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Physiological effects of hyperprotein diets with the addition of *Eisenia foetida* in broilers. Proposal for a model for heart disease

Efectos fisiológicos de las dietas hiperproteicas con la adición de *Eisenia foetida* en pollos de engorde. Propuesta de modelo de cardiopatía

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Abstract

Proposing animal models that allow predicting results in humans becomes critical when the analogies in physiology between both entities are reviewed. About heart disease, the heart rate in humans is more similar to that of chickens than that of the mouse, rat or other mammalian models generally used to study this disease. In the present work, the ethology on the attraction of chickens to earthworms as a food source was reviewed, in addition hematological, organ and urological parameters were measured in chickens fed with double and triple the protein percentage supplied with *Eisenia foetida* live added to the feed. commercial for the Cobb500 line. The results show a marked attraction depending on the nutritional status of the birds for *Eisenia foetida* and differences in hematological parameters, but not for urological parameters. The morphological characteristics of the heart showed a clear association between three times the protein load in the food and cardiac damage in 2 of 7 animals fed during 7 weeks of study. The present work represents the first contribution with the animal model approach in chickens to study cardiac damage and its possible prediction for humans.

High protein diet, *Eisenia foetida*, Heart disease

Resumen

Proponer modelos animales que permitan predecir resultados en humanos se vuelve crítico cuando se revisan las analogías en fisiología entre ambas entidades. Con respecto a la enfermedad cardíaca, la frecuencia cardíaca en humanos es más similar a la de los pollos que a la del ratón, rata u otros modelos de mamíferos generalmente utilizados para estudiar esta enfermedad. En el presente trabajo se revisó la etología sobre la atracción de los pollos por las lombrices de tierra como fuente de alimento, además se midieron parámetros hematológicos, orgánicos y urológicos en pollos alimentados con el doble y el triple del porcentaje de proteína aportado con *Eisenia foetida* viva adicionada al pienso. comercial para la línea Cobb500. Los resultados muestran una marcada atracción en función del estado nutricional de las aves para *Eisenia foetida* y diferencias en los parámetros hematológicos, pero no en los urológicos. Las características morfológicas del corazón mostraron una clara asociación entre tres veces la carga de proteínas en los alimentos y el daño cardíaco en 2 de 7 animales alimentados durante 7 semanas de estudio. El presente trabajo representa la primera contribución con el enfoque del modelo animal en pollos para estudiar el daño cardíaco y su posible predicción para humanos.

Dieta hiperproteica, *Eisenia foetida*, Cardiopatía

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Introduction

The proposal to use new animal models to predict what will happen in humans (Shanks et al., 2009) makes sense when the analogies between the entities involved are taken into account. Currently high protein and low carbohydrate diets are considered to have positive effects on functional capacity in patients with heart damage, although studies are believed to be necessary and since they are not carried out in animal models, their realization is not very feasible. Excessive consumption of these diets and for prolonged periods it can have some adverse effects on the body, these are aggravated when associated with a sedentary lifestyle (Dos Reis et al., 2018). These effects occur mainly in the liver, kidney, skin and on the intestinal microbiota (Vasconcelos, 2021). However, in the short term, diets high in protein, low in carbohydrates, and intermittent fasting promote greater weight loss and could be adopted as a starter for treatments. However, due to undesirable effects, caution is required. There are controversies in the application of diets, for example, to treat being overweight and obese have become popular and are generally widely adopted. However, they are based primarily on personal impressions and reports published in "gray literature" books and magazines, rather than scientific evidence. Human clinical trials and animal models to study changes in body composition and metabolism to determine efficacy meet many limitations and must be carefully analyzed according to Freire (2020). Regarding dietary patterns, it is important to mention that even with technological advances when meta-analysis studies are intended to be carried out, the limitations are recognized given the heterogeneity of the data (Sanches Machado et al., 2018), and it cannot be conclusive in different perspectives of investigation.

For Gout and Heart Failure, developing study models is very important since it is practically impossible to address them from diagnosis to treatments and their consequences. Many mouse models have been established to investigate the causal mechanisms of hyperuricemia, a condition directly associated with Gout, but no mouse model of spontaneous gout exists, despite the availability of animals with stable and increased serum urate concentrations (Lu et al., 2019).

An alternative can be a Gout model in chickens since it represents a health problem that affects poultry and appears frequently enough (Ejaz et al., 2005), this coupled with the fact that hyperprotein diets develop a picture similar to that of humans (Singh et al., 2013). Combining evolutionary and clinical biological studies, Hong's (2020) group concludes that chicken is a suitable animal model for hyperuricemia.

On the other hand, the development of Gout is associated, among other risk factors, with this type of diet (Ejaz et al., 2005). In addition, that diets directed towards Arterial Hypertension try to minimize the consumption of red meat, among other recommendations, since it is well known that high blood pressure is associated with functional changes in the heart and blood vessels, including altered function of the left ventricle (Sanches Machado et al., 2018). Both are diseases that can be addressed with animal models that can reproduce these health problems from diets rich in protein.

One metabolic explanation for the disease and the association between the quail and chicken models is that as in humans, the gene encoding uricase is also inactive in bird species (Remy et al., 1951). This physiological similarity with humans makes it possible to propose models with birds for the study of Gout. In addition, and given that uric acid is the final degradation product of dietary or endogenous purines and that its metabolism varies from one species to another, but is similar in man, primates and birds (Ejaz et al., 2005). For the problem of Heart Failure, the failure of numerous subsequent clinical studies is recurrent, which demonstrated efficacy in animal models, but not in patients. Although it is not considered among the candidate models for Heart Failure, one analogies that the chicken presents with respect to the human is the closeness in the heart rate values, between 95 and 105 beats per minute and 60 to 80, in man (Riehle and Bauersachs, 2019). Values much closer than those of mouse or rat.

Eisenia foetida is the most widely used earthworm in captivity conditions in vermicomposting due to its wide distribution, tolerance to temperature fluctuation, resistance to handling, ability to live in organic waste, with different degrees of humidity, in addition to its high reproductive capacity and rapid growth, among other advantages (Schubert et al., 2019).

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The acceptance among farmers in the application of vermite technology based on the use of earthworms allows to see its greater impact with the increase in sustainable development and strengthening the macroeconomy, as well as at the social and the underlying ecological level (Singh et al., 2020). Another area of knowledge of *Eisenia foetida* that has gained importance is in the diet given its nutritional characteristics when worm flours are made to market as protein ingredients that allow farmers not only to reduce their production costs, but also to improve the efficiency of production (Bahadori et al., 2017; Bollido, 2021), and for poultry farmers an excellent alternative to soy and fish meals, for Khan's group (2016) there is very little information on the use of worm meal in the diet of poultry. Mimicking wildlife conditions and providing a preferred feed as part of environmental enrichment whereis included *Eisenia foetida* can enhance the natural behavior of broilers without causing frustration. Although poultry are considered predatory of worms and risky for vermicomposting (https://lombritec.com/lombriz-roja-californiana-depredadores), no ethological studies of attraction for this type of food have been carried out in chickens fattening. The purpose of the present work is to study the effects of based hyperprotein diets *Eisenia foetida* live in broilers on physiological parameters that may influence the development of Heart Failure and/or Gout.

Metodology

Experimental test

The experimental test was developed in the Behavioral Test Booth of the facilities of the University Center for Biological and Agricultural Sciences, of the University of Guadalajara, which is located at the coordinates of 20º25'30 " to 20º57'00 " north latitude. and 103º19'30 " to 103º39'20 " west longitude, at a height of 1,548 meters above sea level. The average annual temperature is 22 ° C, with a maximum of 36.1 ° C and a minimum of 11 ° C. It has an average rainfall of 906.1 millimeters (García, 2004).

Test attraction by Eisenia foetida

In the experiment attraction Eisenia foetida from broilers line cobb500. 100 chickens 24 hours old were used in pens 2 m^2 where they were provided 20 g in trays feeder type live earthworm. Three tests were carried out with worms for three consecutive days twice/day. It is important to mention that in the first contact the group that responded was that of the chickens that presented nutritional deficiency with an average of 10.5% g less in weight. Chickens that showed interest and took worms and later ate them were marked with a black marker on the nape of the neck. In the 6 attraction experiments, the same 17 chickens plus 4 that showed interest until the third day of contact, allowed to establish a 21% attraction towards *Eisenia foetida* as a preliminary test to start the hyperprotein test with a different flock.

Groups with diets

From a different flock, 21 33-day-old birds of the Cobb500 genetic line were used, with an average weight of 1.26 Kg, randomly distributed in 3 batches with 7 birds each, the control group received the recommended diet for chickens fattening according to the cobb500 manual, 2018. The second group also received 12.5 g of earthworm, *Eisenia foetida*, alive and the same weight of feed was withdrawn. The third group received in addition to the fattening feed 25 g of worm and the same 25 g of commercial feed was withdrawn. It is important to mention that of the groups fed with *Eisenia foetida*, an excessive attraction was noted for two of the birds in the 25 g group and another 8 with moderate attraction between both groups; the other 4 birds showed almost no such behavior. The birds were housed on a floor with a chip bed, which when they reached the age of 33 days they were transferred to quail cages and 7 were housed per cage. At 45 days of age, they were placed in cages for laying hens and one per cage was placed to administer the corresponding diets. At day 60, given that the birds were unable to stay in the "laying" cages, and their weight gain, they were moved one per pen on a floor with chip bed until slaughter. The experiment lasted 7 weeks and the birds were euthanized at the end of the test.

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Ingredient (%)	Start	Finish
Oil	6.00	6.00
Sorghum	48.18	59.28
Soybean paste	35.92	26.34
Ca Carbonate	1.21	1.10
Dicalcium Phosphate	0.68	0.28
Premix	8.00	7.00

Table 1 Dietary characteristics broilers inclusion percentages for ingredients of the diets provided

Nutrient	Initiation	Completion
Protein (%)	21.46	18.01
Metabolizable energy (Mcal / Kg of weight)	2.92	3.07
Calcium (%)	0.96	0.78
Phosphorus	0.48	0.39

Table 2 Nutritional content in the diets provided

Preparation of Fresh Vermi (Eisenia foetida)

The vermi was grown in animal manure, such as livestock waste, poultry excrement. The cultivation was carried out in cement beds 1 m long x 40 cm high x 1 m wide, with integrated drainage through a 3-inch diameter pvc pipe, with a cement bed and covered with shade mesh and in the open. The food was prepared by precomposting to eliminate pathogens with a temperature of 20 to 40 °C and a pH of 6 to 9, added every 15 days and watered with tap water. With a volume of approximately 50 Kg/bed.

To feed the chickens, the corresponding grams of *Eisenia foetida* of any stage of development were taken indiscriminately.

Bromatological Analysis of Eisenia foetida

From 200 g of Live earthworms were desiccated at 40 ºC in a drying oven for 48 hrs and the corresponding tests were carried out for: Moisture and volatile matter using the method of the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC) 934.01; Protein (% NX6.26) AOAC 655.04; Ethereal extract AOAC 920.39; Ashes AOAC 942.05; Crude fiber AOAC 962.09; ELN by difference and Dry matter by difference

Feeding System

During the development of the birds the feed was provided in the morning in a controlled way, according to a feeding program where they were increased according to their requirement and an appropriate consumption was guaranteed of nutrients according to the NRC (1992) tables.

The food was served in tray-type feeders for the first 33 days, and later it was offered in quail cage feeders. The water was initially supplied in vitrolero drinkers and later bell drinkers were used. After 33 days the weight gain was recorded weekly.

The live earthworm *Eisenia foetida* was included in the diet in such a way that it was ingested by the birds prior to consumption of the forage to guarantee total and controlled consumption, and according to the technical reference of the avian lineage. A vaccination schedule was carried out according to the study area and they received a dose of vaccine with live Newcastle virus, intraocularly.

Body Weight Gain

The birds of the three treatments were weighed every seven days with an OHAUS® model T21P digital scale, to obtain weight gain up to the week of slaughter.

Sampling

At the beginning of the experimental test, 21 days later and at the time of sacrifice, blood was obtained to evaluate the hemograms acquired using the MISTIQ18 equipment (Orphée, Switzerland). At the beginning and 21 days later during the test, the blood was extracted by puncture of the vein (clavicular) and in the sacrifice it. By means of a ventral incision, the organs were exposed and the interior of the body was photographed to record any evidence of evident damage related to the treatments. The dimensions of the joint were measured, it was cut in its proximal portions. The heart, liver, spleen were removed. The organs were previously weighed and in the case of the heart an apical cut was made to observe the internal chambers and they were photographed for their registration.

Joint Dimension

The dimensions of the femuro-tibial were measured with a vernier, first antero-posterior and then lateral to calculate the difference in the total dimension with an approximation that allows measurement in mm^2.

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Obtaining the serum

After obtaining the serum by intravenous puncture or by gravity in the carotid vein, it was cooled at 4 ° C for 24 hrs and subsequent centrifugation at 2,500 rpm for 10 min. Serum was obtained and the clot was discarded.

Hematology

The heparinized blood sample was analyzed with the MISTIQ18 (Orphée, Switzerland) chemical veterinary accountant of hemograms. The equipment works by automatically sucking through an exposed needle in the apparatus approximately 70 μL of blood sample, it is an automated multi-parameter hematology analyzer designed for diagnostic use in vitro, which analyzes blood cells from different species, which they provide the necessary reference to the clinical diagnosis. The reading methods of the MISTIQ18 (Orphée, Switzerland) are through electrical impedance and colorimetry to run the parameters of leukocytes, erythrocytes, platelets and hemoglobin appropriate for the qualitative and quantitative analysis of the visible components in the blood of the individual.

Procedure for Faeces

For the collection of excreta, a plastic was spread in the pens where the birds were placed and the faeces deposited on the plastic were absorbed with a rubber pipette. They were transferred into 15 mL conical tubes and frozen until their use for urinalysis.

Urinalysis

The stool samples were placed in a water bath for thawing at 35 °C for ~ 3hrs. Each sample was diluted ~ 1:2 w/v, homogenized and allowed to settle, then the test strip was immersed for at least 10 seconds, allowed to dry and was introduced into the slot of the automatic equipment "KONTROLab 11Vet", which was thrown the results after one min. The parameters that are measured are: color, leukocytes, ketone, nitrites, urobilinogen, bilirubin, proteins, glucose, specific gravity, blood cells, pH, and ascorbic acid.

Statistic analysis

The effect of the treatments was analyzed with a repeated measures anova, where the experimental unit was 7 animals, and for each treatment 7 repetitions were used, when there were statistically significant differences, the means were compared with the Holm-Sidak procedure with an $\alpha = 0.05$.

Results

Ethology by Eisenia foetida

It seems common to know that all chickens accept worms 100% without restrictions. However, when we added to the diet of the 33-day-old chickens, it became apparent that not all the animals accepted said food. The result was that of one n = 14 exposed animals, only 6 showed interest and of these 2 were voracious, they even seemed to have addiction to *Eisenia foetida*. Therefore, an additional test was performed with 100 10-day-old chicks and no response was found on the first exposure to worms. Since 44 chicks were found to have an average weight of 10.5% (257 vs 230 g) less, the worms were exposed separately to a ration of 20 g of earthworm. The experiment was carried out in six-fold 3x at day 10 and 3x at day 11 of age. In the first contact, and without such a forceful attraction, an n = 17 animals attracted by the food was obtained and in the third contact with the worms another 4 chicks joined the group adding 21 in total, that is, 47.7%, It should be noted that in the 3 subsequent contacts the same behavior was confirmed by the total number of chickens.

Bromatological Analysis

Table 2 shows the results of the Bromatological study for *Eisenia foetida* carried out in our laboratory where it can be seen that the moisture and volatile matter content expressed as a percentage represents more than 84%, followed by the dry matter obtained by weight difference. It is interesting to note that although the percentage of Wet Base for Protein occupies 9.41%, it is equivalent to 60.25% in Dry Base, the highest content in the sample.

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Determination	Method	Result BH (%)	Result BS (%)
Moisture and volatile matter	AOAC 934.01	84.38	0.00
Protein (% NX6.26)	AOAC 655.04	9.41	60.25
Ethereal extract	AOAC 920.39	1.21	7.54
Ashes	AOAC 942.05	1.54	9.90
Fiber crude	AOAC 962.09	0.10	0.62
ELN (Nitrogen Free Extract)	By difference	3.36	21.69
Dry matter	By difference	15.62	100

Table 3 Results of the Bromatological Analysis for *Eisenia foetida*

Weight gain

In Fig. 1, regarding total body weight, there were no statistically significant differences between the treated groups. Feed conversion was measured weekly but It did not present statistically significant differences (Data not shown).

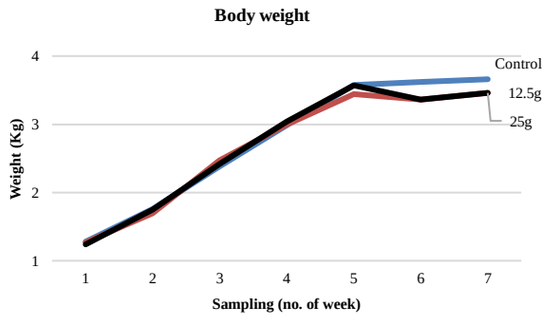

Figure 1 Body weight in Kg of the treatment groups during the 7 weeks of the study. The groups did not show representative statistical differences

Weight of Organs at Sacrifice

Parameter	Treatments					
	Control		12.5 g		25 g	
	Average	Dev. Std.	Average	Dev. Std.	Average	Dev. Std.
Body weight (Kg)	3.66	0.21	3.46	0.21	3.46	0.25
Heart (g)	16.3	2.4	15.78	2.0	17.17	2.7
Liver (g)	46.45	6.0	44.82	3.2	42.05	3.2
Spleen (g)	4.1	1.2	3.44	0.5	3.24	0.7

Table 4 Body and organ weight at slaughter

In table 3 it can be observed that, although there are no statistically significant differences in the total body weight of the chickens in the three experimental groups, the differences in weight of the organs show a tendency to increase the size of the heart in the group of 25 g of *Eisenia foetida*. added to the diet.

The difference is 5.33% higher compared to the control group and 8.52% compared to the group added with 12.5 g of worm, which showed the lowest value and was 3.19% lower than the control group. It is very important to show the photographs that show the lateral deterioration of the myocardium Fig. 2a and 2b, corresponding to the chickens that showed the greatest voracity for worms. As well as Fig. 2c of a bird from the control group.

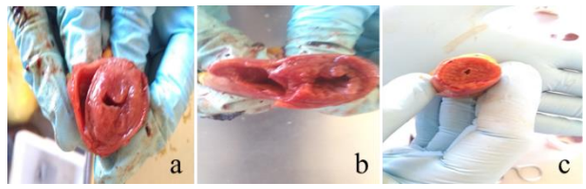

Figure 2 Photographs showing the left lateral myocardial damage of chickens with the addition of 25 g of Eisenia foetida (a and b) and of a chicken fed the commercial diet

In the same table 3 it is observed how the liver shows a slightly more considerable decrease in weight (9.47%) for the group added with 25 g of worm compared to the control (46.45 g) and a little less decrease, more than double, for the group with 12.5 g (3.5%) of the roundworm added to the diet.

The spleen shows a similar trend with 20.97% less weight for the group added with 25 g of earthworm and almost 16.1% in the group added with 12.5 g of *Eisenia foetida*, With a weight of 4.1 g for this organ of the control group (Table 3). The relative weight of the spleen in broilers of the control group was significantly higher compared to the worm treatments.

Treatment											
		Control				12.5 g of E. f.				25 g of E. f.	
Chicken no.	Body weight (Kg)	thickness (mm²)	weight (g)/thickness (mm²)	Chicken no.	Body weight (Kg)	thickness (mm²)	weight (g)/thickness (mm²)	Chicken no.	Body weight (Kg)	thickness (mm²)	weight (g)/thickness (mm²)
Chicken no 66	3.48	590	169.5	Chicken no 93	3.64	540.33	148.4	Chicken no 88	3.26	556.26	170.6
Chicken no 68	4.1	857.49	209.1	Chicken no 92	3.48	595.11	171	Chicken no 86	3.3	570.21	172.7
Chicken no 76	3.54	646.6	182.6	Chicken no 96	3.2	629.01	196.5	Chicken no 84	3.72	558	150
Chicken no 77	3.64	570.18	156.6	Chicken no 94	3.58	686.66	191.8	Chicken no 85	3.24	553.04	170.6
Chicken no 98	3.46	599.76	173.3	Chicken no 97	3.2	588.53	183.9	Chicken no 87	3.28	564.75	172.2
Averages		652.806	178.2			607.928	178.3			560.452	167.2
Desv. Std.		117.8	19.62			54.2	19.31			6.93	9.69

Table 5 Ratio of total body weight to the thickness of the femoro-tibial joints of chickens treated with *Eisenia foetida*

Joints

Table 4 Ratio of total body weight to the thickness of the femoro-tibial joints of chickens treated with *Eisenia foetida*.

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Joints thickness

Table 4 shows the results of the relationship that exists between the total body weight of the chickens with respect to the thickness of the femoro-tibial joints, comparing the weight in g with respect to the dimensions in mm^2. Since there is no uniform weight-to-joint ratio, this ratio is averaged and the group fed 25 g of *Eisenia foetida* shows a decrease in joint thickness of almost half (9.69 mm^2) compared to the other two treatment groups (control 19.62 mm^2 and 19.31 mm^2 for the group fed 12.5 g).

Hematology

Table 5 shows the results obtained from the hematological test where we could divide the parameters to be determined into three blocks, all of which show an average plus-minus the standard deviation.

Parameters							
	Erythrocytes	Hemoglobin	Hematocrit	MCV	MCHC	(STD)RDW_SD	RDW_CV
	Average/DS	Average/DS	Average/DS	Average/DS	Average/DS	Average/DS	Average/DS
Control	2.66/.33	16.35/2.5	25.7/3.9	95.6/10	66.53/10	46.22/2.9	14.5/1.8
12.5 g of *E. f.*	2.94/.24	15.35/6.0	25.43/9.2	98.75/5.9	59.37/4.4	47.31/6.7	13.95/2.4
25 g of *E. f.*	2.92/.41	16.8/2.2	30.64/5.2	104.3/6.8	55.12/3.4	54.78/15	14.7/2
	% Leukocytes	% Lymphocytes	% Monocytes	% Granulocytes	# Lymphocytes	# Monocytes	# Granulocytes
	Average/DS	Average/DS	Average/DS	Average/DS	Average/DS	Average/DS	Average/DS
Control	14.45/7.5	94.67/1.7	4.45/1.4	0.375/.28	13.7/6.0	0.5/.14	0.25/.36
12.5 g of *E. f.*	84.01/31	94.5/2.1	4.72/1.6	0.72/.6	20.03/25	1.29/2.2	0.36/.47
25 g of *E. f.*	11.06/3.8	94.3/2.6	4.35/1.2	1.35/1.5	10.57/4.2	0.45/.25	0.22/.13
	Platelets x10^3/µL	MPV (*fL*)	PDW (*fL*)	Thrombocytes (%)	P_LCR (%)	P_LCCx10^3/µL	
	Average/DS	Average/DS	Average/DS	Average/DS	Average/DS	Average/DS	
Control	108.25/16	13.25/1.1	17.05/5.3	0.137/.02	46.12/7.8	49.5/11	
12.5 g of *E. f.*	108.25/23	13.39/.57	16.54/3.8	0.144/.03	44.4/4.2	49.4/14	
25 g of *E. f.*	115.2/15	13.76/.27	20.12/3.3	0.152/.02	48.96/2.9	56/9.4	

Table 6 Hematological parameters of Cobb500 chickens fed with Eisenia foetida at the time of slaughter

Results of the hematological test in chickens that received diets supplemented with 25 g/day or 12.5 g/day of *Eisenia foetida*, at the time of slaughter. Represented as an average and its Standard Deviation of an n = 7 and in some cases lower, since the sample was not processed properly. (Red blood cell distribution width) RDW_CV; (Red Cell Distribution Repeated Precision) (STD) RDW_SD; (Mean corpuscular hemoglobin concentration) MCHC; (Mean Corpuscular Volume) MCV; (Mean platelet volume) MPV; (Platelet distribution width) PDW; (Higher platelet range) P_LCR; (Major platelets) P_LCC.

Group of 25 g of *Eisenia foetida* seems to present anemia at the beginning of the experiment and the results show a clear recovery when the number of erythrocytes per µL of blood is measured, in addition, in the final part of the experiment approximately 300,000 more erythrocytes are observed than the control for each µL of fluid and only 20,000 cells more than group fed 12.5 g of worm.

The hemoglobin (HB) test measures, in this case, the amount of the molecule in the blood. Here, almost 0.5 g/dL more HB was obtained in group 3 compared to the control and 1.5 g/dL compared to group 2. The hematocrit measures the amount of blood made up of red blood cells. At the end of the experiment, approximately 5% more erythrocytes were observed in group of 25 g of added worm compared to the other two groups. The MCV blood test measures the average cell size of the erythrocytes and in our results it was found that the size of the erythrocytes is more than 10 femptoL greater than the control and almost 5 femptoL than group of 12.5 g of *Eisenia foetida*. In the amount of intracellular Hemoglobin (Humans = 28 to 32 pg/cell). It is possible to see 4.3 pg of HB more in the erythrocytes of the control group compared to group 2 and 5.1 compared to group of 25 g of added worm. According to these results, it seems that HB is found in the extracellular space in chickens treated with 25 g of *Eisenia foetida*.

Regarding immune cells, one of the individuals showed disproportionately high values for the 12.5 g group and the DS extends too much except for the percentage of lymphocytes. An immunostimulatory effect is observed in the dose of 12.5 g of added worm and an immunosuppressive effect in the diet with 25 g of *Eisenia foetida*. If the number of immune cells obtained with the cytometer is observed, there are 3,000 more lymphocytes/µL in the control group compared to group 3 and 10,000 cells/µL, that is, twice in group of 12.5 g of *Eisenia foetida* compared to group of 25 g of *Eisenia foetida*. In Monocytes, 500 were obtained cells/µL less in group 3 compared to the control and almost three times in group of 12.5 g of *Eisenia foetida* compared to group of 25 g of *Eisenia foetida*. For Granulocytes an average of 300 cells/µL less was found in group 3 or fed 25 g of worms with respect to the control and 140 cells/µL more in group 2 or fed with 12.5 g of worm compared to group of 25 g of *Eisenia foetida*.

In terms of platelet and thrombocyte values, an increase in said parameters was found directly related to the amount of *Eisenia foetida*. included in the diet. Although slightly higher the values are constant except for larger platelets (P_LCC), when the control group is compared with the one fed with 12.5 g more of worm, see Table V.

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For Platelets in group 3 of 25 g of *Eisenia foetida* it is observed almost $7x10^3$ cells more than the other two groups.

Stool	Treatment		
Parameter (average)	Control (N = number of individuals)	12.5 g (N = number of individuals)	25g (N = number of individuals)
Leukocytes (cells /µL)	57.8571429	65.7142857	42.1428571
Ketones (mmol / L)	ND-	ND-	ND-
Nitrites	ND	ND	ND
Urobilinogen (µmol / L)	Normal + 33 (1)	Normal + 33 (1)	Normal + 33 (2)
Bilirubin (µmol / L)	NA + 8.6 (1)	NA	NA
Proteins (g / L)	0 + 0.15 (1)	0 + 0.15 (1)	0 + 0.15 (2)
Glucose (mmol / L)	NA + 0.28 (1)	NA	NA
Specific Gravity	1.01785714	1.02071429	1.02071429
Blood cells (cells / µL)	18.57	56.42	38.57
pH	5.85	6	6
color	Transparent + orange (1)	Transparent + yellow (3)	Transparent + orange (2)

Table 7 Results of the urinalysis performed on chicken feces fed with *Eisenia foetida*

In the Uroanalysis carried out on the faeces of the Cobb500 line chickens, a difference of 3 hundredths more was found in the birds fed with worm compared to the controls, triple and double the number of blood cells was observed. in the group supplemented with 12.5 g and 25 g, respectively. The pH remained acidic and only increased by almost 11 tenths to reach 6 in the groups treated with *Eisenia foetida*. No ketones, bilirubin, protein, or glucose were found in the three experimental groups. Urobilinogen showed a value of 33 mg/dL only in a control animal and one more in the 25 g group, none for 12.5 g. For bilirubin, only one individual was found with 8.6 mg/dL belonging to the control group. Regarding the color of the sample, it was found that one individual of the controls presented one orange, 5 transparent and one was not detected; of the group treated with 12.5 g, 3 yellow and 4 transparent were registered, finally for the 25 g group, 2 orange and 5 transparent were obtained.

Discussion

Since our group did not find any literature that demonstrates the attraction of chickens to *Eisenia foetida*, we could propose this study as a pioneer in reporting such contributions.

It is important to mention that after 6 exposures to *Eisenia foetida* the same 17 chicks plus four, for a total of 21 birds, showed interest and consumed the worms provided.

According to the theories that propose how food may have influenced the evolutionary process of species prior to hominids, it is proposed that the provision of an environment with better food resources promoted greater physical and intellectual development (Martínez-Rincón and Cisneros-Redríguez, 2002). It could be said that this theory is in agreement with the results obtained in the pilot test carried out by the attraction of the worm to the chickens, since the birds with 10.5% (257 vs 230 g) less body weight showed their avidity in 5 tests by the consumption of *Eisenia foetida* and that it did not appear in any case in the chickens with greater weight. Similarly, when chickens with 33 days of age were exposed, only 5 showed moderate attraction to *Eisenia foetida* and 2 plus an excessive or voracious attraction.
The results of the bromatological study are shown in table II and are in accordance with what was published by Köse and Ozturk (2017) regarding protein content and where their group proposes worms as a protein source to feed farm birds. On the other hand, the group of Zhenjun et al. (1997) mention that this roundworm contains 20 of the 24 main amino acids that are very important in poultry production. Depending on the 18% protein content, according to the manual for the Cobb500 line, it was decided in the present work to increase the protein content to approximately double, adding 12.5 g of live earthworm, and approximately to triple with 25 g of said worm, given the result of the Bromatological study (62% protein) and according to that reported by Köse and Öztürk (2017). Although we do not adjust to the hyperprotein proportions reported for humans (Buendia et al., 2015; Stamler et al., 2002), we do adjust to the usual in chickens.

In Fig. 1 it can be seen that, in terms of total body weight, there were no statistically significant differences between the treated groups. The results obtained in the present work are consistent with those obtained by Bollido (2021), although an important difference is the amount of worm used, from 2 to 5% compared to our birds that received a supplement of approximately 36 and 54%, in addition that were not found confined to a single development site.

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On the other hand, the group of Vargas López (2004) treating non-confined laying hens in family production units in Puebla, Mexico, also found no differences between the commercial diet and that supplemented with *Eisenia foetida* and it does not appear that they controlled the quantity of worm supplied.

The study of risk factors associated with left ventricular mass carried out by Heckbert et al. (2006) find the Body Mass Index and hypertension as the most related in humans and discuss the need for additional studies of risk factors and their treatment in relation to the new development or progression of left ventricular dysfunction, and the association of subclinical changes in cardiac size and function with outcomes such as myocardial infarction and sudden cardiac death. In this regard, it was found in the present study that the two individuals who showed greater voracity towards worms developed greater weight gain (3.86 and 3.6 Kg) at the time of slaughter, as well as frank deterioration on one side of the heart (images a and b) compared to the rest of the experimental chickens (image c).

For the group of Díaz-Rúa (2017), long-term hyperprotein diets in rats show signs of health risk, when the liver transcriptome is studied, with increased inflammation and alterations in acid-base balance and oxidative stress, given the persistence of the altered metabolic state. Although our results only measure total weight and a decrease is observed in this proportional to the consumption of worm protein, it is likely that this weight loss is related to liver deterioration. Although in rats this decrease is not observed and the excess of protein is with casein (Díaz-Rúa et al., 2017). The lack of studies in chickens that compare liver weight with respect to body weight forces us to confront our results with those obtained in models such as mice, where a decrease in liver weight and its relationship with body weight in "nock out" mice is observed. Of proteins involved with the metabolism of fatty acids and in individuals fed with phytol (Landrock et al. 2017), although in our case it is required to study analogous parameters with said metabolic pathway to compare the effect of the diet based on *Eisenia foetida*.

On the other hand, when protein derived from vegetables such as soy or canola flours is used, no significant changes are observed for the heart, spleen and other organs, but an increase in the size of the liver and, as the group of Payvastegan et al. (2017), the group of Manyeula et al. (2020) agree that said increase corresponds to chemical compounds in the food that require detoxification.

The spleen shows a similar trend with 20.97% less weight for the group added with 25 g of earthworm and almost 16.1% in the group added with 12.5 g of *Eisenia foetida*, with a weight of 4.1 g for this organ of the control group (Table III). Our results agree with those obtained by the group of Chen et al., (2019) and where they find a relationship with an immunosuppression phenomenon.

Joint inflammation in humans has tried to relate the parameters of total body mass with blood levels of uric acid and with damage to the cartilage of the knee or its general condition (Crowson et al., 2011), on the other hand, If the diagnosis of Joint Inflammatory Syndrome (AIS) is reviewed, it is found that deepening the evaluations of joint damage, and understanding that AIS has a generally similar behavior according to the disease that gives it origin, facilitates, once correctly interpreted , the way to achieve a nosological diagnosis and certain treatments (Martínez-Larrarte et al., 2007).

In support of this uncertainty, perhaps the model with chickens will allow the carrying out of studies at both histological, cellular and molecular levels that allow associating the consequences of hyperprotein diets with AIS and with uric acid levels in any of the sources to analyze.

If we take into account that HB values are almost 0.5 g/dL more than HB in group of 25 g of *Eisenia foetida* compared to the control and 1.5 g/dL compared to group of 12.5 g of *Eisenia foetida*, and having hematocrit levels that are too high or low may indicate a problem with the blood, dehydration, or other medical conditions and since observations from clinical use of recombinant, pure, artificial hemoglobin solutions support a causal relationship between excess cell-free hemoglobin in the bloodstream with cardiovascular symptoms and events (Rother et al., 2005).

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It is not unreasonable to think of one of the causes that generated the deterioration of the heart. If we take into account that HB has a harmful role in stressful or pathological situations and that they can fall into the following categories: (a) Autooxidation of hemoglobin within the rbc, (b) Release of hemoglobin outside its confines in rbc to act as a source of Reactive Oxygen Species (ROS) in the vasculature and tissues, (c) Nitric Oxide (NO) uptake by hemoglobin and (d) Release of HB heme to increase the generation of free radicals. Perhaps we are talking about a kind of generalized low intensity inflammatory reaction (Minihane et al., 2015).

First, the released hemoglobin is closer to the vascular endothelium and can also leak into the tissues as dimers so that the ROS generated in autoxidation easily access the macromolecules and cause damage.

Perhaps similar to the pathogenesis of false cell disease that in addition to inflammation involves heterocellular adhesion between erythrocytes, platelets, endothelium, and leukocytes (de Azevedo-Quintanilha et al., 2020). In the same sense, Kato et al. (2017) hypothesize that intravascular hemolysis products damage the vascular system, reducing nitric oxide (NO) given the release of HB and that they react through dioxygenation to form inert nitrate. Quaye's group (2015) also mentions that HB disaggregates and becomes a dangerous molecule for the survival of cells.

The controlled diet in the omnivorous wild bird Pycnonotus barbatus has demonstrated its effect on physiological parameters (decreased haptoglobin or α-globin concentration, earlier molting and immunostimulation) when fruits or invertebrates, the latter considered hyperprotein, are fed as food. The decrease in haptoglobin is associated with the initial evaluation parameters of intravascular hemolysis and oxidation (Nwaogu et al., 2020).

These conditions can explain a picture of hemolytic anemia caused by malignant hypertension, perhaps prompted by the increase in uric acid levels due to the hyperprotein diet and that exceeds the protective mechanisms of erythrocytes (Phillips and Hendersone, 2018); with the consequent release of hemoglobin that increases oxidative stress in the vessels.

In humans, kidney damage is associated as a cause of Malignant Arterial Hypertension, but also the increase in intravascular hemoglobin levels is associated with hyperproteinuria, which seems to explain a systemic and feedback problem (Morales et al., 2011) that probably generates a long-lasting state of low intensity inflammation (Castro et al., 2017), but no one associates these complications with lifestyle or high protein diets in humans. Probably the reason is that institutions such as the European Food Safety Authority (EFSA) and the European Union Register of Health Claims (http://ec.europa.eu/nuhclaims) do not provide for health claims that specifically address the area of health benefits of suppressing or controlling low-intensity inflammation (Minihane et al., 2015). For Kato group (2017) understanding how these components' complexity of sickle erythrocytes is compounded by genetic and environmental modulation provides insight into the well-known clinical heterogeneity of sickle cell disease (SCD). Perhaps the best way to address the consequences of this inflammation is through animal models such as broilers in which it can be studied at any point in development and under controlled conditions.

The Nwaogu group (2020) proposes the existence of a causal effect of the composition of the diet on the innate immune function, since in thebirds *Pycnonotus barbatus* that were found in better conditions when they were fed with fruits than when they were fed with invertebrates, confirming that innate immunity is specific to nutrients and, furthermore, it is well established that nutrients modulate intestinal immunity (Farré et al., 2020). In our results the possibility is observed that the amount of nutrients with 12.5 g of *Eisenia foetida*. it induces immunostimulation, but not to a greater quantity of protein from this source (Table V).

Thrombocythemia or increase in the number of platelets, induced by supplementation with 25 g of *Eisenia foetida*, is a factor that can contribute to the formation of clots and with it to high blood pressure and a high risk of arterial thrombosis (Tefferi et al., 2018). Our results are in agreement with epidemiological studies that present leukocytosis and high levels of total HB as risk factors and increase in episodes of acute pain in acute chest syndrome (de Azevedo-Quintanilha et al., 2020).

ISSN-On line: 1390-9959

Added to all the hematological parameters collected in this study, they allow a more complete view of the physiological alterations suffered by hyperprotein diets and in an environmental enrichment model (Ipema et al., 2020).

Trying to establish study models, as in this case, requires making determinations in a longer period of time, that is, 7 weeks. Since the general approach in the experimental work with broilers is given in function of productive parameters, they are estimated in much shorter treatment time, for example the group of Bollido (2021) during 8 weeks; Vargas López (2004) for 90 days in free-range hens measuring these productive parameters. However, the toxicological evaluation for 4 generations of the worm meal in the feed, with an experimental diet containing 200 g of earthworm protein per kg. It did not reveal any deleterious effect on the health of the rats (Ibáñez et al., 1993). Therefore, if the experimentation time is extended further, the effect of the addition of live earthworms in broilers could be reported.

The use of animals by the biomedical sciences as models to help understand and predict responses in humans, in toxicology and pharmacology in particular, remains the primary tool for biomedical advancements and a source of significant controversy. In general, animals have performed exceptionally well as predictive models for humans when used correctly (Shanks et al., 2009).

In the case of urinalysis, and although the detection equipment is designed for pure urine, the present study attempted to search for biomarkers within the 10 parameters determined by the device. Perhaps observation by phase contrast microscopy should be attempted according to the Recommendations for Urinary Sediment of Chile (Lagos and Pinto, 2013) and compared with our results in the different parameters of the urinalysis. The primary objective should be to separate elements of urine that can be disturbed by contact with solid waste material (Baños-Laredo et al., 2010). The pH of our results is suggestive of the formation of urates in their form of sodium, potassium, magnesium and ammonium salts of uric acid, since their generation occurs between 5.5 and 6 of this parameter (Jiménez and Ruiz, 2010).

On the other hand, in humans the reductions in urinary pH ($p = 0.07$) in studies with meta-analysis for hyperprotein diets (HP) are considered an independent risk factor for developing nephrolithiasis (Schwingshackl and Hoffmann, 2014), we consider that This contradiction requires further study in the broiler model.

A limitation of the earthworm in the chicken feed ration is its dry matter content, since large volumes would be needed to satisfy the requirements or include it in commercial concentrates in the form of flour (Rodríguez et al., 1995). Its carbon footprint implications and environmental impact should probably also be analyzed (Vauterin et al., 2021).

In this way the use of *Eisenia foetida* live on diets represents a better option with multiple extra benefits as a live food. In addition, the worm is the only animal in the world that does not transmit or suffer from diseases (Pineda, 2006). Although exposure to fluoroquinolone and organophosphate pesticides is known to cause oxidation of their proteins through carbinylation, thus decreasing nutritional quality (Márquez-Lázaro et al., 2021). On the other hand, the humus produced in its cultivation could be recommended as other humic substances that have proven to provide quality to broiler meat (Hudák et al., 2021).

The importance of this organism has been recognized since the time of Hansen and Darwin; Currently it is exploited by means of a technique called vermiculture that consists of the breeding and management of earthworms in captivity conditions with the basic purpose of obtaining with it two products of great importance for man: humus as a fertilizer-amendment for agricultural use and protein, either as fresh meat or flour for a food supplement (Nalunga et al., 2021; http://ri.ues.edu.sv/id/eprint/1624/3/13101281TT.pdf

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Conclusions

There is a differential behavior regarding the attraction to *Eisenia foetida* of broilers of the Cobb500 line. Those who showed voracity and were fed 25 g/day of worm have the probability of developing marked heart damage, perhaps from the geometry of the left ventricular mass due to diet. Diets with 12.5 and 25 g/day of *Eisenia foetida* induce low-intensity inflammation symptoms when measuring joints, organ size, hematological and probably urological parameters. Determining the management and the conditions to develop a model of cardiac damage in the Cobb500 chicken line and according to the results obtained in the present work seems plausible and requires more studies to compare the consequences of hyperprotein diets based on *Eisenia foetida* live compared to diets of the same type in humans.

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