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ORIGINAL ARTICLES

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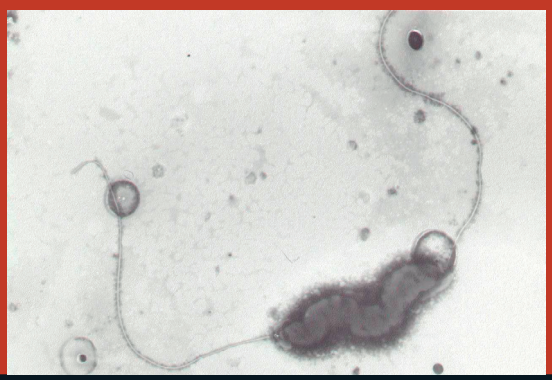

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ORIGINAL ARTICLE

Ultrasonographic study of the right coronary artery in performance horses

Cristóbal A. Dörner[abc*], Constanza Chavarría[a]

ABSTRACT. Historically, echocardiography has focused on the assessment of cardiac dimensions and indices of cardiac function. However, when referred to ultrasonographic coronary arteries characterisation in the horse, information is scarce. The purpose of this study was to describe the right coronary artery in both long and short axis and to analyse its variability between horses with different levels of performance. Sixty healthy horses were included in the study. The animals were eventing horses at different levels of performance and were allocated into two groups according to their athletic level. The internal lumen diameter and the area of the right coronary artery (RCA) were measured in the right parasternal long and short axis views in the 3rd and 4th intercostal space respectively, during systole and diastole. The results were compared between groups using the analysis of variance (ANOVA) and Student's t-test. The correlation between the physiological parameters and the RCA was assessed using the Pearson correlation coefficient. Statistically significant differences were found when the RCA internal diameter was assessed between groups in both short and long axis. There were no statistically significant differences intra-group when short and long axis measurements were compared between each other. A positive correlation was observed between RCA and age, height, left atrium diameter, aortic valve diameter, sinus of Valsalva diameter, and aorta diameter. In conclusion, the level of training might affect the ultrasound measurement of the RCA in sport horses. Non-invasive echocardiography can be used to consistently assess the size of the right coronary artery in athletic horses in both short and long axis.

Key words: coronary artery, echocardiographic, heart, horses, eventing.

INTRODUCTION

The heart is a complex and fascinating organ with a variety of physiological and anatomical properties that aim to pump blood to the body. To make this possible, the heart muscle and its inner structures need to be well irrigated and oxygenated. The blood is distributed through the myocardium thanks to two coronary arteries arising from the right and left sinus of Valsalva (Bright *et al* 2010, Marr and Patteson 2010).

Echocardiography, including two-dimensional (2D), M-mode and various Doppler modalities, have revolutionised equine cardiology. These technologies provide a better understanding of the normal physiology and morphology of the equine heart and an improved ability to diagnose and assess the severity of many forms of heart disease, especially in performance horses. Historically, echocardiography has centred on the assessment of cardiac dimensions and indices of cardiac function. However, when referred to ultrasonographic coronary arteries characterisation in the horse, information is scarce and to our knowledge only one recent pilot study has been published (Siwinska *et al* 2019).

Giving the importance of myocardial irrigation in the normal function of the heart, the evaluation of the coronary arteries in selected cases could be considered during cardiac evaluation, as routinely done in humans (Dodge *et al* 1992, Arnoudse *et al* 2007, Kumar Raut *et al* 2017). Anatomical and morphological studies of the heart in different species have shown the predominance of the right coronary artery (Gómez *et al* 2017) and its ultrasonographic dimension was recently studied in normal horses (n=36) (Siwinska *et al* 2019). The ultrasonographic characterisation of the right coronary artery in the horse is performed in the right parasternal window, long axis at the level of the right outflow tract (Siwinska *et al* 2019). Nonetheless, the technique and normal ultrasound image of the right coronary artery in short axis at the level of the sinus of Valsalva has not been described.

This study aimed to describe the right coronary artery in both long and short axis in the right parasternal window, evaluate its presence repeatability during cardiac evaluation, evaluate if there are any differences in the diameter and/or area when the artery is measured in different axis intra-group, and finally to analyse its variability between horses of different levels of performance.

MATERIAL AND METHODS

Sixty eventing performance horses (21 mares, 39 geldings), clinically healthy and competing at their intended level of performance, aged 5-16 years (mean 8.33 ± 2.589 years old), weighing 495-569 kg with a wither's height of 159-168 cm were included in the study. All horses were Warmblood horses (Holsteiner, Selle Français and Holsteiner x Selle Francais crossbred). A complete physical examination and an echocardiographic examination were performed of each animal included in the study. The exclusion criteria were as follows: systemic illness, dysrhythmia (excluding a physiological second-degree atrioventricular block),

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audible heart murmurs or any heart abnormality found during echocardiography.

The animals were divided into two groups according to their level of performance. Group I contained 30 adult horses ranging from 5 to 8 years old and they were starting at eventing or were competing at CCI1* according to the categorisation provided by the Fédération Équestre Internationale (2020). Group II contained 30 adult animals from 8 to 16 years old that regularly participated in CC2*-S to CCI4*L level, according to the same categorisation. All horses in both groups had undergone regular training for at least one year.

Echocardiography was performed using an Mindray® M-5 ultrasound scanner, equipped with a phased array 2.5-5.0MHz transducer. Prior to echocardiography, the right parasternal window was clipped and the site was washed with chlorhexidine-based soap and warm water. Isopropyl alcohol was then applied as a coupler between the transducer and the skin. Two-dimensional (B-Mode) and M-Mode imaging were conducted at an imaging depth of 30 cm from the right hemithorax. To obtain the left ventricle (LV) measurements, M-Mode was used at the level of papillary muscles. Heart rate (HR), Interventricular septal thickness at the end-diastole and systole (IVSd and IVSs respectively), LV internal diameter (LVIDd and LVIDs), LV free wall thickness (LVFWd and LVFWs) were measured using the ultrasound calliper. Using Teichholz's formula included in the scanner's software, LV fractional shortening (FS), end diastolic volume (EDV), end systolic volume (ESV), LV ejection fraction (EF), stroke volume (SV) and cardiac output (CO) were calculated. Measurements of the RCA were done in B-Mode on both the short and long axis. The right coronary artery lumen was measured in the long axis at the 3rd intercostal space at the right outflow tract (figure 1). The coronary artery was also measured in a short axis at the 4th intercostal space at the level of the aortic sinus of Valsalva (figure 2).

Statistical analyses were run on SPSS, version 19 for Windows (SPSS Inc, Chicago IL, USA). Kolmogorov-Smirnov test was used to determine the normal distribution of the data. ANOVA was run to determine statistical differences between groups on both the long and short axis. A Pearson correlation test was run to determine the association between the RCA and the Two-dimensional (B-Mode) and M-Mode cardiac measurements. Additionally, a paired sample T-test was used to compare the RCA measurements obtained between the long and short axis. The significance level was set at $P<0.05$.

RESULTS

All data were normally distributed. Echocardiographic parameters for both groups are presented in table 1. Group II showed greater measurements when compared with the horses allocated in group I, nonetheless significant differences ($P<0.05$) were found only in the aortic valve, sinus of Valsalva and aorta.

Table 1. Mean values ± and standard deviation (SD) of clinical data and measurements of the left ventricle study using two-dimensional and M-mode ultrasonography.

Parameter	Group I	Group II
Age (years)	6.67 ± 1.35	10.1 ± 2.46
Heart Rate (bpm)	42.1 ± 14.06	44.53 ± 11.86
LA diameter (mm)	104.56 ± 8.82	111.82 ± 12.47
Aortic annulus (mm)	54.40 ± 6.55*	58.82 ± 4.44*
Sinus of Valsalva (mm)	78.32 ± 5.32*	81.02 ± 5.11*
Aorta diameter (mm)	59.74 ± 6.57*	62.77 ± 6.10*
LVIDs (mm)	62.18 ± 14.39	62.09 ± 12.48
LVIDd (mm)	109.09 ± 14.60	112.79 ± 11.36
IVSDs (mm)	45.21 ± 5.33	47.86 ± 5.37
IVSDd (mm)	27.59 ± 3.28	28.27 ± 3.54
LVFWs (mm)	39.78 ± 6.05	38.83 ± 6.41
LVFWd (mm)	22.67 ± 5.08	22.84 ± 5.56
ESV (mL)	201.06 ± 133.14	205.49 ± 105.65
EDV (mL)	697.16 ± 221.16	741.36 ± 166.02
FS (%)	43.16 ± 7.79	45.12 ± 7.11
EF (%)	70.74 ± 8.92	73.14 ± 8.06
SV (mL)	486.68 ± 125.63	536.55 ± 104.57
CO (L/min)	20.35 ± 8.06	24.25 ± 9.33

*Statistically significant difference between groups ($P<0.05$).

In the right parasternal long axis view, the RCA was visible in the form of a centrally located circle with an anechogenic lumen surrounded by a hyperechogenic rim, located ventrally to the right atrium (RA), dorsally to the PA and laterally to the right ventricle (RV) (figure 1). In the right parasternal short axis view, the RCA was visible as parallel hyperechogenic lines with an anechogenic lumen emerging from the right sinus of Valsalva ventrally to the RA, dorsally to the left atrium (LA) and laterally to the Aorta (figure 2). The mean diameter of the RCA for horses in group I was 13.48 ± 1.22 mm in systole and 10.87 ± 0.94 mm in diastole when evaluated on the short axis and 13.5 ± 1.46 mm in systole and 10.48 ± 1.61 mm in diastole when evaluated on the long axis. The RCA calculated mean values for the short axis were 1.437 ± 0.275 cm^2 and 0.94 ± 0.158 cm^2 in systole and diastole respectively. The mean RCA area values for the long axis were 1.448 ± 0.324 cm^2 and 0.881 ± 0.242 cm^2 in systole and diastole, respectively. The mean diameter of the RCA for horses allocated in group II was 15.09 ± 1.46 mm in systole and 11.91 ± 1.22 mm in diastole when evaluated on the short axis and 15.44 ± 1.84 mm in systole and 11.82 ± 1.45 mm in diastole when evaluated on the long axis. When the area was calculated, the mean values for the short axis were 1.805 ± 0.364 cm^2 and 1.126 ± 0.228 cm^2 in systole and diastole, respectively. For the long axis the mean values were 1.896 ± 0.463 cm^2 and 1.111 ± 0.285 cm^2 in systole and diastole, respectively. ANOVA showed statistically significant differences ($P<0.05$) between groups when the RCA diameter and area were analysed in both the short and long axis views at diastole and systole, respectively (table 2). There were no differences between the RCA measurements when compared between long and short axis views.

Pearson coefficient showed a positive correlation between the RCA measurements and age, height, LA diameter,

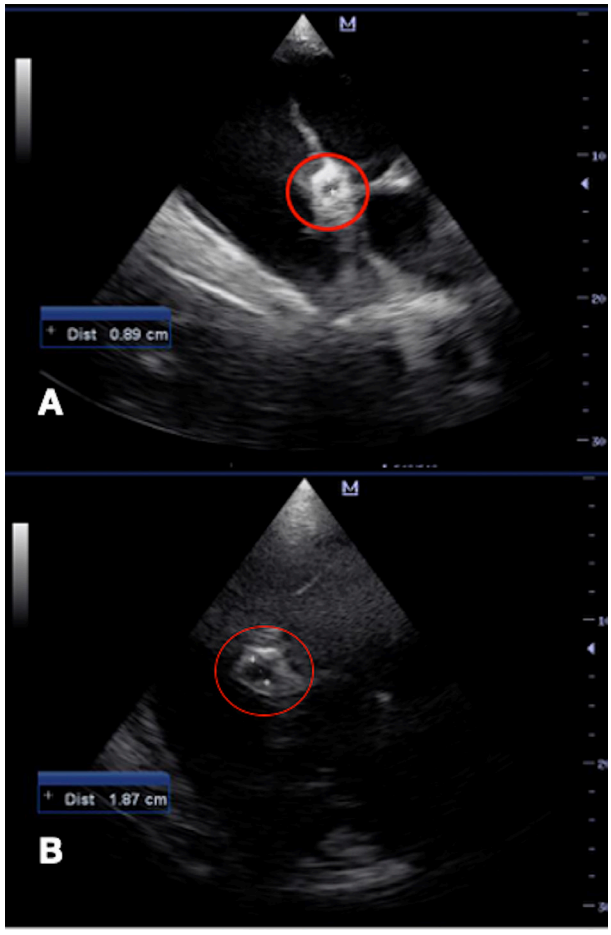

Figure 1. Right parasternal long axis ultrasonographic view of the right outflow tract showing the right coronary artery in diastole (A) and systole (B) as a round hyperechoic image with an anechoic lumen in the centre of the picture. A) Measure of the diameter using the machine calliper with the inner to inner method (red circle). Notice the diastole moment marked by an open tricuspid valve. B) systole marked by a closed tricuspid valve.

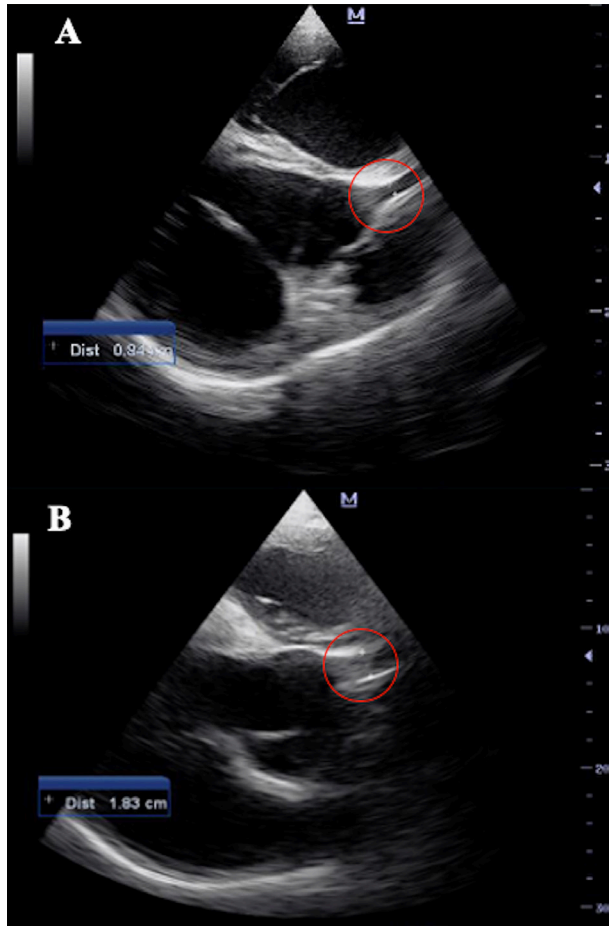

Figure 2. Same horse than figure 1. Right parasternal short axis ultrasonographic view at the level of the aorta (sinus of Valsalva) showing the right coronary artery in diastole (A) and systole (B) as a parallel hyperechoic line emerging from the right sinus of Valsalva. A) Measure of the diameter using the machine calliper with the inner to inner method (red circle). Notice the diastole moment marked by an open mitral valve and closed aortic valve. B) systole marked by an open aortic valve.

Table 2. Mean values ± standard deviation (SD) of the right coronary artery diameter and area obtained from two-dimensional echocardiography.

Parameter	Group I	Group II
Short axis		
RCAs diameter (mm)	13.48 ± 1.22*	15.09 ± 1.46*
RCAd diameter (mm)	10.87 ± 0.94*	11.91 ± 1.22*
RCAs area (cm^2)	1.437 ± 0.275*	1.805 ± 0.364*
RCAd area ((cm^2)	0.94 ± 0.158*	1.126 ± 0.228*
Long axis		
RCAs diameter (mm)	13.50 ± 1.46*	15.44 ± 1.84*
RCAd diameter (mm)	10.48 ± 1.61*	11.82 ± 1.45*
RCAs area (cm^2)	1.448 ± 0.324*	1.896 ± 0.463*
RCAd area (cm^2)	0.881 ± 0.242*	1.111 ± 0.285*

*Statistically significant difference between groups (P<0.05).

Table 3. Correlation between RCA values and age, height, and left ventricle measurements.

	Short axis				Long axis			
	RCA diameter Systole	RCA diameter Diastole	RCA area Systole	RCA area Diastole	RCA diameter Systole	RCA diameter Diastole	RCA area Systole	RCA area Diastole
Parameter	r	r	r	r	r	r	r	r
Age	0.594**	0.470**	0.585**	0.461**	0.472**	0.244	0.459**	0.282*
Height	0.359*	0.260	0.363**	0.268	0.377**	0.272	0.388**	0.184*
LA diameter (mm)	0.412**	0.504**	0.413**	0.509**	0.539**	0.405**	0.534**	0.441**
Aortic valve (mm)	0.442**	0.439**	0.448**	0.430**	0.403**	0.351**	0.413**	0.371**
Sinus of Valsalva diameter (mm)	0.546**	0.574**	0.549**	0.565**	0.450**	0.407**	0.454**	0.440**
Aorta annulus diameter (mm)	0.400**	0.417**	0.412**	0.414**	0.351**	0.278*	0.367**	0.322*
LVIDs (mm)	0.004	–0.020	–0.002	–0.011	0.131	0.084	0.123	0.079
LVIDd (mm)	0.026	0.100	0.023	0.105	0.143	0.173	0.136	0.169
IVSDs (mm)	0.136	0.250	0.138	0.236	0.133	0.188	0.137	0.188
IVSDd (mm)	0.278*	0.216	0.288*	0.217	0.164	0.169	0.178	0.186
LVFWs (mm)	0.198	0.251	0.207	0.241	0.123	0.170	0.131	0.213
LVFWd (mm)	0.289*	0.273*	0.290*	0.266*	0.241	0.139	0.236	0.169
ESV (mL)	0.003	0.076	–0.003	0.076	0.137	0.162	0.127	0.152
EDV (mL)	0.019	0.096	0.014	0.099	0.131	0.168	0.123	0.154
FS (%)	–0.008	0.121	–0.002	0.110	–0.092	0.024	–0.085	0.019
EF (%)	0.004	0.122	0.009	0.110	–0.089	0.015	–0.082	0.013
SV (mL)	0.047	0.168	0.046	0.166	0.111	0.184	0.106	0.167
CO (L/min)	0.050	0.228	0.040	0.217	0.011	0.061	0.001	0.068

r= Pearson Correlation Coefficient
*Correlation present at P<0.05.
**Correlation present at P<0.01.

aortic annulus diameter, sinus of Valsalva diameter, aortic root, and inconstantly with the left ventricle free wall diameter (table 3).

DISCUSSION

Cardiac ultrasound allows measuring the heart structures and the combination of B-Mode, M-Mode and Color Doppler-Mode helps to evaluate its functionality and improves the ability to diagnose and assess the severity of many forms of heart disease (Marr *et al* 2010). However, there is limited information concerning the echocardiographic characteristics of the RCA in horses. This is the first study that evaluates the RCA on both the short and long axis and its characteristics associated with different levels of training in Eventing horses. Fortunately, the RCA is fairly easy to visualise and evaluate during the routine cardiac ultrasound examination in long axis as previously described (Siwinska *et al* 2019) and in the short axis at the level of the aortic valve/ sinus of Valsalva as described in our study.

The diameter of the RCA for horses in group II was larger than the measurements obtained by Siwinska *et al* (2019) or the values obtained by other authors when measuring the RCA in isolated equine hearts *post-mortem* (Thüroff *et al* 1984, Gómez *et al* 2017). These differences can be attributed to the methodology used for measurement under different conditions; *in vivo* echocardiography (Siwinska *et al* 2019), *post-mortem* coronary angiography (Thüroff *et al* 1984) or coronary perfusion with semi-synthetic resin (Gómez *et al* 2017). Additionally, the type of breed used and the study design play an important role. For example, the measurements obtained by Rawling (1977) were based on 10 ponies with an average body weight of 139 kg while the horses analysed by Siwinska *et al* (2019) were horses from different breeds with a wide range of age and with a body weight ranging from 336 to 660 kg. Differences in the echocardiographic measurements between our study and the pilot study published by Siwinska *et al* (2019) could be explained by the factors mentioned previously, given the positive correlation between the echocardiographic measurements and body weight (Rawlings 1977, Al-Haidar *et al* 2013, Siwinska *et al* 2019), breed (Al Haidar *et al* 2013) and height (Siwinska *et al* 2019).

To date, the vast majority of published work examining the heart's remodelling in humans and horses have focused mostly on the left ventricle (Kubo *et al* 1974, Young *et al* 2005, Iskandar and Thompson 2013, Iskandar *et al* 2015, Shave *et al* 2017). This is likely a consequence of the important role the LV plays in generating the cardiac output required to meet the demands during exercise (Shave *et al* 2017). Notwithstanding, cardiac adaptations to exercise/training beyond the LV have been studied and recent evidence in elite human athletes suggests that the left atrium (Iskandar *et al* 2015) and the aortic root diameter at the sinuses of Valsalva and aortic valve annulus respond to athletic training, nevertheless this response to training is not statistically significant and marked aortic root dilatation could likely represent a pathological process rather than a physiological adaptation to exercise (Iskandar and Thompson 2013), therefore, larger sinuses of Valsalva and aortic valve annulus diameter should be evaluated carefully by clinicians.

During training, the heart suffers a variety of physiological and structural changes as a part of the adaptation process to exercise. In human athletes and sport horses, the typical adaptations to training include lower heart rates than average, increase in end-diastolic dimension and maximal stroke volume (Crawford 1983, Bayly *et al* 1983). These cardiac adaptations are coupled with peripheral adaptations in skeletal muscle which increase maximal arteriovenous oxygen content difference during exercise (Bayly *et al* 1983, Thornton *et al* 1983, Tyler *et al* 1996). The increases in cardiac output and arteriovenous oxygen content difference during maximal exercise result in increased maximal oxygen uptake (Thornton *et al* 1983, Tyler *et al* 1996). Also, trained horses have slightly higher relative heart masses (1.1% of body weight) than untrained horses (0.94%), suggesting that training causes hypertrophy of cardiac muscle (Kubo *et al* 1974). This hypothesis has now been supported by a number of longitudinal and cross-sectional echocardiographic studies that have demonstrated increased calculated LV mass and wall thickness following training and a decrease following detraining. Absolute and relative internal cardiac dimensions of equine athletes are also affected by race discipline (Young *et al* 2005).

In human athletes, whilst the existence of a specific phenotype characterised as 'athlete's heart' is generally acknowledged, the question of whether athletes exhibit characteristic vascular adaptations according to the increases in oxygen consumption by cardiac muscle, which is biologically relevant to meet the requirements during exercise, is poorly understood and that is why the concept of 'athlete's artery' as a vascular adaptation has been proposed (Green *et al* 2012). According to the above, there is some evidence for an impact of athletic status on arterial structure and function, so it is tempting to speculate that similar mechanisms for arterial adaptation may be at play in the equine species. In other words, it is thought that athletes have larger coronary arteries and a better vasodilation reservoir (Green *et al* 2012) and according to the aforementioned and the results presented in our study, the same mechanisms may be at play in the horses of our study. Clinically significant diseases of the coronary arteries in animals such as horses and dogs are rare, as opposed to what happens with humans, however, coronary artery anomalies (CAA) may lead to subsequent myocardial ischemia, which is often associated with other underlying diseases such as ventricular tachycardia, atrial fibrillation, and premature ventricular contractions (Driehuys *et al* 1998, Falk and Jönsson 2000, Scansen 2017) and that is why understanding coronary artery anatomy and physiology is

essential in the study of the cardiac response to exercise and training and of other cardiovascular diseases (Cieslinski 1993, Ozgel *et al* 2014, Scansen 2017). Moreover, it would be also interesting to crosslink results from performance dogs and their RCA changes related to exercise with the ones in the present study.

The findings of the present work regarding the echocardiographic measurements according to the level of training/performance are very similar to those of previous reports on different types of breeds and/or discipline (Young *et al* 2005, Shave *et al* 2017). In our study, although the cardiac measurements obtained appear to be larger according to the adaptation process to training, the only measurements significantly larger were the aortic annulus diameter, sinus of Valsalva and aortic root diameter. The above can be explained by the greater pressure in the left outflow tract after systole and the capacity of the aorta to adapt to this increased pressure. On the other hand, the adaptation of the outflow tract of the left ventricle is extremely important in athletes if the entire body and musculoskeletal system have to be correctly oxygenated. Additionally, the RCA was significantly larger in horses competing at a higher level of performance in our study, which is another interesting finding that adds to the scarce information available about right coronary artery characteristics in horses. This situation has been also described in humans where athletes demonstrate increased aortic root dimensions compared with nonathlete controls (Iskandar and Thompson 2013). The aforementioned could be explained by higher oxygen demand in bigger hearts observed in trained horses, possibly due to vascular adaptation to fulfil higher cardiac muscle requirements.

Our study was not limitations-free. The study design is always a limitation when specific animals are used and outer factors need to be controlled. For example, gender distribution (21 mares versus 39 males) might have influenced the results because of the described differences in cardiac morphology between females and males in different species (Dodge *et al* 1992, Evans and Young 2010), nonetheless, it has been also described that dimensional echocardiographic measurements are not affected by gender in horses (Al-Haidar *et al,* 2013, Gomez et al 2017, Siwinska *et al* 2019). Also, the sample size may be low in our study (n=60) but it is higher than the size used by Siwinska *et al* (2019) who considered only 36 horses. Moreover, in that same study, horses from different breeds, wide age range, and wide body weight were used having a variety of factors that could influence their results. In our report, a more specific and less dispersed sample of horses was selected in order to control other factors that might influence our measurements. Age bias between groups could also play an important role in the differences encountered in this study, but there is information available on the correlation between two-dimensional echocardiographic measurements and age being not significantly related in horses (Al-Haidar *et al* 2013, Siwinska *et al* 2019). Al-Haidar *et al* (2013) concluded that dimensional echocardiographic reference values should be established using regression equations as a function of body weight rather than age. In accordance to the above mentioned regarding the correlation between the RCA, age, height, and body weight, in our study we cannot exclude the possibility that part of the differences between groups may be due to these factors, nonetheless, the results show a positive association of RCA measurements and training/performance. Although we found a positive association between the RCA size and level of training/performance, it will probably not be always directly related to the athlete's performance level hence further research under more controlled conditions is warranted to elucidate the clinical impact of larger RCA in performance.

In conclusion, the use of 2D echocardiography to evaluate the size and function of the heart in horses has shown low variability (Rose and Kriz 2002, Decloedt *et al* 2016) and it is a reliable tool to study the RCA in both short and long axis. We have established references values of the right coronary artery in a cohort of sport horses used for the eventing discipline, which complement the information already available for a more detailed cardiovascular evaluation in healthy and ill horses.

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ORIGINAL ARTICLE

Vaccination of nulliparous gilts against porcine epidemic diarrhoea can result in low neutralising antibody titres and high litter mortality

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ABSTRACT. Porcine epidemic diarrhoea (PED) is a disease caused by an alphacoronavirus and the symptoms include watery diarrhoea and vomiting, with more than 80% mortality amongst newborn piglets. The placentation in sows hinders the transference of antibodies to the foetus, therefore, the vaccination of pregnant females and transference of antibodies to piglets through colostrum are essential to protect them against virus particles. The aim of the study was to determine whether vaccination of nulliparous gilts could induce a high colostrum antibody titre and lower litter mortality, in comparison with vaccinated multiparous sows previously exposed to the virus. Samples of colostrum were obtained from 11 nulliparous gilts with two previous vaccinations (inactivated vaccine) and from 9 multiparous sows with three or more vaccinations (inactivated vaccine) that had been exposed to the virus. The IgG antibody titre was determined through anti-PED enzyme-linked immunosorbent assays (ELISA) and the neutralisation of antibodies was evaluated through plaque reduction neutralisation tests (PRNT). The colostrum of nulliparous gilts, when compared to the multiparous sows, presented a lower anti-PED IgG antibody titre as well as fewer neutralising antibodies. Furthermore, the piglets of multiparous sows experienced higher survival in comparison with those of nulliparous gilts ($P<0.01$), and mortality was dependent on the 'farrowing' variable ($P<0.01$). In conclusion, these results show that vaccinating nulliparous gilts does not increase the survival of their piglets in comparison with multiparous sows and that the IgG titres and neutralising antibodies are significantly lower in the former. These results suggest that a modified vaccine strategy is needed for nulliparous gilts to increase piglet protection.

Key words: vaccine, PED, nulliparous, colostrum.

INTRODUCTION

Porcine epidemic diarrhea (PED) is a disease caused by a +ssRNA virus from the *Coronaviridae* family, genus *Alphacoronavirus* (Jung *et al* 2020). It causes watery diarrhoea, vomit, anorexia, and depression in pigs of all age groups. In production units with naïve individuals, which have not been infected nor vaccinated, this disease causes morbidity of up to 100% and 80-100% mortality in newborn piglets, the youngest being the most vulnerable due to their slow enterocyte replacement (Shibata *et al* 2000, Song and Park 2012, Park and Shin 2014, Jung and Saif 2015).

This disease was first identified in May 2013 in the USA (Stevenson *et al* 2013). That same year it spread to Canada and the first outbreak in Mexico was described in 2014, in the State of Mexico. This disease has not been controlled or eradicated in many states of Mexico (Trujillo-Ortega *et al* 2016).

Amongst adult pigs, the most effective adaptative immune response is given by secretory IgA antibodies in the intestinal mucosa and serum IgG antibodies that can cross the epithelial mucosa (Horton and Vidarsson 2013). The type of placentation in sows hinders the transference of antibodies to the foetus, therefore, the transference of antibodies through colostrum and milk is essential for the survival of lactating piglets (Borghesi *et al* 2014). The sow secretes colostrum which contains IgG as the most abundant immunoglobulin during the first 48 hours after farrow, then colostrum is gradually replaced by milk in which secretory IgA is predominant. It is known that maternal antibodies can protect piglets up to 14 days against PED through the neutralisation of virus particles, which stops them from entering their target cell (enterocytes), however, this can vary depending on the sow's level of immunity (Salmon *et al* 2009, Chattha *et al* 2014, Langel *et al* 2016, 2020). To increase this protection, vaccines are designed to promote the transference of immunity to piglets through colostrum and milk. Many studies have reported the effectiveness of using inactivated vaccines in increasing antibodies and diminishing piglet mortality. Even though vaccines have been administered for a few months after the emergence of the disease, studies indicate only a decrease of prevalence from 4.4 to 1.4% in two years at herd-level (Ajayi *et al* 2018) and in some cases the prevalence remain steady between 50 and 60% (Zhang *et al* 2019). In addition, most of these vaccines are inactivated and administered parenterally and

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significant field efficacy has not been observed (Gerdts and Zakhartchouk 2017, Rapp-Gabrielson *et al* 2014).

Therefore, it is important to establish whether there are differences in antibody protection against PED according to the number of vaccinations received or previous exposure to the disease. In this study, we evaluated the response between vaccinated nulliparous gilts and vaccinated and previously exposed multiparous sows, and the association with the titre of neutralising antibodies that are produced after vaccination. It is also important to determine whether the titre of neutralising antibodies is correlated with piglet mortality.

MATERIAL AND METHODS

PIG PRODUCTION UNIT

Colostrum samples were obtained at a pig production unit with a farrow-to-finish system in Puebla, Mexico from a total of 20 females: 11 nulliparous gilts and 9 multiparous sows. According to the sample size calculated in the program "OpenEpi: Open Source Epidemiologic Statistics for Public health" for comparing two means with the results reported by Poonsuk *et al* (2016), we needed at least 4 sows per group for an 80% of power (Poonsuk *et al* 2016). Nulliparous gilts were vaccinated twice before farrowing; the first dose was administered 4 weeks before farrowing and the second dose 2 weeks after. Multiparous sows had been vaccinated at least three times, firstly during their first pregnancy following the same protocol as nulliparous and a booster vaccination was applied after the second pregnancy 2 weeks before each farrowing. An inactivated vaccine was delivered intramuscularly in all cases. The production unit was being affected by a yearly PED outbreak. This protocol was approved by the Institutional Subcommittee for the Care and Use of Experimental Animals (DC2018/2-4).

EVALUATION OF PIGLET MORTALITY UNTIL WEANING DURING A PED OUTBREAK

To calculate the pre-weaning mortality, the reports of deaths amongst piglets that were born alive and had died during lactation were evaluated after weaning.

ANTIBODY DETERMINATION

Colostrum. Al least 8 mL of colostrum was obtained for each sow. Colostrum was treated following the Gomes protocol with the following modifications: centrifuged at 6000g for 60 minutes and inactivated at 56 °C for 30 minutes (Gomes *et al* 2011).

Anti-PED IgG antibodies determination through ELISA (anti-PED immunoenzymatic assay). An immunoenzymatic assay was standardised in which a 96 well Maxisorp plate was coated with PED from the isolate described below. Each well was coated with 50 µL of a 1 µg/mL viral protein in a carbonate buffer solution (pH 9.6) and incubated for 18 hours at 4 °C. Fifty µL of a sample (50 µL of a 1:300 colostrum serum/phosphate buffer solution) were added to each well, followed by incubation for 2 hours at 30 °C. HRP (horseradish peroxidase) protein A was added as a secondary conjugate at a 1:8000 dilution in 50 µL of phosphate buffer solution, and the samples were incubated for one hour at 30 °C. The plates were read 20 minutes after TMB application through a 650 nm filter.

As a quantifiable positive control, 4 mL of serum from two sows that had been immunised against PED on multiple occasions and previously exposed to the virus circulating in the state of Puebla, were precipitated with ammonium sulphate and later dialysed. This precipitate was used to construct a standard curve covering 120 µg/mL to 0.23 µg/mL total protein content. The optical densities of the standard curve and sample readings were analysed with the AssayZap software. It was modelled on a positive sigmoid curve and it was accepted based on the value of b> 0.80 on three repeated occasions.

DETERMINATION OF NEUTRALISING ANTIBODIES

Cell line. For this assay, VERO cells, passage number 25, were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco 12800-017) with 10% foetal bovine serum at 37 °C with 5% CO_2.

Virus isolation. A section of the small intestine was obtained from a piglet, also from the state of Puebla, that tested positive for PEDv through RT-PCR. It was macerated in 500 µL of DMEM and later filtered and used to inoculate VERO cells with 5 mL of infection medium, composed of DMEM with 10 µg/mL trypsin (TB0626) and 1% penicillin-streptomycin-neomycin (100x 15640-055). This mixture was incubated for 72 hours until the characteristic cytopathic effect (syncytia) was observed on more than 50% of the cell monolayer. After incubation, 500 µL of the supernatant were inoculated into another VERO cell culture with infection medium. This procedure was repeated four times. In the end, the presence of the virus was confirmed through end-point RT-PCR with the Verso 1-step RT-PCR ReddyMix kit following the instructions of the manufacturer using the primers 5'-GAATGCAAAACCCCAGAGAA-3'/5'-GTGTCACCACCATCAACAGC-3', which amplify a 156 bp fragment of the viral nucleocapsid, with an annealing temperature of 55 °C.

Virus titration. After the fifth pass, the virus was titrated through a plaque assay. In each well of a 12-well plate, 100,000 VERO cells were plated, and 24 hours later with 80% confluency the virus was inoculated at 1:2, 1:10, 1:100, 1:200, and 1:1000 dilutions with infection medium onto them. The plate was incubated for 2 hours

before the inoculum was removed. Later, each well was supplemented with 1 mL of infection medium with 1.5% carboxymethylcellulose and left incubating for five days. On the fifth day, the medium was removed, and 1 mL of 100% formalin was added. After two hours of incubation at 4 °C, this was replaced by 10% formalin and left to incubate for 15 minutes at room temperature. The plate was washed and each well stained with 1% crystal violet. The titre was calculated following Baer's (2014) formula: the average number of plaques divided by the virus dilution times the quantity of inoculum. The virus was determined to have a 4.3×10^4 PFU/mL titre.

Plaque reduction neutralization test (PRNT). Double dilutions of colostrum from 1:4 to 1:128 were made. Fifty µL of every dilution were incubated with 50 µL of the previously isolated and titrated virus to a 100 $TCID_{50}$ at 37 °C for 30 minutes. Each well of a plate was inoculated with 10,000 VERO cells in 100 µL of DMEM + 10% FBS medium and incubated for 24 hours. Later, 100 µL of the colostrum-virus mixture were added. Every dilution was prepared twice and the mixture was left with the cells for two hours and later removed. Each well was supplemented with 100 µL of infection medium. The plates were checked at 48 and 72 hours. The plates were read and the reciprocal of the percentage of plaque reduction at the average dilution in relation with the virus positive control was used to determine the neutralisation index ([1-(plaques in sample/ plaques in the control)] *100).

STATISTICAL ANALYSES

The results of the analysis in AssayZap were used in the Shapiro-Wilk test for normality and the Levene test for homoscedasticity. A non-parametric Mann-Whitney U test for independent samples, a Kaplan-Meier survival analysis and the Spearman correlation coefficient were used to determine if there were differences between groups. Statistical analyses were carried out on SPSS Statistics v. 24 software.

RESULTS

The colostrum of nulliparous gilts shows fewer antibodies against PED. The determination of PED-specific IgG antibodies in colostrum through ELISA showed the presence of antibodies in all females with an average of 1.3455 g/mL (95% CI=2.2026-0.4884) in nulliparous gilts and 11.7479 g/mL (95% CI=19.2969-4.1989) in multiparous sows (figure 1). This difference was significant ($P<0.01$) according to the Mann-Whitney U test. Furthermore, a positive correlation was observed between the amount of IgG antibodies in colostrum and the number of farrowing of each female (correlation coefficient = 0.821, $P<0.01$).

A significant difference ($P<0.01$) was found through the Mann-Whitney U test in the neutralising antibody titre of colostrum between nulliparous gilts and multiparous sows. The average neutralisation index for nulliparous gilts was 10.00 (95% CI=3.0073-16.99), compared to 26.16 (95% CI=17.83-34.49) amongst multiparous sows. However, the correlation between the neutralisation index and the number of births was 0.577 with $P=0.05$ (figure 2).

The Spearman correlation coefficient between the titres obtained through ELISA and those obtained through PRNT was 0.860 ($P<0.001$), which indicates a high level of correlation between both assays.

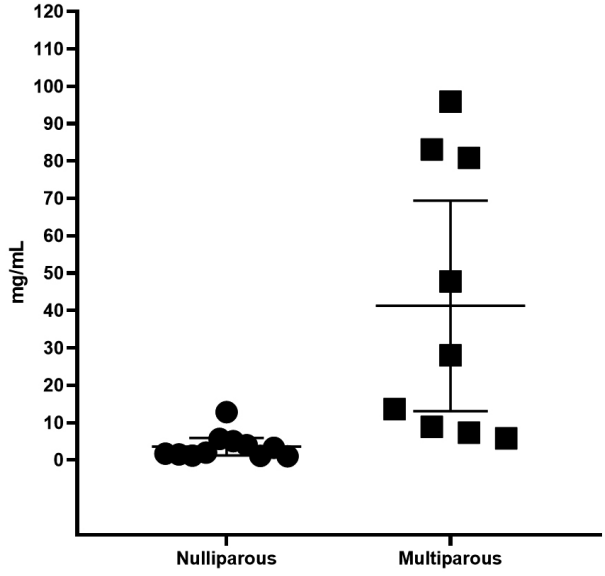

Figure 1. Colostrum anti-PED antibodies detected through indirect ELISA (mg/mL) in nulliparous gilts and multiparous sows. Scatter plot showing the average and 95% confidence interval. $P<0.01$ according to the Mann-Whitney U test.

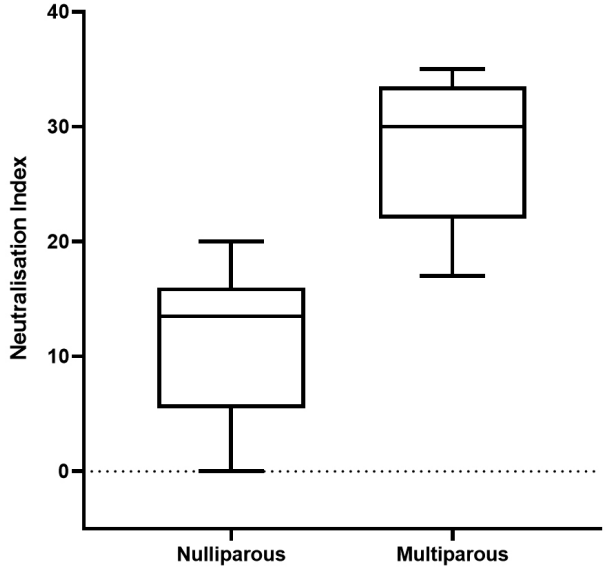

Figure 2. Neutralisation index calculated through plate reduction neutralisation test for PED in cell cultures, using nulliparous gilt and multiparous sow colostrum. $P<0.01$ according to the Mann-Whitney U test.

The percentage of piglet mortality during lactation was evaluated for each female and the cause of death was determined by clinical signs and a positive RT-PCR from a pool of intestinal samples. The litters of five nulliparous gilts had 100% mortality and the litter of another had 50% mortality. Meanwhile, the litter of one multiparous sow had 100% mortality and another had 43% mortality (figure 3A). Overall, the litters of nulliparous gilts had 41.8% mortality while mortality in the litters of multiparous sows was 15.63% (figures 3B and 3C).

The litters of nulliparous gilts tend to experience higher mortality (figure 3), therefore, we decided to evaluate the dependence of piglet mortality to the farrowing variable and its risk, as well as the number of dead piglets in each group, with a piglet survival curve. A χ^2 test for independence concluded that there was no independence between mortality and farrowing (P<0.01). The relative risk between mortality and nulliparity was 2.675 (95% CI=1.700-4.212).

A significant difference was found in piglet survival between nulliparous gilts and multiparous sows (P<0.01). The average survival time of the piglets of nulliparous gilts was 16.479 (15.170-17.789) days, compared to 18.627 (17.679-19.575) days among piglets from multiparous sows (figure 4).

Since multiparous sows present a higher antibody titre, a correlation between that variable and piglet survival is to be expected. A correlation analysis between the number of weaned piglets and the amount of anti-PED IgG antibodies as determined through ELISA was carried out, finding near-significant results (P=0.055).

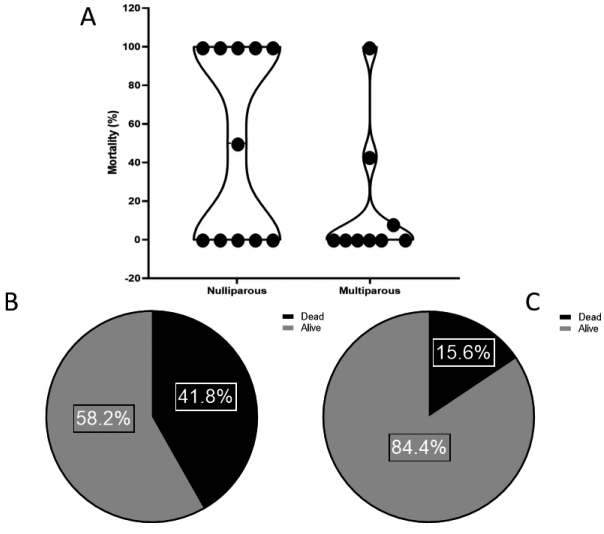

Figure 3. a) Violin plot showing piglet mortality percentage due to PED in nulliparous gilts and multiparous sows. Each dot represents one female's litter. b) Pie chart showing the percentage of piglets from nulliparous gilt litters that died due to PED. c) Pie chart showing the percentage of piglets from multiparous sow litters that died due to PED.

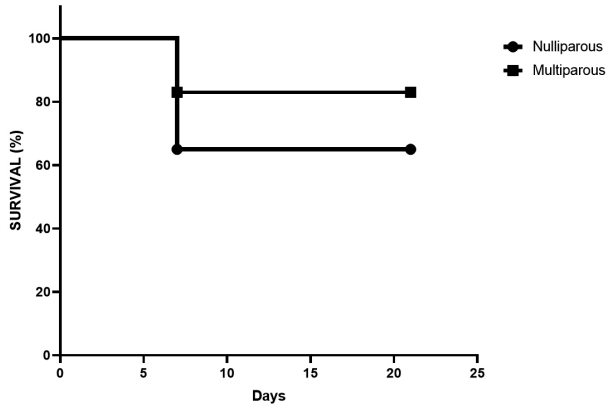

Figure 4. Kaplan-Meier survival curve of piglets born from nulliparous gilts and multiparous sows until weaning (at 21 days old) in a pig production unit undergoing an active PED outbreak P<0.01.

DISCUSSION

In this study, the level of antibodies in the colostrum of nulliparous gilts was lower than that of multiparous sows, despite two immunisation treatments and having been exposed to the virus before farrowing. Furthermore, a low titre of antibodies was correlated with the neutralisation index, suggesting that females which have been immunised following this vaccination schedule may not generate an appropriate response. These results disagree with those reported by Paudel *et al* (2014) who evaluated the vaccination and revaccination of nulliparous gilts with three different protocols: two immunisations using inactivated virus, two immunisations using attenuated virus, or the first immunisation using active virus and the second immunisation using inactivated virus; the best response was obtained with two immunisations using inactivated virus (Paudel *et al* 2014). It is worth noting that in that study, the neutralising capacity of the antibodies generated was not measured and neither was the protection they provided to litters during outbreaks.

In this study, multiparous sows which had been previously exposed to the virus and vaccinated showed a higher antibody titre when compared to nulliparous gilts that had been vaccinated but not previously exposed to the virus. These results are consistent with those obtained by Shwartz (2016), who evaluated the efficacy of an inactivated virus vaccine in two different groups, females previously exposed to the virus (vaccinated and unvaccinated) and females that had not been exposed to the virus (vaccinated and unvaccinated). In that study, a difference in serum antibodies was found across all groups, with females that had been both exposed to the virus and vaccinated showing

the highest average IgG antibody titre. As for IgA, only previously exposed groups had levels above the threshold for positivity. However, for an adequate antibody (IgG and IgA) titre in colostrum to be attained, a field exposure to the virus was necessary regardless of vaccination. This was reflected in piglet mortality, as the litters of females that had not been exposed to the virus had 100% mortality regardless of vaccination, while those of previously exposed females who had not been vaccinated had less than 10% mortality[1]. In our study, piglet mortality among nulliparous gilt litters was not 100% in all litters but was nonetheless significantly higher than that of multiparous sow litters. This could be the result of gilts not being exposed to the field virus before receiving the vaccination.

Around 20% of females are replaced annually in pig production systems. These females undergo quarantine before being immunised against various diseases, including PED. If immunisation is not effective, replacement females and their litters remain susceptible to the disease and newborn piglets may suffer high mortality and morbidity (Kweon *et al* 2000). Colostrum and milk are the most important sources of protection for piglets, particularly of neutralising antibodies (Langel *et al* 2016). This study compared the neutralising capacity of the colostrum of vaccinated nulliparous gilts and multiparous sows, finding that the former had a lower neutralisation index (IN =10.00) in comparison with the latter (IN = 26.16). Furthermore, a correlation with the results of the ELISA assay of 0.86 was found, similar to that reported by Oh *et al* (2005) (84.2%) across 1,024 serum samples. Song *et al* (2007) immunised sows with an attenuated virus vaccine orally and intramuscularly and found that colostrum antibody titres and their neutralising capacity were correlated in both groups, however, upon challenging their piglets, mortality amongst those born from females that had been vaccinated orally was 13%, while the litters of intramuscularly vaccinated females experienced 60% mortality. This could be due to the production of IgA in animals immunised through mucosae and could also explain why the females in our study, which had been exposed to the virus (multiparous sows), were able to better protect their litter, similar to the results by Song *et al*. To demonstrate this, IgA levels in females should be measured.

In our study, the litters of nulliparous gilts experienced on average 50% mortality, while those of multiparous sows averaged 17.66% mortality. However, Rapp-Gabrielson *et al* (2014) administered an inactivated virus vaccine and a placebo in a farm undergoing an active PED outbreak and saw a 90% decrease in mortality among the vaccinated group when compared to the placebo. Nonetheless, piglet mortality in the placebo group was only 6.3%, which does not reflect the conditions of an outbreak such as the one in our study. Studies by Mogler (2014) and Crawford (2015) reported that subunit vaccines reduce the clinical signs and viral excretion post-challenge amongst weaned piglets. They observed that mortality in litters of nulliparous females was reduced to 69% after three pre-delivery immunisations, compared to 91% in the control group. Previously exposed females showed similar results, with piglet mortality going down from 59% to 45% with vaccination. These mortality rates are similar to those reported in our study, however, the infection in that study was controlled and not due to a natural outbreak (Crawford *et al* 2015, Mogler *et al* 2014, Rapp-Gabrielson *et al* 2014).

The results obtained in this study show that mortality is dependent on farrow number, with nulliparous gilt litters being at the highest risk. Furthermore, a statistical difference in survival length was observed, even when some nulliparous gilt litters showed no mortality. It is known that the main source of infection amongst newborn piglets is the mother's faeces due to the excretion of virus particles via this route. This factor, along with the number of antibodies transferred to piglets by females, will determine the development of this disease in piglets. Brown *et al* (2019) measured the excretion of PED viral particles amongst four nulliparous gilt groups with four different treatments: not previously exposed to the virus (Control), previously exposed to the virus (Nv), previously vaccinated twice with an inactivated virus vaccine (Pre), and vaccinated twice after being challenged with the virus (Pos). They found that only the group that had been previously exposed to the virus showed a shortened period of virus excretion, with only 10% of individuals still being positive after three weeks and a relative risk of 4.022 compared to the control. In contrast, more than 40% of females in vaccinated groups was still excreting the virus on the fourth week. Variations in mortality seen in our study could be the result of the different period of time in which each female was infected as the production unit was undergoing an uncontrolled active outbreak of the disease (Brown *et al* 2019). However, as the viral load of each female upon delivery was not determined, this variable cannot be correlated to antibody titres and piglet mortality and also the amount of antibodies that the new-borns adsorb was not determined.

In this study, we used only one isolated virus to determine the quantity of antibodies. Nonetheless, Lara-Romero *et al* (2018) characterised the spike gene from several isolates collected in México, showing a 99% homology between them (Lara-Romero *et al* 2018). Moreover, Wang *et al* (2016) found 55% of cross neutralisation between antibodies generated against the G1 and the G2 strain (Wang *et al* 2016).

In conclusion, this study shows that the colostrum of nulliparous gilts, despite being vaccinated twice, has

[1] Schwartz TJ, Rademacher CJ, Gimenez-Lirola LG, Sun Y, Zimmerman JJ. 2016. Evaluation of the effects of PEDV vaccine on PEDV naïve and previously PEDV exposed sows in a challenge model comparing immune response and preweaning mortality. *American Association of Swine Veterinarians*, 363-366. Available at https://www.aasv.org/foundation/research/Schwartz_QReport_2015_12.pdf

lower antibody counts and neutralising activity than the colostrum of multiparous sows. This suggests a poor response to vaccination in nulliparous sows, associated with a lesser passive immunity amongst piglets and lower survival in the litters. Multiparous sows showed a good antibody response to PED that is most likely associated with the multiple vaccinations or the previous exposure to the disease. Therefore, to improve immune response in sows the vaccines should be applied along with immune modulators that protect since the first delivery, in addition to the regular biosafety measures employed in pig commercial farms to reduce viral dissemination in the farrowing area.

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ORIGINAL ARTICLE

Effects of *Moringa oleifera* and *Brosimum alicastrum* partial feed substitution in intramuscular fat and adipose tissues and on the expression of lipogenic genes of Mexican hairless pigs

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ABSTRACT. The present study evaluated the effects of the inclusion of ground *Moringa oleifera* and *Brosimum alicastrum* leaf meal in the diet of Mexican hairless pigs (MHP) on the amount of intramuscular fat, subcutaneous fat, leg muscle (*Biceps femoris*) fat, loin muscle (*Longissimus dorsi*) fat, leg and back fat, and the expression of lipid metabolism genes. Hairless pigs are reared in the Mexican tropics and are characterised by their body and intramuscular fat accumulation. Eighteen male pigs fed for 82 d were randomly allotted to three experimental isoenergetic and isoproteic diets, where *M. oleifera* or *B. alicastrum* (six pigs per diet) replaced wheat bran. The diets used were a control diet, a diet with 10% *M. oleifera* leaf meal, and a diet with 10% ground *B. alicastrum* leaf meal. The *M. oleifera* diet decreased ($P<0.05$) the fat ratio in the *Longissimus dorsi* muscles, back fat, rib fat, total carcass fat, and the carcass fat: meat ratio. The *B. alicastrum* diet only decreased fat in the *Biceps femoris* muscle, back fat, and rib fat. *Moringa oleifera* and *B. alicastrum* diets also promoted the overexpression of mRNA from the stearoyl-CoA desaturase (SCD), fatty acid synthase (FASN), acetyl-CoA carboxylase alpha (ACACA), sterol regulatory element-binding protein 1 (SREBP1) and acyl carrier protein (ACP) lipogenic genes in the *Biceps femoris* muscle and leg fat ($P<0.001$). In addition, lower ACACA and SREBP1 mRNA expression in the *Longissimus dorsi* muscle and back fat ($P<0.001$) were related to the lower amount of fat in pigs fed *M. oleifera* and *B. alicastrum*. The inclusion of *Moringa oleifera* and *Brosimum alicastrum* meals 10% in the diet of the pig MHP reduces fat, this is an important finding because fat is abundant in this type of pig.

Key words: *Brosimum alicastrum*, gene expression, meat quality, Mexican creole pig.

INTRODUCTION

Mexican hairless pigs (MHP) have an obese phenotype because it tends to accumulate fat from an early physiological stage (Santos *et al* 2011). MHP are adapted to tropical rural environments in Mexico, where they have lived for more than 500 years. They are mainly fed unconventional food resources, but also consume typical forages of the region (Becerril *et al* 2009, Ramos-Canché *et al* 2020). Despite their social and economic importance in rural and backyard communities, these pigs are considered endangered (Hernández *et al* 2020). However, there is a growing demand for their products (Ramos-Canché *et al* 2020).

The body fat accumulation of MHP and the nutritional value of their meat have been previously studied (Dzib-Cauich *et al* 2020). They differ from commercial pigs in terms of performance and genetic background (Lemus-Flores *et al* 2001, Lemus-Flores *et al* 2020). According to Becerril *et al* (2009) and Aboagye *et al* (2019), their body fat accumulation can be attributed to genetic reasons. Specifically, the expression of lipogenic genes in, for example, adipose and muscular tissues has been found to affect fat accumulation (Duran-Montgé *et al* 2009, Benítez *et al* 2016, Fernández *et al* 2017, Wang *et al* 2020). However, the fat content of the meat is also influenced by diet composition, as in other types of pigs (Duran-Montgé *et al* 2009, Mohan *et al* 2012, Benítez *et al* 2016, Wood and Enser 2017).

On the other hand, the increase in back and intramuscular fat is a characteristic of local pigs of Iberian origin, which is important for the elaboration of meat products of high economic value. In these pigs, fattening is generally promoted to improve the quality of fatty acids in meat. This is the reason why, these pigs are often fed with acorns or oils with oleic fatty acids (Fernández *et al* 2007, Benítez *et al* 2016). However, feeding MHP with conventional corn-soybean diets further increases back fat, and the carcass yield is not as high as other commercial pigs (Dzib-Cauich *et al* 2020). Therefore, different feeding approaches should be applied to commercial and local pigs to modulate the content and quality of fat (Mohan *et al* 2012, Albuquerque *et al* 2017, Aboagye *et al* 2019).

An alternative to commercial pig productions in rural areas is backyard or semi-intensive pig production systems. Such systems could reduce feed and production costs, while still produce good-quality meat products (Ramos-Canché *et al* 2020). When social, economic, and environmental challenges are considered, tree foliage and feeding patterns are clearly sustainable for animal husbandry such as pigs. Two forage sources in tropical and subtropical environments are *Moringa oleifera* and

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Brosimum alicastrum trees. Their use as forage for pigs has been previously studied (Moyo *et al* 2011, Dzib-Cauich *et al* 2016, Ly *et al* 2016, Zhang *et al* 2019). They are alternative protein sources in animal feeds because of the protein content of their leaves (Castro-González *et al* 2008, Teixeira *et al* 2014). In addition, they are rich sources of fatty acids, soluble fibre, and antioxidants (Moyo *et al* 2011). A limitation of forages in pigs feed is that high fibre content is associated with impaired nutrient utilisation and reduction of net energy values (Thacker and Haq, 2008). However, MHP can eat up to 40% *M. oleifera* leaf meal without effect on its productive response (Ortiz *et al* 2015); this could be associated with its large intestine and more active microflora. The present research was carried out to determine the effect of the inclusion of *M. oleifera* or *B. alicastrum* leaf meal in the diet of MHP on the amount of intramuscular fat, subcutaneous fat, leg muscle (*Biceps femoris*) fat, loin muscle (*Longissimus dorsi*) fat, leg and back fat, and on the expression of genes involved in lipid metabolism in tissues.

MATERIAL AND METHODS

ANIMALS AND DIETS

The present research was carried out at the Agricultural and Livestock Production and Research Unit of the "Instituto Tecnológico de Conkal" in Yucatan, Mexico (21° 05´ N and 89° 32´ W). The dominant climate of the region is warm sub-humid with rain mostly in summer. The ethics, care and welfare and handling of the pigs followed the guidelines of the official Mexican standards (NOM-062-ZOO-1999[1], NOM-051-ZOO-1995[2] and NOM-033-SAG/ZOO-2014[3]). In addition, the experiment was carried out following the procedures related to the handling of animals approved by ITConkal in 2015. This study is also part of a report of doctoral studies.

Moringa oleifera and *Brosimum alicastrum* leaves were collected from a forage bank after 55 d of growth. The harvested material was dehydrated in a conventional oven[4] at 50 °C for 48 h. Subsequently, it was ground to a particle size of 1.0 mm. The meal obtained was stored in hermetic containers until reaching 750 kg, and the chemical composition was calculated according to the standard method of the AOAC (1997). Each value was then a mean of three measures (table 1).

Eighteen castrated MHP males with an average initial live weight of 22 ± 1.11 kg were evaluated. They were distributed in a completely randomised design, each pig, representing an experimental unit. They were housed in 2.1 m x 1.1 m individual pens with slatted floor and were allowed *ad libitum* access to water and feed for 82 d. The animals were identified with ear tags. All pigs were slaughtered at the end of the experimental period, regardless of their weight. The animals fasted at least 16 h before slaughter. The animals were sacrificed at the slaughterhouse of the Autonomous University of Yucatán by exsanguination after electrical stunning.

The control diet was based on corn, soybean meal and wheat bran. The diet of *M. oleifera* or B. *alicastrum* contained corn, soybean meal, wheat bran and 10% flour *M. oleifera* or 10% *B. alicastrum* leaf meal. The diets were isoenergetic and isoproteic, where wheat bran was replaced with *M. oleifera* or *B. alicastrum* (table 2 and 3). Diets were formulated for pigs with a live weight of 22-50 kg according to N.R.C. (1998). Another article reported consumption and performance productive in growth.

Samples, fat measurement, and gene expression analysis

Forty-five min after slaughter, the carcasses (meat, fat, and bones without the skin, head, legs, and viscera) of each pig were weighed immediately. Back fat was measured at the height of the 10th rib (mm), and the primary pieces were weighed, separating the meat, bone, and fat to obtain the weight of the cold carcass (kg), performance of the carcass in relation to live weight (kg), and carcass fat: meat ratio (kg fat/kg meat). Rib weight (kg) and fat (kg) were also measured. Fifty grams of the *Longissimus dorsi* and *Biceps femoris* muscles were obtained to quantify intramuscular fat following the methodology described by the AOAC (1997) for lipids.

In addition, at the moment of slaughter, three 0.5 g samples of the *Longissimus dorsi* muscle, *Biceps femoris* muscle, back fat, and intramuscular fat of the leg muscles were taken for analysis of gene expression. The samples were collected in 2.0 mL cryotubes with a stabilising solution (DNA/RNA Shield, Zymo Research, USA), placed on ice and stored at −20 ºC. Of the collected tissues, 75 mg were weighed and the Direct-zolTM RNA MiniPrep kit[5] was used for RNA extraction according to manufacturer instructions. The concentration and purity were quantified using Nanodrop spectrophotometry[6]. Subsequently, cDNA synthesis was carried out with 1000 ng of RNA from each

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[4] Terlab, (Jalisco, México)

[5] Zymo Research (California, USA)

[6] Thermo Fischer Scientific (Wilmington, USA)

Table 1. Chemical composition of ground *Moringa oleifera* and *Brosimum alicastrum* leaf meal (dry matter %), each value was mean of three measures.

Leaf meal	Fat	Protein	Ash	Moisture
Brosimum alicastrum	3.60±0.17	20.63±0.01	1.17±0.02	9.55±0.21
Moringa oleifera	4.97±0.14	23.68±0.37	1.12±0.01	7.97±0.09

Table 2. Ingredients and calculated composition in experimental diets (%).

Ingredients	Diet		
	CD	MOD	BAD
Corn	35.96	38.37	37.95
Soybean meal	11.52	11.28	11.73
Wheat bran	48.07	35.91	36.5
Sunflower oil	2	2	2
Moringa oleifera leaf meal	0	10	0
Brosimum alicastrum leaf meal	0	0	10
Calcium phosphate (monocalcium)	1.08	1.13	1.11
Calcium carbonate	0.75	0.72	0
Vitamin premix[a]	0.05	0.05	0.05
Mineral premix[b]	0.1	0.1	0.1
Lysine	0.22	0.16	0.28
Methionine	0	0.03	0.03
Salt	0.25	0.25	0.25

CD = control diet; MOD = *Moringa oleifera* diet; BAD = *Brosimum alicastrum* diet.
[a]Content in one kg: Vitamin A, 8 000 000 IU; Vitamin D3, 500 IU; Vitamin E, 35 000 IU; K3, 1.250 g; Thiamine, 500 mg, Riboflavin, 2 g; Piridoxin, 500 mg, Niacin, 10 g, pantoteneic acid, 5 g, Antioxidant, 125 g, Vitamin B12, 7.5 mg, Biotin, 25 mg; Methionine was added on MOD and BAD diets to make it similar to CD diet.
[b]Content in one kg: Iron, 100 g; Manganese, 100 g; Zinc, 100 g; Copper, 10 g; Iodine, 300 mg; Selenium 200 mg; Cobalt, 100 mg.

Table 3. Calculated chemical composition of experimental diets (% of dry matter).

	Diet[a]		
	CD	MOD	BAD
Crude protein	16.00	16.00	16.00
ME[b] (Mcal kg^{-1} of feed)	2.91	2.86	2.88
Ether extract	5.67	5.62	5.69
Neutral detergent fiber	24.71	22.90	23.63
Acid detergent fiber	7.88	8.95	8.95
Ca	0.60	0.60	0.62
P	0.50	0.50	0.50
Lysine	0.96	0.96	0.96
Methionine	0.26	0.26	0.26
Ca/P	1.20	1.22	1.23

[a]CD = control diet; MOD = *Moringa oleifera* diet; BAD = *Brosimum alicastrum* diet.
[b]ME = metabolizable energy.

Table 4. Sequence of primers used for gene expression analysis.

Genes	Primer sequence 5'→ 3'	Amplicon length (bp)	Tm °C	Access number
ACACA	F- ATGTTTCGGCAGTCCCTGAT R- TGTGGACCAGCTGACCTTGA	133	62	AF175308
FASN	F- CGTGGGCTACAGCATGATAG R- GAGGAGCAGGCCGTGTCTAT	108	64	AY954688
ACP	F-CAGCAGGCCAGGTCAGCATT R- GTCGACATGCCAACGCAGGA	236	60	XM_001924222
SCD	F- GCCGAGAAGCTGGTGATGTT R-CAGCAATACCAGGGCACGAT	95	56	AY487829
SREBP1	F- CGGACGGCTCACAATGC R-GACGGCGGATTTATTCAGCTT	114	64	NM_214157
RNA S18	F-GGCCTCACTAAACCATCCAA R-TAGAGGGACAAGTGGCGTTC	98	64	XM_012100710

SCD = stearoyl-CoA desaturase; FASN = fatty acid synthase; ACACA = acetyl-CoA carboxylase alpha; SREBP1 = sterol regulatory element-binding protein 1; ACP = acyl carrier protein; F = forward primer; R = reverse primer; bp = base pair; Tm °C = melting temperature; RNA S18 = endogenous gene.

sample using the Maxima H Minus First Strand cDNA Synthesis Kit and dsDNase kit[7].

Real-time polymerase chain reaction (PCR) was performed with a Step-One Plus Real-Time PCR kit[8] and the SYBR Green/ROX qPCR Master Mix (2x) kit[9], with a final volume of 20 uL per reaction. The expression of the genes acetyl-CoA carboxylase alpha (ACACA), stearoyl-CoA desaturase (SCD), sterol regulatory element-binding protein 1 (SREBP1), acyl carrier protein (ACP), and fatty acid synthase (FASN) were evaluated (table 4). These genes have been associated with lipid metabolism in pigs (Duran-Montgé *et al* 2009). The endogenous RNA18S gene was used to normalise gene expression. The triplicate real-time amplification of each sample was carried out in 40 cycles under the following conditions: initial denaturation at 95 °C for 8 min and cycling at 95 °C for 15 s and 60 °C for 30 s. The specificity of the amplification of each array was confirmed by the dissociation curve analysis and the temperature ramp of this analysis was 60 °C to 95 °C over 5 s. The reading of the dissociation curve yielded only one peak for each sample, confirming the amplification of each gene. No amplification was detected for the negative samples used as quality controls.

[7] Thermo Fischer Scientific (Wilmington, USA)

[8] Applied Biosystems (Stockholm, Sweden)

[9] Fermentas Thermo Fischer Scientific (Wilmington, USA)

STATISTICAL ANALYSIS

The carcass and fat content variables were analysed using the model: $y_{ij} = D_i + e_{ij,}$ where D_i is the i^{th} diet (control diet [CD], *M. oleifera* diet [MOD], and *B. alicastrum* diet [BAD]). Mean differences were tested using the Duncan test ($P<0.05$). Principal component analysis was carried out to associate the measured fat variables with the diets.

A statistical analysis of the gene expression data of each tissue was performed following the method of Steibel *et al* (2009) and Benítez *et al* (2016), which consists of the analysis of the cycle threshold value (Ct) of the target and endogenous genes. In addition, a linear mixed model with univariate analysis of the gene expression registered for the SCD, FASN, ACACA, SREBP1, and ACP genes was used: $y_{gijk} = G_i + A_j + e_{ijk}$, where $y_{gijk} = -\log2 (E_g - Ct_{gijk})$, E_g is the PCR efficiency (E) of each gene (g), Ct_{gijk} is the value obtained from the thermal cycler software for the gene corresponding to the k^{th} repetition of the j^{th} animal belonging to the i^{th} group, G_i is the i^{th} group-specific effect on gene expression, A_j is the specific random effect on the quantitative polymerase chain reaction (qPCR) expression of the gene in the j^{th} pig, and e_{ijk} is the residual effect. Three different groups corresponding with the CD, MOD, and BOD diets were considered in the model. Differences were obtained between groups in the expression rate of the genes of interest (diffG = control group-test group) normalised by the endogenous gene. Contrasts were performed to obtain differences between groups for each gene (Steibel *et al* 2009). The adjusted *P*-values of the diffG were calculated using the Bonferroni correction method. The relative change values in gene expression (FC) were

calculated from the estimated diffG values according to the following equation: $FC = 2^{-diffG}$. All calculations were performed in SPSS v. 20 (2011).

RESULTS

INTRAMUSCULAR AND SUBCUTANEOUS FAT

Carcass weight and performance were not affected by the experimental diets. The MOD diet decreased the ratio of intramuscular fat of the *Longissimus dorsi* muscle (IMFLD) and *Biceps femoris* muscle (IMFBF), back fat at the 10th rib, total carcass fat, carcass fat: meat ratio, and rib fat (table 5). The MOD and BAD diets had a similar decreasing effect on IMFBF, back fat, and rib fat compared to the control diet. The multivariate analysis of the main components shows the association of higher amounts of fat with the control diet (figure 1).

GENE EXPRESSION IN LONGISSIMUS DORSI AND BICEPS FEMORIS MUSCLES, INTRAMUSCULAR FAT OF LEG MUSCLES, AND BACK FAT

As shown in table 6, the FC of gene expression shows statistical differences between different tissue samples. In general, in the *Biceps femoris* muscle and the internal

Table 5. Differences mean in intramuscular and body fat of pigs fed *Moringa oleifera* and *Brosimum alicastrum*.

Variable	Diet			SEM	P<
	CD	MOD	BAD		
Slaughter weight, kg	51.18[a]	51.80[a]	51.04[a]	0.63	Ns
IMFLD, %	13.45[a]	11.02[b]	11.92[ab]	0.70	0.05
IMFBF, %	10.10[a]	7.57[b]	8.24[b]	0.64	0.05
Back fat at 10th rib, mm	2.90[a]	2.28[b]	2.43[b]	0.18	0.01
Cold carcass weight, kg	32.97[a]	34.08[a]	33.21[a]	0.58	Ns
Carcass yield, %	64.4[a]	65.79[a]	65.07[a]	0.51	Ns
Carcass total fat, kg	12.91[a]	11.26[b]	11.55[ab]	0.51	0.09
Carcass fat: meat ratio, kg/kg	0.87[a]	0.70[b]	0.74[ab]	0.05	0.05
Rib fat, %	35.36[a]	30.14[b]	28.94[b]	1..89	0.01

[a,b]Values with different superscripts differ within a file ($P<0.05$).
CD = control diet; MOD = *Moringa oleifera* diet; BAD = *Brosimum alicastrum* diet; IMFLD = intramuscular fat of the *Longissimus dorsi muscle*; IMFBF = intramuscular fat of the *Biceps femoris muscle*.

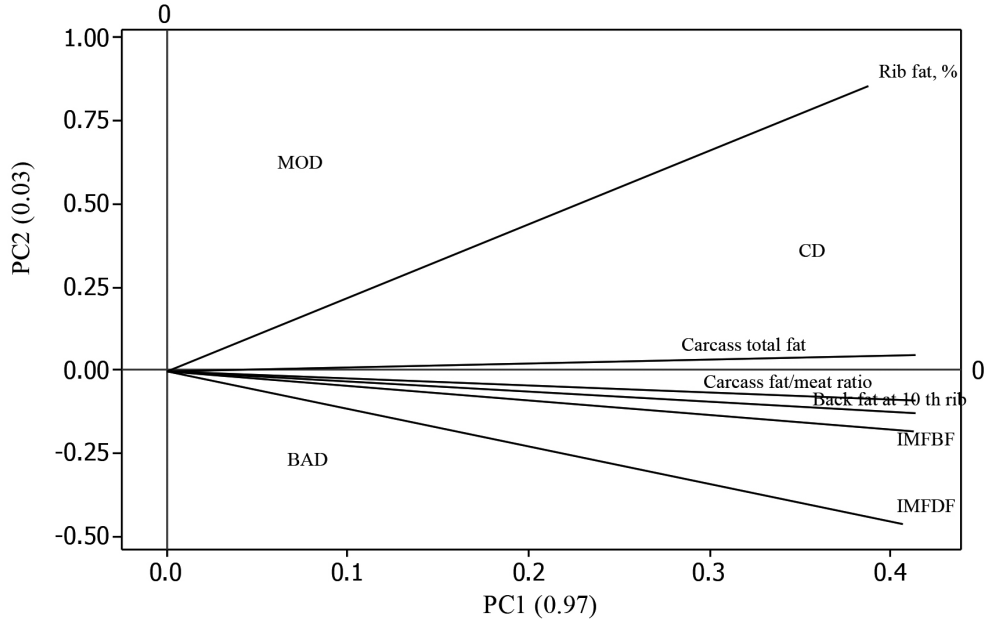

Figure 1. Principle component analysis between diets and fat measurement variables. CD = control diet; MOD = *Moringa oleifera* diet; BAD = *Brosimum alicastrum* diet; IMFLD = intramuscular fat of the *Longissimus dorsi* muscle; IMFBF = intramuscular fat of the *Biceps femoris* muscle.

Table 6. Fold change (FC=2^{-diffG}) of gene expression in different tissues between diets.

Contrast	Genes				
	ACACA	SCD	SREBP1	ACP	FASN
Biceps femoris muscle					
MOD-CD	1.10**	1.25**	0.91	1.09**	1.08**
BAD-CD	1.93**	1.12**	1.18*	1.75**	1.97**
SEM	0.01	0.02	0.08	0.01	0.03
Internal fat of leg muscles					
MOD-CD	1.21**	1.24**	1.55**	1.07**	1.07*
BAD-CD	1.49**	2.10**	1.44**	1.69**	1.34**
SEM	0.02	0.01	0.09	0.02	0.03
Longissimus dorsi muscle					
MOD-CD	0.60**	0.79**	0.34**	0.75**	0.84**
BAD-CD	0.87**	1.17**	0.51**	0.90**	1.10**
SEM	0.02	0.01	0.02	0.01	0.02
Back fat					
MOD-CD	0.58**	0.70**	0.42**	0.80**	0.74**
BAD-CD	0.61**	0.67**	0.47**	0.79**	0.74**
SEM	0.02	0.01	0.02	0.01	0.01

SCD = stearoyl-CoA desaturase; FASN = fatty acid synthase; ACACA = acetyl-CoA carboxylase alpha; SREBP1 = sterol regulatory element-binding protein 1; ACP = acyl carrier protein.
Values with different superscripts differ within a file *=P<0.05, **=P<0.001

fat of the leg muscles, it was observed that the mRNA expression of lipogenic genes was more abundant compared to the *Longissimus dorsi* muscle and back fat. Meanwhile, in the *Biceps femoris* muscle, no difference in the expression of the SREBP1 gene was observed between the MOD and CD diets. In addition, the highest expression of SCD and FASN in the *Longissimus dorsi* muscle was in the BAD diet followed by the MOD diet, both with respect to the CD.

DISCUSSION

These results agree with those of Mukumbo *et al* (2014), who found that the fat ratio of the *Longissimus thoracis* muscle decreased in pigs fed 7.5% *M. oleifera* leaves. Zhang *et al* (2019) did not observe an effect on the fat percentage of the *Longissimus thoracis* muscle in commercial pigs fed 3% to 9% *M. oleifera* for 45 d. However, the fatty acid content was modified, with an increase in monosaturated fatty acids and omega-3. These results are similar to those of Dzib-Cauich *et al* (2016) in MHP fed 20% to 40% *M. oleifera* in the diet. Pérez and García (2017) in Cuba fed 20% *M. oleifera* for 60 d and found a decrease in back fat without an effect on weight and carcass performance.

The inclusion of these forage species in animal feed is multipurpose because they have good bromatological characteristics, good palatability and digestibility, and a low amount of antinutritional compounds (Ly *et al* 2016, Rojas-Schroeder *et al* 2017). In addition, *M. oleifera* and *B. alicastrum* forages are characterised by a large amount of bioactive compounds, including vitamins, carotenoids, and polyphenols (Moo-Huchin *et al* 2019). Thus, the consumption of their seeds and leaves has been shown to have beneficial effects (Moyo *et al* 2011, Rojas-Schroeder *et al* 2017). In addition, *M. oleifera* leaves reduced the levels of phospholipids, triglycerides, and cholesterol in the serum of growing pigs (Adegbenro *et al* 2016), suggesting a general reduction in lipid mobilisation and lipogenesis and confirming its relationship with a decrease in body fat. Adisakwattana and Chanathong (2011) evaluated *M. oleifera* leaf extract (in distilled water) *in vitro* and found that it led to a decrease in the enzymatic activity of pancreatic cholesterol esterase, which is related to lipid digestion and absorption. Specifically, the hydrolysis of cholesterol ester is catalysed by pancreatic cholesterol esterase, which releases cholesterol to the small intestine. Therefore, the inhibition of cholesterol esterase activity limits the absorption of cholesterol in the diet. Furthermore, phenolic compounds present in *M. oleifera* and *B. alicastrum*, such as phenolic acids and flavonoids (Moo-Huchin *et al* 2019), could contribute to the fat-reducing effects and play an important role in lipid regulation (Ezzat *et al* 2020). Zhang *et al* (2020) showed that the inclusion of 0.50% and 0.75% fermented *M. oleifera* leaves in the diet of white Peking ducks had a positive effect on the productive performance and characteristics of the carcass, reducing lipid deposition in liver and adipose tissues. This result

was associated with a reduction in serum adiponectin levels and an increase in leptin and insulin.

As shown here and confirmed by other studies, the incorporation of *M. oleifera* and *B. alicastrum* forage to the diet is one alternative for reducing body fat that does not affect weight or carcass performance.

The expression of lipogenic genes seems to differ according to tissue and diet, which affect lipid metabolism (Duran-Montgé *et al* 2009, Benítez *et al* 2016, Fernández *et al* 2017, Wang *et al* 2020). SCD is related to fatty acid esterification and ACP, ACACA, and FASN with lipogenesis, whereas, SREBP1 is a transcription factor. In the present study, the increase in the mRNA expression of lipogenic genes in pigs fed MOD and BAD diets in *Biceps femoris* muscle and internal fat of leg muscles was not related to a decrease in the amount of fat, indicating possible changes in novo fatty-acid synthesis, which was not studied here. However, MHP meat is known for its nutritional value, specifically, its high proportions of polyunsaturated fatty acids, omega-6, omega-3, docosahexaenoic acid. It also contains high proportions of palmitoleic acid/palmitic acid, oleic acid/stearic acid, linoleic acid/oleic acid, linoleic acid more linolenic acid/oleic acid, and lower values of saturated fatty acids and monounsaturated fatty acids (Dzib-Cauich *et al* 2020). Similar results were found in Iberian pigs (Benítez *et al* 2016, Fernández *et al* 2017). This effect could be explained through the SCD gene, which is the main enzyme involved in the synthesis of fatty acids. This enzyme is involved in the desaturation process necessary for MUFA biosynthesis, particularly for the synthesis of oleic acid from stearic and for the synthesis of palmitoleic from palmitic fatty acids (Fernández *et al* 2017).

Guillevic *et al* (2009) obtained identical results after feeding pigs sunflower or flaxseed oil. The carcass characteristics were not affected, and the lipid content of tissues did not increase, indicating that the enzymatic activity of lipid metabolism-related enzymes was poorly altered. However, they found that the fatty acid content was affected by the ingredients in the diet. In the present study, the decrease in the mRNA expression of the five lipogenic genes in the *Longissimus dorsi* muscle and back fat suggests an effect of the decrease in IMFLD, back fat at the 10th rib, total carcass fat, carcass fat: meat ratio and rib fat. According to Wang *et al* (2020), the low expression of FASN could be reflected in a lower intramuscular fat deposition because it is a predictor of the intramuscular fat content of the *Longissimus dorsi* muscle. The low expression of SREBP1, a transcription factor, is associated with the low expression of ACP, FASN, and SCD, as occurred in MOD (Mohan *et al* 2012). According to Mohan *et al* (2012) and Benítez *et al* (2018), a sunflower oil diet decreased the expression of FASN, SCD, and SREBP1. The decrease in SREBP1 could be due to an increase in the omega-6 polyunsaturated fatty acids, which in turn would reduce the expression of FASN and SCD. Duran-Montgé *et al* (2009) observed that the expression of the FASN and SCD genes was reduced in the liver and muscle of pigs fed with polyunsaturated fatty acids, as similarly found here for MOD.

The expression of ACACA and SREBP1 could be related to the lower amount of fat present in the pigs fed the MOD and BAD diets. ACACA plays a fundamental role in the metabolism of fatty acids and acts as an intermediary in the de novo synthesis of long-chain fatty acids (Muñoz *et al* 2007), and the SREBP1 gene is a regulator of the action of other genes in lipid metabolism and accumulation (Chen *et al* 2008, Mohan *et al* 2012). The over-expression of SREBP1 in MHP has been related to an increase in the intramuscular fat of the *Longissimus dorsi* muscle compared to commercial pigs fed a corn-soy diet (Dzib-Cauich *et al* 2020). Also, in the present study, the SCD and FASN genes were overexpressed in the *Longissimus dorsi* muscle and back fat and showed differences between the BAD and MOD diets, probably in association with the decrease in the amount of fat.

Internal fat of leg muscles, *Biceps femoris* muscle, loin back and *Longissimus dorsi*, the expressions of the genes differed as previously reported, indicating that the lipogenic action varies according to tissue and diet (Duran-Montgé *et al* 2008, Duran-Montgé *et al* 2009, Benítez *et al* 2016). The present study demonstrates that the *M. oleifera* diet decreased the ratio of intramuscular fat of the *Longissimus dorsi* muscle, intramuscular fat of the *Biceps femoris* muscle, back fat, total carcass fat, carcass fat: meat ratio, and rib fat. The *B. alicastrum* diet only decreased IMFBF, back fat, and rib fat compared with the control diet. In addition, the inclusion of *M. oleifera* and *B. alicastrum* leaf meal in the diet increased the mRNA expression of the lipogenic genes (SCD, FASN, ACACA, SREBP1, and ACP) in the *Biceps femoris* muscle and in the intramuscular fat of the leg muscles and decreased gene expression in the *Longissimus dorsi* muscle and back fat. The low expression of ACACA, mRNA, and SREBP1 in the *Longissimus dorsi* muscle and back fat was associated with the lower amount of fat in these tissues in pigs fed both the *M. oleifera* and *B. alicastrum* diets.

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ORIGINAL ARTICLE

Effect of the inclusion of herbal phosphatidylcholine on palatability, digestibility and metabolisable energy of the diet in dogs

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ABSTRACT. This study aimed to evaluate the palatability, nutrient digestibility, metabolisable energy (ME) and faecal characteristics of diets in dogs fed increasing levels of herbal phosphatidylcholine (herbal mix) *versus* an unsupplemented diet (with only 377 mg choline provided by 1 kg food) or choline chloride[1] (2000 mg choline/kg food) in 40 adult dogs. In experiment 1, a palatability test was conducted to make two pairwise comparisons: 0 versus 200; and 0 versus 400 mg/kg herbal mix. In experiment 2, a digestibility test was performed to evaluate herbal mix at 0, 200, 400 and 800 mg/kg and 2000 mg choline provided by choline chloride. Results from experiment 1 indicated that the dogs preferred diets containing herbal mix to the unsupplemented diet ($P<0.05$). In experiment 2, nutrient digestibility and faecal characteristics were not influenced by the treatment ($P<0.059$). The inclusion of 400 mg/kg of herbal mix increased the ME (quadratic effect, $P<0.01$). In conclusion, the results of this study indicate that the inclusion of a herbal mix rich in phosphatidylcholine (1.6%) and other methylated metabolites at 400 mg/kg can fully replace choline chloride in dog diets.

Key words: *Canis familiaris*, feed plant additive, pet food, taste preferences.

INTRODUCTION

Choline is a "quasi-vitamin" (Morrison *et al* 2018) and one of its metabolic pathways is the formation of phosphatidylcholine, the main component of the cell membrane and lipoproteins (Vance and Vance 2008). The NRC (2006) recommendations for choline are based on the results of experiments conducted in the 1930s and 1940s (AAFCO 2015). An evaluation of 75 diets for healthy adult dogs (homemade diets) showed that more than 50% did not meet the choline requirements (Pedrinelli *et al* 2019). However, the needs could be overestimated (German *et al* 2015) and, consequently, both the requirement and different forms of the nutrient must be reviewed in a broader sense. Feeds supply choline in different forms: free choline (Cho), glycerophosphocholine (GPC), phosphocholine (Pcho), phosphatidylcholine (Ptdcho) and sphingomyelin (SM)[2] all of which may differ in bioavailability (Cheng *et al* 1996, Lewis *et al* 2016), transport mechanisms (Sheard and Zeisel 1986), lost by microbial metabolism (Zeisel *et al* 1989) and ATP expenditures to be available in the cells (Fagone and Jackowski 2013).

Phosphatidylcholine is a central metabolite in the functions associated with choline (Zeisel and da Costa 2009). There is evidence that a herbal mix containing *Achyranthes aspera*, *Azadirachta indica*, *Citrullus colocynthis*, *Trachyspermum ammi* and *Andrographis paniculata* contributes with phosphatidylcholine (16 g/kg herbal mix) and other methylated metabolites (Roque *et al* 2020). Several species of domestic animals replace choline chloride by the herbal mix. In broilers, bird performance was similar with herbal choline and synthetic choline (Calderano *et al* 2015). In finishing lambs, Godínez-Cruz *et al* (2015) reported the same performance with 4 g/d of herbal mix versus 4 g/d of a rumen-protected choline (25% choline), whereas Crosby *et al* (2017) used the same source choline levels and reported a large bodyweight gain during gestation in suckling ewes. In dairy cows, the herbal mix (17 g/d) increased milk production, and improved fertility and health (Gutiérrez *et al* 2019).

Synthetic choline (choline chloride) has high hygroscopicity (Calderano *et al* 2015) and is a highly reactive compound that requires attention during the preparation of premixes. Herbal choline, on the other hand, is stable but has aromatic volatile compounds (Mendoza *et al* 2019) that could affect the palatability of canines (Wynn and Fougere 2007). Herbal mix has the advantage of providing nutraceutical metabolites (Roque *et al* 2020) that can be beneficial for health (Di Cerbo *et al* 2017). Therefore, the objective of this experiment was to evaluate the effects of increasing the dietary levels of a herbal mix rich in phosphatidylcholine and other methylated metabolites on food palatability, nutrient digestibility, metabolisable energy content, as well as on faecal characteristics, compared to a

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[1] Surfamex S.A. de C.V.

[2] USDA, United States Department of Agriculture. 2008. Database for the choline content of common foods; release 2. Nutrient Data Laboratory, Beltsville Human Nutrition Research Center, USDA. Availble at: https://data.nal.usda.gov/dataset/usda-database-choline-content-common-foods-release-2-2008

not supplemented diet or a diet with supplemented choline chloride in dogs.

MATERIAL AND METHODS

The experiments were approved by the Academic Committee for Animal Use of the Doctoral Program in Animal Science and Agriculture of the Universidad Autónoma Metropolitana and were conducted in the Centro de Investigación en Alimentos para Mascotas (CIAM), in Tepeji del Rio, Hidalgo, México.

The basal diet (table 1) was extruded to kibbles (Dosky PT290, Canis Food Nutrition) and it was formulated following the nutritional recommendations of NRC (2006) and the Association of American Feed Control Officials (AAFCO 2015) for adult dogs. The vitamin and mineral premixes were manufactured by DSM Nutritional Products of Mexico. Herbal mix (BioCholine®, Nuproxa México -Switzerland) and choline chloride (52% choline, Surfamex SA de CV) were used to prepare the following dietary treatments: unsupplemented diet (with only 377 mg choline/kg food), choline chloride (2000 mg choline/kg of diet) and three levels of herbal mix (200, 400 and 800 mg/kg) with a phosphatidylcholine concentration of 1.6%.

The inclusion content of choline (provided by choline chloride) in the diet was established according to the choline requirements for an adult dog (NRC 2006). The amount of 400 mg/kg of herbal mix was based on a previous experiment where it was concluded that a unit of herbal mix can replace five units of choline chloride (Mallo and Paolella 2017). Productive performance in calves (Díaz-Galvan *et al* 2021) has shown a quadratic response, therefore herbal mix concentrations included a low (200 mg/kg) and upper (800 mg/kg) concentration.

Diets were stored for 15 days in sealed bags protected from direct sunlight at a room temperature of 20.5 ± 1.2 °C and relative humidity of 68.5 ± 2.3% before being supplied to the dogs. The ingredient composition of the basal diet and the analysed chemical composition of the experimental diets are shown in table 1.

Forty (twenty males and twenty females) healthy adult dogs (*Canis lupus familiaris*: 4.6 ± 1.6 years old, dewormed and vaccinated) were individually housed in concrete kennels (2 m width x 5 m long). The dogs were Beagles (23), Schnauzer (8), Bichón Friseé (3), Dachshund (3), Airedale Terrier (2) and Jack Russell (1).

EXPERIMENT 1 - PALATABILITY STUDY

Two palatability tests were performed in two pairwise comparisons: 0 versus 200 mg/kg and 0 versus 400 mg/kg herbal mix using a pair-wise diet comparison with trained dogs as described by Felix *et al* (2012) in 30 min periods

Table 1. Ingredient composition of basal diet (g/kg, as fed).

Ingredient	g/kg
Maize	467.0
Meat meal	250.0
Corn gluten meal	60.0
Regular soybean meal (460 g crude protein/kg)	50.0
Poultry fat	50.0
Wheat meal	44.0
Wheat bran	40.0
Swine liver hydrolysate	20.0
Experimental premix (vitamins, minerals and NaCl)	11.44
Sand	3.0
Yeast cell wall and beta glucan	1.5
Calcium propionate	1.0
Sodium butirate	1.0
Caramel coffee	0.4
Titanium dioxide	0.66
Total	1000
Chemical composition	%
Dry matter intake	90.50
Crude protein	24.05
Crude fiber	2.56
Ether extract	12.05
Ash	12.71

for two consecutive days calculating the food preference according to the intake ration of diet A as:

$$Intake\ ratio = \left[\frac{g\ of\ diet\ A\ or\ B\ intake}{g\ of\ total\ food\ offered\ (A+B)}\right]\times 100$$

The dogs used in food preference were evaluated to discard left and right-sided laterality according to record tests and quality tests where dogs were fed the same food and significant differences were not expected (Tobie *et al* 2015).

Statistical analysis. Data were analysed with R software (v 2.15.3, Auckland, New Zealand) package fdANOVA (Górecki and Smaga 2019). The results of palatability were analysed considering 40 observations per test (20 dogs × two days per test). The data obtained on the relationship or proportion of consumption were first analysed with the Kruskal-Wallis test, which did not reveal any influence (P>0.05) of breed, gender (male and female), or test day on consumption preference. The normality of the data was tested and then a Kruskal Wallis test and a t-test were performed to determine whether the consumption preference data of the herbal mix diets were different from 0.50 with a significance level of 1%.

EXPERIMENT 2 - DIGESTIBILITY ASSAY

Dogs were randomly allocated into 5 dietary treatments which consisted of the unsupplemented diet (negative control), the diets supplemented with the herbal mix at different concentrations (200, 400 and 800 mg/kg) and the diet supplemented with choline chloride (positive control, 2000 mg choline/kg food), with eight repetitions per treatment. The assay lasted 17 days, leaving the last 5 days of this period for total faeces collection (AAFCO 2015). Dogs were weighed to allocate enough food to meet their metabolisable (ME) energy requirements $\left[ME, Mcal/d = 130 \times \left(Body\ weight, kg^{0.75}\right)\right]$ (NRC 2006) by providing food once a day (10:00 h). Water was freely available and feed consumption was recorded daily.

Digestibility was performed using the total faecal collection method for five days (Corsato and Gregory, 2018) after 12 days of adaptation to the experimental diets. Total faeces from dogs were collected twice daily with spatula and forceps, faeces were stored in plastic bags and stored at -20 °C until analysis (Corsato and Gregory 2018). Feed (kibbles) and faeces were oven dried and analysed for dry matter (AOAC method 934.01), crude protein (AOAC method 2001.11), ether extract (AOAC method: 954.02 for feed with acid hydrolysis, and 920.39 for faeces with diethyl ether), and ash (AOAC method 942.05) (AOAC 2015). Neutral detergent fibre (NDF) was analysed with thermostable amylase and acid detergent fibre (ADF) according to Van Soest *et al* (1991). Gross energy was determined with an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL). The apparent digestibility of crude protein, ether extract, ash, and ADF was estimated. The ME was calculated using the equations of the NRC (2006), estimating energy losses in the urine based on the protein content of the food (Duque-Saldarriaga *et al* 2017).

The faecal score was also evaluated for five consecutive days using conventional scores from 1 to 5, considering a score 1 as hard and dry faeces, 2 as hard, formed and dry faeces, 3 as soft, formed and moist faeces (it retains shape), 4 as soft and unformed faeces (faeces take the shape of container) and 5 as watery (liquid) faeces without shape (Clapper *et al* 2001).

Statistical analysis. Data were analysed with R software (v 2.15.3, Auckland, New Zealand) package fdANOVA (Górecki and Smaga 2019). The normality was analysed using Shapiro-Wilk test and then data were analysed according to a complete randomised design, with 5 treatments and a total of eight replicates per treatment. The means of dry matter intake, nutrients digestibility and metabolisable energy with herbal mix (200, 400 y 800 mg/kg) and choline chloride (2000 mg choline/kg basal diet) were compared with orthogonal contrasts. Linear and quadratic effect of treatments inclusion levels on response variables were evaluated with orthogonal polynomial contrasts. Faecal scores were analysed with a Kruskal-Wallis test ($P<0.05$). The faecal score was reported as means as according to Carciofi *et al* (2009).

RESULTS

EXPERIMENT 1 - PALATABILITY STUDY

Dogs consumed a greater quantity of food when the diets contained herbal mix which resulted in a higher intake ratio ($P<0.001$) in the two pairwise comparisons (table 2). The consumption index was higher than 0.5 and the t-test confirms that the dogs had a preference for foods with herbal mix ($P<0.001$) in the two trials.

EXPERIMENT 2 - DIGESTIBILITY ASSAY

The results of the digestibility test and the metabolisable energy values are shown in table 3. Intake was similar among treatments ($P>0.05$) and nutrient digestibility was

Table 2. Preference test of dogs$^{\gamma}$ fed paired diets with different concentrations of herbal mix.

	Intake index	CI	
		Lower	Upper
Assay 1			
Control	0.3384^{b}	0.281	0.395
Herbal mix**, 200 mg/kg	0.6615^{a}*	0.604	0.718
Assay 2			
Control	0.3697^{b}	0.295	0.444
Herbal mix, 400 mg/kg	0.6303^{a}*	0.556	0.705

CI: Confidence interval

$^{\gamma}$: Forty dogs per test.

* The value means that the intake ratio of the diets with herbal mix was different from 0.50 according to the T test ($P<0.001$), ^{ab} Values with different literals within the column are different ($P<0.0001$).

** The phosphatidylcholine concentration in herbal mix is 1.6%.

Table 3. Effect of herbal mix level *versus* choline chloride on nutrient digestibility and metabolisable energy in dog diets.

	Herbal mix*, mg/kg				Choline chloride, mg/kg		*P*-value		
Item	0	200	400	800	2000**	SEM	L	Q	Contrast***
Dry matter intake, g/d	231.1	236.3	239.0	227.2	239.9	22.5	0.97	0.66	0.90
Nutrients digestibility, %									
Dry matter	76.7	77.5	79.0	77.4	77.6	1.71	0.67	0.49	0.87
Crude protein	86.8	86.6	85.1	84.7	86.4	1.65	0.29	0.93	0.62
Ether extract	83.3	76.2	87.8	80.9	83.8	2.98	0.72	0.97	0.64
Acid detergent fibre	32.6	37.0	32.2	38.3	35.3	5.56	0.61	0.87	0.92
Gross energy	88.3	88.7	86.7	87.3	88.6	1.25	0.40	0.95	0.41
Metabolisable energy									
Mcal/kg	3.67	3.73	3.86	3.70	3.8	0.04	0.23	0.01	0.99

* The phosphatidylcholine concentration in herbal mix is 1.6%.
** This is equivalent to 2000 mg choline/kg food. The choline is supplied with a choline chloride product with 60% of choline.
*** Contrast: Herbal mix vs. choline chloride
SEM: Standard error of the mean, L: Linear effect, Q: Quadratic effect.

Table 4. Consistency of faeces by treatment and statistical information.

	Herbal mix*, mg/kg				Choline chloride, mg/kg
	0	200	400	800	2000**
Faecal score	2.70	2.95	2.90	2.80	2.62
Coefficient of variation, %			12.9		
Kruskal-Wallis test					
Chi-square test			4.46		
P value			0.34		
Shapiro-Wilk test			0.57		

* The phosphatidylcholine concentration in herbal mix is 1.6%.
** This is equivalent to 2000 mg choline/kg food. The choline is supplied with a choline chloride product with 60% of choline.
Index scale: 1 = very hard and dry stools; score; 5 = pasty stools.

not affected by the treatments. The inclusion of the herbal mix showed an increase ($P<0.01$, quadratic response) in the metabolisable energy of the ration with the highest values with the intermediate concentrations of the herbal mix (400 mg/kg). Faecal characteristics of the dogs fed diets with increasing levels of herbal mix or choline chloride were not different ($P>0.05$, table 4).

DISCUSSION

EXPERIMENT 1 - PALATABILITY STUDY

A previous study comparing the dietary supplementation of 500 mg/kg of herbal mix against 2000 mg of choline chloride showed the feasibility of replacing the synthetic product without affecting dog acceptability (Mallo and Paolella 2017). The herbal mix is composed, among other plants, by *Azadirachta indica*, a medicinal plant recognised for its bitter taste (Ogbuewu *et al* 2011). This characteristic, together with the carnivorous nature of the dogs and their olfactory capacity, made us expect a lower intake of the food supplemented with herbal mix. In contrast, there was a higher preference defined as a relatively high probability of ingesting one of two available foods under specific conditions (Griffin and Bleider 1984), presumably associated with the secondary metabolites of other plants in the mixture. The herbal mix includes *Trachyspermum ammi* which contains thymol (Vitali *et al* 2016) which is an essential oil that has significantly stimulated consumption in pigs (Michiels *et al* 2012). The herbal mix contains more than 100 volatile compounds among which is (Z) -2-octenal (Mendoza *et al* 2018, Mendoza *et al* 2019),

a volatile compound present in many plants, fruits and flowers (Chen *et al* 2016, Tietel and Masaphy 2017) that has shown to stimulate the olfactory epithelium in rats (Araneda *et al* 2004).

Food preference can be associated with intrinsic feed factors but also with external conditions in dogs; Koppel *et al* (2013) pointed out that the presence of ingredients with secondary metabolites such as alcohols, aldehydes, ketones, esters, sulfur compounds, pyrazines, furans, alkanes, derived from benzene and terpenes resulted in complex odour characteristics that can be associated with the acceptability of the food by the dog and the results showed that the probability of dogs rejecting food with herbal mix is very low, but information on the intake for longer periods is required since there are some limitations presented by the short tests or others related to preference procedures as described by Griffin and Bleider (1984).

EXPERIMENT 2 - DIGESTIBILITY ASSAY

The digestibility of dry matter in commercial dog foods has been reported in the range of 89 to 94% (Alvarado *et al* 2008). However, similar values to those observed in this study have been reported particularly for maize or sorghum based diets (de Oliveira *et al* 2012). Nevertheless, the effects of choline, choline-contributing compounds or herbal sources on DM digestibility have not been reported.

In this experiment, the possibility of some effect of the herbal mix on digestibility was considered, since it provides phosphatidylcholine (also known as lecithin) (Demattê Filho *et al* 2015). Phosphatidylcholine is a metabolite that participates in the solubilisation of bile acids (Barrios and Lichtenberger 2000) and it is recognised that choline phospholipids are important for lipid digestion in the small intestine by the combined action of pancreatic phospholipase A2 IB and mucosal enzymes (jejunoileal brush border phospholipase B/lipase and mucosal secreted phospholipase A2 X) (Nilsson and Rui -Dong Duan 2019). The phospholipase A2 is the predominant digestive enzyme in the pancreatic juice and with the colipase, cleaves the ester linkages in the triacylglycerol releasing two free fatty acids and monoacylglycerol (Murota 2020). The lack of response in digestibility suggests that the contribution of choline-related compounds (free choline, Cho), glycerophosphocholine (GPC), phosphocholine (Pcho), phosphatidylcholine (Ptdcho) and sphingomyelin (SM) in the basal diet have been sufficient to contribute to the dietary phospholipids for the various functions in the digestion and absorption of essential fatty acids (Murota 2020). Based on tables provided by the USDA (2008) we estimated a concentration of total choline of 377 mg/kg (calculated as the sum of Cho, GPC, Pcho, Ptdcho, and SM) in the unsupplemented diet which would have resulted in an intake lower than the dog nutrient requirement (NRC 2006). However, the nutritional requirement for choline in dogs requires a review as other studies have observed that intakes below 40 mg/d of choline give similar results (German *et al* 2015). The results of fat digestibility were lower than those reported in other studies with dogs, with values above 90.1% (Donadelli and Aldrich 2019, Jackson *et al* 2020) and even values of 94% (de Souza *et al* 2019).

The absence of changes in the digestibility coefficients coincides with the faecal score since these two variables are related (Brambillasca *et al* 2010). This indicated that there were no differences in faecal matter excreted, consistency (Sunvold *et al* 1995), or fermentation in the colon (Donadelli and Aldrich 2019) presumably with no changes in water flow to the lumen (Felix *et al* 2013), which determines moisture in the stool (Donadelli and Aldrich 2019). The average faecal scores observed in this study (close to 3) is desirable for pet owners (Clapper *et al* 2001).

The use of herbal mix (400 mg/kg food) increased the ME by 5.03%, compared to the negative control. Similar results were observed in lambs, where the use of the same herbal additive translated in a higher daily gain due to an increment of the available energy in the feed (Martínez-Aispuro *et al* 2019). The higher ME content in the dog food with intermediate doses of herbal mix could be explained by three mechanisms that may be occurring at the same time. First, phosphatidylcholine from herbal mix follows a different metabolic pathway than free choline from choline chloride, expending less metabolic energy to be available for cells. In addition, free choline requires transporters that use ATP and also requires an ATP molecule in the formation of phosphocholine (Fagone and Jackowski 2013), while phosphatidylcholine is absorbed with other products of fat digestion, it is transported in the blood as lipoproteins and is directly available to cells and tissues directly (Tocher *et al* 2008). The second hypothesis that explains the higher content of ME could be related to the presence of methyl groups and hexadecanoic acid (C16: 0) in the herbal mix (Roque *et al* 2020) that act as methyl group donors (Hui-Chao *et al* 2016). The latter could have improved fat utilisation at the cellular level (Pissios *et al* 2013) or could have saved methionine as observed in other species (Dilger *et al* 2007, Sales *et al* 2010). A study in dogs with low methionine levels showed how the oxidation of choline was increased, presumably for methionine synthesis (Harrison *et al* 2020). The third possible mechanism involves the regulation of the gene expression of key allosteric effector enzymes of lipid and glucose metabolism as demonstrated by White et al (2019) in broiler chickens fed herbal mix. These authors observed a 39.03% and 14.61% increase in the gene expression of PPAR receptors and adiponectin, respectively, in liver tissue when compared with choline chloride. They concluded that the herbal mix increased the efficiency of nutrients utilisation.

The quadratic response on ME can be explained by different metabolites contained in the herbal mix which, in other animal species, have shown that growth is improved until an optimum dietary herbal additive inclusion level

and then performance decreases with increasing the levels of inclusion (Gabriel 2019, Razo *et al* 2020). *Achyrantes aspera* is a source of oleanolic acid linked to oligosaccharides (Goyal *et al* 2007), *Azadirachta indica* contains acarbose (Mukherjee and Sengupta 2013) and *Citrullus colocynthis* contains lectin, heterogeneous proteins linked to specific oligosaccharides (Ramzi *et al* 2013). All of them are present in the herbal mix which, at a certain threshold level, could negatively affect and reduce the values of ME (Chen *et al* 2013).

Choline chloride is commonly used to provide choline in balanced dog foods, but it is known to affect the activity of other elements of the premix due to its hygroscopic properties (Moghimi and Roosta 2019). Substitution of choline for phospatydilcholine in dogs (originating from a crustacean) indicated that it is feasible to use this source to maintain physiological levels of choline and its metabolites in plasma (Burri *et al* 2019). Studies in lactating rats fed with phospatydilcholine have confirmed the previous statement through phospatydilcholine measurements in the plasma of suckling pups and immune response, due to its contribution to splenocytes (Lewis *et al* 2016). Our findings should be complemented with long-term evaluations to assess whether choline chloride can be replaced with a herbal source without affecting long-term health, particularly liver and cardiovascular health, and to confirm the protective effects of this nutrient related to glucose and lipid metabolism. In conclusion, the results of this study showed that the herbal mix rich in phosphatidylcholine (1.6%) and other methylated metabolites can fully replace choline chloride in dog diets.

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SHORT COMMUNICATION

Mycoplasmal infection in a guigna (*Leopardus guigna*) from central Chile

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ABSTRACT. Routine blood analysis indicated the presence of *Mycoplasma*-like bodies in a guigna (*Leopardus guigna*). Evidence of infection with *Candidatus* Mycoplasma haemominutum was found in blood samples using PCR and DNA sequencing of the 16S rRNA gene of *Mycoplasma*. *Mycoplasma* spp. are documented in cats but their role in the transmission of *Mycoplasma* to guigna populations requires investigation.

Key words: bacteria, domestic animals, Felidae, vulnerable.

INTRODUCTION

Urbanization and natural environments is an opportunity for the transmission of infectious diseases to native wildlife (Valenzuela-Sanchez & Medina-Vogel 2014). The illegal possession of wildlife and the free-roaming of domestic species in rural areas is well known to have a substantial impact on several aspects of wild animal ecology including habitat use, activity patterns, and host-pathogen interactions (Hwang *et al* 2018). Currently, information about pathogens in populations of Chilean wildlife is limited and further research is necessary to properly understand the consequences of these infections (Llanos-Soto & González-Acuña 2019).

Haemotropic mycoplasmas (haemoplasmas) are small epierythrocytic bacteria that infect a wide variety of mammalian species, including domestic cats (Sykes 2010). Four haemoplasmas species are identified to infect domestic cats: *Mycoplasma haemofelis* (Mhm), *Candidatus* Mycoplasma the associated introduction of domestic animals in haemominutum (CMhm), *Candidatus* Mycoplasma turicensis (CMt) and *Candidatus* Mycoplasma haematoparvum-like (Sykes 2010). In Chile, mycoplasmal infection is common among domestic animals, with findings from a report by Walker *et al* (2016) indicating a prevalence of 15.1% in cats living in the southern region of the country. Nonetheless, the detection in wild species such as Darwin's Fox (*Lycalopex fulvipes*) and guigna (*Leopardus guigna*), is rather recent (Cabello *et al* 2013, Walker *et al* 2016, Di Cataldo *et al* 2020). The guigna inhabits the temperate rainforests of central and southern Chile and it is currently categorised as Vulnerable by the IUCN (International Union for Conservation of Nature) (Gálvez *et al* 2013). Here, we reported the evidence of infection with *Candidatus* Mycoplasma haemominutum (CMhm) in a guigna illegally kept as a pet.

MATERIAL AND METHODS

In August 21, 2018, a male adult guigna (*Leopardus guigna*) was confiscated by the Livestock and Agriculture Service (SAG) and brought in to the Wildlife Rehabilitation Centre, Universidad de Concepción, Chillán. The animal was being illegally kept in a warehouse by a local family from El Carmen (36°53'S, 72°01'W). On arrival, the individual did not exhibit any behavioural or physical anomalies and was unafraid of human handling. An anaesthesia protocol with 20 mg/kg of demedetomidine and 0.3 mg/kg of methadone IM was performed to collect blood from the saphenous vein for biochemical and haematological testing, as it is routinely carried out for all animals entering quarantine on their arrival to the rehabilitation centre (Tayari *et al* 2015). Blood smears were also prepared and stained with Giemsa stain for microscopic observation.

DNA extraction from blood was carried out using the DNAeasy Blood & Tissue kit (Qiagen) and according to the manufacturer's instructions. DNA templates obtained through this protocol were amplified in a thermocycler (MultiGene™ OptiMax Thermal Cycler, Labnet) and the 16S rRNA and RNAseP gene of *Mycoplasma* was targeted using primers described in table 1. The DNA sample was also analysed to detect the presence of other pathogens (table 1). The amplificated PCR products were run in 1% agarose gel, purified using the SigmaSpin ™ Post-Reaction Purification Columns (Sigma-Aldrich), and analysed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequence corresponding to the 16S rRNA was aligned to a single consensus sequence by ProSeq 3.5 software and subject to comparison with the GenBank database of National Center for Biotechnology Information (NCBI).

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Table 1. Primer pairs used for amplification of pathogens of DNA sample from guigna (*Leopardus guigna*).

Primer	Sequence	Size (pb)	Organims	Reference
gltA CS78F CS323R	5'-GCAAGTATCGGTGAGGATGTAAT-3' 5'-GCTTCCTTAAAATTCAATAAATCAGGAT-3'	401	Rickettsia	Labruna *et al* 2004
EHR12SD EHR16SR	5'-GGTACCYACAGAAGAAGTCC-3' 5'-TAGACTCATCGTTTA-3'	345	Anaplasmataceae	Parola *et al* 2000
HBT-F HBT-R	5'-ATACGGCCCATATTCCTACG-3' 5'-TGCTCCACCACTTGTTCA-3'	618	*Candidatus* Mycoplasma haemominutum	Criado-Fornelio *et al* 2003
RNAsePF RNAsePR	5'-CTGCGATGGTCGTAATGTTG-3' 5'-GAGGAGTTTACCGCGTTTCA-3'	175	*Candidatus* Mycoplasma haemominutum	Tasker *et al* 2003
RNAseP-Cmh F RNAseP-Cmh R	5'-CTCTCGTCATTTCTGCAGAACGTC-3' 5´-CGCTTGCACAGTCTGAGATGA-3'	175	*Candidatus* Mycoplasma haemominutum	This study

Mycoplasma sequences reported in domestic and wild felines in the GenBank were used for phylogenetic analysis. Twenty sequences were used to perform the alignment together with the consensus sequence using the ClustalW algorithm (Thompson *et al* 1994) and phylogenetic trees were constructed based on neighbour-joining, maximum likelihood and Bayesian methods using a GTR + I + G model. Maximum-likelihood analysis was conducted using MEGA 7.0 (Kumar *et al* 2016) and Bayesian inference analysis was performed with Mr. Bayes 3.1.253 (Ronquist *et al* 2012). The data set was resampled 1,000 times to generate bootstrap values. Software FigTree 1.4.4 was used for visualisation.

RESULTS AND DISCUSSION

Results from the blood analysis showed no haematological or biochemical alterations, however, blood smears revealed the presence of *Mycoplasma*-like bodies in erythrocytes (figure 1). The sequence obtained from the guigna in this study belongs to *Candidatus* Mycoplasma haemominutum (CMhm) (Accession number: MT772012) (figure 2). Primers reported and designed for the *Mycoplasma sp* RNAseP gene did not amplify any sequences. To our knowledge, there are no reports on the amplification of the RNAseP gene for CMhm. During the analysis, sequences were clustered in different groups, namely group A (CMhm), group B (Mhf), group C (*Candidatus* Mycoplasma turicensis - CMt) and group D (*Mycoplasma sp*).

The sequence obtained in this study was positioned within group A, which is also shared with haemoplasma sequences found in *Felis silvestris catus* from Chile. The context in which the guigna was found could have resulted in increased exposure to pathogens from cats, including *Mycoplasma*. Currently, there are few studies accurately addressing *Mycoplasma* transmission in wild animals in Chile. However, the mechanism of transmission of haemoplasmas among wild and domestic cats has not been elucidated, a recent study suggests that their interaction might be not linked to exposure to haemoplasma-transmitting vectors (Sacristan *et al* 2019). *Mycoplasma* infections can cause disease in domestic cats but just in rare cases there have been reports documenting clinical signs associated with this pathogen in wild carnivores (Criado-Fornelio *et al* 2003). In this report, the guigna showed no clinical signs which is also the case for most studies in carnivores. However, the animal died two weeks

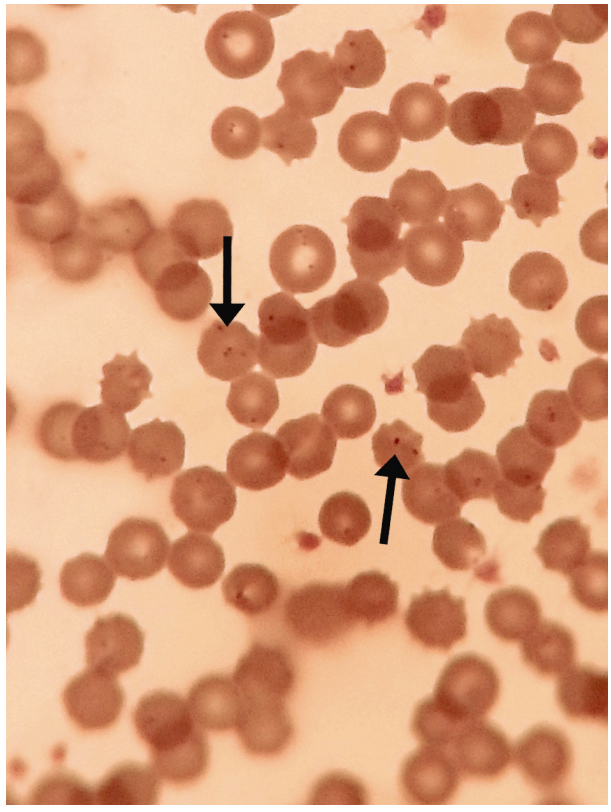

Figure 1. *Mycoplasma*-like bodies in blood of guigna (*Leopardus guigna*).

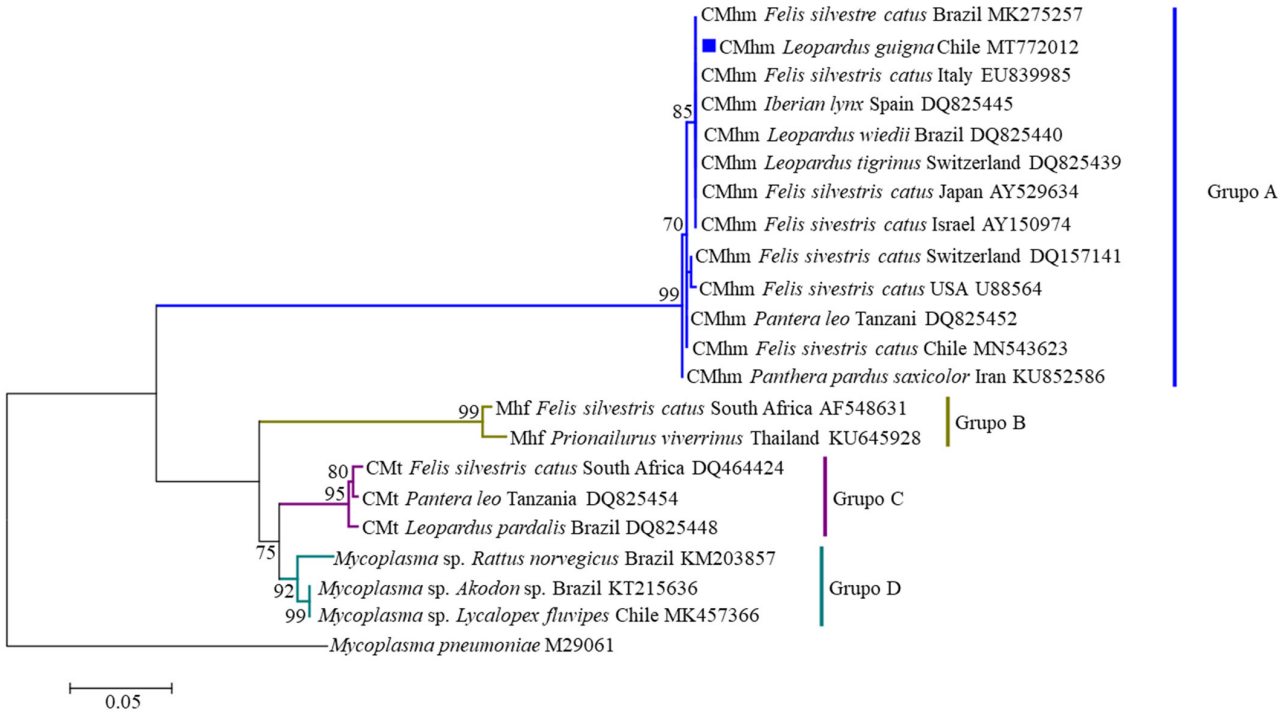

Figure 2. Maximum likelihood tree of 505 bp of the 16S rRNA *Mycoplasma* gene for guigna. *M. pneumonia* sequence was used as outgroup. The data set was resampled 1000 times to generate bootstrap percentage values and bootstrap values of ≥70 are printed at the nodes of the tree. (■) Blue square mark guigna ntST from the present study (Genbank access number MT772012). The Bayesian phylogenetic tree was congruent. The four phylogenetic (taxonomic) groups are labelled (A-D).

later of unknown causes. Mycoplasma infection was not cause of death according to necropsy. Some studies have indicated that domestic cats infected with retroviruses are more susceptible to acquire haemoplasmas and likely to develop more severe clinical signs (Luria *et al* 2004), which is concerning considering that retroviral infection is already documented in guignas in Chile (Mora *et al* 2015). Nonetheless, there is no information about guignas co-infected with haemoplasmas and retroviruses. Further investigations are needed to evaluate the health status of guigna populations along its distribution and determine whether infection with *Mycoplasma* spp. could pose a threat to guigna conservation.

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