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

[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[γ] 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

[γ]: 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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