptoms (Traversa, 2012). The frequency of *T. leonina* (1.35%) in our study differed from the results reported globally (2.9%) (Rostami *et al*., 2020) as well as from other states of Mexico, such as Querétaro (11.91%), Mexico City (4.16%), Oaxaca (7.22%) (Fernández & Canto, 2002; Eguía-Aguilar *et al*., 2005; Vélez-Hernández *et al*., 2014), and Mexicali, Baja California (5.5%) (Trasviña-Muñoz *et al*., 2017). The low frequency found in this study could be explained by the fact that the dogs in this study were domestic, whereas the previously mentioned studies investigated stray dogs, which are generally exposed to various risk factors that facilitate their transmission, such as the presence of rodents, which are paratenic hosts for this parasite, and the absence of health care for dogs (Trasviña-Muñoz *et al*., 2017). In addition, climate factors, as this nematode has a lower development in extreme climates (Rostami *et al*., 2020), such as those we have in this region during summer and winter.

It is important to note that there was a higher proportion of protozoa in this study, since in some countries "preventive" antiparasitic protocols are used, applying anthelmintic drugs, such as praziquantel and pyrantel (Stull *et al*., 2007; Matos *et al*., 2015) with no effects on the protozoa. The spectrum of these drugs could explain the absence of other helminths, such as *Toxocara canis* and *Ancylostoma caninum,* or cestodes such as *Dipylidium caninum,* which had been previously recorded in this region (Trasviña-Muñoz *et al*., 2017; Ramírez-Rubio *et al*., 2019). This highlights the importance of diagnosis prior to the initiation of any treatment as well as post-treatment diagnosis to evaluate its efficacy based on differences in parasite loads (D´Ambroso *et al*., 2022). If owners lack information regarding the possibility of transmission and how improper disposal of feces can affect the environment, there is an increased risk of infection; therefore, the role played by these animals as reservoirs must be considered (Raičević *et al*., 2021). In addition, diagnostic work together with the development of scientific communication strategies focused on the owners of these animals represents an important step in the prevention of intestinal parasites. (Cortes, 2020). It is also necessary to reinforce the role of veterinary clinicians as educators about animal welfare and human-animal bonds. (Dolby & Litster, 2015).

CONCLUSIONS

The predominance of protozoa in owned dogs compared to helminths is of utmost importance because preventive administration of antiparasitic drugs without previous diagnosis is a common practice in some countries, and the spectrum of administered drugs does not include protozoa such as *Cystoisospora* sp. or *Cryptosporidium* sp. Consequently, diagnosis is fundamental before and after antiparasitic treatment. Regarding risk factors, mongrel dogs presented a significantly higher frequency of infection, which could be attributed to the lower veterinary care related to their low economic value, compared to purebred dogs (Ojo *et al*., 2019).

The presence of *Cryptosporidium* sp. is of great interest, mainly because of its zoonotic potential and lack of treatment options. Further studies are needed in both owned and stray dogs with a larger sample size to identify the factors that contribute to the distribution of these parasites throughout the northwestern regions of Mexico.

STATEMENTS

Conflicts of interest: the authors declare that they have no conflicts of interest.

DECLARATION OF ETHICS

All the samples were collected using standard collection method, without harming the dogs. The research was conducted with the approval of the Ethics Committee of the Veterinary Sciences Research Institute from the Autonomous University of Baja California. The personal data provided by the owners of the pets sampled in this study were handled in accordance with the Federal Law for the Protection of Personal Data in Possession of Private Parties (DOF 05-07-2010).

FUNDING

Secretaria de Educación Pública (SEP) - Programa para el Desarrollo Profesional Docente (PRODEP).

ACKNOWLEDGMENTS

This work is part of the Master´s degree in Veterinary Sciences of the first author (Autonomous University of Baja California). We thank the Small Species Veterinary Hospital of the Veterinary Science Research Institute (UABC) for support with the collection and identification of samples, even in times of a global pandemic.

REFERENCES

Acute Communicable Disease Control. (2014). *Anual Morbidity Report. Cryptosporidiosis*. http://publichealth.lacounty.gov/acd/Diseases/Crypto1.pdf

Aguillón-Gutiérrez, D., Meraz-Rodríguez, Y., García-de-la-Peña, C., Ávila-Rodríguez, V., Rodríguez-Vivas, R., & Moreno-Chávez, M. (2021). Prevalencia de parásitos en heces fecales de perros de Gómez Palacio, Durango, México. *Abanico Veterinario*, *11*. http://dx.doi.org/10.21929/abavet2021.39

Bowman, D. (2014). *Georgi's Parasitology for Veterinarians*. 10th edition. Elsevier.

Chalmers, R., & Davies, A. (2010). Minireview: Clinical Cryptosporidiosis. *Experimental Parasitology*, *124*(1), 138-146. https://doi.org/10.1016/j.exppara.2009.02.003

CDC (Centers for Disease Control and Prevention). (2021). Parasites. https://www.cdc.gov/parasites/crypto/prevention-control.html

Cortes N. (2020). Coronavirus disease 2019 (COVID-19): The Importance of scientific communication and updated teaching about zoonoses. *Revista Peruana de Investigación en Salud*, *4*(2), 87-88. https://doi.org/10.35839/repis.4.2.697

D'Ambroso, F., Rojas, R., Ries, A., Cargnelutti., Sangioni, L., & Vogel, F. (2022). Gastrointestinal helminths in dogs: occurrence, risk factors, and multiple antiparasitic drug resistance. *Parasitology Research*, *121*, 2579-2586. https://doi.org/10.1007/s00436-022-07599-0

DOF (2010) DOF 05-07-2010. Ley Federal de Protección de Datos Personales en Posesión de los Particulares. Diario Oficial de la Federación, Mexico. 05 de julio de 2010.

Dolby, N., & Litster, A. (2015). Understanding veterinarians as educators: an exploratory study. *Teaching in higher education*, *20*(3), 272-284. http://dx.doi.org/10.1080/13562517.2014.1001835

Eguía-Aguilar P., Cruz-Reyes, A., & Martínez-Maya, J. (2005). Ecological Analysis and description of the intestinal helminths present in dogs in Mexico City. *Veterinary Parasitology*, 127(2), 139-146. https://doi.org/10.1016/j.vetpar.2004.10.004

El-Tras W., Holt, R., & Tayel, A. (2011). Risk of *Toxocara canis* eggs in stray and domestic dog hair in Egypt. *Veterinary parasitology*, *178*(3-4) https://doi.org/10.1016/j.vetpar.2010.12.051

Fernández, F., & Canto, G. (2002). Frecuencia de helmintos en intestino de perros sin dueño sacrificados en la ciudad de Querétaro, Querétaro, México. *Veterinaria México*, *33*(3). http://dx.doi.org/10.21753/vmoa.33.003.70

Hendrix C., & Robinson E. (2012). *Diagnostic Parasitology for Veterinary Technicians*. (4th ed.). Elsevier.

Innes, E., Rachel, C., Beth, W., & Mattie, P. (2020). One health aproach to tackle cryptosporidiosis. *Trends in parasitology*, *20*(20). https://doi.org/10.1016/j.pt.2019.12.016

Lara-Reyes E., Figueroa-Ochoa, J., Quijano-Hernández, I., Del-Ángel-Caraza, J., Barbosa-Mireles, M., Victoria-Mora, J., & Beltrán-León, T. (2019). Frecuencia de parásitos intestinales de perros en parques públicos de dos municipios vecinos del Estado de México. *Nova, 17*(32), 75-81. https://doi.org/10.25058/24629448.3634

Lucio-Forster A., Griffiths, J., Cama, V., Xiao, L., & Bowman, D. (2010). Minimal zoonotic risk of cryptosporidiosis from pet dogs and cats. *Trends in parasitology*, *26*(4), 174-179. https://doi.org/10.1016/j.pt.2010.01.004

Maggi, R. G., & Krämer, F. (2019). A review on the occurrence of companion vector-borne diseases in pet animals in latin america. *Parasites & Vectors*, *12*(145). https://doi.org/10.1186/s13071-019-3407-x

Martín A. (2020). *Programa de educación para la salud: Zoonosis en mascotas, ¿un riesgo para la salud?* [Trabajo de fin de grado]. Universidad de Zaragoza. Zaragoza, España. https://zaguan.unizar.es/record/90066

Martínez-Barbosa, I., Gutiérrez, M., Ruiz, L., Fernández, A., Gutiérrez, E., Aguilar, J., Shea, M., & Gaona, E. (2015). Detección de *Cryptosporidium* sp y otros parásitos zoonóticos entéricos en perros domiciliados en la Ciudad de México. *Archivos de Medicina Veterinaria*, *47*(3). http://dx.doi.org/10.4067/S0301-732X2015000300012

Matos, M., Alho, A., Owen, S., Nunes, T., & De Carvalho, L. (2015). Parasite control practices and public perception of parasitic diseases: a survey of dog and cat owners. *Preventive Veterinary Medicine*, *122*(1-2). https://doi.org/10.1016/j.prevetmed.2015.09.006

Medina-Pinto, R., Rodríguez-Vivas R., & Bolio-González, M. (2018). Nematodos intestinales en perros en parques públicos de Yucatán, México. *Biomédica, 38*(1). https://doi.org/10.7705/biomedica.v38i0.3595

Morales, M., Soto, S., Villada, Z., Buitrago, J., & Uribe, N. (2016). Helmintos gastrointestinales de perros en parques públicos y su peligro para la salud pública. *CES Salud Pública*, 7(2). http://dx.doi.org/10.21615/cessp.7.2.6

Murnik, L., Daugschies, A. & Delling, C. (2022). *Cryptosporidium* infection in young dogs from Germany. *Parasitology Research*, *121*, 2958-2993. https://doi.org/10.1007/s00436-022-07632-2

OIE (Organización Mundial de Sanidad Animal), 2018. *Manual Terrestre de la OIE*. https://www.oie.int/fileadmin/Home/esp/Health_standards/tahm/3.09.04_CRYPTO.pdf

Ojo, G. A., Adekeye, T. A., & Awobode, H. O. (2019). Prevalence of single and mixed parasitic infections of dogs in Egbeda communities, Ibadan, Oyo State, Nigeria. *Sokoto Journal of Veterinary Sciences*, *17*(4). http://dx.doi.org/10.4314/sokjvs.v17i4.4

Raičević J., Ivan, P. & Tamara, G. (2021). Canine intestinal parasites as a potential source of soil contamination in the public areas of Kruševac, Serbia. *Journal of Infection in Developing Countries*, *15*(1). https://doi.org/10.3855/jidc.12694

Ramírez-Rubio, L., García-Cueto, O., Tinoco-Gracia, L., Quintero-Núñez, M., Cueto-González, S. A., & Trasviña-Muñoz, E. (2019). Frecuencia de huevos de *Toxocara canis* en parques públicos de Mexicali, Baja California, México. *Revista internacional de contaminación ambiental*, *35*(3). https://doi.org/10.20937/RICA.2019.35.03.06

Robertson, L., Johansen, H., Kifleyohannes, T., Efunshile, A., & Terefe, G. (2020). *Cryptosporidium* infections in Africa- How important is zoonotic transmission? A review of the evidence. *Frontiers in veterinary science*, *7*. https://doi.org/10.3389/fvets.2020.575881

Rostami, A., Seyed, M., Vahid, F., Tao, W., Andreas, H., Aliyar, M., Masoud, F., Yadolah, F., Calum, M., & Robin, G. (2020). Global prevalence estimates of *Toxascaris leonina* infection in dogs and cats. *Pathogens*, *9*(6), 503. https://doi.org/10.3390/pathogens9060503

Ryan, U., Zahedi, A., Feng, Y., & Xiao, L. (2021). An update on Zoonotic *Cryptosporidium* species and genotypes in humans. *Animals*, *11*(11), 3307. https://doi.org/10.3390/ani11113307

Serrano A., Frontera, E., Gómez, L., Martínez-Estellez, M., Pérez, J., Reina, D., Calero, R., Carcelén, J., Fernández, J., Gamito, J., Iniesta, V., Pariente, F., Suarez, I., Gómez, M., Monroy, I., Baz, V., & Pajares, P. (2010). *Manual Práctico de Parasitología Veterinaria* (1ra ed.). Universidad de Extremadura.

Smith, A., Semeniuk, C., Kutz, S., & Massolo, A. (2014). Dog-Walking behaviors affect gastrointestinal parasitism in park-attending dogs. *Parasites & Vectors*, *7*. https://dx.doi.org/10.1186%2F1756-3305-7-429

Stull, J., Carr, A., Chomel, B., Berghaus, R., & Hird, D. (2007). Small animal deworming protocols, client education, and veterinarian perception of zoonotic parasites in western Canada. *Canadian Veterinary Journal*, *48*(3), 269-276. https://doi.org/10.4141/cjas68-037

Stull, J. W., Brophy, J., & Weese, J. S. (2015). Reducing the risk of pet-associated zoonotic infections. *Canadian Medical Association Journal*, *187*(10), 736-743. https://doi.org/10.1503/cmaj.141020

Thrusfield, M. (2005). *Veterinary Epidemiology*. (3rd ed.). Blackwell Science Ltd.

Torrecillas, C., Fajardo, M., Córdoba, M., Sánchez, M., Mellado, I., Aleixandre-Gorriz, I., & Sánchez-Thevenet, P. (2021). Parásitos zoonóticos caninos de dos barrios costeros de Comodoro Rivadavia, Chubut, Argentina. *Revista Argentina de Salud Pública*, *13*(46). http://www.scielo.org.ar/scielo.php?script=sci_arttext&pid=S1853-810X2021000100181

Tortolero Low, L. J., Cazorla Perfetti, D. J., Morales Moreno, P., & Acosta Quintero, M. E. (2008). Prevalencia de enteroparásitos en perros domiciliarios de la ciudad de La Vela, Estado Falcón, Venezuela. *Revista Científica*, *18*(3). http://www.redalyc.org/articulo.oa?id=95918312

Trasviña-Muñoz, E., López-Valencia, G., Álvarez, P., Cueto-González, S., Monge-Navarro, F., Tinoco-Gracia, L., Núñez-Castro, K., Pérez-Ortiz, P., Medina-Basulto, G., Tamayo-Sosa, A., & Gómez-Gómez, S. (2017). Prevalence and distribution of intestinal parasites in stray dogs in the northwest area of México. *Austral Journal of Veterinary Sciences*, *49*(2). http://dx.doi.org/10.4067/S0719-81322017000200105

Trasviña-Muñoz, E., López-Valencia, G., Monge-Navarro, F., Herrera-Ramírez, C., Haro, P., Gómez-Gómez, S., Mercado-Rodríguez, J., Flores-Dueñas, C., Cueto-González, S., & Burquez-Escobedo, M. (2020). Detection of intestinal parasites in stray dogs from a farming and cattle region of Northwestern México. *Pathogens*, *9*(7), 516. https://dx.doi.org/10.3390%2Fpathogens9070516

Traversa, D. (2012). Pet roudworms and hookworms: A continuing need for global warming. *Parasites & Vectors*, *5*(91). https://dx.doi.org/10.1186%2F1756-3305-5-91

Vega, S., Serrano-Martínez, E., Grandez, R., Pilco, M., & Quispe, M. (2014). Parásitos gastrointestinales en cachorros caninos provenientes de la venta comercial en el Cercado de Lima. *Salud Tecnológica Veterinaria*, *2*(2). https://doi.org/10.20453/stv.v2i2.2242

Vélez-Hernández, L., Reyes, K., Rojas, D., Calderón, M., Cruz, J., & Arcos, J. (2014). Riesgo potencial de parásitos zoonóticos presentes en heces caninas en Puerto Escondido, Oaxaca. *Salud Pública de México*, *56*(6). http://dx.doi.org/10.21149/spm.v56i6.7389

Villeneuve A., Polley, L., Jenkins, E., Schurer, J., Gilleard, J., Kutz, S., Conboy G., Benoit, C., Seewald W. & Gagné, F. (2015). Parasite prevalence in fecal samples from shelter dogs and cats across the Canadian provinces. *Parasites & Vectors*, *8*(281). https://doi.org/10.1186/s13071-015-0870-x

Vitela-Mendoza, I., Padilla, K., Cruz-Vázquez, C., Medina-Esparza, L., & Ramos-Parra, M. (2019). Frecuencia de Cryptosporidium en perros asociados a establos lecheros y en áreas urbanas del estado de Aguascalientes, México. *Revista Mexicana de Ciencias Pecuarias*, *10*(1). https://doi.org/10.22319/rmcp.v10i1.4758

Wells, D., Clements, M., Elliot, L., Meehan, E., Montgomery, C. & Williams, G. (2022). Quality of the human-animal bond and mental wellbeing during a COVID-19 lockdown. *Anthrozoös*, *35*(6). https://doi.org/10.1080/08927936.2022.2051935

Yee, H., Arruda, A. G., Rudinsky, A. J., Iazbik, C., Millward, L., & Marsh, A. (2021). Risk factors and impact of COVID-19-related clinic closures on the detection of gastrointestinal parasites in dogs, a cross-sectional study. *Veterinary Parasitology: Regional Studies and Reports*, *26*. https://doi.org/10.1016/j.vprsr.2021.100647

Zajac, A., & Conboy, G. (2012). *Veterinary Clinical Parasitology*. Willey-Blackwell.

Zanzani, S., Gazzonis, A., Scarpa, P., Berrilli, F., & Manfredi, M. (2014). Intestinal parasites of owned dogs and cats from metropolitan and micropolitan areas: prevalence, zoonotic risks, and pet owner awareness in Northern Italy. *Biomed Research International*, *2014* https://doi.org/10.1155/2014/696508

Austral J Vet Sci 55, 137-141 (2023)

SHORT COMMUNICATION

Locomotor injuries morbidity data analysis in Chilean sport horses: a retrospective study (2016-2021)

Cristóbal A. Dörner[a,b*]

ABSTRACT. The equine industry in Chile is small but constantly growing, being the Chilean Rodeo the most important discipline. Problems associated to the musculoskeletal system are the most frequent condition in the equine veterinary practice. Economic losses for the equine industry associated to injuries related to the locomotor system are significant and have been estimated in different parts of the world. Five hundred and eighty-one (581) Chilean purebred horses (Caballo Raza Chilena) performing or training for Chilean Rodeo discipline fulfilled the inclusion criteria. Results showed that in Chilean purebred horses, forelimbs injuries were recorded in 73.1% and hindlimbs 26.9% being a statistically significant difference ($P = 0.001$). Unilateral lameness was the most frequent situation seen in 74.9% (n=424), bilateral lameness was observed in 21.7% (n=102) and lameness in 3 or more areas was recorded in 9.5% of horses (n=55). When comparing frequency of distribution between right and left sides, no differences were observed between forelimbs ($P = 0.645$) or hindlimbs ($P = 0.853$). The forelimb digit (hoof, pastern, and fetlock) had a prevalence of 59.6%. When specific diagnoses were recorded the most prevalent causes of lameness in Chilean purebreds were navicular disease (12.9%), fetlock osteoarthritis (11.9%) and distal tarsal joints osteoarthritis (11.7%). In conclusion, due to the activity and specific physical demands suffered by Chilean Rodeo horses, veterinarians must be familiar with injuries affecting Chilean sport horses, as the morbidity of musculoskeletal injuries varies drastically between different disciplines.

Key words: musculoskeletal, horses, Chilean purebred, forelimb, digit.

INTRODUCTION

The horse industry in Chile is mostly supported by the Chilean Rodeo, a discipline solely practiced by Chilean purebred horses (Caballo Raza Chilena), the most popular breed of horses in Chile. Problems associated to the musculoskeletal system are well known to be the most frequent condition in the veterinary practice (Egenvall *et al*., 2005; Penell *et al*., 2005) and the Chilean purebred is not the exception. Most epidemiological studies have focused in the Thoroughbred racing industry (Barroeta *et al*., 2019; Ramzan & Palmer, 2011; Morales *et al*., 2009, 2018) although a few recent studies have analysed different populations of horses (Dabareiner *et al*., 2005a, 2005b; Dyson, 2000; Egenvall *et al*., 2009; Fortini, 2011; Jackman, 2001; Lewis, 2001).

Economic losses for the equine industry associated to injuries related to the locomotor system are significant and have been estimated in different parts of the world (Seitzinger *et al*., 2000, Egenvall *et al*., 2006; Egenvall *et al*., 2009). However, in Chile, there is still scarce information on this subject. Although the equine industry in Chile is small, it is constantly growing and the most important discipline in the country is the Chilean Rodeo. Only one study focused on the epidemiology of the main injuries of the locomotor system in Chilean Rodeo horses has been published (Mora-Carreño *et al*., 2014).

Understanding the biomechanics and demands of each discipline is extremely important to understand the pathophysiology of the specific injuries, as a basis for prevention of lesions in the equine athlete. Moreover, proper knowledge of biomechanics of a specific discipline directly aids the clinician in achieving a proper diagnosis and establishing a correct treatment plan when lameness is present. The Chilean Rodeo is a highly demanding discipline in which biomechanics forces predisposed to an increased stress to different joints and soft tissues such as metacarpophalangeal joint, distal interphalangeal joint, distal tarsal joints, and collateral ligaments. Thus, the aim of this study was to conduct a new morbidity data analysis with a larger cohort of Chilean sport horses to elucidate the most common conditions causing lameness in this breed of horses.

MATERIAL AND METHODS

Data was gathered from the medical records from horses presented to Equestria Equine Medical Center, located in Quillota, Chile, between the years 2016 and 2021. Medical records from eight hundred and ninety horses (890) diagnosed with an associated musculoskeletal injury with an age ranging from 3-18 years-old were included in this study for morbidity analysis. Horses diagnosed with a locomotor problem associated with the head and neck such as temporomandibular joint, cervical spine stenosis or cervical facet conditions were excluded from the analysis. From the eight hundred and ninety horses that fulfilled the inclusion criteria, six hundred and thirteen (613) were Chilean purebred horses performing or training for Chilean

Received: 19.07.2022.

Accepted: 28.11.2022.

[a]Escuela de Medicina Veterinaria, Sede Viña del Mar, Facultad de Ciencias de la Vida, Universidad Andrés Bello, Quillota 980, Viña del Mar. Chile.

[b]Equestria Equine Medical Center, Región de Valparaíso, Quillota 2260000. Chile.

*Corresponding author: e-mail: cdorner@gmail.com, Fundo el Castaño lote 8, Lo Mardones, Quillota, Chile, Telephone number: +56 9 74990538

Rodeo discipline from which thirty-two horses (32) were excluded because the diagnosis was associated to a neck or head problem giving a total of 581 Chilean horses finally included in the morbidity analysis. Information obtained from clinical records included: name, age, gender, affected limb(s), and diagnosis. Horses with any missing information mentioned above were excluded from the analysis.

Statistical analyses were run on SPSS, version 19 for Windows (SPSS Inc, Chicago IL, USA). Frequencies were calculated for description of the qualitative data and for quantitative variables a descriptive statistical analysis based on mean and standard deviation was performed. Additionally, qualitative data was analysed with the non-parametric Pearson Chi-square test to determine differences between lameness distribution. The significance level was set at $P<0.05$.

RESULTS AND DISCUSSION

From a total of 890 medical records gathered from horses diagnosed with one or more locomotor injuries, 68.9% were Chilean purebred horses, 25.3% Warmbloods, 2.6% Thoroughbreds, 2.4% Polo horses, 0.8% Arabians, 1% Crossbred and 0.9% Quarter horses (Table 1). Records obtained from Chilean purebred horses examined (n= 581) showed an average age of 10.78 ± 3.15 years distributed in geldings (n=291, 50.1%), mares (n= 199, 34.3%), and stallions (n=91, 15.7%). The information presented regarding horse breeds diagnosed in our hospital, shows a clear predominance of Chilean Horses; nonetheless, the breed is probably overrepresented, and this distribution might vary between veterinarians and veterinary practices. Forelimbs injuries were recorded in 72.9% of cases and hindlimbs injuries in 27.1% of cases, with a statistically significant difference among them ($P = 0.001$). Unilateral lameness was the most frequent situation seen in 74.9% of cases (n=424), bilateral lameness was observed in 21.7% (n=102), and lameness in 3 or more areas was recorded in 9.5% of horses (n=55). Several publications in different breeds have shown that the distribution of lameness is most prevalent in the forelimbs than the hindlimbs (Barroeta *et al*., 2019; Dabareiner *et al*., 2005a; Dabareiner *et al*., 2005b; Mora-Carreño *et al*., 2014; Morales *et al*., 2009). This differences in lameness distribution between forelimbs and hindlimbs in most equine disciplines is explained by the body-weight distribution, of approximately of 60% on the forelimbs (Hood *et al.,* 2001). This is similar to what we observed in our study where 72.9% of lameness were located in the forelimbs. This could be explained by different factors such as horse body conformation (muscled but small horse, height <145cm to the withers, relatively short limbs, with small hooves, and weighting ≤ 370kg), poor hoof balance often seen in Chilean horses and increase in body-weight distribution on forelimbs related to heavy riders (>80 kg).

When comparing frequencies of distribution between right and left sides, no differences were observed between forelimbs ($P = 0.645$) or between hindlimbs ($P = 0.853$). The most frequently affected areas were the fore hoof (33.2%), fore fetlock (20.2%), hock (13.8%) and fore pastern (8.0%). According with this data, the forelimb digit (hoof, pastern, and fetlock) had a prevalence of 61.4%. This percentage coincides with what has been described in previous studies in different breeds such as Quarter Horses used for western performance (Dabareiner *et al*., 2005a; Dabareiner *et al*., 2005b). This similarities in forelimb digit lameness prevalence might be due to similar body conformation between the Chilean purebred and Quarter horses, an even if the Quarter Horse is a larger horse, their proportions are alike. Similarly to the Chilean Rodeo, western performance horses are under a very strenuous demands in the fore digit joints and hock because of sudden movements of accelerations, turns and stops at high speed during competition (Jackman, 2001; Lewis, 2001; Dabareiner *et al*., 2005a; Dabareiner *et al*., 2005b).

When specific diagnoses were recorded, the most frequent causes of lameness in Chilean purebred horses were navicular disease (12.9%), fetlock osteoarthritis (11.9%) and distal tarsal joints osteoarthritis (11.7%). The recorded diagnoses are shown in table 2. Navicular disease is a common condition in all breeds and in the Chilean purebred is one of the most prevalent condition (Mora-Carreño *et al.,* 2014). Accordingly, in our report, navicular disease was recorded as the most prevalent affection in the

Table 1. Breed distribution from horses diagnosed with one or more locomotor injuries between the years 2016-2021 (before exclusion of diagnosis including head and neck problems).

Breed	N	%	Mean age ± SD
Chilean purebred	613	68.9	10.78 ± 3.15
Warmblood	209	23.5	11.52 ± 3.98
Thoroughbred	23	2.6	5.45 ± 3.03
Polo Horse	21	2.4	12.24 ± 2.43
Arabian	7	0.8	7.86 ± 3.58
Crossbred	9	1.0	14.44 ± 3.74
Quarter horse	8	0.9	9.38 ± 2.87
Total	890	100	

Table 2. Musculoskeletal diagnoses from Chilean purebred horses (n=581) obtained from records between the years 2016-2021.

Limb (n, %)	Area (n, %)	Recorded Diagnosis (n, %)
Forelimbs (n=424, 72.9%)	Hoof (n=194, 33.2%)	Navicular disease (n=75, 12.9%) DIP osteoarthritis (n=22, 3.7%) Laminitis (n=21, 3.6%) Solar contusion (n=18, 3.2%) Collateral ligament desmitis (n=17, 3.0%) Hoof abscess (n=15, 2.5%) Others (n=26, 4.4%)
	Pastern (n=46, 8.0%)	DDFT (n=18, 3.2%) PIP osteoarthritis (n=15, 2.5%) PIP subluxation (n=5, 0.9%) Others (n=8, 1.4%)
	Fetlock (n=118, 20.2%)	Fetlock osteoarthritis (n=70, 11.9%) Fetlock osteochondral fragment (n=19, 3.4%) Collateral ligaments (n=8, 1.4%) Others (n=21, 3.6%)
	Cannon/Carpus (n=57, 9.9%)	Suspensory Desmitis (n=16, 2.8%) Carpus OA (n=16, 2.8%) Splint bones fracture (n=11, 1.8%) SDFT (n=5, 0.9%) Others (n=9, 1.6%)
	Elbow/Shoulder (n=9, 1.6%)	Elbow/Shoulder Trauma (n= 4, 0.6%) Shoulder osteoarthritis (n=2, 0.4%) Elbow osteoarthritis (n=1, 0.2%) Others (n=2, 0.4%)
Hindlimbs (n=157, 26,9%)	Hoof (n=2, 0.4%)	Hoof abscess (n=2, 0.4%)
	Pastern (n=17, 2.9%)	PIP osteoarthritis (n=5, 0.9%) P1 - P2 fracture (n=4, 0.6%) Plantar pastern queloid (n=3, 0.5%) Others (n=4, 0.6%)
	Fetlock (n=26, 4.4%)	Fetlock osteoarthritis (n=8, 1.4%) Fetlock osteocondral fragment (n=6, 0.9%) Collateral ligaments (n=4, 0.6%) Others (n=8, 1.4%)
	Cannon/Hock (n=80, 13.8%)	Hock osteoarthritis (n=68, 11.7%) Suspensory Ligament desmitis (n=6, 1.1%) Others (n=6, 1.1%)
	Stifle (n=19, 3.4%)	Stifle osteoarthritis (n=8, 1.4%) Medial femoral condyle cyst (n=2, 0.4%) Upward fixation of the Patella (n=2, 0.4%) Others (n=6, 1.1%)
	Thoracolumbar spine/pelvis (n=13, 2.2%)	Kissing Spines (n=7, 1.2%) Coxo femoral osteoarthritis (n=2, 0.4%) Others (n=3, 0.5%)

Chilean horse, which could be related, among other things, to the discipline itself strenuous demands to anatomical structures within the hoof capsule. The biomechanical forces applied to the navicular bone due to its anatomical location between the third phalanx, second phalanx, and the deep digital flexor tendon (DDFT) are enormous and it has to withstand great forces, in particular during the propulsive phase of the stride when there is a greater tension of the DDFT (Rijkenhuizen, 2006; Dörner *et al*., 2017). Other factors that could be related with the high prevalence in the Chilean horses are the small hooves and narrow heel bulbs characteristic in this type of breed, which could impair the normal physiology and distribution of biomechanical forces inducing more stress to the inner structures within the hoof capsule. Additionally, there are genetic factors involved and it has been reported that the shape of the proximal articular border of the navicular bone has been found to be inherited and to predispose to the pathogenesis of navicular disease (Rijkenhuizen, 2006). Many different diagnoses were recorded in our data morbidity analysis and that is mostly due to the advancements in the diagnostic techniques and imaging technologies significantly improving the specificity and diagnostic accuracy. Digital radiography (RX) and high-definition ultrasonography (US) are both widely available in our country for the diagnose of most osteoarticular (osteoarthritis, osteochondral fragments, bone remodelling, fractures, among others) and soft tissue injuries (tendons, ligaments, muscles, and articular), respectively. Recently, magnetic resonance imaging (MRI) became available in Chile improving even more the diagnostic capabilities and enabling the diagnosis of injuries not detected by conventional imaging modalities. However, other imaging modalities such as computed tomography (CT) and gamma scintigraphy (bone scan) are not readily available in Chile, but efforts should be made to offer these advanced imaging modalities to the Chilean equine industry. Although there is one study published related to musculoskeletal injuries in Chilean Rodeo horses (Mora-Carreño *et al*., 2014), our study has a larger number of animals, being a better representation of the main locomotor injuries in purebred horses and enriching the scarce information available on pathologies causing poor performance and lameness in Chilean Rodeo horses.

In conclusion, the most prevalent conditions observed in Chilean Rodeo horses in this study were navicular disease (12.9%), fetlock osteoarthritis (11.9%) and distal tarsal joint osteoarthritis (11.7%). The above is due to the specific biomechanical forces and strenuous physical demands of the Chilean Rodeo horses, consequently veterinarians must be familiar with injuries affecting the Chilean horse and imaging modalities available for diagnosis of each specific injury, as the morbidity of musculoskeletal injuries varies drastically between different disciplines. The above, is important to stablish preventive measures, especially focused to hoof trimming and shoeing to diminish the biomechanical strenuous forces to the structures most frequently affected.

COMPETING INTERESTS STATEMENT

The author declare that he does not have competing interests.

ETHICS STATEMENT

No experimental animals or animals for clinical research were used in this study.

AUTHOR CONTRIBUTIONS

CD participated in the design, data gathering, data analysis and writing of the manuscript.

FUNDING

This study received no funding.

ACKNOWLEDGEMENTS

The author wishes to acknowledge Drs. J. Encalada, C. Castellón and V. Vergara for assistance with the data gathering.

REFERENCES

Barroeta, C., Espinal, A., Morales, A., Villoria, D., & Julia, O. (2019). Risk factors for catastrophic limb injury incurred during racing for Venezuela's thoroughbred racehorses 2000-2011. *International Journal of Applied Research in Veterinary Medicine*, *17*(2), 100-107.

Dabareiner, R.M., Cohen N.D., Carter G.K., Nunn, S., & Moyer, W. (2005a). Lameness and poor performance in horses used for team roping: 118 cases (2000-2003). *Journal of the American Veterinary Medical Association*, *226*(10), 1694-1699.

Dabareiner, R.M., Cohen N.D., Carter G. K., Nunn, S., & Moyer, W. (2005b). Musculoskeletal problems associated with lamenessand poor performance among horses used for barrel racing: 118 cases (2000-2003). *Journal of the American Veterinary Medical Association*, *227*(10), 1646-1650.

Dörner, C., Fueyo, P., & Olave, R. (2017). Relationship between the Distal Phalanx Angle and Radiographic Changes in the Navicular Bone of Horses: A Radiological Study. *Global Journal of Medical Research*, *17*(2).

Dyson, S. (2000). Lameness and Poor Performance in the Sports Horse : Dressage, Show Jumping and Horse Trials (Eventing). *2000 AAEP Convention Proceedings*, *46*, 308-315. https://www.ivis.org/sites/default/files/library/aaep/2000/308.pdf

Egenvall, A., Lönnell, C., & Roepstorff, L. (2009). Analysis of morbidity and mortality data in riding school horses, with special regard to locomotor problems. *Preventive Veterinary Medicine*, *88*(3), 193-204. https://doi.org/10.1016/j.prevetmed.2008.10.004

Egenvall, A., Penell, J.C., Bonnett, B.N., Olson, P., & Pringle, J. (2005). Morbidity of Swedish horses insured for veterinary care between 1997 and 2000: Variations with age, sex, breed and location. *Veterinary Record*, *157*(15), 436-443. https://doi.org/10.1136/vr.157.15.436

Egenvall, A., Penell, J.C., Bonnett, B.N., Olson, P., & Pringle, J. (2006). Mortality of Swedish horses with complete life insurance between 1997 and 2000: Variations with sex, age, breed and diagnosis. *Veterinary Record*, *158*(12), 397-406. https://doi.org/10.1136/vr.158.12.397

Fortini, G.A. (2011). *Caracterización clínica y radiográfica de claudicaciones en equinos de tiro en las ciudades de Valdivia y Puerto Montt* [Tesis de título profesional]. Universidad Austral de Chile. http://cybertesis.uach.cl/tesis/uach/2011/fvf742c/doc/fvf742c.pdf

Hood, D.M., Wagner, I.P., Taylor, D.D., Brumbaugh, G.W., & Chaffin, M.K. (2001). Voluntary limb-load distribution in horses with acute and chronic laminitis. *American Journal of Veterinary Research*, *62*(9), 1393-1398. https://doi.org/10.2460/ajvr.2001.62.1393

Jackman, B.R. (2001). Common Lameness in the Cutting and Reining Horse. *Proceedings of the Annual Convention of the AAEP*, *47*, 1-11.

Lewis, R.D. (2001). Lameness in the Rodeo Horse. *AAEP Proceedings*, *47*, 1-5.

Mora-Carreño, M., Briones, R., Parra, D., Rosenfeld, C., Schmeisser, A., & Menarim, B. (2014). Main musculoskeletal injuries associated with lameness in Chilean Rodeo horses [Principales afecciones musculoesqueléticas asociadas a claudicación en equinos del rodeo chileno]. *Archivos de Medicina Veterinaria, 46*(3), 419-424.

Morales, A., Bermúdez, V., García, F., Perdigón, M., & Naranjo, R. (2009). Catastrophic musculoskeletal injuries in thoroughbred horses, Caracas-Venezuela. *Revista de Investigaciones Veterinarias del Perú*, *20*(2), 243-248.

Morales, A., Méndez-Angulo, J. L., & Méndez, A. (2018). A retrospective study of causes for mortality of Thoroughbred horses in Caracas, Venezuela (2008-2012). *Pesquisa Veterinaria Brasileira*, *38*(5), 817-822. https://doi.org/10.1590/1678-5150-PVB-4673

Penell, J.C., Egenvall, A., Bonnett, B.N., Olson, P., & Pringle, J. (2005). Specific causes of morbidity among Swedish horses insured for veterinary care between 1997 and 2000. *Veterinary Record*, *157*(16), 470-477. https://doi.org/10.1136/vr.157.16.470

Ramzan, P.L., & Palmer, L. (2011). Musculoskeletal injuries in Thoroughbred racehorses: A study of three large training yards in Newmarket, UK (2005-2007). *Veterinary Journal*, *187*(3), 325-329. https://doi.org/10.1016/j.tvjl.2009.12.019

Rijkenhuizen, A. (2006). Navicular disease: a review of what's new. *Pferdeheilkunde*, 22(5), 579-586. https://doi.org/10.21836/PEM20060509

Seitzinger, A.H., Traub-Dargatz, J.L., Kane, A.J., Kopral, C.A., Morley, P.S., Garber, L.P., & Losinger, G.H. (2000). A comparison of the economics costs of equine lameness, colic, and equine myeloencephalitis (EPM). *Proceedings des International Symposium on Veterinary Epidemiology and Economics,* Colorado (USA).

Austral J Vet Sci **55, 142-146 (2023)**

SHORT COMMUNICATION

Serosurvey of canine distemper virus in culpeo (*Lycal*opex *culpaeus*) and chilla (*Lycalopex griseus*) foxes of the Araucanía region, Chile

Felipe A. Hernández[a,b], Dominique Surot[c], Antulemu Vallverdú[a], Michelle Cueva[a,d], Isabel M. Aguirre[e], Edith A. Mancilla[e], Gerardo Acosta-Jamett[a,b*]

ABSTRACT. Our goal was to assess whether free-ranging foxes have been exposed to canine distemper virus (CDV) in the Araucanía region in Chile. The study was conducted at three sites in rural areas where free-ranging foxes were trapped and bled from 2009 to 2012. We sampled two species of foxes: the culpeo (*Lycal*opex *culpaeus*) (n=13) and the chilla (*Lycalopex griseus*) (n=14). A serum virus neutralization assay was used to detect the presence and magnitude of functional systemic antibodies to CDV. Overall, CDV seroprevalence in culpeo and chilla foxes was 7.7 and 21.4%, respectively. Exposure to CDV did not differ among the sites. Despite the relatively low seroprevalence found in free-ranging foxes, the presence of CDV-seropositive dogs previously reported in rural sites nearby, suggests a potential risk of pathogen spill over from domestic dogs to foxes in the area.

Keywords: Araucanía, canine distemper virus, seroprevalence, *Lycalopex griseus*, *Lycalopex culpaeus*, pathogen spill over.

INTRODUCTION

Several disease outbreaks have affected wild carnivores worldwide, involving several multi-host pathogens carried by domestic dogs (*Canis familiaris*), such as canine distemper virus (CDV) (Alexander *et al*., 1996; Berentsen *et al*., 2013; Cleaveland *et al*., 2000; Lembo, 2006; Roelke-Parker *et al*., 1996). For example, pathogen spill over from dogs was suggested as the likely cause of CDV emergence in lions (*Panthera leo*) (Cleaveland *et al*., 2000; Lembo, 2006; Roelke-Parker *et al*., 1996) and African wild dogs (*Lycaon pictus*) (Alexander *et al*., 1996), which caused a drastic reduction in these wild carnivore populations. Similarly, spotted hyenas (*Crocuta crocuta*) and African wild dogs have shown relatively low CDV exposure in and around protected areas, potentially originated from dogs (Berentsen *et al*., 2013). Despite the compelling evidence of CDV spill over between domestic and wild carnivores worldwide, additional studies are needed to understand the exposure to this pathogen through rural landscapes in the Neotropics (e.g. Fiorello *et al*., 2007; Furtado *et al*., 2016; Megid *et al*., 2009, 2010), where contact between dogs and free-ranging carnivores is likely to occur.

The Araucanía region of Chile consists of a formerly forested landscape that is currently fragmented by farmlands, exotic plantations, pastures and human settlements (Echeverria *et al*., 2006). This region is inhabited by three species of free-ranging foxes: the culpeo (*Lycal*opex *culpaeus*), the chilla (*Lycalopex griseus*) and one of the most endangered canid species in the world, the Darwin's fox (*Lycalopex fulvipes*). The latter is classified as endangered by IUCN (Silva-Rodríguez *et al*., 2016[1]) and inhabits the Nahuelbuta National Park (NNP) (37º 47' S, 72º 59' W). Recent studies have reported its presence in the locality of Lastarria (Gorbea district, 39º 11' S, 72º 6' W – D'elía *et al*., 2013) and other areas south to the NNP (Farías *et al*., 2014; Vilà *et al*., 2004). Culpeo and chilla foxes are solitary species occurring mainly in mountainous areas and lowland sites, respectively (Jimenez *et al*., 1995). Chilla foxes are habitat-generalists regularly approaching households and getting into contact with domestic dogs and therefore at high-risk of infection with pathogens (Acosta-Jamett *et al*., 2011; Acosta-Jamett *et al*., 2015a, Hernández *et al*., 2021).

We previously reported that urban and rural domestic dogs were exposed to CDV in the Araucanía (Acosta-Jamett *et al*., 2015b), but we still lack data on disease exposure on free-ranging foxes to estimate the potential risk of pathogen spill over from domestic to wild canids in the region. In this study, we explored whether free-ranging foxes inhabiting rural sites of the Araucanía have been exposed to CDV, a pathogen of potential conservation concern for threatened carnivores such as Darwin's fox.

Received: 13.01.2023.

Accepted: 21.03.2023.

[a]Instituto de Medicina Preventiva Veterinaria, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile.

[b]Center for Surveillance and Evolution of Infectious Diseases, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile.

[c]Departamento de Ciencias Biológicas Animales, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile.

[d]Escuela de Graduados, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile.

[e]Instituto de Patología Animal, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile.

*Corresponding author: gerardo.acosta@uach.cl

[1] Silva-Rodríguez E, Farías A, Moreira-Arce D, Cabello J, Hidalgo-Hermoso E *et al* (2016) *Lycalopex fulvipes* (errata version published in 2016). The IUCN Red List of Threatened Species 2016: e.T41586A107263066. http://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T41586A85370871.en. Accessed 9 January, 2023

MATERIAL AND METHODS

The study was conducted between 2009 and 2012 in the Araucanía region in south-central Chile, including three sites: a) a more remote site inserted at the Santa María forestry company lands located 32 km north-west of Angol city and 15 km north of the Nahuelbuta National Park (37° 39' S; 73° 03' W) (hereafter Angol); and two sites nearby towns as b) Conguillío National Park, located 15 km south-east of Curacautín city (38° 42' S; 71° 37' W) (hereafter Curacautín), and c) Arauco forestry company lands located close to Lastarria locality, 20 km south-west of Gorbea city (39° 11' S; 72° 46' W) (hereafter Gorbea) (Figure 1). Angol site was chosen instead of NNP since a capture license was not available from the National Forest Service (CONAF) that administers the Chilean protected areas.

We captured foxes with padded leg-hold traps (Victor Soft Catch No. 1.5, Chagnons Trapping Supply, Manistique, MI, USA) and Tomahawk traps (Tomahawk Live Trap Co., Tomahawk, WI), which were consistently checked for captures every four hours (i.e., two checks/trapping night), to avoid injuries. Animals were anesthetised as reported by Acosta-Jamett *et al*. (2010) and blood was sampled from the cephalic vein. Foxes were marked with unique ear tags to avoid re-sampling during recaptures, and all of them were safely released at the corresponding capture site. To determine humoral immune response to CDV infection, neutralizing antibody titres were obtained from serum samples through a virus neutralization (SVN) assay using the reference strain Onderstepoort from a commercial CDV vaccine (Novibac Puppy DP; Intervet International B.V., Netherlands). Neutralizing antibody titres at serum dilution ≥1:12 was considered positive for CDV exposure.

DATA ANALYSIS

Ninety-five percent confidence limits were calculated for all seroprevalence values. Data analysis was carried out using the software R v. 4.1.0 (R Core Team, 2021).

RESULTS AND DISCUSSION

Overall, 27 foxes were trapped, of which 13 were culpeos (5 males/8 females; 4 juveniles/9 adults) and 14 chillas (7 males/7 females; 4 juveniles/10 adults). One (7.7%; CI 0.2-36.0%) of the 13 culpeos and 3 (21.4%; CI 4.7-50.8%) of the 14 chillas were CDV seropositive (Table 1). CDV-seropositive foxes were evenly distributed according to sex and age; the CDV-seropositive culpeo corresponded to an adult female captured at Curacautín,

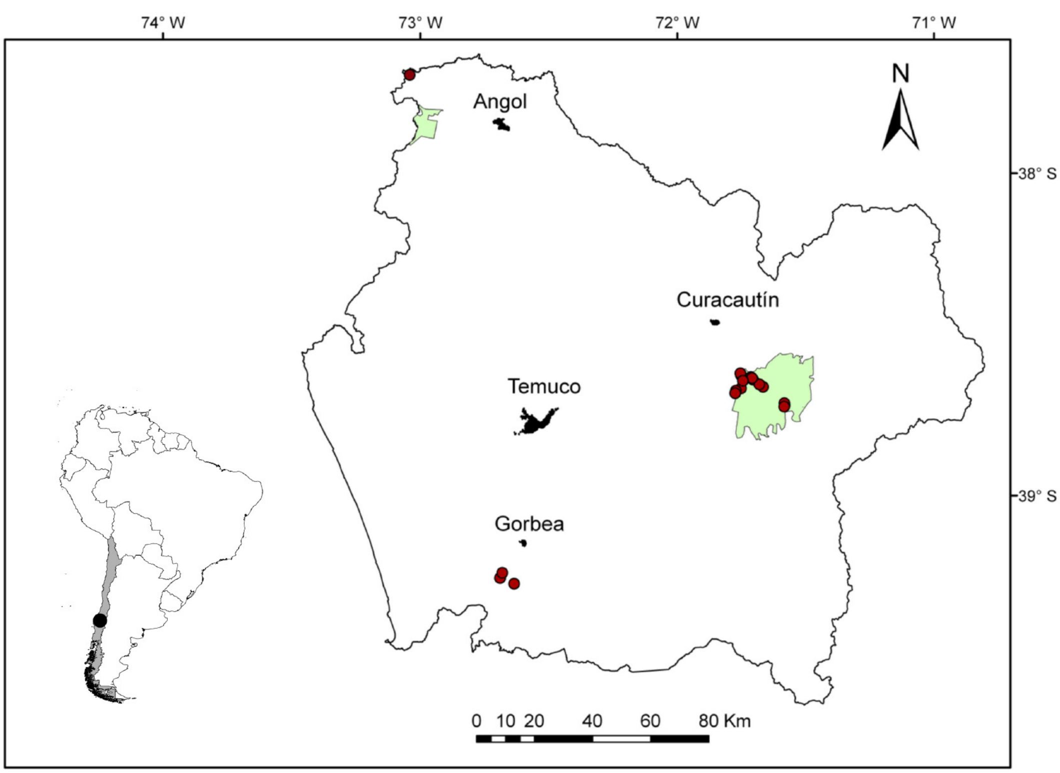

Figure 1. Location of sampled free-ranging foxes (red circles) at three sites of the Araucanía region (Chile) (2009-2012). Zones colored in black denote cities and zones colored in green denote protected areas (Nahuelbuta National Park and Conguillío National Park) in proximity to sampling sites.

Table 1. Percentages of CDV seropositive culpeo and chilla foxes sampled at three sites of the Araucanía region (Chile) (2009-2012). NA denotes that no individual of a particular species was sampled.

	CDV		
Site	Culpeo	Chilla	Overall
Angol	0/1	NA	0/1
Curacautín	1/12 (8.3)	2/11 (18.2)	3/23 (13.0)
Gorbea	NA	1/3 (33.3)	1/3 (33.3)
Total	1/13 (7.7)	3/14 (21.4)	4/27 (14.8)

while the CDV-seropositive chillas were 2 juvenile males captured at Curacautín, and 1 adult female captured at Gorbea. Due to the limited number of sampled animals, CDV seroprevalence data of both culpeos and chillas were pooled to compare pathogen exposure among foxes across sites (Table 1). Overall, exposure to CDV did not differ among the sites.

Our study is the first attempt to explore whether free-ranging foxes (i.e., culpeo and chilla) have been exposed to CDV in the Araucanía region of Chile. Our findings confirmed the exposure to CDV in both culpeos and chillas, similarly to previous studies that reported exposure to these pathogens in other fox populations (Acosta-Jamett *et al*., 2011; Acosta-Jamett *et al*., 2015a; Martino *et al*., 2004). Habitat-generalists as chillas may have increasingly higher opportunities for interaction with domestic dogs compared to culpeos (Hernández *et al*., 2021), likely enhancing the likelihood of interspecific pathogen exposure (i.e., CDV-exposed wild foxes corresponded to 1 culpeo vs. 3 chillas), which may be relatively recent in the two CDV seropositive juvenile chillas of Curacautín.

Compared to previous studies, our CDV seroprevalences in culpeo (7.7%) and chilla (21.4%) foxes were lower than the values reported in north-central Chile (i.e., 42% in both chillas and culpeos combined) (Acosta-Jamett *et al*., 2015a), but higher than the values reported for free-ranging culpeo and chilla foxes in a remote area of the Argentinean Patagonia, where 3.6% of tested foxes had CDV antibodies (which corresponded to chillas only) (Martino *et al*., 2004). This could be explained by a higher closeness to urban dog populations in foxes from the first study compared to the second study, but limited sample size prevents us to overinterpret our findings. Thus, our numbers of foxes sampled were too limited for meaningful comparisons of CDV seroprevalences across the study area (particularly at the remote Angol area), and only relying on serosurvey data we cannot determine whether CDV was actually transmitted between wild and domestic canids.

According to our previous spatial analyses at the Araucanía, there was a wide presence of domestic dogs exposed to CDV in both urban and rural areas across the region (Acosta-Jamett *et al*., 2015b), and they are present even in close proximity to protected areas and sites where CDV-seropositive chilla and culpeo foxes were trapped. Dogs sampled at rural sites had a higher overall seroprevalence of CDV (47%) (Acosta-Jamett *et al*., 2015b) than free-ranging foxes (14.8%), which would be a factor of concern given the high mortality rates caused by pathogens as CDV in chillas and culpeos (Acosta-Jamett *et al*., 2011; González-Acuña *et al*., 2003; Moreira & Stutzin, 2003). Considering that dog population has grown and spread continuously in Chile's urban and rural areas over the last decades (Acosta-Jamett, 2015), it is highly probable that pathogens such as CDV are transmitted from dogs to wild foxes through occasional interspecific contact. Alternatively, a guild of wild carnivores may naturally maintain and transmit generalist pathogens among species; however, because foxes, felids and mustelids generally exhibit lower densities than dogs through their geographic distribution, this hypothesis seems to be less likely (Acosta-Jamett, 2009; Acosta-Jamett *et al*., 2011; Acosta-Jamett *et al*., 2015a).

Our findings suggest that free-ranging foxes, such as culpeo and chilla, have been exposed in some cases recently to CDV across their range in the Araucanía. Domestic dogs from urban areas had a higher risk of being CDV seropositive than rural dogs, suggesting increased force of infection in more dense urban dogs with increased contact rates (Acosta-Jamett *et al*., 2015b); however, whether pathogen exposure in wild foxes has occurred due to spill over from domestic dogs living in proximity is unclear yet. Although we did not sample Darwin's foxes, continued health surveillance should be also recommended for this endangered, immunologically naïve wild canid, given high population mortality could be expected in an eventual CDV spill over event from dogs (or potentially other foxes or carnivores acting as "bridge hosts") to Darwin's foxes in the region (Hidalgo-Hermoso *et al*., 2020; Sepúlveda *et al*., 2014). In this regard, Hidalgo-Hermoso *et al*. (2020) suggested that lack of exposure to CDV in Darwin's foxes may be related to either their forest specialist habits, or the lack of detection of exposed individuals due to unnoticed mortality in foxes that already had contact with the virus. Further long-term longitudinal studies using a combination of PCR and serological tests to assess potential routes of infection from domestic dogs to free-ranging foxes with emphasis in the endangered Darwin's fox are needed to understand the spatial dynamic of infection and prevent CDV outbreaks in wild carnivores at the Araucanía region of Chile.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

Animal capture and handling procedures were approved by the Ethics Committee at the Universidad Austral de Chile and authorized by the Animal Health Service (SAG: N°3245/2011).

AUTHOR CONTRIBUTIONS

GAJ conceived the ideas of the study. DS, AV and GAJ carried out the field work and sampling. MC, IMA and EAM carried out the laboratory assays. FAH and GAJ led the writing of the manuscript. GAJ obtained the funding for this study. All authors revised the manuscript, contributed critically to the drafts and approved the final version for publication.

FUNDING

This work was funded by ANID/CONICYT Fondecyt Iniciación Nº 11100303, The Rufford Small Grants Foundation Nº 39.07.09 and DID-UACH S-2010-58. FAH was funded by ANID/CONICYT Fondecyt Postdoctorado Nº 3180111. MC was funded by ANID Becas Doctorado Nº 21201298.

ACKNOWLEDGEMENTS

The support and permits provided by the National Forest Service (CONAF) and Animal Health Service (SAG) personnel were essential for conducting the trapping of foxes and questionnaire survey. We thank the logistic support provided by the Santa María forestry company, which allowed the fieldwork within their lands. Finally, we thank the support of more than forty volunteers from seven different countries within America and overseas.

REFERENCES

Acosta-Jamett, G. (2009). The role of domestic dogs in diseases of significance to human and wildlife health in central Chile. *Ph.D. Dissertation*. University of Edinburgh, Edinburgh, Scotland.

Acosta-Jamett, G., Astorga-Arancibia, F., & Cunningham, A. A. (2010). Comparison of chemical immobilization methods in wild foxes (*Pseudalopex griseus* and *P. culpaeus*) in Chile. *Journal of Wildlife Diseases*, *46*, 1204-1213. https://doi.org/10.7589/0090-3558-46.4.1204

Acosta-Jamett, G., Chalmers, W. S. K., Cunningham, A. A., Cleaveland, S., Handel, I. G., & Bronsvoort, B. M. deC. (2011). Urban domestic dog populations as a source of canine distemper virus for wild carnivores in the Coquimbo region of Chile. *Veterinary Microbiology*, *152*, 247-257. https://doi.org/10.1016/j.vetmic.2011.05.008

Acosta-Jamett, G. (2015). Increased dog population and potential for bat-borne rabies spillover in Chile in response to ‘‘Dog management, abundance and potential for bat-borne rabies spillover in Chile’’ by Astorga et al. *Preventive Veterinary Medicine*, *120*, 246-247. https://doi.org/10.1016/j.prevetmed.2015.04.001

Acosta-Jamett, G., Cunningham, A. A., Bronsvoort, B. M. deC., & Cleaveland, S. (2015a). Serosurvey of canine distemper virus and canine parvovirus in wild canids and domestic dogs at the rural interface in the Coquimbo Region, Chile. *European Journal of Wildlife Research*, *61*, 329-332. https://doi.org/10.1007/s10344-014-0886-0

Acosta-Jamett, G., Surot, D., Cortés, M., Marambio, V., Valenzuela, C., Vallverdu, A., & Ward, M. P. (2015b). Epidemiology of canine distemper and canine parvovirus in domestic dogs in urban and rural areas of the Araucanía region in Chile. *Veterinary Microbiology*, *178*, 260-264. https://doi.org/10.1016/j.vetmic.2015.05.012

Alexander, K. A., Kat, P. W., Munson, L. A., Kalake, A., & Appel, M. J. G. (1996). Canine distemper-related mortality among wild dogs (*Lycaon pictus*) in Chobe National Park, Botswana. *Journal of Zoo and Wildlife Medicine*, *27*, 426-427.

Berentsen, A. R., Dunbar, M. R., Becker, M. S., M’soka, J., Droge, E., Sakuya, N. M., Matandiko, W., McRobb, R., & Hanlon, C. A. (2013). Rabies, canine distemper, and canine parvovirus exposure in large carnivore communities from two Zambian ecosystems. *Vector Borne Zoonotic Diseases*, *13*, 643-649. https://doi.org/10.1089/vbz.2012.1233

Cleaveland, S., Appel, M. G. J., Chalmers, W. S. K., Chillingworth, C., Kaare, M., & Dye, C. (2000). Serological and demographic evidence for domestic dogs as a source of canine distemper virus infection for Serengeti wildlife. *Veterinary Microbiology*, *72*, 217-227. https://doi.org/10.1016/s0378-1135(99)00207-2

D’elía, G., Ortloff, A., Sánchez, P., Guiñez, B., & Varas, V. (2013). A new geographic record of the endangered Darwin’s fox *Lycalopex fulvipes* (Carnivora: Canidae): filling the distributional gap. *Revista Chilena de Historia Natural*, *86*, 485-488. https://doi.org/10.4067/S0716-078X2013000400010

Echeverria, C., Coomes, D., Salas, J., Rey-Benayas, J. M., Lara, A., & Newton, A. (2006). Rapid deforestation and fragmentation of Chilean Temperate Forests. *Biological Conservation*, *130*, 481-494. https://doi.org/10.1016/j.biocon.2006.01.017

Farías, A. A., Sepúlveda, M. A., Silva-Rodríguez, E. A., Eguren, A., González, D., Jordán, N. I., Ovando, E., Stowhas, P., & Svensson, G. L. (2014). A new population of Darwin’s fox (*Lycalopex fulvipes*) in the Valdivian Coastal Range. *Revista Chilena de Historia Natural*, *87*, 1-3. https://doi.org/10.1186/0717-6317-87-3

Fiorello, C.V., Noss, A.J., Deem, S.L., Maffei, L., Dubovi, E.J. (2007). Serosurvey of small carnivores in the Bolivian Chaco. *Journal of Wildlife Diseases*, *43*, 551-557. https://doi.org/10.7589/0090-3558-43.3.551

Furtado, M.M., Hayashi, E.M.K., Allendorf, S.D., Coelho, C.J., Jacomo, A.T.D., Megid, J., Ramos, J., Silveira, L., Torres, N.M., Neto, J.S.F. (2016). Exposure of Free-Ranging Wild Carnivores and Domestic Dogs to Canine Distemper Virus and Parvovirus in the Cerrado of Central Brazil. *EcoHealth*, *13*, 549-557. https://doi.org/10.1007/s10393-016-1146-4

González-Acuña, D., Ortega-Vásquez, R., Rivera-Ramírez, P., & Cabello-Caballín, J. (2003). A presumed case of canine distemper in a gray fox (*Pseudalopex griseus*) from central Chile. *Zeitschrift Fur Jagdwissenschaft*, *49*, 323-326. https://doi.org/10.1007/BF02189641

Hernández, F. A., Manqui, J., Mejías, C., & Acosta-Jamett, G. (2021). Domestic dogs and wild foxes interactions in a wildlife-domestic interface of north-central Chile: implications for multi-host pathogen transmission. *Frontiers in Veterinary Science*, *8*, 631-788. https://doi.org/10.3389/fvets.2021.631788

Hidalgo-Hermoso, E., Cabello, J., Vega, C., Kroeger-Gómez, H., Moreira-Arce, D., Napolitano, C., Navarro, C., Sacristán, I., Cevidanes, A., Di Cataldo, S., Dubovi, E. J., Mathieu-Benson, C., & Millán, J. (2020). An eight-year survey for canine distemper virus indicates lack of exposure in the Endangered Darwin’s Fox (*Lycalopex fulvipes*). *Journal of Wildlife Diseases*, *56*, 482-485. https://doi.org/10.7589/2019-08-195

Jimenez, J.E., Yanez, J.L., Tabilo, E.L., Jaksic, F.M. (1995). Body size of Chilean foxes - a new pattern in light of new data. *Acta Theriologica*, *40*, 321-326. https://doi.org/10.4098/AT.arch.95-31

Lembo, T. (2006). *An investigation of disease reservoirs in complex ecosystems: rabies and canine distemper in the Serengeti.* [PhD Dissertation]. University of Edinburgh, Edinburgh, Scotland.

Martino, P. E., Montenegro, J. L., Preziosi, J. A., Venturini, C., Bacigalupe, D., Stanchi, N. O., & Bautista, E. L. (2004). Serological survey of selected pathogens of free-ranging foxes in southern Argentina, 1998-2001. *Revue Scientifique et Technique*, *23*, 801-806. https://doi.org/10.20506/rst.23.3.1521

Megid, J., de Souza, V.A.F., Teixeira, C.R., Cortez, A., Amorin, R.L., Heinemman, M.B., Cagnini, D.Q., & Richtzenhain, L.J. (2009.) Canine Distemper Virus in a Crab-eating Fox (*Cerdocyon thous*) in Brazil: Case Report and Phylogenetic Analyses. *Journal of Wildlife Diseases*, *45*, 527-530. https://doi.org/10.7589/0090-3558-45.2.527

Megid, J., Teixeira, C.R., Amorin, R.L., Cortez, A., Heinemman, M.B., de Paula, J.M.A., da Costa, L.F., Fornazari, F., Bosso, J.R., Cremasco, A., Richtzenhain, L.J. (2010.) First identification of canine distemper virus in hoary fox (*Lycalopex vetulus*): pathologic aspects and virus phylogeny. *Journal of Wildlife Diseases, 46*, 303-305. https://doi.org/10.7589/0090-3558-46.1.303

Moreira, R. & Stutzin, M. (2005). Epidemiologic preliminary report on mortality of foxes in IV region, Chile. Boletín Veterinario Oficial. Servicio Agrícola y Ganadero, http://www2.sag.gob.cl/Pecuaria/bvo/marzo_mayo_2005/articulos/mortalidad_zorros_IV_region.pdf [Spanish].

R Core Team. 2021. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

Roelke-Parker, M. E., Munson, L., Packer, C., Kock, R., Cleaveland, S., Carpenter, M., O'Brien, S. J., Pospischil, A., Hofmann-Lehmann, R., Lutz, H., Mwamengele, G. L. M., Mgasa, M. N., Machange, G. A., Summers, B. A., & Appel, M. J. G. (1996). A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). *Nature*, *379*, 441-445. https://doi.org/10.1038/379441a0

Sepúlveda, M. A., Singer, R. S., Silva-Rodríguez, E. A., Eguren, A., Stowhas, P., & Pelican, K. (2014). Invasive American mink: linking pathogen risk between domestic and endangered carnivores. *Ecohealth*, *11*, 409-419. https://doi.org/10.1007/s10393-014-0917-z

Vilà, C., Leonard, J. A., Iriarte, A., O'Brien, S. J., Johnson, W. E., & Wayne, R. K. (2004). Detecting the vanishing populations of the highly endangered Darwin's fox, *Pseudalopex fulvipes*. *Animal Conservation*, *7*, 147-153. https://doi.org/10.1017/S1367943004001271

Austral J Vet Sci 55, 147-151 (2023)

SHORT COMMUNICATION

Neosaxitoxin, a Long-Lasting Local Anesthetic and its Potential Clinical Applications in Horses

Cristóbal Dörner[a,b*], Miguel Del Campo[a], Nestor Lagos[a]

ABSTRACT. Neosaxitoxin (NeoSTX) is a toxin that binds to the voltage-gated sodium channels therefore, inhibiting the neuronal impulse. The present study was conducted to explore the properties of NeoSTX and to evaluate its effects when injected as a perineural nerve block in horses. A group of five client-owned mature Warmblood horses exhibiting clinical signs of unilateral foot pain were enrolled in the study. For inclusion, lameness should subside after a palmar digital nerve block (PDNB) using 2 mL of 2% lidocaine administered over the medial and lateral palmar digital nerves of the affected limb (Day 0). Lameness was assessed using the AAEP lameness grading scale and skin sensitivity was judged objectively using a pressure algometer. On day 1, 5µg of NeoSTX was injected, then on day 4, 10 µg of NeoSTX was administered. Lameness examination and skin sensitivity were evaluated at 3, 5,10, 15,30, 60, 90 minutes, and every hour until the effects of the nerve block were no longer detectable. When effects of NeoSTX was compared to effects of lidocaine at 2% there were no statistical differences in the onset of the anesthetic effect, measured as the time of start of desensitization of the skin and the time of complete desensitization or lameness resolution, nonetheless there was a significant difference in the return of skin sensation or lameness, showing a clear long-lasting nociceptive blocker effect of NeoSTX. In conclusion, results of this study suggest NeoSTX can potentially be used as an alternative to conventional local anesthetics drugs when a long-lasting effect is desired, for example as a part of a multimodal approach for pain management, as a local anesthetic for surgical procedures or to control chronic pain in some musculoskeletal disorders. However, more studies are needed to evaluate its use as long-lasting anesthetic effects in the aforementioned situations.

Keywords: neosaxitoxin, NaV channels, equine, foot pain, welfare.

INTRODUCTION

Animal welfare is a standard consideration that refers to animal well-being under different situations, such as disease prevention and veterinary treatment, appropriate shelter, management, nutrition, humane handling, and even humane euthanasia. The most frequent painful conditions in horses are the ones related to the locomotor system (Pennel *et al*., 2005; Egenvall *et al*., 2009). Horses can suffer several orthopedic conditions throughout their lives and on many occasions those conditions require surgery or progress to chronic disorders, deteriorating the horse's quality of life (McGovan, 2011; Kelemen *et al*., 2021). For years, the use of conventional local anesthetics (e.g., lidocaine, mepivacaine, or bupivacaine) for diagnostic or local anesthetic to perform surgical procedures have been an excellent tool for equine practitioners. Furthermore, studies have been conducted in horses to evaluate the ability of adjuvants such as epinephrine and sodium bicarbonate to either increase the intensity, hasten the onset, or prolong the duration of local anesthesia in some desired situations (Alvarez *et al*., 2018; Boone *et al*., 2019).

Received: 12.12.2022.

Accepted: 21.03.2023.

[a]Membrane Biochemistry Laboratory, Department of Physiology and Biophysics, Faculty of Medicine, University of Chile, Independencia 1027, 8380000, Santiago, Chile.

[b]Equestria Centro Médico Equino, 2260000, Quillota, Chile.

*Corresponding author: e-mail: cdorner@gmail.com Fundo El Castaño lote 8, Lo Mardones, Quillota, Chile Telephone number: +56 9 74990538, ORCID Nº 0000-0003-3755-9740.

The pharmaceutical industry continues to explore the development of safer and more effective local anesthetics. In the last twenty-five years, researchers have used different toxins obtained from plants and microorganisms in the search for a new drug with a potential property to be used as long-lasting local anesthetic. In recent publications, local injection of Paralytic Shellfish Poison (PSP) toxins such as Gonyautoxins and Neosaxitoxin (NeoSTX) have shown its properties *in vitro* (Montero *et al*., 2020) and *in vivo* in pilot studies and case reports showing to be safe and effective in several clinical applications in humans to control chronic and post-operative pain (Lagos, 2014; Manriquez *et al*., 2015; Hinzpeter *et al*., 2016) and potentially in veterinary medicine in different species (Riquelme *et al*., 2018; Valenzuela *et al*., 2019; Varela *et al*., 2019; Montero *et al*., 2021). NeoSTX biological activity lies in the reversible inhibition of excitable cells impeding neuronal impulse propagation at the neuronal synapses or neuromuscular junction (Catterall, 2000; Lagos & Andrinolo, 2000; Lagos, 2014). The inhibition of the neuronal impulse is due to closure of the voltage-gated sodium channels by high affinity binding of NeoSTX to the site 1 of the alfa unit of the channel, blocking the sodium influx through the channel (Lagos, 2014).

Thus, due to the preliminary information available regarding the NeoSTX, the aim of this study was to evaluate the effect of NeoSTX when injected perineurally in horses to gather more evidence of its safety and capability to be included as a part of a multimodal approach for chronic and surgical pain management or to be used alone as a regional anesthetic for standing surgical procedures in horses.

MATERIAL AND METHODS

A prospective clinical study was conducted to evaluate NeoSTX when administered as PDNB. Five client-owned mature Warmblood horses (3 mares and 2 geldings), with an age between 8-14 years-old, and a weight ranging from 485-535 kg were included in the study. Horses selected presented clinical signs consistent of unilateral foot pain. The study was approved by the Ethical Committee of the Medicine Faculty, University of Chile (FM 0551) and informed consent was given by horse owners enrolled in the study. Horses were selected from clinical cases presented to the equine hospital (Equestria Equine Medical Center, Quillota, Chile) and as inclusion criteria, the lameness had to disappear after a palmar digital nerve block (PDNB) using 2 mL 2% lidocaine on each nerve. For injection, the distal pastern region was cleaned with an antiseptic solution, and the nerve block was performed inserting subcutaneously a 25-gauge, 5/8-inch needle over the palpable medial and lateral neurovascular bundles of the affected limb. The needle was placed just proximal to the border of the lateral and medial ungular cartilage (Moyer *et al*., 2007). Finally, 2 mL of 2% lidocaine was administered in each point. Lameness assessment was evaluated subjectively using the American Association of Equine Practitioners (AAEP) grading system (Ross, 2011). For lameness evaluation, all horses were walked and trotted in a straight line in a flat hard surface (concrete). Horses were observed from the front, back, and from both sides. Additionally, horses were trotted in circles, on a lunge line. Only one clinician performed the lameness evaluations (CD). The skin sensitivity was judged objectively using a pressure algometer (Baseline® push-pull force gauge, Model: 12-0804. White Plains, NY, USA), this model has been used previously in equine pain/pressure assessment (Riquelme *et al*., 2018). The algometer with a circular push tip supplement was applied over both heel bulbs, pressure was applied until withdrawal response happened and their maximum pressure thresholds were recorded in kg/cm^2 (maximum pressure of 10 kg/cm^2). The pressure measuring procedure consisted in applying pressure on both, the lame and control, limbs in a non-weight-bearing position. As a standardized technique in this study, the algometer was applied 5 consecutive times to the lame limb in each heel bulb before and after the perineural block, then the mean pressure tolerated was documented. The contralateral limb served as the negative control. On day 0, a PDNB using 2 mL of 2% lidocaine was performed. On day 1 (24 hours post lidocaine treatment), the PDNB was repeated by administering 5 μg of highly purified NeoSTX diluted in 1 mL of NaCl 0.9% at pH 6.2 obtained from *cyanobacteria Aphanizomenon sp*. Finally, on day 4 (96 hours post lidocaine treatment and 72 hours post 5 μg NeoSTX treatment) PDNBs for each horse were repeated by administering 10 μg of NeoSTX. Horses were examined at 3, 5, 10, 15, 30, 45, 60, 90 minutes, and every hour until the effect of the injection wore off. Daily clinical examination was performed to all horses over a one-week period to assess for any local inflammation or systemic illness.

Four time points were established (Figure 1). T0 corresponded to time 0 or baseline, T1 to the start of desensitization of the skin measured by the increase of the threshold in the algometer after pressure was applied and/or by partial improvement in the grade of lameness. T2 was the time when complete desensitization of the skin or lameness resolution was achieved and T3 corresponded to the time when the return of skin sensation or lameness was first observed. The duration of the anesthetic effect was calculated from the time points T3 and T1.

Statistical analyses were performed using GraphPad Prisma 5 software (USA). Normality assumptions were verified with the Kolmogorov-Smirnov test. Time of start of desensitization (T1), complete desensitization / lameness resolution (T2), and return of skin sensation / lameness (T3) were compared between treatments from the baseline starting point (T0) using a repeated-measures analysis of variance (ANOVA) and the Bonferroni`s multiple comparison test. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

All treatments given were consistently efficient abolishing lameness and skin sensation measured by the subjective gait analysis and objective measurement of skin sensation after administration of the drugs as a PDNB (Table 1). Results analysis showed no statistical differences when T1 and T2 were compared between 2% lidocaine and 10 μg of NeoSTX, nonetheless there was a significant difference in T3 and the duration of the anesthetic effect ($p < 0.05$) showing a clear long-lasting nociceptive blocker effect of NeoSTX. No local or systemic adverse reaction was observed after subcutaneous injection of NeoSTX over a one-week period of daily evaluation.

Even though mepivacaine and lidocaine are the most used local anesthetics, it has been reported that their efficacies vary and that variations could be attributed to the horse, time of interpretation, and deficient technique (Arkell *et al*., 2006; Nagy *et al*., 2009). Furthermore, Silva *et al*. (2015) showed that lidocaine inconsistently alleviated skin sensation, but not lameness and contradictory, Hoerdemann *et al*. (2017) showed that lidocaine inconsistently alleviated lameness, but not skin sensation. Despite the aforementioned, in our study we observed a good correlation between the alleviation of lameness caused by foot pain and skin sensation for both treatments. Other factors to be considered in the efficacy of local anesthetics is the one related to the pharmacology and mechanism of action of each drug. The speed of the onset, potency and duration are directly related to the pKa, lipid solubility, and protein binding site of the drug (Taylor & Mcleod, 2020). Local anesthetics activity lies on interruption of neural conductivity by binding to the

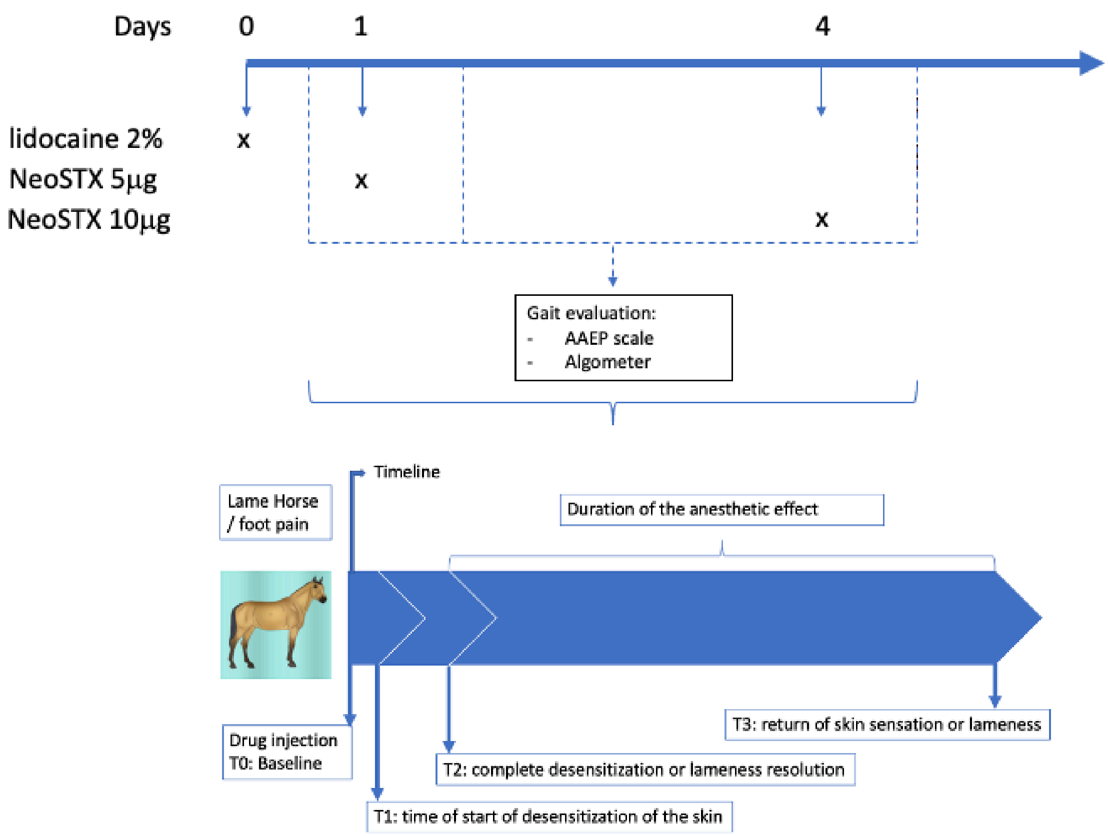

Figure 1. Scheme of timing of palmar digital nerve blocks and evaluation of gait and skin sensation.

Table 1. Mean values recorded of horses with foot pain at different time points.

Local anesthetic	Baseline (T0)			Time of start of desensitization of the skin (T1)			Complete desensitization / Lameness resolution (T2)			Return of complete skin sensation / lameness (T3)		
	Time (hours)	AAEP scale	Algometer (kg/cm^2)	Time (minutes)	AAEP scale	Algometer (kg/cm^2)	Time (minutes)	AAEP scale	Algometer (kg/cm^2)	Time (hours)	AAEP scale	Algometer (kg/cm^2)
lidocaine 20 milligrams	0	3	5.90 ± 0.141	3.42 ± 0.78	0	9.70 ± 0.142	5.58 ± 2.58^{ac}	0	10 ± 0	1.4 ± 0.224^{a}	3	5.90 ± 0.316
NeoSTX 5 micrograms	0	3	5.56 ± 0.205	6 ± 1.98	2	5.66 ± 0.282	16 ± 2.22^{b}	0	10 ± 0	5.8 ± 0.447^{b}	3	5.50 ± 0.228
NeoSTX 10 micrograms	0	3	6.10 ± 0.200	3.42 ± 0.9	1	8.10 ± 0.245	4.98 ± 0.6^{ac}	0	10 ± 0	11.6 ± 0.548^{c}	2	5.96 ± 0.316

Different letters indicate statistically significant differences ($P \leq 0.05$).

voltage-gated sodium channels (NaV channel), preventing depolarization, and avoiding further neuronal transmission (Taylor & Mcleod, 2020). Amino amides (e.g., lidocaine and mepivacaine) and amino esters (e.g., procaine), bind to the NaV channel inner pore, in other words the binding occurs from the intracellular side of the cell (Catterall, 2000). More specifically, the binding site for local anesthetics is in domain IV, loop S6 and is only accessible when the NaV channel is open and depolarized (Taylor & Mcleod, 2020). On the other hand, NeoSTX binds with high affinity to site 1 of the alpha unit on the NaV channel, impeding the sodium ion entrance to the nerve cell. Consequently, the foremost effect of NeoSTX is related to their blocking action at the axonal level, inhibiting nerve depolarization and impulse conduction (Lagos, 2014). The mechanism of action of NeoSTX explains the rapid onset of the effect because firstly, NeoSTX does not have to pass through the phospholipid membrane to bind the NaV channel (binding to its site facing in extracellular part of the channel), secondly, is not affected by the intracellular pH, and finally there is no need for the NaV channel to be opened for it to bind, as it must happen when amino amides and amino esters are used. Accordingly, NeoSTX has shown to be a long-lasting dose-dependent local anesthetic when administered subcutaneously around a painful area without the need of any adjuvant to prolong the duration of the anesthetic effect (Riquelme *et al*., 2018) as also shown in this report where the capability of NeoSTX to abolish the nerve impulse through peripheral nerves when administered perineurally is demonstrated. Therefore, NeoSTX could potentially be used as a local anesthetic to perform surgical procedures in the standing horse due to its dose dependent long-lasting nociceptive blocker properties and in the recumbent anesthetized horse as a part of the regional pain management after a surgical procedure.

This pilot clinical study was not limitations-free, and probably the most important one is the small sample size used which directly affects the representativeness of the statistical results. Additionally, despite we did not specifically evaluated the effect of NeoSTX in the surrounding tissues, according to the previous studies in different animal models no detrimental effects have been reported (Rodriguez-Navarro *et al*., 2007, 2011; Wylie *et al*., 2012; Manriquez *et al*., 2015; Hinzpeter *et al*., 2016; Riquelme *et al*., 2018; Valenzuela *et al*., 2019; Varela *et al*., 2020; Montero *et al*., 2021), however, none of the studies mentioned above did an histopathological evaluation of the injected tissues so it should be considered for future studies.

According to our results, NeoSTX has a rapid anesthetic effect when a dose of 1 mL with 10 μg solution is used to decrease pain arising from the foot. All the above data shows that NeoSTX can be effectively used in a very low volume (1 mL) and can abolish lameness as rapidly as 5 minutes when a PDNB is performed. It is demonstrated that the local anesthetic properties of NeoSTX, when used as a perineural nerve block in horses, are an excellent alternative when a long-lasting anesthetic effect is desired.

ACKNOWLEDGMENTS

This study was supported by grants from the Fondo Nacional de Ciencias FONDECYT #1130037, Chile.

AUTHORS CONTRIBUTIONS

CD participated in the design and implementation of the clinical trial, data analysis and writing of this manuscript. MdC in the analysis of data and writing of this manuscript. NL participated in the clinical design and writing of this manuscript.

CONFLICT OF INTEREST STATEMENT

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

REFERENCES

Alvarez, A., Schumacher, J., & DeGraves, F. (2018). Effect of the addition of epinephrine to a lidocaine solution on the efficacy and duration of palmar digital nerve blocks in horses with naturally occurring forefoot lameness. *American Journal of Veterinary Research*, *79*, 1028-1034. https://doi.org/10.2460/ajvr.79.10.1028

Arkell, M., Archer, R., Guitian, F., & May, S. (2006). Evidence of bias affecting the interpretation of the results of local anaesthetic nerve blocks when assessing lameness in horses. *Veterinary Record*, *159*, 346-349. https://doi.org/10.1136/vr.159.11.346

Boone, L., Schumacher, J., DeGraves, F., & Cole, R. (2019). Buffering mepivacaine with sodium bicarbonate speeds and potentiates analgesia of median and ulnar nerve blocks performed on horses. *Veterinary Record*, *184*, 155. https://doi.org/10.1136/vr.104962

Catterall, W. (2000). From ionic currents to molecular mechanism: the structure and function of voltage-gated sodium channels. *Neuron*, *26*, 13-25. https://doi.org/10.1016/s0896-6273(00)81133-2

Egenvall, A., Lönnell, C., & Roepstorff, L. (2009). Analysis of morbidity and mortality data in riding school horses, with special regard to locomotor problems. *Preventive Veterinary Medicine 88*, 193-204. https://doi.org/10.1016/j.prevetmed.2008.10.004

Hinzpeter, J., Barrientos, C., Zamorano, A., Martinez, A., Palet, M., Wulf, R., Barahona, M., Sepúlveda, J., Guerra, M., Bustamante, T., Del Campo, M., Tapia, E., & Lagos, N. (2016). Gonyautoxins: First evidence in pain management in total knee arthroplasty. *Toxicon*, *119*, 180-185. https://doi.org/10.1016/j.toxicon.2016.06.010

Hoerdemann, M., Smith, R., & Hosgood, G. (2017). Duration of action of mepivacaine and lidocaine in equine palmar digital perineural blocks in an experimental lameness model. *Veterinary Surgery*, *46*, 986-993. https://doi.org/10.1111/vsu.12689

Lagos, N., & Andrinolo, D. (2000). Paralytic shellfish poisoning (PSP): toxicology and kinetics. In *Seafood and Freshwater Toxin: Mode of Action, Pharmacology and Physiology* (1st ed.). Botana L (ed.). Marcel Dekker Inc, New York, USA. pp. 203-215.

Lagos, N. (2014). Clinical applications of paralytic shellfish poisoning toxins. In *Toxins and Biologically Active Compound from Microalgae, Volume 2* (1st ed.). Rossini, G-P (ed). CRC Press Taylor & Francis Group, New York, USA. pp. 309-329.

Kelemen, Z., Grimm, H., Long, M., Auer, U., & Florien, J. (2021). Recumbency as an equine welfare indicator in geriatric horses and horses with chronic orthopedic disease. *Animals*, *11*, 3189. https://doi.org/10.3390/ani11113189

Manriquez, V., Castro, D., Guzmán, R., Naser, M., Iglesias, V., & Lagos, N. (2015). First evidence of neosaxitoxin as a long-acting pain blocker in bladder pain syndrome. *International Urogynecology Journal*, *26*, 853-858. https://doi.org/10.1007/s00192-014-2608-2

McGowan, C. (2011). Welfare of aged horses. *Animals*, *1*, 366-376. https://doi.org/10.3390/ani1040366

Montero, C., Del Campo, M., Bono, M., Simon, V., Guerrero, J., & Lagos, N. (2020). Neosaxitoxin Inhibits the Expression of Inflammation Markers of the M1 Phenotype in Macrophages. *Marine Drugs*, *18*, 283. https://doi.org/10.3390/md18060283

Montero, C., Riquelme, G., Del Campo, M., & Lagos, N. (2021). Neosaxitoxin, a paralytic shellfish poison phycotoxin, blocks pain and inflammation in equine osteoarthritis. *Toxicon*, *204*, 5-8. https://doi.org/10.1016/j.toxicon.2021.10.006

Nagy, A., Bodo, G., Dyson, S., Szabo, F., & Barr, A. (2009). Diffusion of contrast medium after perineural injection of the palmar nerves: an in vivo and in vitro study. *Equine Veterinary Journal*, *41*, 379-383. https://doi.org/10.2746/042516409x372502

Pennel, J., Egenvall, A., Bonnett, B., Olsen, P., & Pringle, J. (2005). Specific causes of morbidity among Swedish horses insured for veterinary care between 1997 and 2000. *Veterinary Record*, *157*, 470-477. https://doi.org/10.1136/vr.157.16.470

Riquelme, G., Sepúlveda, J., Ghumgham, Z., Del Campo, M., Montero, C., & Lagos, N. (2018). Neosaxitoxin, a Paralytic Shellfish Poison toxin, effectively manages bucked shins pain, as a local long-acting pain blocker in an equine model. *Toxicon 141*, 15-17. https://doi.org/10.1016/j.toxicon.2017.11.004

Rodriguez-Navarro, A., Lagos, N., Lagos, M., Braghetto, I., Csendes, A., Hamilton, J., Figueroa, C., Truan, D., García, C., Rojas, A., Iglesias, V., Brunet, L., & Alvarez, F. (2007). Neosaxitoxin as a local anesthetic: preliminary observations from a first human trial. *Anesthesiology*, *106*, 339-345. https://doi.org/10.1097/00000542-200702000-00023

Rodriguez-Navarro, A.J., Berde, C.B., Wiedmaier, G., Mercado, A., García, C., Iglesias, V., & Zurkowski, D., (2011). Comparison of neosaxitoxin versus bupivacaine via port infiltration for postoperative analgesia following laparoscopic cholecystectomy: a randomized, double-blind trial. *Regional Anesthesia & Pain Medicine 36*, 103-109. https://doi.org/10.1097/aap.0b013e3182030662

Ross, M. (2011). Movement. In *Diagnosis and management of lameness of the horse*. (2nd ed.). Ross M, Dyson S (eds.). Elsevier Saunders, St Louis, MO. Pp. 64-80.

Silva, G., De La Corte, F., Brass, K., & Azevedo, M. (2015). Duration and efficacy of different local anesthetics on the palmar digital nerve block in horses. *Journal of Equine Veterinary Science*, *35*, 749-755. https://doi.org/10.1016/j.jevs.2015.07.013

Taylor, A., & Mcleod, G. (2020). Basic pharmacology of local anaesthetics. *BJA Education 20*, 34-41. https://doi.org/10.1016/j.bjae.2019.10.002

Valenzuela, C., Torres, C., Muñoz, V., Simbaina, J.C., Sánchez, A., Bustamante, T., Sepúlveda, J., Piron, R., Del Campo, M., & Lagos, N. (2019). Evaluation of Neosaxitoxin as a local anesthetic during piglet castration: A potential alternative for Lidocaine. *Toxicon 164*, 26-30. https://doi.org/10.1016/j.toxicon.2019.03.021

Varela, X., Del Campo, M., Piron, R., Sepúlveda, J., Bustamante, T., Hinzpeter, J., & Lagos, N. (2019). Effect of Neosaxitoxin on Epidural Anesthesia in Cats: A Promising Alternative to Conventional Anesthetics. *International Physiology Journal 2*, 4-10.

Wylie, M., Johnson, V., Carpino, E., Mullen, K., Hauser, K., Nedder, A., Kheir, J., Rodriguez-Navarro, A., Zurakowski, D., & Berde, C. (2012). Respiratory, neuromuscular, and cardiovascular effects of Neosaxitoxin in Isoflurane-anesthetized sheep. *Regional Anesthesia & Pain Medicine 37*, 152-158. https://doi.org/10.1097/AAP.0b013e3182424566

Universidad Austral de Chile

Facultad de Ciencias Veterinarias

PROGRAMA DOCTORADO

DOCTORADO EN CIENCIAS VETERINARIAS
https://veterinaria.uach.cl/doctorado

DIPLOMADOS

INOCUIDAD DE ALIMENTOS
https://veterinaria.uach.cl/diplomado_ia

DESARROLLO GANADERO: MEJORAMIENTO GENÉTICO. PRODUCTIVO Y REPRODUCTIVO
https://veterinaria.uach.cl/dgmgpr

TÉCNICAS APLICADAS PARA LA INVESTIGACIÓN Y GESTIÓN DE LA FAUNA SILVESTRE
https://veterinaria.uach.cl/taigfs

PROGRAMA MAGISTER

MAGISTER EN CIENCIAS MENCIÓN SALUD ANIMAL
https://veterinaria.uach.cl/magister-en-ciencias-mencion-salud-animal-2

POSTÍTULO

POSTÍTULO EN CIENCIAS CLÍNICAS VETERINARIAS
https://veterinaria.uach.cl/postitulo_cc

INFORMATION AND APPLICATIONS

Escuela de Graduados – Facultad de Ciencias Veterinarias – Universidad Austral de Chile
Tel. 56-63-2221548 – Valdivia – Chile
e-mail: postgvet@uach.cl – https://veterinaria.uach.cl/postgrado

Universidad Austral de Chile

Facultad de Ciencias Veterinarias

FACULTAD DE CIENCIAS VETERINARIAS

VALDIVIA

VOLUME 55 / VALDIVIA - CHILE / 2023 / Nº 2