UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

BIOMARCADORES EM CORDEIRAS (*Ovis aries*) NATURALMENTE INFECTADAS POR PARASITOS GASTRINTESTINAIS SUPLEMENTADAS COM CAROÇO DE ALGODÃO EM SISTEMA INTEGRADO DE PRODUÇÃO AGROPECUÁRIA

VITOLDO ANTONIO KOZLOWSKI NETO

Botucatu – SP
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VITOLDO ANTONIO KOZLOWSKI NETO

Dissertação apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para a obtenção do título de Mestre.

Orientador: Prof. Dr. Alessandro Francisco Talamini do Amarante

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LISTA DE TABELAS

LISTA DE FIGURAS

SUMÁRIO

KOZLOWSKI NETO, V. A. **Biomarcadores em cordeiras (*Ovis aries*) naturalmente infectadas por parasitos gastrintestinais suplementadas com caroço de algodão em sistema integrado de produção agropecuária.** Botucatu, 2022. 100p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho".

RESUMO

Em animais de fazenda, as perdas econômicas são amplamente descritas devido aos parasitos. Para auxiliar no monitoramento dessas infecções e na suplementação alimentar, as análises bioquímicas e biomarcadores do estresse oxidativo estão se tornando ferramentas importantes na Medicina Veterinária. A presente investigação teve como objetivo avaliar os níveis séricos de biomarcadores do estresse oxidativo e análises bioquímicas em cordeiras cruzadas, naturalmente infectadas por nematódeos gastrintestinais e *Eimeria* spp., com e sem a inclusão do caroço de algodão nas dietas, em sistema integrado de produção agropecuária (SIPA). O experimento foi realizado na Fazenda Experimental do Lageado, pertencente à FMVZ/UNESP, com 36 cordeiras cruzadas (cruzamento: Ile de France x White Dorper x Texel), separadas em 18 animais para cada tratamento (com inclusão de caroço de algodão e controle, sem inclusão de caroço de algodão) na fase de recria. O peso corporal, coleta de sangue e análise fecal de contagens de ovos e oocistos por grama de fezes (OPG e OoPG, respectivamente) foram realizados para cada animal a cada 14 dias (total de sete tempos). O soro obtido foi avaliado quanto às concentrações das análises bioquímicas: haptoglobina, albumina, globulina, proteína total, colesterol e de biomarcadores do estresse oxidativo: capacidade antioxidante cúprica redutora (CUPRAC: *cupric reducing antioxidant capacity*), capacidade de redução férrica do plasma (FRAP: *ferric reducing ability of plasma*), capacidade antioxidante equivalente ao Trolox (TEAC: *Trolox equivalent antioxidant capacity*), tiol, ácido úrico, paraoxonase-1 (PON-1), estado oxidante total (TOS: *total oxidant status*), método férrico-xilenol (FOX: *ferric-xylenol orange*), produtos da oxidação avançada de proteínas (AOPP: *advanced oxidation protein products*) e derivados de metabólicos reativos de oxigênio (d-ROMs: *reactive oxygen metabolites derived compounds*). A haptoglobina estava abaixo do limite de detecção do ensaio para todos os animais em todos os tempos. O tratamento com inclusão de caroço de algodão apresentou maiores ($p < 0,05$) concentrações de TEAC, AOPP e d-ROMs. Enquanto que a contagem de OoPG, albumina, tiol e FOX foram maiores ($p < 0,05$) para o tratamento controle. Não foram observadas diferenças significativas entre os tratamentos ($p > 0,05$) para a contagem de OPG, peso corporal, ganho de peso médio diário e outras variáveis. A inclusão de caroço de algodão sugeriu o benefício no controle de *Eimeria* spp. além de induzir aumento de oxidantes e antioxidantes em cordeiras naturalmente infectadas por parasitos gastrintestinais. A combinação de caroço de algodão e SIPA pode ser útil no controle de infecções por endoparasitos sem afetar o desempenho da produção.

Palavras-chave: Nematódeos gastrintestinais; *Eimeria* spp.; Estresse oxidativo; Inflamação.

KOZLOWSKI NETO, V. A. **Biomarkers in lambs (*Ovis aries*) naturally infected by gastrointestinal parasites supplemented with whole cottonseed in an integrated crop-livestock system.** Botucatu, 2022. 100p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista “Júlio de Mesquita Filho”.

ABSTRACT

In farm animals, economic losses are widely described due to parasites. To assist in monitoring these infections and the feed supplementation, the biochemical analytes and the oxidative stress biomarkers are becoming important tools in Veterinary Medicine. The present investigation aimed to evaluate serum levels of oxidative stress biomarkers and biochemicals analytes in crossbred lambs naturally infected by gastrointestinal nematodes and *Eimeria* spp., with and without the dietary inclusion of whole cottonseed (WCS) in an integrated crop-livestock system (ICLS). The experiment was carried out at the Experimental Farm of Lageado, belonging to the FMVZ/UNESP, with 36 crossbred lambs (cross: Ile de France x White Dorper x Texel), separated into 18 animals for each treatment (with WCS inclusion and control, without WCS inclusion) in the rearing phase. Body weight, blood collection and faecal analysis of egg and oocyst counting per gram of faeces (EPG and OPG, respectively) were performed for each animal every 14 days (total of seven time points). The serum obtained were evaluated for concentrations of biochemical analytes: haptoglobin, albumin, globulin, total protein, cholesterol and biomarkers of oxidative stress: cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), trolox equivalent antioxidant capacity (TEAC), thiol, uric acid, paraoxonase-1 (PON-1), total oxidant status (TOS), ferric-xylenol orange (FOX), advanced oxidation protein products (AOPP) and reactive oxygen metabolites derived compounds (d-ROMs). The haptoglobin were below the detection limit of the assay for all animals at all time points. The treatment with the WCS inclusion had higher ($p < 0.05$) TEAC, AOPP, and d-ROMs concentrations. Whereas the OPG counting, albumin, thiol and FOX were higher ($p < 0.05$) for the control treatment. No significant differences were observed between treatments ($p > 0.05$) for the EPG counting, body weight, average daily weight gain and other variables. The inclusion of WCS suggested the benefit in controlling *Eimeria* spp. infection as well as inducing increase in oxidants and antioxidants in lambs naturally infected by gastrointestinal parasites. The combination of WCS and ICLS could be useful in controlling endoparasite infection without affecting the production performance.

Keywords: Gastrointestinal nematodes; *Eimeria* spp.; Oxidative stress; Inflammation.

CAPÍTULO I

1 INTRODUÇÃO

O principal problema sanitário envolvendo a ovinocultura são as infecções por nematódeos gastrintestinais, e *Eimeria* spp., outro importante parasito que também pode estar envolvido. Como resultado das infecções, perdas econômicas consideráveis ocorrem e por conseguinte a produção pode se tornar inviável (CHARTIER; PARAUD, 2012; AMARANTE, 2014a).

O sistema integrado de produção agropecuária (SIPA) permite o uso de alternativas e estratégias para aumentar a renda da criação de ovinos (Costa et al., 2019). Para suplementar e aprimorar a performance de ovinos neste sistema, um potencial componente na formulação de dietas de ruminantes que pode ser utilizado é o caroço de algodão, em virtude da combinação da alta energia, proteína e fibra no mesmo ingrediente (ROGERS; POORE; PASCHAL, 2002; KNUTSEN et al., 2017).

Piechota-Polanczyk e Fichna (2014) demostraram o envolvimento do estresse oxidativo na patofisiologia de doenças gastrintestinais e o potencial uso de diferentes biomarcadores no monitoramento de tratamento. As diversas funções das proteínas de inflamação e suas utilizações, também são descritas na literatura, auxiliando na proteção e suscetibilidade de diversas doenças infecciosas e não infecciosas (DI MASI et al., 2020). Desta forma, diferentes biomarcadores do estresse oxidativo e da inflamação podem ser utilizados para avaliar e monitorar possíveis alterações fisiopatológicas em ovinos naturalmente infectados por parasitos gastrintestinais.

Dentro do cenário atual, o SIPA visa a produção sustentável, incluindo o bem-estar animal, a diminuição do uso de tratamentos indesejados que levam à resistência de diversos microrganismos e a diminuição dos impactos ambientais causados pela produção animal para o consumo humano. Portanto, torna-se relevante estudar biomarcadores aplicados na saúde de ovinos, naturalmente infectados por parasitos gastrintestinais, suplementados com inclusão do caroço de algodão em dietas em SIPA.

2 REVISÃO DE LITERATURA

2.1 Sistema Integrado de Produção Agropecuária

Para maior precisão científica, pretende-se neste presente estudo, adotar a referência determinada pela *Food and Agriculture Organization of the United Nations* (FAO), onde o sistema integrado é denominado em inglês como *Integrated Crop-Livestock System* (ICLS) e em português, conforme a recomendação de Carvalho et al. (2014), sugere-se utilizar Sistema Integrado de Produção Agropecuária, com acrônimo SIPA. Contudo, de acordo com Balbino et al. (2011), os sistemas de integração podem ser definidos em quatro modalidades: agropastoril ou integração lavoura-pecuária (iLP), na qual integra pecuária e lavoura na mesma área, em consórcio, rotação ou sucessão, no mesmo ano ou múltiplos anos; silvipastoril ou integração pecuária-floresta (iPF), na qual integra floresta e pecuária em consórcio; agrossilvipastoril ou integração lavoura-pecuária-floresta (iLPF), na qual integra floresta, pecuária e lavoura na mesma área, em consórcio, rotação ou sucessão; e silviagrícola ou integração lavoura-floresta (iLF), na qual integra lavoura e floresta em consórcio.

O conceito de integração é definido como “[...] A integração bem-sucedida envolve uma integração intencional que reflete na relação de sinergismo entre os componentes [..], culturas, pecuária e/ou árvores e esta relação de sinergismo quando gerenciado corretamente resulta em melhoria social [...], econômica e um ambiente sustentável [...]” (FAO, 2010).

O SIPA apresenta muitos benefícios para a planta, solo e animais. Não é apenas uma combinação no sistema, mas algo complexo que cresce em propriedades com novas funcionalidades, que ainda não foram estudadas e promove a intensificação com sustentabilidade, preservando e recuperando a qualidade ambiental (MORAES et al., 2019).

A diversidade de paisagem e clima da região Sudeste permite a utilização de alternativas produtivas, envolvendo a ovinocultura de corte e leite. A produção de ovinos com o SIPA, permite estratégias para o aumento da renda daqueles que produzem leite, carne, grãos e diminui a sazonalidade de animais para o

abate (COSTA et al., 2019). Outro fator interessante, benéfico para a ovinocultura e produtores, é o controle de nematódeos gastrintestinais no sistema, estudos demonstram a eficácia em produzir pastos livres de contaminação por larvas infectantes destes parasitos (ALMEIDA et al., 2018; COSTA et al., 2019). A utilização de uma boa nutrição associada com estas pastagens, resultam em uma diminuição progressiva da infecção por nematódeos gastrintestinais durante o crescimento do indivíduo, assim como um desempenho considerável no final da terminação e por fim, pode auxiliar na redução de tratamentos com antiparasitários, que aumentam a resistência e elevam os gastos (ALMEIDA et al., 2018).

2.2 Caroço de algodão

A variedade de produtos oriundos do processamento do algodão podem ser ingredientes alimentares valiosos para a formulação de dietas de ruminantes, justamente pelo seu conteúdo nutricional. Como resultado do processamento, incluem os produtos a partir do descaroçamento (*e.g.* caroço de algodão e lixo do descaroçamento), processamento das sementes de algodão (*e.g.* línter de algodão, sementes de algodão deslintadas, casca e farelo de sementes de algodão), e moagem têxtil do algodão (*e.g.* resíduos de cardagem e do moinho) (ROGERS; POORE; PASCHAL, 2002). Desta forma, o caroço de algodão é considerado um subproduto da indústria têxtil a partir do descaroçamento do algodão, proveniente da planta algodoeira (*Gossypium* L.). Possui alta fonte de energia associada com alta proporção de proteína e fibra (ROGERS; POORE; PASCHAL, 2002; KNUTSEN et al., 2017), e ainda considerado como uma fonte suplementar às forragens para ruminantes (ROGÉRIO et al. 2003).

O caroço de algodão é composto por fibra (línter e sobras de plumas), casca e amêndoa (ROGÉRIO et al., 2003). De acordo com o *National Research Council* – NRC (2007) o caroço de algodão com línter apresenta 23% de proteína bruta, 95% de nutrientes digestíveis totais, 17,8% de extrato etéreo, 39% de fibra em detergente ácido e 47% de fibra em detergente neutro, respectivamente na matéria seca. A amêndoa do caroço de algodão é composta por óleo rico em ácidos graxos essenciais (ácido linoleico), que por sua vez possui efeitos positivos nas funções imunomoduladoras e na saúde cardiovascular, assim

como, tocoferóis (conferindo propriedades antioxidantes naturais), carboidratos, minerais, proteína e entre outros (SIHAG; PATEL; KUMAR, 2021). Contudo, o caroço de algodão também apresenta um composto conhecido como gossipol.

O gossipol é um composto polifenólico de cor amarela, secretado por glândulas pigmentares localizadas nas raízes, hastes, folhas e nas sementes (maiores concentrações encontradas) do algodoeiro, além de promover resistência contra danos por insetos (BOTTGER; SHEEHAN; LUKEFAHR, 1964; ROGÉRIO et al., 2003; SIHAG; PATEL; KUMAR, 2021). Para animais monogástricos é altamente tóxico, porém, ruminantes são mais resistentes ao composto devido à fermentação ruminal, ligando-se ao gossipol e consequentemente reduzindo os efeitos colaterais tóxicos (KNUTSEN et al., 2017; SIHAG; PATEL; KUMAR, 2021). A toxicidade ao gossipol em ruminantes é predisposto de acordo com a idade, função ruminal e conteúdo de proteína, duração da ingestão (pelo efeito cumulativo) e de minerais da ração (ROGÉRIO et al., 2003).

Diante do exposto, o caroço de algodão tem sido descrito e utilizado como um potencial componente para a formulação de dietas para ovinos (CUNHA et al., 2008; MADRUGA et al., 2008; GERON et al., 2012). Kandylis et al. (1998) reportaram o caroço de algodão como um ingrediente alimentar satisfatório para ovinos em crescimento, sendo que sua inclusão nas dietas aumentou o consumo de alimento e ganho de peso vivo. Em adição, se tornou aceito como alternativa a suplementação por grão de cereal nas rações, apresentando diminuição nos custos por quilograma do ganho de peso vivo e consequentemente conferindo vantagem econômica.

2.3 Parasitos gastrintestinais de ovinos

2.3.1 Nematódeos

A classificação dos parasitos provém de análises morfológicas e moleculares, possibilitando o estudo pela taxonomia destes organismos. Uma vez identificados, são classificados por táxon de acordo com o padrão internacional, sendo os principais: reino, filo, classe, ordem, família, gênero e

espécie. Alguns organismos não podem ser alocados de forma precisa nos táxons principais, então há uma classificação de táxons intermediários. Um exemplo de táxon intermediário é a superfamília Trichostrongyloidea, com os representantes desta superfamília sendo denominados tricostrongilídeos (TAYLOR; COOP; WALL, 2017).

Estas classificações apresentam constantes alterações de acordo com os avanços científicos, desta forma, é indicada a atualização de profissionais que necessitam reportar organismos (MATHISON; BRADBURY; PRITT, 2021). A classe Nematoda é um exemplo (TAYLOR; COOP; WALL, 2007; AMARANTE, 2014a; MONTEIRO, 2017) que em outras literaturas, é possível encontrar como filo Nematoda (ANDERSON, 2000; BOWMAN, 2010; TAYLOR; COOP; WALL, 2017), de qualquer forma, os autores se referem aos nematódeos que apresentam boca, esôfago, corpo cilíndrico com pseudoceloma, intestino, sistema genital feminino ou masculino e ânus (BOWMAN, 2010; AMARANTE, 2014a; MONTEIRO, 2017; TAYLOR; COOP; WALL, 2007, 2017).

Em pequenos ruminantes, os nematódeos gastrintestinais com maior importância pertencem a ordem Strongylida e a maioria são da superfamília Trichostrongyloidea. *Haemonchus contortus* é o estrongilídeo mais importante nos Estados Unidos da América (EUA) e a nível global. Além deste, *Ostertagia* (*Teladorsagia*) *circumcincta* é o segundo nematódeo mais importante nos EUA e em terceiro, o gênero *Trichostrongylus* com casos de infecções até em seres humanos (ZAJAC, 2006). A infecção por *Trichostrongylus* é mais comum em produtores de ovinos e caprinos, mas também pode ser adquirida pelo contato com fezes de outros animais infectados, como bovinos, camelídeos e muares. Na Austrália indivíduos infectados usavam fezes de caprinos como fertilizantes para vegetais consumidos (RALPH et al., 2006) e casos similares em pequenas propriedades nos EUA também ocorreram (ZAJAC, 2006).

No Brasil, *H. contortus* seguido de *Trichostrongylus colubriformis*, são os principais nematódeos gastrintestinais em ovinos (AMARANTE, 2014b). Em um estudo realizado por Almeida et al. (2018) em São Paulo, com ovinos adquiridos no Rio Grande do Sul, a maior porcentagem de larvas infectantes recuperadas

de coproculturas foram as do gênero *Haemonchus*, seguido de *Trichostrongylus* e *Cooperia*.

Haemonchus contortus é um parasito hematófago do abomaso de pequenos ruminantes e quando o hospedeiro apresenta altas infecções, pode ir a óbito pelo resultado da anemia severa (AMARANTE, 2014a). Outras características importantes deste parasito é a alta prolificidade e por não ser a causa primária de diarreia, dificulta a detecção de animais infectados pelos produtores, até o momento em que a morte ocorra (ZAJAC, 2006). *Trichostrongylus colubriformis* infecta o intestino delgado, criam túneis, consequentemente atrofia das vilosidades, enterites severas e comprometimento da absorção (AMARANTE, 2014a).

A compreensão do ciclo e comportamento destes nematódeos é fundamental para aplicação de manejos e a fim de diagnosticar, tratar e controlar as infecções. Partindo do momento em que fezes com a presença de ovos de nematódeos gastrintestinais são eliminados para o meio ambiente, o desenvolvimento destes ovos morulados (classificados como do tipo estrongilídeos) seguem para um estágio denominado de embrião vermiforme (ovos larvados), que por sua vez eclode destes ovos como larvas de primeiro estágio (L1), das quais se alimentam de microrganismos e matéria orgânica. Após o desenvolvimento destas larvas de primeiro estágio, ocorre uma primeira muda, originando uma larva de segundo estágio (L2) que continua se alimentando e realiza uma segunda muda de transição para o terceiro estágio (L3). As L3 são infectantes, ou seja, quando o hospedeiro realiza a ingestão do pasto contaminado contendo estas larvas infectantes, tem a capacidade de continuar seu ciclo evolutivo no trato gastrintestinal do mesmo. Este estágio é resistente ao meio ambiente por possuir duas cutículas, uma nova cutícula deste estágio somada com a do estágio anterior. Neste momento as L3 não se alimentam no meio ambiente e a sobrevivência é devido as reservas acumuladas nos primeiros estágios (L1 e L2). Quando as L3 de *H. contortus* atingem o rúmen, perdem a bainha (cutícula externa) e ao chegarem no abomaso, as L3 penetram na mucosa. Ocorre o desenvolvimento e uma terceira muda para então atingir o quarto estágio larval (L4). Por fim, na luz do abomaso, ocorre a quarta e última

muda de transição para o quinto estágio (L5) que completa seu desenvolvimento em parasitos adultos, os quais realizam a cópula e oviposição, completando o ciclo (BOWMAN, 2010; AMARANTE, 2014a).

Todo o processo do ciclo parasitário que ocorre no hospedeiro, iniciando com a ingestão do parasito, desenvolvimento até atingir a maturidade sexual e eliminação de ovos, é denominado como período pré-patente (PPP) (MONTEIRO, 2017).

2.3.2 *Eimeria* spp.

Dentre outros parasitos gastrintestinais temos como exemplo os protozoários, organismos eucarióticos, unicelulares primitivos (TAYLOR; COOP; WALL, 2017). São caracterizados por apresentar um estágio infectante estável e resistente no ambiente, como oocistos, permitindo a transmissão pela água ou alimentos (CAMA; MATHISON, 2015).

A coccidiose ou eimeriose é a doença causada pelo protozoário do gênero *Eimeria* (AMARANTE, 2014a). Os cordeiros são mais suscetíveis (KEETON; NAVARRE, 2018), podem se infectar logo após o nascimento, acarretando em eliminação de oocistos nas fezes com poucas semanas de vida, com ápice na eliminação de oocistos entre 4 a 8 semanas de idade (AMARANTE; BARBOSA, 1992).

A coccidiose em ovinos leva a redução do bem-estar, aumento da mortalidade e perdas econômicas na produção em todo o mundo (ODDEN et al., 2018). O impacto econômico gerado de forma significativa ocorre tanto pela doença subclínica quanto pela doença clínica (KEETON; NAVARRE, 2018).

Os ovinos podem se infectar simultaneamente por várias espécies de *Eimeria*, porém espécies como *Eimeria crandallis* e *Eimeria ovinoidalis* são consideradas como altamente patogênicas. As lesões intestinais são observadas em infecções maciças, podendo resultar em hemorragia, má absorção, diarreia, desidratação e morte (AMARANTE, 2014a; TAYLOR; COOP; WALL, 2017), contudo, em infecções discretas a absorção também é prejudicada pelo efeito na mucosa, provocando alterações nas vilosidades, diminuindo a altura das

células epiteliais, resultando em menor absorção e menor eficiência alimentar (TAYLOR; COOP; WALL, 2017).

Seu ciclo de vida é composto por multiplicação assexuada e sexuada. No intestino do hospedeiro ocorre a formação de oocistos pela forma sexuada, que posteriormente são eliminados em conjunto com as fezes (BOWMAN, 2010; AMARANTE, 2014a). A forma infectante encontrada no meio ambiente e que possui capacidade de infectar o hospedeiro é o oocisto esporulado, com quatro esporocistos, contendo dois esporozoítas cada (BOWMAN, 2010).

2.4 Proteínas de fase aguda

As proteínas de fase aguda (PFA) foram identificadas em doenças infecciosas como reagentes precoces no início do ano de 1900 (CRAY, 2012) e mostraram ser biomarcadores importantes que aumentam em infecções, inflamações, estresse, trauma e neoplasias (CRAY, 2012; TOTHOVA; NAGY; KOVAC, 2014). Possui aplicabilidade inicial como prognóstico, contudo, estudos demostraram relevância na detecção, diagnóstico e monitoramento de doenças subclínicas (CRAY, 2012).

As PFA são parte das respostas de fases agudas (RFA) sistêmicas, que por sua vez são respostas fisiológicas após danos aos tecidos, iniciado por estímulos externos e internos que envolvem a inflamação. Estas respostas fazem parte e são reconhecidas pela defesa imune inata com o propósito de reestabelecer a hemostasia e promover a cura (Figura 1) (CECILIANI et al., 2012; CRAY, 2012). Acredita-se que o papel principal na RFA sistêmicas da inflamação são os das PFA, que inclui, a regulação dos estágios da inflamação, opsonização de patógenos e eliminação de substâncias tóxicas (CECILIANI et al., 2012). Em seres humanos e animais sadios as PFA apresentam valores insignificantes ou não determinados, o que não acontece em casos de RFA, que durante a resposta, as concentrações de PFA aumentam (ILIEV; GEORGIEVA, 2018).

Com o passar dos anos e avanço de ensaios, os biomarcadores da inflamação estão disponíveis para serem aplicados em seres humanos e todas

as áreas da medicina veterinária, desde pesquisa básica até pesquisas clínicas (CRAY, 2012).

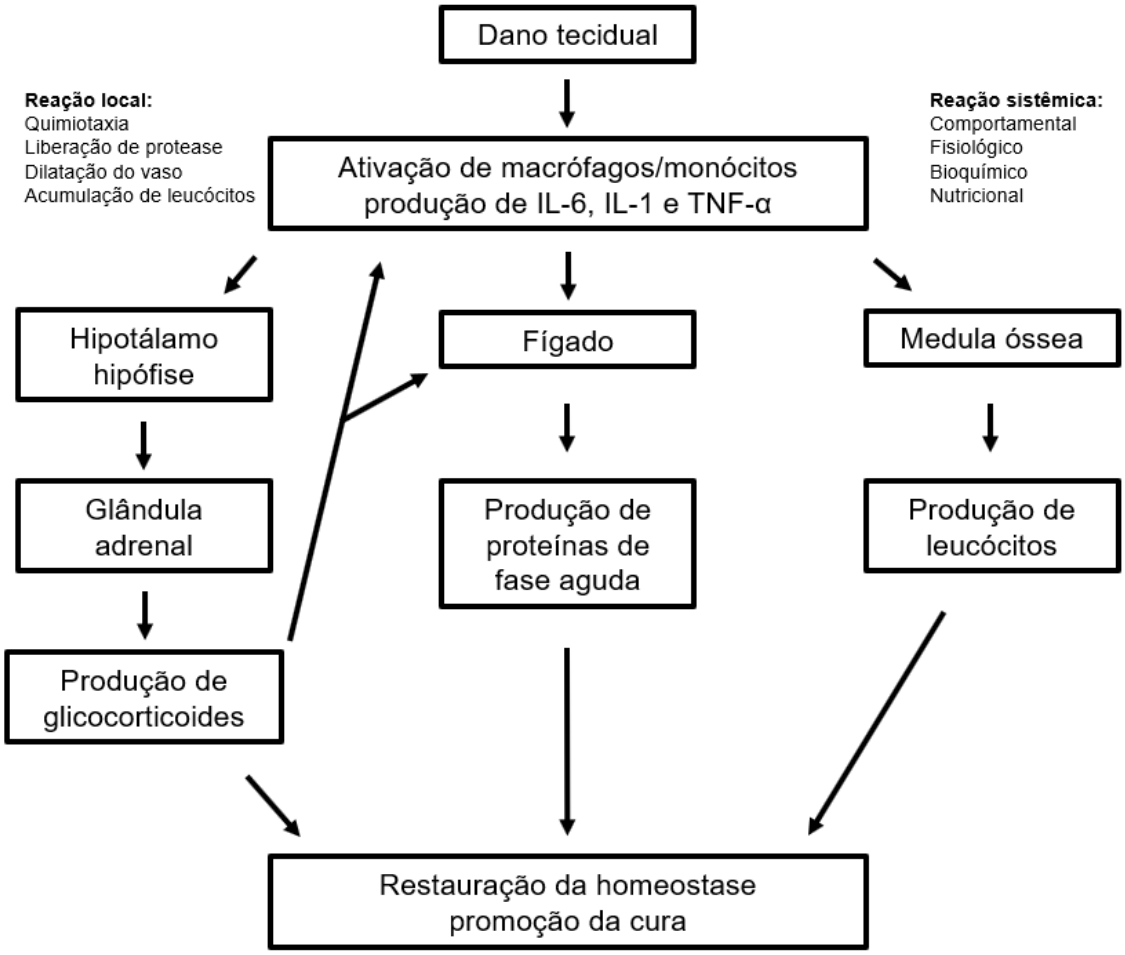

Figura 1. Resposta da fase aguda. Início da produção de proteínas de fase aguda após dano tecidual, envolvendo a ativação de macrófagos/monócitos, liberação de citocinas pró-inflamatórias como interleucina 6, 1 e fator de necrose tumoral alfa. Seguido da ativação do fígado e outros tecidos, induzindo a síntese de proteínas de fase aguda. Fonte: Adaptado de Cray, 2012.

As PFA, de uma forma geral, podem ser classificadas de acordo com sua função e quantidade de variação (ILIEV; GEORGIEVA, 2018). Em relação a diminuição e aumento durante a RFA, são determinadas como negativas ou positivas. Quando há diminuição das concentrações de proteínas, são consideras negativas. As positivas por sua vez, aumentam suas concentrações e são as proteínas mais descritas. A classificação pelo aumento da magnitude das PFA positivas é considerada informal, pois há desacordo com a literatura, depende do tipo de estímulo, do ensaio utilizado e diferem entre espécies. As

PFA principais (*major*) são aquelas que aumentam entre 10 a 1000 vezes e respondem de forma precoce, com grandes aumentos após estímulos entre 24 e 48 horas. Com o organismo hígido, não são detectadas ou se forem, apresentam baixas concentrações. As PFA moderadas aumentam entre 5 a 10 vezes, com picos entre três ou mais dias e apresentam níveis basais maiores do que as anteriormente citadas. As PFA menores (*minor*) apresentam aumentos inferiores a duas vezes e de forma lenta (CRAY, 2012; ILIEV; GEORGIEVA, 2018).

2.4.1 Haptoglobina

A haptoglobina (Hp) faz parte do grupo das PFA positivas. É produzida principalmente pelos hepatócitos e é composta por dois alfa e beta subunidades. Sua principal atividade é a afinidade pela hemoglobina (Hb) livre, onde ocorre ligação após sua liberação em casos de doenças infecciosas, hereditárias ou autoimune. Os macrófagos/monócitos realizam a fagocitose deste complexo formado (Hp-Hb) por meio do receptor CD163 (CECILIANI et al., 2012; CRAY, 2012).

Apresenta várias funções biológicas como: antioxidante, reduzindo o dano oxidativo da Hb pela estabilização do ferro, anti-inflamatória, atuando durante a resposta da imunidade e bacteriostática, que reduz a disponibilidade do ferro utilizado por bactérias (CECILIANI et al., 2012; DI MASI et al., 2020). Durante a hemólise, a Hp sequestra a Hb formando um grande complexo Hp-Hb, que por sua vez não permite a eliminação pela filtragem glomerular (CECILIANI et al., 2012; ILIEV; GEORGIEVA, 2018; DI MASI et al., 2020).

É considerada a principal PFA em caprinos e ovinos (Tabela 1) (CECILIANI et al., 2012; ILIEV; GEORGIEVA, 2018). Em um estudo para validação de métodos analíticos, realizado por González et al. (2008) em caprinos, após aplicação de óleo de terebintina, foi possível obter concentrações de Hp 80 vezes maiores depois de três dias de avaliação, partindo dos valores mínimos de 0,02 g/ L pelo ensaio utilizado, sendo que na maioria dos animais saudáveis, as concentrações de Hp são insignificantes.

Dinler et al. (2017) observaram aumento significativo da concentração de Hp no soro de ovinos neonatos infectados experimentalmente com *Cryptosporidium parvum* no segundo e sexto dia pós-inoculação, assim como uma moderada correlação entre a concentração de Hp e o número de oocistos contabilizados.

Tabela 1. Proteínas de fase aguda em ovinos e caprinos (Fonte: Adaptado de Iliev; Georgieva, 2018).

PFA		Ovinos	Caprinos
Positivas	Principais	Hp, SAA	Hp, SAA
	Moderadas	AGP	Fb, AGP
	Menores	Fb, Cp	Cp
Negativas		Alb	Alb

PFA= Proteínas de fase aguda; Hp= Haptoglobina; SAA= Amiloide A sérico; AGP= α_1-glicoproteína ácida; Fb= Fibrinogênio; Cp= Ceruloplasmina; Alb= Albumina.

2.4.2 Albumina

A principal PFA negativa em ruminantes é a albumina (Alb) sérica (Tabela 1). É encontrada no sangue de seres humanos e animais como a proteína mais abundante, representando 35 – 50% da proteína total. A Alb apresenta funções como: fonte de aminoácidos para síntese de PFA positivas, é responsável por aproximadamente 75% da pressão osmótica no plasma por conta de seu tamanho pequeno, atua como proteína transportadora de diversas substâncias e também é um indicador nutricional, pois apresenta uma meia vida relativamente longa de aproximadamente 14-20 dias (TOTHOVA; NAGY; KOVAC, 2014; ILIEV; GEORGIEVA, 2018).

Diversos fatores acarretam na sua diminuição, como por exemplo: hepatopatias, má nutrição, nefropatias, doenças gastrintestinais, edema ou em casos de RFA, onde a síntese hepática de albumina é reduzida durante a inflamação por conta da demanda da síntese de outras PFA positivas (CECILIANI et al., 2012; CRAY, 2012; ILIEV; GEORGIEVA, 2018).

Caprinos avaliados durante RFA apresentaram diminuição significativa (< 10%) dos valores séricos de Alb após 48 horas, devido as condições inflamatórias (GONZÁLEZ et al., 2008).

Chaichisemsari et al. (2011) avaliaram alguns parâmetros bioquímicos em ovinos naturalmente infectados por nematódeos gastrintestinais e relataram uma correlação positiva significativa entre contagens ovos por grama de fezes (OPG) e concentrações de Alb. Neste mesmo estudo foi possível concluir que em infecções com eliminação de ovos menores do que 850 OPG, não apresentaram efeitos significativos em proteínas totais, globulinas e albumina, mas com o aumento da carga de infecção por nematódeos gastrintestinais, as concentrações de globulinas aumentavam, por outro lado as concentrações de proteína total e Alb reduziam de forma não significativa.

Ovinos da raça Santa Inês infectados artificialmente por *T. colubriformis* quando comparados com o grupo controle, não apresentaram diferenças significativas das concentrações de globulina e Alb, porém foram observadas diferenças significativas na redução de eritrócitos, hemoglobina, volume corpuscular médio, hematócrito e proteínas totais, assim como aumento de eosinófilos e plaquetas (embora todos os parâmetros dentro dos valores de referência, com exceção das proteínas totais) (DIAS-SILVA et al., 2020).

Concentrações significativas de Alb foram aproximadamente 10% menores em ovinos infectados por nematódeos gastrintestinais quando comparados com animais sem infecção, uma provável explicação seria pelo motivo do dano intestinal, causado pelos próprios nematódeos gastrintestinais e uma segunda explicação poderia ser pelo fato da redução da síntese durante RFA para então disponibilizar os aminoácidos e consequentemente realizar a síntese das PFA, como anteriormente já discutido (NAGY et al., 2020).

2.5 Estresse oxidativo e biomarcadores

O desequilíbrio entre antioxidantes (redutores) e oxidantes, nas células ou no organismo, pode ser caracterizado e comumente definido como estresse oxidativo (LYKKESFELDT; SVENDSEN, 2007; RODRÍGUEZ; MENGE; CERÓN, 2011; RUBIO; CERÓN, 2021).

Nos animais de produção, o estresse oxidativo pode estar envolvido nas condições patológicas, de produção e de bem-estar (LYKKESFELDT;

SVENDSEN, 2007). Há um aumento de evidências que o papel principal na patogenia e etiologia de diversas doenças metabólicas na Medicina Veterinária são frutos do estresse oxidativo, além de prejudicar funções orgânicas de formas significativas, contudo ainda não está claro se os oxidantes são produzidos a partir de danos gerais nos tecidos, secundários em consequência de doenças ou se são os próprios que iniciam as doenças (RODRÍGUEZ; MENGE; CERÓN, 2011).

Baptistiolli et al. (2018) avaliaram os biomarcadores do estresse oxidativo em ovinos da raça Suffolk e Santa Inês infectados artificialmente com *H. contortus*. Diferenças significativas foram detectadas durante o período experimental, assim como a diferença do perfil dos biomarcadores entre as duas raças estudadas. Pivoto et al. (2015) concluíram que o estresse oxidativo poderia ser reduzido em ovinos com infecções por *H. contortus*, quando tratados com edetato de zinco. Outro estudo avaliou a relação entre o estresse oxidativo em ovinos naturalmente e artificialmente infectados por *H. contortus* (MACHADO et al., 2014). Alterações nos biomarcadores foram observados, indicando danos celulares e com o propósito de proteção, assim como achados histológicos confirmando a lesão no abomaso. Complementando estes estudos, outras pesquisas também demonstraram a correlação entre estresse oxidativo e infecções experimentais ou naturais por parasitos gastrintestinais (SMITH; BRYANT, 1989; FARID et al., 2008, 2009; PATHAK et al., 2017; RASHID; IRSHADULLAH, 2020).

Os oxidantes são compostos com potencial de oxidar moléculas e podem ser divididos em diversos grupos, dependendo da sua reatividade ou natureza química. O grupo composto por radicais livres, apresentam elétrons desemparelhados em sua última camada (número ímpar), que confere instabilidade e alta reatividade, portanto incluem o radical hidroxila ($OH^{\bullet}$), superóxido ($O_2^{\bullet -}$), óxido nítrico ($NO^{\bullet}$) e entre outros. Outro grupo caracterizado como oxidantes não-radicais (sem radicais livres), mas que confere alta reatividade, incluem o peróxido de hidrogênio (H_2O_2), peroxinitrito ($ONOO^-$), ácido hipocloroso (HOCl) e entre outros (FERREIRA; MATSUBARA, 1997; LYKKESFELDT; SVENDSEN, 2007).

Existem diversas fontes de oxidantes endógenas e de espécies reativas (Figura 2), que fazem parte do metabolismo e das condições fisiológicas (VASCONCELOS et al., 2007), que por sua vez engloba os derivados do metabolismo do oxigênio (O_2) e são determinadas como espécies reativas de oxigênio (ERO) (RUBIO; CERÓN, 2021). A cadeia de transporte de elétrons normalmente na mitocôndria, é composto por ERO, onde o O_2 sofre redução até a formação de água (H_2O) e esta reação é neutralizada pela entrada de elétrons (FERREIRA; MATSUBARA, 1997; LYKKESFELDT; SVENDSEN, 2007). Durante este processo, algumas moléculas de oxigênio se comportam como ERO intermediários, resultando em níveis basais de oxidantes no organismo (LYKKESFELDT; SVENDSEN, 2007).

Outros fatores que podem contribuir na produção de oxidantes são as reações imunológicas, especialmente durante a defesa contra infecções ou pela resposta autoimune, por enzimas que produzem espécies reativas e reações catalíticas de metais, que também geram espécies reativas por meio das reações de Fenton e de Haber-Weiss (FERREIRA; MATSUBARA, 1997; LYKKESFELDT; SVENDSEN, 2007; VASCONCELOS et al., 2007). A carga de oxidantes também pode ser suplementada por fontes exógenas (Figura 2) de forma indireta ou direta. O que inclui poluentes no ar, gases tóxicos, desinfetantes oxidantes, toxinas, químicos, radiação, microrganismos e dietas inadequadas (LYKKESFELDT; SVENDSEN, 2007).

A oxidação pode resultar em danos celulares, sobre as proteínas, enzimas, aminoácidos e sobre os lipídeos, por serem os principais constituintes da membrana celular, por apresentar facilidade de oxidação dos ácidos graxos insaturados, consequentemente desencadear uma reação em cadeia, causando danos na integridade celular, alterando a permeabilidade, fluidez, propriedades químicas e físicas das membranas, por fim, levando a morte celular. Outro dano que também pode ocorrer, é no ácido desoxirribonucleico (DNA: *deoxyribonucleic acid*), acarretando em modificações, mutações, inativação e apoptose (LYKKESFELDT; SVENDSEN, 2007; VASCONCELOS et al., 2007).

Grandes números de oxidantes provenientes de fontes exógenas e endógenas, desempenham papéis importantes na fisiologia e expõem de forma

contínua todas as células que vivem em condições aeróbicas. Desta forma, o aumento da produção de oxidantes não está necessariamente associado ao dano oxidativo (apenas se ocorrer um desequilíbrio) e se os resultados não forem correlacionados (marcadores do estresse oxidativo e danos oxidativos), pode então resultar em uma difícil interpretação (LYKKESFELDT; SVENDSEN, 2007).

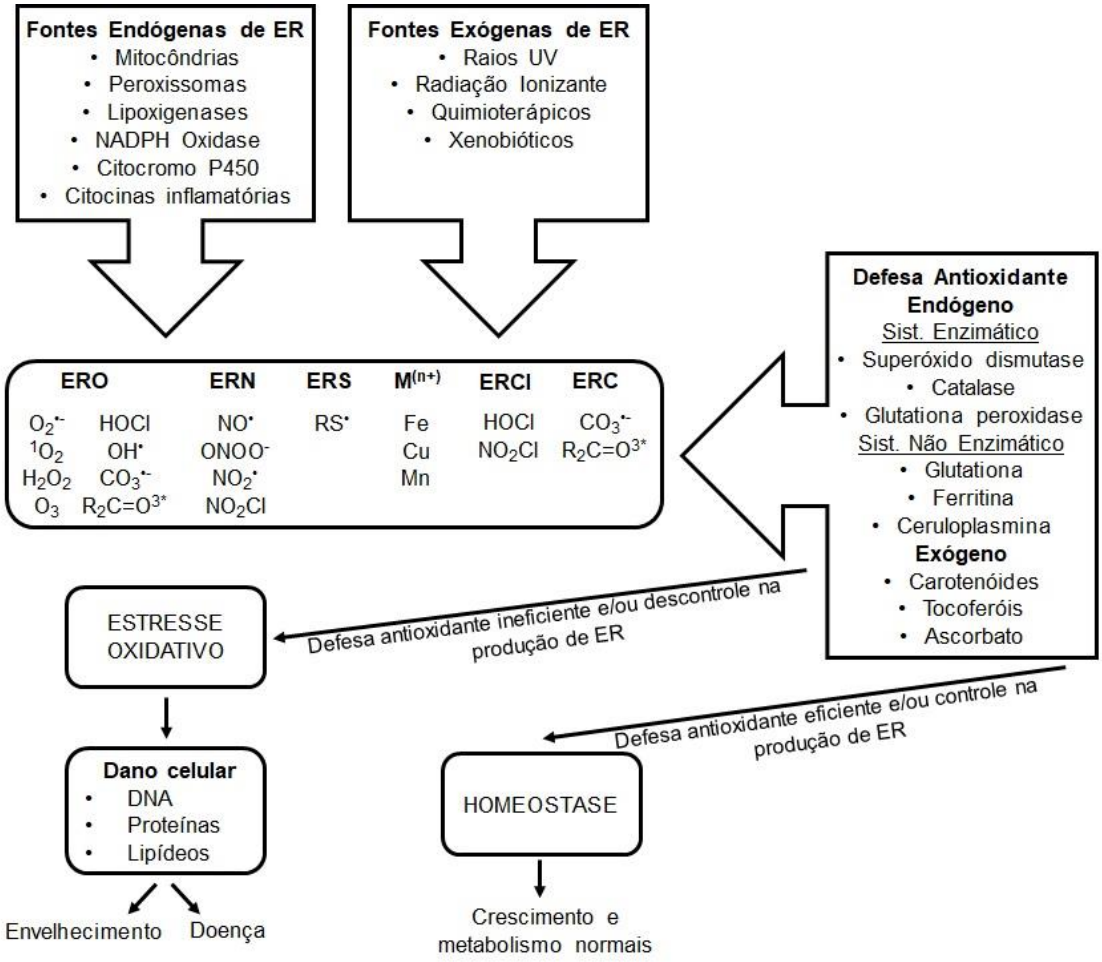

Figura 2. Fontes endógenas e exógenas de espécies reativas (ER). Espécies reativas de oxigênio (ERO), espécies reativas de nitrogênio (ERN), espécies reativas derivados do enxofre (ERS), metais de transição [$M^{(n+)}$], espécies reativas de cloro (ERCl) e espécies reativas de carbono (ERC). Sistema de defesa antioxidante, endógeno e exógeno, atuando para controlar a produção de espécies reativas, a fim de promover a homeostase. Resultado de estresse oxidativo e consequentemente danos celulares a partir da produção descontrolada de espécies reativas. Fonte: Adaptado de Finkel; Holbrook, (2000); Vasconcelos et al. (2007).

No momento em que ocorre uma produção descontrolada de espécies reativas, sendo derivadas do oxigênio, nitrogênio e/ou outras, o organismo apresenta um eficiente mecanismo de controle e restabelecimento do equilíbrio via antioxidantes (VASCONCELOS et al., 2007) endógenos e exógenos, que atuam na proteção, reparação, interceptação e prevenção dos danos causados pelo estresse oxidativo (RUBIO; CERÓN, 2021). Os antioxidantes endógenos são classificados em enzimáticos e não enzimáticos. O superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), são representantes enzimáticos e por sua vez, a glutationa (GSH), ferritina, ceruloplasmina e ácido úrico, são representantes não enzimáticos. Os antioxidantes exógenos são obtidos por meio da dieta, como carotenóides, tocoferóis e ascorbato (VASCONCELOS et al., 2007; RUBIO; CERÓN, 2021).

É importante ressaltar a respeito das funções benéficas e importantes dos antioxidantes como já foram abordados, porém os mesmos podem apresentar danos aos organismos quando em excesso e em casos extremos, morte por conta da suplementação com antioxidantes. Não se sabe exatamente quais mecanismos que podem desencadear tal processo, mas resultados apontam para o desequilíbrio íntimo entre antioxidantes e espécies reativas, quando os antioxidantes neste caso se prevalecem (VILLANUEVA; KROSS, 2012). Portanto, o uso indiscriminado de antioxidantes exógenos deve ser avaliado de forma criteriosa frente a terapêutica de doenças envolvidas com o estresse oxidativo (FERREIRA; MATSUBARA, 1997).

O sangue e outros fluídos biológicos, como o exemplo a saliva e urina, são fontes de biomarcadores, utilizados para avaliar o estresse oxidativo (RUBIO et al., 2016, 2019; GYURÁSZOVÁ et al., 2018; RIVERA-GOMIS et al., 2020; RUBIO; CERÓN, 2021).

No momento de uma avaliação, monitoramento e identificação do estresse oxidativo associadas com doenças, os biomarcadores antioxidantes, oxidantes, elementos traços e proteínas da inflamação deveriam ser utilizados em conjunto, tornando-se ferramentas importantes para tal propósito (RUBIO; CERÓN, 2021).

A determinação dos antioxidantes é realizada por meio de biomarcadores agrupados de acordo com uma estimativa total da capacidade ou por uma determinação precisa por marcadores individuais (LYKKESFELDT; SVENDSEN, 2007).

2.5.1 Capacidade antioxidante total

A capacidade antioxidante total (TAC: *total antioxidant capacity*) é frequentemente utilizada para dar acesso ao estado antioxidante de amostras biológicas de uma forma global, onde pode ser avaliada a resposta de radicais livres produzidos por uma doença (RUBIO et al., 2016). A literatura relata diversos ensaios que depende do tipo de radicais formados reagindo com uma molécula, do método utilizado para a determinação, quantificação e indicadores de oxidação selecionados (VASCONCELOS et al., 2007). Para a avaliação da TAC é recomendada a utilização de vários ensaios de forma integrada, pois dependendo do ensaio utilizado, pode resultar em diferenças e ainda não há método ideal. Desta forma, são utilizados em conjuntos: a capacidade antioxidante cúprica redutora (CUPRAC: *cupric reducing antioxidant capacity*), a capacidade de redução férrica do plasma (FRAP: *ferric reducing ability of plasma*), sendo estas classificadas como ensaios indiretos que por sua vez possuem a capacidade de reduzir complexos de metais. Assim como os ensaios diretos que inibem a oxidação, envolvendo a capacidade antioxidante equivalente ao Trolox (TEAC: *Trolox equivalent antioxidant capacity*) e a capacidade de absorção do radical oxigênio (ORC: oxygen radical absorbace capacity) (RUBIO et al., 2016).

Os ensaios utilizados para o presente estudo experimental foram CUPRAC, FRAP e TEAC. São baseados em mecanismos de transferência de elétron, utilizando reações químicas de forma *in vitro*, análises por meio do instrumento espectrofotômetro e seus resultados devem ser interpretados com cautela (VASCONCELOS et al., 2007; RUBIO et al., 2016).

O ensaio CUPRAC é baseado na mensuração de Cu^{1+} com a presença de um agente quelante, estabilizando o complexo com formação colorimétrica de laranja-amarelada mensurável de absorbância máxima de 450 - 490 nm, após a

redução do Cu^{2+}, em pH 7, por antioxidantes quando presentes nas amostras. O grupo tiol, albumina, ácido úrico, vitaminas C e E, entre outros antioxidantes não enzimáticos são avaliados (HUANG; BOXIN; PRIOR, 2005; CAMPOS et al., 2009; ÖZYÜREK et al., 2011; RUBIO et al., 2016).

O ensaio FRAP é baseado na mensuração de Fe^{2+}-TPTZ (ferritripiridiltriazina), em pH 3,6, que apresenta coloração azul e absorbância máxima de 593 nm, após redução de Fe^{3+}-TPTZ por antioxidantes quando presentes nas amostras. Este ensaio avalia principalmente o ácido úrico, sendo que as vitaminas C e E, bilirrubina, entre outros, também são avaliados, com exceção do grupo tiol que não é avaliado (BENZIE; STRAIN, 1996; HUANG; BOXIN; PRIOR, 2005; VASCONCELOS et al., 2007; RUBIO et al., 2016).

O ensaio TEAC é baseado na incubação de ABTS 2,2'-azino-bis(3-etilbenzo-tiazolina-6-sulfonato, formando um radical $ABTS^{+\bullet}$ de cor azul-esverdeada, com absorbância em 415, 660, 734 e 820 nm, o radical é reduzido ou inibido pela ação dos antioxidantes quando presentes nas amostras, posteriormente é avaliada a capacidade antioxidante por meio da intensidade da descoloração. Este ensaio avalia principalmente albumina, seguido do ácido úrico, assim como, bilirrubina, vitaminas C e E (MILLER et al., 1993; ARNAO et al., 1996; HUANG; BOXIN; PRIOR, 2005; VASCONCELOS et al., 2007; RUBIO et al., 2016).

2.5.2 Tiol

Nas biomoléculas a forma ativa e multifuncional do enxofre reduzido é a sulfidrila (–SH) ou tiol e está presente em proteínas, peptídeos e aminoácidos. Está envolvido em diversos processos fisiológicos e são explorados como biomarcadores nas doenças (OLIVEIRA; LAURINDO, 2018).

Os principais antioxidantes responsáveis por atuar em proteínas plasmáticas são do grupo tiol e ainda apresentam correlação positiva de seus níveis séricos com a TAC, desta forma pode ser utilizado como ferramenta em conjunto com TAC para a determinação estimada do estado antioxidante (COSTA; SANTOS; LIMA, 2006). São extremamente eficientes como protetores

celulares por danos oriundos de radicais livres, porém podem gerar radicais tiil ($RS^{\bullet}$) e a eficiência na sua remoção apresentam efeitos críticos sobre ações pró-oxidativas ou antioxidante de tióis nas células (WŁODEK, 2002).

Para a mensuração sérica do tiol ou grupos sulfidrila é utilizado 5,5′-ditiobis-(2-nitrobenzóico) (DTNB), reagente de Ellman, que interage formando um ânion altamente colorimétrico com absorbância máxima de 412 nm (COSTA; SANTOS; LIMA, 2006).

2.5.3 Ácido Úrico

O ácido úrico é derivado do metabolismo das purinas, sua produção ocorre pela oxidação de xantina e hipoxantina. Apresenta propriedades antioxidantes por quelar metais, remoção de radicais livres, de espécies reativas de nitrogênio e oxigênio (VASCONCELOS et al., 2007; ROSA et al., 2015).

2.5.4 Paraoxonase-1

A paraoxonase-1 (PON-1) é uma enzima sintetizada pelo fígado e está associada com a lipoproteína de alta densidade, possui ação protetora contra estresse oxidativo por meio da degradação dos peróxidos lipídicos. Apresenta diversos papeis em doenças humanas, incluindo aterosclerose e diabetes mellitus, sua baixa atividade está associada como um dos principais riscos em eventos cardiovasculares (MACKNESS; ARROL; DURRINGTON, 1991; MACKNESS; DURRINGTON; MACKNESS, 1998; TVARIJONAVICIUTE et al., 2012; SHUNMOOGAM; NAIDOO; CHILTON, 2018).

Farid et al. (2009) reportaram uma diminuição significativa de PON-1, associada com resposta inflamatória em ratos após serem infectados com *Nippostrongylus brasiliensis* e sugerem investigação para elucidar estas alterações durante a infecção por este nematódeo gastrintestinal.

2.5.5 Estado oxidante total

O ensaio utilizado para o estado oxidante total (TOS: *total oxidant status*) é baseado na oxidação do íon ferroso em férrico na presença de espécies

oxidantes em meio ácido e sua mensuração é realizada por xilenol laranja. Os oxidantes H_2O_2 e LOOH são os principais determinados por este ensaio (EREL, 2005).

2.5.6 Método férrico-xilenol

O método férrico-xilenol (FOX: *ferric-xylenol orange*) apresenta diferença quando comparado com o ensaio TOS, pois se utiliza gluconato de ferro ao invés sulfato de amônio ferroso (ARAB; STEGHENS, 2004).

2.5.7 Produtos da oxidação avançada de proteínas

As proteínas são alvos críticos de oxidantes e por meio de ensaio laboratorial dos produtos da oxidação avançada de proteínas (AOPP: *advanced oxidation protein products*) é possível avaliar estes danos. A AOPP pode ser formada *in vitro* a partir de uma solução de albumina sérica humana purificada e uma segunda forma relatada é pela exposição do plasma por ácido hipocloroso. As concentrações plasmáticas de AOPP quando avaliadas *in vivo*, se correlacionam com níveis de ditrosina, sendo esta um fator da oxidação proteica, assim como com a pentosidina, um marcador de glicação de proteínas enzimáticas e ainda, que a albumina se modifica pela oxidação acarretando em formação de AOPP (WITKO-SARSAT et al., 1996).

2.5.8 Derivados de metabólicos reativos de oxigênio

O ensaio referente aos derivados de metabólicos reativos de oxigênio (d-ROMs: *reactive oxygen metabolites derived compounds*) é baseado na capacidade de metais de transição em catalisar peróxidos gerando desta maneira radicais livres que são capturados por uma alquilamina (CESARONE et al., 1999).

3 OBJETIVOS

3.1 Objetivo geral

Avaliar as concentrações séricas dos biomarcadores do estresse oxidativo e análises bioquímicas em cordeiras recriadas, naturalmente infectadas por nematódeos gastrintestinais e *Eimeria* spp., suplementadas com e sem a inclusão do caroço de algodão em SIPA.

3.2 Objetivos específicos

- Avaliar infecções naturais por parasitos gastrintestinais em cordeiras suplementadas com e sem a inclusão do caroço de algodão;
- Acompanhar o desenvolvimento de cordeiras suplementadas com e sem a inclusão do caroço de algodão;
- Avaliar o perfil dos biomarcadores do estresse oxidativo frente as infecções naturais por parasitos gastrintestinais, em cordeiras suplementadas com e sem a inclusão do caroço de algodão;
- Avaliar o perfil das análises bioquímicas frente as infecções naturais por parasitos gastrintestinais, em cordeiras suplementadas com e sem a inclusão do caroço de algodão.

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CAPÍTULO II – Oxidative stress biomarkers in lambs naturally infected by gastrointestinal nematodes and *Eimeria* spp. supplemented with whole cottonseed

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Oxidative stress biomarkers in lambs naturally infected by gastrointestinal nematodes and *Eimeria* spp. supplemented with whole cottonseed

Abstract

In farm animals, economic losses are widely described due to gastrointestinal parasites infections. To assist in monitoring these infections and the feed supplementation, the oxidative stress biomarkers and biochemical analytes are relevant tools in Veterinary Medicine. The purpose of this trial was to evaluate serum levels of oxidative stress biomarkers and biochemicals analytes in crossbred lambs naturally infected by gastrointestinal nematodes and *Eimeria* spp., with and without the dietary inclusion of whole cottonseed (WCS) in an integrated crop-livestock system (ICLS). The experiment was carried out with 36 crossbred lambs (cross: Ile de France x White Dorper x Texel), in the rearing phase. Body weight, blood collection and faecal analysis of eggs and oocysts counting per gram of faeces (EPG and OPG, respectively) were performed for each animal every 14 days, with a total of seven time points within 85 days of experiment. Serum biochemical analytes were determined: total protein, albumin, globulin, cholesterol, haptoglobin, and oxidative stress biomarkers: cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), trolox equivalent antioxidant capacity (TEAC), thiol, uric acid, paraoxonase-1 (PON-1), total oxidant status (TOS), ferric-xylenol orange (FOX), advanced oxidation protein products (AOPP), and reactive oxygen metabolites derived compounds (d-ROMs). The treatment with the WCS inclusion had higher ($p < 0.05$) TEAC, AOPP, and d-ROMs concentrations. Whereas the OPG counting, albumin, thiol and FOX were higher ($p < 0.05$) for the control treatment (without the WCS). No significant differences were observed between treatments ($p > 0.05$) for EPG counting, body weight, average daily weight gain, and other biomarkers of oxidative stress and biochemical analytes. The inclusion of WCS suggested the benefit in controlling *Eimeria* spp. infection as well as inducing an increase in oxidants and antioxidants in lambs naturally infected by gastrointestinal parasites. The combination of WCS and ICLS could be useful in controlling gastrointestinal parasites infection without affecting the production performance.

Keywords: *Haemonchus*; *Trichostrongylus*; antioxidant; oxidant; biochemical analytes; integrated crop-livestock system

1. Introduction

Sheep farming faces a constant battle against gastrointestinal nematodes (GIN) and *Eimeria* spp. infections, which results in considerable economic losses. *Haemonchus contortus* followed by *Trichostrongylus colubriformis* are the main GIN infecting sheep raised in southeastern Brazil (Wilmsen et al., 2014; Starling et al., 2019). *Haemonchus contortus*, known as barber pole or wireworm, infects the abomasum in small ruminants and has important characteristics that must be considered, such as high prolificacy, as they are hematophagous, however for not being the primary cause of diarrhea, it becomes difficult for producers to detect infected animals, until death occurs (Zajac, 2006). *Trichostrongylus colubriformis*, on the other hand, infects the small intestine, and causes severe lesions in the intestinal mucosa. In addition, these parasites cause villous atrophy, epithelial erosion, and consequently loss of performance (Cardia et al., 2011). The protozoan *Eimeria* spp. is another gastrointestinal parasite that infects the small and large intestines. Therefore, leading to losses by clinical and subclinical disease (Chartier and Paraud, 2012). Lambs are more susceptible, as they could become infected soon after birth, resulting in the elimination of a high number of oocysts in faeces within a few weeks of life (Amarante and Barbosa, 1992). Thus, alternatives for the control of gastrointestinal parasites infections must be considered.

The diversity of landscape and climate in the southeast region in Brazil allows the use of sheep farming alternatives. The production of sheep in an integrated crop-livestock system (ICLS) enables strategies for increasing the income of those who produce milk, meat, grains and decreases the seasonality of animals for slaughter (Costa et al., 2019). Furthermore, the use of good nutrition associated with pastures in ICLS, aids in the control of GIN infection and performance of sheep (Almeida et al., 2018). To supplement the animals and to improve the productive performance in this system, the whole cottonseed (WCS) can be used as a potential component for the formulation of diets. The WCS is a byproduct of the cotton ginning industry, from the cotton plant (*Gossypium* L.). It combines high energy with high proportion of protein and effective fiber for ruminant supplementation (Rogers et al., 2002; Knutsen et al., 2017).

Additionally, it has been reported as a satisfactory feed ingredient for growing sheep (Kandylis et al., 1998).

The imbalance between oxidants and antioxidants, in cells or in the body, are defined as oxidative stress (Lykkesfeldt and Svendsen, 2007; Rubio and Cerón, 2021). The oxidative stress may be involved in pathological conditions, relevant conditions for production and general welfare of farm animals, and as consequence, the oxidative damage affects cellular macromolecules, in particular proteins, lipids, and deoxyribonucleic acid (Lykkesfeldt and Svendsen, 2007). When there is an uncontrolled production of oxidants, such as those from reactive species, derived from oxygen, nitrogen and/or others, the organism offers an efficient mechanism to control and restore balance through antioxidants, endogenous and exogenous, which act in the protection, repair, interception, and prevention of damage caused by oxidative stress (Rubio and Cerón, 2021).

In animal health, biomarkers are less widespread and less developed than in human health. However, when applied from the One Health perspective, animal biomonitoring could provide important information, supporting the prevention and management of health risks (Frazzoli et al., 2015). Antioxidant, oxidant, trace elements, and acute phase proteins biomarkers should be used together, becoming important tools when assessing, monitoring, and identifying the oxidative stress associated with diseases (Rubio and Cerón, 2021).

Recent reports indicated the involvement of parasitic infections (Machado et al., 2014; Pathak et al., 2017; Baptistiolli et al., 2018; Alam et al., 2020; Schmidt et al., 2021), as well as the intake of cottonseed (Moretti et al., 2019) in the oxidative status of sheep. However, to the authors knowledge there is a lack of data in the literature regarding the use of a panel of oxidative stress biomarkers to assist and interpret results, targeting the health of sheep when infected by gastrointestinal parasites in combination with the inclusion of WCS in the diet in an ICLS. In addition, we hypothesize that changes in oxidative status could be observed in sheep under these conditions. Thus, the purpose of this trial was to evaluate serum levels of oxidative stress biomarkers and biochemicals analytes in crossbred lambs naturally infected by GIN and *Eimeria* spp., with and without the dietary inclusion of WCS in an ICLS.

2. Material and Methods

All procedures performed in this experiment were approved by the Ethics Committee on Animal Use (CEUA) of the São Paulo State University (UNESP) at the School of Veterinary Medicine and Animal Science (FMVZ), Botucatu, São Paulo, Brazil, under protocol number 0099/2019 – CEUA.

2.1. Experimental area description

This experiment was conducted between December 2018 and July 2019, at Lageado Experimental Farm (22°51'01”S and 48°25'28”W; altitude, 777 m), belonging to FMVZ - UNESP, Botucatu, São Paulo, Brazil. The soil was a clay, kaolinitic, thermic Typic Haplorthox (FAO, 2006) with 630, 280 and 90 g kg^{-1} of clay, sand and silt, respectively. According to the Köppen climate classification system, the region’s climate is the Cfa type, which covers 6.5% of the Brazilian territory and 33.4% of the São Paulo state territory, characterized by warm temperate (mesothermic) humid climate (Cunha and Martins, 2009; Alvares et al., 2014). The average accumulated monthly precipitation is highest (260.7 mm) in January and lowest (38.2 mm) in August. The average monthly temperature ranges from 23.2 ºC in February to 17.1 ºC in July (Escobedo et al., 2011).

The experimental area is part of an ICLS established since 2010. Before the present study, the area was used to produce maize (*Zea mays* L.) or soybean [*Glycine max* (L.) Merr.] silage in summer/autumn, and black oat (*Avena strigosa* Schreb) oversown in winter/spring, with different treatments made with intercropped marandu palisade grass (*Urochloa (syn. Brachiaria*) *brizantha* cv. Marandu), black oat, pigeon pea [*Cajanus cajan* (L.) Millsp.], and aruana guinea grass (*Megathirsus* (syn. *Panicum*) *maximum* cv. Aruana). During the winter the black oat pastures were grazed by lambs (Almeida et al., 2018; Pariz et al., 2020).

In December 2018, soybean (cv. BMX Potência RR) was sown at a 3 cm depth at a density of 350,000 seeds ha^{-1}, with a row spacing of 0.45 m, using a seeder-fertilizer machine (PDCP model, Marchesan Implementos e Máquinas Agrícolas "TATU" S.A., Matão, São Paulo, Brazil). The aruana guinea grass was intercropped with soybean, at depths of 6 cm, amounts of 600 points of cultural

value ha^{-1}, as Kluthcouski and Yokoyama (2003) recommended. After mechanical harvesting (soybean and aruana guinea grass) in March 2019, the pastures of aruana guinea grass reached its ideal management point (presenting 95% of light interception on average), which were grazed by lambs from May 8 to July 31, 2019.

2.2. Animals and management

Details about animals and management were described by Tardivo (2021). Briefly, 36 crossbred (Ile de France x White Dorper x Texel) female lambs were used, with a mean age of 105 days, mean body weight of 25.6 ± 5.3 kg, acquired from a commercial farm located approximately 160 km from Botucatu, São Paulo, Brazil.

Before starting the experimental trial (Figure 1), the animals were tagged and vaccinated against *Clostridium* spp. (Poli-Star®, Vallée, Brazil). Faecal samples were collected for counting of nematode eggs and *Eimeria* spp. oocysts per gram of faeces (EPG and OPG, respectively) using a modified Gordon and Whitlock (1939) technique (Ueno and Gonçalves, 1998). All animals were treated subcutaneously with 1% moxidectin (Cydectin®, Zoetis, São Paulo, Brazil), single application, dose of 0.2 mg/ kg, on May 06, 2019, and due to the outbreak of eimeriosis on June 26, 2019, it was carried out an orally treatment with 5% toltrazuril suspension (Baycox®, Bayer Saúde Animal), single application, dose of 20 mg/ kg.

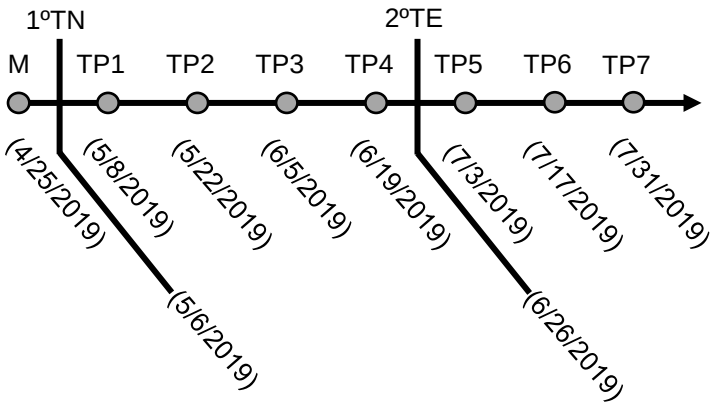

Figure 1. Timeline of the experimental trial. Management (M) of crossbred lambs that were tagged, vaccinated, adapted to the diets (whole cottonseed and control), and evaluated through faecal examination. Two treatments were performed, first treatment (1°TN) with 1% moxidectin against gastrointestinal nematodes infection and second treatment (2°TE) with 5% toltrazuril against *Eimeria* spp. infection. The experimental trial samples were collected at seven different time points (TP1 – TP7). Between each time point and management there was an interval of 14 days.

Eighteen lambs (Figure 2) were housed in four pens (two pens with five animals and two pens with four animals each), which were supplemented with concentrate, maize silage and WCS inclusion in their diets (whole cottonseed treatment). Another eighteen lambs (Figure 2) were also housed in four pens (two pens with five animals and two pens with four animals each), which were similarly supplemented, however without the WCS inclusion in their diets (control treatment). Details about the diet ingredients are presented in Table 1. Daily at 7:00 a.m. all lambs were released from the pens and allocated in their respective ICLS paddocks. At 5:00 p.m., they returned to a covered shed which was lined with rice straw and had eight pens, each one with 5 m x 5 m (25 m^2). The animals always had free access to water. They were supplemented according to the treatment and groups were balanced as closely as possible by body weight. The lambs were adapted to the diets for 14 days, prior the sampling.

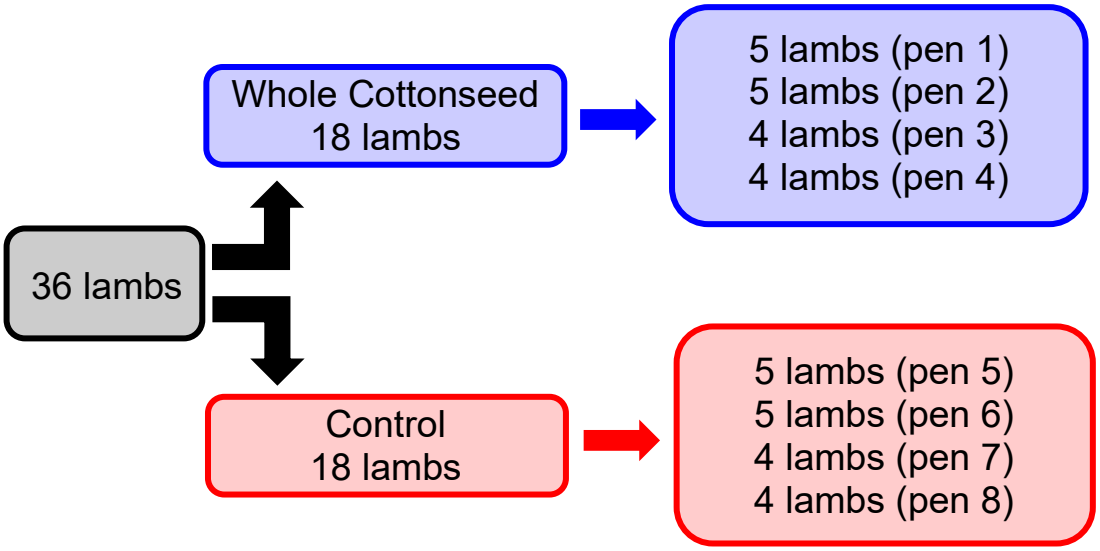

Figure 2. Distribution of crossbred lambs according to treatment.

At the paddocks, the grazing of the aruana guinea grass was rotational with a fixed stocking rate, distributed in groups in the same way as previously determined for the pens in the covered shed. The area was divided by an electric fence into twenty-four paddocks of 16 m x 30 m (480 m²), twelve paddocks for each treatment. Each group rotated in the same three paddocks during the trial, where they grazed for seven days each.

The diets were formulated using the Small Ruminant Nutrition System (SRNS), computer program based on the Cornell Net Carbohydrate and Protein System (2000) for sheep. The estimated weight gain was approximately 200 g/day (NRC, 2007).

Table 1. Formulation and chemical composition of the experimental trial diets.

Diets	Whole Cottonseed	Control
Ingredients (%)		
Corn ground	17.66	35.07
Soybean meal	4.93	1.15
Mineral[1]	1.83	1.83
Limestone	0.80	0.86
Urea	-	1.26
Monensin[2]	0.03	0.03
Whole cottonseed	14.91	-
Maize silage	29.93	29.91
Aruana guinea grass	29.93	29.91
Chemical composition		
Dry matter (%)	52.78	54.60
Net Energy for Maintenance (Mcal/ kg)	2.25	2.23
Net Energy for Gain (Mcal/ kg)	0.87	0.87
Crude Protein (%)	14.21	14.88
Metabolizable Protein (%)	8.50	8.40
Ether Extract (%)	3.96	3.08
Neutral Detergent Fiber (%)	36.19	24.04
Acid Detergent Fiber (%)	21.24	11.69
Calcium (%)	0.70	0.70
Phosphorus (%)	0.40	0.40

[1]Mineral composition (kg of product) 155 g Ca, 65 g P, 110 g Mg, 210 g S, 380 mg Se, 83.500 mg Zn, 26.300 mg Mn, 2500 mg I, 2500 mg Co; (Maximicrominer®, Maxi Nutrição Animal,Brazil); [2]Rumensin® (Elanco Animal Health, Greenfield). Adapted from: Tardivo (2021).

2.3. Body weight, faecal and blood samples

On seven occasions (Figure 1), faecal and blood samples were collected from each animal at every 14 days, and body weight was recorded on the same day. The time points were: TP1 (5/8/2019), TP2 (5/22/2019), TP3 (6/5/2019), TP4 (6/19/2019), TP5 (7/3/2019), TP6 (7/17/2019), and TP7 (7/31/2019).

The faecal samples, blood samples, and body weights at the TP1, TP2, TP3, TP4, TP5, TP6, and TP7 were obtained in the morning, always at 6:00 a.m., with the animals fasting, still in the shed.

The faecal samples were collected directly from the rectum of each lamb, packed in identified plastic bags, transported in thermal boxes with recyclable ice (temperature of 4 °C) and sent to the Laboratory of Parasitic Diseases of Animals, FMVZ - UNESP, campus of Botucatu, São Paulo, Brazil.

Blood samples were collected via jugular puncture, transported within an hour in thermal boxes with recyclable ice (temperature of 4 °C) and sent to the Laboratory for Research on Acute Phase Proteins and Non-Invasive Monitoring of Animal Reproduction and Welfare at FMVZ - UNESP, campus of Botucatu, São Paulo, Brazil. Four milliliters (mL) of blood were collected from each animal in plain tubes with clot activator (BD Vacutainer® Blood Collection Tube; Becton, Dickinson and Company, USA). The serum was obtained by centrifuging the tubes at 1500 x *g* for 10 minutes. Once the serum was obtained, the samples were stored in Eppendorf® tubes (Eppendorf, Hamburg, Germany) and frozen at -20 °C, until the laboratory analysis of the biochemical analytes and biomarkers of oxidative stress.

2.3.1 Eggs and oocysts counting

The feacal counting of EPG and OPG were determined for each sample using a modified Gordon and Whitlock (1939) technique (Ueno and Gonçalves, 1998). The faecal samples were kept under refrigeration and processed within 24 hours. Briefly, the recommendations of this technique for sheep are: two grams of faeces, macerate with 58 mL of saturated NaCl solution with a density of 1,2, filter with gauze or sieve, followed by filling the right and left side of the McMaster chamber (without the formation of “air bubbles”) and count the two cells subdivided by dashes. Each counted egg or oocyst (right and left side of the chamber) represented 100 nematode eggs or *Eimeria* spp. oocysts per gram (g) of feaces (Ueno and Gonçalves, 1998).

2.3.2 Biochemical analytes

A commercially available colorimetric method (Haptoglobin Tridelta® phase range, Tridelta Development Ltd., Maynooth, Country Kildare, Ireland) was used to determine serum haptoglobin (Hp). Serum cholesterol was determined using a commercial kit (Beckman Coulter® Inc., Fullerton, CA, USA), total protein and albumin concentrations were determined by Olympus commercial kits (Olympus Life® and Material Science Europe GmbH, Hamburg, Germany), following the instructions of the manufacturer. The estimated concentrations of globulins were calculated by the difference between total proteins and albumin concentrations.

2.3.3. Biomarkers of oxidative stress

Total antioxidant capacity (TAC) in serum was determined by using three different assays (Rubio et al., 2016): Trolox equivalent antioxidant capacity (TEAC), ferric reducing ability of plasma (FRAP), and cupric reducing antioxidant capacity (CUPRAC), according to methods described by Arnao et al. (1996), Benzie and Strain (1996), and Campos et al. (2009) respectively. Thiol antioxidative effects were determined by an automated method, based on Ellman's method (Costa et al., 2006). Individual antioxidants, such as uric acid and paraoxonase-1 (PON-1) were also evaluated. The uric acid concentration was determined according to the instructions of a commercially spectrophotometric method (OSR6698 Beckman Coulter AU analyzers, Switzerland) based on a protocol described by Fossati et al. (1980). Serum PON-1 activity was determined by an assay that used p-nitrophenyl acetate as substrate (Tvarijonaviciute et al., 2012).

Oxidant biomarkers such as advanced oxidation protein products (AOPP), a marker of oxidative damage to proteins, was based on di-tyrosine containing cross-linked proteins and oxidatively modified albumin as Witko-Sarsat et al. (1996) described. Total oxidant status (TOS) was determined by Erel (2005) method, based on the oxidation of the ferrous ion–o-dianisidine complex. Ferric-xylenol orange (FOX) was determined by using an assay based on the ferrous oxidation by xylenol orange (Arab and Steghens, 2004). Reactive oxygen metabolites derived compounds (d-ROMs) was determined by an assay (Cesarone et al., 1999) based on monitoring the N-dyethyl-paraphenyldiamine radical cation concentration.

All sera analysis were performed using an automated chemistry analyzer (Olympus AU600, Olympus Diagnostic Europe GmbH, Ennis, Ireland).

2.4. Statistical analysis

Data were evaluated for normality with graphic analysis of histogram, QQ plot and Shapiro-Wilk test. The data which did not present normal distribution (EPG and OPG) were transformed to log10 (x + 1). All data were analyzed by

analysis of variance and for this purpose, the Statistical Analysis System - SAS® Studio (SAS Institute Inc., Cary, NC, USA) with the General Linear Model (GLM) procedure were used. Tukey's test at a 5% significance level was used to compare means. The EPG and OPG transformed data results were reported in tables with arithmetic means (± standard deviation), and for the graphs, the raw data with the medians (interquartile range) were used. The results of the variables that had normal distributions were reported in tables with arithmetic means (± standard deviation), and for the graphs, the arithmetic means (± standard error of the mean) were used. A p value of < 0.05 was considered statistically significant for all statistical analysis.

3. Results

Gastrointestinal nematodes eggs were detected in lambs during the trial. Significant time points effects ($p < 0.0001$) and no significant treatment effect ($p = 0.348$) were observed (Supplementary File 1). However, at TP1 the lambs in the WCS treatment (Figure 3) had higher EPG counting. At TP2 and over the next time points the EPG counting decreased in both treatments.

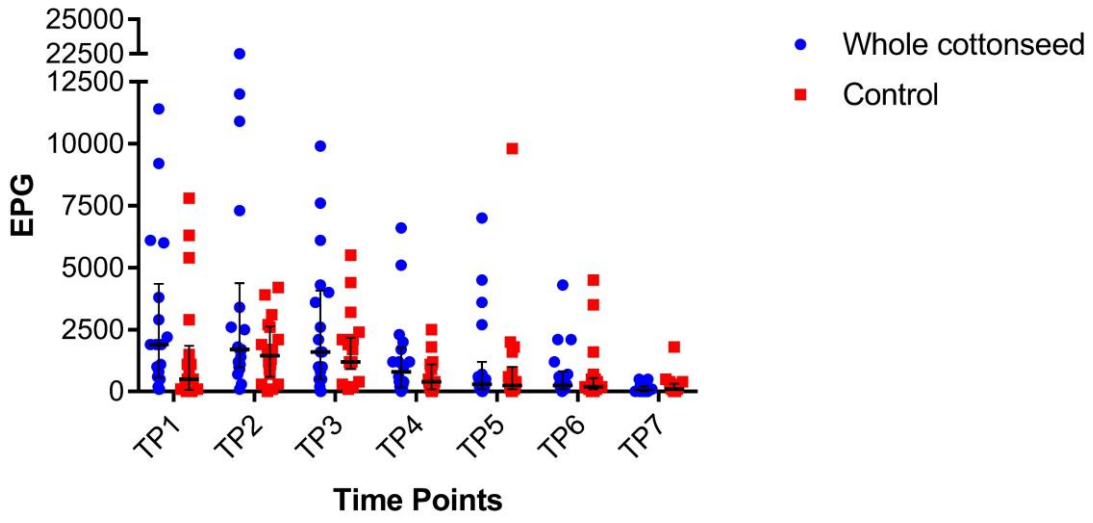

Figure 3. Median (interquartile range) of eggs per gram of faeces (EPG) of crossbred lambs naturally infected with gastrointestinal nematodes, receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

Infections by *Eimeria* spp. were detected in the lambs during the trial. Low OPG counting were observed at TP1 for both treatments (Figure 4). However, at the following time points there was a considerable increase of oocysts counting, with a peak at TP4, followed by a pronounced drop of the counting due to treatment with toltrazuril performed between TP4 and TP5. The OPG remained in low counting until TP6, and at TP7 there was a slight increase in the counting. The WCS treatment had lower general mean of OPG (p = 0.039) during the trial (Supplementary File 1) and significant time points effects (p < 0.0001) were observed.

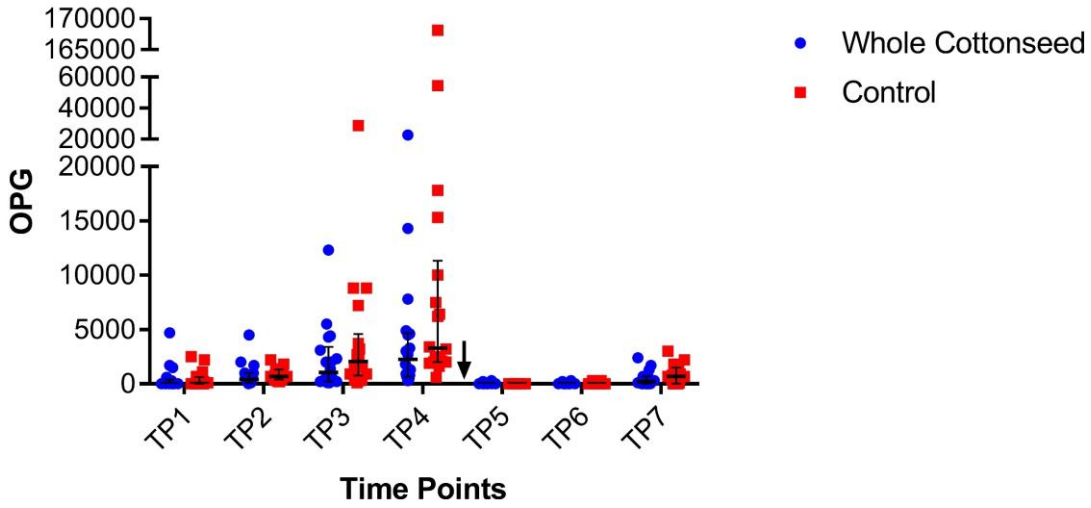

Figure 4. Median (interquartile range) of *Eimeria* spp. oocysts per gram of faeces (OPG) of crossbred lambs naturally infected, receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days. The arrow indicates the treatment with 5% toltrazuril.

The lambs in the WCS and control treatments had a similar body weight gain over the experimental period (Figure 5), therefore, without statistical differences (p = 0.743) between the treatments (Supplementary File 2). Significant time points effects (p < 0.0001) were observed for body weight gain, with a general mean of body weight at TP1 of 25.6 kg and 36.6 kg at TP7 (Supplementary File 2). The animals in both treatments had low average daily weight gain (ADG) between TP1 and TP3 (Figure 6), with a pronounced drop between TP4 and TP5 in the WCS treatment, and between TP5 and TP6 in the

control treatment. Afterwards, high mean values for both treatments were observed between TP6 and TP7. There was no treatment effect (p = 0.391) for ADG, however, significant treatment x time points interaction (p = 0.028) and time points effects (p < 0.0001) were observed (Supplementary File 3).

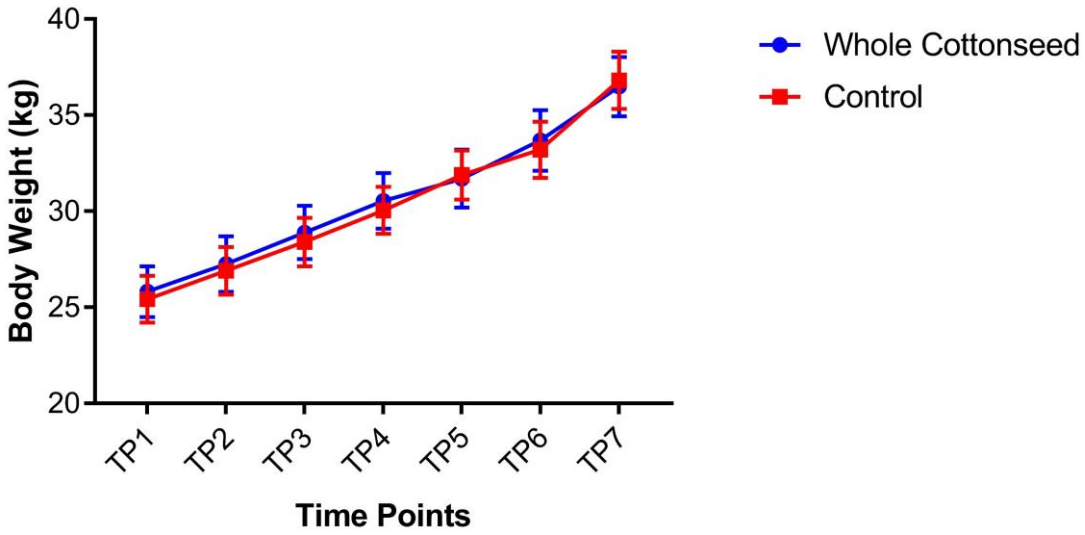

Figure 5. Mean (± standard error of the mean) of body weight (kg) values of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

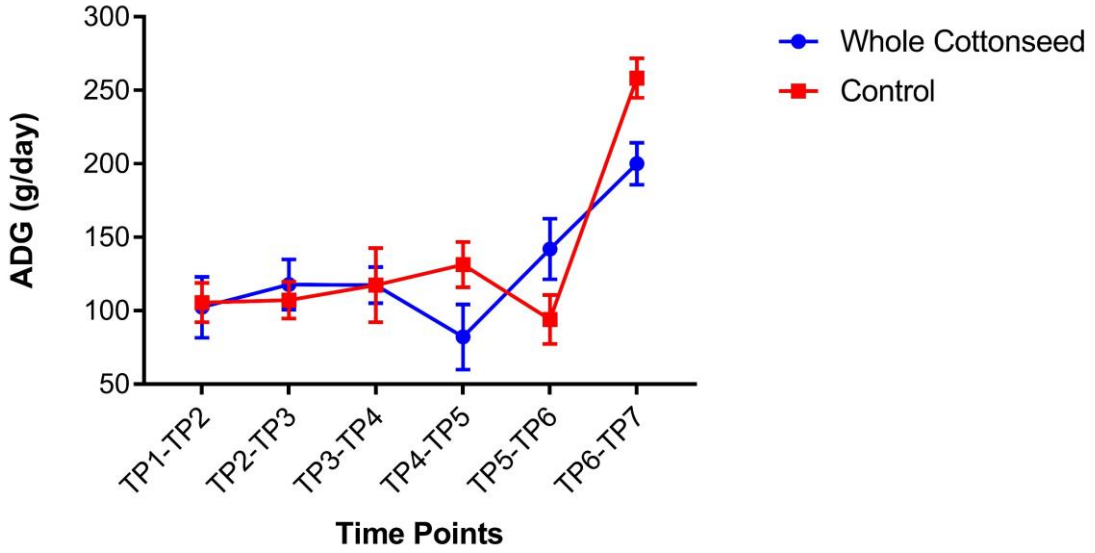

Figure 6. Mean (± standard error of the mean) of average daily weight gain (ADG) values of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

Significant treatment effect was observed (p = 0.005) for albumin concentrations (Supplementary File 4), where the lambs in the control treatment had higher general mean throughout the trial. There were no significant time points effects (p = 0.116), however, albumin concentrations decreased for both treatments at TP4 and TP5 (Figure 7).

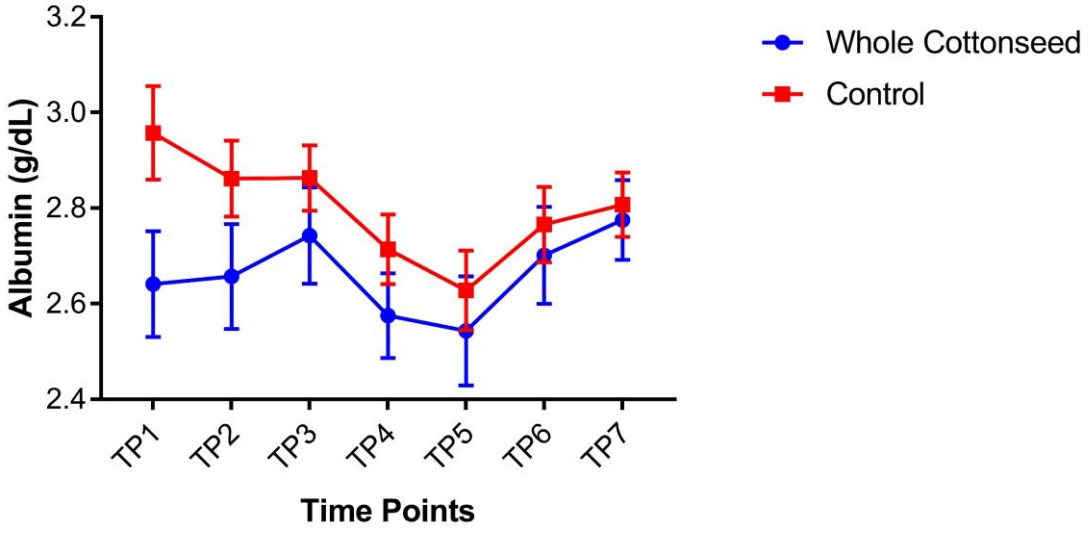

Figure 7. Mean (± standard error of the mean) of albumin concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

Total protein concentrations had oscillations throughout time (Figure 8). At TP4 and TP5 a decrease in the total protein concentrations were observed for both treatments and at TP6 and TP7 an increase occurred, reaching higher concentrations. Significant time points effects ($p = 0.047$) and no treatment effect ($p = 0.785$) of total protein concentrations are shown in Supplementary File 4.

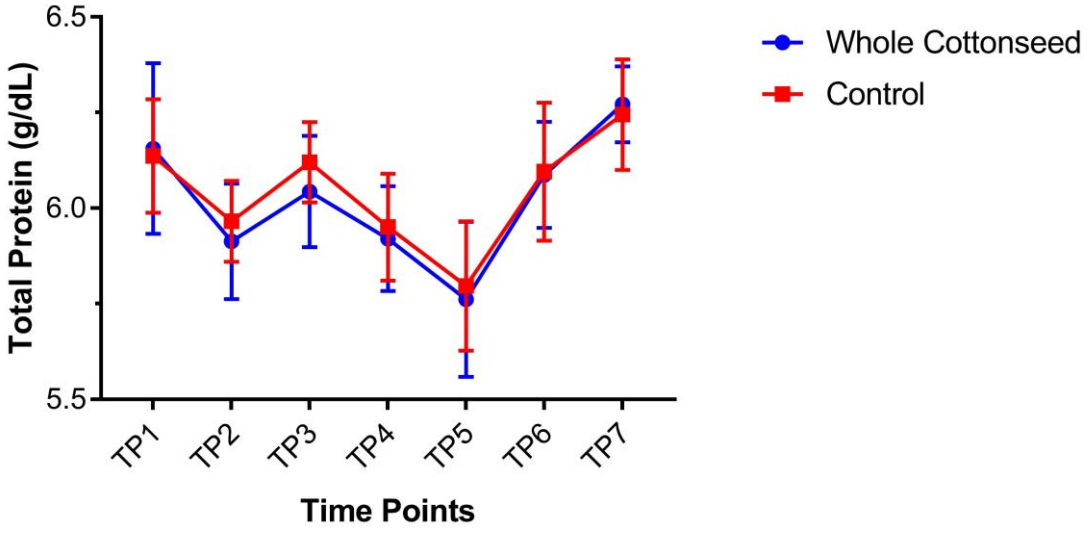

Figure 8. Mean (± standard error of the mean) of total protein concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

There were no significant time points effects (p = 0.167) and no significant treatment effect (p = 0.059) of globulins concentrations (Supplementary File 4), although the WCS treatment had slightly higher means at all time points (Figure 9).

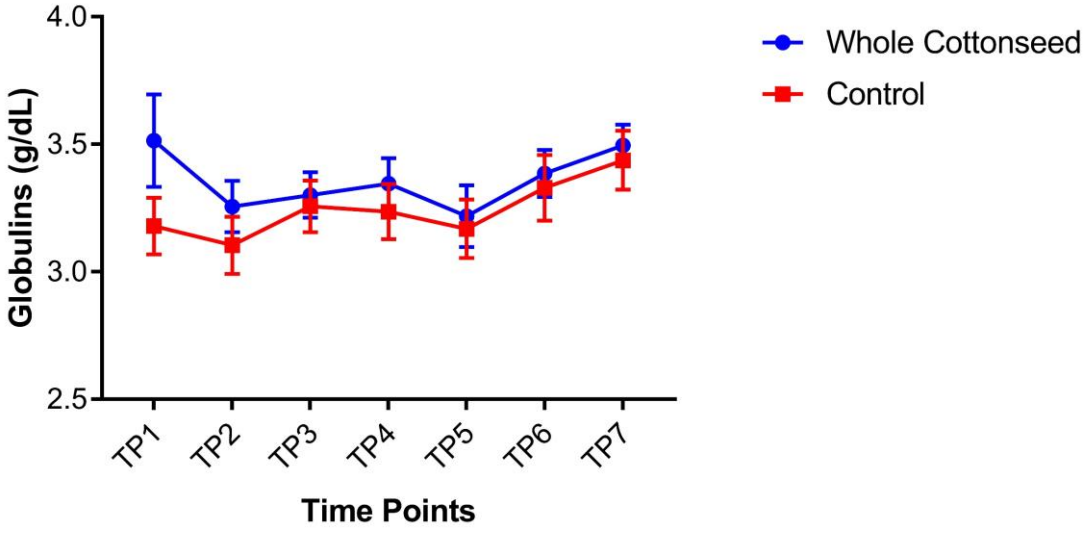

Figure 9. Mean (± standard error of the mean) of globulins concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

Higher means of cholesterol concentrations for both treatments were observed at TP3 and TP7 (Figure 10). There was no significant treatment effect (p = 0.263), however, time points effects were significant (p = 0.002) and are shown in Supplementary File 4.

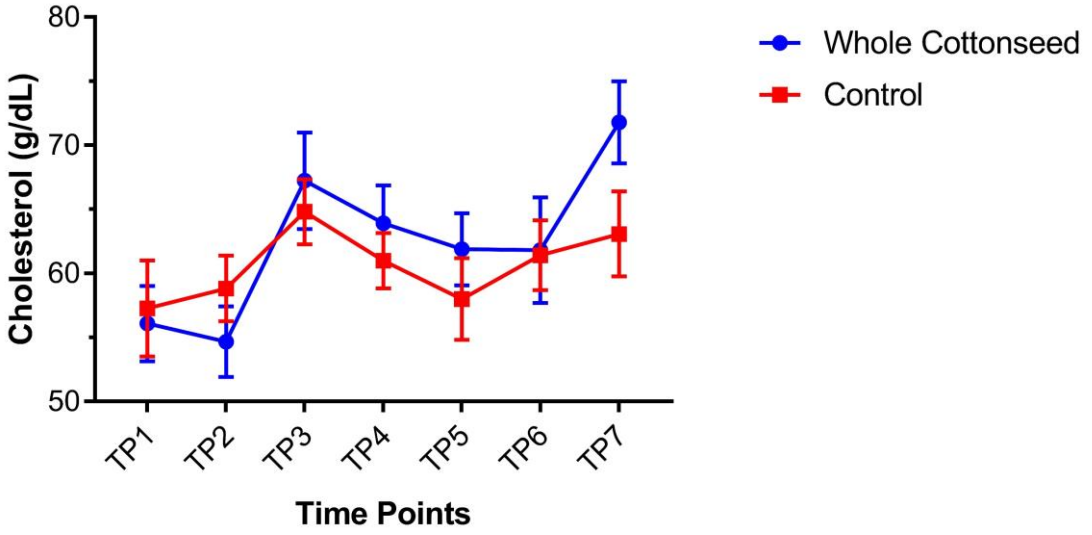

Figure 10. Mean (± standard error of the mean) of cholesterol concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

The Hp concentrations in serum were below the detection limit of the assay for all animals at all time points (data not shown).

Treatment effect was observed (p = 0.015) for the TEAC concentrations (Supplementary File 5) as the WCS treatment had higher general mean of TEAC concentration (0.601 mmol/L). Time points effects were not detected (p = 0.148), however, lower concentrations of TEAC were observed at TP5 for both treatments, followed by an increase, with higher concentrations at TP7 (Figure 11).

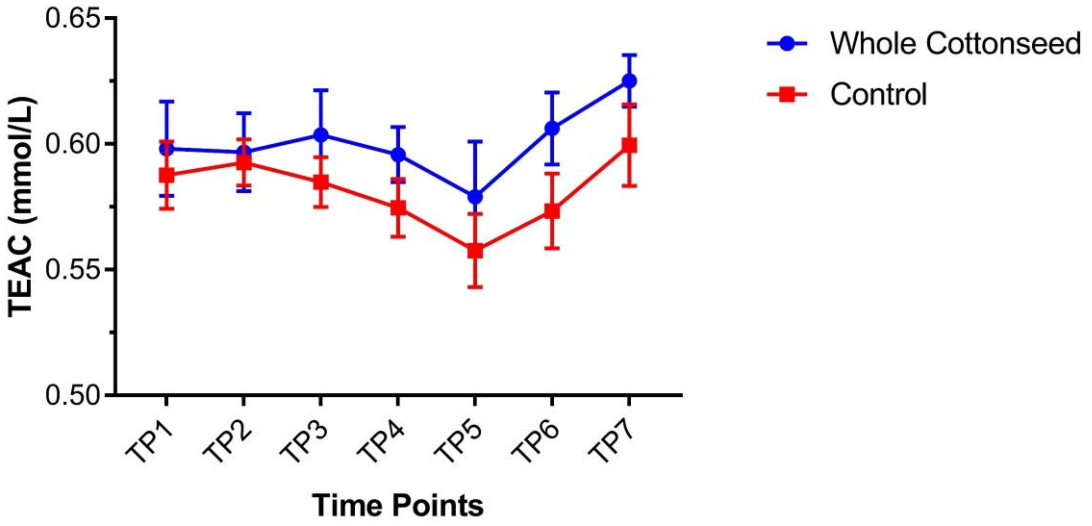

Figure 11. Mean (± standard error of the mean) of trolox equivalent antioxidant capacity (TEAC) concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

No significant treatment and no significant time points effects were observed for the concentrations of CUPRAC and FRAP (Supplementary File 5). However, CUPRAC concentrations were higher for the control treatment at TP1 and TP2 (Figure 12), and an increased concentration for the WCS treatment was observed at TP3. Throughout the experiment there was a decrease in CUPRAC concentration for both treatments and lower general mean concentration was observed at TP5. Subsequently, increases in CUPRAC concentrations for both treatments were observed at the last time points, with a higher general mean at TP7. Concentrations for FRAP were slightly similar for both treatments over the trial period and higher concentrations means were observed only at TP7 (Figure 13).

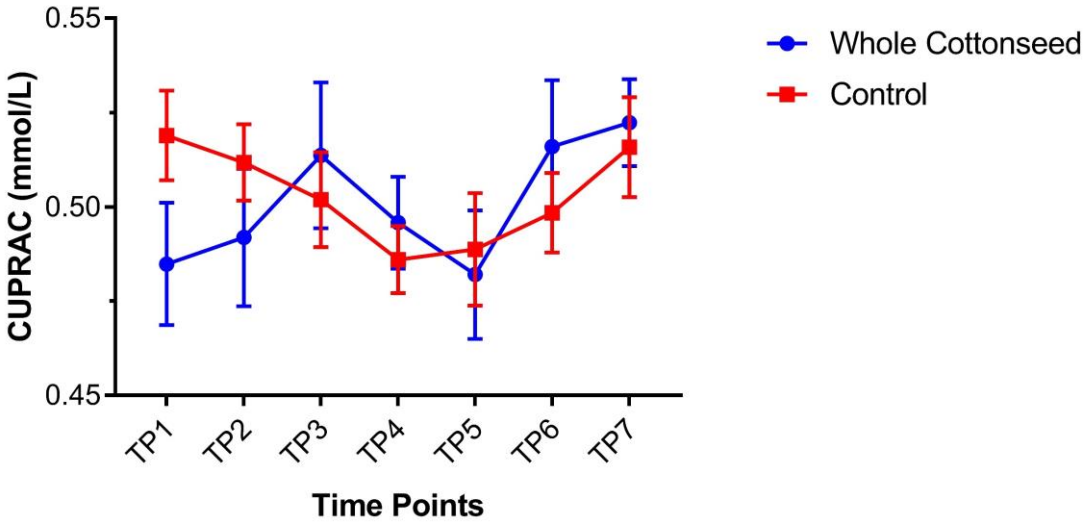

Figure 12. Mean (± standard error of the mean) of cupric reducing antioxidant capacity (CUPRAC) concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

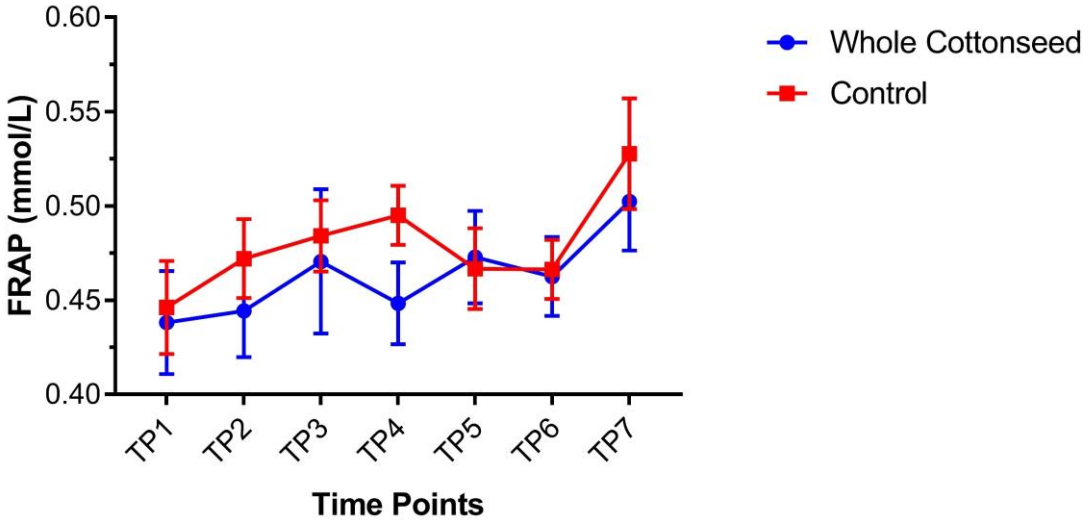

Figure 13. Mean (± standard error of the mean) of ferric reducing ability of plasma (FRAP) concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

There were significant time points effects ($p < 0.0001$) and significant treatment effect ($p = 0.0009$) for thiol concentrations (Supplementary File 5). At TP1 and TP2 the control treatment had higher means of thiol concentrations (Figure 14), followed by a decrease in the concentrations for both treatments at TP4. At the last time points, thiol concentrations increased, and higher means were observed at TP7 for both treatments.

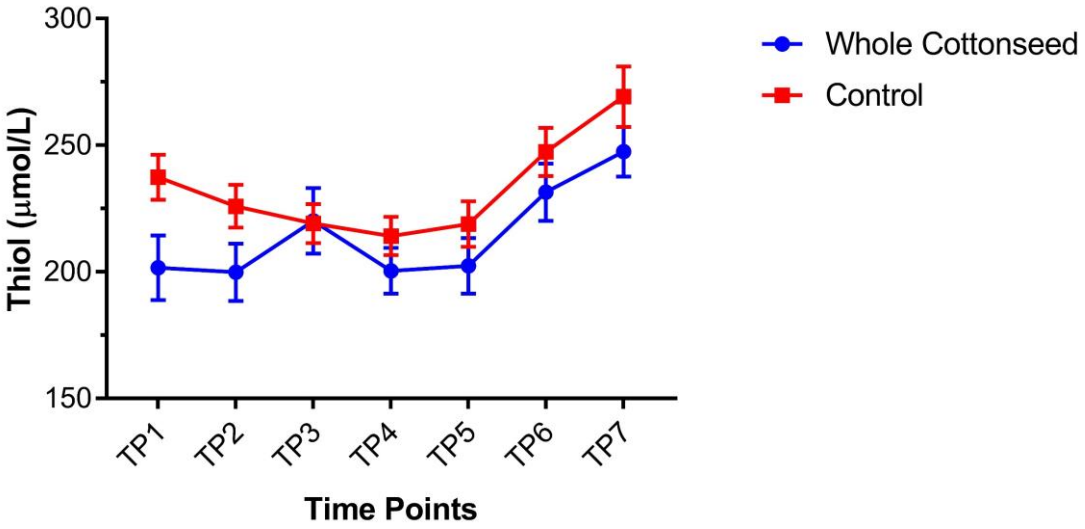

Figure 14. Mean (± standard error of the mean) of thiol concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

There were no significant effects of treatment ($p = 0.175$) and time points ($p = 0.439$) for uric acid concentrations (Supplementary File 5). However, higher general mean of uric acid concentration was observed at TP5 (Figure 15).

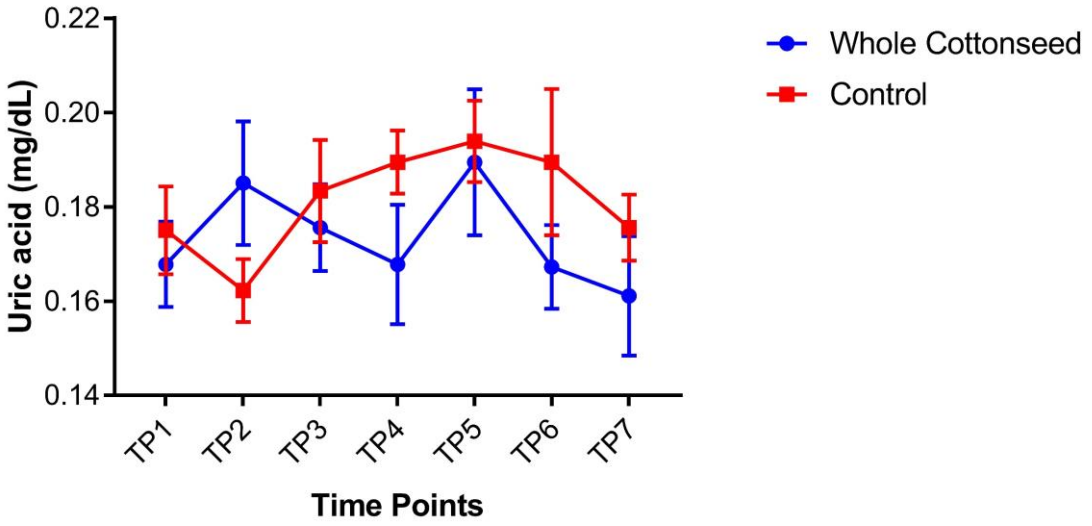

Figure 15. Mean (± standard error of the mean) of uric acid concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

The PON-1 had lower concentrations at TP1 for both treatments (Figure 16), followed by an increase of general mean concentrations from TP2 to TP4 (Supplementary File 5). From TP3 to TP5 the WCS treatment had higher means for PON-1, although no treatment effect (p = 0.382) was observed (Supplementary File 5). On the other hand, time points effects were significantly different (p = 0.006).

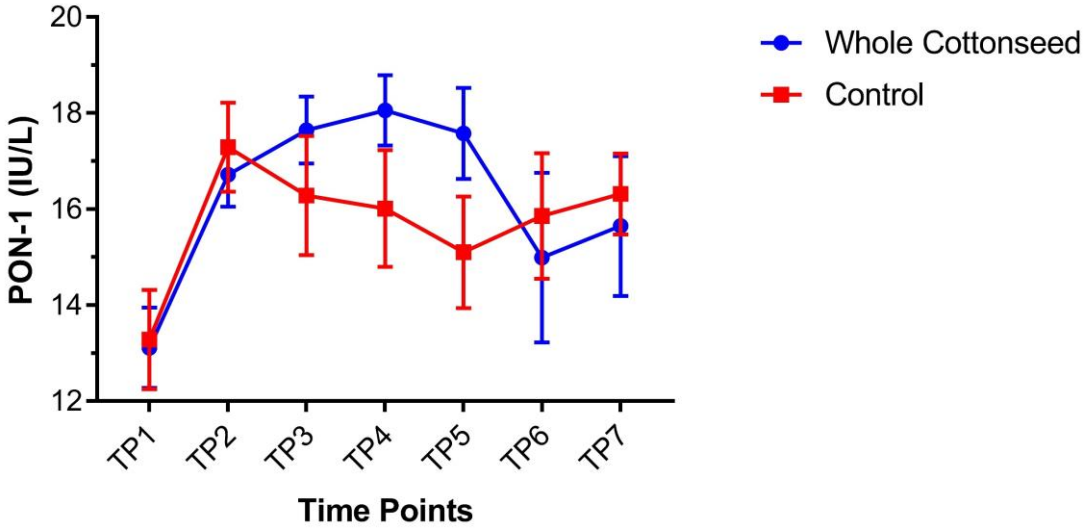

Figure 16. Mean (± standard error of the mean) of paraoxonase-1 (PON-1) concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

Treatment effect (p = 0.011) was detected for AOPP concentrations and the WCS treatment had the higher general mean (Supplementary File 5). At TP6, AOPP had the lowest concentrations (Figure 17) for both treatments and significant time points effects were observed (p = 0.0002).

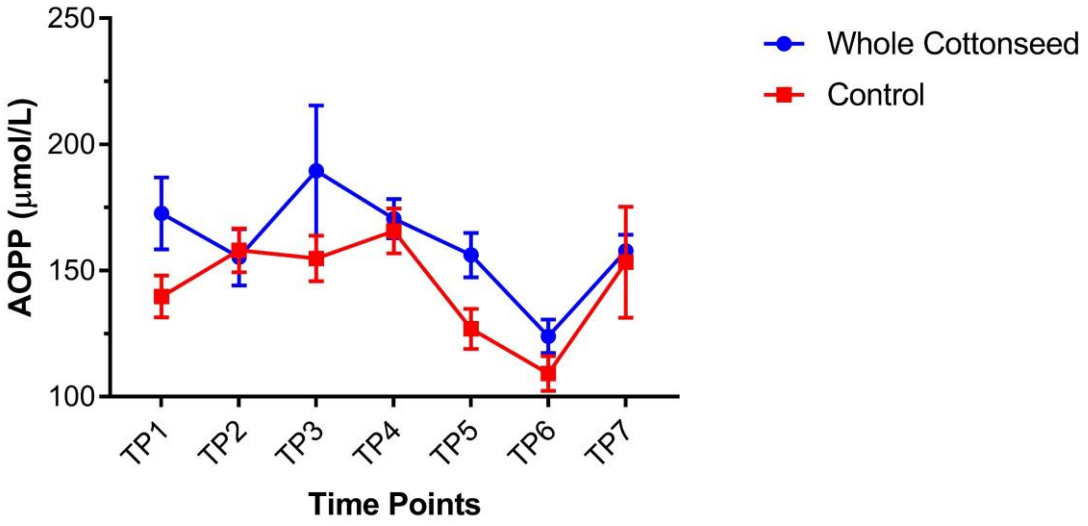

Figure 17. Mean (± standard error of the mean) of advanced oxidation protein products (AOPP) concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

Treatment effect (p = 0.253) was not observed for TOS concentrations (Supplementary File 5). On the other hand, time points effects (p = 0.031) were significantly different for TOS concentrations. Throughout the trial period, a higher mean concentration of TOS was observed for the WCS treatment at TP3 and a higher concentration for the control treatment at TP7 (Figure 18).

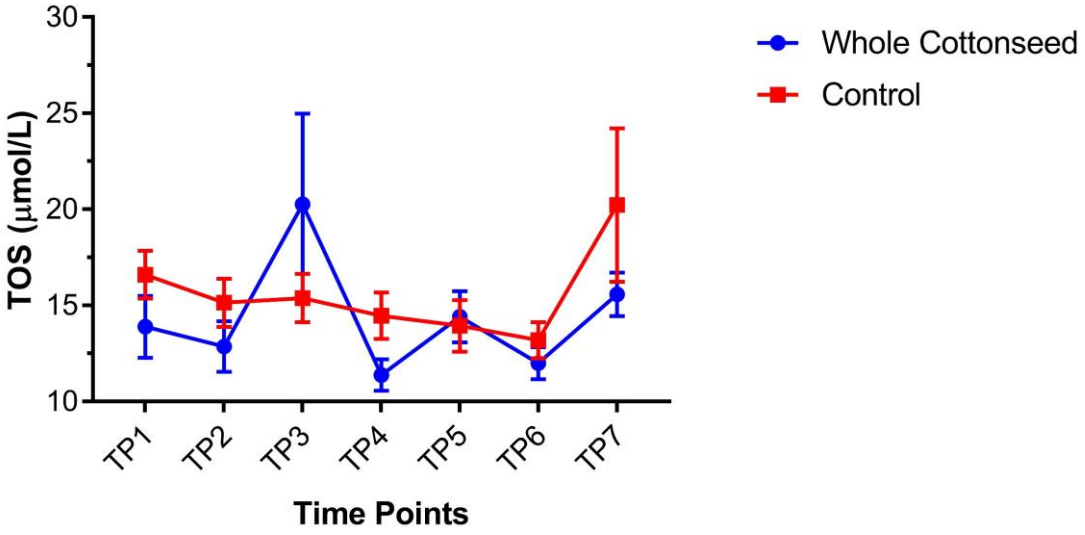

Figure 18. Mean (± standard error of the mean) of total oxidant status (TOS) concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

Treatment effect (p = 0.040) for FOX concentration was detected and the control treatment had the higher general mean (Supplementary File 5). No significant time points effects (p = 0.078) for FOX concentrations were observed. However, higher general means were observed at TP3 and TP7 (Figure 19).

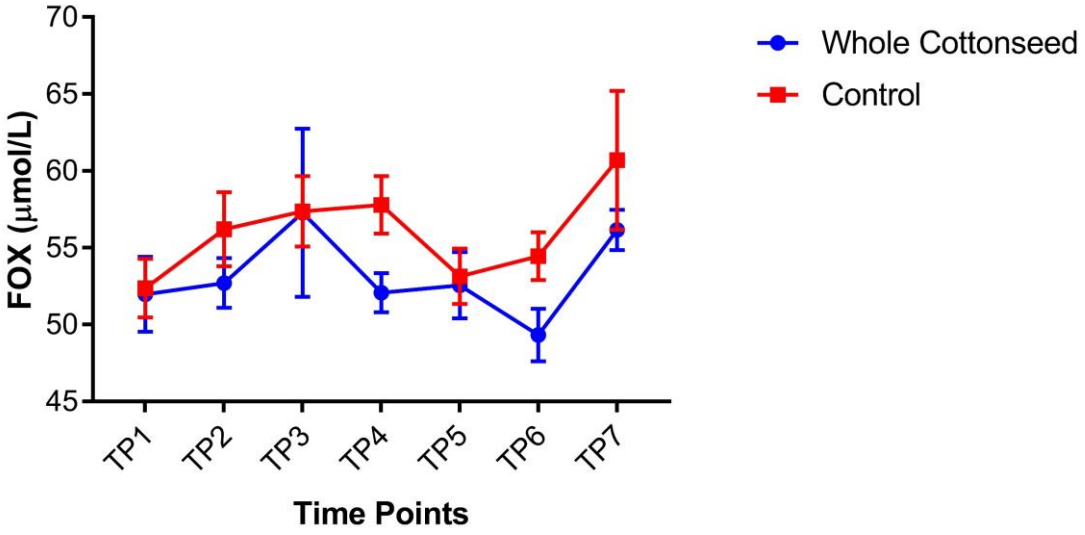

Figure 19. Mean (± standard error of the mean) of ferric-xylenol orange (FOX) concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

Significant treatment effect (p = 0.0008) and significant time points effects (p < 0.0001) were observed for the concentrations of d-ROMs (Supplementary File 5). The WCS treatment had the higher general mean of d-ROMs concentration. The higher general mean of d-ROMs concentration was observed at TP1, followed by a decrease and lower concentrations at TP4, TP5 and TP6 for both treatments (Figure 20).

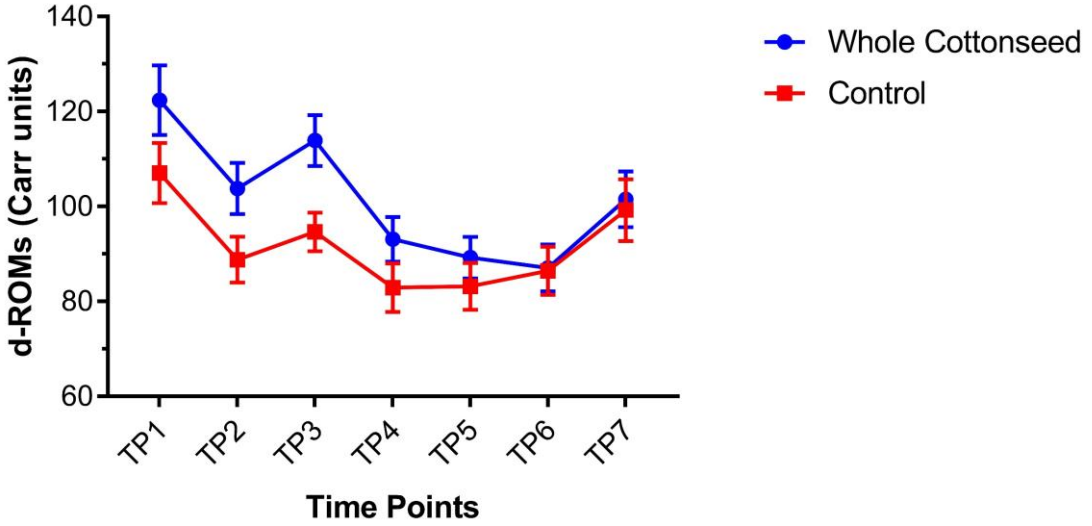

Figure 20. Mean (± standard error of the mean) of reactive oxygen metabolites derived compounds (d-ROMs) concentrations of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

Higher concentrations of AOPP, TOS, and FOX biomarkers at different time points were found for three lambs. At TP3, one animal from the WCS treatment had concentrations of 594.4 µmol/L for AOPP, 93.9 µmol/L for TOS, and 140.05 µmol/L for FOX. At TP7, two animals from the control treatment had concentrations of 376.8 and 422 µmol/L for AOPP, 59.9 and 68.5 µmol/L for TOS, and 96.39 and 117.68 µmol/L for FOX. The concentrations of the biomarkers (AOPP, TOS and FOX) for each treatment, at each time point, with means and standard deviations were reported in Supplementary File 5.

4. Discussion

At the beginning of the experimental trial higher general mean of EPG counting were observed. Afterwards, the faecal egg count reduced along the experimental period. Almeida et al. (2018), observed high means of EPG counting at the beginning of an ICLS trial with crossbred male lambs, with a poor efficacy of closantel treatment, and subsequent decrease of the EPG counting throughout the trial. Moreover, these same authors reported that the pastures

were free of environmental contamination by GIN infective larvae, due the fact that the area was employed as an ICLS, and the same area was not occupied by infected animals. In our trial the environmental contamination was not evaluated, however, the area was not grazed by infected animals for at least 300 days prior the beginning of the experiment. An important factor that could have played a major role in decreasing the faecal egg counts was the good quality of the diets, as immunity possibly developed efficiently in controlling the GIN infection, increasing the body weight gain (Almeida et al., 2018; Starling et al., 2019), and the edaphoclimatic conditions, as the lack of moisture could interfere in the development of GIN larvae (Almeida et al., 2018).

Although there was no significant difference between treatments for GIN infection in this study, according to Teye et al. (2010) the supplementation with WCS had significant effects for several categories of sheep that were naturally infected by GIN, therefore lower faecal egg counts were recorded for those animals.

The lambs were naturally co-infected by *Eimeria* spp. throughout the experimental trial period, with peak of OPG counts at TP4 for both treatments. The treatment protocol performed between TP4 and TP5 with toltrazuril was effective in controlling the coccidial infection, with a pronounced decrease of OPG counts observed at TP5. According to Amarante and Barbosa (1992), animals raised in high densities contribute for the transmission of *Eimeria* spp., which leads to significant elimination of oocysts in the faeces, especially in lambs between four and eight weeks old. However, over time, it is suggestive that the development of resistance to the infection by this protozoan occurs, thus reducing the excretion of oocysts. Further risk factors in addition to high stocking are: breeding intensification, no rotation of pasture, stress, slatted floors, dirt, and poor hygiene (Chartier and Paraud, 2012; Carneiro et al., 2022). The possible cause of this infection during the trial was when the animals returned to the covered shed every day in the late afternoon and were housed in up to 5 animals per pen. Besides the treatment with toltrazuril, bedding changes, cleaning measures of pens and troughs were adopted to control the infection in the animals and to reduce the environmental contamination.

Significant lower overall mean of faecal oocyst counts for the WCS treatment were recorded in this trial suggesting, a possible positive effect for WCS in being useful to control *Eimeria* spp. infection. Our data agrees with Teye et al. (2010), that observed differences in rams, ewes, and lambs naturally infected by gastrointestinal parasites and supplemented with WCS. Lower OPG counts were reported for the supplemented treatment when compared to the non-supplemented treatment, and the decrease of faecal oocyst counts as well as faecal egg counts could be attributed for the presence of gossypol in the WCS. In addition, gossypol has been reported to have an inhibitory action on enzymes and on the growth of *Trypanosoma cruzi* (Montamat et al., 1982), as well as an anti-amoebic effect on *Entamoeba histolytica* (González-Garza et al., 1993). Gossypol is an antinutrient polyphenolic compound secreted by pigment glands distributed throughout the cotton plant, with higher concentrations in the seeds, and in plants promotes resistance against insects (Bottger et al., 1964; Sihag et al., 2021). For monogastric animals it is highly toxic, however, ruminants are more resistant to the compound due to ruminal fermentation, binding with gossypol and consequently reducing the toxic side effects (Knutsen et al., 2017; Sihag et al., 2021).

For both treatments, there was a linear increase in the body weight of lambs over time, however, when the average daily weight gain data was analyzed, low gains were observed between TP1 and TP6 regardless of the treatment. Without statistical differences for treatments for the variables body weight and average daily weight gain, the diets did not influence the initial poor performance of the animals, but at the end of the trial the expected averages of daily weight gain were reached. The sum of the co-infection by GIN and *Eimeria* spp., triggered limitations for these animals, impairing the performance and health status. Starling et al. (2019) evaluated productive losses in Dorper lambs naturally infected by GIN receiving basal and supplemented diets. Losses were observed in the infected groups regardless the diet when compared with the animals in the control group (without GIN infection).

In addition to the damage caused by GIN, *Eimeria* infection played a role in this scenario. Infection by *Eimeria* protozoa in sheep contributes to economic

losses, as the low weight gain is due to clinical and subclinical disease (Chartier and Paraud, 2012). Lambs in confinement, between three and four months old, with severe diarrhea, presented whitish lesions in the duodenum, proximal jejunum, severe atrophy, and epithelial desquamation by *Eimeria* (Amarante et al., 1993).

In this study the supplementation with the inclusion of WCS and partial replacement of ground corn fed to lambs resulted in similar productive gains when compared to the control diet. However, Tardivo et al. (2021) evaluated these same animals from this study soon after the infection by gastrointestinal parasites was controlled, and they observed a higher average daily weight gain and a lower cost/ kg in the production for the treatment with the WCS supplementation. Demir and Can (2019) reported that there was no difference between average daily weight gain, feed intake, dry matter intake, feed efficiency, and water consumption, in Awassi male lambs supplemented with 8.5% and 17% of WCS. In addition, the same authors indicated that the concentrate could be replaced by WCS in up to 8.5% without negative effects on the health of the animals, and might reduce the production cost, in view that the WCS costs less than concentrate. Thus, the inclusion of the WCS in lambs' diets is an alternative as a co-product, which may decrease the production costs through partial replacement of ground corn with satisfactory results in performance.

There were no significant differences between treatments for total protein and globulins. However, higher albumin concentrations were observed for the control treatment. The decrease in albumin concentration for the WCS treatment could be caused by the co-infection of GIN and *Eimeria* spp.. Although there was a pronounced decrease for the albumin and total protein in both treatments at TP5, at the end of the trial the concentrations increased. Experimental trials reported that infections by GIN (Starling et al., 2019; Carvalho et al., 2021; Schmidt et al., 2021), as well as by *Eimeria* spp. (Catchpole and Gregory, 1985; Mohamaden et al., 2018), decreased total protein and albumin concentrations in sheep. In addition, although the gastrointestinal parasites were infecting the animals in this present study, according to Kaneko et al. (2008) the albumin concentrations were within the reference range interval for sheep, except for total

protein, which concentrations were slightly below 6 g/dL for both treatments at TP2, TP4, and TP5.

The treatments did not influence cholesterol concentrations in this trial. On the other hand, Demir and Can (2019) observed increases only in cholesterol levels in lambs supplemented with WCS, and no changes in albumin, total protein, urea, glucose, and potassium levels. In another trial, intoxication was reported in calves receiving cottonseed meal containing 400 and 800 ppm of free gossypol, and in some animals the albumin, globulin, and total protein concentrations decreased. However, no signs of disease were detected in calves consuming 100 and 200 ppm of free gossypol, and the authors reported that the changes in the profiles of the serum biochemistry analytes were insufficient to determine safe or unsafe dietary concentrations of free gossypol fed to young calves (Risco et al., 1992). Corroborating with the previous trial, Câmara et al. (2016) evaluated effects of gossypol from cottonseed cake in sheep and concluded that there were no consistent changes in biochemical analytes.

Biomarkers of oxidative stress are becoming important tools in veterinary medicine to assess various infectious and non-infectious diseases that affect domestic and production animals. Moreover, it provides data regarding the monitoring of different procedures to reduce the parasite load (Schmidt et al., 2021), stress monitoring (Rubio et al., 2019), and evaluation of diets fed to lambs (Pathak et al., 2017). Our current scenario aims at a sustainable production, including animal welfare, decreasing the use of undesired treatments that lead to the resistance of several microorganisms such as parasites, and in the decrease of environmental impacts caused by animal production for human consumption. Thus, a panel analysis of biomarkers of oxidative stress was performed.

The TEAC, FRAP, and CUPRAC assays, involve several antioxidant molecules and were used to evaluate TAC. Significant differences were only observed for TEAC among treatments, with higher mean concentration recorded for the WCS treatment. A possible change in TEAC may have occurred due to increased production of oxidants during the experimental trial. Rubio et al. (2019) reported increased concentrations of salivary FRAP, CUPRAC, TEAC, and uric acid in sheep experimentally induced to stress, indicating that there was an

increase in antioxidants to maintain the redox balance, thus preventing further damage to cells after exposure to oxidants. However, the gastrointestinal parasites infection could have influenced the oscillation of TEAC concentrations over the period in this experimental trial, since the concentration of TEAC reduced at TP5 despite the treatment, and after the parasite load decreased, the concentration of TEAC increased at TP7. Our results are in line with Schmidt et al. (2021), that observed a decrease in CUPRAC and TEAC concentrations in lambs in an ICLS and were naturally infected by GIN. After 70 days of closantel treatment and under the influence of the ICLS, there was an increase in CUPRAC and TEAC concentrations, with a decrease in GIN infection, and a negative correlation of these biomarkers with EPG. In addition, these same authors observed that the ICLS, besides decreasing the parasite infection, improved the inflammatory and oxidative response of the animals. Thus, another factor that could be related to the increase in TEAC concentration for the WCS treatment, although not measured in this trial, would be the tocopherol that is part of the cottonseed.

According to Sihag et al. (2021), cottonseed oil is rich in tocopherol, and has an antioxidant activity by reacting against free radicals. Lactating dairy cows fed with 15% WCS inclusion had higher plasma α-tocopherol concentrations when compared to animals that did not receive the WCS, additionally, the increase of α-tocopherol in blood could have antioxidant effects (Risco et al., 2002). Another study, also with lactating dairy cows, reported that with increasing intake of free gossypol from WCS, there is an increase in serum Vitamin E (Mena et al., 2004).

No significant differences were observed for uric acid and PON-1 analytes between treatments; however, significant differences and higher thiol concentrations were recorded for the control treatment. According to Schmidt et al. (2021) lambs naturally infected by GIN with EPG counts higher than 5000, had a strong negative correlation of total thiol. Therefore, animals with higher parasites burdens showed a decrease in total thiol concentrations, indicating that GIN infection probably resulted in changes in the balance between reduced and oxidized thiol. A further hypothesis that could be attributed is through albumin,

since is the major portion of the plasma thiol pool (Yılmaz et al., 2021). As discussed above, lower albumin concentrations were observed for the WCS treatment when compared to the control treatment. To prevent the albumin possible effect, it is indicated to use the ratios of disulphide/total thiol, disulphide/native thiol, and native thiol/total thiol (Schmidt et al., 2021; Yılmaz et al., 2021). For this reason, future studies should consider the use of total thiol, disulphide and native thiol in combination, aiming to obtain refined results and to prevent the albumin effects.

For oxidants investigation, the biomarkers AOPP, TOS, FOX and d-ROMs were evaluated. Higher means were observed for the AOPP and d-ROMs analytes for the WCS treatment, however, the control treatment had higher mean for FOX. There were no significant differences for TOS between treatments. We hypothesized that WCS inclusion may have stimulated a higher host immune response against parasitic infection, which resulted in increased oxidants. Furthermore, although without statistical differences, an increase was observed for the analytes AOPP, TOS, FOX and d-ROMs at TP3 for the animals fed with WCS. The dynamics of increased concentrations for these oxidant biomarkers were not observed at TP3 for the control treatment. Our results are similar to other studies that reported increased oxidants in sheep infected with GIN (Machado et al., 2014; Baptistiolli et al., 2018; Alam et al., 2020), which induce local oxidative stress and mucosal damage, resulting in systemic changes depending on the intensity of infection (Schmidt et al., 2021). Machado et al. (2014) observed a significant increase in AOPP levels in crossbred lambs, 75 days post-experimental infection with *H. contortus* larvae, and reported that naturally infected animals also had similar increases in AOPP levels associated with an inflammatory infiltrate in the mucosa of the abomasum. Moreover, these same authors reported changes in the levels of antioxidants with the purpose of cellular protection, which in turn was observed by the increase of FRAP.

The diet may have influenced the production of oxidants, as according to Moretti et al. (2019), Santa Ines ewes fed with cottonseed concentrate during the reproductive period may increase oxidative stress biomarkers, indicating redox imbalance, since oxygen radical absorption capacity and catalase were lower for

this treatment when compared to the control treatment that received soybean concentrate. However, the authors reported probable positive effects on colostrum from ewes that consumed cottonseed concentrate, such as antibacterial activity, antioxidant potential, and immune quality.

According to the findings of this experimental trial, to assist in the investigation of the role of the WCS inclusion in lambs' diets regarding oxidative stress, further studies should be conducted without the influence of infection by gastrointestinal parasites and with different percentages of WCS inclusion in the diet.

5. Conclusion

The diet with WCS inclusion in this experimental trial suggested the benefit in controlling the *Eimeria* spp. infection as well as inducing increase in oxidants and antioxidants in lambs naturally infected by GIN and *Eimeria* spp., as the biomarkers TEAC, AOPP and d-ROMs were higher for the WCS treatment. The combination of WCS and ICLS can be useful in controlling gastrointestinal parasites infection, without affecting the animal's performance. In addition, WCS can be used for the formulation of lambs diets, without negative effects according to this trial, although it did not increase weight gain when compared to the control diet, but apparently improved the body's oxidative immune response.

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SUPPLEMENTARY FILE

FILE I

Supplementary File 1. Mean (± standard deviation) of eggs per gram of faeces (EPG) and oocysts per gram of faeces (OPG) of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

		Time Points								Effects (*p*-value)		
	T	TP1	TP2	TP3	TP4	TP5	TP6	TP7	**General Mean**	T	TP	TxTP
EPG	WCS	3.141 (± 0.615)	3.275 (± 0.572)	2.900 (± 1.146)	2.691 (± 0.869)	2.374 (± 1.055)	2.152 (± 1.112)	1.149 (± 1.200)	2.526A	0.348	< 0.0001	0.069
	Control	2.310 (± 1.376)	2.921 (± 0.843)	3.065 (± 0.457)	2.376 (± 0.966)	2.224 (± 1.149)	2.138 (± 1.102)	1.819 (± 1.053)	2.408A			
	General Mean	2.726abd	3.098b	2.983ab	2.534abd	2.300ad	2.145cd	1.484c				
OPG	WCS	1.075 (± 1.437)	2.249 (± 1.129)	2.949 (± 0.654)	3.294 (± 0.554)	0.377 (± 0.871)	0.711 (± 1.041)	1.535 (± 1.445)	1.741A	0.039	< 0.0001	0.371
	Control	1.344 (± 1.430)	2.836 (± 0.318)	3.260 (± 0.604)	3.715 (± 0.597)	0 (± 0)	0.721 (± 1.057)	2.138 (± 1.402)	2.002B			
	General Mean	1.209ad	2.543b	3.105bc	3.505c	0.188e	0.716ae	1.837d				

Data transformed to log10 (x + 1); TP = Time point; T = Treatments; WCS = Whole cottonseed. For each variable, means followed by different uppercase letters in the column and means followed by different lowercase letters in the row differ statistically by Tukey test ($p < 0.05$).

FILE II

Supplementary File 2. Mean (± standard deviation) of body weight (kg) values of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

		Time Points							General Mean	Effects (*p*-value)		
	T	TP1	TP2	TP3	TP4	TP5	TP6	TP7		T	TP	TxTP
Body Weight (kg)	WCS	25.817 (± 5.646)	27.250 (± 6.113)	28.900 (± 5.892)	30.544 (± 6.168)	31.694 (± 6.404)	33.683 (± 6.649)	36.483 (± 6.565)	30.625A	0.743	< 0.0001	1.000
	Control	25.422 (± 5.174)	26.900 (±5.277)	28.400 (± 5.327)	30.044 (± 5.187)	31.883 (± 5.387)	33.200 (± 6.164)	36.817 (± 6.323)	30.381A			
	General Mean	25.619a	27.075ae	28.650ace	30.294bce	31.789bc	33.442bd	36.650d				

TP = Time Point; T = Treatments; WCS = Whole cottonseed. For each variable, means followed by different uppercase letters in the column and means followed by different lowercase letters in the row differ statistically by Tukey test ($p < 0.05$).

FILE III

Supplementary File 3. Mean (± standard deviation) of average daily weight gain values of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

		Time Points						Effects (*p*-value)		
	T	TP1-TP2	TP2-TP3	TP3- TP4	TP4-TP5	TP5-TP6	TP6-TP7	T	TP	TxTP
ADG (g/day)	WCS	102.381Aa (± 87.892)	117.857Aa (± 72.274)	117.460Aa (± 52.273)	82.143Aa (± 94.069)	142.063Aab (± 87.546)	200.000Ab (± 60.311)	0.391	< 0.0001	0.028
	Control	105.556Aa (± 56.326)	107.143Aa (± 53.114)	117.460Aa (± 106.967)	131.349Aa (± 65.012)	94.048Aa (± 70.721)	258.333Ab (± 57.261)			

ADG = Average daily weight gain; TP = Time Point; T = Treatments; WCS = Whole cottonseed. For each variable, means followed by different uppercase letters in the column and means followed by different lowercase letters in the row differ statistically by Tukey test ($p < 0.05$).

FILE IV

Supplementary File 4. Mean (± standard deviation) concentrations of the biochemicals analytes of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

		Time points								Effects (*p*-value)		
	T	TP1	TP2	TP3	TP4	TP5	TP6	TP7	**General Mean**	T	TP	TxTP
Albumin (g/dL)	WCS	2.641 (± 0.470)	2.657 (± 0.466)	2.742 (± 0.427)	2.575 (± 0.377)	2.543 (± 0.483)	2.701 (± 0.430)	2.775 (± 0.354)	2.662A	0.005	0.116	0.761
	Control	2.957 (± 0.415)	2.862 (± 0.338)	2.863 (± 0.290)	2.714 (± 0.308)	2.628 (± 0.353)	2.765 (± 0.335)	2.807 (± 0.286)	2.779B			
	General Mean	2.799a	2.759a	2.802a	2.644a	2.585a	2.733a	2.791a				
Total Protein (g/dL)	WCS	6.155 (± 0.945)	5.913 (± 0.640)	6.043 (± 0.618)	5.920 (± 0.582)	5.761 (± 0.858)	6.087 (± 0.590)	6.271 (± 0.420)	6.021A	0.785	0.047	1.000
	Control	6.136 (± 0.629)	5.965 (± 0.449)	6.119 (± 0.445)	5.950 (± 0.592)	5.796 (± 0.717)	6.095 (± 0.764)	6.244 (± 0.613)	6.044A			
	General Mean	6.146ab	5.939ab	6.081ab	5.935ab	5.779a	6.091ab	6.258b				
Globulins (g/dL)	WCS	3.514 (± 0.767)	3.256 (± 0.426)	3.301 (± 0.381)	3.345 (± 0.425)	3.218 (± 0.516)	3.385 (± 0.394)	3.496 (± 0.344)	3.359A	0.059	0.167	0.862
	Control	3.179 (± 0.472)	3.104 (± 0.476)	3.257 (± 0.430)	3.236 (± 0.460)	3.168 (± 0.485)	3.329 (± 0.545)	3.437 (± 0.490)	3.244A			

Cholesterol (g/dL)	**General Mean**	3.347a	3.180a	3.279a	3.291a	3.193a	3.357a	3.467a			
	WCS	56.084 (± 12.455)	54.669 (± 11.650)	67.225 (± 15.975)	63.905 (± 12.526)	61.878 (± 11.940)	61.806 (± 17.487)	71.793 (± 13.573)	62.480A	0.263	0.002	0.518
	Control	57.263 (± 15.890)	58.823 (± 10.862)	64.804 (± 10.792)	60.993 (± 9.133)	57.996 (± 13.511)	61.406 (± 11.573)	63.069 (± 14.051)	60.622A			
	General Mean	56.674a	56.746a	66.015b	62.449ab	59.937ab	61.606ab	67.431b			

TP = Time point; T = Treatments; WCS = Whole cottonseed. For each variable, means followed by different uppercase letters in the column and means followed by different lowercase letters in the row differ statistically by Tukey test ($p < 0.05$).

FILE V

Supplementary File 5. Mean (± standard deviation) concentrations of the biomarkers of oxidative stress of crossbred lambs naturally infected with gastrointestinal nematodes and *Eimeria* spp., receiving two different treatment diets (whole cottonseed and control) at seven different time points (TP1 – TP7). Between each time point there was an interval of 14 days.

		Time points								Effects (*p*-value)		
	T	TP1	TP2	TP3	TP4	TP5	TP6	TP7	**General Mean**	T	TP	TxTP
TEAC (mmol/L)	WCS	0.598 (± 0.079)	0.597 (± 0.066)	0.604 (± 0.075)	0.596 (± 0.046)	0.579 (± 0.093)	0.606 (± 0.060)	0.625 (± 0.043)	0.601A	0.015	0.148	0.974
	Control	0.587 (± 0.057)	0.593 (± 0.039)	0.585 (± 0.042)	0.574 (± 0.049)	0.558 (± 0.062)	0.573 (± 0.063)	0.599 (± 0.069)	0.581B			
	General Mean	0.593ab	0.595ab	0.594ab	0.585ab	0.568a	0.590ab	0.612b				
FRAP (mmol/L)	WCS	0.438 (± 0.116)	0.444 (± 0.104)	0.470 (± 0.162)	0.448 (± 0.092)	0.473 (± 0.104)	0.462 (± 0.089)	0.502 (± 0.110)	0.463A	0.191	0.119	0.953
	Control	0.446 (± 0.104)	0.472 (± 0.089)	0.484 (± 0.080)	0.495 (± 0.067)	0.467 (± 0.091)	0.466 (± 0.066)	0.527 (± 0.124)	0.480A			
	General Mean	0.442a	0.458ab	0.477ab	0.472ab	0.470ab	0.464ab	0.515b				
CUPRAC (mmol/L)	WCS	0.485 (± 0.069)	0.492 (± 0.077)	0.514 (± 0.082)	0.496 (± 0.052)	0.482 (± 0.072)	0.516 (± 0.075)	0.522 (± 0.049)	0.501A	0.778	0.290	0.507
	Control	0.519 (± 0.050)	0.512 (± 0.043)	0.502 (± 0.053)	0.486 (± 0.038)	0.489 (± 0.063)	0.498 (± 0.045)	0.516 (± 0.056)	0.503A			

	General Mean	0.502a	0.502a	0.508a	0.491a	0.485a	0.507a	0.519a				
Thiol (µmol/L)	WCS	201.544 (± 54.162)	199.789 (± 48.077)	220.117 (± 55.078)	200.372 (± 38.545)	202.322 (± 46.723)	231.400 (± 48.109)	247.394 (± 41.790)	214.71A	0.0009	< 0.0001	0.714
	Control	237.317 (± 37.795)	225.861 (± 35.637)	219.056 (± 32.634)	214.111 (± 31.969)	218.839 (± 38.139)	247.333 (± 40.284)	269.133 (± 50.826)	233.09B			
	General Mean	219.43ac	212.83ac	219.59ac	207.24c	210.58ac	239.37ab	258.26b				
Uric acid (mg/dL)	WCS	0.168 (± 0.038)	0.185 (± 0.055)	0.175 (± 0.039)	0.168 (± 0.054)	0.189 (± 0.066)	0.167 (± 0.037)	0.161 (± 0.054)	0.173A	0.175	0.439	0.430
	Control	0.175 (± 0.039)	0.162 (± 0.028)	0.183 (± 0.046)	0.189 (± 0.028)	0.194 (± 0.037)	0.189 (± 0.066)	0.175 (± 0.030)	0.181A			
	General Mean	0.171a	0.174a	0.179a	0.179a	0.192a	0.178a	0.168a				
PON-1 (IU/L)	WCS	13.113 (± 3.560)	16.717 (± 2.836)	17.646 (± 2.964)	18.057 (± 3.101)	17.578 (± 4.019)	14.991 (± 7.489)	15.647 (± 6.177)	16.250A	0.382	0.006	0.561
	Control	13.283 (± 4.385)	17.290 (± 3.936)	16.282 (± 5.260)	16.012 (± 5.168)	15.102 (± 4.930)	15.856 (± 5.547)	16.314 (± 3.582)	15.734A			
	General Mean	13.198a	17.004b	16.964b	17.034b	16.340ab	15.424ab	15.981ab				

AOPP (µmol/L)	WCS	172.689 (± 60.568)	155.250 (± 47.443)	189.606 (± 109.427)	170.528 (± 32.968)	156.133 (± 37.443)	123.922 (± 28.236)	157.861 (± 27.055)	160.86A	0.011	0.0002	0.584
	Control	139.744 (± 35.131)	158.017 (± 37.018)	154.822 (± 38.009)	165.722 (± 37.743)	126.906 (± 33.746)	109.172 (± 29.069)	153.361 (± 93.358)	143.96B			
	General Mean	156.22b	156.63b	172.21b	168.13b	141.52ab	116.55a	155.61b				
TOS (µmol/L)	WCS	13.883 (± 6.798)	12.861 (± 5.603)	20.261 (± 19.980)	11.372 (± 3.477)	14.411 (± 5.672)	11.994 (± 3.518)	15.578 (± 4.805)	14.337A	0.253	0.031	0.293
	Control	16.594 (± 5.275)	15.139 (± 5.335)	15.383 (± 5.365)	14.467 (± 5.136)	13.933 (± 5.682)	13.183 (± 3.997)	20.217 (± 16.948)	15.559A			
	General Mean	15.239a	14.000a	17.822a	12.919a	14.172a	12.589a	17.897a				
FOX (µmol/L)	WCS	51.965 (± 10.350)	52.705 (± 6.829)	57.276 (± 23.182)	52.074 (± 5.356)	52.559 (± 9.124)	49.323 (± 7.223)	56.166 (± 5.568)	53.153A	0.040	0.078	0.850
	Control	52.373 (± 8.091)	56.208 (± 10.235)	57.360 (± 9.714)	57.790 (± 7.874)	53.143 (± 7.676)	54.457 (± 6.606)	60.689 (± 19.144)	56.003B			
	General Mean	52.169a	54.457a	57.318a	54.932a	52.851a	51.890a	58.428a				
d-ROMs (Carr units)	WCS	122.356 (± 31.021)	103.789 (± 22.884)	113.883 (± 22.811)	93.089 (± 19.917)	89.217 (± 18.569)	87.055 (± 20.932)	101.489 (± 24.830)	101.55A	0.0008	< 0.0001	0.525
	Control	107.056 (± 26.855)	88.800 (± 20.556)	94.628 (± 17.288)	82.888 (± 21.599)	83.183 (± 20.939)	86.450 (± 21.537)	99.211 (± 27.595)	91.745B			

General Mean	114.71b	96.294ac	104.26ab	87.989c	86.200c	86.753c	100.35abc

TP = Time point; T = Treatments; WCS = Whole cottonseed; TEAC = Trolox equivalent antioxidant capacity; FRAP = Ferric reducing ability of plasma; CUPRAC = Cupric reducing antioxidant capacity; PON-1 = Paraoxonase-1; AOPP = Advanced oxidation protein products; TOS = Total oxidant status; FOX = Ferric-xylenol orange; d-ROMs = Reactive oxygen metabolites derived compounds. For each variable, means followed by different uppercase letters in the column and means followed by different lowercase letters in the row differ statistically by Tukey test ($p < 0.05$).