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CENTRO DE ENERGIA NUCLEAR NA AGRICULTURA

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Passage rate, phosphorus metabolism (^{32}P) and body composition by tomography of lambs infected in single dose of *Trichostrongylus colubriformis*

Piracicaba
2019

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*"Regardless of the challenge, always be **antifragile**"*

ABSTRACT

BOMPADRE, T. F. V. **Passage rate, phosphorus metabolism (^{32}P) and body composition by tomography of lambs infected in single dose of *Trichostrongylus colubriformis*.** 2019. 121 p. Tese (Doutorado em Ciências) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2019.

The aim of this study was to identify physiological, biochemical and metabolic changes in sheep infected with *T. colubriformis*, and possible interaction with the deficient consumption of P. The animals were completely randomized distributed in four treatments, in a split-plot factorial arrangement 2×2, with two levels of P in the diet (sufficient and deficient) and two sanitary conditions with and without *T. colubriformis*. Eighteen Santa Ines lambs with initial body weight of 22.4 ± 2.7 kg, were distributed as: sufficient P content and uninfected (n = 4), deficient P and uninfected (n = 4), sufficient P content and infected (n = 5) and deficient P and infection (n = 5). Deficient P diet animals consumed 53% of P consumed by sufficient treatment. Infected lambs received 40,000 *T. colubriformis* in L_3 larval stage in a single oral dose. For performance the animals were weekly weighed in fasting condition, and the apparent digestibility and methane emission were performed in individual metabolic stalls. Passage rate was determined with external (Yb, Co and Cr) and internal (iNDF) markers. The P metabolism was evaluated by the isotope dilution technique with ^{32}P isotope. Ruminal liquid phase samples were used to determine short chain fatty acids, and urine samples to determine the microbial protein using the purines derivatives technique. Two computered tomography scans were performed to determine tissue density, one at the beginning of the experiment and one just before ^{32}P injection. Histological analyzes were performed in the duodenal region, due to characterize mucosa modifications caused by the infection, methane determination was conducted in respirometric chambers. Blood hematological and biochemical parameters were tested twice a month throughout the experiment. Among the main results obtained, it was found that infection by *T. colubriformis* did not affect sheep consumption and performance. However, P and N metabolism were affected in infected animals, which presented lower absorption values (compatible with the mucosal lesions evaluated in histological trial) and plasma P concentration with higher turnover rate and lower bone density with lower P content in bones ($P < 0.05$). Regarding N metabolism, infected animals presented lower retention with greater N loss via urine. Thus, it was concluded that acute *T. colubriformis* infection negatively affected P and N metabolism, and compromised sheep bone mineralization.

Keywords: Endoparasites. Lambs. Minerals. Radioisotope. Santa Ines. Verminosis.

RESUMO

BOMPADRE, T. F. V. **Taxa de passagem, cinética de fósforo (^{32}P) e composição corporal por tomografia em ovinos com infecção aguda por *Trichostrongylus colubriformis*.** 2019. 121 p. Tese (Doutorado em Ciências) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2019.

Objetivou-se com o presente estudo identificar as alterações fisiológicas, bioquímicas e metabólicas dos ovinos em condições de infecção aguda por *T. colubriformis* associando com a consumo deficiente de P. O delineamento experimental foi inteiramente casualizado com arranjo "split-plot" fatorial 2×2, com dois teores de P na dieta (suficiente e deficiente) e duas condições sanitárias com e sem infecção *T. colubriformis*, utilizando-se 18 cordeiros com peso corporal de 22,4 ± 2,7 kg, distribuídos em quatro tratamentos: Teor suficiente de P e sem infecção (n = 4), deficiente de P e sem infecção (n = 4), teor suficiente de P e infectado (n=5) e deficiente de P e infectado (n=5). Os animais restritos consumiram 53% do P consumido pelo tratamento adequado. Os cordeiros infectados receberam 40.000 larvas em estágio de desenvolvimento L_3 de *T. colubriformis* em dose única via oral. Para avaliação do desempenho os animais foram pesados semanalmente e para a digestibilidade aparente da dieta e emissão de metano foram utilizadas gaiolas para estudo de metabolismo. A taxa de passagem da dieta foi determinada por marcadores externos (Yb, Co e Cr) e internos (FDNi). O metabolismo de P foi avaliado pela técnica da diluição isotópica com o ^{32}P radioativo. Amostras de líquido ruminal foram utilizadas para determinar o perfil de acidos graxos de cadeia curta, e as amostras de urina utilizadas para estimar proteina microbiana, pela técnica de derivados de purina. Duas tomografias computadorizadas foram realizadas para determinação da densidade dos tecidos uma no início do experimento e outra antes da injeção do ^{32}P. Análises histológicas foram realizadas na região do duodeno para caracterizar alterações na mucosa causadas pela infecção. Os parâmetros hematológicos e bioquímicos sanguíneos foram testados ao longo do experimento. Dentre os principais resultados obtidos verificou-se que infeção por *T. colubriformis*, não afetou o consumo e desempenho dos ovinos. No entanto, os metabolismos de P e N foram afetados nos animais infectados, que apresentaram menores valores de absorção (compatível com as lesões da mucosa avaliadas pela histologia) e concentração de P no plasma com maior taxa de renovação e menor densidade óssea com menor teor de P nos ossos ($P < 0.05$). Quanto ao metabolismo de N, animais infectados apresentaram menor retenção com maior perda de N via urina. Sendo assim, conclui-se que a infeção aguda por *T. colubriformis* afeta negativamente os metabolimso de P e N, comprometendo a mineralização óssea dos ovinos.

Palavras-chave: Cordeiro. Endoparasita. Minerais. Radioisótopo. Santa Inês. Verminose.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

-d	Experimental days
^{o}C	Degrees Celsius
^{31}P	Stable phosphorus
^{32}P	Radioactive phosphorus
a*	Redness
ADG	Average daily gain
ADF	Acid detergent fiber
ATP	Adenosine tri-phosphate
b*	Yellowness
Bo	Bone
BW	Body Weight
Ca	Calcium
CCC	Concordance correlation coefficient
CH_4	Methane
Co	Cobalt
CP	Crude protein
Cr	Chromium
Cr_2O_3	Chrome oxide
CT	Computed tomography
Dg	Degraded
DM	Dry matter
DPi	Dietary P deficient and infected
DPui	Dietary P deficient and uninfected
EE	Ether extract
F	Flux (model)
Fe	Feces (model)
FE	Feed efficiency
FEC	Fecal egg counts
GIT	Gastrointestinal tract
H_2SO_4	Sulfuric Acid
He	Helium
HU	Housfield scale
I	Infection treatment
iADF	Indigestible acid detergent fiber
iDM	Indigestible dry matter
In	Intake (model)
iNDF	Indigestible neutral detergent fiber
Kp	Passage rate
L*	Lightness
L_1	First larval physiological stage
L_2	Second larval physiological stage
L_3	Third larval physiological stage (Infectant)
L_4	Fourth larval physiological stage
L_5	Fifth larval physiological stage (Adult)
LI	Large intestine
MN	Microbial nitrogen
MRT	Mean retention time
MSE	Mean square prediction error

N	Nitrogen
NaCl	Sodium Chloride
NDF	Neutral detergent fiber
NH_3	Ammonia
P	Phosphorus
P_{lin}	Linear statistical effect
PxI	Interaction effect diet and infection
PDM	Purines' derivatives metabolites
PHL	Physical half-life
Pi	Phosphorus phytate (Model)
Po	Phosphorus organic non-phytate (Model)
Pp	Phosphorus inorganic (Model)
Pt	Phosphorus total (Model)
Q	Total quantity of phosphorus (Model)
R^2	Determination coefficient
RMSE	Root mean square error
RR	Reticulum-rumen
S	Specific activity (Model)
Sa	Saliva (Model)
SCFA	Short chain fatty acids
SEM	Standard error of the mean
SI	Small intestine
SPi	Dietary P sufficient and infected
SPui	Dietary P sufficient and infected
ST	Soft tissue (Model)
Sv	Superficial density
T	Time random effect
$T_{1/2}$	Biological half-life
Ur	Urine (Model)
Vv	Volume
Y	Dependent variable – statistical equation
Yb	Ytterbium
$F_{ab,cd}$	Flux structure (Model)
a	Phosphorus source from
b	Phosphorus source to
c	Outflow pool
d	Inflow pool

CONTENTS

1. INTRODUCTION

Considering sheep farming in Brazil in the last decade and the relation between meat production and consumption, the per capita consumption of sheep meat exceeds its national production, thus the consequences was the necessity of sheep meat import. Nevertheless, despite the vast potential that Brazil has to produce meat and other products from sheep, the total number of animals produced has been constant over the last years. In this scenario, Brazil could be characterized as a favorable livestock producer, with geographic and climatic potential to increase sheep production in different sectors and products. Sheep has a niche market, with meat production by the northeast region, wool in the southern region and the processed products, like fine cheeses, yogurts, and others across the country.

Brazil has about 40% of the sheep farms destinated for meat production, being this sector the most important from an economic stand point. Moreover, most of the beef herd in the country is composed of animals characterized as "rustic", resistant and resilient to diseases, temperature variations and adverse environments. Due to its rusticity and farms conditions, the main sheep breeding systems in Brazil are extensive and semi-intensive, with pasture being the main nutritional source. In grazing production system, health costs are relevant and should be considered, where the principal health prevention costs are the control of endoparasite infections. Considering the parasitological scenario, the gastrointestinal tract (GIT) colonizing nematodes are highlighted, being these parasites responsible for large economic losses in sheep production, mortality of hosts and socioeconomic impacts related to labor and medication (anthelmintics).

There are several species of endoparasites directly related to the sheep infection, the main ones belong to the family Trichostrongylidae, in which, *Haemonchus sp., Trichostrongylus sp., Cooperia sp., Ostertagia sp.* and *Nematodirus sp.* are responsible for most of the economic losses in sheep farming in tropical countries. Among the genus previously mentioned, currently *Trichostrongylus colubriformis* has been widely studied, due to factors such as: high rates of infection in tropical pastures, cross-species infectant parasite (cattle, goats, sheep, pigs and wildlife) and for been considered even a zoonosis.

The main focus of current studies involving *T. colubriformis* infecting ruminants in pasture conditions, are related to prevention and control methods, especially regarding the pasture where animals are housed and fed. However, studies that evaluate the physiological and metabolic modifications that occur in digestive, absorptive and metabolic mechanisms of

infected animals are still incipient. Considering the *T. colubriformis* physiology, L_3 larval stage is the immature infectant and L_5 is the last stage before adult nematodes, and after infected the nematode inhabit the early small intestine (duodenum and early jejunum). The infection consequences are the possibility of changes in basic GIT functions, such as decrease intake, resulting in changes in digestion flow, and decrease nutrients digestion and absorption. Furthermore, the nematode could degrade the intestinal lumen microvilli, causing the contact surface, and as consequence, decrease absorption, furthermore the nematode could act as a physical barrier affecting the feed transit throughout the GIT. All in all, the *T. colubriformis* could negatively affect the digestive capacity and nutrient availability for the host development.

Among all nutrients that infection could reduce absorption and consequently availability, phosphorus (P) stands out, due to the elevated cost to introduce this mineral in ruminant diets. Phosphorus is an essential micromineral, second most quantitatively required in sheep diets, and P is required in many metabolic activities, like ATP energy, bone composition, increased muscle mass (protein), milk production, buffering, and others. Reduction in P absorption can occur in infected sheep once the main site of P absorption is the early portion of the small intestine, the same portion where the nematode inhabits. Since P has not been absorbed, it will be lost in feces. Thus, the infection impact is tremendous, considering the individual negative impact with sick animals, economical negative impact considering nutrient losses in diets previously formulated for a specific requirement, and environmental negative impact like ground and water contamination from excess of P excretion.

In experimental conditions, there are several direct or indirect methods for determining the minimum P requirements for a given animal class. However, the accuracy and precision of those techniques may be considered when trying to evaluate the minimum P requirement as well as the nematodes influence in mineral metabolism. In order to improve P determination, several authors have used the isotopic dilution technique using radioactive P (^{32}P) as a reference indicator to mapping P metabolism in different ruminant species. However, there are few studies correlating P metabolism with endoparasite infection using the isotopic dilution technique. Moreover, to our knowledge, literature does not present data regarding P metaboism of *T. colubriformis* infected animals, which is the main focus of the present study.

Thus, understanding the physiological changes that occur in animals infected with endoparasites are fundamental to driven decisions about the control of endoparasites in the infected individual, as well as preventive action in herd and pasture. Additionally, understanding these physiological changes can be an important tool to avoid excessive nutrient supply to animals (economic losses) and environmental contamination.

1.1. Hypothesis

The main hypothesis of this study is that infection will negatively impact the nutrient (P) absorption, considering that both, parasite and absorption site are located in the same GIT portion (duodenum). Infected animals will increase P losses via feces, so we hypothetize that the digesta flow throughout the GIT will be modified. Therefore, the passage rate will be reduced before the small intestine, to increase nutrient digestion and availability, to facilitate absorption. However, the consequence will be an increase of entheric methane production and emission. The metabolism will be affected by the infection, where body P deposits (bones) will be required to adjust plasma P concentration, so will increase the P resorption from bones, and most of the P will be driven to mesenteric cell, as energy to control the disease, and as a result the animal performance will decrease.

The second factor tested was the diets in P deficiency, providing almost half of the requirements, and we hypothesize that everything described above will be potentialized in animals fed low P diets, and the low amount of P will result in consumption and performance reduction.

1.2 General objective

The aim of the entire experiment was to evaluate possible physiologic, and metabolic changes in Santa Ines male lambs infected with *T. colubriformis* and fed with different P levels.

1.3 Specific objectives

- To evaluate differences in digestive parameters, such as intake, apparent total tract digestibility, digesta passage rate, reticulo-rumen functionality (methane production, short-chain fatty acids concentration, and microbial protein), and compartmental modification in the gastrointestinal tract of Santa Ines male lambs infected with *T. colubriformis* and fed different P levels.
- To verify differences in metabolic parameters, such as P metabolism, nitrogen balance, tissue direct (bone) and indirect (muscle, fat) specific density of Santa Ines male lambs infected with *T. colubriformis* and fed different P levels.
- After all dataset evaluated verify the real consequences of P deficiency, and give a mathematical interpretation of the deficiency and the relation between consumption and losses, developing a model that can explain the metabolism and predict feces losses.

2. REVIEW: Consequences of dietary phosphorus deficiency, and *Trichostronglylus colubriformis* infection

2.1 Sheep production

The ovine species (*Ovis aries*) were one of the first species to be domesticated by humans. Those animals were chosen by the purpose that they produce multiple products that could be used by humans (meat, milk, wool, and others), and sociable and gregarious behaviors that contributed to the domestication (SILVA SOBRINHO, 2001). In addition, some relevant characteristics like animal size, and capability of adaptation in different climate conditions also contributed to the choice. Sheep livestock provided subsistence for nomadic people in food and protection as alternative to environmental conditions.

Nowadays, sheep farms and wild animals is currently present on all continents, particularly due to its adaptive capability in different climates and vegetation. Being explored for economic, subsistence, and entertainment by farms and industries (VIANA, 2008, AMARANTE, 2015). According to Ávila (2010), Australia and New Zealand stand out worldwide as the biggest sheep producers, with very developed, technified and productive sheep farms, being one of the main livestock activities. The interested final product in these countries are meat and wool. But also, they strongly invest in genetics improvement and breed development.

In Brazil, livestock farms destinated to sheep production is characterized in the Northeast of the country as a profitable and widespread activity, due to a high nutritional level of meat and milk available to farmers and local market. The small ruminants have roughness that promote resistance and resilience to the climatic conditions in northeast regions, moreover, adapt to different production systems, from familiar and small farmers, up to sophisticated and intensive systems (GRANADOS; DIAS; SALES, 2006; SOUSA, 2016). The national folck of sheep in Brazil has been reducing in the last 10 years, and in 2017 was registered a herd of approximately 17.93 million animals (IBGE, 2017; Table 2.1). The number of sheep in the Northeast is relevant, gathering 65% of the Brazilian herd. It is noteworthy that the states of Bahia, Pernambuco, Ceará and Piauí contribute to this concentration of sheep in northeast, with almost 82% of all northeast sheep production (BRAZILIAN INSTITUTE OF GEOGRAPHY AND STATISTICS – IBGE, 2017).

Table 2.1: Regional distribution of sheep production herds, between years 2006 and 2017

Regions	Sheep national herd (million animals)		
	2006	2017	Variation (%)
Northeast	7.79	9.03	+15.9
South	4.18	3.30	-20.9
Center-west	0.92	0.59	-35.2
Southeast	0.79	0.43	-45.9
North	0.48	0.41	-15.2
Brazil	14.2	13.8	-2.80

Source: IBGE, 2017

An important physiological aspect related to sheep production, is the reproductive seasonality, which are known that small ruminants in general are reproductive able when the photoperiod begin to drop (between February and March in south hemisphere), commonly called as short days season (AMARANTE, 2015). Therefore, they present sexual activity in periods of the year with shorter duration of the luminosity. The influence of the photoperiod is outstanding in sheep breeds living in the hemisphere. In this region, the reproductive cycle begins due to the decrease in the intensity and duration of light, usually occurring in the seasons with shorter days, as in the fall (SILVA et al., 2018). However, this seasonal characteristic is not observed in northeast sheep productions, since there is no significant variation in the time of luminosity during the days. In this way, animals that live in this locality are not influenced by the photoperiod, reproducing throughout the year.

Another important aspect to be considered is due to the contrast between production and roughness. In Brazil we can find many herds producing specialized animal, destinated exclusively to meat, or milk, or wool. Many breeds could be considered as specialized for meat production (Dorper, Texel, Suffolk, Ile de France, and others) with characteristics like higher apparent digestibility, better performance, and good quality carcass. However, there is a negative relation between production and roughness, where high production animals commonly are more susceptible to diseases, and climate modifications. In this sense, many

studies are trying to find animals with these both characteristics, and understand the causes and consequences of these resistance and resilience aspects.

In Brazil, there are some breeds classified as “native” (Santa Ines, Morada Nova) that is widely produced in northeast regarding to the rusticity observed in these animals. Considering the Brazilian native breeds, the Santa Ines stands out for its high reproductive efficiency, good productivity and adaptability, low susceptibility to endo and ectoparasites, rusticity and larger body size when compared with others sheep breeds with similar characteristics (SILVA SOBRINHO, 2001). By all these relevant considerations, this breed is being the largest herd in Brazil and still expanding, making it an excellent alternative in meat production, especially in equatorial climate regions.

2.2 Phosphorus in the animal metabolism

Phosphorus (P) is a mineral element with atomic number 15 and atomic mass 30.97. On earth surface, P is responsible for 0.12% of all mineral matrix. The ^{31}P is the stable element, however, some radioactive isotopes could also be observed, the most common isotope is the ^{32}P, presenting one more neutron in the molecular structure, emitting high energy β radiation, and have a physical half-life of 14.8 days, approximately. There are many sources of P, but the most common feed source of P is Phytic acid, phospholipids, nucleic acids, and some other presented in vegetal structure. However, in animal feed diet the most common source of P used is from rocks sources, in general, because of the viability to purchase this ingredient and the better palatability that rock mineral sources provide (DIAZ GONZALEZ; OSPINA; BARCELLOS et al., 1998). Across all mineral sources added in diets, the dicalcium phosphate is the most used.

Phosphorus is the second mineral most presented in live organisms, in animal, P and Calcium are responsible for bones matrix mineralization (MCDOWELL, 1992). Regarding all body weight, 0.9 to 1.1% is P (ANDRIGUETTO, 1990), where, 80 to 85% of total P presented in body is located in bone, 3% is in gastrointestinal tract (GIT), and between 12 and 17% is distributed in different soft tissues (DIAZ GONZALEZ; OSPINA; BARCELLOS et al., 1998). Among its metabolic functions, P participate directly in the energy metabolism (ATP molecules), being also an essential component of amino acids, proteins and lipids (TERNOUTH, 1990), and some other enzymatic cofactors. In ruminants, P represents an important role in the metabolism, rumen microbiota maintenance and development (BREVES; SCHRÖDER, 1991). P deficiency represents an economic loss by farmers, and the

deficiency is not unusual, due to it's high cost, and the reduction of P natural deposits. Being considered a limiting mineral, the correct P balance in the diet promotes better animal performance and reduces over supplementation (DIAZ GONZALEZ; OSPINA; BARCELLOS, 1998).

Some studies recommend consumption of P around 0.15 g/kg of body weight (BW) for adult animals (NRC, 2007), and 0.18 to 0.20 g/kg of BW in animal in growing phase (LOUVANDINI et al., 2008). After intake, the rumen, is the first compartment to receive feed P, however, P from feed is not the only source achieving rumen, the other source is the endogenous salivary secretion, an important recyclable via that contributes with 50 to 70% of P presented in rumen liquid (VITTI; KEBREAB, 2010). In the rumen, P is responsible to ruminal environment homeostasis control, regulating ruminal pH avoiding acidification (less then 6), and moreover, contribute as nutrient to ruminal microorganisms (SCHRÖDER, 1991). Normal P values in sheep saliva are between 2 and 6 mg / 100 mL (THOMPSON JUNIOR, 1978), depending on the dry matter and P intake, as well as the effective fiber content present in the diet (VALK et al., 2000).

An important process that occur in rumen and abomasum is the bioavailability, a kind of digestive process that turns P into an available form to be absorbed. Dietary P is in inorganic (mono, di and tri phosphate) and organic (phytate, phospholipid and phosphoprotein) forms, and endogenous P is exclusively in organic form. Inorganic P is solubilized by the action of gastric juice. However, much of the organic P (phytate) suffers the action of microorganisms at the rumen level (BRAVO et al., 2003a), the organic form is the majority absorbable form. Moreover, the P could be absorbed by rumen, small intestine and large intestine mucosa, however que main P absorption site is the duodenum, first portion of the small intestine (REINHARDT; HORST; GOFF, 1988). An adult sheep is able to absorb 4 g of P daily (PFEFFER et al., 1989), but the absorption efficiency depends on many factors, like source and amount of P intake, ruminal and intestinal pH, interaction with other minerals, age, physiological stage, and health status (BRAITHWAITE, 1984). The P non absorbed or resorbed from the system back to the GIT and others endogenous sources is excreted via feces. This route is the principal way of P excretion, representing up to 70% of all animal P losses (VITTI et al., 1991; BRAVO et al., 2003b).

After absorbed by the mesenteric cells, P achieve the system by blood. The blood could be considered a central pool of P stock and driven the mineral to body deficient spots. in the blood P is ready to be retained by bones, and other tissues. In normal conditions of health animals, the P concentration in blood is between 4 – 9 mg / mL of blood

(MCDOWELL et al., 2003), concentration below 9 mg could indicate P homeostasis because of any disfunction, and concentration below 4 mg is a dietary deficiency indicator (UNDERWOOD; SUTTLE, 1999). An excess of P in blood will promote a physiological answer of GIT resorption, as described before, and urine excretion, that is a secondary excretion via, where an animal in normal conditions will excrete 2 mg of P / Kg of BW per day (TERNOUTH; SEVILLA, 1990). Although the urine P excretion route is not a high excretion way, it is essential to the blood P homeostasis in ruminants (BRAVO et al., 2003b).

The P present in blood could be retained in organs, other tissues, and bones. Bones are the mean stock of P in the body, and the transport of P between blood and bones is continuously happening, but the relation retention / resorption depending on the concentration of P in plasma and the requirements of P in other tissues (BORTOLUSSI; TERNOUTH; MCMENIMAN, 1996). For example, a dietary P restriction will reduce blood P concentration and consequently increase the P resorption from bones. On the other hand, in organs and other soft tissues, P is fundamental to the energy, carbohydrates (by forming coenzymes), and intermediate protein metabolisms. And those soft tissues store around 20% of total P present in the BW (UNDERWOOD, 1981).

The P fluxes, transports and physiological mechanisms are system strategies to preserve the animal homeostasis status. Homeostasis is defined as a regulatory mechanism that animal organism has to balance its metabolism in response to dietary or physiological changes (CHALLA; BRAITHWAITE, 1988). The P regulation is accomplished through absorption, excretion, retention, recycling and secretion in the digestive tract, bone retention and resorption, and the importance of each mechanism depends on the dietary P (LOUVANDINI, 1995), and these processes are responsible to control and regulate the circulant and deposits of P (MATSUI et al., 1984).

2.3. *Trichostrongylus colubriformis* infection

Gastrointestinal helminths are responsible for promoting malnutrition, avitaminoses, gastrointestinal disorders, convulsive states and compromise animal development (SOUZA, 2013). Gastrointestinal nematodes are the most frequently class of parasite observed in ruminants worldwide, especially in humid areas, and in grazing animals at pasture. The principal consequences of the infection are lesions ranging from the abomasum to the intestine (COSTA, 2007), digestive changes, delays in growth, and production reduction (CANTACESSI et al., 2010), moreover, some hematophagous species can promote anemia.

The principal family of parasite that infect sheep flocks is *Trichostrongylidae*, and in this family it is possible to highlight the genus *Haemonchus spp.*, *Ostertagia spp.*, *Trichostrongylus spp.*, *Cooperia spp*. According to Amarante (2015), nematodes that parasitize ruminants and reside in the gastrointestinal tract, most of them have a similar evolutionary aspect, however differ in their effects on the host.

Highlighting the life cycle of the parasite from genus *Trichostrongylus spp.*, it does have a direct life cycle and do not require intermediate host. This class of parasite have two distinct life stages, one free and one parasitic. Adult parasites inhabiting the small intestine of the host lay eggs that are eliminated by feces. After hatching, the larvae start the free life in first stage of development (L_1), the larvae change cuticles to pass the next two development stages (L_2, and L_3). The L_3 stage is when the parasite is capable to infect a new host. The free life phase lasts approximately seven days. L3 larvae are ingested at the time the sheep grazes on contaminated pasture, and complete the evolutionary cycle (L_4, and L_5) in the digestive tract (mainly in the duodenum), originating adult males and females (AMARANTE, 2015). According to Wood et al. (1995), the pre-patent period of *T. colubriformis* can last an average of 21 days, and them the eggs will start to appear in feces. The free-life phase, infective larvae are able to survive for long periods, even under adverse conditions of cold or without rain periods (LETTINI; SUKHDEO, 2006).

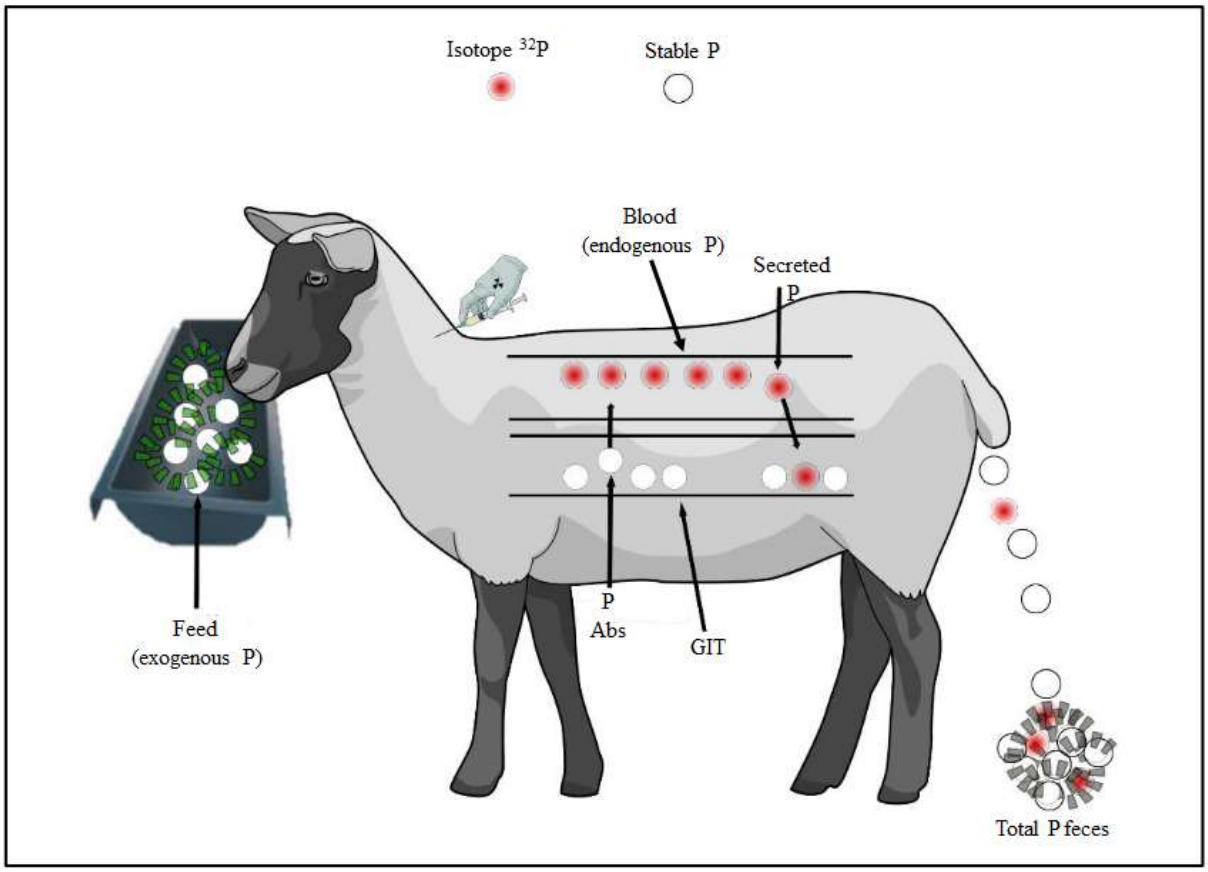
Clinical symptomatology of animals infected with endoparasites is dependent of a triple interaction between the infection challenge, disease establishment, and host immune response (AMARANTE, 2015). The consequences of a severe infection are the generation of tunnels, causing erosion in the intestinal epithelium with villous and microvilli atrophy, intestinal crypt hypertrophy, mucosal thickening, and the formation of leukocyte inflammatory process (TAYLOR et al., 2010; CARDIA et al., 2011). These changes caused by infection, promote increase of vascular permeability (BARKER, 1975), affecting gastrointestinal motility with reduced nutrient consumption (GREER et al., 2005), altering digesta retention time throughout the tract, reduced digestion and nutrient absorption (STEEL; SYMONS; JONES, 1980; POPPI et al., 1985; CANTACESSI et al., 2010), compromising the growth rate and performance.

2.4. Isotope dilution technique in metabolism assays

Metabolism assays are important in animal nutrition for a better comprehension of nutrient requirements and fluxes across animal system. Literature studies have been using different methodological approaches to determine animal requirements and metabolism. In mineral studies, the most common techniques applied are using nutrient free diets, multiple diets using increasing levels of nutrient, or nutrient tracers (COELHO DA SILVA; LEÃO, 1979), for all these techniques, when the study aim to determine minerals metabolism, the animals need to be slaughtered to evaluate bones and soft tissues concentration, and determine mineral system dynamics (LOFGREEN; GARRET, 1968).

Regarding the studies involving tracers to determine metabolism, radioactive tracer is classified as a precise and accurate form to determine the systemic metabolism, true absorption efficiency and endogenous losses (CANTONE; GIUSSANI, 2001). Considering studies involving the P metabolism determination, Kleiber et al. (1951) used ^{32}P to determine fecal endogenous P and total tract true digestibility. The isotopic dilution technique applied to determine P dynamics is based on the use of a known amount of ^{32}P (radioactive isotope) as a tracer substance infused in the animal system, after the isotopic steady-state condition, measurements are performed across the system (blood, bone, soft tissue), in the GIT and excretions (feces, urine). Radioactive tracers are considered the most appropriate marker in metabolism studies, by considering that the isotope will have same chemical, physical or biological properties compared to the stable element. Moreover, radioactive elements have particular characteristic (particle or electromagnetic waves emission) that torns it able to be detected and measured (CANTONE; GIUSSANI, 2001). Figure 2.1 illustrate how the technique is applied in animal metabolism studies.

Figure 2.1: A schematic representation of the radioisotope technique in sheep



Source: Vitti and Kebreab (2010), adapted from Dias e Silva (2017).

Isotopes are elements that have the same chemical properties, but with different atomic masses conditioned by different numbers of electrons. Assuming that the radioactive tracer and the unlabeled element are both in steady-state condition, the tracer will have similar flow behavior of the unlabeled element, being able to determine the kinetics of this element in a given system (VITTI; KEBREAB, 2010). When ^{32}P is carried from the blood to the GIT, it could be used by the digestive microbiota, reabsorbed in the small intestine, or excreted in feces, all routes following the same pattern then unlabeled P. In sheep, the maximum peak of ^{32}P feces excretion occurs 24 hours after intravenous injection (VITTI, 1989), after peak, the ^{32}P in feces decrease in an exponential curve, this mathematical expression is used determine endogenous losses and real P absorption (COMAR et al., 1953).

2.5. Mean retention time and passage rate

In ruminants, food digestion and nutrient availability depends on the fractional degeneration rate, and the fractional passage rate (VAN SOEST, 1994). Several factors can modify digesta passage rate, these being related to the diet and the animal. Dietary causes are particle size, density, specific gravity and dietary intake level (de VEGA; POPPI, 1997; HRISTOV, 2003), and among animal factors, the passage rate could be affected by gender, physiological stage, species, and sanitary status (FAICHNEY, 1996). Clauss et al. (2006) observed differences in the passage rate between grazer and selector herbivores, sheep is classified as a grazer animal, with an intermediate selection capacity, higher than cattle, but lower when compared to goats (HOFFMANN, 1989), however, any of these characteristics reduce the infection susceptibility.

The methodologies to determine mean retention time and consequently, passage rate could be classified as indirect and direct. The indirect methodologies have as their principle the use of markers complexed feed solid fraction, being supplied to the animals in single dose, and multiple defined interval sampling. The samples are analyzed and the marker concentration points fit a nonlinear mathematical model to determine the excretion curve and calculate the passage rate (LASCANO, 1990). Direct methodologies, in turn, consist of ruminal evacuation, which can be performed in slaughtered or cannulated animals. The ruminal evacuation method has been widely used (de VEGA, 1998; HUHTANEN et al., 2006; KRIZSAN et al., 2010) seeking to estimate the passage kinetics and the average retention time for each GIT segment independently.

To determine the passage rate in the indirect methodology, markers infusion should be performed continuously to achieve “steady-state” status, what means that the indicator concentration along the GIT will be constant, thus allowing the measurement of each segment independently. Steady-state means the period during which food and, consequently, marker input and output flow remains constant (FOX, 1991) are important requirement to estimate feed degradation and transit. Fontes et al. (2001) stated that slaughter methodologies although generating a single measurement, is described as more precise and accurate when compared to cannulated methods.

Markers in digesta flux studies, are the main methodological tools for estimating digestion and passage kinetics along the GIT. Uden et al. (1980) and Owens (1992) described that an efficient marker needs to be:

- not be absorbed throughout the tract;
- be nontoxic;
- not affect or be affected by digestive processes;
- be easily determined and measured in laboratory procedures;
- not dissociate from the feed correspondent;
- have an intimate association with feed;
- equal distributed in the GIT digesta;
- do not influence the digestive tract microbiota.

Markers are classified as internal and external (FIRKINS et al., 1986). Internal marker refers to a component present on feed, that does not undergo actions of mechanical (reticulo-rumen, omasum, cecum and colon) and chemical (abomasum) digestion, also called indigestible fraction of the diet. Since indigestible constituents do not undergo digestion action, the mechanism of disappearance of the constituent is only the passage. If the indigestible particles have similar digestion kinetics, the fraction of these digestible particles may be considered as a marker. Advantages regarding the use of internal markers have been reported in the literature been a constituent of feed, low cost, easy measurement and does not need feed labelling process (PIAGGIO et al., 1991). Many indigestible feed portions were tested but the most common applied in trials are the indigestible detergent fibers in acid (iADF) and neutral detergent fiber (iNDF), and the indigestible dry matter (iDM) (DETMANN et al., 2001; ZEOULA et al., 2002).

Regarding external markers, we can divide them by adherence to the solid or liquid digesta fraction. The most common external solid phase markers are, rare earth elements, such as Samarium, Lanthanum, Ytterbium, Dysprosium, Cesium, Chromium, Cobalt, and Ruthenium for estimating GIT particles and fluid passage rate (COMBS et al., 1992; CEZIMBRA, 2010). Determining some of the rare earth elements require specific procedures involving elevate costs and specific laboratories techniques (HARTNELL, 1979). Nevertheless, external markers have been receiving methodological criticism for altering the physical characteristics of the diet interfering in digestion process, as well as a possible dissociation of the labeled material, and labelling other particles or microorganisms, distorting the result (SATTER, 1992).

2.6. Body tissue density and composition via computed tomography

Body composition means the chemical composition of the whole body of the animal, and refers to the composition of the empty body. Its determination is fundamental for the evaluation of the animal nutritional status and give a qualitative view of the animal growth. Some author analyzes the body composition as an estimation of nutrient requirements for animal weight gain (GREENHALGH, 1986; PUTRINO et al., 2006).

There are several methodologies for determining body composition and in general, they can be divided into those who measure live animal, and those who determine it after slaughter process. Direct methods (after slaughter) is considered by some authors as the most accurate and reliable way to evaluate body composition, since it consists in determining the concentration of nutrients in the animal's body (MILLER et al., 1988) by chemical analysis of all animal tissues (muscle, fat and bones). To determine the weight gain (performance) composition by the direct method, the comparative slaughter technique, described by Lofgreen and Garret (1968), is adopted, in which a group of animals is slaughtered at the beginning of the experiment (reference animals) representing the initial body composition. and the other animals are slaughtered at the end of the experimental period, representing the final body composition, and the difference is mathematically interpreted to represent the gain composition. The direct method is criticized by the high costs in the experiment, the necessity of slaughter an elevated number of animals, in large animals the difficult in have a representative sample, and the unfeasibility of multiple measurements (STANFORD et al., 1998).

The methodologies performed in live animal allow to evaluate the body composition several times in the same animal, being considered a nondestructive tissue technique, and provide multiple low-cost analysis at the same time in the same animal. However, some negative aspects need to be cited, like the high cost equipment to provide the results, some results can have inconsistent repeatability, and the animal radiation exposition (STANFORD et al., 1998). Considering all methods described in the literature, the computed tomography (CT) is the most used, regarding to the high-quality image provided, and the availability of evaluate the whole body and multi specific portion of the body at the same image (SEIDELL et al., 1990). The basic CT system consist of an x-ray tube and receiver that rotate in a perpendicular plane of the animal tested, the x-ray beams are attenuated as they pass through tissues (SPRAWLS, 1977), the attenuation is related to the density, and the water volume in the tissue, and the attenuation CT number determined is converted in a gray shades contrast

scale (from -1000 to 1000), named Housfield scale - HU (HEYMSFIELD et al., 1979). The HU contrast has direct linear high correlation with the tissue density (WANG et al., 1993), moreover, identifying the HU range number for each body carcass tissue, is possible to quantify the proportion of each tissue, been characterized as carcass tissue composition (WANG et al., 1995)

2.7. Previous studies interacting Phosphorus metabolism and nematode infection

Wilson and Field (1983) studied the effects of the interaction between P dietary restriction (70% of adequate treatment) and a light *T. colubriformis* infection in the P metabolism of growing lambs, using the ^{32}P isotopic dilution technique. The results indicated a reduction in plasma P concentration, as well as a reduction in the P secretion by saliva, in restricted P diet lambs, but non effect was observed for infection for each variable.

By using the same technique, Louvandini et al. (2009a) tested the infection effect of a single dose of 45,000 infecting *Cooperia punctata* larvae, in calves P metabolism, and the results did not show any reduction in animal consumption, however, the infection reduced the gain of BW in live weight, blood P concentration, and P retention. The consequences of those reductions were a greater P excretion via feces. Moreover, the infection promoted a P resorption from bones and soft tissues to the blood. Louvandini et al. (2009b) also tested the same calves P metabolism effect of *Cooperia punctata,* but a serial infection of 10,000 infective larvae per week for 35 days. The serial infection reduced feed consumption, P absorption in the small intestine, and reduced weight gain. The study showed a reduction in the P exchange between blood and GIT, and a reduction in the P retention by bones and small tissues. These results were obtained when applied the model developed by Vitti et al. (2000).

The literature presents some tools that help metabolism studies, an important tool described is some dynamic mathematical models that mathematically explain the fluxes of P in the metabolism. Lopes et al. (2001) developed a six-pool (rumen, small intestine, blood, saliva, bone, and soft tissues) model, and Louvandini et al. (2008) applied the model to study the effect of increasing levels of dietary P in the performance and metabolism of growing lambs. The model showed an exponential curve by the P intake, where was able to determine the minimum P intake required to give the best performance condition. Authors also found a linear correlation between P intake and the balance (retention/resorption) for bone and soft tissue.

A recent study conducted by Dias e Silva et al. (2018) evaluated the effect of *T. colubriformis* serial infection (5,000 L_3 larval stage per three days, for three weeks) in the P metabolism of growing Santa Ines lambs, also using the isotope dilution technique. The results indicated that infection did not affect feed intake. However, compromised the absorption coefficient as a consequence, the blood P concentration decreased, as well as, the salivary secretion, and the retention in bone and soft tissue. The absorption reduction contributed to the increase of P concentration in feces of infected animals.

All studies described above showed that endoparasite infection affect the P metabolism in lambs and calves, but some other effects could be observed caused by the infection. Dias e Silva et al. (2019) tested the effects of a serial *T. colubriformis* infection (5,000 L_3 larval stage per three days, for three weeks) in the GIT physiology and digesta flow, and the reticulo-rumen was majorly affected by infection, infected animals presented faster digesta (forage and concentrate) passage rate compared to uninfected ones, and consequently, impacted the short chain fatty acids cocentration in the rumen.

Not only the P metabolism could be impared by the endoparasites infection, but the nitrogen metabolism could also be impared. Prichard, Hennessy and Griffiths (1974) evaluated the effect of *T. colubriformis* infection (single dose of 40,000 – 85,000 L_3 larvae per animal) in merino ewes. They concluded that the infection affected endocrine system, and may affect the nitrogen metabolism. Yu et al. (2000) worked with crossbreed sheep and evaluated the leucine metabolism in animal infected with *T. colubriformis*, and the results indicated that a severe nematode infection reduce aminoamides, and consequently, nitrogen blood concentration, and the infection also increased the nitrogen losses via urine. Therefore, based on previous studies that describe there are evidences that not only minerals need to be evaluated, but also other nutrients' metabolism.

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3. Performance, phosphorus metabolism, bone density and blood markers in Santa Ines lambs infected with T*richostrongylus colubriformis* and fed different levels of phosphorus

Abstract: This trial tested the performance, phosphorus metabolism, bone density and blood markers in Santa Ines lambs infected with *T. colubriformis* and fed different levels of phosphorus. Eighteen Santa Ines, castrated male, six-month age, health lambs (initial body weight 22.4 ± 2.7 kg) were distributed in 4 treatments in a 2×2 split-plot arrangement: Sufficient dietary P level and uninfected (SPui; n = 4), Sufficient dietary P level and infected (SPi; n = 5), Deficient dietary P level and uninfected (DPui; n = 4), Deficient dietary P level and infected (DPi; n = 5). Infected lamb received, orally, a single pulse dose of 40.000 *T. colubriformis* infective larval stage (L_3). Animals were fed with Tifton hay cv85 (*Cynodon ssp.*; 60%), and cassava meal and maize gluten meal (40%). For performance, animals were weekly weighted fasten. Blood samples were weekly collected for hemogram description and biochemical (total protein, albumin, urea, and glucose). A computered tomography was performed two times during the experiment. The isotope dilution technique was used to estimate P metabolism, therefore a ^{32}P solution (7.4MBq/0.5mL). Fecal egg counts (FEC) was performed each three days beginning at 18 days after infection, after slaughter the adult nematodes was counted and characterized (gender, length, eggs in reproductive tract). Statistical procedures were performed in R studio, treatments, interaction, and time effects were tested by ANOVA, and means compared by Duncan's test at 5% significance. Results showed that DPi kept high FEC longer compared to SPi. Performance, feed intake, and blood analysis (hematological and biochemical) were not affected by the treatments. Regarding the tissue density, infected animals reduced bone density and uninfected kept. In fat tissues all treatments tested reduced Housfield scale (get darker) across measurements (0-d and 90-d). Bone P concentration decreased ($P<0.05$) in infected animals. The P metabolism, lower endogenous losses ($P < 0.001$) were observed in animals fed deficient P diet, as expected. Plasma, saliva, and ruminal liquid content P concentration, decreased in infected animals ($P < 0.01$). The absorption ($P = 0.07$) and retention ($P = 0.71$) coefficients did not differ by treatments, on the other hand, the biological haf-life ($T_{1/2}$) ($P = 0.04$) on infected animals presented faster ^{32}P removal from blood compared to the uninfected. We concluded that *T. colubriformis* infection can modify animal metabolism and the blood P concentration was an efficient way, to identify animal problems, linking the infection and the bone mineralization.

Key words: absorption, endoparasite, hematological, retention, sheep, verminose

3. Desempenho, metabolismo de fósforo, densidade ossea e parâmetros bioquímicos sanguíneos de cordeiros Santa Inês infectados com *Trichostrongylus colubriformis* e submetido a dietas com diferentes níveis de fósforo.

Resumo: Objetivou-se com o presente estudo avaliar o desempenho, metabolismo de fósforo, a densidade óssea e os parâmetros bioquímicos sanguíneos de cordeiros Santa Inês infectados com *T. colubriformis* e alimentados com diferentes níveis de fósforo. Foram utilizados 18 cordeiros Santa Inês, machos castrados, com seis meses de idade, peso inicial 22,4 ± 2,7 kg, distribuídos em quatro tratamentos em arranjo com parcelas subdivididas 2×2: P adequado e não infectado (PAni; n = 4), P adequado e infectados (PAi; n = 5), P deficiente e não infectados (PDui; n = 4), P deficiente e infectados (PDi; n = 5). Os cordeiros infectados receberam, via oral, a dose única de 40.000 *T. colubriformis* em estágio larval infectante (L_3). Os animais foram alimentados com feno de tifton cv85 (Cynodon ssp.; 60%) e concentrado a base de farelo de mandioca e farelo de glúten de milho (40%). Para o desempenho, os animais foram pesados semanalmente em jejum. Amostras de sangue foram coletadas semanalmente para testes de hemograma e bioquímica (proteína total, albumina, ureia e glicose). Foram realizadas duas rodadas de tomografia computadorizada, no início e ao fim do experimento. A técnica de diluição isotópica foi utilizada para estimar o metabolismo do P, no qual, uma solução de ^{32}P (7,4MBq / 0,5mL). A contagem de ovos nas fezes (OPG) foi realizada a cada três dias a partir de 18 dias pós infecção, após o abate, os nematoides adultos foram contados e caracterizados quanto ao sexo, comprimento, ovos no trato reprodutivo. Os procedimentos estatísticos foram realizados no programa R studio, tratamentos, interação, e os efeitos do tempo foram testados por ANOVA, e as médias comparadas pelo teste de Duncan a 5% de significância. Os resultados indicaram que o PDi manteve um FEC mais alto em comparação com o PAi. Quanto ao desempenho, consume voluntário e análise sanguínea (hematológica e bioquímica), nenhum destes foram afetados pelos tratamentos. Em relação à densidade dos tecidos, os animais infectados reduziram a densidade óssea e os não infectados mantiveram ao longo do tempo. Nos tecidos adiposos, todos os tratamentos testados reduziram a escala de Housfield (tornam-se mais escuras) entre as medidas (0-d e 90-d). A concentração de P no osso diminuiu ($P < 0,05$) nos animais infectados. Quanto ao metabolismo de P, menores perdas endógenas ($P < 0,001$) foram observados em animais alimentados com dieta deficiente em P, como previamente esperado. A concentração de P no plasma, na salivar e no líquido ruminal, diminuída em animais infectados ($P < 0,01$). Os coeficientes de absorção ($P = 0,07$) e de retenção ($P = 0,71$) não diferiram estatisticamente entre tratamentos, por outro lado, a meia vida biológica ($T_{1/2}$) diferiu significativamente pelo tratamento ($P = 0,04$), sendo que animais infectados apresentaram uma saída mais rápida de ^{32}P do sangue quando comparado com não infectados. Concluí-se que a infecção por *T. colubriformis* pode modificar o metabolismo animal e a concentração sanguínea de P pode ser considerada uma maneira eficiente de identificar problemas sanitários nos animais, ligando a infecção e as alterações na mineralização óssea.

Palavras chave: absorção, endoparasita, hematológico, ovinos, retenção, verminose

3.1. Introduction

One of the most important characteristics in ruminants is the capability in transform non-human edible food in energy to attend the requirements, avoiding food competition between humans and animals (MCNEIL; VAN ELSWYK, 2012). Moreover, grazing animals permit indirectly the conversion of fiber in products that can meet human needs as meat, milk, wool, and others. The small ruminant production offers benefits beyond those mentioned above, like the smaller animal size, easy management, and products like low fat meat, high solids milk, wool (of cashmere for goats), and others destinated to the cosmetics and clothes industries (ZYGOYIANNIS, 2006).

Nutrition is one of the most important factors for a sustainable sheep production, as well as other livestock production species. Nutrients are relevant and necessary to attend animal requirements, and therefore, minerals are added in animals' diets to participate in chemical and physiological functions necessary to keep the animal health and alive (KHOL-PARISINI et al., 2015). Among the minerals required, phosphorus (P) is an important one, considered as the second essential macronutrient most quantitatively required in sheeps' diets. Moreover, P is a high cost mineral and increasingly scarce in the world for extraction, however, P dietary addition guarantee energy for vital functions (ATP), enzymatic P dependent activities, avoid bones devises, and many other benefits (HUMER; ZEBELI, 2015).

Economic losses promoted by P deficiency are strongly discussed, and an important cause of economic losses is gastrointestinal helminths which currently represent one of the main limiting factors in all kinds of sheep system(MAVROT et al., 2015). Helminths diseases are even worse in extensive systems, principal sheep production system in tropical countries, and pasture conditions favored and empowered the parasite lifecycle and the host infection. By all of the sheep nematodes parasites described in the literature, *T. colubriformis* is one of the most common parasites observed in field conditions. This endoparasite infects the initial portion of the sheep small intestine (duodenum), for being a non-hematophagous parasite, the *T. colubriformis* main problem is the competition for nutrients with the host (AMARANTE, 2015).

The relation between P and the endoparasite infection is that the gastrointestinal tract (GIT) portion where the endoparasite infect is also responsible for mostly P absorption to attend the animal requirements, moreover, *T. colubriformis* may damage the duodenal mucosa, reducing the absorptive surface, and consequently, reduce the nutrient absorbed (CARDIA et al., 2011). In this scenario, a severe infection could affect all P metabolism and bioavailability of P to maintain all mechanisms (DIAS e SILVA et al., 2018), animals in growing stage, the quantitative (average daily gain and efficiency) and qualitative (modifications in muscle, fat, and bone) performance could be affected (FOX, 1997), and biochemical and hematological parameters, even considering a non-hematophagous parasite (AMARANTE, 2015).

In general, the infection could affect P bioavailability, absorption and retention, affecting all animal mechanisms of P uses in body, when the infection is associated with unbalanced diets, providing less nutrient as required, the animal damage is aggravated, resulting in irreversible metabolic problems and growth deficiency, on the other hand, the excess of P could be flushed out from the GIT, resulting in economic losses, and the excretions will pollute the environment. Therefore, the present trial tested the performance, phosphorus metabolism, bone density and plasma biochemical parameters in Santa Ines lambs infected with *T. colubriformis* and fed sufficient and defficient levels of phosphorus.

3.2. Material and Methods

3.2.1. Location, experimental design, feeding regimen, and infection

The study was performed in the Animal Nutrition Laboratory (22º42'30" South, 47º38'30" West, and 546m Altitude) of the Center of Nuclear Energy in Agriculture, a research center of Sao Paulo University. All procedures were approved by the Ethics Committee on Animal Use (CENA/USP – 004/2015) and conducted in accordance with the rules of Waste Management of ESALQ/USP. The trial was carried out with 18 Santa Ines, castrated male, six-month age, health lambs (average initial body weight (BW) 22.4 ± 2.7 kg). Those animals were distributed in a complete randomized unbalanced experimental design with a 2 × 2 split-plot arrangement: Sufficient dietary P level and uninfected (SPui; n = 4), Sufficient dietary P level and infected (SPi; n = 5), Deficient dietary P level and uninfected (DPui; n = 4), Deficient dietary P level and infected (DPi; n = 5).

Previously, the lambs were checked for endoparasite infection, and health status confirmation. The total experimental period was 118-d, in which, 22-d was for basal diet and stall acclimation. The first 45-d of experiment was allowed to dietary P levels factor, thus, 18 animals were randomly distributed in two treatment groups (SP and DP). On day 46 the endoparasite infection factor was added, where, five animals in sufficient and deficient P treatment were randomly sorted and infected to complete four treatment effects. After infection the trial lasted 51-d. For infection, each lamb received, orally, a single pulse dose of 40.000 *T. colubriformis* infective larval stage (L_3). The noninfected group received distillated water.

During the 96 experimental days, the lambs were individually housed with unrestricted supply of water. Forage (60 % of DM basis) source was Tifton hay cv85 (*Cynodon ssp.*) and the concentrate (40 % of DM basis) source was cassava meal and maize gluten meal (Table 3.1). The diets were offered twice daily (8:00 am and 5:00 pm) in amounts sufficient to provide at least 10% as daily refusal. The diets where formulated based on the Nutrient Requirements for growing lambs gaining 100g/day of BW (NRC, 2007; LOUVANDINI et al., 2008).

Table 3.1: Ingredient partition (as fed percentage), ingredients chemical composition (g/kg of dry matter (DM)), and experimental diets composition (g/kg if DM) provided to the lambs

Ingredients	Diets (% as fed)		g/kg	g/kg DM					
	SP	DP	DM	CP	NDF	ADF	EE	ASH	P
Hay (*Cynodon ssp.*)	60.0	60.0	865	75.2	789	408	2.4	60.2	1.8
Cassava meal	20.4	21.1	897	16.9	53.2	35.5	-	6.7	0.5
Maize gluten meal	17.0	17.0	929	621	15.0	10.0	25.8	18.7	5.0
Limestone	-	0.4	997	-	-	-	-	994	0.1
Dicalcium phosphate	1.1	-	975	-	-	-	-	919	187
Mineral mixture[1]	0.5	0.5	997	-	-	-	-	997	-
Soy bean oil	0.7	0.7	1000	-	-	-	923	-	-
Urea	0.3	0.3	981	2337	-	-	-	-	-
Treatments									
Sufficient P	-	-	874	147.2	422.0	219.9	14.6	45.3	3.8
Deficient P	-	-	873	147.3	422.4	220.1	14.6	39.7	1.8

SP = sufficient Phosphorus, DP = deficient phosphorus, DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber, EE = ether extract, P = phosphorus

[1] Mineral mixture: Mg = 10 mg/g, S = 35 mg/g, Na = 120 mg/g, Cu = 0,756 mg/g, Mn = 2.18 mg/g, Zn = 2.8 mg/g, I = 0.056 mg/g, Co = 0.044 mg/g, Se = 0.14 mg/g, F = 0.85 mg/g.

3.2.2. Performance and intake evaluation

The lambs were weekly weighted before been fed during the 96-d experimental period. The refusal was weighted daily and the given feed was also daily adjusted to attend the unrestricted feed status. The feed intake was calculated with the Eq.1, and the feed efficiency (FE) was calculated with the Eq.2.

$$DFI_{(g)} = Given_{(g)} - Refusal_{(g)} \quad \text{Eq.1}$$

$$FE_{(Unitless)} = BWgain_{(kg)} / TFI_{(kg)} \quad \text{Eq.2}$$

Where, DFI was the daily feed intake in g, FE was the feed efficiency, BWgain was the body weight gain during the experimental period, and TFI was total feed intake calculated for the whole experimental period in kg/d. Feed and refusal were also sampled for bromatological analysis. In addition, the water intake was daily measured for 5-d during the digestibility trial, and adjusted for contamination and evaporation.

3.2.3. Biochemical and hematological parameters, and phosphorus in blood

Blood samples from the lambs with and without anticoagulant (EDTA-2k) were weekly collected in the morning right before feeding by the jugular vein during all experimental period. The hematological analysis was performed in the blood collected with anticoagulant in an automated hematology analyzer for veterinary use (pocH – 100iV Diff, Sysmex corporation – Kobe, Japan). Differential leucocyte count was performed following the method descripted by Sastry (1989).

Plasma and serum were harvested from blood sample with and without anticoagulant, respectively, after centrifuging during 10 minutes in 2000 cycles per minute, and stored frozen at -20 °C. The biochemical parameters were performed by using diagnostic test kits (Labtest diagnostic S.A. – Minas Gerais, Brazil), and the parameters measured were total protein (Labtest Ref.:99), albumin (Labtest Ref.:19), urea (Labtest Ref.:27), and glucose (Labtest Ref.:133). The concentration of P in plasma was determined by the protocol described by Fiske and Subbarow (1925).

3.2.4. Tomography analysis and tissue density determination

The tomography slices were done at the Veterinary Medicine faculty from São Paulo University (USP; São Paulo city). The computered tomography equipment used was an helicoidal, multislice, with 16 detector rows (Model Mx 8000 - Philips Medical Systems). Each lamb was submitted twice to the procedure, during the acclimation period, and one day before radioactive infusion, 90-d between both measurements.

For the procedure, the animals were anesthetized (xylazine: 0.05 mg/kg; ketamine: 4.5 mg/kg, intravenous), just before the procedure, for each lamb, the tomography images were acquired in a transversal plan, with three millimeters of thickness and 1.5 millimeters of increment. In sequence, it was selected the images around the 4^{th} rib, 12^{th} rib and thigh for the measurements (Figure 3.1a, 3.1b, 3.1c), the left side of the images (left side of the animal) was used for measurements.

For those evaluations, the software Osirix MD (DICOM / Pixmeo SARL - Swiss) was used and in each picture, the Hounsfield scale (HU; between 3000 and -1000) was determined (Figure 3.1d). Five points were measured and the mean value was used for statistical analysis. The HU is the linear attenuation water coefficient, the equation that represents HU was described above:

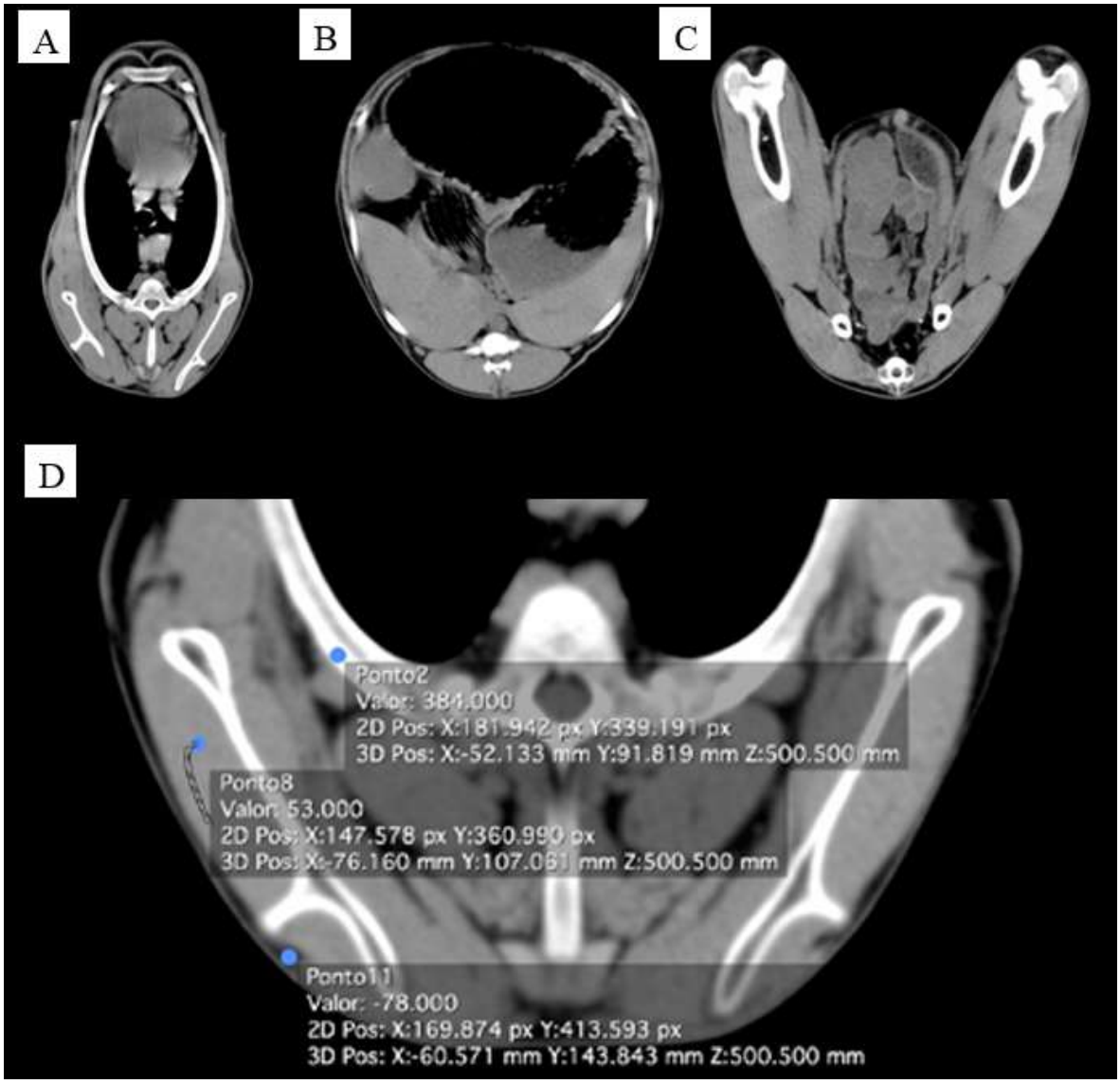
$$HU = 1000 * ((\mu - \mu_{H2O}) / \mu_{H2O}) \qquad \text{Eq.3}$$

where, HU is the Housfield scale, μ is the linear attenuation coefficient of the tissues, and μ_{H2O} is the linear attenuation coefficient of water (Water HU = 0). The HU determined for bone tissues were calculated by linear regression to tissue density by the equation described below (Eq.4; Campbell et al., 2003), muscle and fat tissues were held in HU scale in the result section.

$$\text{Bone Density}_{(g/cm3)} = 1.0062 + 0.00106 * \text{Bone HU} \qquad \text{Eq.4}$$

in which, Bone Density was the tissue density for bones in g/cm^3, and Bone HU was the Hounsfield scale for bones.

Figure 3.1: Osirix MD screen shoot of a tomographic images used for the HU measurements, A, B, C were respectively, images from 4th rib, 12th rib and thigh region (rear legs), and D were the specific regions in which the data were collected



3.2.5. Isotope dilution technique and ^{32}P administration

The radioactive solution was prepared with a sodium phosphate solution with ^{32}P (Na_2HPO_4; Physical half-life ~ 14.8 days), obtained by the Instituto de Pesquisas Energéticas de Sao Paulo (IPEN). The initial material activity was 370 MBq / 460 mL, therefore, the material was diluted with a sterile saline solution (NaCl – 0.85%) to achieve the final solution activity of 7.4 MBq / 0.5 mL, activity and dose standardized to be injected in each lamb.

The ^{32}P was infused in the left jugular vein. Five minutes after the radioisotope infusion, 10 mL of blood were sampled from the right jugular vein in a tube with

anticoagulant (EDTA-2k). The blood was also daily sampled in fasten condition by the same technique during seven consecutive days. In the same period, the total daily excretion of feces and urine were weighted and a 10% representative sample was collected. Six days after ^{32}P infusion 10 mL of saliva were sampled before feeding, and after slaughter, samples of tissues (heart, liver, kidney, thigh region muscle and 12^{th} rib bones) and ruminal liquid fraction were collected. All samples were stored in -20 °C. The samples from urine, blood (Plasma) and rumen liquid content were analyzed without manipulation, on the other hand, for feces and tissues samples, one gram of each was weighted and burned in 500 °C, the ash was totally solubilized in sulfuric acid (18 N), the resultant solution digested was stored. The standard solution used to correct the ^{32}P radioactive decay was prepared by pipetting 0.5 mL of the same radioisotope solution infused in the animals and completing the solution volume to one litter with distillated water.

The radioactivity was determined transferring the total content digested (solid samples), one mL of the liquid sample, or one mL of the standard solution to the Cerenkov counting vials, and complete the volume to 20 mL with distillated water. The radioactivity was determined by liquid scintillation spectrometer (Tri-carb TR, Packard) by the Cerenkov effect (NASCIMENTO FILHO; LOBÃO, 1977; IAEA, 1979).

3.2.6. Faecal egg counts (FEC) and L_5 Nematode characterization

Fecal samples were collected directly from the rectum starting from 18-d followed by three days interval up to 45-d. For FEC analysis, two grams of feces were weighted, and mixed with 18 mL of saturated saline solution, the solution was transported to a McMaster's chambers, and, after 3 to 5 minutes, the endoparasite eggs were counted in a light microscope (10 times objective; Olympus CX31 optical Co. Ltd, Tokyo, Japan), counting method described in Ueno and Gonçalves (1998).

After slaughter, the small intestine content was totally collected, homogenized, and completed to two litters with distillated water. Ten percent of the solution was filtered in a 35 µm sieve, and the leftover was totally collected and stored in a formalin solution (10%). The L_5 larval stage *T. colubriformis* counts was performed with a binocular microscope (Olympus SZ51-LGB, Olympus corporation, Tokyo, Japan), where the nematodes were counted, and sexed in individual bottle with Alcohol (70%). For average length 10 nematodes of each sex were separated and individually measured in a regular ruler, and then calculated the average length for males and females.

3.2.7. Slaughter procedure and tissues sampling

At the end of the experimental period, the animals were slaughtered according the recommendations described in the Ministry of Agriculture, Livestock and Food Supply (BRASIL, 2018). After that, the animals were eviscerated and ruminal, small intestinal and large intestinal contents were separated, weighted and a sample were freeze dried and stored for further analysis. For kidney, liver, and heart samples the whole organ was separated, weighted, and stored at -20^{o}C for further analysis. For bone and muscle sample all region between 12th and 13th ribs were exposed from the left side, and a sample from the *longissimus lumborum* was collected, and for bone a sample from the 12th rib was collected. Therefore, samples were stored in -20^{o}C for further analysis.

3.2.8. Phosphorus metabolism

For P determination, the solid samples of feed, refusal, feces, soft tissues (heart, liver, kidney, muscle), and bone were weighted in a precision scale and determined dry matter and ash as described in AOAC (2016). The ash residue was digested, filtered, and then measured the P (SARRUGE; HAAG, 1974). The P in the liquid samples (plasma, urine, saliva, and rumen liquid content) was determined as described by (FISKE; SUBBAROW, 1925). Endogenous P losses was estimated with the isotope dilution technique (LOFGREEN; KLElBER, 1953) and was mathematically described in Eq.5.

$$\% P_{endogenous} = (AE_{fc} / AE_{pl}) *100 \quad \text{Eq.5}$$

where, AE_{fc} was the fecal specific activity (Eq.6), and AE_{pl} was the plasma specific activity (Eq.7).

$$AE_{fc} = (A_{sample} / A_{std}) / \text{mg of P in 1 g of feces} \quad \text{Eq.6}$$

$$AE_{pl} = (A_{sample} / A_{std}) / \text{mg of P in 1 mL of plasma} \quad \text{Eq.7}$$

where, A_{sample} was the sample (feces or plasma) radioactivity and A_{std} was the radioactivity in the standard sample. Then, the fecal endogenous losses were calculated (Eq.8).

$$P_{endogenous\ (g/day)} = \% P_{endogenous} * P_{feces} \quad \text{Eq.8}$$

$$P_{feces\ (g/day)} = \%\ P_{fc} * Q_{fc} \quad \text{Eq.9}$$

in which, P_{feces} was the total P excreted in feces, in g/day, $\%P_{fc}$ was the percentage of P excreted via feces, and Q_{fc} was the daily feces excreted in g/day.

The true daily P absorption was determined by the Eq.10, and the amount of P retained in the body was determined in Eq.11.

$$P_{absorbed\ (g/day)} = P_{intake\ (g/day)} - (P_{feces\ (g/day)} - P_{endogenous\ (g/day)}) \quad \text{Eq.10}$$

$$P_{retained\ (g/day)} = P_{intake\ (g/day)} - (P_{feces\ (g/day)} - P_{urine\ (mg/day)}) \quad \text{Eq.11}$$

The radioactive physical half-life (PHL) represents the time required to reduce 50% of the chemical element initial activity, approximately 14.8 days is the ^{32}P PHL. However, for live organisms the excretions (feces, urine, and sweat) needed to be added to the PHL, this combination is called biological half-life ($T_{1/2}$) considering endogenous losses and physical decay. The $T_{1/2}$ was determined (Eq.12) by the specific radioactivity determined in plasma during the time, the exponential function (Eq.13) was applied according to the decay law (IAEA, 1979).

$$T_{1/2\ (h)} = \ln 2 / K \quad \text{Eq.12}$$

$$A = A_0 * e^{-k * t} \quad \text{Eq.13}$$

in which, K represented the decay rate, determined by the equation where A represented the radioactivity in time t, A_0 represented the initial radioactivity (in the infusion), and t represented time in days.

3.2.9. Statistical procedures

The trial was tested as a complete randomized statistical design with time dependent repeated measurements split-plot arrangement (two dietary P levels and two infection status – with or without *T. colubriformis* infection). All statistical analysis and procedures were performed in R studio software (3.4.2. version; 2017). The statistical model that described the statistical design was described above (Eq.14):

$$Y_{ijkl} = P_i + I_j + P \times I_{ij} + T_k + P|T_{ik} + I|T_{jk} + P \times I|T_{ijk} + a_l + e_{ijkl} \quad \text{Eq.14}$$

where, Y_{ijkl} was the dependent variables tested, P_i was the fixed effect of dietary P levels, I_j was the fixed effect of infection, $P \times I_{ij}$ was the fixed effect of the interaction between P and infection, T_k was the effect of time (repeated measurements), $P|T_{ik}$ was the effect of P within time, $I|T_{jk}$ was the effect of infection within time, $P \times I|T_{ijk}$ was the interaction effect within time, a_l was the random effect of animal (experimental unit), and e_{ijkl} was the experimental error, following the assumption: e_{ijkl} = iid N $(0,\sigma^2_e)$. time effect was tested only in repeated measurements variables.

All variables were tested for mean (Shapiro-Wilk) and variance (Bartlett) normality, whose variables that not fit the normality criteria (FEC) were adjusted by $Y_{adj} = \ln (Y + 1)$. Outliers and influence data were tested and removed from the dataset when exceed three times the standard deviation of the mean. The model was evaluated as a mixed model effect (fixed and random) with the function lmer in R studio. The effect of treatment was tested by variance analysis with 5% significance criteria, mean differences was compared by Duncan's test with the same significance criteria. Time dependent variables was tested by Duncan's test ($P < 0.05$) and compared treatment effects within time. Time effect was analyzed as linear and quadratic polynomials regression conversion, for each treatment.

3.3. Results

The infection was accompanied by the fecal egg count (FEC) test, did it began 15 days after infection and was performed in a three days interval (Figure 3.2), the infection was well succeed, in which, 24 days after infection was the maximum FEC obtained for sufficient-P (1230 eggs/g of feces) and deficient-P (1050 eggs/g of feces) treatments. However, the deficient-P animals presented high FEC levels in 30-d and 33-d compared to sufficient-P ones ($P < 0.01$). After slaughter L_5 stage nematodes was separated in males and females and counted. The number of nematodes in total and by sex did not differ between levels of P fed (Table 3.2), the nematode length and number of eggs counted in the female reproductive tract did not differ as well.

Figure 3.2: Fecal egg counts (FEC) in feces of Santa Ines castrated male lambs infected by *Trichostrongylus colubriforms* and fed different P levels. Different lowercase letters indicated Duncan's test difference ($P < 0.05$)

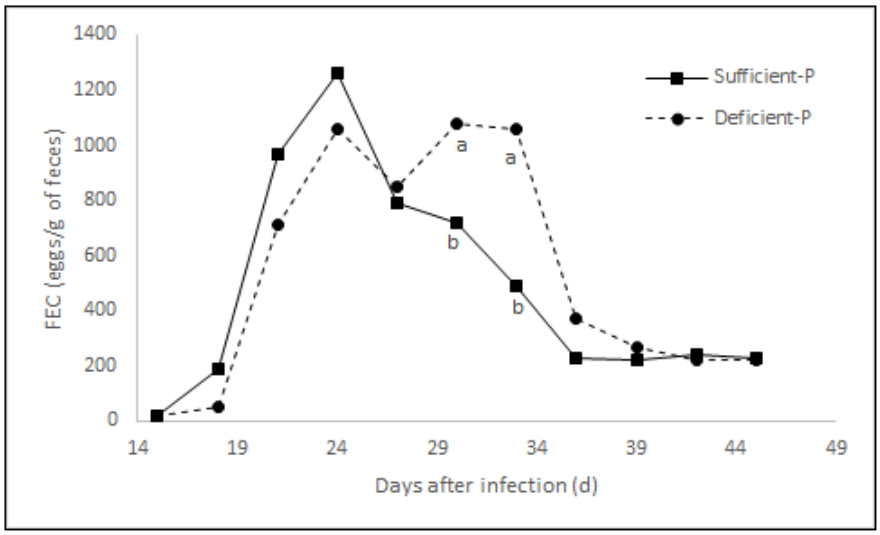


Table 3.2: Mean fecal eggs count (FEC), and nematode L_5 larval stage characterization of Santa Ines male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P	Deficient P	SEM	*P Value*
FEC (eggs/g feces)	240	370	21.0	ns
Total worms (10^3)	12.3	11.0	2.23	ns
Ratio Female/Male	1.27	1.4	0.10	ns
Male length (mm)	6.50	6.38	0.15	ns
Female length (mm)	6.24	6.00	0.17	ns
Length ratio Male/Female	1.05	1.05	0.03	ns

SEM = standard error of the mean; *P value* – ns ($P > 0.10$).

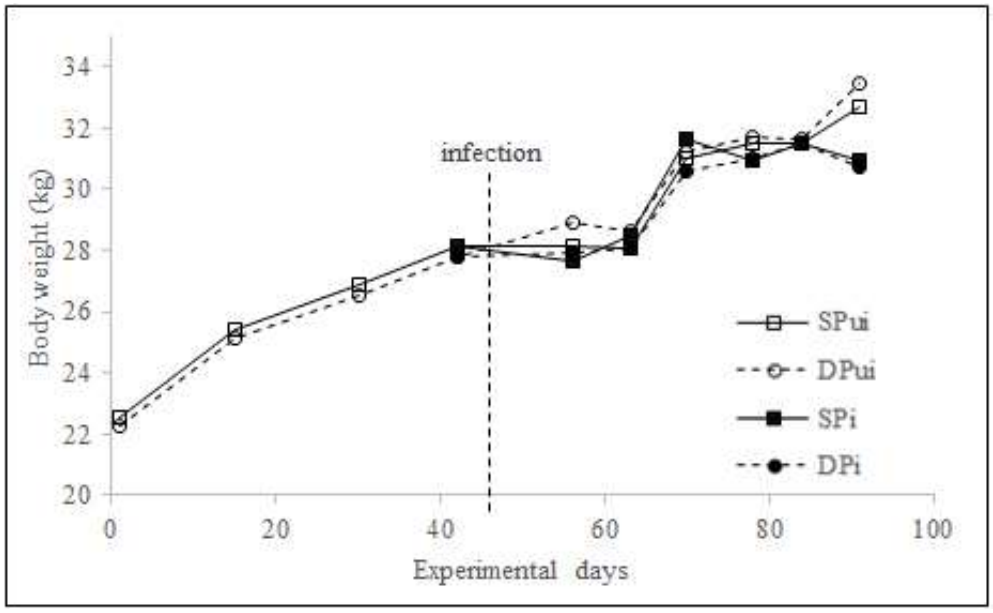
Feed and water intake were not affected by nematode infection and P supplementation (Table 3.3). The performance parameters estimated did not differ by infection status, where the average daily gain during the trial were 90 g/day and 108 g/day for infected and uninfected animals, respectively. The feed efficiency was the same across treatments (Table 3.3). The animals' weight and average weight gain was similar between treatments (Figure 3.3).

Table 3.3: Feed intake, feed efficiency parameters, and water intake of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P		Deficient P		SEM	*P value*		
	Uninfected	Infected	Uninfected	Infected		P	I	P×I
Dry matter intake								
(g/day)	969	964	1024	943	63.9	ns	ns	ns
(g/ kg BW)	29.7	31.7	30.6	30.9	1.19	ns	ns	ns
(% BW)	2.97	3.17	3.06	3.09	0.12	ns	ns	ns
ADG (g/day)	105.6	86.8	110	93.7	14.5	ns	ns	ns
Feed efficiency	0.110	0.101	0.107	0.095	0.025	ns	ns	ns
Water intake								
(L/day)	1.7	2.36	1.92	1.89	0.26	ns	ns	ns
(mL/kg BW)	53.6	79.2	58.1	60.9	9.38	ns	ns	ns

SEM = standard error of the mean, P = phosphorus, I = Infection, P×I = interaction phosphorus and infection, BW = body weight, ADG = average daily gain;
P value – ns (P > 0.10).

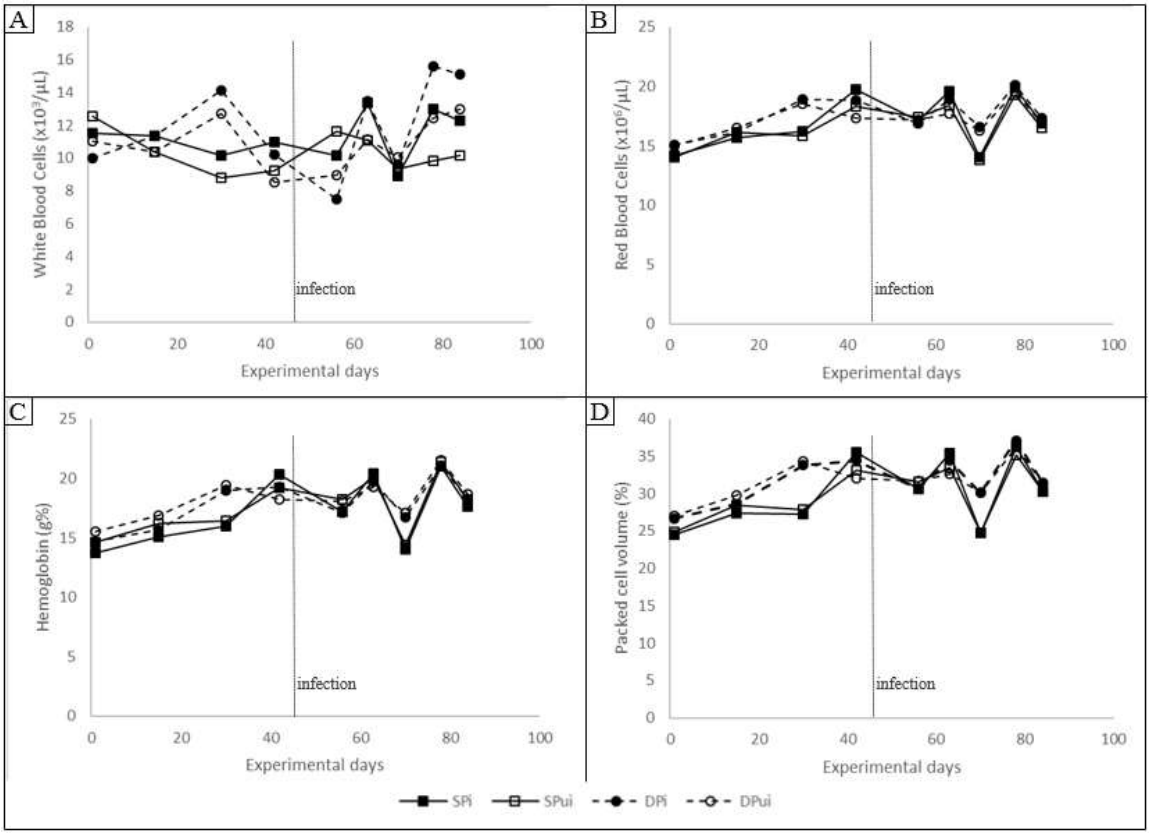
Figure 3.3: Mean body weight (kg) of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels



The hematological components (white blood cells, red blood cells, hemoglobin, and packed cell volume; Figure 3.4) did not differ across treatments and time. The general average packed cell volume was 31%, no difference was observed for the interaction time × P feed

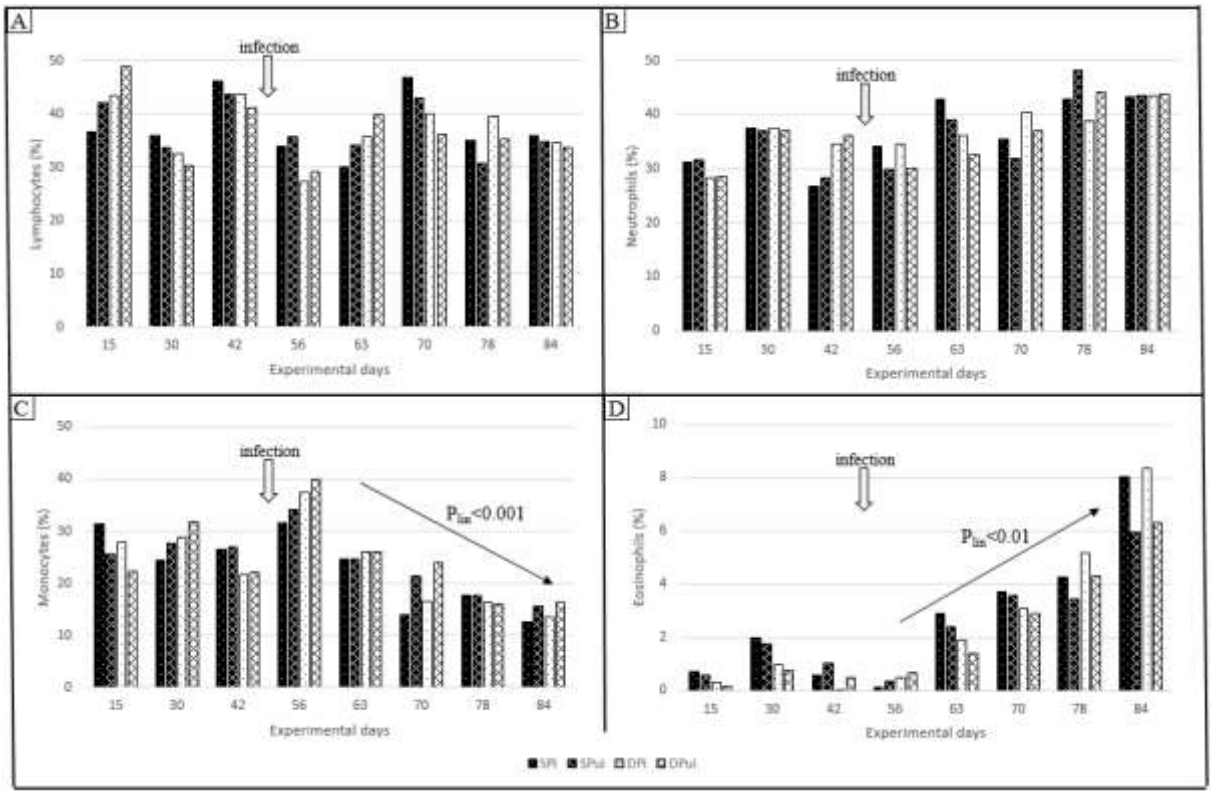
levels (P = 0.16) and time × infection status (P = 0.95). The biochemical parameters observed were glucose, albumin, urea, and total protein, all tested across time (data not shown). However, no difference was observed during the experimental period for diet P levels (P ≥ 0.22), infection (P ≥ 0.21), and the treatments interaction (P ≥ 0.42).

Figure 3.4: Hematological parameters of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels. A = white blood cells (×10^3/µL), B = red blood cells (×10^6/µL), C = hemoglobin (g%), and D = packed cell volume (%). Treatments: SPui = sufficient P uninfected, SPi = Sufficient P infected, DPui = deficient P uninfected, DPi = Deficient P infected



White blood cells were not different as described above, therefore the WBC was subtyped, and tested the percentage of each leucocyte cell and possible variations on time. Lymphocytes, Neutrophils, Eosinophils, and Monocytes were counted and the results did not indicate any treatment (P ≥ 0.41) and interaction (P ≥ 0.81) effect in the subtyped leucocyte cells (Figure 3.5). However, between 10 (56-d) and 38 (84-d) days after infection, the percentage of monocytes cells linearly decreased (P_{lin} < 0.001) while eosinophils cells linearly increased (P_{lin} < 0.01) similarly in the treatments.

Figure 3.5: Mean percentage of differential leucocyte count of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels. A = lymphocytes (%), B = neutrophils (%), C = monocytes (%), and D = eosinophils (%). Treatments: SPui = sufficient P uninfected, SPi = Sufficient P infected, DPui = deficient P uninfected, DPi = Deficient P infected



Housfield contrast scale and bone density indirectly indicated modifications in the composition of the tissue (Table 4). The first measurement (0-d) of tissue density (bone) and HU contrast (muscle and fat) did not show differences between treatments for the tissue tested (bone, muscle, and fat) and body regions (4^{th} rib, 12^{th} rib and thigh) analyzed, these results certified the randomization when the animals were distributed in treatments. For the second measurement (90-d) no differences was observed between treatments. However, when compared the first and the second measurement (Table 3.4), we observed that the bone density measured on the 12^{nd} rib ($P < 0.01$) and thigh ($P = 0.02$) decreased for infected animals and did not change for uninfected animals. Moreover, fat HU contrast differed in all regions measured differed by time across treatments (infection and P levels), where, 90-d measurements presented lower HU contrast values (more negative) compared to 0-d ones.

Table 3.4: Bone tissues density, and muscle and fat tissues Housfield contrast scale of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels, measured at the beginning and the end of the experiment

Body tissue	Region	Exp days	Sufficient P		Deficient P		SEM	*P value*				
			Uninf	Infec	Uninf	Infec		P	I	P×I	P|T	I|T
Bones Density (g/cm^3)	4th rib	0-d	1.35	1.35	1.33	1.32	0.03	ns	ns	ns	ns	ns
		90-d	1.31	1.31	1.35	1.35	0.04	ns	ns	ns		
	12nd rib	0-d	1.46[a]	1.47[a]	1.53[a]	1.49[a]	0.03	ns	ns	ns	ns	**
		90-d	1.52[a]	1.37[b]	1.55[a]	1.38[b]	0.05	ns	ns	ns		
	Thigh	0-d	1.80[a]	1.82[a]	1.79[a]	1.81[a]	0.06	ns	ns	ns	ns	*
		90-d	1.76[a]	1.67[b]	1.76[a]	1.71[b]	0.05	ns	ns	ns		
Muscle HU Contrast	4th rib	0-d	59.7	54.6	59.1	59.2	4.54	ns	ns	ns	ns	ns
		90-d	53.7	55.7	61.0	57.8	4.47	ns	ns	ns		
	12nd rib	0-d	56.2	59.5	56.5	56.0	3.18	ns	ns	ns	ns	ns
		90-d	54.3	53.8	51.0	56.8	12.4	ns	ns	ns		
	Thigh	0-d	60.5	60.2	59.5	57.2	14.8	ns	ns	ns	ns	ns
		90-d	54.8	56.6	43.6	53.9	9.62	ns	ns	†		
Fat HU Contrast	4th rib	0-d	-66.1[b]	-60.8[b]	-53.4[b]	-69.9[b]	5.70	ns	ns	ns	**	**
		90-d	-101[a]	-91.6[a]	-89.1[a]	-97.9[a]	3.69	ns	ns	ns		
	12nd rib	0-d	-63.9[b]	-66.5[b]	-50.0[b]	-70.3[b]	5.37	ns	ns	ns	***	**
		90-d	-96.5[a]	-104[a]	-109[a]	-106[a]	12.5	ns	ns	ns		
	Thigh	0-d	-83.0[b]	-71.5[b]	-77.3[b]	-86.7[b]	11.8	†	ns	ns	**	**
		90-d	-100[a]	-103[a]	-109[a]	-110[a]	8.50	†	ns	ns		

Uninf = Uninfected, Infec = infected, SEM = standard error of the mean, P = Phosphorus, I = Infection, T = time, HU = Housfield scale.
P value – ns ($P > 0.10$), † ($0.05 \leq P \leq 0.10$), * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).
[a,b] different superscript lowercase letter represented interaction differences between infection or dietary P levels with time mean values ($P < 0.05$) according Duncan's test.

Related to the concentration of P in different soft tissues (Table 3.5), the bone P concentration was affected by infection status, infected animals presented lower levels of P in bones ($P < 0.05$) compared to infected animals.

Table 3.5: Soft tissues (heart, liver, kidney, muscle), and bone phosphorus concentration of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P		Deficient P		SEM	*P value*		
	Uninfect	Infected	Uninfect	Infected		P	I	PxI
Phosphorus concentration (g/kgDM)								
Muscle	6.05	6.42	6.59	6.46	0.19	ns	ns	ns
Bones	80.2^{a}	73.1^{b}	81.2^{a}	76.4^{b}	2.61	ns	*	ns
Heart	8.29	9.89	7.68	8.39	1.32	ns	ns	ns
Liver	10.4	10.5	10.7	10.7	0.86	ns	ns	ns
Kidney	9.75	10.4	10.2	10.4	0.39	ns	ns	ns

SEM = standard error of the mean, P = Phosphorus, I = Infection, DM = dry matter
P value – ns (P > 0.10), † (0.05 ≤ P ≤ 0.10), * (P < 0.05), ** (P < 0.01), *** (P <0.001).
^{a,b} different superscript lowercase letter represented infection differences in mean values (P < 0.05) according Duncan's test.

Considering the P metabolism (Table 3.6), losses of P via feces did not differ by treatment, furthermore, P excreted in feces represented 71% of the total P daily consumed, however, lower endogenous losses (P < 0.001) were observed in animals fed deficient P diet. The concentration of P in blood (plasma), saliva, and ruminal liquid content decreased in infected animals (P < 0.01). The absorption (P = 0.07) and retention (P = 0.71) coefficients did not differ by treatments. On the other hand, the biological haf-life ($T_{1/2}$) significantly differed by treatment (P = 0.04), in which, infected animals presented faster ^{32}P removal from blood compared to the uninfected (Table 3.6).

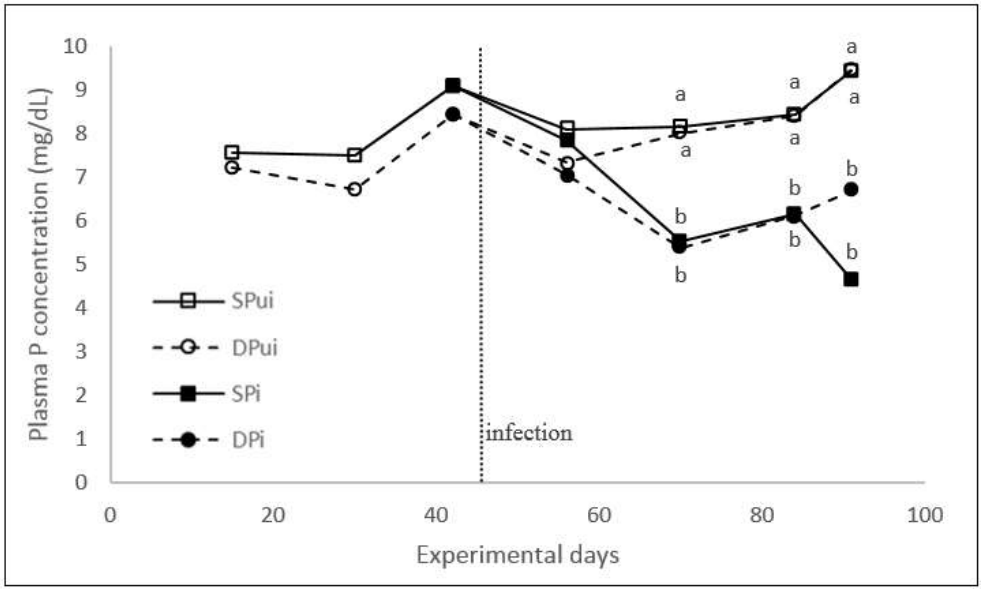
Table 3.6: Phosphorus metabolism of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P		Deficient P		SEM	*P value*		
	Uninf	Infec	Uninf	Infec		P	I	PxI
P intake (g/day)	3.68^{A}	3.66^{A}	1.84^{B}	1.69^{B}	0.15	***	ns	ns
P plasma (mg/dL)	8.13^{a}	5.74^{b}	7.87^{a}	5.63^{b}	0.75	ns	***	ns
P urine (mg/day)	9.31^{A}	9.85^{A}	5.97^{B}	4.81^{B}	1.85	*	ns	ns
P feces (g/day)	2.52^{A}	2.82^{A}	1.17^{B}	1.17^{B}	1.37	***	ns	ns
% P feces (P int)	68.5	77.3	63.6	69.2	9.33	ns	ns	ns
P endogenous fecal (g/day)	1.00^{A}	1.11^{A}	0.17^{B}	0.30^{B}	0.07	***	ns	ns
% P endogenous fecal (P int)	30.8^{A}	29.6^{A}	9.1^{B}	16.6^{B}	2.15	***	ns	ns
P absorbed (g/day)	1.99^{A}	1.79^{A}	0.68^{B}	0.66^{B}	0.2	***	ns	ns
P absorption coefficient	0.54	0.50	0.38	0.38	0.07	†	ns	ns
P retained (g/day)	1.15^{A}	1.08^{A}	0.50^{B}	0.48^{B}	0.24	*	ns	ns
P retention coefficient	0.32	0.31	0.28	0.28	0.09	ns	ns	ns
P saliva (mg/dL)	81.6^{a}	58.2^{b}	68.2^{b}	49.0^{b}	6.33	†	**	ns
P rumen liquid (mg/dL)	104.8^{a}	81.8^{b}	93.7^{a}	86.2^{b}	4.24	ns	**	†
T1/2 (h)	27.6^{a}	26.5^{b}	29.2^{b}	27.4^{b}	0.8	†	*	ns

Uninf = Uninfected, Infec = infected, SEM = standard error of the mean, P = Phosphorus, I = Infection, $T_{1/2}$ = Biological half-life.
P value – ns ($P > 0.10$), † ($0.05 \leq P \leq 0.10$), * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).
^{a,b} different superscript lowercase letter represented infection differences in mean values ($P < 0.05$) according Duncan's test.
^{A,B} different superscript lowercase letter represented dietary P levels differences in mean values ($P < 0.05$) according Duncan's test.

Figure 3.6 reported the results, in which, 24-d after the infection (70 experimental days) induction, infected treatments presented lower P concentration in plasma ($P < 0.01$) compared to uninfected ones. This pattern persisted until 45-d after infection (91 experimental days).

Figure 3.6: Plasma phosphorus concentration (mg/dL) of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels. Treatments: SPui = sufficient P uninfected, SPi = Sufficient P infected, DPui = deficient P uninfected, DPi = Deficient P infected



3.4. Discussion

The overall mean parasitological infection determined in this experiment was approximately 11,600 adult nematodes per animal, an amount that could be characterized as a severe infection profile, according to Ueno; Gonçalves (1998), an infection where the adult endoparasite count overcame 10,000 adult parasites, can be considered a severe infection. Correlating the infection challenge and the voluntary intake, Cantacessi et al. (2010) reported that a severe *T. colubriformis* infection negatively impacted the dry matter intake. This behavior was not observed in this study, where any of treatments tested (infection, and dietary P levels) impacted the feed intake. Several authors also did not observe changes in feed consumption by infected animals (PORTILHO et al., 2006; LOUVANDINI et al., 2008). Some authors explained that infections related to the genus *Tricostrongiloidea* were not directly responsible for the reduction in consumption, but the reduction in nutrients digestion and absorption efficiency (CARDIA et al., 2011; MAVROT et al., 2015; DIAS E SILVA et al., 2018).

Regarding P intake, in the present study, animals consumed 3.68 and 1.69 g of P per animal per day, for sufficient and deficient P diets, respectively. Portilho et al. (2006) tested different dietary P levels to attend the requirements of growing male Santa Ines lambs (22.6 kg of BW), and based on their study, the present experiment caused a dietary P deficiency condition. Although there was a P deficiency in lambs' diets, there was no difference in the performance during the period measured. Sufficient and deficient P diet animals gained an average of 110 and 90 g per animal per day, respectively. Antunes et al. (2006) studied the performance of growing Santa Ines uncastrated males' lambs (initial BW = 13.9 kg) and observed that supplemented animals (3 g of P / day) did not increased the weight gain when compared to non-supplemented animals. However, these results tested the quantitative weight gain, and some authors observed that although the weight does not change quantitatively, P restriction may compromised quality of weight gain, reducing muscle and bone tissue deposition (LOUVANDINI et al., 2006).

Understanding the infection effects in the tissue density, is an important step to understand how the nematode could impact the body composition. The present results showed that the parasite infection reduced bone density. Amarante (2015) reported an indirect effect in the bone density in infected animal, where the infection compromised the P absorption (CARDIA et al., 2011), and consequently the P stored in bones was resorbed to the bloodstream as a compensatory mechanism, and the increase in the resorption reduced the bone density. Moreover, the P concentration in bone decreased in infected animals, justifying the decreased bone density occurred in animals infected by *T. colubriformis*. The present study, also showed a reduction in the HU contrast in all animals, independent of treatment exposed, i.e., there was a reduction in the fat tissue transmittance. The results could be related to the increase amount of fatty acids inside the cells increasing the relation fat / muscle in the adipocites cells (DOREAU; CHILLIARD, 1997), and reducing the adiposite tissue transmittance, and consequenty, the HU contrast.

Evaluating hematological and biochemical compounds in infected animals can indirectly represents the immunity status of those animals (MCRAE et al., 2005), moreover, dietary P deficiency can compromise the ATP levels in the system, contributing to aggravate animal immune system maintenance. The present study did not present any changes in hematological and biochemical compounds of infected animals, diverging from previous studies, where endoparasite infection in sheep caused reduction in hematocrit and hemoglobin of *T. colubriformis* (DIAS E SILVA, 2017), and an increase of eosinophils cells in infected animals' blood (CARDIA et al., 2011).

All these studies previously cited, observed these blood parameters modifications 60 days after infection, and in serial infection condition. Dorchies et al. (1997) reported that infection implementation and time after infection were intimately related to immunity response.

Analyzing P metabolism data, P intake was greater in animals fed sufficient dietary P levels, and consequently, P losses in feces (g of P / day), fecal endogenous P (g of P / day) and urinary P excretion (mg of P / day) were also higher in treatment fed sufficient P levels. These positive correlations between nutrient consumption and excretion was also observed in previous studies (VITTI; KEBREAB, 2010). Phosphorus excretion in feces represented around 70% of P ingested, these results corroborated to previous studies, where the authors also concluded that in ruminants, P excretion occurs mostly through feces (LOUVANDINI et al., 2008). In the present study, infected sheep had lower plasma P concentration and salivary secretion compared to uninfected ones. According to Vitti and Kebreab (2010) there is a high and positive correlation between blood P concentration and salivary P secretion, this previous study supported the result observed in the present experiment. Due to the higher P content secreted via saliva of infected animals, and considering that previous studies concluded that P from saliva represented 50 to 70% of total P concentration observed in ruminal liquid phase digesta (VALK et al., 2000; VITTI; KEBREAB, 2010). However, in this study, the relation between parasite infection and P metabolism was not related to the salivation secretion, but the sequence of effects caused by the infection status, that direct interfere the P metabolism, like the P resorption from bones (previously discussed), GIT resorption from blood, explained by the increase in the $T_{1/2}$, and reduction in P absorption (Table 3.6).

Absorption coefficiency is an important parameter in studies of endoparasites, in studies with *T. colubriformis* studies, the importance is even greater regarding to the infection characteristics and the damages in intestinal lumen, affecting directly the nutrient absorption (WILSON; FIELD, 1983), The present studies did not presented an statistical reduction in the absorption coefficient of infected animals, however, this parameter had many factors that could affect and consequently inflate the individuality variation, and the variation coefficient. In this sense, Dias e Silva et al. (2018) evaluated the absorption coefficient of Santa Ines lambs infected with *T. colubriformis* and the results were similar in 5% probability mean test, in agreement with the present study. Now considering the dietary P levels, the P absorption coefficient was statistically similar, however, sufficient P diets absorbed 14 percent more compared to deficient P diets. The P consumed was directly related to the absorption of P (LOUVANDINI et al., 2008), the source of P and the bioavailability capacity of this mineral

(GODOY; CHICCO, 2005). The source of P used to supplement the animals was dicalcium phosphate, which is highly bioavailable and can be potentially absorbed by the ruminant. Animals fed deficient P diet, the source of P was those naturally presented in the vegetable ingredients of the diet. Most of the P from these sources were less bioavailable due to formation of phytic acid (HUMER; ZEBELI, 2015). The different P sources bioavailability could be resulted in a reduction in the absorption, and the high individual variation observed for this parameter could compromised the statistical results. In turn, the retention coefficient was not affected by the treatments tested in this study.

The biological half-life ($T_{1/2}$) of ^{32}P indicated how fast is the disappearance of P from the bloodstream system, and in our study, uninfected animals presented more hours to achieve the T1/2 compared to infected animals, it means that P leaved the central pool (blood) faster in infected animals, indicating that the endoparasites were determinant in the changes. The disappearance could be interpreted as the P removal from the bloodstream, and the destination could be excretion, retention, secretion (saliva) and energy use (ATP). Larval development of *T. colubriformis* may promotes a greater muscular tissue catabolism, followed by an increase in GIT mineral resorption, and excretion losses (YU, et al., 2000). Moreover, parasites could promote host's intestinal mucosa damage to the intestinal microvilli, resulting in inflammatory processes (FERREIRA et al., 2017). This process require energy input (ATP). The combination of these effects accelerates the P dessapearance from the bloodstream.

3.5. Conclusion

Severe *T. colubriformis* infection may negatively impact the P metabolism of lambs, increasing the P resorption from bones, reducing plasma P concentration and accelerating the P removal from blood. However, hematological and biochemical compounds did not differ among treatments. Furthermore, under the conditions of the present study, Phosphorus restriction did not affect feed consumption, biochemical, and hematological parameters and tissue density of growing lambs. The only exception was the P metabolism that was affected by P dietary reduction, but not sufficient to affect performance.

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4. Digestion physiological parameters in Santa Ines lambs infected with *Trichostrongylus colubriformis* and fed different levels of phosphorus

Abstract: This study aimed to evaluate digestion physiological parameters in Santa Ines lambs infected with *T. colubriformis* and fed different levels of phosphorus Eighteen Santa Ines, castrated male, six-month age, health lambs (initial body weight 22.4 ± 2.7 kg) were distributed in 4 treatments in a 2×2 split-plot arrangement: Sufficient dietary P level and uninfected (SPui; n = 4), Sufficient dietary P level and infected (SPi; n = 5), Deficient dietary P level and uninfected (DPui; n = 4), Deficient dietary P level and infected (DPi; n = 5). Infected lamb received, orally, a single pulse dose of 40.000 *T. colubriformis* infective larval stage (L_3). Animals were fed with Tifton hay cv85 (*Cynodon ssp.*; 60%), and cassava meal and maize gluten meal (40%). The nutrient's apparent digestibility and nitrogen metabolism were performed in individual metabolic stalls. The methane emission was collected in respiratory chambers, and quantified in gas chromatography. The purine derivatives were measured in urine and indirectly represent the microbial nitrogen absorbed. Short-chain fatty acids (SCFA) were tested in rumen liquid, and the passage rate was measured in each gastrointestinal tract segment using external (Yb, Cr, and Co) and internal (iNDF) markers. Duodenum mucosa tissue was sampled and a histological procedure was performed to observe microvilli damages. Statistical procedures were performed in R studio, treatments, and interaction fixed effects were tested by ANOVA, and means compared by Duncan's test at 5% significance. Apparent digestibility was not affected by treatments, however, nitrogen retention coefficient decreased ($P<0.01$) and nitrogen urine losses increased ($P<0.01$) in infected animals. Small intestine digesta content, compartment, and length were higher in infected animals ($P<0.05$). Passage rate was not majorly affected neither by infection or dietary P levels. Methane emission, SCFA, and purine's derivatives were not affected by treatments. Regarding to the histology, the vilosity weight ($P<0.05$), and crypt depth ($P<0.01$) decreased in infected animals. In conclusion, *T. colubriformis* infection can damage intestinal mucosa and affect nitrogen metabolism, but did not affect the digesta transit, and nutrient digestibility. The P dietary levels did not promote any modification in GIT physiological parameters tested in this study.

Key words: absorption, nematode, phosphorus deficiency, sheep, retention, verminose

4. Parâmetros fisiológicos digestivos de cordeiros Santa Ines infectados com *Trichostrongylus colubriformis* e submetidos a dietas com diferentes níveis de fósforo.

Resumo: Objetivou-se com o presente estudo avaliar os parâmetros fisiológicos da digestão de cordeiros Santa Inês infectados com *T. colubriformis* e alimentados com diferentes níveis de fósforo. Foram utilizados 18 cordeiros Santa Inês, machos castrados, com seis meses de idade, peso inicial 22,4 ± 2,7 kg, distribuídos em quatro tratamentos em arranjo em parcelas subdivididas 2×2: P adequado e não infectado (PAni; n = 4), P adequado e infectados (PAi; n = 5), P deficiente e não infectados (PDui; n = 4), P deficiente e infectados (PDi; n = 5). Os cordeiros infectados receberam, via oral, a dose única de 40.000 *T. colubriformis* em estágio larval infectante (L_3). Os animais foram alimentados com feno de tifton cv85 (Cynodon ssp.; 60%) e concentrado a base de farelo de mandioca e farelo de glúten de milho (40%). A digestibilidade aparente dos nutrientes e o balanço de nitrogênio foram realizados em gaiolas para estudo de metabolismo animal. A produção de metano foi avaliada em câmaras respiratórias e quantificada por cromatografia gasosa. Os derivados de purina foram medidos na urina e indiretamente representam o nitrogênio endógeno de origem microbiana. Os ácidos graxos de cadeia curta (AGCC) foram testados no líquido ruminal, e a taxa de passagem foi mensurada separadamente em cada segmento do trato gastrintestinal usando marcadores externos (Yb, Cr e Co) e internos (FDNi). O tecido da mucosa do duodeno foi amostrado a técnica da lâmina histológica foi realizado para observar os danos ao lúmen intestinal. Procedimentos estatísticos foram realizados no R Studio, onde os tratamentos e suas interações foram testadas por ANOVA, e as médias comparadas pelo teste de Duncan a 5% de significância. A digestibilidade aparente não foi afetada pelos tratamentos, entretanto, o coeficiente de retenção de nitrogênio diminuiu ($P < 0,01$) e as perdas de nitrogênio na urina aumentaram ($P < 0,01$) nos animais infectados. O conteúdo e o segmento vazio do intestino delgado foram maiores nos animais infectados ($P <0,05$). A taxa de passagem não foi afetada nem pela infecção nem pelos níveis de P na dieta. A emissão de metano, os AGCC e os derivados de purina não foram afetados pelos tratamentos. Em relação à histologia, a altura dos vilos ($P < 0,05$) e a profundidade da cripta ($P <0,01$) diminuíram nos animais infectados. Em conclusão, a infecção por *T. colubriformis* pode danificar a mucosa intestinal e afetar o metabolismo do nitrogênio, mas não afetou o trânsito da digesta e a digestibilidade dos nutrientes. Os níveis dietéticos de P não promoveram qualquer modificação nos parâmetros fisiológicos do TGI testados neste estudo.

Palavras-chave: absorção, deficiência de fósforo, nematóide, ovinos, retenção, verminose

4.1. Introduction

Trichostrongylus colubriformis is a specie of gastrointestinal nematode that typically infect the duodenum portion (beginning of the small intestine) of the gastrointestinal tract (GIT) of sheep (AMARANTE, 2015). In severe stage of infection, this nematode could damage the intestinal mucosa, resulting in reduction of nutrients absorption. The nutrients absorption will be compromised due to physiological modification in the GIT, and other systems involved in the nutrient harvest process in intestinal lumen. Among the injuries is the intestinal mucosa vilosity atrophy, and crypt depth reduction (CARDIA et al., 2011), formation of tunnels that compromise the correct lumen functionality, duodenal mucosa epithelium erosion, and intestinal lumen serum protein exudation (TAYLOR et al., 2010).

As a result of all these injuries in the GIT, is the reduction in the capacity of digestion and absorption of nutrients (CANTACESSI et al., 2010), but some indirect consequences of the infection can come up, like for example, modification in the flux of digesta across the GIT, as a physiological strategy to increase the nutrient availability. Huhtanen (2006) reported that animal health status is one factor that can modulate the digesta and fluids transit in the GIT, other factor mentioned is the intestinal mucosa integrity. Furthermore, some authors described characteristics observed in infected animals, like faster passage rate in duodenum, and slower in late jejunum, longer small intestine length, and increase of jejunum microvilli in animals infected with *T. colubriformis* as a physiological compensatory response to the infection (POPPI et al., 1985).

Passage rate, is the fractional amount of digesta moving from a compartment to another in a determined period (GROVUM; WILLIAMS, 1973), and the opposite of mean retention time, that is the average period of time that the digesta spend in the GIT or in each compartment. Some authors studied *T. colubriformis* infection in lambs and observed lower reticulo-ruminal passage rate in infected animals (DIAS E SILVA et al., 2019). The more time the digesta stays in reticulo-rumen segment, more the microorganisms could degrade the digesta and convert in nutrients, and consequently, higher production of short-chain fatty acids (ruminant alternative energy source), and methane production (HUHTANEN, 2006).

Therefore, the parasite infection could modify physiological and histological parameters to improve the resilience between the endoparasite and the host, however, these modifications could waste energy that was targeted to growth and development, also, infected animals can produce and eruct more methane, that contribute to the global warning, a

worldwide problem. In this sense this study aimed to evaluate digestion physiological parameters in Santa Ines lambs infected with *T. colubriformis* and fed different levels of phosphorus.

4.2. Material and Methods

4.2.1. Location, experimental design, and experimental timeline

The trial was performed according the Ethics Committee on Animal Use (CENA/USP – 004/2015), and conducted following the rules of Waste Management of ESALQ/USP in the Animal Nutrition Laboratory (22º42'30" South, 47º38'30" West, and 546m Altitude) of the Center of Nuclear Energy in Agriculture, a research center of Sao Paulo University. Eighteen Santa Ines, castrated male, six-month age, health lambs (average initial body weight (BW) 22.4 ± 2.7 kg) were distributed in a complete randomized unbalanced experimental design with a 2×2 split-plot arrangement: Sufficient dietary P level and uninfected (SPui; n = 4), Sufficient dietary P level and infected (SPi; n = 5), Deficient dietary P level and uninfected (DPui; n = 4), Deficient dietary P level and infected (SPui; n = 5).

During the 96 experimental days, the lambs were individually housed, and in the first experimental day, lambs were checked for endoparasite infection, and health status confirmation. The total experimental period was 118-d, in which, 22-d was for basal diet and stall acclimation. The first 45-d of the experiment was allowed to dietary P levels factor, thus, 18 animals were randomly distributed in two treatment groups (SP and DP). On day 46 the endoparasite infection factor was added, where, five animals in each P treatment were randomly sorted and infected to complete four treatment effects. After infection, the trial lasted 51-d.

4.2.2. Experimental design, feeding regimen, and infection procedures

During the infection procedure, each lamb received, orally, a single pulse dose of 40 thousand *T. colubriformis* infective larval stage (L3). The noninfected group received distilled water. The animals were housed with an unrestricted supply of water and feed. The experimental diets description, ingredients used and others diet considerations was previously described in chapter 2.

4.2.3. Nutrient's apparent digestibility and nitrogen metabolism

A metabolism assay was performed between 36-d and 42-d after infection, where the lambs were housed in individual metabolic stalls for seven days. The first two days were used for animal acclimation, and five consecutive days for measurements and sampling. The diet was provided twice a day (8:00 am and 5:00 pm) and daily adjusted for 10% of refusal. The feed ingredients were sampled for bromatological analysis. The feed refusal and feces were daily weighted and 10% of the total were sampled (as a pool) and stored in a -20 ^{o}C freezer for further nutrient analysis. For urine samples, 100 mL of a sulfuric acid solution (H_2SO_4 – 10%) was used to avoid ammonia (NH_3) volatilization, the total urine excreted was daily measured and 10% representative sample was collected (as a pool) and stored in a -20 ^{o}C freezer for further analysis.

Feed offered, refusal and feces were defrosted in natural temperature for analysis, those samples were weighed and dried in an air forced stove (MA 037 – Marconi, Piracicaba – SP, Brazil) in 55^{o}C during 72 hours and determined the first dry matter, the dried samples were grounded (1 mm) for bromatological analysis. The dry matter (DM), ash, and crude protein (CP) were determined according to AOAC (2016), moreover, the neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by the methodology described by Van Soest, Robertson and Lewis (1991). The total tract apparent digestibility was calculated (Eq.1) as:

$$NutAD_{(\%)} = (NutInt - NutFeces) / NutInt * 100 \quad \text{Eq.1}$$

where, NutAD = nutrient apparent digestibility in %, NutInt = nutrient intake in g/kg of DM, and NutFeces = nutrient in the feces in g/kg of DM.

The urine collected was defrosted in natural temperature for protein analysis, the CP was determined in urine with the same method as previously described. The nitrogen balance and metabolism (absorbed and retained) were calculated (Eqs. 2,3,4) using the CP determined in the feed intake, feces, and urine:

$$N_{abs} = N_{int} - N_{feces} \quad \text{Eq.2}$$

$$N_{ret} = N_{abs} - N_{urine} \quad \text{Eq.3}$$

in which, N_{abs} = nitrogen absorbed, N_{int} = nitrogen intake, N_{feces} = nitrogen in feces, N_{ret} = nitrogen retained, and N_{urine} = nitrogen in urine, all variables in g/day.

4.2.4. Methane emission quantification

The methane emission quantification was determined between 30-d and 35-d after infection. The respiratory chamber technique used in this trial was previously described by Abdalla et al. (2012). The animals were splitted in two groups of nine animals, and each group kept in analysis by three days, in which, one day for animals' chambers acclimation, and two days for sample collection. Each group were randomly distributed in nine chambers (157 × 71 × 167 cm – 1.9 m^3 of volume), the gases were daily sampling during 23 hours, totaling 46 hours of gases collection.

The mean temperature (24.2 ± 2.7 ºC) and humidity (79.3 ± 6.9 %) were measured with a digital thermometer inside of each chamber, the mean airway out flux was measured with an anemometer (AD-250 digital – Instrutherm, Sao Paulo – SP, Brazil). and the mean was calculated across six-time measurements during the 23-hour period (7h, 10h, 13h, 16h, 19h, 21h). The gases were withdrawn from the chambers by pipes connected in an exhaust pump (202 L/min flux). The gas samples were continuous collected from the pipes with a peristaltic pump, during the 23-hour period and stored in metallic seven litters volume bladders. At the end of each day, the pool of gases was subsampled in triplicate of 10 mL and stored in glass vials tightly closed for further analysis.

The samples were tested for enteric methane (CH_4) concentration by gas chromatography (GC-2010, Shimadzu, Tokyo, Japan) with a detection column Shincarbon ST 100/120. Helium (He) was used as mobile phase in a constant flux of 10 mL/min. The calibration curve was determined in concentrations of 0, 30, 90, and 120 mL of CH_4 / L (99.5% purity - White Martins gases company SA, Osasco – SP, Brazil), the linear regression from the calibration curve was applied to determine the sample CH_4 concentration.

4.2.5. Purine derivatives' assay.

The urine sampled in the apparent digestibility trial was also used to determine the purine derivatives. The method used to extract the purines from the urine was described by Adibe Filho et al. (2017), and consequently, calculate the microbial protein synthesis as described by Makkar and Chen (2004). The acidified urine samples were defrosted in 8 ºC

refrigerator, homogenized, and subsampled for the analysis. The purines' derivatives were determined by high-efficiency liquid chromatography (HPLC – Agilent 1100 with column Zorbax ODS C18, 5 µm particles – and photodiode detector arrangement, UV-Vis).

To isolate the purine derivates from the other urine components, we used an ammonium monobasic phosphate 2.5 mM ($NH_4H_2PO_4$) solution with methanol in a proportion of 95:5 (v/v), in a constant temperature of 23 ^oC. The analytical curve was developed with solutions of allantoin, uric acid, hypoxanthine, and xanthine in a known concentration between 500 and 1500 µM. Besides, the internal standard compound used was the oxypurinol in the same range of concentration. The wavelength used to determine the purines' derivatives were 225 µm for allantoin, 267 µm for xanthine, 284 µm for uric acid, and 254 µm for hypoxanthine and oxypurinol. The microbial nitrogen (MN) absorbed by the small intestine was calculated as:

$$MN = (PDM * N_{purines}) / ((N_{purines} / N_{microbial}) * MPD * 1000 \quad \text{Eq.4}$$

in which, MN was the microbial nitrogen absorbed by the small intestine in gN/day, PDM was the purines' derivatives metabolites absorbed in mmol/day*$kg^{0.75}$, $N_{purines}$ was the content of the purine nitrogen = 70 mg N/mmol, $N_{microbial}$ was the content of microbial nitrogen, the relation $N_{purines}$: $N_{microbial}$ = 0.116, and MPD was the microbial purines' digestibility = 0.83. The PDM was calculated as:

$$PDM = (M_{area} * [SO] * DF) / ((CF * SO_{area}) * Urine_{volume}) \quad \text{Eq.5}$$

$$CF = (SM_{area} * [Soxipurinol]) / ([SM] * Soxipurinol_{area}) \quad \text{Eq.6}$$

where PDM was the purines' derivatives metabolites absorbed in µM/L, M_{area} was the metabolite's area, [SO] was the standard oxypurinol concentration = 50, DF was the dilution factor = 2.5, CF was the correction factor of purine's metabolites, SO_{area} was the standard oxypurinol area, $Urine_{volume}$ was the urine volume in mL, SM_{area} was the standard metabolite's area, [Soxipurinol] was the standard oxypurinol concentration, [SM] was the standard metabolite's concentration, and $Soxipurinol_{area}$ was the standard oxypurinol area.

4.2.6. Slaughter procedure, GIT measurements, and content sampling

At the end of experimental period, all animals were slaughtered for GIT and tissues sampling. All slaughter procedures were executed following the recommendations described in Ministry of Agriculture, Livestock and Food Supply (BRASIL, 2018). Animals were

slaughtered in right lateral decubitus for avoiding feed transit between segments and eviscerated for sampling.

The GIT was separated from the rest of the tissues, and segmented in six compartments (rumen-reticulum (RR), omasum, abomasum, small intestine (SI), cecum, and colon-rectum), each compartment was weighted with content, and for RR and cecum were also determined the pH with a digital pHmeter (pHmeter PG2000, Gehaka Ltda., Sao Paulo – SP – Brazil) in triplicate. Then, the segments were emptied, and the compartments were cleaned and weighted again, now empty. The content amount was calculated as compartment full – compartment empty. The segments' contents were sampled for further bromatological and mean retention time analysis, and the first portion of the SI (duodenum) was sampled and maintained in ethanol (70%) for further histological analysis.

The soft tissues evaluated in this trial were heart, kidneys, liver, lung, and spleen. Those tissues were separated, cleaned for fat, blood, and other contaminants and weighted. The muscle *longissimus lumborum* near to the 12nd rib was separated and measured the color space (L*, a*, b*) using colorimetric technique with a Minolta hand spectrophotometer (CM-600d, Minolta – Osaka - Japan), with 8mm aperture, spectra range of 360-740 nm with 10nm intervals, triplicate mean, and calibrated every 10 measurements using a standard white plate from the manufacturer.

4.2.7. Short-chain fat acids (SCFA) determination

The rumen liquid sampled after the slaughter was used of SCFA determination, the procedures to determine acetic, propionic, butyric, iso-butyric, valeric, iso-valeric acids was described by Palmquist and Conrad (1971) and adapted by Lima et al. (2018). In this procedure, 1.5 mL of rumen liquid was subsampled and centrifuged by 40 minutes in a speed of 10.400 *g and constant temperature of 4^{o}C (Sorvall RC–5B Plus, USA), the supernatant (800 µL) was pipetted in micro vials and added 100 µL of 2-etilbutyric acid (intern standard) and 200 µL of formic acid (85%). From this solution, one µL was used to determine the SCFA profile by gas chromatography (GC-2014, Shimadzu, Tokyo, Japan), with a flame ionization detector. Helium (He) was used as mobile phase in a constant flux of 25 mL/min, the fluxes of hydrogen and synthetic air for the detector were 40 mL/min and 400 mL/min, respectively (LIMA et al., 2018). The concentration of Acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, and isovaleric acid was estimated by linear regression of internal and external standards.

4.2.8. External and internal marker GIT passage rate

The feed retention time was evaluated with internal and external markers. The internal marker used was the indigestible NDF (iNDF). For external marker we adopted the technique of triple marker described by France et al. (1986) where allowed to estimate the retention time of concentrate, forage, and liquid phase. Concentrate fraction was labeled with Yb (ytterbium acetate) by the method described by de Vega and Poppi (1997), forage was labeled with Cr (Cr_2O_3) as described by Uden et al. (1980), and the liquid phase was labeled with Co-EDTA complex as described by Downes and McDonald (1964).

One g of concentrate, 1.5 g of forage, and 35 mL of Co-EDTA were orally administrated for each animal four times a day in six hours interval (01:00, 07:00, 13:00, and 17:00 h), during five consecutive days period before slaughter, to attend the "steady-state" assumption. During the slaughter (2.8 ± 0.7 h after marker feeding), as described above, the GIT was segmented in the six different compartments. The RR digesta was separated into solid and liquid fractions by straining the contents through 2 layers of cheesecloth, then, these fractions were weighed and sampled according to the proportion determined to obtain a representative sample. The content of the entire GIT was calculated as a sum of each digesta segment weighted. The digesta samples were individually stored as -20ºC for later chemical analysis. The samples were defrosted in 8ºC refrigerator for 36 hours, the digesta was subsampled in plate dishes and covered with parafilm with some roles and dried in a freeze drier during 72 hours. The dry matter of the digesta was calculated as the amount of water withdrawn by the sample.

Determination of Cr, Co, and Yb in the feed, and digesta of each compartment were obtained with a triple quadrupole inductively coupled plasma mass spectrometry (ICP-QQQ 8900, Agilent) and internal standard solution for Bi, Ga, In, 6Li, Sc, Tb and Y (100 mg L-1 – Alfa Ae sar). An assisted microwave (Ethos UP – Milestone) was performed to digest and open the matrix dried sample, then the solution was filtered and tested for the trace elements Cr, Co, and Yb. The elements certified reference materials (988 mg kg-1 ± 3 mg kg-1) used to calibrate the curve was provided from Sigma Aldrich. The iNDF was determined with an *in situ* degradability assay as described by Huhtanen et al. (1994) and Valente et al. (2011), where, 0.5 g of each digesta and feed samples (2 mm particle size) were weighed in filter bags (F57, 25 µm – Ankon Technology Corp.), the samples remained in sheep rumen during 288 hours (24 filter bags per animal), then the filter bags were removed and washed in current water for 30 minutes, then a NDF analysis was

performed as described by Van Soest, Robertson and Lewis (1991) to determine the residual NDF.

Mean retention time (MRT) of forage, concentrate and liquid phase, determined by internal and external markers in different segments of the GIT were determined by the flux/compartmental pool method using Eq.7 and Eq.8, as described by Ellis et al. (1994):

$$K_e \text{ of IE} = \text{Intake rate/compartmental mass} \quad \text{Eq.7}$$

in which, K_e was the fractional rate of escape per hour, IE was the indigestible entity with an intake rate expressed in g/h, the compartmental mass was the segment content expressed in g, and

$$K_{p\ (\%/h)} = K_e * 100 = 1/MRT \quad \text{Eq.8}$$

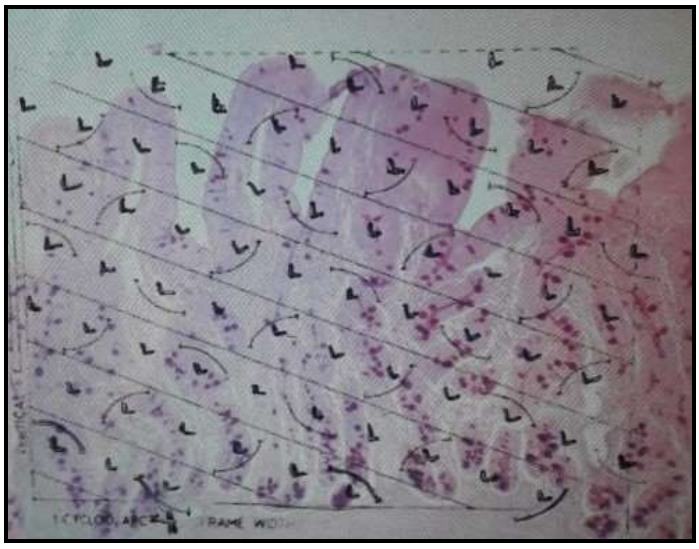
where, K_p was the passage rate expressed in %/h, and MRT was the mean retention time in h. The intake rate estimated for external markers (Cr, Co, and Yb) was calculated as a sum of the 5-d feed and liquid labeled orally administrated for each animal, and the iNDF intake rate was calculated as a proportion of iNDF in total feed intake provided during the 5-d period before slaughter.

4.2.9. Duodenal histology procedures

The samples from the duodenum (SI) kept in 70% ethanol was dehydrated using increasing concentrations of ethanol solutions (70%, 80%, 90%, absolute), then fixed in a resin solution (glycol methacrylate resin). The histological resin blocks were allocated in wood support and branched in a steel razor microtome (Leica RM 2125 RT, with five μm nonsequential thickness cut). After cut, the slices of abomasum were fixed in glass microscope slides and colored. The slides were colored with Alcian Blue (AB, pH 2.5) during 30 minutes, oxidized with peroxide acid (1%) for 10 minutes, and colored with Schiff reagent (pH 2.5) during 10 minutes, then five minutes after the last procedure, the slides were washed in current water.

The histological analysis was performed in the duodenum colored slides with a 100x lens light microscope (Bel) associated with an image analytical system. Caliciform cells with acid and neutral mucins were counted in five duodenal images in a 100x lens light microscope. The total caliciform cells count was calculated as a sum of cells containing acid and neutral mucins. The morphometrical analysis done was the vilosity length, the crypt depth determined with a 100x lens, the final result was obtained by the mean of 10 different images. The ratio between vilosity and crypt was also calculated. We performed a stereological analysis, a technique developed to calculate the superficial density (Sv) and volume (Vv) of the duodenum mucosa, these parameters were determined with a counting grid (Figure 4.1) developed by Baddeley et al. (1986).

Figure 4.1: Counting grid with points and cycloids to estimate the surface density (Sv) and volume (Vv) of duodenal mucosa tissue



4.2.10. Statistical procedures

The trial was tested as a complete randomized statistical design with a split-plot arrangement (two dietary P levels and two infection status – with or without *T. colubriformis* infection). All statistical procedures were performed in R studio software (3.4.2. version; 2017). The statistical design was mathematically described below (Eq. 9):

$$Y_{ijk} = P_i + I_j + P \times I_{ij} + a_k + e_{ijk} \quad \text{Eq.9}$$

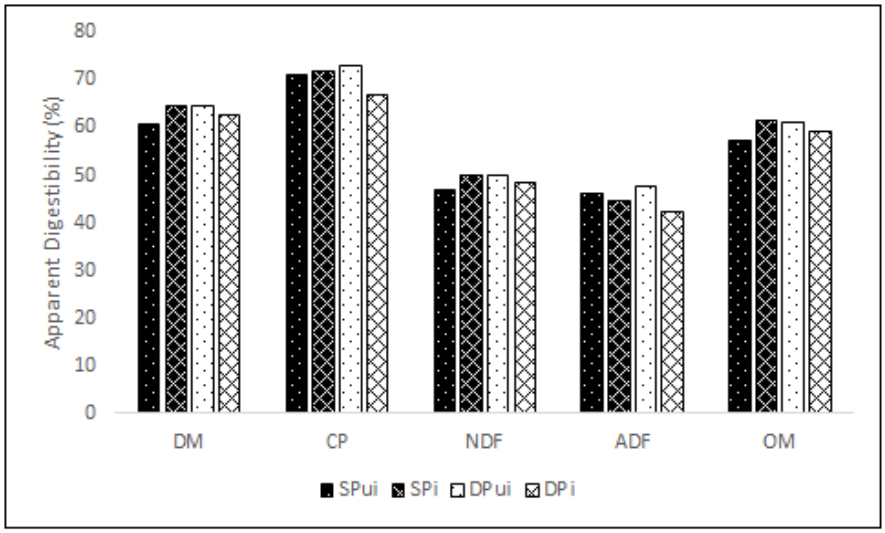
where Y_{ijk} was the dependent variables tested, P_i was the fixed effect of dietary P levels, I_j was the fixed effect of infection, $P \times I_{ij}$ was the fixed effect of the interaction between P and infection, a_k was the random effect of animal (experimental unit), and e_{ijk} was the experimental error, following the assumption: e_{ijk} = iid N $(0,\sigma^2_e)$.

All variables were tested for the mean (Shapiro-Wilk) and variance (Bartlett) normality. Outliers and influence data were tested and removed from the dataset when exceeding three times the standard deviation of the mean. The model was evaluated as a mixed model effect (fixed and random) with the function lmer in R studio. The effect of treatment was tested by variance analysis (ANOVA) with 5% significance criteria, mean differences were compared by Duncan's test with the same significance criteria.

4.3. Results

The apparent digestibility indicated the percentage of nutrients digestion and absorption from the gastrointestinal tract, in this study (Figure 4.2), the infection and the dietary P levels, did not affect the nutrient digestibility ($P \geq 0.11$), and the average crude protein (CP), neutral detergent fiber (NDF), and dry matter (DM) digestibility across treatments were 70.5, 48.8, and 63.1 % of intake, respectively.

Figure 4.2: Mean percentage of dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and organic matter (OM) apparent digestibility of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels. Treatments: SPui = sufficient P uninfected, SPi = Sufficient P infected, DPui = deficient P uninfected, DPi = Deficient P infected



The nitrogen (N) metabolism was also evaluated (Table 4.1), the absorption of crude protein, and consequently the nitrogen, did not differ across treatments, however the N retention coefficient was higher for uninfected animals ($P < 0.01$) when compared to infected ones, consequently, the same pattern was observed for the ratio N retained and N absorbed ($P < 0.01$). The principal consequence of a reduced N retention was the excretion of this N via urine, and in fact, the N excreted in urine was higher in infected animals (Table 4.1) both in absolute amount ($P < 0.05$) or as a percentage of N intake ($P < 0.01$). Purines' derivatives measured in urine provide some informations about microbial metabolizable protein, and in this study (Table 4.1), purine derivatives were similar across treatments ($P \geq 0.17$).

Table 4.1: Nitrogen metabolism and purines' derivatives of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P		Deficient P		SEM	*P value*		
	Uninfec	Infected	Uninfec	Infected		P	I	PxI
Nitrogen balance								
Intake (g/day)	21.6	21.5	21.6	21.0	1.93	ns	ns	ns
Feces (g/day)	6.28	6.09	6.01	7.13	0.79	ns	ns	ns
Feces (% N intake)	29.0	28.3	27.2	33.2	2.29	ns	ns	ns
Urine (g/day)	5.75^{b}	8.96^{a}	6.00^{b}	8.37^{a}	1.22	ns	*	ns
Urine (% N intake)	26.5^{b}	41.1^{a}	26.1^{b}	39.5^{a}	5.21	ns	**	ns
N absorption coefficient	0.71	0.72	0.73	0.67	0.02	ns	ns	ns
N retained coefficient	0.44^{a}	0.31^{b}	0.47^{a}	0.27^{b}	0.06	ns	**	ns
N retained / N absorbed	0.63^{a}	0.42^{b}	0.64^{a}	0.40^{b}	0.08	ns	**	ns
Purines derivates								
PD (mmol/day)	7.72	7.93	7.23	6.6	2.06	ns	ns	ns
PD (µmol/day*$kg^{-0.75}$)	569	603	526	501	57.2	ns	ns	ns
MN (g N/day)	3.15	3.08	3.18	3.06	0.20	ns	ns	ns

SEM = standard error of the mean, P = Phosphorus, I = Infection, PD = purine derivatives collected from urine, MN = microbial nitrogen.
P value – ns ($P > 0.10$), † ($0.05 \leq P \leq 0.10$), * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).
[a,b] different superscript lowercase letter represented infection differences in mean values ($P < 0.05$) according Duncan's test.

For a better understanding of digestion and absorption the gastrointestinal tract (GIT) anatomy and physiology were essential, and about the quantity of digesta content in the total GIT (Table 4.2), there were no differences ($P > 0.05$) observed across treatments, however when the GIT was segmented in different compartments, it was observed that infected animals had a greater amount of digesta in the SI ($P < 0.05$) compared to uninfected ones. Considering now the tissue (empty compartment) of each segment, there was a significant infection effect, where infected animals presented higher tissue weight for SI ($P < 0.01$) and Cecum ($P < 0.05$) compared to uninfected animals. There were no differences ($P \geq 0.44$) across treatments for the rumen-reticulum (RR), and cecum pH, and for the proportion between solids and liquids (solutions) in the RR digesta content.

Table 4.2: Gastrointestinal tract segments' weight, and rumen-reticulum and cecum pH of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P		Deficient P		SEM	*P value*		
	Uninfec	Infected	Uninfec	Infected		P	I	PxI
Digesta weight (g)								
RR	5903	5338	5495	4736	780	ns	ns	ns
Omasum	88.8	80.0	96.2	110.0	31.3	ns	ns	ns
Abomasum	181	407	170	258	89.0	ns	†	ns
Small Intestine	529^{b}	804^{a}	432^{b}	687^{a}	133	ns	*	ns
Cecum	260	348	255	347	79.6	ns	ns	ns
Colon-rectum	295	447	426	505	69.0	ns	ns	ns
Total Tract	7256	6716	7483	6643	950	ns	ns	ns
empty compartment (g)								
RR	715	716	812	699	83.2	ns	ns	ns
Omasum	78.8	79.0	83.8	79.0	12.6	ns	ns	ns
Abomasum	119	140	129	130	17	ns	ns	ns
Small Intestine	398^{b}	771^{a}	478^{b}	844^{a}	58.2	ns	**	ns
Cecum	52.5^{b}	84.0^{a}	50.0^{b}	63.0^{a}	10.0	ns	*	ns
Colon-rectum	325	261	331	421	85.4	ns	ns	ns
Total Tract	1688	2051	1884	2236	233	ns	ns	ns
RR Solids proportion (%)	52.7	49.5	50.3	50.6	4.90	ns	ns	ns
RR pH	6.64	6.55	6.74	6.65	0.16	ns	ns	ns
Cecum pH	7.13	7.07	7.06	7.17	0.13	ns	ns	ns

SEM = standard error of the mean, P = Phosphorus, I = Infection, RR = rumen-reticulum.
P value – ns ($P > 0.10$), † ($0.05 \leq P \leq 0.10$), * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).
^{a,b} different superscript lowercase letter represented infection differences in mean values ($P < 0.05$) according Duncan's test.

Table 4.3 represented the organs (soft tissues) weight and P concentration in the GIT segments' digesta. In this sense, just the kidney weight was affected by dietary P levels, where animals fed sufficient levels of P presented heavier kidneys ($P < 0.05$) compared to deficient diets. Now, considering the GIT segments' P concentration, animals fed sufficient P levels presented the digesta of omasum ($P < 0.001$) and colon-rectum ($P < 0.05$) more concentrated in P when compared to deficient P diets. On the other hand, the infection status affected RR and colon-rectum P concentration, uninfected lambs presented higher P

concentration in RR digesta (P < 0.05), contrarily, infected animals showed higher P concentration in colon-rectum (P < 0.01). The spectrophotometry measurements (Table 4.3) showed an infection effect in variable a* (redness), with greater values for infection treatments (P < 0.05) compared to uninfected ones.

Table 4.3: Soft tissues weight, *Longissimus lumborum* spectrophotometry, and gastrointestinal tract digesta phosphorus concentration of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P		Deficient P		SEM	*P value*		
	Uninfect	Infected	Uninfect	Infected		P	I	PxI
Soft tissues (g)								
Heart	126	125	129	126	10.1	ns	ns	ns
Liver	449	638	446	436	69.0	ns	ns	ns
Kidney	90.0^{A}	88.0^{A}	78.0^{B}	78.0^{B}	5.22	*	ns	ns
Lung	459	507	459	444	55.6	ns	ns	ns
Spleen	55.0	55.0	55.0	56.0	6.97	ns	ns	ns
GIT digesta P concentration (g/kgDM)								
Rumen-reticulum	8.49^{a}	7.03^{b}	8.21^{a}	6.29^{b}	0.77	ns	*	ns
Omasum	8.54^{A}	9.46^{A}	6.74^{B}	5.35^{B}	1.18	***	ns	ns
Abomasum	8.10	9.85	5.26	7.58	2.27	ns	ns	ns
Small Intestine	10.8	13.9	9.75	13.7	2.29	ns	ns	ns
Cecum	7.56	12.1	7.73	10.4	1.99	ns	†	ns
Colon-rectum	7.05^{bA}	11.93^{aA}	4.92^{bB}	8.45^{aB}	1.48	*	**	ns
Muscle Colorimetry								
L*	34.8	34.8	34.1	35.3	0.96	ns	ns	ns
a*	12.6^{b}	15.2^{a}	13.1^{b}	16.6^{a}	1.39	ns	*	ns
b*	10.6	11.4	10.5	12.0	0.67	ns	†	ns

SEM = standard error of the mean, P = Phosphorus, I = Infection, DM = dry matter, L* = lightness, a* = redness, and b* = yellowness.
P value – ns (P > 0.10), † (0.05 ≤ P ≤ 0.10), * (P < 0.05), ** (P < 0.01), *** (P <0.001).
[a,b] different superscript lowercase letter represented infection differences in mean values (P < 0.05) according Duncan's test.
[A,B] different superscript lowercase letter represented dietary P levels differences in mean values (P < 0.05) according Duncan's test.

The amount of digesta, P concentration, and digestibility and absorption availability in a specific segment were directly related to the amount of feed consumed and the time that a nutrient spent to move from and compartment to the subsequent one. Thus, the passage rate

was evaluated and the results (Table 4.4) indicated that the treatments tested did not statisticaly affect most of the feed fractions (concentrate, forage, and liquid phase), considering internal and external markers, however, the forage fraction in the SI, tested even with internal and external marker (P = 0.09), presented around two times greater passage rate for infected animals, compared to uninefected. For total tract forage passage rate, deficient P levels presented faster forage transit across the entire GIT (P < 0.05) compared to sufficient P levels diet. Furthermore, the liquid phase passage rate was faster in the abomasum for infected lambs (P < 0.05), and also faster in the SI for Sufficient P diets (P < 0.05).

Table 4.4: Concentrate, forage (with external and internal markers), and liquid phase passage rate in each gastrointestinal tract segment and total tract of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P		Deficient P		SEM	*P value*		
Passage rate	Uninfect	Infected	Uninfect	Infected		P	I	PxI
Concentrate, %/h								
Rumen-reticulum	9.86	7.54	10.6	8.20	2.15	ns	ns	ns
Omasum	367	236	265	206	51.1	ns	ns	ns
Abomasum	422	442	421	429	19.2	ns	ns	ns
Small intestine	229	295	257	313	55.1	ns	ns	ns
Cecum	90.1	64.0	76.5	91.2	17.9	ns	ns	ns
Colon-rectum	43.3	33.1	32.0	27.3	6.85	ns	ns	ns
Total tract	6.44	5.08	7.62	4.89	1.13	ns	ns	ns
Forage, %/h								
Rumen-reticulum	1.66	2.23	2.58	2.93	0.75	ns	ns	ns
Omasum	67.7	57.2	55.1	76.9	16.8	ns	ns	ns
Abomasum	38.2	26.2	37.2	38.7	13.6	ns	ns	ns
Small intestine	66.0	107.6	42.8	87.0	20.7	ns	†	ns
Cecum	30.1	23.9	24.3	15.9	5.37	ns	ns	ns
Colon-rectum	15.5	13.3	12.1	9.31	2.59	ns	ns	ns
Total tract	0.64^{B}	0.97^{B}	1.53^{A}	1.50^{A}	0.27	*	ns	ns

continue

conclusion

Liquid phase, %/h								
Rumen-reticulum	40.6	36.2	38.9	30.4	10.2	ns	ns	ns
Omasum	1790	1500	1560	1330	462	ns	ns	ns
Abomasum	660[a]	414[b]	587[a]	315[b]	110	ns	*	ns
Small intestine	326[A]	228[A]	153[B]	154[B]	44.0	*	ns	ns
Cecum	82.8	74.0	98.8	97.5	16.1	ns	ns	ns
Colon-rectum	50.4	42.1	49.8	31.7	10.5	ns	ns	ns
Total tract	15.3	13.4	15.7	8.99	3.01	ns	ns	ns
iNDF, %/h								
Rumen-reticulum	2.70	1.78	2.87	2.85	0.62	ns	ns	ns
Omasum	32.5	43.1	39.1	48.3	7.11	ns	ns	ns
Abomasum	75.8	70.9	55.2	107.9	21.5	ns	ns	ns
Small intestine	158	380	278	342	35.1	ns	†	ns
Cecum	46.5	56.8	36.9	42.1	11.9	ns	ns	ns
Colon-rectum	37.1	31.9	14.4	18.0	5.89	ns	ns	ns
Total tract	2.00	1.64	1.95	1.96	0.36	ns	ns	ns

SEM = standard error of the mean, P = Phosphorus, I = Infection, iNDF = indigestible neutral detergent fiber.
P value – ns ($P > 0.10$), † ($0.05 \leq P \leq 0.10$), * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).
[a,b] different superscript lowercase letter represented infection differences in mean values ($P < 0.05$) according Duncan's test.
[A,B] different superscript lowercase letter represented dietary P levels differences in mean values ($P < 0.05$) according Duncan's test.

The results presented in Table 4.5 showed increased ratio between acetic and propionic acids produced in the infected RR segment, compared to the uninfected ones ($P = 0.02$). However, the total SCFA production and individual proportions was not affected ($P \geq 0.12$) across treatments tested. An important result considered was the numerical difference in methane produced and emitted from RR, where sufficient P level diets emitted on average almost five more litters of methane, compared to deficient P levels.

Table 4.5: Methane produced, and rumen-reticulum liquid fraction short-chain fatty acids of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P		Deficient P		SEM	*P value*		
	Uninfect	Infected	Uninfect	Infected		P	I	PxI
CH4 (L/day)	24.4	22.2	18.0	18.7	2.72	†	ns	ns
Total SCFA (mmol/L)	48.8	51.4	59.5	50.7	9.39	ns	ns	ns
Acetate (%)	74.7	71.6	71.5	72.6	1.47	ns	ns	ns
Propionate (%)	12.3	11.4	14.2	11.6	1.59	ns	†	Ns
Butyrate (%)	7.96	9.80	8.00	9.61	1.22	ns	ns	ns
Iso-butiric (%)	1.44	1.46	1.38	1.40	0.34	ns	ns	ns
Valeric (%)	0.82	0.97	1.06	1.00	0.12	ns	ns	ns
Isovaleric (%)	3.65	3.90	3.90	3.80	0.50	ns	ns	ns
Acetic/Propionic ratio	6.07^{b}	6.28^{a}	5.03^{b}	6.26^{a}	0.38	ns	*	ns

SEM = standard error of the mean, P = Phosphorus, I = Infection, CH_4 = methane, SCFA = short chain fatty acids.
P value – ns ($P > 0.10$), † ($0.05 \leq P \leq 0.10$), * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$)

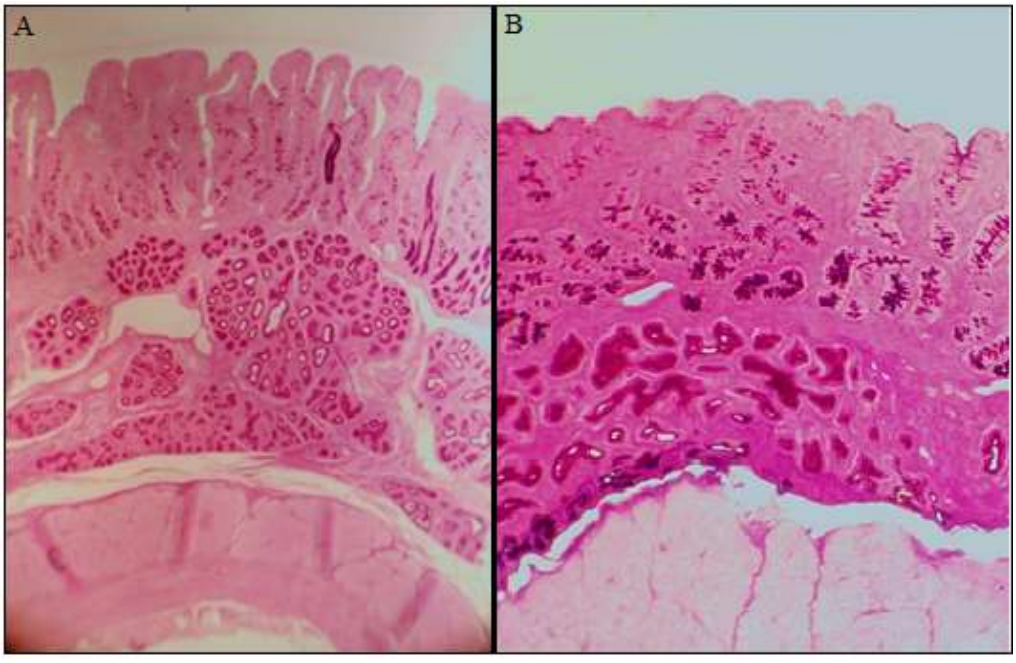
The P and many nutrients were meaning absorbed by the first portion of SI (duodenum), considering this as an important structure, a histological analysis was procedure and the results described in Table 4.6 indicated that the treatments did not affect the number of goblet cells. However, the infection status affected directly the duodenal lumen surface, infected lambs presented lower vilosity height ($P < 0.05$) and crypt depth ($P < 0.01$), compared to uninfected ones, the exfoliation process presented in Table 4.6, could be illustrated in Figure 4.3.

Table 4.6: Histological parameters and caliciform cells count of Santa Ines castrated male lambs infected by *Trichostrongylus colubriformis* and fed different P levels

Parameters	Sufficient P		Deficient P		SEM	P value		
	Uninfect	Infected	Uninfect	Infected		P	I	PxI
Histology								
Vilosity height (µm)	369[a]	318[b]	395[a]	365[b]	57.1	ns	*	ns
Crypt depth (µm)	104.6[a]	91.6[b]	102.6[a]	71.1[b]	19.6	ns	**	ns
Crypt/vilosity ratio	0.28	0.29	0.26	0.20	0.040	†	ns	ns
Sv (($\mu m^2/\mu m^3$) $*10^{-3}$)	10.4	10.4	10.2	10.3	0.47	ns	ns	ns
Vv (%)	73	59	61.2	66.6	20.4	ns	ns	ns
Caliciform cells								
Cell count	75.5	110.4	77.1	72.8	28.4	ns	ns	†
Cell count/mm^2	473	748	483	456	177	ns	ns	†

SEM = standard error of the mean, P = Phosphorus, I = Infection, Sv = mucosa surface density, Vv = mucosa volume.
P value – ns ($P > 0.10$), † ($0.05 \leq P \leq 0.10$), * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).
[a,b] different superscript lowercase letter represented infection differences in mean values ($P < 0.05$) according Duncan's test.

Figure 4.3: Histological image from duodenum (initial portion of the small intestine) luminal surface, colored with Alcian blue/PAS, of Santa Ines castrated male lambs uninfected (A), and infected by *Trichostrongylus colubriformis* (B)



4.4 Discussion

Endoparasite infection, especially those from genus *Tricostrongiloidea* could not be related to the feed consumption decrease in ruminants, but how the animal uses those nutrients provided from feed. Therefore, some authors observed a negative relation between infection and nutrients digestion and absorption, and consequently, an efficiency reduction (CARDIA et al., 2011; MAVROT et al., 2015). Although the nutrient absorption site (duodenum) is the same as that where the *Trichostrongylus* nematode inhabits, in the present study, the nutrients' apparent digestibility was not different between treatments, in agreement with previous studies that evaluated *T. colubriformis* infection in growing lambs and also not observed changes in macronutrients digestibility (DIAS E SILVA, 2017). Considering that the performance data were also not affected by treatments (chapter 2), one possible explanation was compensatory mechanism for the distal portion of the intestine (jejunum and ileum) once the proximal portion (duodenum) is compromised (BARKER, 1975). The mechanism is related to nutrient retention time in each SI portion, and the mucosa microvilli development, Bown et al. (1991) observed that infected animal presented an acceleration in digesta flux in the SI early portion, and a deceleration in the next SI portions. Moreover, Poppi et al. (1985) discussed about a histological modification in late SI portion mucosa microvilli, as a physiological compensatory strategy by the damages caused by *T. colubriformis* infection.

Regarding N metabolism, the infection did not compromise N absorption. Dias e Silva (2017) also not observed N absorption changes in Santa Ines lambs infected with *T. colubriformis*. The explanation for endoparasites infection not affects protein absorption coefficient was previously discussed. In contrast to the absorption coefficient, the N retention coefficient was lower in infected animals, due to higher urinary N losses compared to uninfected animals ($P < 0.05$). Previous trials also identified that *T. colubriformis* infection reduced N retention (COOP; HOLMES, 1996). The explanation may be related to the host's adaptive immune system in parasitological condition, infected animals reduce the tissue N retention and increase tissue N resorption to provide sources to the immune system (SYKES, 2010). In general, there is an increase in the skeletal muscles' catabolism followed by an increase in the liver's anabolism and activity (YU et al., 2000). The liver, together with the kidneys, are responsible for modifying N to an excretion form. Increase in urinary N losses was also observed by these authors. Increase liver activity can promote blood structural and nutritional modifications, like nutrients proportion, oxidation capability and color

intensity (SUMAN; JOSEPH, 2013), increasing the color intensity by reducing nutrients quantity due to the muscular tissue catabolism process. Regarding the blood continuously irrigation muscle tissues, could explain the result of the present study that infection increased muscle redness intensity, showed in Table 4.3.

Nutrients digestibility and absorption is highly correlated to the GIT lumen and mucosa conditions, flux of nutrients and digesta retention time in each segment, therefore each GIT segment digesta and tissue were weighed and our results presented an infection effect in the SI. Although all animals were fed at the same time, infected animal presented higher amount of digesta in the small intestine and also heavier tissue compared to the uninfected animals. Intestinal structural modifications could be observed in animals parasited with *T. colubriformis* as a compensatory and resilient strategy, like increase intestinal mucosa microvilli, mesenteric cells, and other structures related to the absorption (CARDIA et al., 2011), this modification may increase the segment weight, as observed in this study. Regarding to the increased digesta weight in infected animals, may be related to the segment passage rate, like discussed above that may occur an increase of duodenum passage rate, and a decrease on jejunum and ileum passage rate (POPPI et al., 1985), and the digesta flow direct interfere the digesta amount in a segment, the passage rate of each portion of SI was not evaluated in the present study, therefore, a next trial need to be performed to confirm the hypothesis.

However, the total tract and segmental passage rate was performed in the present study, and the results indicated that any treatment tested affected the digesta flow throughout the GIT. Different pattern was observed by Dias e Silva et al. (2019) that studied solid and liquid passage rate in lambs infected with *T. colubriformis*, and observed an increase in the RR passage rate of infected animals. The differences between both studies, although executed in similar conditions, could be related to the infection process, where the previous study made a serial infection, where every three days the host was challenged with a higher number of infectant parasite, and the present study provide a single dose of infectant larval, which mean a single challenge. Another aspect was about the period after infection that the passage rate was tested, 75-d and 51-d after infection, for Dias e Silva et al. (2019) and present study, respectively. These differences can direct interfere the challenge status, immunity, and GIT physiology (CANTACESSI et al., 2010; AMARANTE, 2015). In agreement with a non-treatments effect in the RR passage rate, the ruminal methane production, ruminal SCFA production, and the microbial N measured by purine derivates were also similar across treatments tested, as expected, because same digesta retention time in rumen, will provide

same conditions to bacteria degradation, and consequently, SCFA formation (DIAS E SILVA et al., 2019), and other volatile products of fermentation (methane), moreover, protein absorption from microbial sources are closely related to the digesta flow out of the rumen (ORSKOV; MCDONALD, 1970).

The digesta passage rate was an important parameter to understand nutrients flux in the GIT, and the present study showed an increase in forage passage rate of infected animals, and forage is intimaly related to the motility and nutrients flux thoughtout the GIT. Considering P as a main nutrient tested in this study, the P concentration was measured in each GIT segment, and the results indicated a decreased P concentration in RR and increased concentration of P in the colon-rectum segments of infected animals. Reticulo-rumen was already discussed in chapter 2, where the reduction in plasma P concentration observed in infected animals was caused by a GIT resorption from plasma P, and reduction in the absorption coefficient (VITTI; KEBREAB, 2010). On the other hand, the P concentration in large intestine is strongly negative correlated to the absorption coefficient. The infection reduced the P absorption in the small intestine (Chapter 2), and the consequence was increase P concentration in large intestine, last GIT segment before feces excretion. The absorption coefficient mentioned above could be negatively affect by innumerous factors, including the passage rate, as observed reported above. Therefore, in agreement with several studies in wool sheep (BARKER, 1975) and meat sheep (CARDIA et al., 2011), the growing phase Santa Ines lamb, infected with *T. colubriformis*, showed a reduction in the villous height and also a reduction in crypt depth, but this modification in the intestinal mucosa could be clearly observed in the Figure 4.3, where, the mucosa was severely damaged by the nematode, followed by a generalized villous atrophy. These damages reduced the mucosa absorption site and could negatively impact the entire system (AMARANTE, 2015).

Phosphorus deficiency was tested and results showed a minority dietary P deficiency effect in the physiological parameters related to the GIT, P concentration, nutrients digestion, and absorption. The feed systems used to determine the animal requirements were developed for exotic, and specialized animal using a biased dataset (NRC, 2007). Moreover, among studies conducted in Brazil, few of those used Santa Ines sheep as animal model, in which, were performed more than 10 years ago. All genetic, and nutritional transformations could affect the nutritional requirements of growing Santa Ines lambs, however, future studies need to be conducted to update the Santa Ines P requirements and confirm those assumptions.

4.5. Conclusion

T. colubriformis compromise the nutrients absorption by damaging the mucosa villous, the absorption site in the small intestine, and direct interfere in the N metabolism, reducing the muscle N retention, and increasing urinary losses. However, the parasite did not modify the ruminal environment, and can not be classified as a physical barrier that affect digesta transit through the GIT, and principally in small intestine. Differently to the infection, the dietary P restriction did not affect GIT physiological parameters, however, more studies need to be performed to confirm the real P requirements for growing Santa Ines lambs.

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5. Modeling of phosphorus flux and excretion in growing lambs

Abstract: Prediction of phosphorus (P) fluxes from animals is necessary to reduce its excretion to the environment, which is a major challenge facing animal production systems. The study aimed to adapt a multicompartmental model to predict P fluxes in lambs. Eighteen Santa Ines male lambs (age = 6 months, body weight = 31.8 ± 4.8 kg) were randomly divided into two groups and fed 50 or 100% of their P requirements based on National Research Council (2007) recommendations. All animals were given a bolus infusion of 32P, and a 6-pool model was used to derive P fluxes. Modeled pools were the rumen, small intestine, large intestine, plasma, bone, and soft tissues. Daily intake and excretion of P in feces and urine and the specific activity of P and mass of stable P in each pool were determined and used as inputs to the model. The low-P diet did not change P flux from plasma to bone and to soft tissues. However, the flux from bone to plasma (resorption) was observed in both treatments while the resorption of P from soft tissues occurred only for animals fed the P-restricted diet. Overall, the model was both accurate and precise, being able to predict P excreted in feces with reasonable prediction errors for both treatments. Therefore, the model can be used for predicting P fluxes in lambs fed different diets which can be used as a nutritional tool to reduce the excretion of P in feces.

Key words: Absorption, kinetics, modeling, radioactive, retention, sheep

5. Modelando o fluxo e excreção de fósforo de cordeiros em crescimento

Resumo: A predição matemática dos fluxos de fósforo (P) a partir de testes em animais é necessária para reduzir perdas e consequentemente eliminação no meio ambiente, que é um grande desafio para os sistemas de produção animal. O estudo teve como objetivo adaptar um modelo multicompartimental para predizer o fluxo de P em cordeiros. Dezoito cordeiros machos da raça Santa Inês (idade = 6 meses e peso corporal ao abate = 31,8 ± 4,8 kg) foram aleatoriamente divididos em dois grupos e alimentados com 53 ou 100% de seus requerimentos de P com base nas recomendações do National Research Council (2007). Todos os animais receberam uma infusão única de ^{32}P, e um modelo de 6 compartimentos foi usado para derivar os fluxos de P. Os compartimentos utilizados foram o rúmen, intestino delgado, intestino grosso, plasma, ossos e tecidos moles. A ingestão diária, excreção de P nas fezes e urina, quantidade de P estável e a atividade específica de P em cada compartimento foram determinadas e usadas como entradas do modelo. A dieta com baixo teor de P não alterou o fluxo de P do plasma para os ossos e tecidos moles. No entanto, o fluxo de osso para plasma (reabsorção) foi observado em ambos os tratamentos, enquanto a reabsorção de P a partir de tecidos moles ocorreu apenas para animais alimentados com dieta restrita por P. No geral, o modelo foi preciso e acurado, sendo capaz de predizer o P excretado nas fezes com erros de previsão razoáveis para ambos os tratamentos. Portanto, o modelo pode ser usado para predizer fluxos de P em cordeiros alimentados com diferentes dietas, podendo este ser usado como uma ferramenta nutricional para reduzir a excreção de P nas fezes.

Palavras-chave: absorção, cinética, modelagem, ovinos, radioatividade, retenção

5.1. Introduction

Many small ruminant producers provide supplemental dietary phosphorus (P) due to its importance in maintaining metabolic functions. However, total P intake from supplements plus the basal diet often exceeds animal requirements, and does not improve animal performance or productivity. This can have a negative impact on the environment if the soil is overloaded with the excreted P (TAMMINGA, 1996). Additionally, inorganic P supplements are limited in supply and becoming expensive (GILBERT, 2009).

Ruminants have the ability to absorb P through the rumen wall, small intestine (SI), and large intestine (LI), but its absorption occurs predominantly in the duodenum and jejunum (BREVES; SCHRODER, 1991; GOFF, 2017). Dietary and endogenous P that is not absorbed and retained in tissues is excreted in feces and urine, with fecal excretion being the predominant route of elimination (LOUVANDINI et al., 2009). Urinary excretion is generally almost negligible, but serves as a compensatory mechanism of P elimination when salivary P secretion is not adequate to maintain blood P homeostasis (SCOTT; BUCHAN, 1985). Salivary P secretions also help prevent ruminal pH variation through their buffering activity, and provide nutrients to the rumen microbiome (VITTI; KEBREAB, 2010).

Restriction of dietary P intake induces several metabolic responses, but the main action is to mobilize P from bone to meet the demands of other body tissues. Conversely, excess dietary P intake can affect metabolism by raising plasma P levels, which stimulates salivary secretion of P (TOMAS et al., 1967) and increases fecal excretion.

The use of isotope dilution techniques in metabolism studies provides a method of deriving flux estimates where such measurements cannot be readily determined using other methods. The element ^{32}P is a radioactive isotope with a half-life of 14.8 days. The short half-life makes its use for field experiments possible; moreover, ^{32}P concentrations in samples can be easily measured using a scintillation counter which are relatively inexpensive. Mathematical models have been used in several studies as an aid in interpretation of the data to gain a better understanding of the mechanisms involved in P metabolism, to identify P pathways, to measure flux of P among compartments, and to derive P requirements of small ruminants (VITTI et al., 2000; DIAS et al., 2006; DIAS et al., 2011a; DIAS E SILVA et al., 2018). The prior work has generally used multicompartmental, empirical, dynamic models fitted to the observed isotope dilution data (VITTI; KEBREAB, 2010). The main compartments evaluated in those studies included gastrointestinal tract, plasma, muscle (and other organs), and bone. However, in some studies, the models did not well represent the

observed data well. For those cases, it was hypothesized that an improved representation of the gastrointestinal compartment to represent absorption of P via different routes, and consequently, the flux across the entire system would provide better alignment with the observed data.

Models of P metabolism in animals are necessary to allow dietary P manipulation to achieve reduced P excretion while maintaining animal health and productivity. The aim of this study was to develop and evaluate a multicompartmental model of P metabolism and excretion in Santa Ines growing lambs that better represented the observed data to provide a better understanding of the effect of supplementation P on P metabolism.

5.2. Material and Methods

5.2.1. Animals, design, and treatments

The trial and analyses were carried out at the Center of Nuclear Energy in Agriculture (CENA), University of São Paulo (USP). All procedures were previously approved by the Institutional Animal Care and Use Committee of the CENA/USP. Eighteen growing Santa Ines castrated male sheep averaging 6 months of age and 31.8 ± 4.58 kg of body weight (BW) were used. A complete randomized experimental design was adopted, and the lambs were randomly allocated in a 2 × 2 factorial arrangement; factors were diets formulated to contain 50% (Low-P diet) or 100% (Adequate-P diet) of the animals' P requirements based on the National Research Council (2007) and endoparasite infected or endoparasite free status. The infected lambs received a single oral dose of 40.000 L_3 larvae stage of *Trichostrongylus colubriformis*.

Animals were kept in individual stalls with free access to water for a total period of 120 days with an adaptation period of 24 days, and the last 96 days were used to collect data. Diets were formulated based on the Nutrient Requirements of Small Ruminants (NRC, 2007) for growing sheep gaining 100 g/day of BW. Diets had a forage to concentrate ratio of 60:40, where the forage source was Tifton 85 bermudagrass hay (Cynodon dactylon cv.) and the concentrate was composed of cassava meal and maize gluten meal (Table 3.1 – Chapter 2). The diets were offered twice daily (8:00 am and 5:00 pm) in amounts sufficient to provide at least 10% as daily refusal.

5.2.2. Sampling

Eight days before the end of the experiment, the barn was prepared for radioactive isotope use, and each animal received a single dose of 7.4 MBq ^{32}P in 0.5 mL of sterile saline solution via the jugular vein before feeding. The radioactive ^{32}P molecule ($Na_2H^{32}PO_4$) was purchased from the Instituto de Pesquisas Energéticas e Nucleares (IPEN, São Paulo, Brazil). At the end of the experiment, samples of feed refusals, urine, blood, and feces were collected, processed, frozen, and stored for analysis, followed by slaughter following recommendations described in the Ministry of Agriculture, Livestock and Food Supply (BRASIL, 2018). After slaughter, animals were eviscerated and ruminal, small intestinal, and large intestinal contents were separated. The collected contents were freeze dried and stored for subsequent analyses. After slaughter, the thigh-bone of the left leg was exposed and a sample was collected from the medial location relative to its length, and stored at -20 ^{o}C for further analyses. Muscle, kidney, liver, and heart samples, the whole organ was separated, weighed and stored at -20 ^{o}C for further analyses. The muscle sample was taken from the *longissimus dorsi* between the 12^{th} and 13^{th} ribs. The muscle was separated and stored in -20 ^{o}C freezer for analyses.

5.2.3. Sample analysis

Samples of feed offered and refused were analyzed for dry matter (DM), ash, crude protein (CP), and ether extract (EE) according to the Association of Official Analytical Chemists (AOAC, 2016) procedures. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were analyzed according to Van Soest et al. (1991), and for starch content according to Mertens (2002). Samples of gastrointestinal tract contents, soft tissues, and bone were analyzed for DM and ash.

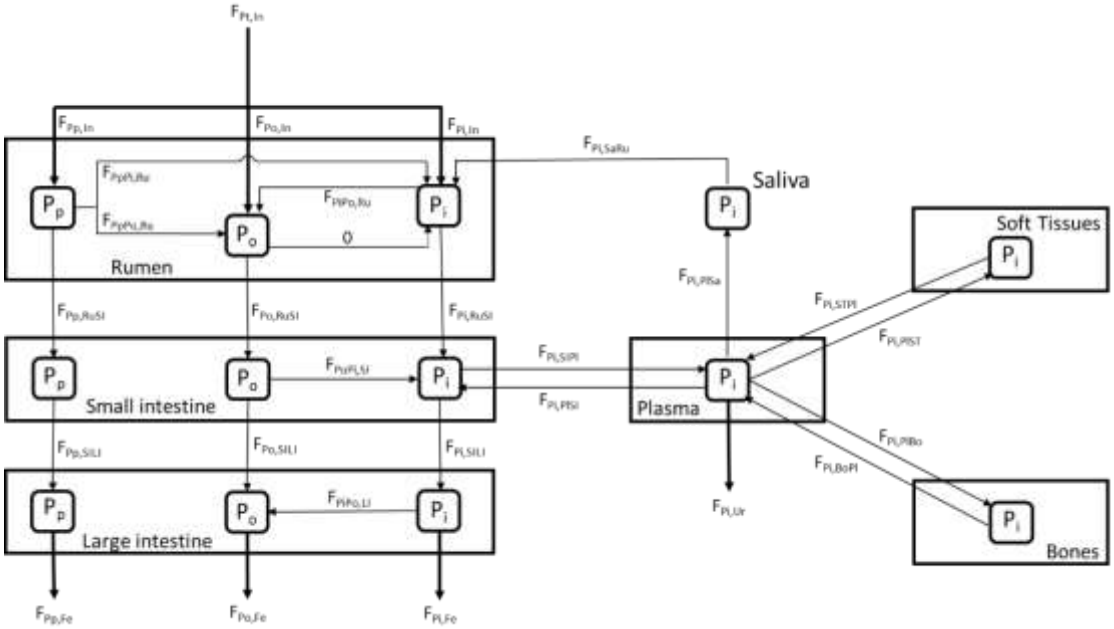
All samples were analyzed for P content with samples from the gastrointestinal tract lyophilized prior to analysis. One gram of each lyophilized solid sample (feed ingredients or refusal, feces, gastrointestinal tract content, soft tissues, and bones) was acid digested and the solution analyzed for P by colorimetry (SARRUGE; HAAG, 1974). Blood samples were centrifuged at 2000 × g for 10 minutes and plasma was separated to determine plasma levels of P. The P concentrations in liquid samples (plasma and urine) were determined by colorimetry (FISKE; SUBBAROW, 1925). The radioactive P (^{32}P) was determined using the Cerenkov effect technique with liquid scintillation spectrometry.

5.2.4. Model development

The model was developed based on prior work. The compartmental structure described by Dias et al. (2011a) model was used in the present model, due to the agreement with the pathways and stokes. Additionally, Dias et al. (2006) and Hill et al. (2008) state different types of P related to the intake and the modification occurred in the gastrointestinal tract before absorption.

The model approach of the present study consisted of six pools [rumen (Ru), small intestine (SI), large intestine (LI), plasma (Pl), bone (Bo), and soft tissues (ST)] and 3 different types of phosphorus [organic phytate P (Pp), organic non-phytate P (Po), and inorganic P (Pi)]. The overall scheme of the model is shown in Figure 5.1.

Figure 5.1: Schematic representation of phosphorus (P) absorption and metabolism in growing lambs. Larger boxes represent the six pools used for the model, small boxes with Pp, Po and Pi inside represents the portion of P in each pool and the arrows represent fluxes. The statement $F_{a,bc}$ means: a = the type of P, b = pool from, and c = pool to. P = phosphorus; p = phytate P; o = organic (non-phytate) P; i = inorganic P; t = total P; In = Intake; Fe = feces; Ur = urine; Ru = rumen; SI = small intestine; Li = large intestine; Pl = plasma; Sa = saliva; Bo = bones; ST = soft tissues



Intake of P (total (Pt), Pp, Po, and Pi, the quantity of P (Q) in each pool (Ru, SI, LI, Pl, Bo, and ST), and the specific activity (S; degradations per minute/g) of P in each pool seven days after ^{32}P infusion were used as inputs to the model. The fluxes (F) were described as $F_{x,yz}$ where x is the form of P (Pt, Pp, Po, or Pi), y is the precursor pool, and z is the product pool. Fluxes denoted with P were assumed values based on scaled observations from dairy cows (KNOWTON et al., 2001) or prior work in sheep (BOMPADRE et al., 2018[1]) adjusted for current experimental conditions.

The first assumption was that 70% of the Pp in the rumen was degraded and split into Po and Pi in a relationship of 5:1, respectively as denoted in Eq.1, 2, and 3.

$$F_{Pp,RuSI} = F_{Pp,In} - F_{Pp,Dg} \qquad Eq.1$$

$$F_{PpPi,Ru} = F_{Pp,Dg} / 6 \qquad Eq.2$$

$$F_{PpPo,Ru} = F_{Pp,Dg} - F_{PpPi,Ru} \qquad Eq.3$$

where $F_{Pp,RuSI}$ = the Pp flux from the rumen to the small intestine in g/day, $F_{Pp,In}$ = the Pp intake in g/day, $F_{Pp,Dg}$ = the Pp degraded to Pi and Po in the rumen in g/day, $F_{PpPi,Ru}$ = the amount of Pp converted to Pi from degradation in g/day, $F_{PpPo,Ru}$ = the amount of Pp converted to Po from degradation in g/day.

The next assumption was related to microbial P in the rumen ($F_{PiPo,Ru}$), considering that 15% of the Pi is converted to Po in the rumen. Thus, the outflux of Po from rumen was calculated as:

$$F_{Po,RuSI} = F_{Po,In} + (F_{Pp,Dg} \times 5/6) - F_{PiPo,Ru} \qquad Eq.4$$

Where $F_{Po,RuSI}$ = the Po flux from the rumen to the small intestine in g/day, $F_{Po,In}$ = the Po intake in g/day, $F_{Pp,Dg}$ = the Pp degraded in the rumen in g/day, $F_{PiPo,Ru}$ = the microbial conversion from Pi to Po in the rumen in g/day.

[1] Personal information

In the small intestine, it was previously observed that 91% of Po was digested or converted to Pi ($F_{PoPi,SI}$), and Pp flux was not affected. In the large intestine, it was assumed the the microflora convert 2.5% of Pi to microbial Po ($P_{PiPo,Li}$).

$$F_{Pp,SILI} = F_{Pp,Fe} = F_{Pp,RuSI} \quad Eq.5$$

$$F_{Po,SILI} = F_{Po,RuSI} - F_{PoPi,SI} \quad Eq.6$$

$$F_{Po,Fe} = F_{Po,SILI} + F_{PiPo,LI} \quad Eq.7$$

where $F_{Pp,SILI}$ = the Pp flux from the small intestine to the large intestine in g/day, $F_{Pp,Fe}$ = the Pp flux excreted via feces in g/day, $F_{Pp,RuSI}$ = the Pp flux from the rumen to the small intestine in g/day, $F_{Po,SILI}$ = the Po flux from the small intestine and large intestine in g/day, $F_{Po,RuSI}$ = the Po flux between rumen and small intestine in g/day, $F_{PoPi,SI}$ = the digestive conversion from Po to Pi in the small intestine in g/day, $F_{Po,Fe}$ = the Po flux excreted via feces in g/day, $F_{Po,SILI}$ = the Po flux from the small intestine to the large intestine in g/day, $F_{PiPo,LI}$ = the microbial conversion from Pi to Po in the large intestine in g/day.

The labeled ^{32}P was administrated intravenously as a single pulse dose on day 0 and the specific activity (S) of pools was measured on day 8 at the end of the trial; thus, the model assumes there was no re-entry of labelled P from external sources. Derivative equations were used to understand the assumptions of the stable and radioactive P flux in the system, and those equations were previously described by Dias et al. (2011a). Eight days after a single dose of ^{32}P, the rumen, small intestine, and large intestine were assumed to be in a complete steady state (isotope and non-isotope P), while plasma was considered in non-isotopic steady state. Therefore, the equations to describe those were:

$$F_{Pi,In} + F_{Pi,SaRu} + F_{PpPi,Ru} - F_{Pi,RuSI} = 0 \quad Eq.8$$

$$F_{Pi,RuSI} + F_{PoPi,SI} + F_{Pi,PlSI} - F_{Pi,SIPl} - F_{Pi,SILI} = 0 \quad Eq.9$$

where $F_{Pi,In}$ = the Pi intake in g/day, $F_{Pi,SaRu}$ = the Pi flux between plasma (saliva) and rumen in g/day, $F_{PpPi,Ru}$ = the amount of Pp converted to Pi after degradation in g/day, $F_{Pi,RuSI}$ = the Pi flux between rumen and small intestine in g/day, $F_{PoPi,SI}$ = the digestive conversion from

Po to Pi occurring in the the rumen, stomach, and small intestine, in g/day, $F_{Pi,PlSI}$ = the Pi flux between plasma and small intestine in g/day, $F_{Pi,SIPl}$ = the Pi flux between small intestine and plasma in g/day, $F_{Pi,SILI}$ = the Pi flux between small intestine and large intestine in g/day

$$S_{Pt,Pl} * F_{Pi,PlSI} - S_{Pt,SI} * (F_{Pi,RuSI} + F_{Pi,SaRu}) = 0 \qquad Eq.10$$

$$(S_{Pt,Pl} - S_{Pt,Bo}) * F_{Pi,PlBo} / Q_{Pt,Bo} = S_{Pt,Bo} / 8 \qquad Eq.11$$

$$(S_{Pt,Pl} - S_{Pt,ST}) * F_{Pi,PlBo} / Q_{Pt,Bo} = S_{Pt,ST} / 8 \qquad Eq.12$$

where $S_{Pt,Pl}$ = the ^{32}P specific activity measured in plasma in dpm/g, $F_{Pi,PlSI}$ = the Pi flux between plasma and small intestine in g/day, $S_{Pt,SI}$ = the ^{32}P specific activity measured in the small intestine in dpm/g, $F_{Pi,RuSI}$ = the Pi flux between rumen and small intestine in g/day, $F_{Pi,SaRu}$ = the Pi flux between plasma (saliva) and rumen in g/day, $S_{Pt,Bo}$ = the ^{32}P specific activity measured in bone in dpm/g, $F_{Pi,PlBo}$ = the Pi flux between plasma and bone in g/day, $Q_{Pt,Bo}$ = the quantity of P in bones in g, $S_{Pt,ST}$ = the ^{32}P specific activity measured in soft tissues in dpm/g. The algebraic manipulation of Eq. (8) to (12) gives:

$$F_{Pi,SaRu} = S_{Pt,Ru} * F_{Pi,In} / (S_{Pt,Pl} - S_{Pt,Ru}) \qquad Eq.13$$

$$F_{Pi,PlSa} = F_{Pi,SaRu} \qquad Eq.14$$

where $F_{Pi,SaRu}$ = the Pi flux between plasma (saliva) and rumen in g/day, $S_{Pt,Ru}$ = the ^{32}P specific activity measured in rumen in dpm/g, $F_{Pi,In}$ = the Pi intake in g/day, $S_{Pt,Pl}$ = the ^{32}P specific activity measured in plasma in dpm/g, $F_{Pi,PlSa}$ = the Pi outflux from plasma via salivation in g/day.

Eq. 14 indicated that the flux of inorganic P into the rumen via saliva is constant and derived entirely from plasma equal to the flux being reabsorbed from plasma to rumen, and the flux from saliva to rumen ($F_{Pi,SaRu}$) to take into account the specific activity of P in the rumen ($S_{Pt,Ru}$) and plasma ($S_{Pt,Pl}$), and the amount of inorganic P intake ($F_{Pi,In}$).

$$F_{Pi,RuSI} = F_{Pi,SaRu} + F_{Pi,In} + F_{PpPi,Ru} - F_{PiPo,Ru} \quad Eq.15$$

$$F_{Pi,Fe} = F_{Pi,SiLi} - F_{PiPo,LI} \quad Eq.16$$

where $F_{Pi,RuSI}$ = the Pi flux between rumen and small intestine in g/day, $F_{Pi,SaRu}$ = the Pi flux between plasma (saliva) and rumen in g/day, $F_{Pi,In}$ = the Pi intake in g/day, $F_{PpPi,Ru}$ = the Pp degraded in rumen in g/day, $F_{PiPo,Ru}$ = the microbial conversion from Pi to Po in rumen in g/day, $F_{Pi,Fe}$ = the Pi flux excreted via feces in g/day, $F_{Pi,SiLi}$ = the Pi flux between small intestine and large intestine in g/day, $F_{PiPo,LI}$ = the microbial conversion from Pi to Po occurring in large intestine in g/day.

Eq. 16 estimated the flux of inorganic P in feces from the small intestinal flux ($F_{Pi,SILI}$) and microbial P degradation in the LI.

$$F_{Pi,PlSI} = (S_{Pt,Ru} - S_{Pt,SI}) / (S_{Pt,SI} - S_{Pt,Ru}) * F_{Pi,RuSI} \quad Eq.17$$

$$F_{Pi,SIPL} = F_{Pi,RuSI} + F_{Pi,PlSI} + F_{PoPi,SI} - F_{Pi,SILI} \quad Eq.18$$

where $F_{Pi,PlSI}$ = the Pi flux between plasma and small intestine in g/day, $S_{Pt,Ru}$ = the ^{32}P specific activity measured in the rumen in dpm/g, $S_{Pt,SI}$ = the ^{32}P specific activity measured in small intestine in dpm/g, $F_{Pi,RuSI}$ = the Pi flux between rumen and small intestine in g/day, $F_{Pi,SIPL}$ = the Pi flux between small intestine and plasma in g/day, $F_{PoPi,SI}$ = the digestive conversion from Po to Pi occurred in the after rumen stomachs and small intestine, in g/day, $F_{Pi,SILI}$ = the Pi flux between small intestine and large intestine in g/day.

Absorption of P from the SI was only in the inorganic form. Thus, while the flux from plasma to the small intestine (F_{P_SI}) accounts for the specific activity of fluxes from the rumen (S_{Rumen}) and the small intestine (S_{SI}), the flux of inorganic P from the rumen to the SI ($F^{(i)}_{R\_SI}$). On the other hand, the absorption of Pi (F_{SI_P}) is the sum of fluxes estimated in Eq. 15, 17 and the bioavailability of P post-rumen, minus the flux of Pi from SI to the LI.

$$F_{Pi,PlBo} = (S_{Pt,Bo} * Q_{Pt,Bo}) / (7 * (S_{Pt,Pl} - S_{Pt,Bo})) \quad Eq.19$$

$$F_{Pi,PlST} = (S_{Pt,ST} * Q_{Pt,ST}) / (7 * (S_{Pt,Pl} - S_{Pt,ST})) \quad Eq.20$$

where $F_{Pi,PlBo}$ = the Pi flux from plasma to bone in g/day, $S_{Pt,Bo}$ = the ^{32}P specific activity measured in bone in dpm/g, $Q_{Pt,Bo}$ = the quantity of P in bones in g, $S_{Pt,Pl}$ = the ^{32}P specific activity measured in plasma in dpm/g, $F_{Pi,PlST}$ = the Pi flux between plasma and soft tissues in g/day, $S_{Pt,ST}$ = the ^{32}P specific activity measured in soft tissues in dpm/g, $Q_{Pt,ST}$ = the quantity of P in soft tissues in g.

Eq. 19 and 20 represented the retention flux of Pi by different tissues, divided into bone and soft tissues, where soft tissues were the weighted average of muscle, kidney, liver, and heart. In Eq. 19. Resorption from bone and ST was represented as:

$$F_{Pi,BoPl} + F_{Pi,STPl} = F_{Pi,Ur} + F_{Pi,PlSI} + F_{Pi,PlSa} + F_{Pi,PlBo} + F_{Pi,PlST} - F_{Pi,SIPl} \quad Eq.21$$

$$F_{Pi,STPl} = ((S_{PtBo,PtST} - S_{Pt,Bo}) * (F_{Pi,BoPl} + F_{Pi,STPl})) / (S_{Pi,ST} - S_{PtBo,PtST}) \quad Eq.22$$

$$S_{PtBo,PtST} = (S_{Pt,Bo} * (Q_{Pt,Bo} / (Q_{Pt,Bo} + Q_{Pt,ST}))) + (S_{Pt,ST} * (Q_{Pt,ST} / (Q_{Bones} + Q_{ST}))) \quad Eq.23$$

where $F_{Pi,BoPl}$ = the Pi flux between bones and plasma in g/day, $F_{Pi,STPl}$ = the Pi flux between soft tissues and plasma in g/day, $F_{Pi,Ur}$ = the Pi flux excreted via urine in g/day, $F_{Pi,PlSI}$ = the Pi flux between plasma and small intestine in g/day, $F_{Pi,PlSa}$ = the Pi flux between plasma (saliva) and rumen in g/day, $F_{Pi,PlBo}$ = the Pi flux between plasma and bones in g/day, $F_{Pi,SIPl}$ = the Pi flux between small intestine and plasma in g/day, $S_{PtBo,PtST}$ = the weighted average ^{32}P specific activity measured in bones and soft tissues in dpm/g, $S_{Pt,Bo}$ = the ^{32}P specific activity measured in bones in dpm/g, $S_{Pi,ST}$ = the ^{32}P specific activity measured in soft tissues in dpm/g, $Q_{Pt,Bo}$ = the quantity of P in bones in g, $Q_{Pt,ST}$ = the quantity of P in small intestine in g.

The flux of Pi from bone to plasma was calculated by subtraction of Eq. 22 from Eq. 2. Eq. 23 the specific activity of the combination of bone and soft tissue.

The model was used to predict fluxes for each animal in the study, and statistical analyses was performed on the resulting estimates to assess the impact of treatment on P metabolism.

5.2.5. Statistical analysis

Statistical analysis was performed using the R software (version 3.4.2., 2017). Tests of data normality were performed and outliers were identified using studentized residuals where values greater than the absolute value of 2 were removed from the dataset; one data point from the Adequate-P diet was removed. The mixed model function lmer from the lme4 package was used for all tests of treatment effects. Fixed effects were dietary treatments (Adequate-P or Low-P) and health status (infected or not), and random effect was the experimental unit (animal).

Measurements of P intake and its excretion in feces and urine, the specific activity of P in each pool (rumen, small intestine, large intestine, plasma, bone, and soft tissues), and mass of stable P in each pool were analyzed by Tukey's test. Fluxes of P were used for model development. The t-test was used to compare observed and estimated fluxes of P where a P value < 0.05 was considered significant.

The model was evaluated using mean squared prediction error (MSE), root mean squared error (RMSE) expressed as a proportion of the observed mean, and MSE values were decomposed to mean and slope error bias as described by Bibby and Toutenburg (1977). Concordance correlation coefficients (CCC) were calculated according to Lin (1989) to evaluate the precision and accuracy of the model. Observed versus predicted values and residual versus predicted values were plotted to evaluate the predictive performance of the model.

5.3. Results

5.3.1. Animal Experiment

Initial analyses indicated that the effect of health condition was not significant ($P = 0.82$), therefore, the data were pooled for further analyses. Animal inputs excretion in feces and urine, the specific activity of each pool, and stable P pool sizes are summarized in Table 5.1 The P intakes of animals fed the adequate-P diet were in agreement with the feeding system for growing male lambs with similar BW. The true restriction of P intake was 49.8% and differences between treatments were associated with inorganic P supplementation, which was offered only to animals fed adequate-P diets. Total P excreted in feces (Table 5.1) was greater than P intake, indicating the animals were in negative P balance and resorbing P from bone which was supported by the flux estimates (Table 5.3) resorption.

The animals had urinary P concentrations of 7.01 and 8.32 mg/dL for both P-adequate and P-restricted treatments, respectively. Urinary excretion of P was less than 0.01% of P intake as expected. Animals fed the adequate-P diet excreted 3.89 g/day of P in feces while animals fed the low-P diet excreted 2.05 g/day of P in feces.

Table 5.1: Phosphorus ingested and excreted via feces and urine, specific radioactivity of each pool, and pool size in each compartment of lambs fed adequate or low phosphorus diets

Parameter	Symbol [a]	Adequate-P diet		Restricted-P diet		*P*-value
		Amount	SEM [b]	Amount	SEM [b]	
Flux		----------g/d----------		----------g/d----------		
Intake	$F_{Pt,In}$	3.27[a]	0.263	1.64[b]	0.146	<0.001
Phytate	$F_{Pp,In}$	0.626	0.0516	0.662	0.0589	0.838
Non-phytate	$F_{Po,In}$	0.346	0.0285	0.365	0.0325	0.848
Inorganic	$F_{Pi,In}$	2.30[a]	0.189	0.611[b]	0.0544	<0.001
Feces	$F_{Pt,Fe}$	3.89[a]	0.641	2.05[b]	0.387	<0.001
Phytate	$F_{Pp,Fe}$	0.593[a]	0.0975	0.312[b]	0.0589	<0.001
Non-phytate	$F_{Po,Fe}$	0.621[a]	0.107	0.327[b]	0.0646	<0.001
Inorganic	$F_{Pi,Fe}$	2.68[a]	0.436	1.41[b]	0.263	<0.001
Urine	$F_{Pi,Ur}$	0.00877	0.00125	0.0104	0.00917	0.138
Specific radioactivity		----------dpm/g---------		----------dpm/g---------		
Rumen	$S_{Pt,Ru}$	5.49	1.97	6.83	1.76	0.452
Small Intestine	$S_{Pt,SI}$	9.28	1.77	11.9	2.48	0.313
Feces	$S_{Pt,Fe}$	10.8	5.46	8.79	2.75	0.360
Plasma	$S_{Pt,Pl}$	28.39	3.76	37.4	6.72	0.574
Bone	$S_{Pt,Bo}$	1.36	0.422	1.38	0.48	0.859
Soft tissues	$S_{Pt,ST}$	2.09	0.399	1.84	0.219	0.627
Bone plus soft tissues	$S_{PtBo,PtST}$	1.42	0.384	1.424	0.435	0.889
Pool size		----------g----------		----------g----------		
Rumen	$Q_{Pt,Ru}$	2.97	0.997	2.74	0.436	0.684
Small Intestine	$Q_{Pt,SI}$	0.501	0.0914	0.322	0.0897	0.696
Plasma	$Q_{Pt,Pl}$	0.118	0.0217	0.117	0.0168	0.764
Bone	$Q_{Pt,Bo}$	151.7	14.3	162.4	19.6	0.596
Soft tissues	$Q_{Pt,ST}$	14.9	1.03	16.7	0.917	0.995

[a] Symbols used in equations and Figure 1. S* was calculated using combined data from bone and soft tissues.
[b] SEM = standard error of treatment means.
All specific radioactivity values were measured or projected for 168 h after the infusion.
[a, b] Means with different superscript lowercase letters indicate the difference between treatments by the t-test ($P < 0.05$).

5.3.2 Model Evaluation

The model predicted fecal excretion of Pp, Po, and Pi with RMSE ranging from 5.36 to 28.9% of the observed mean values (Table 5.2). For Pp, the low-P group was predicted with an RMSE of 0.10 g/day, which was composed of a non-significant mean bias (28.9% of MSE) and moderate to low slope bias value (19.8% of MSE). Predictions of Po from low-P diets, had a similar RMSE value of 0.11 g/day in comparison to the Pp from in low-P diets, but it had a mean bias of 5.45% and a slope bias of 37.2%. However, predictions of Po in animals fed the adequate-P diet (RMSE = 0.14 g/day) had significant mean bias (47.3% of MSE; P = 0.03) and a small slope bias (7.67% of MSE). The RMSE for fecal excretion of Pi was 0.16 g/d for the adequate-P diet and 0.21 g/d for the low-P diet. CCC values ranged between 0.37 and 0.92 for the low-P diet, and 0.36 and 0.96 for adequate-P

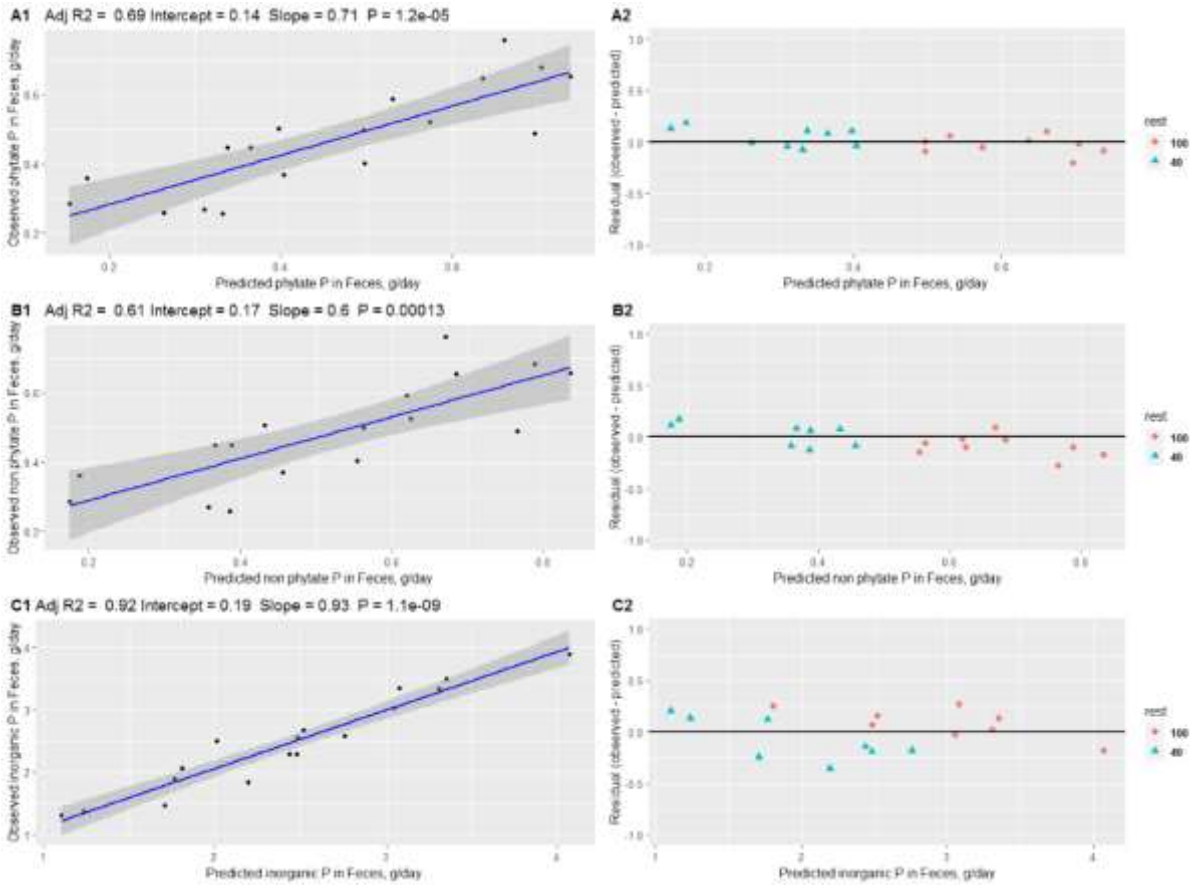
Table 5.2: Model evaluation using observed and predicted amount of phosphorus (P) excreted in feces of growing sheep. Data from 18 animals fed adequate or low-P diets

Type of P[a]	Restriction	Fecal excretion, g/day		RMSE[b], g/d	RMSE, % obs. mean	Mean bias, % of MSE[c]	Slope bias, % of MSE[c]	CCC[d]
		Observed mean	Predicted mean					
F_{Pp}	Adequate	0.581	0.615	0.09	15.926	13.64	5.22	0.57
	Restrict	0.366	0.309	0.10	28.887	28.91	19.76	0.40
F_{Po}	Adequate	0.585	0.679	0.14	23.231	47.32*[e]	7.67	0.36
	Restrict	0.368	0.343	0.11	28.847	5.45	37.22	0.37
F_{Pi}	Adequate	3.044	2.961	0.16	5.363	25.78	36.28	0.96
	Restrict	1.880	1.962	0.21	11.12	15.25	40.11	0.92

[a] $F^{(p)}$ = organic phytate phosphorus excreted in feces, $F^{(o)}$ = organic non-phytate phosphorus excreted in feces, and $F^{(i)}$ = inorganic phosphorus excreted in feces.
[b] RMSE = root mean square error, % of observed mean.
[c] MSE = mean square error, % of MSE.
[d] CCC = Concordance correlation coefficient (Lin, et al., 1989).
[e] signifcance test: * P < 0.05, ** P < 0.01, *** P < 0.001.

As can be observed in Figure 5.2, the bias noted above is minor, and there is no clear indication that the model fits are biasing conclusions regarding treatment effects. The linear regressions relating observed and predicted values were significant to all types of P ($P \leq 0.00013$), and the model showed moderate to high precision ($R^2 \geq 0.60$). Residuals (observed minus predicted values) plotted against predicted (Figure 5.2) values showed that Pi was in agreement with a higher bias shown in Table 5.2.

Figure 5.2: Evaluation of the model used for estimating the excretion of phosphorus (P) in feces of growing sheep. A, B and C, represent phytate P, non-phytate P, and inorganic P, respectively. 1= Observed versus predicted values; 2 = Residues versus predicted values; Adj R2 = Adjusted determination coefficient; P = significance of the linear model, which was considered significant when *P* value < 0.05.



5.3.3. Modelled Fluxes

Daily P fluxes through the rumen, SI, LI, and plasma were greater for animals fed the adequate-P diet as compared to those fed the low-P diet (Table 5.3, $P < 0.001$). Surprisingly, fluxes between plasma and bone ($P = 0.896$), and plasma and ST ($P = 0.416$) were not different between treatments although the numerical differences were as expected with a reduction in bone deposition and an increase in resorption. These numerical differences are more apparent when examining the balance of P between plasma and bone where the balance was more negative for the P-restricted treatment (- 0.74 g/day; $P = 0.011$) than animals fed adequate P-diets (-0.35 g/d). Similar results were observed for the balance of P between ST and plasma ($P = 0.004$) where the adequate-P treatment showed a positive balance of P (0.0046) representing retention of P in tissues, and the low-P diet had negative values (-0.0769) which indicated a P resorption from tissues.

Regarding the low-P treatment, the P-balance between bone and plasma was 0.701 g/day ($P = 0.005$), and between ST and plasma was 0.064 g/day ($P = 0.011$), which indicated a resorption from the tissues (bone and soft tissues) to the plasma.

The fact that both treatments had negative P balance may indicate some bias in the measurements of P intake or P excretion. As this is an accumulator for measurement errors, it is not uncommon to have bias in the balance measurement. Any losses of P would result in an underestimate of balance.

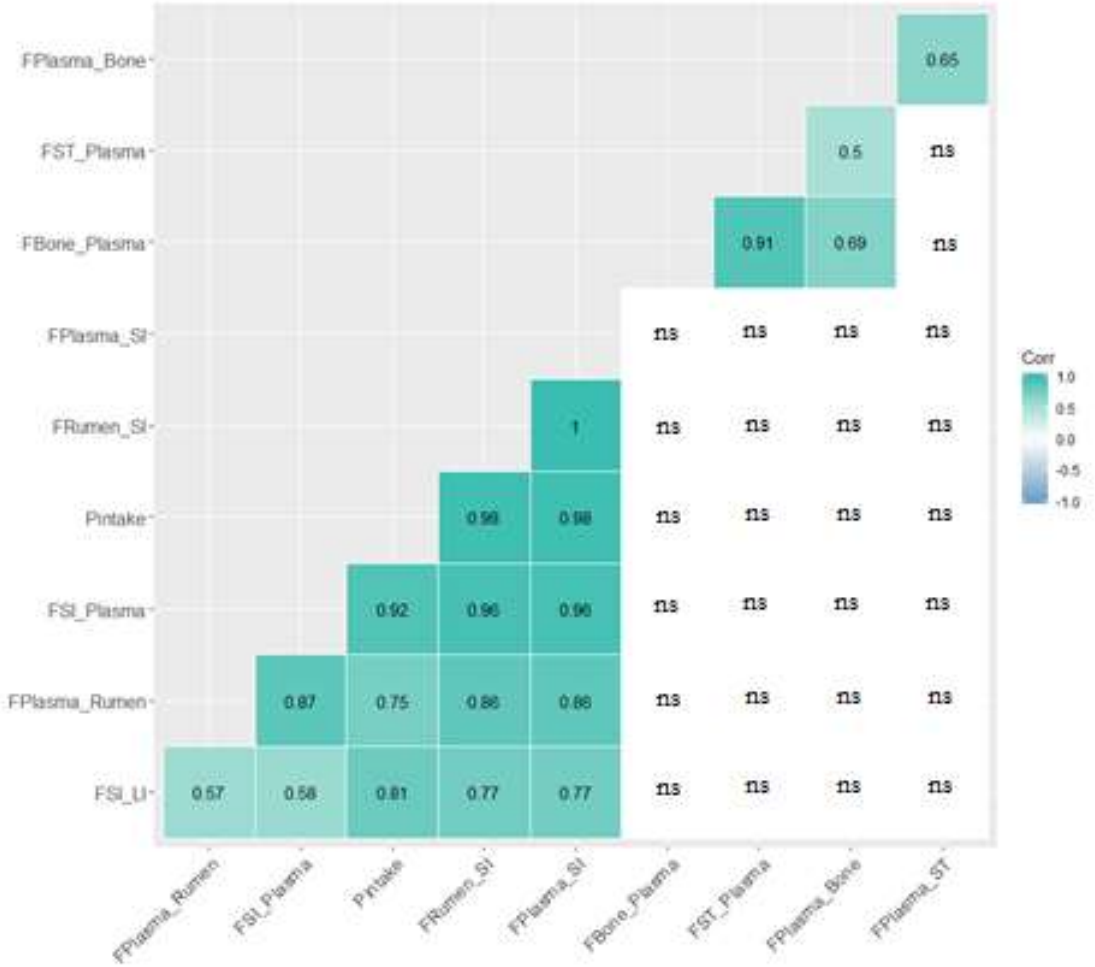
Table 5.3: Predicted flows of phosphorus through the gastrointestinal tract, plasma, muscle, and bone compartments of lambs

Flow, g/d		Eqn # [a]	P-Adequate		P-Restricted		*P* value
From	To		Amount	SEM[b]	Amount	SEM[b]	
Rumen	Small intestine	15	2.87[a]	0.114	0.743[b]	0.0620	<0.001
Small intestine	Large intestine	16	2.65[a]	0.436	1.40[b]	0.263	0.001
Small intestine	Plasma	18	3.15[a]	0.405	0.119[b]	0.337	<0.001
Plasma	Small intestine	17	2.87[a]	0.114	0.761[b]	0.0609	<0.001
Plasma	Rumen	13	0.587[a]	0.276	0.144[b]	0.0599	<0.001
Plasma	Bone	19	0.966[1]	0.332	0.789[2]	0.289	0.896
Bone	Plasma	21	1.29[1]	0.483	1.49[2]	0.172	0.459
Plasma	Soft tissues	20	0.152[3]	0.0385	0.112[4]	0.0295	0.416
Soft tissues	Plasma	22	0.144[3]	0.0596	0.176[4]	0.0384	0.205
$F_{Pi,PlBo} - F_{Pi,BoPl}$			-0.349[a]	0.375	-0.738[b]	0.270	0.011
$F_{Pi,PlST} - F_{Pi,STPl}$			0.0046[a]	0.0337	-0.077[b]	0.0246	0.004

[a] Equation number used to determine the flow.
[b] Standard error of treatment means.
[1] Statistical t-test within P adequate treatment between plasma ~ bone (P = 0.003).
[2] Statistical t-test within P restricted treatment between plasma ~ bone (P = 0.005).
[3] Statistical t-test within P adequate treatment between plasma ~ soft tissue (P = 0.516).
[4] Statistical t-test within P restricted treatment between plasma ~ soft tissue (P = 0.011).
[a, b] Means with different superscript lowercase letters indicate the difference between treatments by the t-test (P < 0.05).

The Pi fluxes obtained from the model (Table 5.3) were also tested to determine correlations among the fluxes (Figure 5.3). There were high Pearson correlation coefficients among $F_{Pi,In}$, $F_{Pi,RuSI}$, and $F_{Pi,SILI}$ (Corr. = 0.98 and 0.81, respectively), indicating that the fluxes through the gastrointestinal tract were dependent on intake as one would anticipate. The same correlation pattern was observed among $F_{Pi,In}$, $F_{Pi,SIPl}$, and $F_{Pi,PlSI}$ (Corr. = 0.92 and 0.98, respectively), indicating that intake and absorption of Pi were strongly related. However, there was no correlation among $F_{Pi,In}$ and the fluxes related to bone and soft tissues (P > 0.05) which was not anticipated.

Figure 5.3: Pearson correlation coefficient across the flows determined by the model. The green squares indicate significant correlation ($P < 0.05$). the symbol that describe the flow was described as Fa_b: where F = the flow, a = the outflow pool, and b = the inflow pool



5.4. Discussion

We adapted and re-parametrized two prior compartmental models to represent P digestion, metabolism, and excretion. Daily intake of P and its flux passing through six compartments (rumen, small intestine, large intestine, plasma, bone, and soft tissues) were described by the model. The primary model input was P intake which is readily determined from dietary P concentrations and dry matter (DM) intake; thus, DM intake and concentrations of dietary P are essential observations for predicting excretion of P (SCHIAVON et al., 2019).

Very few models have been developed to describe P flux and excretion by small ruminants. A model to estimate the fluxes of P in growing goats aged between 4 and 5 months with BW varying from 20 to 30 kg was described by Vitti et al. (2000). This model had similar input variables and represented a similar physiological state as our work; however, our multicompartmental model considered three pools (rumen, small intestine, and large intestine) to describe the gastrointestinal tract (GIT) while the model developed by Vitti et al. (2000) used only one pool (GIT). Thus, P exchange between the GIT and plasma was represented by a single flux in the prior work, whereas the current work represented separate fluxes for GIT resorption (from plasma to small intestine) and salivation (from plasma to rumen). Additionally, the current work considered of the impact of different forms of GIT P on bioavailability. Salivary P is an important component of P metabolism in ruminants representing 45% of the total ruminal P pool (KEBREAB; VITTI, 2005). This flux also provides ruminal pH buffering and supplies P required by the GIT microbiome (COUNOTTE et al., 1979).

A revised version of the model proposed by Vitti et al. (2000) was published in 2006 (DIAS et al., 2006). This model used four pools (GIT, plasma, bones, and soft tissues) which included the effects of different sources of P ingested and absorption from the small intestine. Data from Santa Ines male sheep (31.6 kg of BW and 8 months of age) were collected to test that model, but the model did not align well with the observed data. A limitation of that model was the lack of consideration of salivary P flux. It also represented P digestion using a single total P pool, whereas Hill et al. (2008) found that Pp, Po, and Pi had different bioavailabilties, and thus the form of P in the diet contributes to absorbability. Dias et al. (2011a) hypothesized that the latter may be the reason their six-pool model did not accurately represent P fluxes in their sheep experiment.

The mean P excretion in urine and feces of lambs in the current work was 8.97 mg/d and 2.97 g/d, respectively. Therefore, our data indicated that the major route of excretion for P was via feces in agreement with Bravo et al. (2003) and Vitti and Kebreab (2010). For healthy animals, the standard concentration of P in plasma is between 6.5 and 9.5 mg/dL (CHALLA; BRAITHWAITE, 1988), and the overall average for plasma P in the current study was 7.6 mg/dL.

A descriptive study found that P excretion, secretion, retention, and resorption were driven by the concentration of P in blood (CHALLA et al., 1989). They hypothesized that plasma could be considered as a regulatory pool of the entire system with P concentrations in plasma determining the direction and magnitude of flux in the system. The homeostasis

concept could be applied in this scenario. Penido and Alon (2012) described two distinct homeostatic scenarios: the first one indicated regulation was via the gastrointestinal tract, and the second indicated storage tissues mobilization of P as the compensatory mechanism.

If plasma P concentrations are related to the regulation P flux in the system, then plasma P concentrations should be directly correlated with plasma flux. However, our results suggested that animals fed adequate-P (high P input) or low-P (low P input) diets had no differences in plasma pool size. The model developed for fluxes between pools is time-dependent, being classified as a dynamic model (TEDESCHI, 2006). Even though the intakes of P were different between treatments, the total P concentration in a specific pool would not change, but their fluxes may change resulting a steady-state flux conditions of P in the blood. Therefore, our results indicated that plasma may be considered as a pool in steady-state, where a low level of P intake would result in physiologic response to maintain the same P pool size in the plasm of lambs.

Predicted P fluxes through the GIT (rumen, small intestine, and large intestine) and daily intakes of P were positively correlated. Our results are in agreement with the data observed by Dias et al. (2011b), who developed a compartmental model of P fluxes for sheep fed different levels of P and found a linear relationship between P intake and ruminal P outflux, absorption of P in the small intestine, and salivary secretion of P. On the other hand, the positive relationship between dietary P intake and pool size has not been reported for bone. Several studies showed no significant association between levels of P ingested and concentration of P in the bone (BELONJE; VAN DEN BERG, 1983; WU; SATTER; SOJO, 2000; GEISERT et al., 2010). In the current study, we observed similar results in which the relationship between P in plasma and bone or soft tissues indicated no effects of different levels of P ingested on concentrations of P in the bone, muscular tissues, and organs. Thus, the signals that elicited the large changes in return of plasma P to the SI and rumen apparently did not affect P cycling in bone.

All animals had a negative bone P balance. However, the low-P treatment had a greater negative P balance than the adequate-P group (Table 5.3). Bone serves as a reserve of minerals, mainly Ca and P, which can be used by animals in a mineral deficient state (DIAS et al., 2009). Therefore, in terms of P homeostasis, our results for animals fed low-P diets were expected. However, it was surprising that the adequate diet did not have neutral or positive balance. For the soft tissues, net balance ($F_{Pi,PlST} - F_{Pi,STPl}$, Table 5.3) was positive for the adequate treatment and negative for the restricted treatment, however the differences from 0 and between treatments were very small, and biologically negligible relative to bone flux.

Daily P intakes and the flux through the six compartments of lambs were used for the model development while only data of P excreted in feces was used for model evaluation due to daily observations from feces versus single end-point observations for the remaining, at slaughter time. In addition, once the P fluxes from animals with different heath conditions (infected with endoparasite or not) were similar ($P > 0.05$), the data was pooled. Animal manure P is a huge environmental challenge across the world, which can result in soil and water contamination with a considerable reduction in farm income due legal restrictions on P application. Thus, models to for predicting P excretion by animals are a powerful tool to support a reduction in nutrients excretion from animal production farming systems.

Evaluations of models in terms of accuracy and precision are essential to verify if the model can be applied on further studies. In terms of accuracy, models with less than 20% prediction error are desired (TEDESCHI, 2006). Predictions of P excretion by animals fed adequate-P using all types of P fluxes and for animals fed P-restricted treatment using only Pi form were accurate (RMSE $\leq$ 11.12% of observed mean). However, predictions Pp or Po fluxes were less accurate and precise (RMSE range = 15.9 – 28.8% of observed mean, mean bias range = 5.45 – 47.32% of MSE, and CCC < 57) for animals fed low-P diets.

Hill et al. (2008) also reported a low accuracy when a model was evaluated using the same types of P fluxes used in our study. That study suggested that some adaptative response in the rumen allows greater phytase activity when the intake of phytate increases or the total intake of P decreases. This potential physiological mechanism needs to be better elucidated which required additional data. Once the data have been collected, the model can be re-evaluated using an independent database of Pi, Po, and Pp fluxes. A regression approach using a pooled dataset (n = 18 animals) was adopted for evaluating the model, and the observed and predicted amounts of P in feces were used. The Figure 5.2 showed significant linear regressions between the observed and predicted values, in which the goodness of fit (R2) were greater than 0.60 indicating a moderate precision of the model. Overall, the residual values for the Pi form showed higher variation, which can be explained by a greater, but non-significant, mean and slope bias observed in the low-P treatment. However, the CCC values of 0.92 and 0.96 for the low-P and adequate-P treatments using Pi fluxes represents an accurate and precise model.

5.5. Conclusion

Considering the P and N regulatory requirements facing farmers across the world, the model developed provides accurate estimates of P excretion by lambs, the knowledge of P regulation can be applied on small ruminant production systems as a nutritional tool to support programs for reducing the P excretion by animals. The predictions of fluxes of P in plasma and the fluxes between plasma and tissues (bone, muscle, organs) is also an important tool for predicting and evaluating the P requirements of small ruminants. We would recommend evaluating the current model using independent datasets from animals fed different feed ingredients, age, and sheep production systems in different climate conditions. Over time, further adaptations of this model may be needed to improve its predictions.

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6. FINAL CONSIDERATIONS

The present study tested two variables that are important to the livestock in Brasil and overseas. The first variable was the dietary P restriction, many farms in brasil works in extensive system, with grazing animals, by understanding the importance of grazers in reducing feed competition with humans, and the capability of convert high fiber diets in a rich protein source. Work in extensive systems, the mineral supplementation is essential, and across all mineral supplied, phosphorus is important because of all metabolic and physiological benefits, but from an economic stand point, it is an expensive mineral to supplement. Excess amount of P in the diet will promote an increase in P losses via feces, an economic loss for the farmer and an environmental issue by the contamination of soil and underground water. But, providing diet deficiency in P, will reduce metabolic and physiological aspects, that can reduce performance, such as body weight gain and fat deposits, resulting also in economic losses. The present study was not conclusive about the effects of dietary P deficiency for growing Santa Ines, with lack of differences, and similar animal growth between deficient and sufficient dietary P. Therefore, a next study could evaluate the actual basal and gain requirements for Santa Ines sheep.

The second variable tested was the *T. colubriformis* infection. This endoparasite was chosen because, along with *H. contortus*, they are the most relevant GIT endoparasite for sheep production in extensive system. However, *H. contortus* are much more studied, due to be a hematophagous parasite. So, we evaluated the infection with *T. colubriformis* the objective of understanding all damages that this parasite can promote in the host small intestine, and the consequences of intestinal damages are absorption reduction, and animal system disorder. This infection is classified as a disease, therefore, some of the animal nutrients requirement will be used to the immunological system to act in the disease, increasing all nutrients requirements, resulting in unnecessary excess of feed provide to maintain animal and the parasites. It is important to contextualize that by *T. colubriformis* not been too dangerous to the host and have a small size, the parasite load in the host could be high, therefore, the pasture contamination will also be high, resulting in a fast growth infection cycle. The present study showed the histological damages promoted by this nematode, however the digesta flow was not affected and probably because a mucosa development in other portion of the small intestine (jejunum, ileum) as a compensatory absorption strategy, therefore, next studies the laboratory will sample other small intestine mucosa portions to observe if the hypothesis cited really occur or not.