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Curcumina como aditivo na alimentação de cães: Produção da ração e seus benefícios a saúde dos animais

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CURCUMINA COMO ADITIVO NA ALIMENTAÇÃO DE CÃES: PRODUÇÃO DA RAÇÃO E SEUS BENEFÍCIOS A SAÚDE DOS ANIMAIS

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Elaborada por
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como requisito parcial para obtenção do grau de
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RESUMO

Dissertação de Mestrado
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CURCUMINA COMO ADITIVO NA ALIMENTAÇÃO DE CÃES: PRODUÇÃO DA RAÇÃO E SEUS BENEFÍCIOS A SAÚDE DOS ANIMAIS

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A curcumina é um componente biológico presente na planta *Curcuma longa L.*, conhecido como açafrão, tem ação em diferentes funções no organismo, onde destacamos as propriedades ação anti-inflamatória, anti-tumoral, antioxidante, antimicrobiano, anticoccidiano, hepatoprotetor, assim como uma molécula funcional, capaz de favorecer o ganho de peso. Em virtude disso, o objetivo foi verificar se a adição de curcumina na ração tem efeito antioxidante, prolongando a validade desse pro0duto, assim como se tem efeito benéficos sobre a saúde de cães na fase de crescimento, quando alimentados diariamente. Esse estudo foi realizado em dois experimentos distintos (Experimento 1 e 2). Para o Experimento 1, uma ração foi produzida de forma comercial em uma fábrica de ração, sendo a curcumina (100 mg/kg) adicionada após a extrusão da ração, isto é, foi adicionada durante o banho de gordura, junto como os outros micronutrientes. Na ração, embalada e fresca, foi mensurado os níveis de curcumina, sendo constatado que após processo de produção o nível real foi de 32.9 mg/kg. Uma ração controle foi produzida, com os mesmos ingredientes, mas sem curcumina. Em avaliações mensais por 6 meses, verificamos que a composição e pH não diferiram, apesar da ração com curcumina apresentar menor oxidação proteica e peroxidação lipídica, assim como maior capacidade antioxidante total. Após 2 meses da produção da ração, foi dado início ao experimento que utilizou 10 cães jovens da raça Beagle. Os animais foram alojados em canil de experimentação e divididos em dois grupos: cães alimentados com ração contendo curcumina (n=5) e cães alimentados com ração controle (n=5). As alimentações foram realizadas duas vezes ao dia em canis individuais. As coletas de sangue foram realizadas nos dias 1, 35 e 42. Durante a fase de adaptação os animais passaram por desafios infeciosos naturais, isto é, apresentaram giardíase e gastroenterite bacteriana controlados com anti-protozoário e antimicrobiano, respectivamente. Foi observado um maior número de células vermelhas no sangue nos cães alimentados com curcumina (dias 35 e 45), assim como número de leucócitos elevado em consequência do aumento de neutrófilos no dia 42. No final do experimento foi observado uma redução significativa no número de linfócitos nos cães que ingeriram curcumina (dia 42), o que caracteriza um efeito anti-inflamatório, que foi confirmado pela redução dos níveis de globulina no sangue. Nos últimos 15 dias de experimento, os animais estavam aparentemente saudáveis, momento em que verificamos maiores níveis séricos de glicose, ureia, triglicerídeos e colesterol nos cães alimentados com curcumina. A menor atividade da alanino-aminotransferase sérica pode caracterizar um efeito hepatoprotetor da curcumina. Alimentação dos cães com ração contendo curcumina aumentou a atividade de enzimas antioxidantes (catalase, superóxido dismutase e glutationa peroxidase), tióis não-proteicos e a capacidade antioxidante total no soro ao final do experimento, consequentemente reduziu os níveis de espécies reativas ao oxigênio. Para Experimento 2, houve a produção dos petiscos utilizando carne enlatada comercial para cães, onde a curcumina foi adicionada e homogeneizada. Em seguida foi confeccionado os petiscos contendo 15 mg de curcumina, os quais foram congelados e oferecido aos cães duas vezes ao

dia. Para avaliar os efeitos da curcumina na saúde de cães nos utilizamos 10 cães da raça Beagle, com seis meses de idade. Os animais foram alojados em canil de experimentação e dividido em dois grupos (n=5), isto é, um dos grupos de cães recebeu o petisco contendo curcumina (30 mg curcumina/animal/dia) e o outro grupo recebeu os mesmos petiscos sem curcumina. Coletas de sangue foram realizadas nos dias 1, 15 e 30 do experimento, a fim de avaliar variáveis hematologia e bioquímicas. Não houve diferença entre grupos para as variáveis glicose, ureia, triglicerídeo, colesterol, proteína total, albumina, globulina e alanino aminotransferase. No dia 15, o número de eritrócito e hematócrito foi maior nos cães que consumiram petiscos com curcumina. Já o número de leucócitos total foi menor nos cães alimentados com curcumina no dia 30; assim como houve redução no número de neutrófilos (dia 15) e linfócitos (dia 30) quando comparado aos cães controle. Os níveis de oxido nítrico (NOx) também foi menor nos cães que ingeriram petiscos com curcumina. Os níveis de espécies reativas ao oxigênio, lipoperoxidação e proteína carbonila foram menores nos cães suplementados com curcumina no dia 30. Também houve aumento da capacidade antioxidante total (ACAP), tiós proteicos (PSH) e não proteicos (NPSH), assim como as enzimas antioxidante glutationa peroxidase e superóxido dismutase nos cães alimentados com petiscos contendo curcumina quando comparado ao grupo controle (dia 30). Portanto, com base nos resultados dos dois experimentos concluímos que a curcumina ingerida pelos cães tem efeito antioxidante e anti-inflamatório, capaz de minimizar os impactos causados pelos níveis exacerbados de radicais livres e peroxidação lipica no sangue dos cães, assim como na ração produzida.

Palavras-chave: Cães, curcumina, saúde animal, alimentação, nutrição animal.

ABSTRACT
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Universidade do Estado de Santa Catarina

CURCUMINE AS AN ADDITIVE IN DOG FEEDING: FEED PRODUCTION AND ITS BENEFITS IN ANIMAL HEALTH

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Chapecó, 25 February 2019

Curcumin is a biological component found in the *Curcuma longa L.* plant, known as saffron, which performs different functions in the body, among which stand out the anti-inflammatory, anti-tumor, antioxidant, antimicrobial, anticoccidial properties and hepatoprotective action, as well as a functional molecule, capable of promoting weight gain. Therefore, we aimed to verify if the addition of curcumin in dog food has antioxidant effect, prolonging the validity of this product, as well as if it has beneficial effect on the health of dogs in the growth phase fed daily. This study was carried out in two different experiments (Experiment 1 and 2). For Experiment I, a feed was commercially produced in a feed mill, adding curcumin (100 mg/kg) after feed extrusion, i.e., added during the fat bath, together with other micronutrients. In packed and fresh dog food the curcumin levels were measured, and it was verified that after the production process the actual level was 32.9 mg/kg. A control feed was produced, with the same ingredients but without curcumin. In monthly evaluations for 6 months, we verified that the composition and pH did not differ, although the feed with curcumin showed lower protein oxidation and lipid peroxidation, as well as higher total antioxidant capacity. After 2 months of feed production, the experiment was started, using 10 young Beagle dogs. The animals were housed in experimental kennel and divided into two groups: dogs fed a feed containing curcumin (n=5) and dogs fed a control feed (n=5). Feeding was done twice a day in individual kennel. Blood samples were taken at 1, 35 and 42. During the experiment, the animals went through natural infectious challenges, that is, they presented giardiasis and bacterial gastroenteritis controlled with anti-protozoa and antimicrobial, respectively. A higher number of red blood cells were observed in dogs fed with curcumin (days 35 and 45), as well as increased leukocyte number as a consequence of the increase of neutrophils at day 42. At the end of the experiment, a significant reduction in the number of lymphocytes was observed in dogs that ingested curcumin (day 42), which means an anti-inflammatory effect, which was confirmed by the reduction of blood globulin levels. In the last 15 days of the experiment, the animals were apparently healthy, at which point we verified higher serum levels of glucose, urea, triglycerides and cholesterol in dogs fed with curcumin. The lower activity of serum alanine aminotransferase may characterize a hepatoprotective effect of curcumin. Feeding of dogs with feed containing curcumin increased the activity of antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase), non-protein thiols and total antioxidant capacity in the serum at the end of the experiment, consequently reduced the levels of oxygen reactive species. For Experiment 1, there was the production of snacks using commercial canned meat for dogs, where curcumin was added and homogenized. Then, there was the production of snacks containing 15 mg curcumin, which were frozen and offered to the dogs twice a day. To evaluate the effects of curcumin on dog health we used 10 Beagle dogs, six months old. The animals were housed in experimental kennel and divided into two groups (n=5), that is, one of the groups of dogs received the curcumin-containing snack (30 mg curcumin/animal/day) and the other group

received the same snacks without curcumin. Blood samples were taken on days 1, 15 and 30 of the experiment in order to evaluate hematology and biochemical variables. There was no difference between groups for the variables glucose, urea, triglyceride, cholesterol, total protein, albumin, globulin and alanine aminotransferase. At day 15, the number of erythrocyte and hematocrit was higher in dogs that consumed curcumin snacks. Yet, the total number of leukocytes was lower in dogs fed curcumin on day 30; as well as a reduction in the number of neutrophils (day 15) and lymphocytes (day 30) when compared to control dogs. The nitric oxide levels (NOx) were also lower in dogs that ingested snacks with curcumin. The levels of oxygen-reactive species, lipoperoxidation and carbonyl protein were lower in dogs supplemented with curcumin at day 30. There was also an increase in total antioxidant capacity (ACAP), protein thioses (PSH) and nonprotein thioses (NPSH), as well as the antioxidant enzymes glutathione peroxidase and superoxide dismutase in dogs fed with curcumin-containing snacks when compared to the control group (day 30). Therefore, based on the results of the two experiments, we concluded that the curcumin ingested by dogs has antioxidant and anti-inflammatory effects, capable of minimizing the impacts caused by exacerbated levels of free radicals and lipid peroxidation in the blood of dogs, as well as in the feed produced.

Key words: Dogs, curcumin, animal health, feeding, animal nutrition.

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1. CAPÍTULO I

REVISÃO DE LITERATURA

1.1 Alimentação de cães

1.1.1 Histórico

Com a domesticação, os cães foram afastados da alimentação estritamente carnívora que vinha de seus ancestrais selvagens e passaram a ter uma alimentação fornecida pelo homem. Até o século XIX, os cães de caça e de pastoreio (regiões pobres) eram alimentados com pão de diversos cereais, laticínios e miúdos de carne fornecidos apenas quando estavam muito fracos ou doentes, e possuíam expectativa de vida curta (Grandjean e Vaissaire, 2006). Conforme houve uma elevação do nível das sociedades, a carne passou a ser incorporada na alimentação dos cães substituindo os cereais. A partir do século XIX, a carne passou a ser considerada a panaceia nutricional do cão, passando a ser considerado pelo homem como um carnívoro exclusivo, mudando inclusive a condição dele nesse período e saindo do papel funcional para um mais social e sendo integrado como membro da família (Grandjean e Vaissaire, 2006).

Desde então, as famílias começaram a alimentar os cães com fórmulas caseiras, até que em 1860 foi produzida a primeira ração comercial criada por James Spratt. O produto formulado era uma ração granulada seca conhecida como “bolo para cães” (Case et al., 2000; Cowell et al., 2000). No início do século XX, devido ao sucesso de Spratt, outras fórmulas surgiram e começaram a ser comercializadas como os biscoitos Milk-Bone de Bennett (Cowell et al., 2000). Com o tempo, surgiram as rações enlatadas que se tornaram mais populares que as secas. A comercialização na época se dava/ em lojas de alimentos humanos e mercearias, mesmo sendo discutida a questão higiênica, já que eram feitas de subprodutos. No entanto, a conveniência e praticidade superaram as preocupações e devido essa facilidade na comercialização houve um aumento na venda e na popularidade das rações para animais de estimação (Case et al., 2000; Wortinger, 2009).

Naquela época, pouco se sabia sobre as exigências nutricionais de cães e gatos, e muitas rações eram feitas da mesma forma, só mudando o rótulo. Durante os anos 1990 a Association of American Feed Control Officials (AAFCO) foi criada e desenvolveu os perfis de nutrientes para serem utilizados na formulação de rações de cães e gatos (Wortinger, 2009). Anteriormente, eram utilizadas as recomendações do NRC (National Research

Council) que se baseavam em alimentos purificados e consideram 100% de disponibilidade de seus nutrientes, além de considerar apenas uma fase da vida (Wortinger, 2009).

1.1.2 Ingredientes e nutrientes na alimentação

As exigências nutricionais de um cão não se resumem ao fornecimento de carne, ele precisa muito mais do que isso na sua alimentação. As principais necessidades são proteína usadas na síntese dos ossos, músculos, formação do corpo, estruturas nervosas, entre outros. Destacamos a importância de outros nutrientes na obtenção de energia como carboidratos e gorduras, estas últimas são também necessárias para a saúde da pele e pelos. Ademais as vitaminas e minerais são necessárias para reações químicas que ocorrem no organismo, e as fibras para manutenção das funções intestinais, assim como mais é essencial animal estar hidratado, uma vez que a água é essencial para a grande maioria das funções no corpo (Taylor, 2006).

Os alimentos possuem três tipos de moléculas: glicídios, protídeos e os lipídeos. Para ocorrer a digestão, o organismo usa diferentes mecanismos e processos enzimáticos no tubo digestivo (Grandjean e Vaissaire, 2006). Seu estomago é grande quando comparado ao intestino, devido seu hábito alimentar carnívoro, e, portanto, ao alimentar-se seu estomago pode aumentar de tamanho e ocupar até metade da cavidade abdominal, órgão onde ocorre a digestão mecânica e química ao mesmo tempo (Grandjean e Vaissaire, 2006). O intestino do cão é como em todas as espécies, rico em microflora, composta por microrganismos que realizam a digestão, porém é uma flora extremamente sensível às variações de alimentos. Em consequência disso, os cães não devem mudar diariamente sua alimentação, pois pode ocorrer uma destruição da flora resultando em diarreia. Por isso, é necessária uma transição alimentar de oito dias quando se troca ou altera a alimentação (Grandjean e Vaissaire, 2006).

O estômago é o local onde ocorre a maior parte da digestão mecânica e da absorção de nutrientes, através da contração das camadas musculares que possuem diversas dobras e vão continuar misturando o alimento, aumentando a exposição das partículas à superfície do intestino (Case et al., 2000; Grandjean e Vaissaire, 2006). A absorção dos aminoácidos de forma complexa ocorre pelas células do intestino, outras formas podem ocorrer na “luz intestinal como os peptídeos (cadeias +/- longas de aminoácidos), já as mais curtas podem ser absorvidas por sistema ativo (Grandjean e Vaissaire, 2006). Os glicídios e os lipídeos são absorvidos pelas células intestinais; onde os glicídios na forma de oses que são encontradas nos vasos sanguíneos e maior número no intestino delgado; já os lipídeos em diferentes

constituintes das micelas, remanejados para produzir triglicerídeos que serão fixados por proteínas e outras moléculas nos vasos linfáticos do intestino delgado (Grandjean e Vaissaire, 2006).

O principal objetivo de uma dieta é a nutrição, além de manutenção e diminuir os fatores de risco com doenças. Os cães adultos possuem uma exigência relativamente pequena comparadas as fases reprodutivas (Buffington et al., 2004). A maioria dos cães não necessitam de uma variedade de alimentos, mas uma alimentação com ração balanceada e água a vontade (Case et al., 2000). Uma recomendação importante é pesar os cães com certa regularidade, a fim de ajustar o consumo do animal de acordo com exigência, além de observar o formato das fezes, que precisam ter formato regular e cor marrom (Wortinger, 2009). Importante ressaltar que a cor das fezes pode alterar, se na ração for usado corantes como ingredientes.

1.1.3 Ração comercial para pets

No Brasil a classificação das rações se dá pelo propósito de uso, processamento, teor de água, qualidade da matéria prima e segmentação do mercado. Além disso, as rações comerciais podem ser 1) completas quando atendem todas as exigências nutricionais; 2) complementares (biscoitos e petiscos) que servem como agrado e 3) especiais, usadas para animais que apresentam distúrbios e tem particularidades e/ou restrições (Volpato, 2014). Além disso, as rações podem ser separadas pela quantidade de umidade, isto é, 1) alimentos úmidos (70 a 85%) são os enlatados, carnes e legumes; 2) semiúmidos (20 a 60%) que são os cozidos e 3) estabilizados devido ao uso de conservantes e colocados sobre refrigeração; e 4) alimentos secos (menos de 12%), isto é, biscoitos, croquetes e flocos de cerais (Grandjean e Vaissaire, 2006).

Os alimentos extrusados são os mais vendidos no mercado, devido a sua qualidade nutricional, custo e praticidade (Grandjean e Vaissaire, 2006). Os ingredientes antes de serem utilizados são testados para verificar se não há qualquer adulteração ou que sejam de qualidade ruim, a fim de evitar que esse afete negativamente o produto final, que depois de pronto também passa por testes para garantir a qualidade (França et al., 2011).

Na maioria das vezes são fornecidos os alimentos secos aos cães, porém estes também podem se deteriorar, devido à grande quantidade de gordura, crescimento bacteriano e a oxidação (Jones et al., 1999). Os lipídeos têm importância grande na nutrição, pois exercem três funções: fornecer energia, ácidos graxos essenciais e flavor (ligado ao aroma e paladar), e

para cães é o principal regulador de consumo (França et al., 2011). No entanto, o problema dessa grande quantidade de gordura é a rancidez oxidativa que também ocorre em farinhas e produtos armazenados incorretamente (Racanicci et al., 2000). A rancidez inicia pela produção de peróxidos e radicais livres que são reativos, são formados através de uma reação do oxigênio nas duplas ligações dos ácidos graxos que compõe um lipídeo (Coneglian et al, 2011). Essas reações negativas na ração podem ser minimizadas pela utilização de acidificadores, antioxidantes e conservantes (Volpato, 2014). De acordo com a literatura, um antioxidante na ração deve ser eficiente na conservação da gordura, não apresentar toxidade e ser viável economicamente (Coneglian et al., 2011).

Antioxidantes podem ser sintéticos, os mais comumente utilizados na fabricação de rações são BHT (butilhidroxitolueno), BHA (butilhidroxianisol) e toxiquim, assim como antioxidantes naturais como extratos vegetais, além das vitaminas E e C (Volpato, 2014; França et al., 2011). Segundo OGOSHI et al. (2016), o uso de antioxidantes na alimentação de gatos adultos teve uma importância fundamental para manutenção da saúde, e promoveu efeitos benéficos relacionados ao equilíbrio ácido-básico e na concentração de hemoglobina quando induzidos ao estresse. Passoto et al. (1998) observaram adição de vitamina A (beta-caroteno e acetato de retinol) na ração leva a uma estabilização de lipídeos, os quais foram efetivos mesmo com ação menor que o BHT. Em virtude disso, nos últimos anos cresceu no mercado rações com diferentes tipos de antioxidantes. O ministério da Agricultura Pecuária e Abastecimento (MAPA) liberou o uso de curcumina como aditivo em dietas animais, pois estudos mostram que esse componente é excelente na alimentação de humanos (Sandur et al., 2007) e frangos (Yarru et al, 2009) e ovinos (Jaguezeski et al., 2018; Molose et al. 2019). Apesar de não ter comprovação científica, existem rações comerciais para cães que usam como ingrediente extrato de *Curcuma longa*, que tem um componente com potente ação antioxidante conhecido como curcumina.

1.2 *Curcuma longa:* curcumina

1.2.1 Origem e composição

A curcumina é um componente biológico presente na planta *Curcuma longa L.*, uma monocotiledônea que pertence à família Zingiberaceae. Conhecido como açafrão, açafrão-da-terra, batatinha amarela, entre outros. Oriunda da Índia onde é muito utilizada na medicina e na culinária asiática (Maia et al., 1995). O açafrão foi consumido inicialmente pela sua

capacidade corante, aroma picante a sabor característico (Péret-Almeida, 2006), mas também pelos seus benefícios a saúde, a curcumina só foi liberado para uso na alimentação animal em 2010 pela Instrução Normativa Nº42 de 17 de dezembro de 2010. A curcumina pode ser comercializada em pó (elevada pureza) ou como oleoresinas e extrato de curcumina purificado, possui substâncias corantes e também óleos essenciais com ótima qualidade técnica e organoléptica (Martins, Rusig, 1992; Antunes, Araujo, 2000). Extrato de cúrcuma é facilmente encontrado em mercados brasileiros, destinado a alimentação de humanos. No entanto, esse extrato geralmente tem entre 3 a 7 % de curcumina. Hoje, tem empresas internacionais que conseguem produzir curcumina em diferentes níveis de concentração (exemplo: 50, 70, 80 ou 96% de pureza) para comercialização no uso de indústrias ou alimentação animal. Um limitante do uso de curcumina na alimentação animal ainda é a necessidade de importação e o baixo número de informações sobre seus efeitos na alimentação de animais.

1.2.2 Propriedades biológicas da curcumina

A curcumina é um eliminador de espécies reativas de oxigênio (EROs), consequentemente protegendo a hemoglobina de oxidação induzida por nitrito, inibe a peroxidação lipídica, devido sua propriedade antioxidante (Hewlings e Kalman, 2017). Além dessa propriedade, a curcumina tem ação anti-inflamatória e anti-tumoral (Hatcher et al., 2008; Gupta et al.; 2012). O sistema de defesa antioxidante pode ser enzimático ou não enzimático, sendo o enzimático composto por enzimas com destaque para catalase (CAT), superóxido dismutase (SOD), glutationa (GSH) e a glutationa peroxidase (GPx); e os não enzimáticos são formados pelos grupos das vitaminas, minerais e compostos fenólicos, como a curcumina (Barbosa et al, 2010). O efeito antioxidante da curcumina ocorre pela sua capacidade em sequestrar EROs e quelar íons metálicos, pois ela doa elétrons ou átomos de hidrogênio permitindo assim estabilizar espécies reativas, impedindo reações em cadeia como a peroxidação lipídica (Scotti et al., 2007; Itokawa et al., 2008).

As enzimas antioxidantes como SOD, CAT, GSH e GPx tem um importante papel no organismo, pois protegem as células contra os radicais livres, sendo de fundamental importância para animais e humanos (Matés et al., 1999). Pesquisas mostram que a suplementação com curcumina gera efeitos sobre o estresse oxidativo, que pode ocorrer por diferentes mecanismos, modulando atividade das enzimas SOD, GSH e CAT que irão neutralizar os radicais livres, ou inibir enzimas geradoras de EROs (Hewlings e Kalman,

2017). Estudo com suplementação de curcumina em cordeiros mostrou o aumento dos níveis das enzimas antioxidantes como CAT, SOD, GPx, dessa forma ocorrendo um estímulo do sistema antioxidante (Molosse et al., 2019). Resultados semelhantes foram encontrados em frangos de corte e ovelhas, também suplementadas com curcumina, que tiveram o aumento dos níveis das enzimas antioxidantes no sangue e no leite das ovelhas (Rahmani et al., 2018; Jaguezeski et al., 2018). A curcumina também tem efeito redutor de oxidantes e renoprotetor, assim como pode agir diretamente sobre EROs, conforme já mencionado, e enzimas como a lipoxigenase e xantina hidrogenase, além de reduzir a formação de malondialdeído e o hidroperóxido lipídico que são indicadores de excesso de peróxidos lipídicos (Hatcher et al., 2008; Hewlings e Kalman, 2017) Estudos com cordeiros e carpas mostraram a ação da curcumina na redução nos níveis de EROs e lipoperoxidação (LPO) e aumento da capacidade antioxidante total (ACAP), eliminando os radicais livres que causam a peroxidação lipídica (Molosse et al., 2018; Jiang et al., 2016).

A ação anti-inflamatória da curcumina se deve pela presença de grupos fenólicos na molécula, que garante a sua capacidade de regular negativamente a ativação de fatores de transcrição no processo inflamatório, como por exemplo o NF-kB e o AP-1, que desempenham um papel importante na iniciação da resposta inflamatória, inibindo a ativação do complexo IKK e por fim, impedindo a fosforilação e degradação da proteína IkB-α (Bastos et al, 2009; Khalaf et al, 2010). Outra via importante que é modulada pela ação da curcumina é a via do ácido araquidônico, que gera mediadores pró-inflamatórios como prostaciclinas, tromboxanos, leucotrienos e prostaglandinas. Desse modo, inibe as enzimas COX-2 e LOX-5 (Gupta et al., 2013). Como exemplo desse efeito e também na atuação antitumoral, Zhao et al. (2017) demonstrou que a curcumina teve um efeito potencializador do cloridrato de nimustina (agente quimioterápico) contra o glioblastoma, suprimindo as vias de sinalização PI3K, AKT, NF-kB e COX-2, podendo ser um agente quimiopreventivo para o câncer. A curcumina também tem ação em fatores de transcrição que são superativados em células cancerosas, podendo ser assim considerada um composto que pode ser utilizado no tratamento e prevenção do câncer (Sung et al., 2012).

Liu et al. (2015) mostraram o efeito anti-inflamatório da curcumina em camundongos asmáticos, pois houve um efeito de ativação da Nrf2/HO-1 que regulou negativamente a expressão de TNF-α, IL-1β e IL-6. Em um estudo recente pesquisadores verificaram que camundongos tratados com LPS e curcumina tiveram a expressão de citocinas induzidas pelo LPS (TNF-α e IL-6) e microRNA-155 (miR-155) reduzidas; o que pode interferir no controle de infecção nesses animais (Ma et al., 2016).

A curcumina também se mostrou eficiente na redução de níveis séricos de índices lipídicos aterogênicos, aumento de concentrações de HDL-Colesterol em pacientes com diabetes tipo 2, podendo ser um suplemento útil no tratamento de dislipidemia da diabetes, além de apresentar também efeito hipoglicemiante e capacidade de aumentar insulinemia (Panahi et al., 2017; Aggarwal, 2009). A obesidade é um problema que está diretamente ligado a diabetes, e de acordo com a literatura a curcumina age nos adipócitos, células do pâncreas, macrófagos, e também é responsável por suprimir fatores pró-inflamatórios, e em consequência disso é capaz de reverter resistência à insulina, hiperglicemia entre outras variáveis que estão ligadas a diabetes (Aggarwal, 2010). Além disso, estudos realizados com humanos mostraram um efeito da curcumina na redução dos níveis de açúcar no sangue. Essa diminuição pode estar relacionada com a diminuição da enzima responsável pela conversão de sorbitol em frutose, que está ligado também ao estresse oxidativo (Aggarwal, 2010). Outro efeito encontrado em ratos alimentados com grandes quantidades de gordura e suplementados com curcumina foi a diminuição da concentração plasmática de AGL (ácidos graxos livres), a qual é responsável por causar morte súbita quando associada a hiperlipidemia (Aggarwal, 2010).

A curcumina também tem ação antimicrobiana (Péret-Almeida et al., 2008; Hewlings e Kalman, 2017). Devido a essa propriedade, pesquisadores já usaram a curcumina na dieta de frangos de corte em substituição a antimicrobianos convencionais, que foram posteriormente desafiados com *Salmonella* Typhimurium. De acordo com esses autores, a curcumina teve a capacidade de impedir a colonização intestinal, promovendo um desequilíbrio na população de bactérias da microbiota e principalmente contra as inoculadas, além de preservar a integridade intestinal, e favorecer ganho de peso, consumo de ração e conversão alimentar (Nascimento, 2016). Martins et al. (2009) observaram efeito *in vitro* sobre microorganismos como *Paracoccidioides brasiliensis, Sporothrix schenckii, Cryptococcus neoformans* e *C. dubliniensis*. De acordo com a literatura essa ação antimicrobiana e antifúngica deve-se a sua capacidade de regular negativamente a expressão do gene ERG3 (ergosterol 3) causando alterações na permeabilidade da membrana, associadas com a atividade da ATPase e secreção de protease (Collino, 2014; Neelofar et al., 2011).

A curcumina também tem efeitos coccidiostáticos, em cordeiros tratados com 200 mg/kg de *Curcuma longa,* pois apresentaram significativa redução de oocistos de Eimeria spp, sendo crescente pelos dias tratados, chegando a 100 % de eficácia aos 42 dias, com redução de nitritos e peroxidação lipíca (Cervantes-Valencia et al., 2016). Em outro ensaio *in vitro* com *Cryptosporidium parvum* a curcumina reduziram a infecção por esporozoítos em 65%

(Shahiduzzaman et al., 2009). Da mesma forma, outro trabalho com *Eimeria tenella* resultados semelhantes foram encontrados, uma vez que a curcumina reduziu a infecciosidade de 41,6% e 72,8% nas concentrações de 100 e 200 μM de curcumina (Khalafalla et al., 2010). Sua ação antiparasitária é devido a geração de EROs e a inibição de acetilação de histonas, tornando-se tóxica aos parasitas, como já demonstrado seu efeito em *Leishmania* spp, *Trypanosoma* spp, e *Giardia lamblia* (Collino, 2014). O efeito coccidiostático e antimicrobiano da curcumina deve-se pela sua ação em modificar a estrutura intestinal, afetar o desenvolvimento dos organismos patogênicos e dessa forma diminuir sua capacidade de colonização no trato digestivo dos animais, além de modular as respostas imunes inata e adaptativa (Campagnolo et al., 2013) A curcumina também induz a apoptose pela presença de precipitados nos esporozoítos afetando sua morfologia, a capacidade de adesão e a viabilidade dos parasitas (Cervantes-Valência et al., 2016).

Um alimento para ser considerado funcional deve atuar beneficamente diferentes funções no organismo que auxilie em doenças ou faça bem ao organismo (Collino, 2014). A curcumina como demonstrado em vários trabalhos apresenta diferentes funções nos organismos humanos e animais, mostrando-se cada vez mais útil na alimentação. Coelhos tratados com paracetamol, mostraram um efeito hepatoprotetor da curcumina devido a redução das enzimas marcadoras de dano hepático como aspartato aminotransferase (AST) e alanina aminotransferase (ALT), assim como também diminuiu níveis das enzimas biomarcadoras séricas, além de auxiliar na regeneração de células hepáticas pela expressão das citocinas (El-Agamy, 2010; Soliman et al., 2014; Sayed e El-kordy, 2014). Cabe ressaltar que cordeiros e frangos de corte suplementados com curcumina apresentaram maior ganho de peso e peso corporal comparados a cordeiros não suplementados, isso deve-se ao aumento de absorção de nutrientes e melhor eficiência alimentar (Cervantes-Valência et al., 2016; Molosse et al., 2019; Rahmani et al., 2018).

A curcumina já teve efeitos positivos frente a diversas situações desafiadoras, onde destacamos as micotoxinas, comumente presentes nos cereais e rações. Pesquisa publicada em 2015 testou o efeito da curcumina para ratos intoxicados com aflatoxina B1, e mostrou que houve uma regulação positiva da expressão do gene de todas as enzimas antioxidantes, assim como aumentou os níveis de GSH e demais enzimas antioxidantes (El-Bahr, 2015). Devido a esse efeito positivo que a curcumina apresentou nas micotoxinas, ela pode ser uma alternativa para rações de cães, principalmente quando malconservadas, podendo diminuir as micotoxinas que estarão presentes nessa ração e o risco a saúde dos animais.

Também foi testada em cordeiros e ovelhas leiteiras onde a curcumina apresentou uma redução no estresse oxidativo, ação antioxidante e anti-inflamatória, além de uma melhora no perfil de ácidos graxos no leite das ovelhas, nos cordeiros além da ação antioxidante foi observado ganho de peso nos animais (Jaguezeski et al., 2018; Molosse et al, 2019). Liu et al. (2017) forneceram curcumina e induziram lesões coronárias em ratos, os animais que foram suplementados tiveram redução no risco da doença através do estimulo da via do sinal JAK2/STAT3, com redução no dano oxidativo e inibiu a apoptose do miocárdio. Os efeitos da curcumina foram demonstrados por Yarru et al. (2009) em frangos de corte onde teve efeito sobre a expressão dos genes hepáticos associados ao sistema imunológico e também ao sistema antioxidante, observou uma melhora no desempenho, peso do fígado e na expressão dos genes que codificam as enzimas antioxidantes. Outro experimento realizado com galinhas poedeiras que receberam curcumina na dieta apresentaram aumento nos níveis de antioxidantes nos ovos, redução na peroxidação lipídica tanto nos ovos frescos como nos armazenados, sendo assim melhorou a qualidade dos ovos, além de ter promovido efeitos benéficos na saúde das galinhas devido o controle da coccidiose e o estímulo da resposta imune (Galli et al., 2018). Ainda destacamos, resultados de estudo com ratos diabéticos que foram tratados com curcumina incorporada ao iogurte por 31 dias e obtiveram melhora dos parâmetros fisiológicos e bioquímicos (Gutierres et al., 2012).

1.3 Hipótese científica

Acreditamos que a curcumina na ração vai proporcionar maior estabilidade da ração, assim como ela será um alimento com propriedade nutracêutica. Dessa forma, os cães alimentados com a ração contendo curcumina responderão melhor aos desafios diários. Pois como já demonstrado em diversas pesquisas a curcumina tem diversas propriedades biológicas sendo especialmente importantes para cães em crescimento, os quais inclusive será objeto desse estudo. A fase de filhote é uma das mais importantes, pois é quando eles passam por maiores desafios e adquirem imunidade duradoura, assim como necessitam de uma quantidade de nutrientes muito maior, isso porque, além da mantença precisam também para seu crescimento, desenvolvimento e ganho de peso. Dessa forma, acreditamos que um componente como a curcumina pode auxiliar no metabolismo, melhorar a absorção de nutrientes e melhorar o sistema de defesa, e assim essa molécula torna-se uma opção de ingrediente para conservação da ração a ser incluída na alimentação de cães.

1.4 Objetivo

1.4.1 Geral

Verificar se a adição de curcumina na ração tem efeito antioxidante, prolongando a validade desse produto, assim como se tem efeitos benéficos sobre a saúde de cães na fase de crescimento alimentados diariamente.

1.4.2 Específicos

- Produzir as rações comerciais com curcumina e analisar sua composição bromatológica, viabilidade e qualidade (reações oxidativas e peroxidação lipídica) após armazenamento da ração, assim como níveis de curcumina após produção industrial da ração.
- Determinar se o consumo de ração com curcumina afeta positivamente o metabolismo proteico, lipídico e de carboidrato sérico em cães.
- Analisar se o consumo de ração contendo curcumina na dieta reduz os níveis oxidativos e aumenta os níveis de antioxidantes em cães.
- Avaliar se a ingestão de ração contendo curcumina na dieta de cães tenha ação anti-inflamatória e anticoccidiana.

2 CAPÍTULO II

MANUSCRITO

Os resultados desta dissertação são apresentados na forma de dois manuscritos, com sua formatação de acordo com as orientações da revista ao qual foi submetido:

2.1 MANUSCRITO I

Curcumina como aditivo na ração de cães e seus efeitos sobre o crescimento e saúde dos animais

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Curcumin as an additive in dog feed and its effect on animal growth and health

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Abstract. The objectives of this study were to produce a feed for dogs containing curcumin and to determine the impacts of it on feed conservation due to its antioxidant properties, as well as to evaluate its beneficial effects on animal growth and health. Curcumin (100 mg kg) was added after the extrusion process, that is, during the fat bath along with the other micronutrients. Curcumin levels were measured in packed, fresh and stored feed. The final concentration of curcumin was 32.9 mg/kg in ration. A control ration was produced, with the same ingredientes, but without curcumin. Monthly evaluations were performed for six months, and feed composition, and pH did not differ throughout this period, although the feed with curcumin showed lower protein oxidation and lipid peroxidation, as well as higher total antioxidant capacity. After 2 months of feed production, 10 young Beagle dogs were housed in an experimental kennel and divided into two groups: dogs fed a feed containing curcumin (n = 5), and dogs fed a control diet (n = 5) without curcumin. The animals were fed twice a day using individual kennels. Blood samples were taken on days 1, 35 and 42. In the first 30 days of the study, the animals had natural infectious diseases (giardiasis and bacterial gastroenteritis) that were controlled with anti-protozoal and antibiotics, respectively. A greater number of red blood cells was observed in dogs fed with curcumin (days 35 and 45), as well as increased white blood cell counts as a consequence of increased neutrophils at day 42. At the end of the experiment, a significant reduction in the number of lymphocytes was observed in dogs that ingested curcumin (day 42), suggesting an anti-inflammatory effect, mainly because we also observed a decrease on globulin levels. In the last 15 days of the experiment, the animals were apparently healthy, when higher serum levels of glucose, urea, triglycerides and cholesterol were observed in dogs fed with curcumin. The lower activity of serum alanine aminotransferase may characterize a hepatoprotective effect of curcumin, already described in other animals fed with this additive. Curcumin increased the activity of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, in addition to non-protein thiols and the total antioxidant capacity in the serum, which consequently reduced the levels of oxygen reactive species. Curcumin supplementation of dogs did not favor growth and weight gain, as there was no difference between groups (P>0.05). However, we concluded that it caused beneficial effect on animal health, with emphasis on the stimulation of the antioxidant system and anti-inflammatory effect.

Key words: Antioxidant, anti-inflammatory, canines, *Curcuma longa*, feed, health.

1. Introduction

The increased presence of pets as part of the Brazilian families has required more attention regarding the feeding habits of these animals and boosted the dog and cat feed industry. A survey conducted by Mathias (2018), who interviewed 500 dog owners, showed that 49% of them still buy bulk rations (in kg without a commercial brand), standard or other rations, and 51% buy premium rations for their animals. Rations labeled as standard or of low price adds feather or blood meal, which characterizes products of low-quality, low nutrient availability, and lower digestibility (Saad et al., 2014), besides the risk of mycotoxins that could affect the liver, kidneys and other organs (Santin and Bona, 2009) of these animals. One of the problems encountered in animal feed is the amount of lipids, responsible for the enhancement of essential fatty acids, smell and consumption (França et al., 2011), but this ingredient may contribute to oxidative reactions and rancidity.

One way to control oxidative reactions in feed is the use of acidifiers, preservatives or antioxidants (Volpato, 2014), ingredients that can be of synthetic or natural origin. Commercial rations have included in their formula plant extracts, among them the *Curcuma longa* extract, popularly known as saffron that contains curcuminoids, where the curcumin component stands out. Curcumin is a molecule widely researched in recent years because of its potent antioxidant, anti-inflammatory and antimicrobial action (El-Bahr, 2013; Santos et al., 2003; Hewlings and Kalman, 2017; Hatcher et al., 2008). The protective effect of curcumin to exacerbated oxidative reactions is widely studied in several animal species and has recently been included in the diet of production animals such as chickens, lambs and dairy sheep (Molosse et al., 2019; Galli et al., 2018; Jaguezeski et al., 2018). Curcumin showed positive effects against several challenging situations, where we highlight the minimization of the negative effects of mycotoxins commonly present in cereals and feed (El-Bahr, 2015). Lambs and broilers supplemented with curcumin showed greater weight gain since the curcumin is related to increased absorption of nutrients and the activity of enzymes that act in the digestion, besides increasing the superficial area of the villi, and thus, it is considered a molecule with functional properties (Rahmani et al., 2018; Molosse et al., 2019). Due to these properties we believe that curcumin may favor the growth of dogs, as well as minimize negative effects of feed quality and common infectious agents in the environment.

It should be emphasized that for the production of animal feed, ingredients of plant and animal origin are used, which need to be properly conserved. In this context, we believe that curcumin may reduce oxidative reactions, lipid peroxidation and consequently maintain the stability and quality of the feed. In addition, the antioxidant, anti-inflammatory and

antimicrobial effects can be active in the metabolism of dogs, and thus, beneficial to their health. Therefore, the objective of this study was to produce a commercial dry feed for dogs supplemented with curcumin and to evaluate whether this additive has beneficial effects on their health, as well as on the conservation of feed quality.

2. Materials e Methods

2.1 Curcumin

Curcumin was purchased from Shaanxi Jiahe Phytochem Ltd. (China) with 98% of purity; levels dectected by Jaguezeski et al. (2018). The curcumin dosage (100 mg per kg of feed produced) was based on recent studies in other animal species (Molosse et al., 2019; Galli et al., 2018; Jaguezeski et al., 2018), since there are no scientific papers that used curcumin in the diet of dogs.

2.2 Feed production

The feed was manufactured using the extrusion technology at 100 °C for 2.5 minutes in the pre-extrusion, at 120 °C for 20 seconds in the extrusion and 120 °C in the drying for 25 minutes, leaving 9% of moisture. Following this procedure, the ration particles were given a flavor bath using liver hydrolyzate through in-line sprinkler nozzles, followed by a fat bath with poultry oil, in which curcumin was dissolved, also through in-line sprinklers. Subsequently, the feed was subjected to the cooling process reducing its temperature to 10 °C above room temperature with subsequent packging (Table 1). All process was carried out in a commercial dog food industry, and therefore, 1000 kg of feed was produced which corresponds to the minimum capacity of the facility. In this study a ration was commercially classified as economic, that is, that uses ingredients of lower quality and digestibility and consequently with lower cost price. According to a previous survey, this type of ration is one of the most sold in Brazil. We chose to produce this feed in order to increase the challenge for dogs, as well as to verify wether the curcumin used as an additive could minimize the negative effects of consuming a lower quality feed.

2.3 Determining the concentration of curcumin

Samples of fresh, crushed and frozen (-20ºC) feed were collected. The curcumin content (mg/kg) was determined by high performance liquid chromatography (HPLC) following the methodology described by Coradini et al. (2014) with modifications. For this,

the curcumin-containing feed (0.6 g) was diluted in 10 mL of acetonitrile, then the homogenization of the mixture was performed using ultrasound (30 min), magnetic stirring (30 min) and centrifugation (30 min at 15.000 rpm), and the sample was filtered (0.45 μm) and injected into the chromatograph. The chromatographic system (y = 2836198x - 612806, R^2 = 1) consisted of a Shimadzu® CLC-ODS (M) column (15 cm x 4.6 mm x 5 μm), pre-column, mobile phase composed of acetonitrile: (70:30 v/v), flow rate of 0.6 mL/min, detection at 427 nm and injection volume of 20 μL (Coradini et al., 2014). As a control, a feed containing a known concentration of curcumin (1000 μg), Sigma standard (99% purity) was prepared. For this, 0.1 g of standard feed and 0.001 g of curcumin were weighed and diluted in 10 mL of acetonitrile, following the same homogenization procedure and the same chromatographic conditions mentioned above.

2.4 Feed chemical composition

The feed produced was stored in commercial sacks, opened at a 30 day interval for sampling and analysis of its composition. The analyzes of dry matter, mineral matter, crude protein and pH were performed following the methodology of Silva and Queiroz (2002). The ethereal extract by acid hydrolysis was performed using two grams of dry batch of becker-heavy ration, 40 mL of distilled water and 50 mL of 8M hydrochloric acid solution. Subsequently, it was heated in 100 °C up to boiling. At room temperature it was filtered with filter paper and washed with distilled water. Finally, the sample was taken to a 60 °C oven for one day, after which fat extraction was carried out following the methodology of Silva and Queiroz (2002).

2.5 Feed levels of oxidants and antioxidants

Feed samples collected for composition analysis (days 1, 30, 60, 90, 120 and 150) were also used for analysis of oxidants and antioxidants. The feed was triturated, homogenized in Tris-HCl solution (1:10), and centrifuged at 6500 g for 10 min. The supernatant was collected and stored in microtubes under freezing temperatures (-20 °C). The protein concentration in the feed was determined by the Coomassie Blue method following the methodology described by Read and Northcote (1981) using bovine serum albumin as standard.

Lipid peroxidation in the homogenate was obtained as described by Giampietro et al. (2008) by the measurement of thiobarbituric acid reactive substances (TBARS) in the diet. The levels of carbonyl protein were analyzed by spectrophotometer according to Reznick and

Packer (1994). It was used 100 µL of supernatant which were mixed with 200 µL of 10 mM of 2,4-dinitrophenylhydrazine (DNPH) prepared in 2.5 N HCl or 2.5 N HCl (white) and left in the dark for 1 hour. Then, 0.5 mL of 20% trichloroacetic acid was added to the samples to precipitate the proteins, and the tubes were centrifuged at 9000 rtpm for 5 min. The pellet was washed with 1 mL of ethanol: ethyl acetate (1:1 v/v) and dissolved in 300 µL of 6 M guanidine prepared in 2.5 N HCl at 37 °C for 5 min. The difference between samples treated with DNPH and with HCl were used to calculate the carbonyl formation at 365 nm. The levels of oxygen reactive species (ROS) were evaluated by determining the oxidation concentration of DCFH (LeBel et al., 1992). DCFH-DA hydrolyzed by intracellular esterases to form DCFH fluorescence, which then rapidly oxidized to form fluorescence of 2',7' dichlorofluorescein (DCF) in the presence of ROS. The fluorescence intensity of the DCF was correlated to the amount of ROS when measured using excitation and emission wavelengths of 480 and 535 nm, respectively. The calibration curve was performed with standard DCF (0.1 to 1 µM). The antioxidant capacity against peroxyl radicals (ACAP) was determined according to the method described by Amado et al. (2009), with modification. This method uses a fluorescent substrate (2',7' dichlorofluoresceindiacetate - H2DCF-DA) and the production of peroxyl radicals by thermal decomposition of ABAP (2,2' azobis 2-methylpropionamidine hydrochloride). Fluorescence was determined in the supernatant of the feed homogenate through a microplate reader (Spectramax I3) at 37 (excitation: 485 nm; emission: 530 nm) with readings every 5 min for 30 min. Fluorescence detection (with and without ABAP) was performed for 40 min at 37 °C and the results were expressed by the relative fluorescence area (fluorescence × time) and the ACAP was calculated according to the following equation: ACAP = 1 [(area of fluorescence with ABAP – area without ABAP)/area without ABAP].

2.6 Animal and experimental design

Ten dogs, Beagle, male, four-months old, same father from two different mothers, born a few days apart were used as experimental models. The animals were housed in an experimentatal kennel under controlled temperature (24ºC), with two collective kennels and 10 small kennels for individual feeding. Externally, there was a shaded and lawned area where the animals had access during the day. Two groups were formed with five animals each, being one of the groups of dogs fed with feed containing curcumin and the other group of dogs fed with control diet (without curcumin). The groups were divided according to the body weight of the animals. Feeding was also based on the body weight of animals averaging

4.0 kg. Therefore, 180 grams of feed divided into two fractions were given at the beginning of the experiment, one in the morning and one in the afternoon, representing 4.5% of live weight. As they were growing, the amount of feed was adjusted weekly according to their body weight.

2.7 Sampling

Blood samples were collected on days 1, 35 and 42 of the experiment after 12 hours of fasting. For that, the dogs were manually contained, and blood collection was done by the jugular vein using syringe (3 mL) and needle (25/7 gauge). The blood collected was placed in a tube containing EDTA for blood count analysis. After the hemogram, EDTA tubes were centrifuged (5500 g for 10 min) to obtain the plasma used in biochemical and immunological analyzes. Tubes without anticoagulant, also were centrifuged (5500 g for 10 min) to obtain the serum. Plasma and serum were placed in microtubes and frozen at -20 °C until analysis.

2.8 Hemogram

The number of erythrocytes and leukocytes and hemoglobin concentration were obtained through CELM semi-automatic counter analysis (CC530). Hematocrit was performed by the technique described by Feldman et al. (2000) with microhematocrit capillaries. The leukocyte differential was performed using blood smears stained by the Romanowsky technique, with 100 cells/slide identified under an optical microscope (1000x).

2.9 Seric biochemistry

Serum levels of glucose, cholesterol, triglycerides, total proteins, albumin, urea and alanine aminotransferase (ALT) activity were measured on a semi-automatic equipment (Bioplus 2000®) with commercial kits (Gold Analisa®). The globulin values were obtained through a calculation: total protein - albumin.

2.10 Serum free radicals and antioxidants

2.10.1 ROS

The levels of reactive oxygen species (ROS) in plasma were analyzed by the method described by Ali et al. (1992). Plasma (10 μL) was incubated with 12 μL of dichlorofluorescein (DFC) per mL at 37 °C for 1 h in the dark. Fluorescence was determined

using 488 nm for excitation and 520 nm for emission. The results were expressed as U DCF/mg of protein.

2.10.2 Non-protein thiols

In order to measure non-protein thiols (NPSH) and protein (PSH), the method using DTNB (5,5-dithiobis acid (2-nitrobenzoic; Sigma) was used as described by Sedlak and Lindsay (1968). NPSH in the samples was measured after deproteinization with trichloroacetic acid (TCA 50%). The pellet formed by the precipitated protein was resuspended with homogenization buffer to determine the PSH content. The absorbance readings (405 nm) were performed using a spectrofluorimeter (Biotek, Synergy HT).

2.10.3. Antioxidant enzymes

2.10.3.1 Glutatione S-tranferase activity (GST)

The GST activity was measured according to Mannervik and Guthenberg (1981), with modifications. Briefly, GST activity was measured by the rate of dinitrophenyl-S-glutathione formation at 340 nm in a medium containing 50 mM of potassium phosphate at pH 6.5, 1 mM of GSH, 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB) as substrate and tissue supernatants (approximately 0.045 mg of protein). The results were calculated and expressed as U GST/mg of protein.

2.10.3.2 Glutatione peroxidase activity (GPx)

GPx activity was measured using tert-butyl hydroperoxide as substrate (Wendel 1981). Enzyme activity was determined by monitoring the disappearance of NADPH at 340 nm in a medium containing 100 mM of potassium phosphate buffer/1 mM EDTA at pH 7.7 and 2 mM GSH, 0.1 U/mL GR, 0.4 mM azide, 0.5 mM tert-butyl hydroperoxide, 0.1 mM NADPH, and tissue supernatants. The results were calculated and expressed as U GPx/mg of protein.

2.10.3.3 Superoxide dismutase activity (SOD)

The activity of SOD was determined according to the methodology described by Beutler (1984), which aims at the auto oxidation principle of pyrogallol, inhibited in the presence of SOD. The variation of the optical density was determined kinetically for two minutes at 420 nm at ten second intervals. Activity was expressed as U SOD/mg of protein.

2.10.3.4 Catalase activity (CAT)

CAT activity was measured in plasma according to Nelson and Kiesov (1972). The buffer for the CAT assay was 50 mM potassium phosphate buffer (TFP) at pH 7.5, and CAT activity was determined by the decomposition of H_2O_2 at 240 nm. The enzymatic activity was expressed in nmol CAT/mg of protein.

2.10.4 Total antioxidant capacid

ACAP was determined in plasma according to the method described by Amado et al. (2009) as described in section 2.5.

2.11 Statistical analysis

First, the data were submitted to the normality test (Shapiro-Wilk). Data without normal distribution were transformed using logarithm. Subsequently, the data were submitted to two-way analysis of variance in order to compare groups and measures repeated over time in each group. It was considered significant when $P \leq 0.05$. Results were shown as mean and standard deviation.

3. Results

3.1 Feed levels of curcumin

Levels of curcumin analyzed in the diet are shown in Figure 1. The technique used had sensitivity of 94.4% using ration with known concentration of curcumin (Figure 1a). Figure 1b shows the detected levels of curcumin in the diet. The feed production process reduced the levels of curcumin in the feed, that is, 100 mg/kg was added, however curcumin concentration was reduced to 32.9 mg/kg after feed processing. This result was obtained by a mathematical calculation that considered the sensitivity of the chromatographic technique used according to the control sample (experimentaly contaminated feed with curcumin). Knowing the amount of feed consumed by each dog per day and animal weight, as well as the concentration of real curcumin, the curcumin dose consumed daily and also the dose per kg of body weight could be calculated, that is, approximately 6 mg curcumin/dog/day; and 1.5 mg of curcumin/day/kg of animal body weight.

3.2 Feed chemical composition

The results of the feed composition are shown in Table 2. The values for dry matter, crude protein, pH and ashes did not differ between the two rations, regardless of the presence of curcumin. Over time, these variables were also not altered in both groups.

3.3 Feed oxidant and antioxidant levels

The results of oxidants and antioxidants are shown in Table 3. Differences between feed types (control and test) were observed in all variables. The carbonyl protein (days 1 (fresh feed) and day 30), ROS (days 1, 30 and 60), TBARS (days 1, 30, 60, 90, 120 and 150), and LPO (days 1, 30, 120 and 150) showed lower values in the curcumin diet. The ACAP was higher in the feed with curcumin after 120 and 150 days of storage.

3.4 Body weight and experimental challenges

No significant difference was observed between groups for body weight at all moments evaluated (Figure 2). However, over time a weight gain was observed in both groups ($P<0.05$), which was expected since the dogs were in the growth phase.

The adaptation period was approximately of 30 days and in that period several natural events were observed in the dogs of both groups. Five days after starting the feed, it was observed that all the animals had diarrhea and in some cases it was more intense. Feces from all animals were collected, analyzed by centrifugation flotation with sugar-hypersaturated solution, and *Giardia* spp. were observed in both groups (control: 552 ± 256 oocysts/g; supplemented: 276 ± 86 oocysts/g of feces - $P>0.05$). Thus, all animals in the experiment were treated with a dose of 10 mg/kg of the antiprotozoal secnidazole. The treatment was effective to eliminate diarrhea and the parasite (fecal examination negative), however the feces remained softened. On day 10 of the experiment, the animals had diarrhea, anorexia and hyperthermia again when they received a clinical diagnosis of gastroenteritis. Thus, all animals were submitted to enrofloxacin treatment (5 mg/kg) for five consecutive days.

Three days after starting the treatment, the animals were eating normally, and the diarrhea disappeared within a few days. On day 16 of the experiment, again, some animals showed signs of diarrhea, and parasitological examinations were performed and *Giardia* spp. infection was confirmed in all animals (no difference between groups - $P>0.05$). Again, they were treated with antiprotozoal, but this time the active principle fenbendazole at 50 mg/kg (Panacur) was used for 3 consecutive days. The animals recovered, and did not show any more clinical signs or complications throughout the rest of the experiment. We also observed that some dogs (n= 1, 3, 5, 6 and 10), independently of the group, developed skin lesions,

which was considered an allergic response of the ration produced, since all lesions disappeared at the end of the experiment when the experimental ration was replaced by a commercial one. Therefore, during the experimental period the animals had a number of challenges, which even with curcumin additive in the diet, were not minimized.

3.5 Hematological analysis

Figure 3 shows the hematological resuls. The total number of erythrocytes (days 35 and 42), hematocrit (day 42) and the hemoglobin concentration (day 42) was higher in the animals that received curcumin. On day 42, the number of leukocytes was higher in the animals of the supplemented group ($P<0.05$), due to the increase of neutrophils and monocytes ($P<0.05$). Also on day 42 of experiment, the number of lymphocytes was lower in the animals of the supplemented group compared to the control ($P<0.05$). The number of eosinophils did not differ between groups ($P>0.05$). Over time, erythrocytes and hemoglobin concentration increased ($P<0.05$) in the supplemented group (day 1 to 42). The number of monocytes decreased over time in both groups ($P<0.05$), but with a greater decrease in the control group from day 1 to 42 ($P<0.001$). The overall profile of these variables over time can be seen in Figure 3.

3.6 Seric biochemical analysis

Results of serum biochemistry are shown in Figure 4. Levels of glucose, cholesterol (day 42), triglycerides and urea (days 35 and 42) were higher in dogs in the supplemented group ($P<0.05$). On the other hand, total protein and globulin levels and ALT activity (day 42) were lower in animals fed curcumin containing feed ($P<0.05$) compared to control. Levels of albumin did not differ between groups, as well as over time ($P > 0.05$). Emphasis was given to the reduction of globulins over time (day 1 to 42) in the supplemented group. ALT also reduced over time in the supplemented group (day 1 to 35 and day 1 to 42).

3.7 Plasma oxidant and antioxidant status

ROS levels were lower in the animals of the supplemented group (day 42) compared to the control group ($P<0.05$; Figure 5a). Over time, ROS levels reduced (day 1 to 35 and 42 of the experiment) in dogs that consumed feed with curcumin ($P<0.05$). Antioxidant enzyme results are shown in Figure 5 (b, c, d, e). The activity of CAT (days 35 and 42), SOD (day 42) and GPx (day 42) was higher in dogs of the supplemented group ($P<0.05$) compared to control. GST did not differ between groups and over time ($P>0.05$). Over time, CAT activity

decreased in the plasma of dogs in the control group (day 1 to 35 and 42), as well as reduced SOD activity in that group (day 7 to 35 and 42 of experiment). GPx increased over time (day 35 to 42) in the animals of the supplemented group ($P<0.05$). The results of the non-protein thiols were shown in Figure 5 (f, g). NSPH levels (days 35 and 42) and PSH (day 42) were higher in the dogs of the supplemented group ($P<0.05$) compared to the control group. Over time no difference ($P>0.05$) was observed for NSPH and PSH in both groups. The total antioxidant capacity (ACAP) was higher on days 35 and 42 of the experiment in the plasma of dogs that consumed curcumin in the diet compared to the control group ($P<0.05$). ACAP levels decreased over time in the control group, that is, from day 1 to 35 and day 1 to 42 of experiment ($P<0.05$).

4. Discussion

This is the first scientific study of commercial feed production with curcumin that evaluates its effects on the health of dogs. As mentioned, other experiments have already been carried out on chickens and sheep and have shown positive effects on animal health (Rahmani et al., 2018; Molosse et al., 2019). The biological and medicinal properties of curcumin are well described in the literature, with emphasis on the antioxidant activity capable of inhibiting lipid peroxidation, and the anti-inflammatory and antimicrobial effects (Aggarwal and Harikumar 2009; Hewlings and Kalman, 2017; Hatcher et al., 2008). As a consequence of these curcumin properties, it was possible to observe a lower lipid peroxidation in the test ration, as well as the dogs that consumed the curcumin feed had higher antioxidant activity in the dogs plasma, as well as reduced inflammatory markers, which characterizes an anti-inflammatory response.

Antioxidants can be considered substances responsible for delaying the deterioration and rancidity that occur through oxidation, being free radical inhibitors (Coneglian, 2011; Decker and Xu, 1998). When the oxidation variables of the ration were analyzed, the presence of curcumin gave a lower lipid peroxidation compared to the control ration. Likewise, this ration had lower levels of ROS in the first three months and lower levels of TBARS in all collections for six months. A study by Racanicci (2000) tested the addition of 500 mg/kg BHT (butylated hydroxytoluene) in meat-and-bone meal and this was effective in preventing oxidative rancidity. Some natural antioxidants such as vitamin E and C are already used for lipid oxidation termination (Coneglian, 2011). Therefore, the use of additives such as curcumin is an alternative to improving and preserving dog food. The activity of the ALT enzyme was lower in animals supplemented with curcumin, and high concentrations of ALT

and AST (aspartate aminotransferase) indicated hepatic damage or overload of this organ. A study of AFB1 intoxicated rats showed a reduction in ALT levels after consumption of curcumin in the diet (El-Bahr, 2015), in the same way as rabbits treated with paracetamol, a drug that causes damage to the liver, i.e. animals who received the drug along with curcumin showed a reduction of ALT and AST. This effect is due to curcumin inducing a hepatoprotective activity, which is able to decrease the level of serum biomarker enzymes, as well as to aid in the regeneration of liver cells by the expression of cytokines (El-Agamy, 2010; Soliman et al., 2014; Sayed and El-kordy, 2014).

There was also a reduction in ROS levels and an increase in ACAP activity in studies with lambs (Molosse et al., 2018) and with carps (Jiang et al., 2016) fed with curcumin. According to these authors, this increase in ACAP is due to the increase in antioxidant enzymes and non-enzymatic molecules capable of eliminating free radicals, as in our study. Jiang et al. (2016) detected an increase in the enzymes catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in carp intestines fed with curcumin, which enzymes also increased in the serum of dogs in the current study. These enzymes play an important role in the body, as they are the most important endogenous antioxidant defense in the fight against free radicals (Barbosa et al., 2010). SOD converts the superoxide anion produced by the interaction of oxygen and transport chain electrons in the hydrogen peroxide mitochondria, which will have subsequent action of GPx and CAT by detoxifying the organism, cells or tissues (Remmen et al., 2004).

The values of total erythrocytes, hemoglobin and hematocrit were higher in the group supplemented with curcumin on the last day of sampling. Rats treated with paracetamol showed a decrease in red blood cells, hemoglobin and hematocrit, and the authors justified that when there is damage in liver cells does not occur the production of the hormone erythropoietin responsible for forming the red blood cells, but after being supplemented with curcumin there was an increase of all of these parameters due to its protective function, increasing erythropoiesis and avoiding oxidative damage (Sayed and El-kordy, 2014). This mechanism may have been the same in the dogs of this study who fed ration containing curcumin.

In this study, supplemented animals showed higher concentrations of total leukocytes, neutrophils and lower concentrations of lymphocytes on the last day sampling day, contrary to Molosse et al (2019) while studying lambs supplemented with curcumin that showed a reduction of these variables, demonstrating an anti-inflammatory effect. Lymphocytes were at lower levels, and since these cells are responsible for the production of immunoglobulins, this

would explain the lower levels of globulins in the blood. Protein and globulin levels were also lower in lambs supplemented with curcumin (Molosse et al., 2019); and according to these authors this may be a negative impact of curcumin on the immune response, since globulins are proteins associated with the innate response; but on the other hand avoiding exacerbated inflammatory response also contributes to dogs health. Already Galli et al. (2018) showed an increase in globulins and total protein in laying hens supplemented with curcumin, and the authors concluded that this increase improved the immune response. Based on these findings, it is clear that inflammatory and immunological variables differ after curcumin treatment according to the animal species by mechanisms not known yet which deserves future research.

Conclusion

The use of curcumin as an additive in the diet of dogs had a positive effect on the preservation of this product by reducing the lipoperoxidation and increasing the levels of antioxidants, favoring feed quality. In addition, curcumin had beneficial effects on the health of dogs by stimulating erythropoiesis and the antioxidant system, facts that may have generated a protective effect on the liver, requiring further analysis to prove. However, it also had a direct action on lymphocytes, affecting globulin levels, which might be a positive or negative effect of curcumin, as already mentioned.

Ethical Committee

This work was approved by the Committee on Ethics in Animal Use (CEUA) of Universidade do Estado de Catarina (UDESC), under protocol number 4831301117.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Ingredients and nutritional composition of feed

Ingredients (g/kg)	Proportion
Corn	373.25
Bone and meat meal 45%	210.00
Degreasing rice brand	200.00
Bean bands	140.00
Oil of broiler offal	40.00
Hydrolyzed broiler liver	30.00
Mineral premix[1]	2.00
Vitamin premix[2]	2.00
Salt	1.00
Antifungi	1.00
Antioxidant butyl-hydroxytoluene	0.50
Yucca Schidighera extract	0.25
Calculated chemical compositon	
Crude protein, %	18.17
Ethereal extract, %	10.28
Crude fiber, %	5.00
Calcium, %	2.40
Available phosphorus, %	1.33
Sodium, %	0.20

Note: 1 - Mineral supplement content per kg of product: Iron - 200 g; Cobalt - 2 g; Copper - 20 g; Manganese - 200 g; Zinc - 250 g; Iodine - 0.4 g; Selenium - 250.0 mg and Excipient q.s.p - 1000 g;
2 - Vitamin Supplement content per kg of product: Vit. A – 4.000.000 U.I.; Vit. D3 – 800.000 U.I.; Vit. E – 30.000 U.I.; Vit. B1 - 2.0 g; Vit. B2 - 3.0 g; Vit. B6 - 4.0 g; Vit. B12 - 0.015 g; Pantothenic acid – 4.0 g; Biotin - 0.1 g; Vit. K3 - 1.0 g; Folic acid - 0.8 g; Nicotinic acid 8 g and Excipient q.s.p - 1000 g.

Table 2: Feed composition with and without curcumin after 1, 30, 60, 90, 120 and 150 days of production and storage.

Chemical composition	Days after production	Feed without curcumin	Feed with curcumin supplementation
Dry Matter	1	93.27	93.76
	30	92.73	94.01
	60	93.35	93.59
	90	93.05	93.69
	120	92.81	93.62
	150	92.77	93.44
Crude protein, %	1	18.8	17.2
	30	19	17.9
	60	17.3	17.1
	90	17.6	19.7
	120	19.6	20.7
	150	18	17.5
pH	1	6.23	6.28
	30	6.26	6.31
	60	6.24	6.30
	90	6.26	6.32
	120	6.25	6.32
	150	6.22	6.30
Ash, %	1	0.210	0.207
	30	0.204	0.207
	60	0.198	0.200
	90	0.206	0.218
	120	0.203	0.221
	150	0.204	0.220
Ethereal extract	1	10.38	11.71
	30	10.28	10.66
	60	11.48	12.43
	90	10.66	10.29
	120	11.26	10.35
	150	10.69	11.12

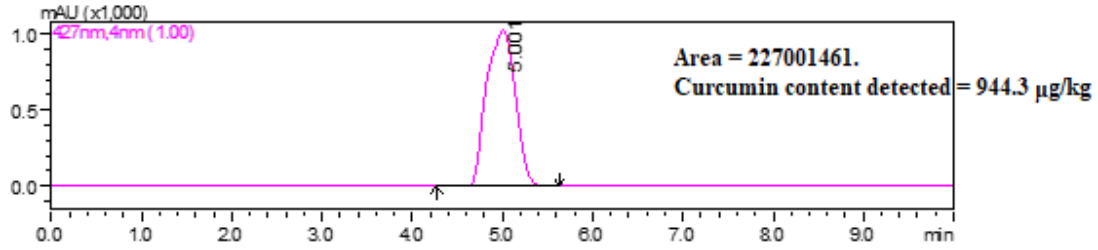
Note: No difference was observed between groups and over time for both types of feed ($P > 0.05$).

Table 3: Levels of carbonyl protein, oxygen reactive species (ROS), thiobarbituric acid reactive substances (TBARS), lipid peroxidation (LPO) and serum antioxidant capacity against peroxyl radicals (ACAP) in diets with and without curcumin on days 1, 30, 60, 90, 120 and 150 after production and storage.

Variable	Days after production	Feed without curcumin	Feed with curcumin supplementation
Protein carbonil	1*	99.83 ± 6.9	17.06 ± 9.7
(nmol of carbonyl/	30*	78.92 ± 7.6	26.69 ± 3.8
mg of protein)	60	51.36 ± 8.0	46.29 ± 5.5
	90	54.60 ± 1.9	58.28 ± 5.6
	120	58.80 ± 7.1	60.25 ± 2.9
	150	60.12 ± 4.3	71.89 ± 11.6
ROS	1*	4.06 ± 1.14	1.28 ± 0.87
(DCF/mg protein)	30*	3.67 ± 1.21	1.34 ± 1.02
	60*	2.85 ± 0.79	1.61 ± 0.65
	90	2.03 ± 1.14	2.13 ± 0.74
	120	2.44 ± 0.54	2.33 ± 0.87
	150	3.10 ± 1.74	3.05 ± 1.08
TBARS	1*	1.25 ± 0.09	1.05 ± 0.04
(nmol MDA/mg	30*	1.49 ± 0.40	0.95 ± 0.22
protein)	60*	1.69 ± 0.32	0.97 ± 0.34
	90*	1.85 ± 0.08	1.48 ± 0.18
	120*	2.09 ± 0.24	1.36 ± 0.17
	150*	3.00 ± 0.74	1.16 ± 0.36
LPO	1*	69.83 ± 14.9	47.32 ± 7.60
(nmol CHP/g of feed)	30*	60.18 ± 7.10	49.06 ± 8.74
	60	58.94 ± 2.9	55.83 ± 7.36
	90	55.32 ± 4.56	52.76 ± 2.98
	120*	60.54 ± 8.57	45.65 ± 3.91
	150*	73.70 ± 3.87	47.34 ± 10.4
ACAP	1	0.21 ± 0.08	0.18 ± 0.04
(UF/mg protein)	30	0.61 ± 0.14	0.40 ± 0.21
	60	0.62 ± 0.08	0.48 ± 0.07
	90	0.40 ± 0.07	0.42 ± 0.10
	120*	0.12 ± 0.04	0.34 ± 0.06
	150*	0.25 ± 0.09	0.53 ± 0.13

Note: * $P<0.05$ indicates significant difference at each sampling time.

a) Chromatogram control using feed (ration) contaminated with curcumin (1000mg)



b) Chromatogram of the feed (ration) containing curcumin.

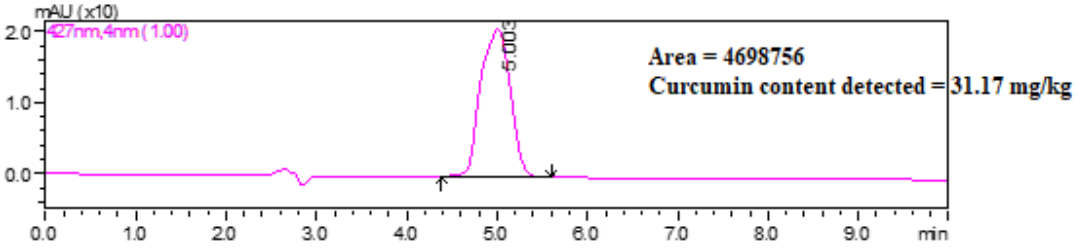


Figure 1: Levels of pet food produced. (a) Chromatography of feed contaminated with curcumin in the laboratory, used as control of the reaction. (b) Chromatography of feed produced with curcumin additive, ingredient used at the dose of 100 mg/kg.

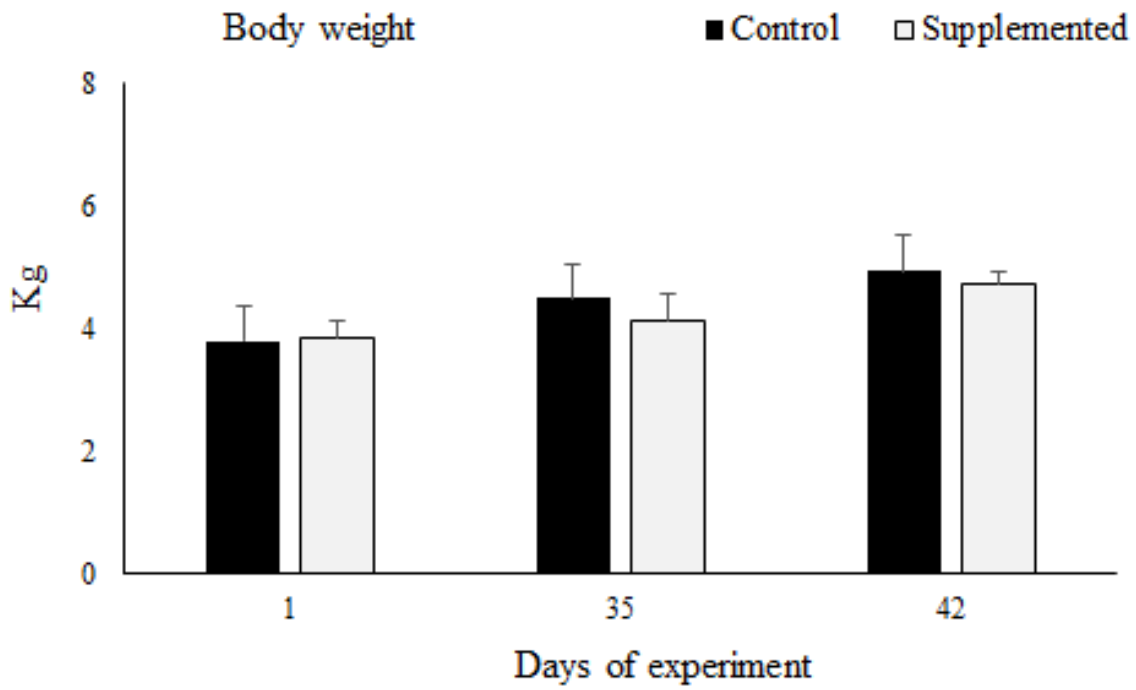


Figure 2: Dogs weight after feeding feed supplemented with curcumin (the suplemented group) and the control dogs without supplementation (the control group). Note: P>0.005.

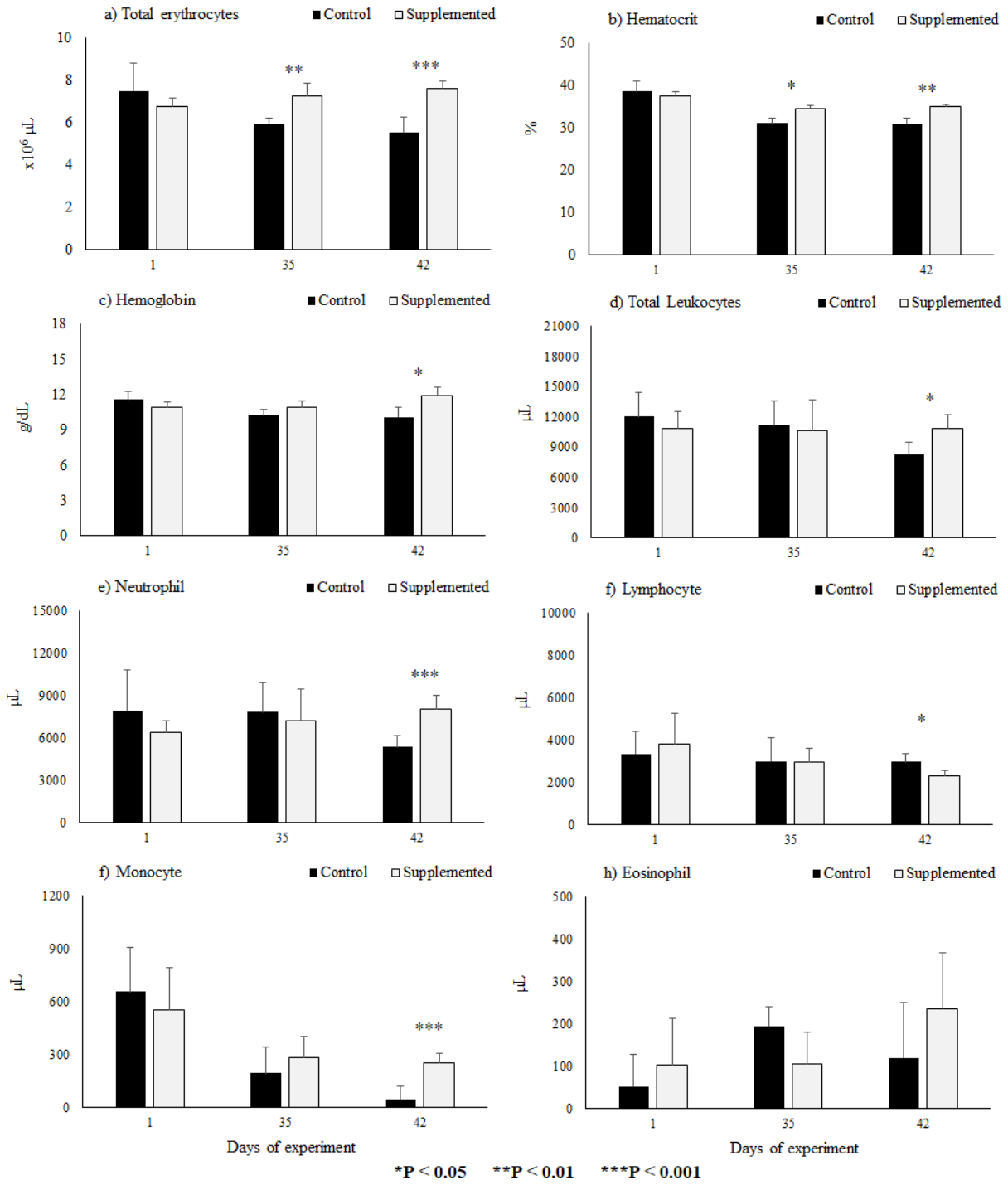


Figure 3: Dogs fed with feed containing curcumin (supplemented group) and without curcumin (the control group): dog blood count. Asterisk (*) indicates difference between groups at any given time.

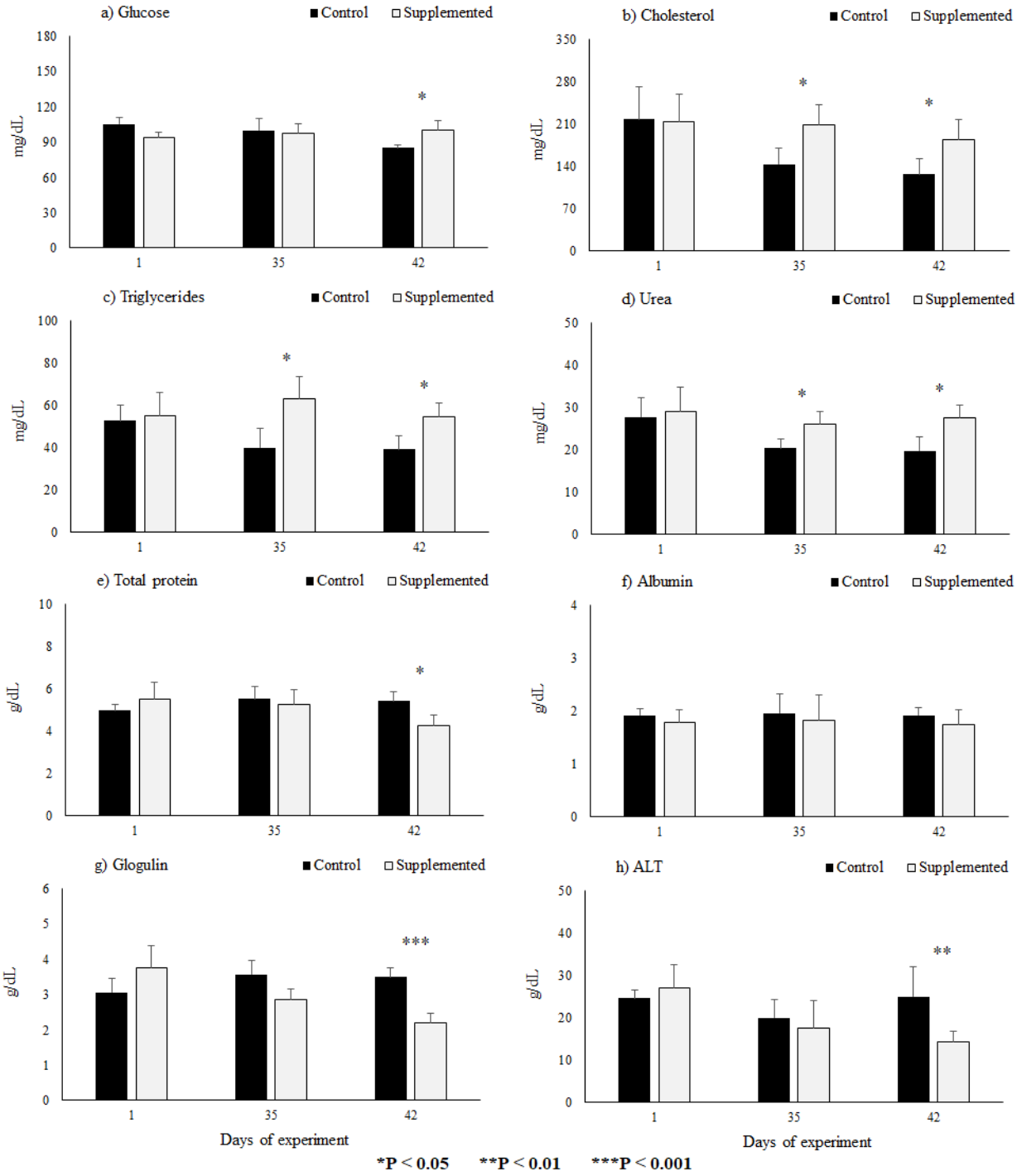


Figure 4: Dogs fed a feed containing curcumin (the supplemented group) and without curcumin (the control group): Levels of glicose (a), cholesterol (b), triglycerides (c), urea (d), total protein (e), albumin (f), globulin (g) and ALT (h). Asterisk (*) indicates difference between groups at any given time.

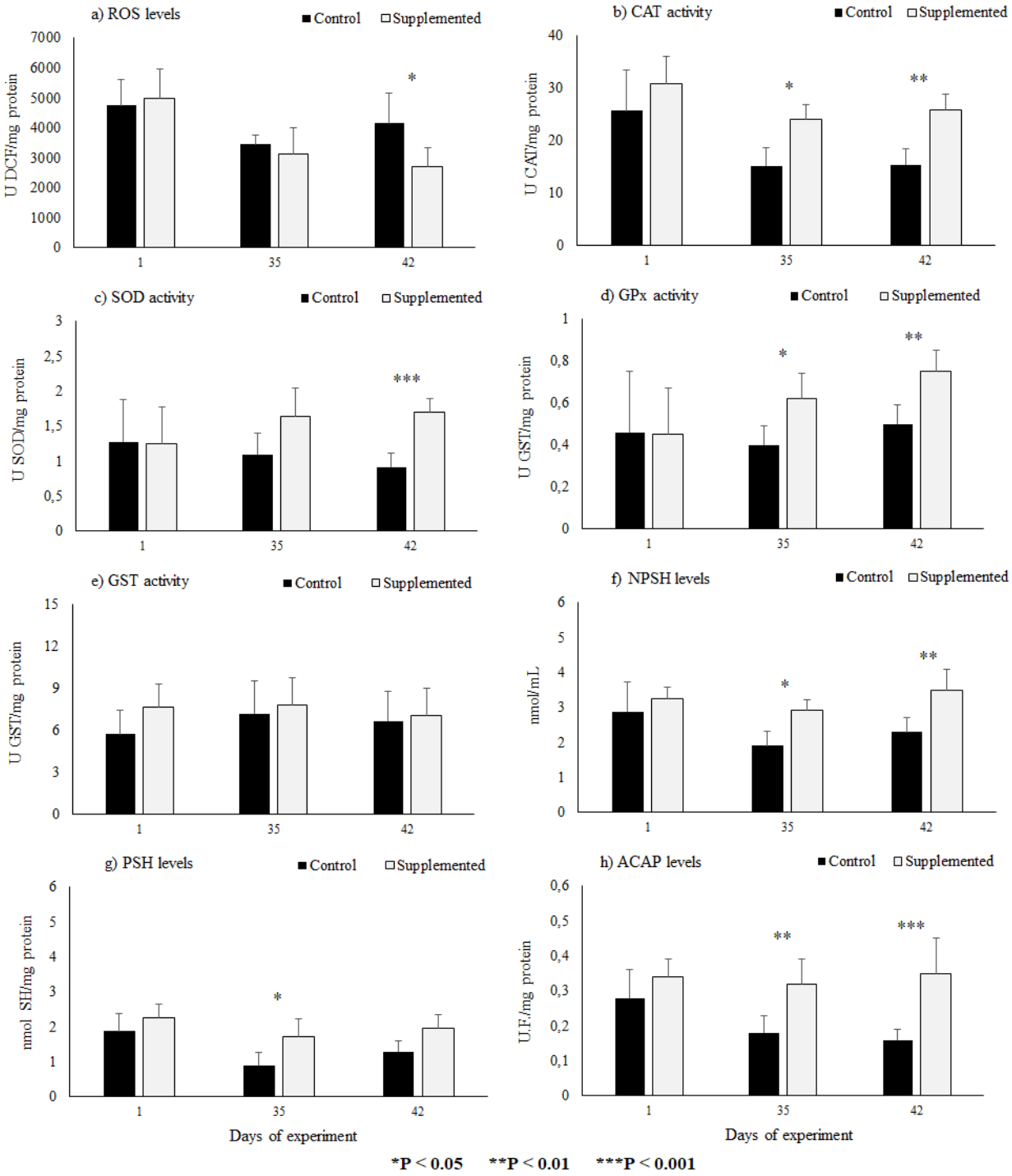


Figure 5: Dogs fed a feed containing curcumin (the supplemented group) and without curcumin (the control group): levels of oxygen reactive species - ROS (a), catalase activity - CAT (b), superoxide dismutase - SOD (c), glutathione peroxidase - GPx (d), glutathione S - transferase - GST (e), nonprotein thiols - NPSH (f), protein thiols - PSH (g) and antioxidant capacity - ACAP (h). Asterisk (*) indicates difference between groups at any given time.

2.2 – MANUSCRITO II

Titulo do artigo: Petiscos contendo curcumina oferecido diariamente a cães estimula a resposta antioxidante e anti-inflamatória

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Snacks containing curcumin stimulates the antioxidant and anti-inflammatory responses in dogs: beneficial effects on animal health

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ABSTRACT

The objective of this study was to evaluate if the supply of snacks containing curcumin to dogs exerts beneficial effects on health. The snacks were produced from commercial canned meat for dogs, where the curcumin was added and homogenized. Then, snacks containing 15 mg of curcumin were produced, frozen and then offered to the dogs twice a day. Ten Beagles (6 months of age) were used as the experimental unit. The animals were allocated in an experimental kennel and assigned randomly to 1 of 2 treatments (5 dogs/treatment): snacks containing curcumin (Curcumin; 30 mg of curcumin/animal/day) or not (Control). Blood samples were collected on days 1, 15 and 30 to evaluate hematological and biochemical variables. No effects of treatment were observed for plasma concentration of glucose, urea, triglycerides, cholesterol, total protein, albumin, globulin and alanine aminotransferase. On day 15, the number of erythrocytes and hematocrit were greater in dogs fed with curcumin compared to control. However, dogs fed with curcumin had lower number of leukocytes (day 30), neutrophils (day 15) and lymphocytes (day 30), compared to control. In addition, dogs fed with curcumin had lower plasma levels of nitric oxide, reactive oxygen species, lipoperoxidation, and protein carbonylation on day 30, compared to control. Further, dogs fed with curcumin had greater plasma total antioxidant capacity and concentration of protein thiol, non-protein thiol, glutathione peroxidase, and superoxide dismutase on day 30, compared to control. Thus, we conclude that the addition of curcumin in the food of dogs stimulated the antioxidant system and consequently reduced the oxidative reactions, which is benefic to animal health. In addition, the dose of 30 mg of curcumin/dog/day induced mild anti-inflammatory action, which is also a desirable property for health.

Keywords: food, curcumin, snacks, dogs, antioxidant.

1. Introduction

The dog food is classified in complete, special or complementary classifications (Volpato, 2014). Dry rations are part of the complete food and today is the most sold, occupying 80% of the sales in the pet market in America (Grandjean and Vaissaire, 2006). Generally, pet food products are formulated using agricultural products from animal origin, which may have problems with contaminants or storage, thereby increasing the possibility of the presence of mycotoxins, as an example (Alves, 2003). A way to increase the shelf life of these rations is with the utilization of acidifiers and antioxidants associated with packaging and low humidity (Fortes, 2015).

Among these antioxidants, the synthetic BHT (butylhydroxytoluene), BHA (butylhydroxyanisole) and ethoxyquin are the most used as food preservatives (Volpato, 2014). However, there are several doubts about reliability and security of these products, and consequently, alternatives as natural antioxidant from plants have gained attention and highlight in the scientific research (Volpato, 2014; França et al., 2011). In addition, many studies showed that the utilization of curcumin in the diet improved the animal health, which is principally linked to its potent antioxidant properties (El-Bahr, 2015; Jaguezeski et al., 2018; Molosse et al., 2019).

Curcumin is a curcuminoid present in the *Curcuma longa*, a molecule that possesses different mechanisms of antioxidant, anti-inflammatory and antimicrobial actions (El-Bahr, 2013; Santos et al., 2003; Hewlings, Kalman.,2017; Hatcher et al., 2008; Hewlings and Kalman, 2017). In this way, curcumin can be used as an additive in rations and snacks to dogs, to improve the animal health and to preserve the quality of pet food products. Thus, the objective of this study was to evaluate if the supply of snacks containing curcumin to dogs exerts beneficial effects on health, related to the antioxidant system.

2. Material and methods

2.1. Curcumin

Curcumin (powder) was purchased from Shaanxi Jiahe Phytochem Ltda (China) and had 98 % of purity. The dosage of curcumin used in the snacks was defined according to a recent study conducted by Galli et al. (2018) with laying hens, since there is no scientific literature that used curcumin in the diet of dogs.

2.2. Snacks production

The snacks were produced utilizing 200 g of canned meat, where was added 4.5g of curcumin. Further, the snacks were prepared at circular format with a weight of 0.70 g and consequently containing 15 mg of curcumin. Snacks with the same size and composition, but without the addition of curcumin were produced to be used as control. All snacks produced were stored (-20 ºC) and then were defrosted (10 min in room temperature) before the feedings (twice a day), characterizing 30 mg curcumin/animal/day.

2.3. Animals and experimental study

Ten Beagle dogs (6 months of age) were used as the experimental unit. The animals were fed with a commercial ration (AUK VIP puppies; BioBase; 30 % of protein) and the amount provided was based in the body weight of the dogs (average of 6 kg at beginning of the experiment). Two hundred and seventy grans of commercial ration were provided twice a day (divided in two meals - morning and afternoon), representing 4.5 % of the body weight.

The animals were assigned randomly to 1 of 2 treatments (5 dogs/treatment): snacks containing curcumin (Curcumin) or not (Control) provided twice a day. The snacks were offered to the animals before supply the commercial ration, and the snacks intake was accompanied by the researchers. These animals were kept in a maintenance kennel, with an indoor heated area (24ºC) and an outdoor area with lawn and shaded areas where they stayed approximately 4 h per day. The animals had free access to water.

2.4. Sample collection

Blood samples were collected on days 1, 15 and 30 with fasting for approximately 12 hours. The animals were manually contained and blood samples were collected from the jugular vein using syringes (3 mL) and needles (25/7). The blood was allocated in two tubes containing EDTA, and refrigerated until arrived in the laboratory (same day). In the laboratory, one tube was used for hemogram analyses and the other one for plasma collection. One tubes without anticoagulante was centrifuged (5500 g; 10 min) and then the serum was stored in microtubes and frozen at – 20 ºC until the analyzes.

2.5. Hemogram

The concentration of erythrocytes, total leukocytes and hemoglobin were measured using a semi-automated analyzer (CELM CC530). Hematocrit was determined according to the microhematocrit technique (Feldman et al., 2000). Leucocyte differential counts were

performed in blood smears stained with commercial dye (Romanowsky method) using a light microscope at 1000x magnification.

2.6. Serum biochemistry

The serum concentration of total protein, albumin, triglycerides, cholesterol, urea, glucose and alanine aminotransferase (ALT) were evaluated using a semi-automated analyzer (Bio Plus 2000®) with commercial kits (Analisa®). The concentration of globulin was obtained subtracting the concentration of albumin from total protein.

2.7. Levels of nitric oxide (NOx)

The levels of NO_x were measured according to the Griess method that indirectly quantifies the levels of nitrite/nitrate as previously described in detail by Tatsch et al. (2011), and the results were expressed in μmol/L.

2.8. Oxidant variables

2.8.1. Levels of reactive oxygen species (ROS)

The levels of ROS were determined by the DCFH oxidation method as described by Ali et al. (1992) using excitation and emission of the wavelengths of 485 and 538 nm, respectively, and the results were expressed in U DCF/mL.

2.8.2. Levels of lipoperoxidation (LPO)

The levels of LPO were measured as proposed by Monserrat et al. (2003), and recently published in detail by Da Silva Barreto et al. (2018), and the results were expressed in μmol CHP/mL.

2.8.3. Levels of carbonyl protein

The content of carbonyl protein was determined in the supernatant as described by Reznick and Packer (1994), and the results were expressed in nmol of carbonyls formed/ mg of protein.

2.9. Non-enzymatic antioxidants: thiols

The levels of non-proteic (NPSH) and proteic (PSH) were evaluated according to Sedlak and Lindsay (1968) and reported in details by Maltez et al. (2018). The results were expressed in nmol SH/mL and nmol SH/mg of protein, respectively.

2.10. Enzymatic antioxidants

2.10.1. Glutathione S-transferase (GST) activity

The GST activity was measured based on the method described by Habig et al. (1974) and reported in detail by Biazus et al. (2017). Enzymatic activity was expressed in U GST/mg of protein.

2.10.2. Glutathione peroxidase (GPx) activity

The GPx activity was measured according to the methodology described by Paglia and Valentine (1967) and reported in detail by Souza et al. (2018). Enzymatic activity was expressed in U GPx/mg of protein.

2.10.3. Superoxide dismutase (SOD) activity

The SOD activity was evaluated spectrophotometrically as described by Marklund and Marklund (1974), and the enzymatic activity was expressed in U SOD /mg of protein.

2.11. Levels of total antioxidant capacity

The levels of ACAP were measured according to Amado et al. (2009) using wavelengths of 485 nm (excitation) and 520 nm (emission) for 40 min at 37 °C, and the results were expressed in fluorescence units/mg of protein.

2.12. Statistical analysis

Firstly, data were submitted to a normality test (Shapiro-Wilk). Data that did not have normal distribution were transformed to logarithm for the purpose of normalization. Further, the comparison between groups and the effect over time was performed using a two-way ANOVA. Values were considered significant at $P \leq 0.05$. Results were presented as mean and standard deviation.

3. Results

3.1. Body weight

No effects of treatment were detected for body weight after 30 days of experiment ($P > 0.05$; Control: 5.78 ± 0.34 kg; Treated: 6.01 ± 0.5 kg). However, over time, both groups had weight gain ($P < 0.05$). The weight gains from day 1 to 30 were 1.20 kg and 1.06 kg for control and treated groups, respectively.

3.2. Hemogram

Hematological results were presented in Table 1. Curcumin dogs had a greater number of erythrocytes and the hematocrit on day 15, compared to Control dogs ($P < 0.05$). However, Curcumin dogs had a lower number of leukocytes on day 30, neutrophil on day 15 and lymphocytes on day 30, compared to Control dogs ($P < 0.05$). Over time, the number hematocrit increased from day 1 to 15 and the number of total leukocytes and lymphocytes decreased from day 1 to 30, only in dogs that received curcumin ($P < 0.05$).

3.3. Plasma biochemistry

Plasma biochemistry results were presented in Table 2. No effects of treatment or time of collection were detected for all parameters analyzed ($P > 0.05$).

3.4. Levels of NOx

Plasma levels of NOx were presented in Figure 1a. Curcumin dogs had lower plasma levels of NOx on days 15 and 30, compared to Control dogs ($P < 0.05$). Over time, the plasma levels of NOx were reduced (days 1 to 15; 15 to 30) only in the dogs that received curcumin ($P < 0.05$).

3.5. Oxidative reactions

The results of the oxidative profile were presented in Figure 1 (b, c, and d). Curcumin dogs had lower plasma levels of ROS, LPO and protein carbonyl on day 30, compared to Control dogs ($P < 0.05$). Over the time, the plasma levels of protein carbonyl were reduced in dogs that received curcumin ($P < 0.05$), and no significant differences were observed over the time for ROS and LPO in both groups ($P > 0.05$).

3.6. Antioxidant status

The results of antioxidant status were presented in Figure 2. Curcumin dogs had greater plasma levels of ACAP, NPSH, and PSH, and also greater GPx and SOD activities on day 30, compared to Control dogs ($P < 0.05$). No effects of treatment were detected for plasma GST activity. Over time, the plasma GPx activity increased (days 1 to 30, 15 to 30) only in dogs that received curcumin. However, over time, the other variables did not differ in both groups ($P > 0.05$).

4 Discussion

In this study, the supplementation with curcumin showed positive effects on some hematological variables, increasing the number of erythrocytes and hematocrit, and reducing the number of leukocytes, which indicates possible stimulatory effects on erythropoiesis, as observed by Yonar et al. (2019) in common carp (*Cyprinus carpio*) exposed to pesticide chlorpyrifos and treated with curcumin. In addition, Bagheri et al. (2018) described that the supply of curcumin caused protective effects on erythrocytes of rats exposed to radiation, and concluded that this effect is linked to the stimulation of erythropoiesis in the bone marrow. Sayed and El-Kordy (2014) showed that the use of curcumin was able to protect the hepatic cells and regulated the production of erythropoietin, which regulated the erythrocytes and hematocrit values.

A significant decrease on plasma levels of ACAP and LPO were observed in dogs that received snacks containing curcumin, in agreement to observed by Molosse et al. (2019) in lambs fed with a diet containing curcumin. According to these authors, these effects occur due to an increase in the antioxidant capacity to remove the free radicals responsible by lipid peroxidation, as ROS. The utilization of curcumin as an antioxidant has been widely publicized, and this occurs because curcumin acts on various signaling pathways at the cellular level, and its anti-oxidant and anti-inflammatory properties are the main pathways associated with their protective effects that entail benefits to animal health (Hewlings and Kalman, 2017). A study conducted by Sahebkar et al. (2015) revealed that the supplementation with curcumin reduced the production of free radicals, and increased the activity of antioxidant enzymes, like catalase, SOD, and GPx, as observed in our present study. Similar to our results, Hosseinzadehdehkordi et al. (2015) observed that the supply of curcumin reduced the levels of LPO in rats with lung cancer. The supplementation with curcumin in dairy sheep increased the SOD, CAT and GPx activities, revealing that curcumin is capable to activate several enzymatic antioxidant pathways (Jaguezeski et al., 2018), in agreement with the results observed in our study. Similarly, Jiang et al. (2016) showed that the supply of curcumin increased the intestinal CAT, GPx and SOD activities in common carp, which contributes to neutralize the excessive production of free radicals (Barbosa et al., 2010).

The NOx can be involved in several histopathological lesions, including ischemia and cerebral reperfusion. According to Yu et al. (2012), the use of curcumin decreased the excessive production of NOx in rats submitted to ischemia, which reveals the protective effects of curcumin linked to anti-inflammatory and anti-antioxidant systems. Further, Soto-

Urquieta et al. (2014) demonstrated that curcumin prevented to increase the levels of LPO and NOx in streptozotocin-induced diabetes in rats. Curcumin also had protective effects against memory deficits by increasing the activity of the nNOS/NO pathway in the prefrontal cortex, amygdala and hippocampus thereby improving memory deficits (Yu et al., 2013).

The increase on levels of protein carbonylation is a parameter that is directly linked to greater ROS formation, and indicates damage to proteins and consequently impairs the physiological functions of the proteins and the health status of the animals (Friguet, 2002). A study conducted by Dkhar and Sharma (2013) revealed an increase on plasma levels of protein carbonylation concomitantly with greater animal age, and treatment with curcumin reduced the damage to proteins, revealing the protective effects of curcumin against protein oxidation.

Conclusion

The addition of curcumin in snacks provided to dogs increased the levels of antioxidant and consequently decreased lipid peroxidation and protein oxidation. Thus, curcumin is an option for supplementation in the diet of dogs, especially in the puppy's stage when the health challenges are greater, as the inflammatory responses are exacerbated, which in a way harms the health of the growing animals.

Ethics Committee

The methodology used in the experiment was approved by the Ethical and Animal Welfare Committee of the Universidade do Estado de Santa Catarina (protocol 4831301117.

Conflict of interest:

The authors declare no conflict of interest.

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Table 1: Mean and standard deviation of hematological analyses of dogs supplemented with snacks containing curcumin.

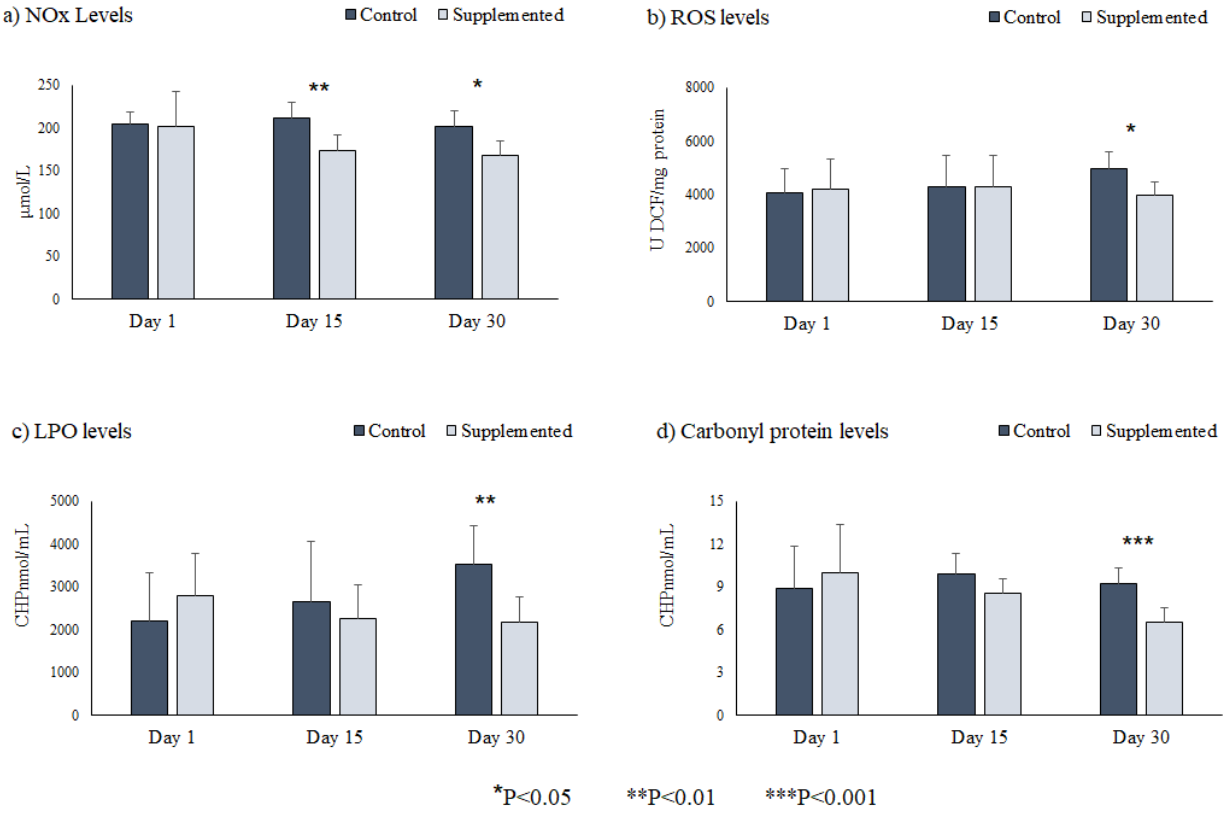
Variables	Day	Control	Curcumin	P value
Hematocrit (%)	1	29.2 (1.5)	30.8 (1.3)	>0.05
	15	31.8 (0.4)	34.1 (1.4)	<0.05*
	30	33.8 (1.3)	32.2 (2.2)	>0.05
Erythrocytes	1	6.05 (1.1)	5.88 (0.9)	>0.05
	15	5.47 (0.4)	6.37(0.7)	<0.05*
	30	5.98 (0.4)	6.57 (1.0)	>0.05
Hemoglobin	1	9.28 (0.4)	9.46 (0.5)	>0.05
	15	9.42 (0.3)	9.72 (0.3)	>0.05
	30	9.98 (0.4)	9.6 (0.7)	>0.05
Total leukocytes	1	10.4 (0.8)	9.8 (2.0)	>0.05
	15	8.9 (1.1)	7.7 (1.0)	>0.05
	30	8.2 (1.1)	6.3 (1.2)	<0.05*
Lymphocytes	1	3.2 (1.2)	3.6 (1.2)	>0.05
	15	2.2 (0.8)	3.1 (0.7)	>0.05
	30	3.1 (0.4)	2.1 (0.6)	<0.05*
Neutrophils	1	6.8 (0.9)	5.6 (1.4)	>0.05
	15	6.3(0.8)	4.3(0.4)	<0.05*
	30	4.8 (1.0)	4.0 (0.6)	>0.05
Monocytes	1	0.15 (0.13)	0.19 (0.12)	>0.05
	15	0.04 (0.05)	0.08 (0.05)	>0.05
	30	0.10 (0.08)	0.08 (0.10)	>0.05
Eosinophils	1	0.20 (0.19)	0.23 (0.07)	>0.05
	15	0.37 (0.30)	0.13 (0.07)	>0.05
	30	0.16 (0.20)	0.12 (0.05)	>0.05

Note: * P <0.05 indicates a significant difference between groups.

Table 2: Mean and standard deviation of plasma biochemistry of dogs supplemented with snacks containing curcumin.

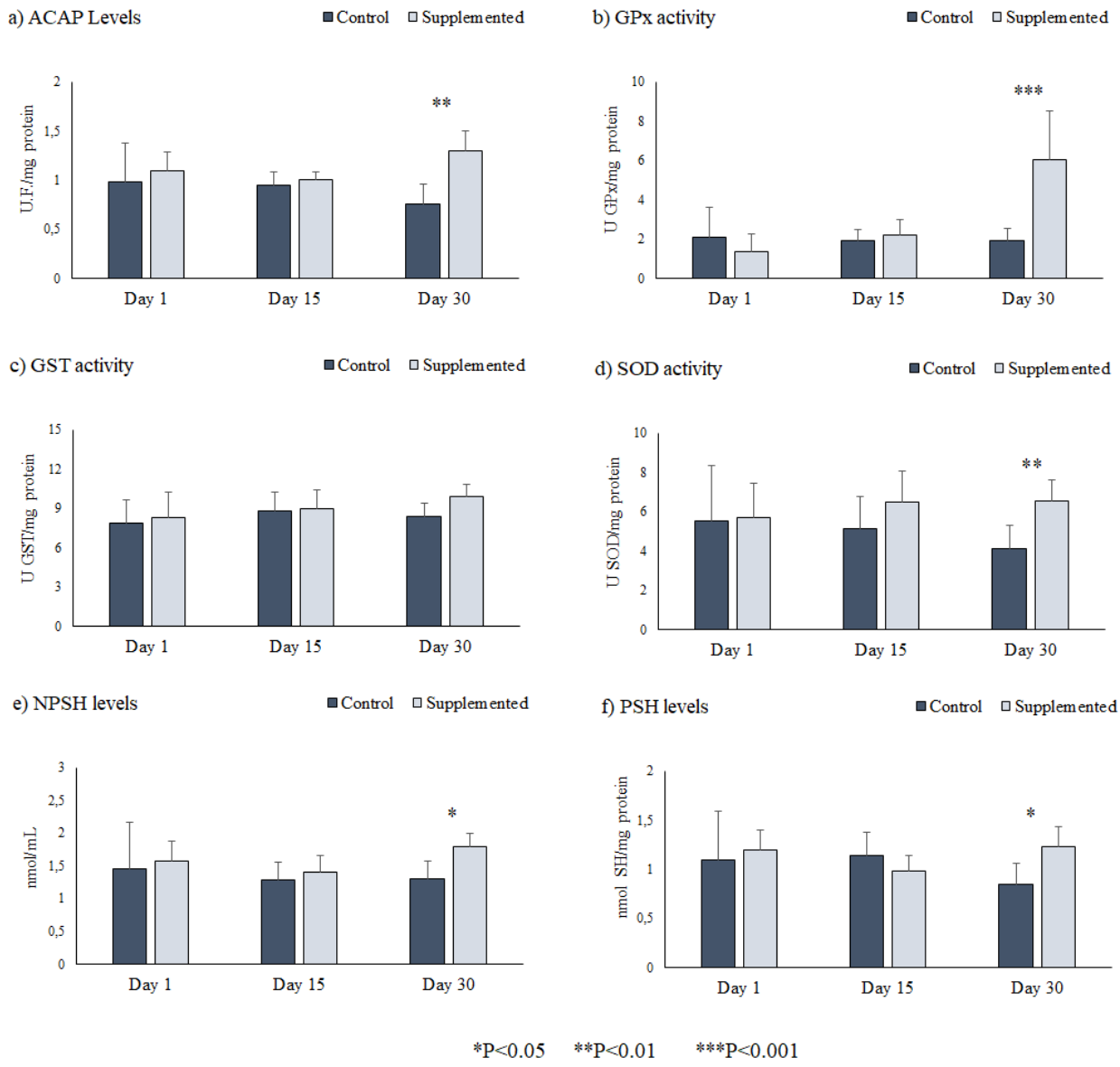
Variables	Day	Control	Curcumin	P value
Glucose	1	103 (18.5)	85.4 (10.5)	>0.05
	15	104.2 (5.0)	104.8 (10.4)	>0.05
	30	94.2 (10.8)	104.8 (7.3)	>0.05
Urea	1	26.4 (2.5)	24.4 (2.6)	>0.05
	15	27 (2.7)	27.2 (4.9)	>0.05
	30	30.2(4.7)	30.4 (2.6)	>0.05
Triglycerides	1	50.6 (8.9)	43.8 (8.3)	>0.05
	15	49.8 (4.9)	46.4 (7.7)	>0.05
	30	47.4 (4.3)	44.6 (5.5)	>0.05
Cholesterol	1	115 (23.7)	119 (29.1)	>0.05
	15	131.6 (34)	157 (31.2)	>0.05
	30	138 (31.1)	152 (27)	>0.05
Total protein	1	4.84 (0.32)	4.48 (0.39)	>0.05
	15	5.06 (0.34)	5.20 (0.43)	>0.05
	30	5.26 (0.32)	5.30 (0.04)	>0.05
Albumin	1	1.94 (0.26)	2.02 (0.2)	>0.05
	15	2.34 (0.45)	2.44 (0.26)	>0.05
	30	2.42 (0.22)	2.44 (0.22)	>0.05
Globulin	1	2.6 (0.34)	2.46 (0.4)	>0.05
	15	2.72 (0.6)	2.76 (0.2)	>0.05
	30	2.84 (0.3)	2.84 (0.13)	>0.05
ALT	1	37 (1.1)	35.4 (10)	>0.05
	15	43.2 (4.2)	36.2 (6.3)	>0.05
	30	38.6 (4.3)	40. (15)	>0.05

Note: * $P < 0.05$ indicates a significant difference between groups.



*P<0.05 **P<0.01 ***P<0.001

Figure 1: Plasma levels of nitric oxide (NOx) [a], reactive oxygen species (ROS) [b], lipid peroxidation (LPO) [c] and protein carbonylation [d] in dogs supplemented with 30 mg curcumin/day compared to control group on days 1, 15 and 30 of the experiment.



*P<0.05 **P<0.01 ***P<0.001

Figure 2: Plasma levels of total antioxidant capacity (ACAP) [a] levels, glutathione peroxidase (GPx) [b], glutathione S-transferase (GST) [c], superoxide dismutase (SOD) [d] activities, and non-protein thiol (NPSH) [e] and protein thiol (PSH) [f] in dogs supplemented with 30 mg curcumin/day compared to control group on days 1, 15 and 30 of the experiment.

3 CONSIDERAÇÕES FINAIS

Os dois experimentos conduzidos nessa dissertação permitiram concluir de modo geral que a curcumina tem efeitos benéficos relacionados a qualidade da ração e saúde dos cães. O segundo experimento foi conduzido objetivando animais sem desafio sanitário e consumindo a curcumina sem passar por processos que envolvem calor, que poderia alterar suas propriedades, como foi o caso do banho de gordura quente, previamente a peletização da ração.

No processo de produção da ração, perdeu-se 67,1 mg de curcumina. No entanto, os 32,9 mg/kg presentes na ração foram capazes de aumentar a capacidade antioxidante da ração, e consequentemente reduzir a níveis de radicais livres, a oxidação proteica e a peroxidação lipídica, sem alterar a composição bromatológica e pH da ração por até seis meses de armazenamento.

Como os cães receberam ração controlada e individualmente sabe-se que consumo médio diário de curcumina foi 6 mg/animal ou 1,5 mg/kg de peso corporal dos cães (manuscrito 1). Apesar do baixo nível de curcumina consumido, observou-se nesses cães efeitos da molécula relacionados a eritropoiese na primeira semana pós fornecimento, ação antioxidante e anti-inflamatória e também reduziu atividade da ALT, que pode sugerir o efeito hepatoprotetor já conhecido. Como os cães jovens estavam em desafio natural constante, também constatamos que o metabolismo lipídico, proteico e de carboidratos responde positivamente com consumo de ração contendo curcumina.

O consumo de petiscos contendo curcumina na dose de 30 mg/dia/cão teve resultados similares aos apresentados no manuscrito 1, isto é, verificou-se que a curcumina na dieta de cães estimula eritropoiese, identificada pelo aumento de eritrócitos e hematócrito, assim como estimulou a ação antioxidante e anti-inflamatória no sangue.

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UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Curcumina como aditivo na alimentação de cães ", protocolada sob o CEUA nº 4831301117 (ID 000527), sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 07/03/2018.

We certify that the proposal "Curcumin as a feed additive for dogs", utilizing 12 Dogs (males and females), protocol number CEUA 4831301117 (ID 000527), under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 03/07/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 01/2018 a 12/2018 Área: Zootecnia

Origem:	Animais de proprietários						
Espécie:	Cães	sexo:	Machos e Fêmeas	idade:	18 a 24 meses	N:	12
Linhagem:	Bulldog Francês			Peso:	5 a 12 kg		

Local do experimento: O experimento será realizado em um canil localizado no município de Chapecó- Santa Catarina.

Lages, 30 de janeiro de 2019

Marcia Regina Pfuetzenreiter
Coordenadora da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

Prof. Dr. Ubirajara Maciel da Costa
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

Lages, 11 de julho de 2018
CEUA N 4831301117

IImo(a). Sr(a).
Responsável: Aleksandro Schafer Da Silva
Área: Zootecnia

Título da proposta: "Curcumina como aditivo na alimentação de cães ".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais UDESC (ID 000224)

A Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 13/junho/2018) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Resumo do Projeto Aprovado em reunião do CEUA A rotina imposta sobre os animais e o aumento do tempo de vida trazem consigo alguns problemas como a diminuição da capacidade de resposta ao estresse, para evitar isso há a necessidade de uma boa nutrição, com alimento de qualidade que atenda suas exigências nutricionais, com boa digestibilidade e principalmente palatabilidade, o uso de produtos como antioxidantes tem auxiliado na nutrição e qualidade das rações. Entre os aditivos usados na alimentação animal tem-se recentemente a curcumina, um componente extraído da planta Curcuma longa que tem entre diversas propriedades a ação antimicrobiana, anti-inflamatória e antioxidante. E, virtude disso, este estudo tem como objetivo verificar se a adição de curcumina na ração tem efeito antioxidante, prolongando sua validade desse produto, assim como se tem efeito benéficos sobre a saúde de cães adultos. Primeiramente será produzida a ração usada nesse estudo, sendo a única diferença entre dois lotes de ração é que um deles vai conter 100 mg/kg de curcumina. Teste bromatologicos, teste rancidez e níveis oxidativos serão avaliados na ração por 150 dias. O experimento será realizado em um canil localizado no município de Chapecó- Santa Catarina. Serão utilizados 12 animais com idades entre 1,5 e 2 anos, da raça Bulldog Francês. Inicialmente terá um período de adaptação de 7 dias com a ração controle (sem curcumina) para todos os animais. Na sequência, os animais do grupo controle continuarão recebendo a ração controle, enquanto os animais do grupo testes passarão a receber a ração contendo curcumina por um período de 30 dias. Durante o experimento todos os animais receberão 128 g de ração por dia por animal de forma individual em três arraçoamentos Sangue será coletado para realizar os testes que vão avaliar a saúde dos animais, isto é, bioquímico sérico (colesterol, triglicerídeos, uréia, proteínas totais, albumina, globulina e ALT), hemograma completo, exame de fezes e contagem bacteriana. Também será avaliado as enzimas antioxidantes: SOD (Superóxido Dismutase) e CAT (Catalase), GST e GPx (Glutationa Peroxidase), assim como os agentes oxidantes como EROs (Espécies Reativas de Oxigênio) e TBARS (Substâncias Reativas ao Ácido Tiobarbitúrco) que também será realizado nas rações como o teste rancidez nos dias 0, 30, 60, 90 e 150. Os animais serão pesados no início e final do experimento. ALTERAÇÃO PROPOSTA e JUSTIFICATIVA No projeto já aprovado estava previsto usar 12 cães raça Bulldog Francês. No entanto, o canil que iria disponibilizar os animais teve problemas de sanidade dos animais (cinomose) e informou que não vai colaborar com a pesquisa. Em virtude disso, nos compraremos cães da raça Beagle machos, a raça mais usada em pesquisas de nutrição animal. Nenhum outro procedimento será alterado. As únicas alterações são referentes a raça (Beagle) do cão, assim como usaremos somente machos na fase de crescimento. O NÚMERO DE ANIMAIS É O MESMO. Os animais serão mantidos em canil comercial próximo a UDESC em Chapecó, e futuramente serão alojados em canil previsto para ser construído na fazenda experimento do CEO. ".

Comentário da CEUA: "".

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