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FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

DESEMPENHO PRODUTIVO DE CORDEIROS ILE DE FRANCE E SANTA INÊS SUBMETIDOS A TRATAMENTO SUPRESSIVO OU SELETIVO COM ANTI-HELMÍNTICO

ANA CLÁUDIA ALEXANDRE DE ALBUQUERQUE

Botucatu, SP

Dezembro 2019

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Tese apresentada junto ao Programa de pós-graduação em Medicina Veterinária para a obtenção do título de Doutora.

Orientador: Alessandro Francisco Talamini do Amarante.
Co-orientadores: Fabiana Alves de Almeida e César Cristiano Bassetto.

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DEDICATÓRIA

À minha mãe e minha madrinha,
por estarem de mãos dadas
comigo nessa jornada.

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Aos animais, que permitiram que esse estudo fosse possível.

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Campus de Botucatu

ATESTADO

Atesto que o Projeto de Pesquisa "Desempenho produtivo de cordeiros Ile de France e Santa Inês submetidos a tratamento seletivo com anti-helmíntico" **Protocolo CEUA 47/2016**, a ser conduzido por **Ana Cláudia Alexandre de Albuquerque**, orientador Prof. Alessandro Francisco Talamini do Amarante, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal – CONCEA.

Vigência do projeto	12/09/2016 a 17/04/2017
Finalidade	Pesquisa Científica
Espécie/Linhagem	Ovina/Ovis aries
Nº de animais	40
Peso/Idade	22 kg/60-90 dias
Sexo	Macho
Origem	Propriedades no Estado de SP

Projeto de Pesquisa aprovado em reunião da CEUA em 13/05/2016

Profª.Ass.Drª. Ibiara Correia de Lima Almeida Paz

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ALBUQUERQUE, A.C.A. **Desempenho produtivo de cordeiros Ile de France e Santa Inês submetidos a tratamento supressivo ou seletivo com anti-helmíntico.** Botucatu, 2019. 109p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

RESUMO

A infecção por nematódeos gastrintestinais (NGI) está entre os principais problemas sanitários e econômicos na ovinocultura. Como alternativa contra intensa utilização de anti-helmínticos surgiram os tratamentos seletivos. O presente estudo teve por objetivo avaliar e comparar o desempenho produtivo e a resposta imunológica de cordeiros Santa Inês e Ile de France, naturalmente infectados por NGI, submetidos a tratamento supressivo ou seletivo com anti-helmíntico. Trinta e oito cordeiros foram alocados em dois tratamentos anti-helmínticos: supressivo - 9 Santa Inês e 10 Ile de France tratados com monepantel a cada 14 dias; seletivo - 10 Santa Inês e 9 Ile de France tratados quando apresentaram volume globular ≤ 20%. A pesagem e as amostras de sangue e fezes foram coletadas semanalmente, para realização de contagem de ovos por grama de fezes (OPG), determinação do volume globular (VG), proteína plasmática total (PPT) e contagem de eosinófilos sanguíneos, além de aferidos imunoglobulina G (IgG) e IgE no plasma sanguíneo. Após o abate, foram retirados 10% do conteúdo gastrointestinal e armazenados; posteriormente os parasitas foram contados e identificados. Adicionalmente, células inflamatórias foram quantificadas nas mucosas abomasais e intestinais, e níveis de IgA aferido no muco, além de realização de imunohistoquímica. Ocorreu redução da eficácia do monepantel para 0% no tratamento supressivo e 76% no seletivo. Os animais sob tratamento seletivo apresentaram desempenho produtivo inferior, com menores valores de peso vivo, ganho em peso médio diário, peso de carcaça e rendimento de carcaça quente. Os animais Santa Inês apresentaram melhor resposta imunológica em comparação aos Ile de France, principalmente contra *Haemonchus contortus*, com menores valores de OPG e carga parasitária, além de maiores contagens células inflamatórias e níveis de IgG e IgA. Concluímos que o tratamento seletivo não retardou o aparecimento de resistência anti-helmíntica e não preveniu perdas produtivas. A raça Santa Inês apresentou grande potencial em melhorar a resistência dos ovinos aos NGI em programa de seleção de animais, como uma medida de controle estratégico alternativo.

Palavras-chave: Nematódeos gastrointestinais; Pequenos ruminantes; Performance produtiva; Resposta imune.

ALBUQUERQUE, A.C.A. **Productive performance of Ile de France and Santa Ines lambs under suppressive or targeted selective treatment with anthelmintic.** Botucatu. 109p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

ABSTRACT

The gastrointestinal nematodes (GIN) infections are among the main health and economic problems affecting sheep farming. The targeted selective treatment (TST) arises as an alternative approach for worm control to reduce the anthelmintic use. The present study aimed to assess and compare productive performance and immunological response of Santa Ines and Ile de France lambs naturally infected with GIN under TST or suppressive anthelmintic treatments programs. Thirty eight lambs were allocated in two anthelmintic groups: suppressive - 9 Santa Ines and 10 Ile de France drenched with monepantel every 14 day; targeted selective treatment (TST) - 10 Santa Ines 9 Ile de France drenched when presented packed cell volume (PCV) ≤ 20%. Animals were weighing and faecal and blood samples were collected weekly to perform eggs per gram of faeces (EPG), PCV, total plasma protein, blood eosinophils counts and immunoglobulin G (IgG) and IgE levels were measured in plasma. Post mortem parasites species were counted and identified. In addition, inflammatory cells were quantified in abomasums and intestine mucosa, and IgA were dosed in mucus, besides immunohistochemistry was performed. Monepantel showed reduction to 0% and 76% in efficacy in suppressive and TST group, respectively. Animals under TST presented inferior productive performance, with low body weight gain, daily weight gain, carcass weight and hot carcass yield. Santa Ines lambs showed greater immunological response than Ile de France, mainly to *Haemonchus contortus*, with lower EPG and worm burden, besides high inflammatory cells count and IgG and IgA levels. In conclusion, the targeted selective treatment does not prevent anthelmintic resistance and animal productive losses. Santa Ines breed presented potential value in improving the resistance of sheep to GIN infection in breeding program as sustainable alternative control strategy.

Key words: Gastrointestinal nematodes; Small ruminants; Productive performance; Immune response.

CAPÍTULO 1

1. INTRODUÇÃO

O Brasil esteve no auge da ovinocultura no ano de 1991 com 20.127.952 cabeças de ovinos (FAO, 2016). No entanto, a queda no preço da lã nos anos 90 acarretou em considerável diminuição no rebanho (AMARANTE, 2014). Atualmente, o Brasil possui um rebanho de ovino com 18 milhões cabeças (IBGE, 2017), indicando um retorno no investimento na ovinocultura, voltado para a comercialização da carne de cordeiro.

A infecção por parasitas gastrintestinais é considerada um dos maiores obstáculos para criadores de ovinos e está entre os principais problemas sanitários que acometem essa espécie (CHAGAS et al., 2011; VALCÁRCEL et al., 2015). Os prejuízos econômicos são elevados, devido à redução na produtividade, apresentando menor eficiência de conversão alimentar, menor ganho em peso, diminuição na produção de leite e lã, qualidade de carcaça inferior, além de maiores despesas com produtos veterinários e mão de obra, além do aumento na mortalidade (CARDIA et al., 2011; BRITO et al., 2013; AMARANTE, 2014; COSTA JUNIOR; AMARANTE, 2015).

O Brasil possui um clima favorável para a sobrevivência e desenvolvimento das larvas de parasitos nas pastagens ao longo do ano, contribuindo para taxas elevadas de contaminação dos rebanhos (AMARANTE, 2009). Animais com altas cargas parasitárias apresentam significativa diminuição na produtividade (AMARANTE, 2014). Para o controle parasitário a principal medida adotada pelos produtores é a utilização de anti-helmínticos, no qual é realizada de forma repetitiva e indiscriminada sem orientação especializada (BRICARELLO, 2015; CARDOSO et al., 2015). Este tipo de estratégia visa maximizar a produção, porém não é economicamente sustentável, devido a presença de resíduos químicos nas carcaças e no ambiente, e ao aparecimento de populações de nematódeos resistentes (BRITO et al., 2013; AMARANTE, 2014; VALCÁRCEL et al., 2015).

A resistência de NGI à drogas anti-helmínticas é um problema global na ovinocultura que vem se intensificando com o passar dos anos, ocorrendo a diminuição da eficácia desses compostos (GILLEARD, 2006). Em cordeiros infectados artificialmente com *Haemonchus contortus* e *Trichostrongylus colubriformis*, observou-se significativa diminuição da eficácia dos principais princípios ativos utilizados na ovinocultura: albendazol, levamisol, moxidectin, ivermectina, triclorfon e closantel (ALMEIDA et al., 2010). Evidenciando o cenário alarmante em que a produção de ovinos se encontra em relação à resistência aos antiparasitários.

Em 2008, o monepantel foi lançado como uma alternativa para o tratamento das helmintoses em rebanhos de ovinos onde havia caso de múltipla resistência (KAMINSKY et al., 2008). Contudo, a partir de 2013 surgiram relatos de populações de nematódeos resistentes a esse princípio ativo em vários países, principalmente envolvendo a espécie *H. contortus* (SCOTT et al., 2013; MEDEROS et al., 2014; VAN DENBROM, 2015; SALES; LOVE, 2016; MARTINS et al., 2017), até mesmo quando tratados seletivamente (ALBUQUERQUE et al., 2017). De forma que, medidas alternativas como o tratamento seletivo, não estão sendo suficientes para frear o rápido aparecimento de populações de nematódeos resistentes.

A não utilização de anti-helmínticos na rotina produtiva de ovinos é utópico, pois há momentos em que sua utilização é necessária, como: no periparto em que as fêmeas se tornam mais suscetíveis (ROCHA et al., 2004), quando há infecções maciças em cordeiros antes ou após a desmama (ROCHA et al., 2005) ou em adultos com manifestação clínica de parasitose (AMARANTE et al., 2009). Dessa forma, é importante que as drogas anti-helmínticas sejam utilizadas de forma racional em conjunto com outras práticas alternativas a fim de minimizar a seleção de populações resistentes (BRICARELLO, 2015).

Já existem alternativas para amenizar a utilização de anti-helmínticos, como: pastejo integrado com diferentes espécies de herbívoros (AMARANTE, 2014); sistemas integrados de produção agropecuária, que consiste na associação na produção de animais com produção agrícola de forma integrada, intercalada ou sucessão (MACEDO, 2009); seleção e cruzamento de raças

resistentes com raças comerciais (AMARANTE, 2009), maior aporte nutricional aos animais (AMARANTE, 2014; AMARANTE, 2015) e tratamentos seletivos (MEDINA-PÉREZ et al., 2015); vacinação dos animais contra *Haemonchus contortus*, porém infelizmente esta pratica ainda não pode ser adotada no Brasil devido a vacina ainda não estar disponível comercialmente no país (BASSETTO; AMARANTE, 2015).

A utilização de animais mais resistentes a infecções por nematódeos gastrointestinais (NGI) como método de controle alternativo permitirá a redução do número de larvas nas pastagens e a infecção dos animais por esses parasitas, reduzindo assim, a necessidade de administrar anti-helmínticos e por consequência o aparecimento de resistência à essas drogas (AMARANTE; AMARANTE, 2003; ZVINOROVA et al., 2018).

Apesar de tratamentos seletivos terem surgido como uma alternativa para desacelerar o aparecimento de resistência anti-helmíntica, ainda não foi observada mudança significativa que mostre alteração no cenário da produção de ovinos no Brasil (AMARANTE, 2014).

Este estudo tem por objetivo avaliar o desenvolvimento da resposta imunológica e a influência do tratamento seletivo no desempenho produtivo de cordeiros das raças Santa Inês e Ile de France, naturalmente infectados por NGI, comparando-os com animais tratados de forma supressiva, mantidos em condições de baixa exposição a infecções helmínticas.

2. REVISÃO DE LITERATURA

2.1- Parasitas gastrintestinais em ovinos

O parasitismo por NGI é considerado o principal obstáculo ao desenvolvimento da ovinocultura responsável por grandes perdas produtivas e econômicas (KAPLAN et al., 2004; AMARANTE, 2014).

Os ovinos comumente são parasitados simultaneamente por várias espécies de NGI, ocorrendo o somatório de efeitos patogênicos de cada espécie de parasita (AMARANTE, 2015). A diversidade de espécies que são encontradas nesses animais depende do manejo utilizado, das condições ambientais e do tratamento anti-helmíntico (AMARANTE, 2009).

No Brasil, os nematódeos mais importantes presentes na produção de ovinos são *Haemonchus contortus* e *Trichostrongylus colubriformis*, possuem elevada relevância sanitária por serem prevalentes e patogênicos, provocando diminuição do desempenho produtivo e em infecções severas podem levar o animal à óbito (AMARANTE, SALES, 2007; AMARANTE, 2014).

Haemonchus contortus habita o abomaso de ruminantes e é o principal causador de problemas de parasitismo clínico, acompanhado de significante mortalidade. Esse parasita pode ser encontrado na região sudeste ao longo do ano e sua ação sobre o hospedeiro causa anemia, hipoproteinemia, edema submandibular, podendo levar a óbito em infecções severas. Assim, essas características clínicas tornam a haemoncose facilmente reconhecida pelos produtores (AMARANTE; SALES, 2007; AMARANTE, 2014; WILMSEN et al., 2014).

Trichostrongylus colubriformis está entre os NGIs de importância para a ovinocultura, habita no intestino delgado, é encontrado presente em boa parte das criações de ovinos. Responsável por provocar lesões na mucosa intestinal dos hospedeiros causando lesões teciduais importantes, ocasionando intensa exsudação de proteínas para a luz do órgão, distúrbios na digestão e na absorção dos nutrientes, além de redução do apetite (AMARANTE; SALES, 2007). Em cargas parasitárias elevadas o animal pode apresentar enterite severa e anorexia (AMARANTE, 2015).

Outro nematódeo importante é o *Oesophagostomum columbianum*. Possui relativa frequência no rebanho ovino, alta patogenicidade e causa lesões nodulares no intestino grosso. Os sinais clínicos apresentados pelos animais são anorexia, perda de peso, diarreia e anemia. Outras espécies encontradas em ovinos são: *Strongyloides papillosus* e *Cooperia* spp., que ocorrem no intestino delgado, causando lesões na mucosa e *Trichuris* spp., que ocorre no intestino grosso, causando infecções leves nos hospedeiros (AMARANTE, 2015).

2.2- Monepantel

Uma nova classe de anti-helmíntico foi lançada na Nova Zelândia em 2008 chamada monepantel (Zolvix®, Novartis), derivado do amino-acetonitrilo (MEDEROS et al., 2014). Esse anti-helmíntico chegou ao mercado como uma alternativa para o tratamento das helmintoses em rebanhos de ovinos, onde havia casos de resistência a outras classes de anti-helmínticos (FORTES; MOLENTO, 2013; CIUFFA et al., 2013).

Este princípio ativo atua no receptor nicotínico nAchR, encontrado apenas em nematódeos. Age como agonista dos canais iônicos, causando intensa contração da parede muscular do corpo do parasita e consequente paralisia, além de causar contração espástica da porção anterior da faringe, levando o nematódeo a morte (KAMINSKY et al., 2008).

É uma droga bem tolerada e com baixa toxicidade para ruminantes, com período de carência curto e sem contra-indicações. Possui um amplo espectro contra nematódeos gastrointestinais de ovinos, com ação comprovada contra *Haemonchus contortus, Cooperia oncophora, Cooperia curticei, Trichostrongylus colubriformis, Trichostrongylus axei, Ostertagia ostertagi, Teladorsagia circumcincta, Chabertia ovina* e *Nematodirus spathiger* (KAMINSKY et al., 2008).

Com a disponibilidade do monepantel, que apresenta elevada eficácia contra os principais parasitas de ovinos, criou-se a possibilidade de avaliar os prejuízos causados pelos helmintos em animais mantidos no pasto, livres de parasitos (submetidos a tratamentos supressivos) em comparação com animais parasitados, tratados seletivamente.

2.3- Tipos de tratamento

Na produção de pequenos ruminantes existem variados tipos de tratamentos com anti-helmínticos que podem ser utilizados, cada um com seu objetivo, vantagens e desvantagens (COSTA et al., 2011). São eles: Tratamento Preventivo, o anti-helmíntico é administrado em todo o rebanho em períodos previamente estabelecidos para prevenir infecções clínicas ou subclínicas (MOLENTO, 2005); Tratamento Curativo, princípio ativo administrado apenas quando o animal que apresentar sinais clínicos

perceptíveis (HASSUM, 2009); Tratamento Tático, utilizando em períodos do ano em que as condições ambientais favorecem as infecções por NGI, no qual todos ou alguns animais são tratados (TORRES-ACOSTA; HOSTE, 2008); Tratamento Supressivo, todos os animais são tratados a cada duas a quatro semanas com anti-helmínticos, com o objetivo de o parasita não atingir o período pré-patente da infecção (HASSUM, 2009), porém, sabe-se que com a utilização do mesmo princípio ativo de forma frequente em um período prolongado, ocorre a pressão de seleção, sobrevivendo as populações de nematódeos mais resistentes (BRITO et al., 2013; ARECE-GARCÍA et al., 2016); Tratamento não intencional, quando é realizado tratamento com droga para outra afecção, mas também possui efeito anti-helmíntico (TORRES-ACOSTA; HOSTE, 2008); e o Tratamento seletivo, se baseia em tratar com anti-helmíntico apenas os animais que apresentem alguma alteração no estado fisiológico, baseada em critérios como grau de anemia pelo exame da conjuntiva ocular (FAMACHA©), aferição do volume globular (VG), condição corporal, indicadores de diarreia e contagem de ovos por grama de fezes (OPG) (AMARANTE, 2014; MEDINA-PÉREZ et al., 2015; VALCÁRCEL et al., 2015).

Com o aparecimento da resistência anti-helmíntica em rebanhos de ovinos, surgiu à necessidade de buscar novas alternativas de controle das helmintoses gastrintestinais, uma delas é o tratamento seletivo (BEVILAQUA et al., 2015). Atualmente, no Brasil, o método de tratamento seletivo mais utilizado é o FAMACHA© (MAIA et al., 2013).

O método FAMACHA© consiste no tratamento de animais que apresentem anemia clínica causada por *H. contortus*, no qual o grau de anemia é avaliado pela coloração da conjuntiva ocular com auxílio de um cartão padrão com categorias de cores que variam de 1 a 5, cada categoria representa um intervalo de valores de VG (COSTA et al., 2011; AMARANTE, 2014; ARECE-GARCÍA et al., 2016).

Dentre as vantagens do tratamento utilizando o FAMACHA© está a redução do número de animais tratados, diminuição na utilização de anti-helmínticos e dos custos com essas drogas, além de preservação da refugia, desacelerar o aparecimento da resistência parasitária e não ser um método

invasivo (ROSALINSKI-MORAES; SOTOMAIOR, 2015; VALCÁRCEL et al., 2015). Os fatores limitantes decorrentes da utilização desse método são: sensibilidade apenas para animais infectados por *H. contortus*, sendo que os ovinos geralmente apresentam infecções parasitárias mistas, aumentando a necessidade da realização de culturas de fezes periódicas para verificar os parasitas presentes no rebanho; a avaliação do grau de anemia é subjetiva, sendo funcional somente para animais com sinais clínicos, passando despercebidos os animais resilientes; há a necessidade dos animais serem avaliados individualmente e de forma frequente; necessidade de uma infra-estrutura adequada e maior organização no manejo para a manipulação dos animais, além de mão de obra treinada para realizar as avaliações, elevando os custos de implementação e manutenção deste método (MAIA et al., 2013; ROSALINSKI-MORAES; SOTOMAIOR, 2015; VALCÁRCEL et al., 2015).

Além da infecção por *H. contortus*, existem também outros fatores que podem interferir na coloração da mucosa dos animais, mostrando que o método não deve ser utilizado de forma isolada, sendo necessário adotar outras medidas de controle em conjunto (ROSALINSKI-MORAES; SOTOMAIOR, 2015).

Apesar de esta metodologia ter sido introduzida no Brasil há mais de 10 anos, ainda não foi possível observar nenhuma mudança no cenário da resistência parasitária no Brasil (AMARANTE, 2014).

2.4- Raças

Na década de 90 houve uma queda significativa no preço da lã, que levou os produtores de ovinos a mudarem o foco da produção, voltando os esforços para a produção de carne, devido ao aumento no consumo. Com o aumento na demanda na produção de carne, foram introduzidas no país raças comerciais que possuem maior ganho em peso e rendimento de carcaça como Ile de France, Texel, Suffolk e Dorper (AMARANTE, 2014). Essas raças apresentam qualidade de carcaça e ganho em peso superior em relação às raças locais, porém são mais sensíveis a infecções por NGI, o que influencia diretamente no seu desempenho produtivo (AMARANTE et al., 2009).

A produtividade das raças de ovinos pode ser influenciada por fatores intrínsecos e extrínsecos. Os fatores intrínsecos estão relacionados diretamente ao animal, à capacidade individual que o animal tem a resistir a infecções por NGI. Os fatores extrínsecos estão relacionados a fatores externos, ao ambiente em que o animal será criado, o tipo de criação, se será intensivo, semi-intensivo ou extensivo, ao tipo de manejo sanitário e nutricional, e tipo de produção. Animais de raças comerciais, que possuem maior susceptibilidade, quando criados em condições de baixa exposição a larvas infectantes e manejo nutricional adequado, apresentam alta produtividade. Já em situações de maior exposição a larvas infectantes, as raças locais possuem como vantagem maior resistência, apresentando maior produtividade em relação as raças comerciais (AMARANTE, 2015). Já é conhecido que as raças nativas apresentam maior resistência à infecção do que as raças comerciais introduzidas (AMARANTE et al., 2004; AMARANTE et al., 2009; ROCHA et al., 2004), porém os mecanismos subjacentes à resistência natural são mal compreendidos.

A capacidade de algumas raças apresentarem resposta imunológica mais eficiente ao parasitismo é decorrente do processo de seleção natural, no qual sobreviveram os indivíduos que tiveram melhor resposta imunológica aos parasitas comuns ao seu ambiente, repassando geneticamente essa habilidade aos herdeiros (AMARANTE, 2014).

A utilização de raças que apresentam maior grau de resistência a NGI, como Santa Inês, Crioula e Morada Nova, é um fator relevante que pode ser aproveitado para diminuir as perdas econômicas na produção de ovinos (AMARANTE et al., 2009; AMARANTE, 2014).

Nos estudos desenvolvidos no Brasil, a maioria utilizou a raça Santa Inês em comparação com outras raças, e esta se demonstrou mais resistente ao parasitismo, independente da idade e categoria em que se encontravam os ovinos (AMARANTE et al., 2004; ROCHA et al., 2005; AMARANTE et al., 2009; AMARANTE, 2015).

Estudos comparando a resistência a NGI de cordeiros da raça Santa Inês e Ile de France, mostrou que os cordeiros Santa Inês foram mais resistentes tanto nas fases de amamentação quanto na de desmame, mesmo

apresentando maior contagem de OPG em alguns momentos, com valor de VG e número de eosinófilos maior em todas as idades, além de não necessitarem de tratamentos anti-helmínticos para prevenir a mortalidade dos animais em comparação aos animais da raça Ile de France (AMARANTE et al., 2004; ROCHA et al., 2005; AMARANTE et al., 2014).

Estudo em ovelhas peri-parturientes e em lactação das raças Santa Inês e Ile de France mostrou que as ovelhas Santa Inês, embora no período de maior suscetibilidade, apresentaram uma alta capacidade de suportar o parasitismo, mesmo estando em um ambiente altamente contaminado com grande quantidade de larvas infectantes de NGI (ROCHA et al., 2011). Embora alguns indicadores de resistência à parasitas tenham sido detalhados, pouco se sabe sobre as respostas imunes dessas raças.

2.5- Resposta imunológica aos nematódeos gastrointestinais

Os parasitas gastrointestinais desencadeiam resposta imune inata com hiperplasia de mastócitos na mucosa, aumento do número de leucócitos globulares, elevação no número de eosinófilos circulantes e maior produção de muco (HUNTLEY, 1984; BALIC et al., 2000; SOROBETEA et al., 2018), com posterior resposta adaptativa ocorrendo a ativação de células T $CD4^+$ "*naive*", que se diferenciam em T "*helper*" 2 (Th2), com produção de citocinas específicas incluindo interleucina 4 (IL-4), IL-5, IL-10 e IL-13, e posterior produção de anticorpos específicos (MEEUSEN et al., 2005; BALIC et al., 2002; TAYLOR et al., 2012; SOROBETEA et al., 2018).

As principais imunoglobulinas que participam da resposta ao parasitismo pelos NGI são a IgE, IgA e o IgG. Esses anticorpos poderão agir através da opsonização, em que se fixam ao parasita atraindo células efetoras, como eosinófilos e mastócitos, para liberação de grânulos sobre os parasitas; ou se fixar em áreas que possam dificultar sua alimentação, estabelecimento, fixação e reprodução (ALEXANDER; HILNTON, 2004; MOREAU; CHAUVIN, 2009; TAYLOR et al., 2012; ALLEN; MAIZELS, 2004; TIZARD, 2014; SOROBETEA et al., 2018).

Estudos têm sido desenvolvidos com o objetivo de esclarecer os mecanismos envolvidos na resposta imune ao parasitismo por NGI (TEREFE et al., 2007; GONZALEZ et al., 2011; SHAKYA et al., 2011; JACOB et al., 2016; SOROBETEA et al., 2018). No entanto, ainda não foi totalmente esclarecida a dinâmica que ocorre na iniciação e desenvolvimento da resposta imunológica a esses patógenos.

As células epiteliais gastrointestinais são as primeiras células em que os parasitas entram em contato, e recentemente foram associadas ao processo de indução e ativação da resposta antiparasitária (TAYLOR et al., 2012; GERBE; JAY, 2016; SOROBETEA et al., 2018), porém faltam informações para elucidar como esse processo se desenvolve.

As células "*tuft*" são células epiteliais quimiossensoriais encontradas na mucosa gastrointestinal (GERBE et al., 2012; SOROBETEA et al., 2018), que expressam o fator de transcrição POU2F3 (MATSUMOTO et al., 2011) responsáveis por conduzir sinais precoces para iniciação da imunidade do tipo 2 (Th2) contra a infecção por helmintos em camundongos (GERBE et al., 2016). Essas células são a fonte dominante de IL-25, citocina responsável por induzir a produção de citocinas específicas Th2 através da ação de células linfóides inatas do grupo 2 (ILC2s) (SMITH et al., 2018; TING; VON MOLTKE, 2019). Estudos realizados em camundongos observaram que animais que tiveram ausência dessa célula apresentaram defeito na resposta Th2 e consequentemente comprometimento na expulsão dos vermes (GERBE et al., 2016; VON MOLKTE et al., 2016). Atualmente, um grupo de pesquisa da Escócia tem buscado detalhar a dinâmica dessas células durante a infecção por endoparasitas além de investigar o perfil de expressão gênica das células POU2F3+ no abomaso de ovinos para determinar se essas são equivalentes às células "*tuft*" de camundongo (HILDERSLEY et al., estudo ainda em andamento).

2.6 – Resistência aos nematódeos gastrointestinais

A habilidade do animal em desenvolver melhor resposta imunológica aos NGI irá variar entre os indivíduos (STEAR; MURRAY, 1994). Fatores como

raça, idade, período da gestação podem influenciar na suscetibilidade dos animais, além da variação genética que ocorre dentro desses grupos (STEAR; MURRAY 1994; ROCHA et al., 2004; AMARANTE et al., 2004; ROCHA et al., 2005).

Animais com maior resistência aos NGI possuem habilidade em desenvolver resposta imune mais rápida e robusta, limitando o estabelecimento de larvas infectantes, diminuindo a fecundidade das fêmeas e/ou promovendo a expulsão de parasitas (MOWEN; GLIMCHER, 2004; AMARANTE et al., 2009; SHAKYA et al., 2011; DE LA CHEVROTIÈRE et al., 2012).

Hospedeiros que apresentaram maior resistência aos NGI exibiram maior capacidade e velocidade de desenvolver a resposta imune Th2, caracterizada pelo aumento de citocinas importantes como IL-4 e IL-5, recrutamento de células efetoras e produção de anticorpos específicos (JACOBS et al., 2016; SOROBETEA et al., 2018). Além de animais com maior resistência serem capazes de manter valores de variáveis fisiológicas como o VG e as proteínas plasmáticas totais (PPT) dentro dos valores normais para espécie (VG: 27-45%; PPT 6.0-7.5 g/dL WEISS; WARDROP, 2010) (AMARANTE et al., 2004; ROCHA et al., 2004; 2005).

A resistência aos NGI é uma característica genética com herdabilidade de moderada a alta, que pode ser utilizada em programas de seleção de animais como método de controle alternativo, com intuito de aumentar a resistência do rebanho (HUNT et al., 2013; AMARANTE, 2014).

Apesar de já terem sido realizados vários estudos diferenciando raças resistentes de suscetíveis (AMARANTE et al., 2004; TEREFE et al., 2007; GONZÁLES et al., 2011; BOWDRIDGE et al., 2015), pesquisas com finalidade de identificar os possíveis marcadores genéticos relacionados à resistência (BENAVIDES et al., 2002; KEMPER et al., 2011; BENAVIDES et al., 2015; ZVINOROVA et al., 2018) e avaliação da expressão gênica de diferentes raças submetidas à infecções por NGIs (GOSSNER et al., 2013; GUO et al., 2016; ZHANG et al., 2019), ainda não é bem esclarecido quais os principais fatores que conferem resistência aos animais.

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CAPÍTULO 2 - Development of *Haemonchus contortus* resistance in sheep under suppressive or targeted selective treatment with monepantel.

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Research paper

Development of *Haemonchus contortus* resistance in sheep under suppressive or targeted selective treatment with monepantel

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ABSTRACT

This study examined the development of resistance to anthelmintics in *Haemonchus contortus* in lambs under suppressive or selective treatment regimens that included monepantel. Twenty Ile de France and 20 Santa Ines lambs were allocated to two anthelmintic treatment regimens, based on body weight and nematode faecal egg counts (FEC): targeted selective treatment (TST) or suppressive treatment, both with monepantel. Lambs of the TST group were treated individually when they presented with a packed cell volume (PCV) ≤20%. On 7 October 2016, the lambs were allocated to clean pastures, where they grazed in separated paddocks by group until late February 2017. The experimental area was contaminated with nematodes that were introduced with the experimental Ile de France and Santa Ines lambs, naturally infected with gastrointestinal nematodes. To maintain the grazing lambs in the suppressive treatment group and their pasture as free of worms as possible, these lambs were treated with anthelmintics before being allocated to their paddock and then were periodically treated with monepantel. However, the use of a suppressive treatment regimen that included monepantel over a period of 3 months resulted in the emergence of a population of resistant *H. contortus*. In the TST group, there was a rapid and progressive reduction in the efficacy of monepantel, which at the end of the experiment was only 76%. The Ile de France lambs were all treated one or more times during the experiment, whereas only two Santa Ines lambs in the TST required treatment. In conclusion, a population of *H. contortus* resistant to monepantel emerged quickly during the rainy season, even when sheep were submitted to selective treatment.

1. Introduction

Parasitism by gastrointestinal nematodes (GIN) is an important cause of economic losses in the sheep industry. Prophylaxis against GIN infections is based on anthelmintic treatment. However, lack of professional guidance and ease of access to anthelmintics have led farmers to frequently and indiscriminately use these drugs on their flocks. Therefore, parasites with resistance to most important chemical compounds (benzimidazole, macrocyclic lactone, imidazothiazoles, tetrahydropyrimidines, and salicylanilides) have emerged, resulting in a global problem that jeopardizes the control of nematode infections in ruminants (Kaplan et al., 2004; Gilleard, 2006; Amarante, 2014; Bartley et al., 2015).

A new class of anthelmintic, derived from amino-acetonitrile (AAD) and called monepantel (Kaminsky et al., 2008), was introduced into the market in 2009 and is considered an important alternative treatment when there is multiple resistance to other classes of antiparasitics used to treat sheep (Ciuffa et al., 2013). In 2013, two nematode species (*Teladorsagia circumcincta* and *Trichostrongylus colubriformis*) resistant to monepantel were first reported in New Zealand (Scott et al., 2013). Since then, resistance in *Haemonchus contortus* has been reported in Uruguay (Mederos et al., 2014), the Netherlands (Van den Brom et al., 2015), Australia (Sales and Love, 2016; Lamb et al., 2017), and Brazil (Martins et al., 2017).

The mechanisms that underlie the emergence of resistance are complex and may include mutations in the genomes of parasites (Rufener et al., 2013). In *Caenorhabditis elegans* resistant to monepantel, a spontaneous mutation in the *acr-23* allele was identified, whereas in *H. contortus*, a mutation was found to occur in *Hco-mptl-1* and *Hco-des-2H* (Rufener et al., 2009; Bagnall et al., 2017).

Frequent treatment is considered one of the main risk factors for the development of anthelmintic resistance, especially when the anthelmintic is administered to all of the sheep in a flock. Resistant parasites, which survive treatment, are selected and reproduce, resulting in a

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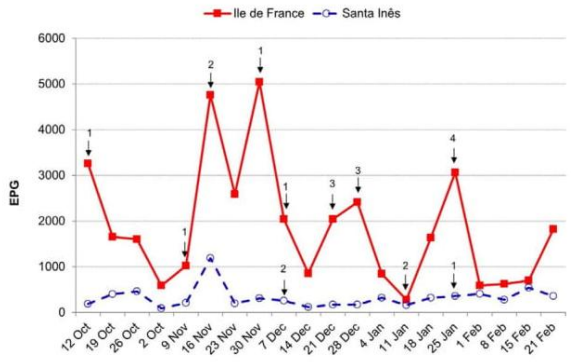

Fig. 1. Average of eggs of strongyles per gram of faeces (EPG) of lambs under targeted selective treatment with monepantel. Arrows indicate the number of animals treated on each occasion owing to PCV ≤ 20%.

progressive increase in the frequency of genes in the population that confer resistance (Besier, 2012; Falzon et al., 2014). To increase the size of the worm population in refugia, targeted selective treatment (TST) has emerged as an alternative treatment regimen that can be used to delay the onset of anthelmintic resistance (Besier, 2012).

From October 2016 to February 2017 (the rainy season), in an experiment designed to determine the productive performance of lambs, a decrease in the efficacy of monepantel was observed. Thus, in the present study, we aimed to describe the development of *H. contortus* resistance in lambs naturally infected by GIN and submitted to suppressive or selective treatment with monepantel.

2. Material and methods

2.1. Animals and pasture

All the procedures involving animals in this study are in accordance to international ethical standards and were approved by the Animal Use Ethics Committee of the FMVZ/Unesp (47/2016).

Twenty Ile de France and 20 Santa Ines 3-month-old uncastrated male lambs were purchased from farms located in São Manuel and Vitoriana, respectively, in São Paulo state, Brazil. The lambs were raised indoors at their farms of origin. The Santa Ines and Ile de France lambs arrived in the experimental area of the University on September 29 and 30, 2016, respectively, where they were housed until the beginning of the experiment. On the day of arrival, blood and faecal samples were collected for haematological and parasitological examinations. The Ile de France and Santa Ines lambs presented averages of 435 (range of 0–1100) eggs per gram (EPG) and 1553 (0–7300) EPG, respectively. Based on faecal culture, Santa Ines and Ile de France lambs were found to harbour *Haemonchus* spp. (63% and 86%, respectively) and *Trichostrongylus* spp. (37% and 14%, respectively) infections. All lambs received a clostridiosis vaccine (Poli-Star®, Vallée), with a booster dose administered 2 months later, and toltrazuril (14 mg/kg, Farmacox®, Farmabase Animal Health) to prevent coccidiosis.

The experiment was in a 2 × 2 factorial, with two anthelmintic treatment regimens (TST or suppressive) and two sheep breeds (Santa Ines and Ile de France). The animals of each breed were allocated to two groups balanced as far as possible for body weight and nematode faecal egg counts (FEC). The Ile de France showed average of 300 EPG (range 0–1100 EPG) and the Santa Ines average of 950 (0–7300 EPG) in the Group under suppressive treatment, while in the TST group, the Ile de France presented average of 300 (0–1100 EPG) and the Santa Ines 600 (0–6700 EPG).

At the beginning of the experiment, one Santa Ines and one Ile de France lamb died. For this reason, the experimental groups had the following number of animals:

- Group of lambs with low exposure to GIN that received suppressive treatment: 10 Ile de France and 9 Santa Ines lambs maintained in the same paddock.
- Group of high exposure to GIN that received TST: 9 Ile de France and 10 Santa Ines lambs maintained in the same paddock.

On 7 October 2016, the lambs were allocated in groups to separate paddocks in a pasture of *Cynodon* spp. and *Urochloa decumbens* that was maintained without ruminants from 1 March 2016 until the beginning of the present experiment. At the study site, nematode infective larvae were recovered for a maximum of 3–4 months after pasture contamination (Carneiro and Amarante, 2008). Because the pasture

Table 1
Faecal egg count reduction (FECR) in lambs under target selective treatment with monepantel (high-exposure group). Date of the treatment, number of eggs of strongyles per gram of faeces (EPG) on the day of the treatment (pre-treatment) and 14 days post-treatment.

Month	Lamb identification	Date	EPG		FECR (%)
			Pre-treatment	14 days post-treatment	
October	Ile de France – 33	12-Oct	15400	0	100%
November	Ile de France – 33	09-Nov	2700	400	98% (100; 91)
	Ile de France – 34	16-Nov	6700	0	
	Ile de France – 38	16-Nov	6200	100	
	Ile de France – 40	30-Nov	15500	0	
December	Santa Ines – 22	07-Dec	1100	0	83% (98; 0)
	Santa Ines – 29	07-Dec	200	0	
	Ile de France – 33	07-Dec	4200	0	
	Ile de France – 35	07-Dec	7600	0	
	Ile de France – 31	21-Dec	1800	0	
	Ile de France – 38	21-Dec	400	4800	
	Ile de France – 34	28-Dec	10100	300	
	Ile de France – 36	28-Dec	4500	100	
January	Ile de France – 31	11-Jan	100	0	76% (95; 0)
	Ile de France – 39	11-Jan	300	3800	
	Ile de France – 33	25-Jan	6300	600	
	Ile de France – 34	25-Jan	4400	400	
	Ile de France – 35	25-Jan	8000	400	
	Ile de France – 40	25-Jan	1900	100	
	Santa Ines – 22	25-Jan	1500	0	

FECR (%) in each month = 100 × (1–post-treatment mean EPG/pre-treatment mean EPG). Upper and lower confidence interval limits (95%) are in parentheses.

Table 2
Third stage larvae (%) in faecal cultures of lambs under target selective treatment with monepantel.

Data	Breed	*Haemonchus*	*Trichostrongylus*	*Cooperia*	*Oesophagostomum*
05-Oct	Ile de France	94	6	0	0
	Santa Inês	55	44	0	1
12-Oct	Ile de France	91	6	0	3
	Santa Ines	63	34	1	2
19-Oct	Ile de France	92	8	0	0
	Santa Ines	50	43	0	7
26-Oct	Ile de France	95	1	0	4
	Santa Ines	74	26	0	0
02-Nov	Ile de France	83	6	11	0
	Santa Ines	37	58	4	1
09-Nov	Ile de France	78	22	0	0
	Santa Ines	46	52	2	0
16-Nov	Ile de France	95	0	5	0
	Santa Ines	67	30	3	0
23-Nov	Ile de France	60	40	0	0
	Santa Ines	38	62	0	0
30-Nov	Ile de France	98	0	2	0
	Santa Ines	43	55	2	0
07-Dec	Ile de France	94	6	0	0
	Santa Ines	54	46	0	0
14-Dec	Ile de France	88	7	5	0
	Santa Ines	59	39	2	0
21-Dec	Ile de France	98	1	1	0
	Santa Ines	41	54	5	0
28-Dec	Ile de France	96	4	0	0
	Santa Ines	42	50	8	0
04-Jan	Ile de France	61	28	11	0
	Santa Ines	40	46	14	0
11-Jan	Ile de France	92	2	6	0
	Santa Ines	56	39	5	0
18-Jan	Ile de France	83	9	8	0
	Santa Ines	56	36	8	0
25-Jan	Ile de France	75	20	5	0
	Santa Ines	35	52	13	0
01-Feb	Ile de France	86	14	0	0
	Santa Ines	62	31	7	0
08-Feb	Ile de France	97	0	3	0
	Santa Ines	51	46	3	0
15-Feb	Ile de France	84	7	9	0
	Santa Ines	69	29	2	0
21-Feb	Ile de France	82	15	3	0
	Santa Ines	31	54	15	0

remained without animals for a period of 7 months, the grazing area used was considered “clean”. Therefore, gastrointestinal nematodes in the experimental area were introduced by the naturally infected experimental Ile de France and Santa Ines lambs. In the paddocks, the lambs had free access to tap water and mineral salt (Presencefós, Presence Animal Nutrition) and received a daily dietary supplement (Supplementa Ovinos, Presence Animal Nutrition). It is important to emphasize that the groups always remained in the separate paddocks.

2.2. Suppressive anthelmintic treatment in the group with low exposure to GIN

Just after lambs were assigned into groups (7 days before being allocated to the paddocks), they were treated with the following combination of anthelmintics: albendazole (10 mg/kg, Endazole® 10% CO, Hipra), levamisole (9.4 mg/kg, Ripercol® L 150 F, Zoetis), and monepantel (2.5 mg/kg, Zolvix®, Novartis). Following this treatment, no nematode eggs were detected in a series of faecal examinations. Then, to keep the grazing lambs and their pastures as free of worms as possible, the lambs were treated periodically with monepantel (details in Fig. 2). In addition to periodic treatments with monepantel, lambs in the suppressive group received moxidectin (0.4 mg/kg, Cydectin® Zoetis) on November 16, because some of them were shedding *Strongyloides papillosus* eggs (mean of 42 and range of 0–200 EPG). On January 14, lambs also received a combination treatment of monepantel, levamisole (9.4 mg/kg), and albendazole (10 mg/kg), because they were shedding strongyle eggs (379, 0–1000 5300EPG) and *S. papillosus* (874, 0–5300 EPG).

2.3. TST in the group with high exposure to GIN

The lambs in the TST group (high exposure) were not treated on arrival at the experimental facilities. Instead, they were introduced into the clean pasture harbouring natural infections of parasites acquired at

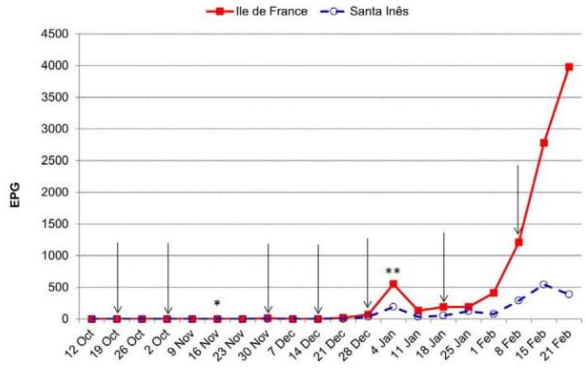

Fig. 2. Average number of strongyles eggs per gram of faeces (EPG) from lambs under suppressive anthelmintic treatment. The black arrows indicate the dates of treatment with monepantel, the orange arrow indicates the date of treatment with moxidectin, and the blue arrow indicates the date of treatment with a combination of monepantel, levamisole, and albendazole. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

their farm of origin. These lambs underwent selective treatment with monepantel when they showed a packed cell volume (PCV) ≤20%.

2.4. Faecal examination, haematological tests, and body weight measurement

Blood samples were collected weekly from each lamb to determine the PCV by micro-haematocrit centrifugation. Faecal egg counts were determined using a modified McMaster technique, in which each worm egg counted represented 100 EPG. A composite faecal culture to obtain infective larvae of gastrointestinal nematodes was performed separately for each group and breed (Ueno and Gonçalves, 1998). These larvae were identified according to descriptions by Ueno and Gonçalves (1998). At the same time as blood and faecal sampling, body weights were recorded to determine the precise amount of anthelmintic to be administered to each animal.

2.5. Faecal egg count reduction test (FECRT)

A FECRT was performed to evaluate the efficacy of the anthelmintic treatments (Coles et al., 1992). RESO 2.0 software (Wursthorn and Martin, 1990) was used to calculate anthelmintic efficacy. Resistance was considered present when the percentage reduction in egg count was less than 95% 14 days after anthelmintic treatment.

3. Results

At the arrival in the experimental facilities (September 30), the Ile de France lambs presented 435 EPG on average. However, one week later, the TST group displayed 3110 EPG on average. This increase in the EPG values indicates that the animals were harbouring immature worms that reached patency at the beginning of the trial. For example, the animal 33 presented 1100 EPG at the arrival and 15,400 EPG two weeks later (October 12).

Of the two Santa Ines lambs under TST that presented PCV < 20% and therefore required anthelmintic treatment, one of them was treated twice. On the day of treatment, the EPG counts of these animals were relatively low, between 200 and 1500 EPG (Fig. 1; Table 1). After treatment, no eggs were found in these Santa Ines lambs, indicating high treatment efficacy (Table 1). In contrast, all nine Ile de France lambs required anthelmintic treatment at some point in the experiment: three animals required one treatment; three animals needed two treatments; two animals needed three treatments; and one animal required four treatments (Table 1). The efficacy of monepantel varied from animal to animal, but there was a clear tendency toward a reduction in efficacy as the experiment progressed (Table 1). At the end of the experiment, based on seven animals treated in January, a monepantel efficacy of 76% was recorded (Table 1). The predominant genera of strongyles in the faecal cultures were *Haemonchus* spp. and *Trichostrongylus* spp. (Table 2). On some occasions, individual post-treatment faecal cultures were performed: on December 7 and on January 1, a culture of faeces from lamb 33 showed 100% *Haemonchus* spp. larvae; on January 12, culture of faeces from animal 23 showed 81% *Haemonchus* spp. and 19% *Cooperia* spp. larvae; and, on the same day, culture of faeces from animal 31 showed 93% *Haemonchus* spp. and 7% *Cooperia* spp.

No eggs were detected in the faeces of lambs in the group under suppressive treatment up to December 14 (Fig. 2). However, larvae were detected in faecal cultures on November 16 (Table 3), indicating that mild infections, not yet detected by egg counting, had already been established in animals. On December 21, two animals presented detectable eggs in their faeces (100 EPG) for the first time. Combined treatment with monepantel, levamisole, and albendazole on January 4 showed high efficacy against *S. papillosus* infection, with an FECR of 99% (mean of 874 EPG before treatment and 5 EPG after treatment). However, this regimen showed less efficacy against *H. contortus* infection, with animals shedding an average of 126 EPG (range of 0–500) two weeks after treatment on January 18 (Fig. 2). In February, monepantel treatment did not reduce EPG counts in lambs in the suppressive group; EPG counts increased progressively until the end of the experiment (Fig. 2). In faecal cultures, larvae of *Haemonchus* spp. and *Cooperia* spp. were detected; however, only *Haemonchus* larvae were identified in the animals in the suppressive treatment group from December 28 to the end of the trial (Table 3).

4. Discussion

The use of a suppressive treatment regimen with monepantel over a period of 3 months resulted in the rapid emergence of a resistant *H. contortus* population in the present study. When the population of parasites in refugia is small, hosts are reinfected with the progeny of worms that survived the anthelmintic treatment, which, even with a small number of survivors, produce offspring that are the only individuals available for reinfection (Bartley et al., 2015). The rapid emergence of resistance in animals under suppressive treatment observed in this study was not surprising. At the end of the experiment, the selected population consisted only of parasites that survived the treatment, i.e., resistant worms.

In the TST group, there was a rapid and progressive reduction in efficacy, which, at the end of the experiment, was only 76%. This rapid emergence of resistant parasites in the paddocks where there was a nematode population in refugia was unexpected, although in sheep under TST, Cintra et al. (2016) and Mederos et al. (2014) observed the appearance of monepantel-resistant *T. colubriformis* and *H. contortus*, respectively. Selective treatment of animals has been suggested as an important alternative strategy to delay the onset of resistance to anthelmintics. By reducing the number of animals treated, there should be reduced selection pressure and an increase in the proportion of nematodes in refugia. This population consists of parasites at stages within the host and those of free-living stages in the pastures that are not exposed to anthelmintic treatment and can pass on susceptible alleles to the next generation of parasites (Besier, 2012). To a certain extent, this was demonstrated in the present study, because in the group under TST, a considerable percentage of the parasite population, approximately 76% based on FECRT, was still susceptible. If the experiment lasted longer, it is possible that a resistant population would come to predominate. Also noteworthy was the fact that, although lambs submitted to each treatment regimen were held in separate paddocks throughout the experiment, the appearance of resistance to monepantel occurred at similar times, with a difference of approximately 1 month between the groups.

In conclusion, the emergence of a population of *H. contortus*

Table 3
Third stage larvae (%) in faecal cultures of lambs under suppressive treatment with monepantel (low-exposure group).

Data	Breed	*Haemonchus*	*Trichostrongylus*	*Cooperia*	*Oesophagostomum*
05-Oct	Ile de France	89	11	0	0
	Santa Ines	67	33	0	0
12-Oct	Ile de France	*	*	*	*
	Santa Ines	*	*	*	*
19-Oct	Ile de France	*	*	*	*
	Santa Ines	*	*	*	*
26-Oct	Ile de France	*	*	*	*
	Santa Ines	*	*	*	*
02-Nov	Ile de France	*	*	*	*
	Santa Ines	*	*	*	*
09-Nov	Ile de France	*	*	*	*
	Santa Ines	*	*	*	*
16-Nov	Ile de France	89	0	11	0
	Santa Ines	80	0	20	0
23-Nov	Ile de France	100	0	0	0
	Santa Ines	*	*	*	*
30-Nov	Ile de France	97	0	3	0
	Santa Ines	100	0	0	0
07-Dec	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0
14-Dec	Ile de France	100	0	0	0
	Santa Ines	*	*	*	*
21-Dec	Ile de France	99	1	0	0
	Santa Ines	100	0	0	0
28-Dec	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0
04-Jan	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0
11-Jan	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0
18-Jan	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0
25-Jan	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0
01-Feb	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0
08-Feb	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0
15-Feb	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0
21-Feb	Ile de France	100	0	0	0
	Santa Ines	100	0	0	0

* No larvae were recovered from the faecal culture.

resistant to monepantel was shown to occur quickly during the hot and wet season, even when sheep are selectively treated. With a decrease in the efficacy of anthelmintics, prophylaxis of GIN infections in sheep has become more challenging. It is of utmost importance that the efficacy of new compounds such as ADD is preserved through the use of sustainable control measures that minimize the use of anthelmintics.

Conflicts of interest

The authors have no conflicts of interest.

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CAPÍTULO 3 – Differences in immune response to *Haemonchus contortus* infection in the susceptible Ile de France and the resistant Santa Ines sheep under anthelmintic different regimens

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VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

Differences in immune responses to *Haemonchus contortus* infection in the susceptible Ile de France and the resistant Santa Ines sheep under different anthelmintic treatments regimens

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Abstract

Understanding the immunological basis of resistance to gastrointestinal nematode infections in livestock is important in order to develop novel methods of parasite control such as vaccination or genetic selection for parasite resistance. The present study aimed to investigate differences in immune response between parasite resistant Santa Ines and susceptible Ile de France sheep breeds to natural *Haemonchus contortus* infection. Parasitological parameters, humoral immunity, local and circulating cellular immune responses were evaluated in 19 Santa Ines and 19 Ile de France lambs undergoing different anthelmintic treatments regimens: suppressive treatments (SUP) or targeted selective treatments (TST) over a 5-month grazing period. Santa Ines lambs had significantly lower *Haemonchus* faecal egg count and worm burden compared to Ile de France regardless of treatment regime. In addition, circulating blood eosinophils count and parasite-specific IgG levels were significantly higher and more rapidly induced in Santa Ines lambs. Abomasal immune responses were generally greater in the resistant breed, which had significantly higher levels of parasite-specific IgA in mucus, and elevated number of globule leukocytes and CD3+ T cells within the abomasal mucosal. Furthermore, numbers of POU2F3+ epithelial cells, a tuft-cell specific transcription factor, were also elevated in the Santa Ines breed, suggesting that this breed is better able to initiate T-helper type 2 immune responses within the abomasum. In conclusion, the differential immunological responses detailed here are relevant to understanding resistance to gastrointestinal nematodes in other host breeds, as well as to resistance breeding as a sustainable control approach for parasitic infections.

Introduction

Gastrointestinal nematode (GIN) infections are among the main health problems affecting ruminants and are responsible for huge economic loss to the livestock industry [1]. Control of GIN is heavily dependent on anthelmintic treatment [2], however the high frequency of dosing increases the prevalence of anthelmintic-resistant nematode populations, even with new compounds, such as monepantel [3, 4]. Insight into mechanisms involved in the appropriate gastrointestinal immune response to GIN is fundamental for the development of sustainable approaches, such as selective breeding and vaccination to reduce anthelmintic use [5–11].

Haemonchus contortus is an important GIN in sheep husbandry across the humid temperate and tropical regions worldwide. It is a highly pathogenic, blood-feeding parasite, responsible for massive economic loss, due to reduced productive performance, compromised

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reproduction and high mortality [3, 7, 12]. The emergence of multi-drug resistance *H. contortus* has focussed research on development of alternative control strategies, such as selective breeding for animals resistant to infection [5] and vaccination [8]. A vaccine, Barbervax®, comprising native proteins extracted from the nematode gut has been introduced in Australia [13] and tested with promising results in Brazil [14–16]. However, its widespread use is limited, mainly due to cost, licensing, requirement for multiple vaccinations and additional strategies, such as nutritional improvement [15].

Host resistance to nematode species is related to the ability to develop strong innate and acquired immunity, limiting the establishment of infective larvae and/or eliminating the worm population [9, 17–21]. This differs from resilience, defined as the ability of animals to produce and reproduce in the face of parasite infection [22]. Resistance against GIN infection is a moderately heritable trait, and selection of animals with an efficient immune response to GIN may increase flock resistance and potentially be exploited as an alternative control measure [3, 23].

Protection against GIN infection is mediated by type 2 immune responses, involving induction of cytokines and antibody production, and expansion and mobilization of innate and adaptive immune cells [6, 10, 11, 24]. Parasite clearance is associated with innate and adaptive responses characterised by mucosal mast cells hyperplasia, globular leukocyte appearance, increased eosinophil concentration and induction of goblet cell hyperplasia with mucus production, while humoral responses involve IgG, IgE and IgA production [23, 25, 26]. However, the mechanisms involved in the initiation and the development of the immune response to GIN have not been fully elucidated. Some studies indicated that in the gut mucosa of resistant animals, upregulation of a T helper 2 (Th2)-type response is responsible for protection, while in susceptible animals, with chronic nematode infections, a Th1-type response is enhanced [19, 27, 28].

More recently, it was demonstrated in murine models that epithelial cells called tuft cells might be responsible for initiating type 2 responses to nematode infection, and release IL-25 to stimulate IL-4 production by Th2 cells through a feed-forward loop involving Group 2 Innate Lymphoid Cells (ILC2) [29, 30]. Wild-type mice with experimental *Nippostrongylus brasiliensis* infection showed worm clearance 13 days after infection. In contrast, mice deficient in tuft cells, through knockout of tuft cell specific transcription factor POU2F3, presented numerous worms 42 days post-infection. This demonstrated the importance of tuft cells in the immune response against GIN in mice [29]. Our ongoing work is detailing the dynamics during infection and gene expression profile of ovine abomasal POU2F3+ cells to determine if these cells are equivalent to mouse tuft cells (Hildersley et al. unpublished data).

Our previous work identified Santa Ines hair sheep as naturally resistant to *H. contortus* infections at different age categories, in which they presented a high number of blood eosinophils, high production of IgG, low eggs per gram of faeces (EPG), normal packed cell volume (PCV) and low worm burden, in addition to requiring fewer anthelmintic treatments in comparison with other commercial wool-sheep breeds [26, 31]. The present study aimed to elucidate and compare the innate and adaptive immunological response between Santa Ines and Ile de France lambs. Humoral and cellular responses were compared during infection and at post-mortem to determine in detail the mechanisms of resistance against natural *H. contortus* infection in the field.

Materials and methods

Ethical considerations

The experiment was carried out at São Paulo State University (UNESP), Botucatu-SP, Brazil. All animal procedures were in accordance with the ethical standards and were approved by the Animal Use Ethics Committee of the FMVZ/UNESP (47/2016).

Experimental design

The experimental design was described previously [4] and was carried out over 5 months, from October 2016 to February 2017, during the rainy season. Briefly, the experiment was in a 2×2 factorial design, with 19 Santa Ines (SI) and 19 Ile de France (IF) 3-month-old uncastrated male lambs purchased from commercial farms located in São Paulo state, Brazil. The lambs were raised indoors at their farms of origin. The lambs were allocated to the two anthelmintic treatment groups: suppressive (SUP)—animals drenched every 2 weeks with monepantel (2.5 mg/kg; Zolvix®, Novartis) in order to maintain the grazing lambs and their pastures as free of worms as possible; targeted selective treatment (TST)—animals under continuous challenge infection for 5 months and underwent monepantel treatment only when they showed a PCV $\leq$ 20% corresponding to category 3 of FAMACHA© method [32], in order to prevent the occurrence of mortality and/or severe haemonchosis. Animals were allocated into each group by stratified randomization, balanced as far as possible taking into consideration body weight and nematode faecal egg counts (FEC) at start of the trial prior to drenching.

Suppressive groups (SUP-SI and SUP-IF) were drenched on arrival with the following combination of anthelmintics: albendazole (10 mg/kg, Endazole® 10 Cobalto, Hipra), levamisole (9.4 mg/kg, Ripercol® L 150 F,

Zoetis), and monepantel (2.5 mg/kg, Zolvix®, Novartis). Afterwards, they were allocated to the paddock after no nematode eggs were detected in a series of faecal examinations. The last anthelmintic drench for lambs under suppressive treatment was administered on week 19 and they were slaughtered on week 21. The lambs in the TST group (TST-SI and TST-IF) were not treated on arrival at the experimental facilities. Instead, they were introduced onto clean pasture that became contaminated with eggs from the nematodes acquired at their origin farm.

Faecal examination

Faecal samples were collected upon animals' arrival and then weekly, directly from the rectum of each animal in polyethylene bags previously labelled and kept refrigerated until processing. The modified McMaster technique [33] was used to measure faecal eggs count (FEC), in which each nematode egg counted represented 100 eggs/g of faeces (EPG). Faecal cultures were prepared separately for each group for production of GIN infective third-stage larvae (L3) on animals' arrival and then weekly to identified morphologically and counted [33]. Based on the proportion of L3 identified as *H. contortus* in the cultures, the *Haemonchus* EPG of each lamb was estimated.

Worm burden

All lambs were slaughtered and the gastrointestinal tract removed. The abomasum was opened along its greater curvature and the contents placed in graduated buckets. Aliquots of 10% of the total abomasum were collected individually, stored in plastic flasks and preserved in −20 °C freezer. All nematodes present in the 10% preserved material were identified morphologically and quantified, according to their developmental stage [33, 34] and stored in 70% ethanol.

Haematology

Blood samples (5 mL) were collected weekly by jugular vein puncture into Vacutainer® tubes containing anticoagulant (EDTA). The blood in the tube was centrifuged to allow plasma separation. Aliquots of plasma samples were stored at −20 °C and −80 °C prior to ELISA. Eosinophil counts in the peripheral blood were performed in a Neubauer's chamber after staining with Carpentier's solution [35], and the counts expressed as the number of eosinophil cells per µL of blood.

Histology

Abomasal tissue samples were fixed in 4% buffered formaldehyde for 48 h, then moved to 70% ethanol, and paraffin-embedded. Tissue sections were cut to 5 µm thick and mounted on glass slides. Eosinophils and globule leukocytes were counted on Haematoxylin and Eosin (H&E) stained sections whereas mast cells were counted on sections stained with 1% toluidine blue. All cells were counted in sixty randomly selected fields of view per animal in a 0.01 mm^2 area at 1000× magnification [adapted from [6]. The counts were expressed as cells/mm^2 tissue surface.

Immunohistochemistry (IHC)

Immunohistochemistry sections were mounted on Superfrost Plus glass slides (Thermo Fisher Scientific). The tissues were dewaxed in xylene, rehydrated and heat-induced antigen-retrieval was performed by autoclaving at 121 °C for 10 min in 10 mM citrate buffer pH 6.0. This was followed by two washes in PBS and one in PBS/0.5% Tween 80 (PBS/T80) for 5 min, and then endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS/T80 for 20 min at room temperature (RT). Following a further two PBS/T80 washes for 5 min each, slides were loaded into a Sequenza immunostaining centre rack (Thermo Fisher Scientific). The sections were incubated with 25% goat serum in PBS/T80 for 1 h at RT to reduce nonspecific binding and background staining. The primary antibodies and their appropriate isotype control were diluted in PBS/T80 containing 10% normal goat serum (NGS) and incubated overnight at 4 °C. Slides were washed twice in PBS and incubated with the secondary antibody at RT for 30 min. This was followed by PBS wash and incubation with 3,3′-diaminobenzidine (DAB) for 7.5 min at RT. Thereafter, the slides were washed in distilled water, counterstained with haematoxylin, dehydrated and mounted in Shandon synthetic mountant (Thermo Fisher Scientific). Detailed information of all antibodies used in immunohistochemistry are specified in Additional file 1.

Images were taken from 10 random areas of each slide for POU2F3+ (putative tuft cell) counting at 400× magnification using microscope Olympus BX50 with digital camera Olympus DP70. POU2F3+ epithelial cell frequency was calculated as the proportion of epithelial cells positive for POU2F3, expressed as a percentage (adapted from [30]). Ten random areas of each slide were used to perform the T and B cell counting using calibrated graticule at 250× magnification on Dialux 20 EB Microscope (Leitz Wetzlar, Germany). T and B cells counts were expressed as the number of positive cells per mm^2.

Enzyme-linked immunosorbent assay (ELISA)

Haemonchus contortus-specific IgG

Plasma samples collected at 11 time-points were used to determine IgG levels against L3-soluble extract of *H. contortus*. The L3 extract was prepared as previously

described [17]. A previously described protocol was applied to determine the parasite-specific plasma IgG levels [36], with some modifications: the plates were coated with 2 µg of antigen/mL; each wash was done three times, rotating through 180 and re-washing three more times; the negative control (NC) sample used was from a worm free animal, as previously described [37]; the plasma positive control (PC) sample used was from a sheep artificially infected with both *H. contortus* and *Trichostrongylus colubriformis* every 3 days for 84 days. Results were expressed as the percentage of optical density (OD) value of the PC plasma sample [38].

Haemonchus contortus-specific IgA

A 5 cm piece from the greater curvature of abomasum was sampled and stored at −20 °C until processing for the extraction of mucus. Tissues were thawed and mucus was scraped off with a glass slide and stored in a falcon tube on ice, followed by addition of PBS supplemented with protease inhibitors (Complete®, Roche) to each sample in a proportion of 4:1 (4 mL PBS + 1 mL of mucus). The samples were shaken for 1 h at 4 °C and centrifuged for 30 min at 4 °C and 3000 × *g*. The supernatant was collected and centrifuged again for 30 min at 4 °C and 15 000 × *g*.

ELISA assays for parasite-specific IgA recovered from abomasal mucous were carried out against *H. contortus* L3 and adult extract as previously described [36] with a few differences: plates were coated with 5 µg of antigen/mL; each wash was done three times, rotating through 180° and re-washing three more times; mucus samples were diluted in PBS-GT (1:20) and rabbit anti-sheep IgA peroxidase-conjugated antibody was diluted at 1:20 000. The results were expressed as OD value minus OD-blank sample [38].

Total plasma IgE

Total IgE antibody levels were determined in plasma from six time-point samples by sandwich enzyme immunoassay using a Sheep Immunoglobulin E ELISA kit (My BioSource©, San Diego, USA). The data were expressed in µg/mL.

Statistical analysis

All data were submitted to normality test and transformed using $\log_{10}(x+1)$ when necessary. Data with single measures and data for repeated measures at several time points were analysed by ANOVA using the General Linear Model (GLM) and groups means were compared by Tukey's test using Statistical Analysis System, version 9.2 (SAS Institute, Inc., Cary, NC, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Parasitology

Before the beginning of the trial, the young lambs had been infected with *Haemonchus* spp. and *Trichostrongylus* spp. on their farms of origin. Then, during the trial, the lambs under suppressive treatment were maintained with low exposure of strongyle infection during the first 4 months of the trial, with increase in EPG values and L3 detected in the faecal culture in the last month of the trial, due to development of anthelmintic resistance [4]. The animals under TST were continuously challenged by natural GIN infections for 5 months as determined by the presence of parasite eggs within faeces throughout the study period (Figure 1). *Haecmonchus contortus* at different developmental stages, was the only species found in the abomasum at post-mortem, with significantly lower worm burdens in the Santa Ines lambs in both suppressive and TST treated groups ($P < 0.0001$, Table 1).

Mean total *H. contortus* burden was lower in TST groups of both breeds, suggesting that continuous infection for 5 months induced some protection in the TST-lambs in comparison with lambs undergoing suppressive anthelmintic treatments, in which patent GIN infections were only seen at the end of the trial (Table 1), related to the development of anthelmintic resistance in surviving worms [4]. There was a breed effect on all worm stages of development (Table 1), with Santa Ines lambs presenting a lower burden for all stages recovered ($P < 0.0001$). No significant breed × treatment interaction was identified in *H. contortus* burdens. The intestinal GIN species *T. colubriformis*, *Strongyloides papillosus*, *Cooperia curticei* and *Trichuris* spp. were also found in the lambs of the both breeds.

There was a substantial difference between the breeds in the number of salvage drenches required in the TST groups: all Ile de France lambs needed to be treated with monepantel due to PCV < 20%, treatments ranging from one to four times, totalling 19 drenches during the trial. In contrast, only two Santa Ines lambs needed to be treated: one once and another twice, detailed in [4]. One of these Santa Ines presented elevated *Haemonchus* worm burden (1680 specimens) in relation to animals in the same group (group average = 283). Therefore, our results showed that besides a significant breed difference, there was also genetic variability in each breed influencing the degree of resistance.

Antibody responses

Plasma IgG and IgE levels

The mean levels of anti-*H. contortus* L3 IgG in plasma showed changes over time, resulting in significant time × breed ($P < 0.04$) and time × treatment ($P < 0.0001$)

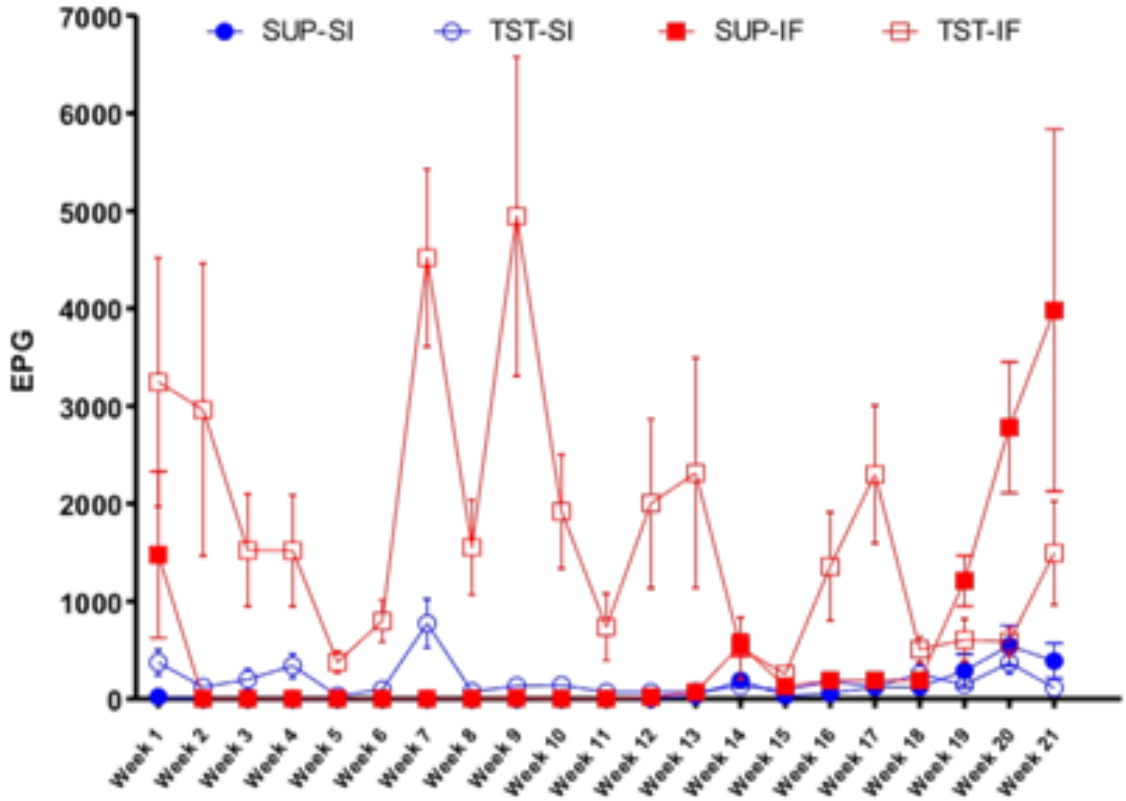

Figure 1 Means of *Haemonchus contortus* eggs per gram of faeces (EPG). EPG counting of the Santa Ines (SI) and Ile de France (IF) lambs naturally infected with *H. contortus* and under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Values represent mean ± standard error.

Table 1 Developmental stages of *Haemonchus contortus* means (minimum–maximum values) of the Santa Ines and Ile de France lambs under suppressive or targeted selective treatment (TST) programme with anthelmintics

Development stages	Santa Ines		Ile de France		Effects (*P* value)	
	Suppressive ($n=9$)	TST ($n=10$)	Suppressive ($n=10$)	TST ($n=9$)	Breed	Treatment programme
Early L4	31 (0–80)	114 (0–650)	141 (30–600)	597 (10–142)	< 0.0001	ns
Female late L4	38 (0–120)	60 (0–360)	103 (0–360)	414 (0–1450)	0.0036	ns
Male late L4	20 (0–70)	63 (0–360)	100 (10–420)	456 (0–2010)	0.0002	ns
Female early L5	92 (0–590)	5 (0–30)	389 (100–830)	179 (0–460)	< 0.0001	0.0094
Male early L5	49 (0–270)	16 (0–140)	334 (80–710)	160 (0–530)	< 0.0001	0.0066
Adult female	121 (0–400)	17 (0–110)	921 (210–2620)	279 (0–600)	< 0.0001	0.0015
Adult male	121 (0–420)	8 (0–50)	881 (190–2190)	289 (0–740)	< 0.0001	0.0011
Total worm burden	472 (20–1330)	283 (0–1680)	2869 (720–7100)	2373 (60–7040)	< 0.0001	0.0125

L: larvae, ns: not significant ($P>0.05$).

interactions. Breed had an effect ($P<0.05$) on mean anti-*H. contortus* IgG levels in the beginning of the trial (on week 5, week 7 and week 9), when Santa Ines lambs showed a sharp increase in production of anti-*H. contortus* IgG level. Treatment program had an effect on IgG levels at five time-points, between week 9 and week 17, when TST groups showed the highest IgG levels. In the last month of the trial, the means of all groups were similar (Figure 2). In contrast, we did not detect any breed or treatment effect ($P>0.05$) in total IgE levels in plasma. There was a significant time × treatment interaction ($P<0.02$), with the highest mean IgE levels in groups under TST treatment at the last sampling (Figure 3).

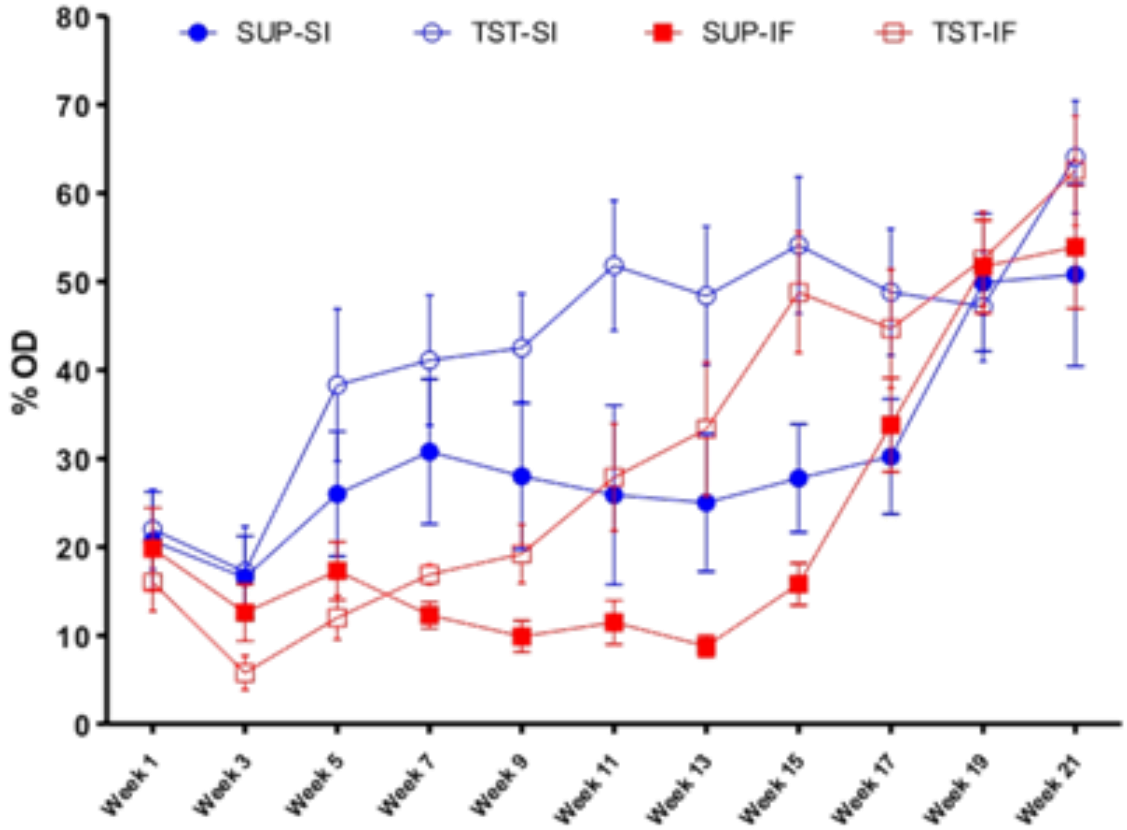

Figure 2 Mean levels of anti-*Haemonchus contortus* L3 IgG (% OD) in plasma. IgG levels of the Santa Ines (SI) and Ile de France (IF) lambs naturally infected with *H. contortus* and under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Values represent mean ± standard error.

Abomasal IgA levels

There was a significant breed effect on mean levels of *H. contortus* L3-specific IgA in abomasal mucus. Levels of L3-specific IgA were significantly ($P<0.05$) higher in Santa Ines lambs compared to Ile de France lambs independent of treatment group (Figure 4). Mean levels of adult *H. contortus*-specific IgA were relatively low and similar among the four groups.

Cellular responses

Blood eosinophils

The mean blood eosinophil count of each animal showed changes over time with significant time × breed ($P<0.02$) and time × treatment interactions ($P<0.0001$). There was breed x treatment interaction at three time-points at the beginning of the trial: week 4, week 5 and week 6, when TST-SI lambs had significantly higher averages than the other groups. Breed had the greatest effect on mean blood eosinophil count, with Santa Ines lambs showing early eosinophilia and the highest mean eosinophil counts during the trial. All means increased progressively until week 16, followed by stabilization until the end of the trial (Figure 5).

Abomasal cellular immune responses

Santa Ines and Ile de France lambs under TST showed the highest number of mast cells (MC) and globular leukocytes (GL). In the case of GL, but not MC, there was a significant ($P<0.05$) effect of breed, with Ile de France under suppressive treatment displaying the lowest GL counts. A significant effect of treatment was found for both cells ($P=0.0001$), with higher MC and GL counts in lambs undergoing TST. A significant breed x treatment interaction was also found for GL counts. Numbers of eosinophils were similar among groups without any breed or treatment effect ($P>0.05$) (Table 2).

IHC revealed positive nuclear staining in the abomasum mucosa of Ile de France and Santa Ines lambs of POU2F3+ epithelial cells (Figures 6A and B) and positive labelling of CD3+ T cells (Figures 6C and D), and CD79α+ B cells (Figures 6E and F). There was a significant breed effect ($P<0.05$) on % POU2F3+ cells in the epithelium and T cell numbers within the abomasal mucosa, with highest mean levels in the Santa Ines breed. The treatment regimen had an effect ($P<0.05$), with greater frequency of POU2F3+ cells in the TST groups.

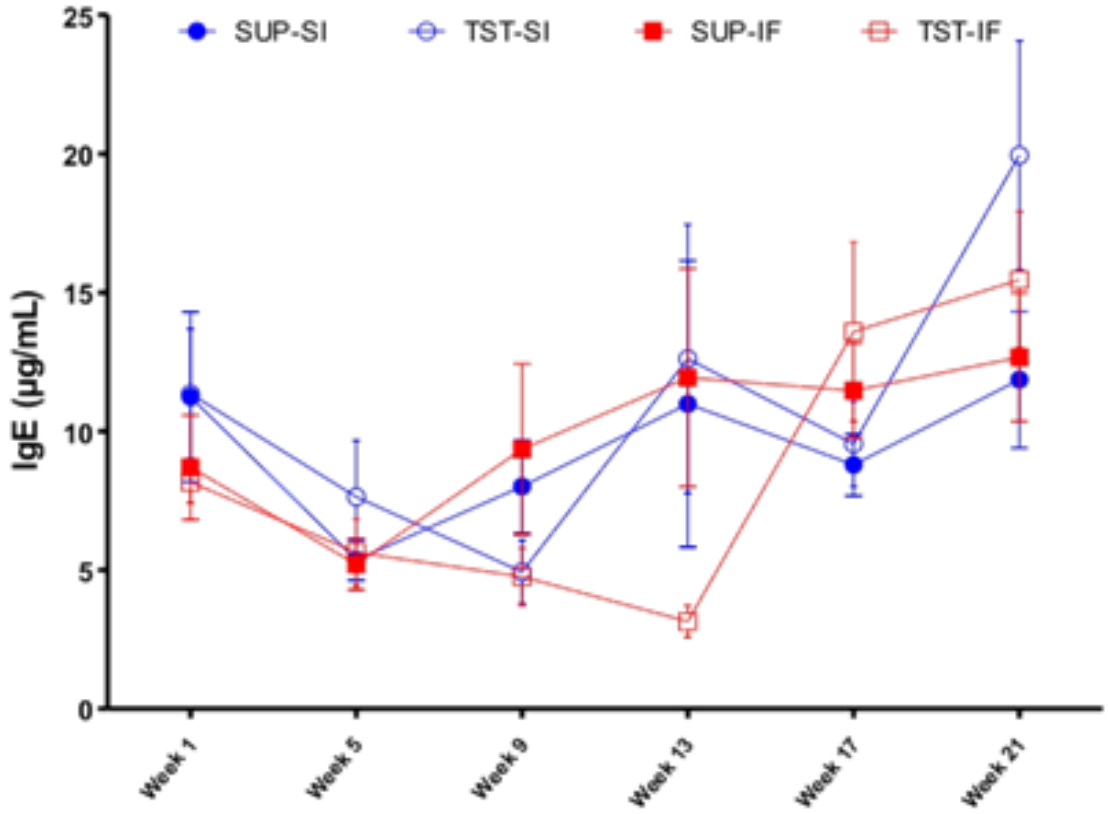

Figure 3 Total IgE plasma concentration (µg/mL). IgE concentration of the Santa Ines (SI) and Ile de France (IF) lambs naturally infected with *H. contortus* and under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Values represent mean ± standard error.

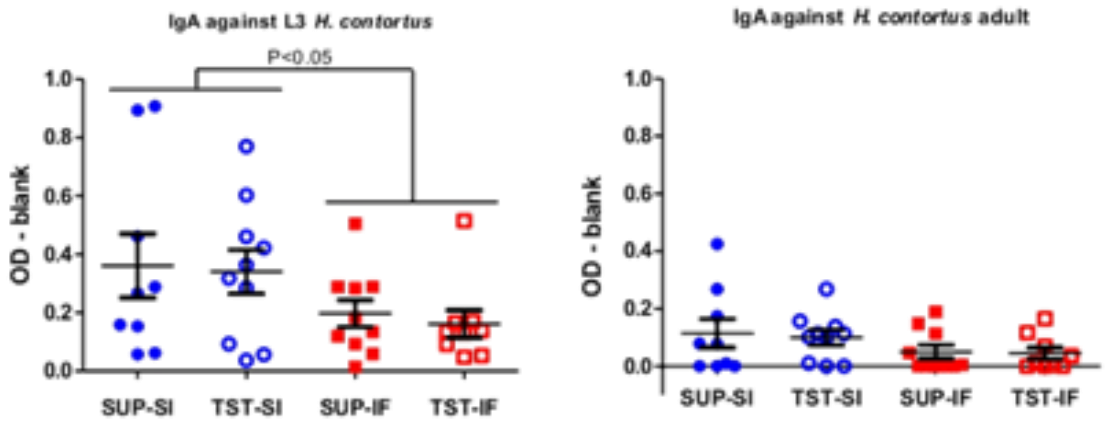

Figure 4 Mean levels of anti-*Haemonchus contortus* L3 and adult—specific IgA (OD—blank). IgA levels measured in the abomasal mucus collected post mortem of the Santa Ines (SI) and Ile de France (IF) lambs naturally infected with *H. contortus* and under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Values represent mean ± standard error.

B cell numbers were similar among groups ($P > 0.05$) (Table 2).

Discussion

The immune response against GIN infection is characterised by reductions in faecal egg count and worm burden and can be influenced by several factors such as age, sex, physiological status (especially parturition and lactation), and breed [26, 39–41]. Previous studies have

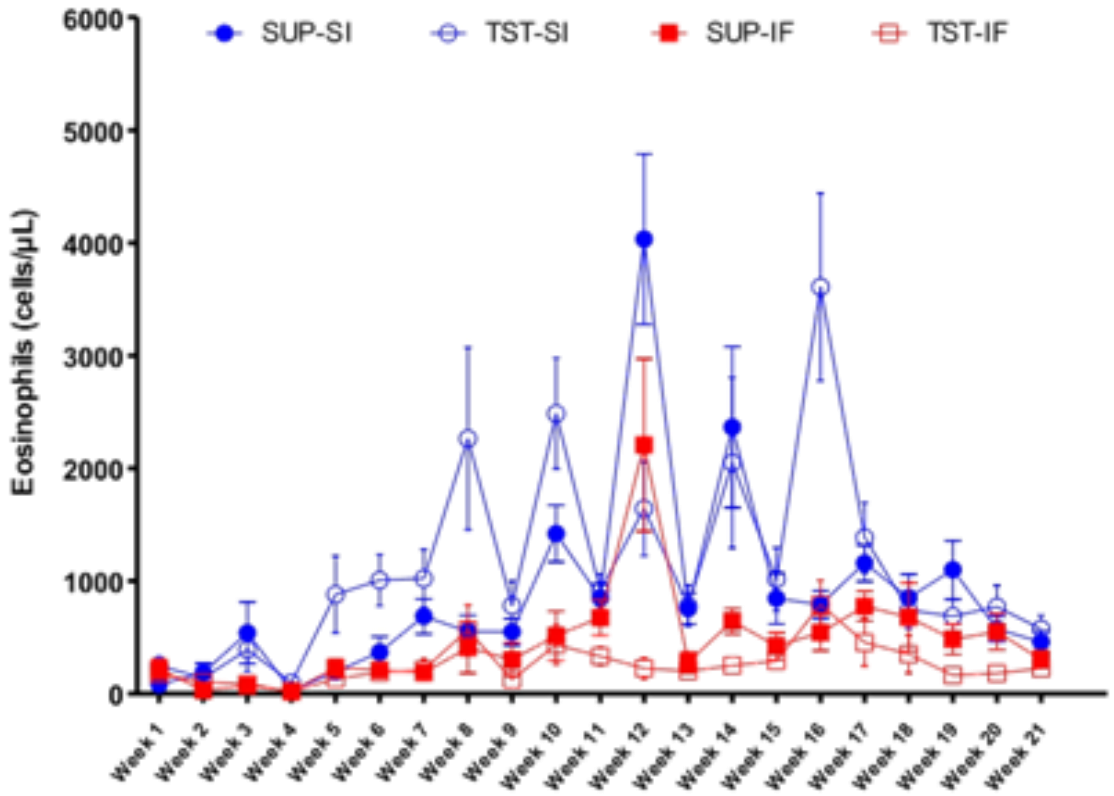

Figure 5 Mean blood eosinophil count (cells/µL). Blood eosinophil counting of the Santa Ines (SI) and Ile de France (IF) lambs naturally infected with *H. contortus* and under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Values represent mean ± standard error.

Table 2 Averages (minimum–maximum values) of eosinophils (cells/mm²), mast cells (cells/mm²), globule leukocytes (cells/mm²), POU2F3 + cells (%), T cells (cells/mm²) and B cells (cells/mm²) in abomasum mucosa of the Santa Ines and Ile de France under suppressive or targeted selective treatment (TST) programme with anthelmintics

Cells	Santa Ines		Ile de France		Effects (*P*-value)		
	Suppressive ($n = 9$)	TST ($n = 10$)	Suppressive ($n = 10$)	TST ($n = 9$)	Breed	Treatment programme	Breed × treatment
Mast cells	51 (17–97)	132 (17–305)	28 (0–83)	113 (15–165)	ns	0.0001	ns
Globule leukocyte	66 (27–145)b	148 (55–252)b	30 (0–118)a	142 (18–238)b	0.0031	< 0.0001	0.0228
Eosinophils	95 (10–277)	90 (5–197)	80 (5–317)	42 (7–60)	ns	ns	ns
POU2F3+ cells	7.33 (3.0–12.0)	11.18 (1.9–13.7)	3.64 (2.0–5.5)	7.62 (4.6–11.3)	< .0001	< .0001	ns
T cells	993.5 (768–1499)	1102.5 (740–1458)	808.0 (481–1167)	788.0 (582–1030)	0.0012	ns	ns
B cells	94.0 (27.2–181.7)	124.5 (50.9–249.4)	99.2 (14.8–260.9)	45.4 (12.8–130.4)	ns	ns	ns

Means followed by different letter in the same line differ from each other by Tukey's test ($P < 0.05$).

ns: not significant ($P > 0.05$).

shown that Santa Ines is a relatively resistant breed to *H. contortus* infections at different stages of life: sucking lambs [42], weaned lambs [26], ewes around parturition and during lactation [39]. However, the mechanisms involved in their greater resistance to GIN infection are not clear.

Taking into consideration that resistance to parasites is largely immune mediated [13, 28], our aim was to look for differences in immune responses that could explain resistance or susceptibility between breeds. The infections were naturally acquired by the lambs during grazing. Therefore, it was not possible to determine the number of L3 they ingested during the trial. Nevertheless, we assume that, in each treatment group, animals ingested a similar number of infective larvae because they were under the same grazing

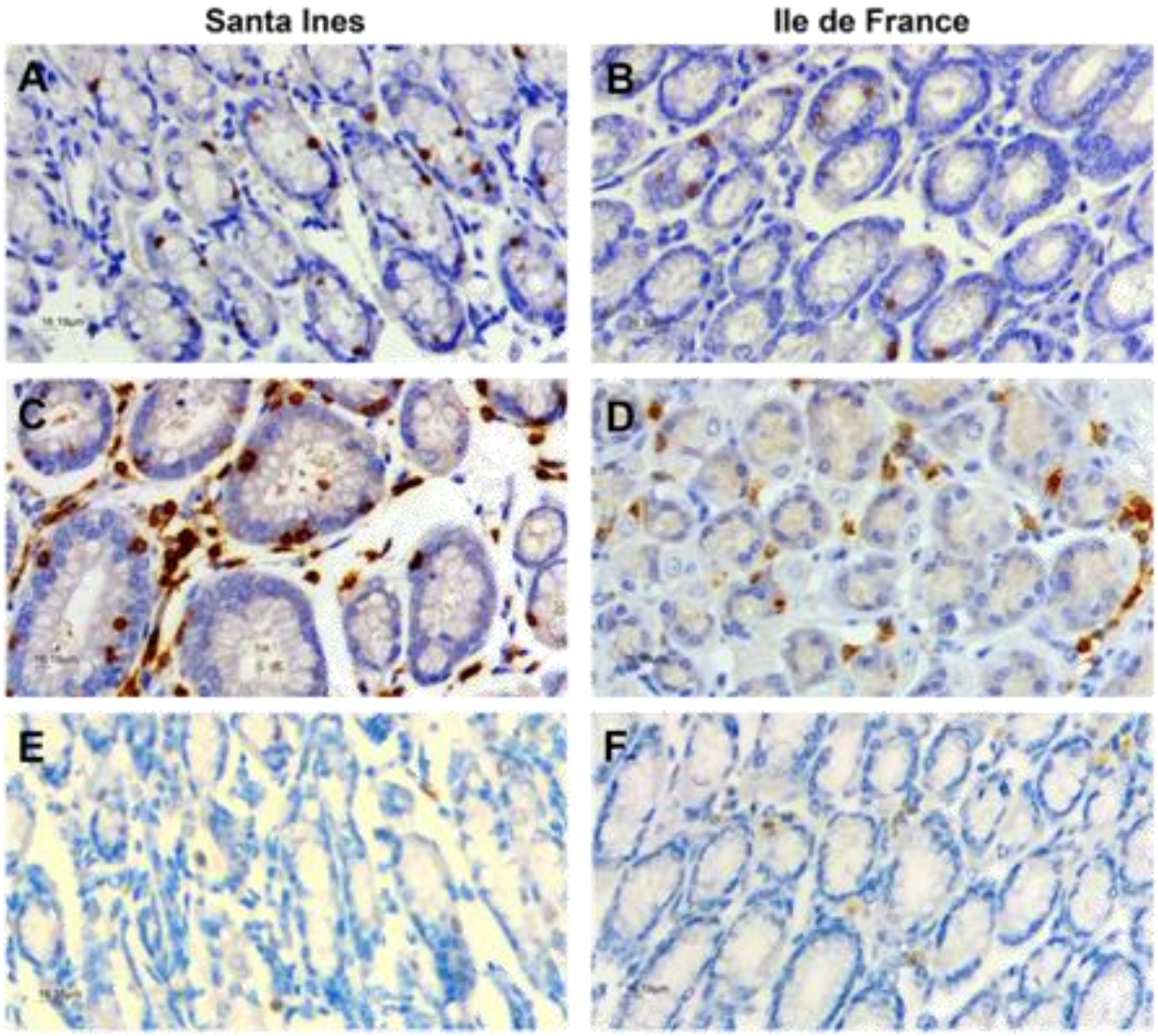

Figure 6 Immunohistochemical labelling of abomasum mucosa sections showing positive cells. A, **B** POU2F3+ cells; **C**, **D** CD3+ T cells; **E**, **F** CD79α+ B cells (×400 magnification). The micrographics **A**, **C**, **E** show tissue from the Santa Ines lamb with no detectable *Haemonchus contortus* worm; while **B**, **D**, **F** show tissue from Ile de France lamb with a *H. contortus* burden of 1860.

management. Our study was divided into two stages: firstly, the evaluation of circulating immune responses in lambs exposed to natural *H. contortus* infection and secondly, the evaluation of local abomasal cellular and antibody responses from the same lambs at post-mortem.

In the first stage, the major differences were higher levels of anti-*H. contortus* L3-specific IgG and number of blood eosinophils in the Santa Ines breed, especially at the beginning of the trial, indicating that the response against *H. contortus* occurs earlier in this breed than in the Ile de France. At post-mortem, there were marked differences between breeds, with Santa Ines showing significantly higher levels of mucus anti-L3 IgA, POU2F3+ cells, T cells and GL. These local responses may be implicated in limiting establishment of infective larvae and/or eliminating adult parasites [6, 11, 20, 30, 44].

Studies of other resistant sheep breeds identified earlier and robust initial type 2 responses against GIN infection, with production of specific antibodies [10, 19, 20, 27]. In the current study, earlier and marked IgG production were observed in Santa Ines lambs, however, both breeds presented similar IgG levels at the end of experiment, demonstrating that with the progression of the trial, the Ile de France lambs were also responding to *Haemonchus* infection. Such level of IgG response, however, was not enough to protect the Ile de France from haemonchosis. Similar results have been reported previously, with no difference in IgG level between resistant and susceptible breeds infected with *H. contortus* [27], suggesting

that resistance to *H. contortus* infection is not related to plasma IgG level.

IgE is the main antibody involved in the type 2 response against GIN infection and its expression is associated with greater resistance and shows moderate to high heritability [23]. We observed that total IgE concentration increased over time, however, there was no significant difference between breeds. This may be due to IgE being present at extremely low serum concentrations with a short half-life. Most of the IgE is found irreversibly attached to specific receptors of mast cells and eosinophils [45, 46]. In future studies, it will be interesting to evaluate IgE attached to inflammatory cells or *H. contortus*-specific IgE level in the blood serum, which can provide more informative results.

In the present study, the resistant breed presented the highest means of mucosal anti-*H. contortus* L3-specific IgA, suggesting that this immunoglobulin had an important regulatory role in the local immune response against *H. contortus* infection in the Santa Ines lambs. IgA is associated with reductions in worm length and fertility in *H. contortus* infection, and early worm expulsion [44, 47, 48].

The early and robust blood eosinophil expansion in the Santa Ines lambs for almost the entire experimental period may contribute to low nematode establishment and elimination [49], and as consequence low FEC and low worm burden in the resistant breed. The magnitude of eosinophil response may be related to resistance to helminth infection. This cell type remains briefly in the bloodstream before migrating to tissues, where they may stay for several weeks, depending on the presence of Th2 cytokines, or undergo apoptosis [49, 50]. Once triggered, eosinophil degranulation may occur by exocytosis, piecemeal degranulation, explosive degranulation with consequent cellular lysis or formation of extracellular traps [51–53]. Unlike in blood, numbers of eosinophils in the mucosa were similar in the two breeds. This may reflect the time-point at which mucosal eosinophil were enumerated, as at post-mortem circulating levels of eosinophils were similar between the two breeds. Another possibility is that the turn-over rate of mucosal eosinophil populations in the Santa Ines breed was more rapid, meaning that higher recruitment of blood eosinophils into the abomasum in the Santa Ines breed would not necessarily result in elevated eosinophil numbers in the mucosa. Enumeration of mucosal eosinophils at time-points of infection where blood eosinophil numbers were significantly different between the two breeds would be required to investigate this further.

Rapid rejection of nematodes in sheep is associated with expansion and mobilization of MC and GL into the gut mucosa [6, 54, 55]. The strength of MC response is linked to Th2 cytokines and IgE production [56, 57], whereby resistant breeds show greater responses [20, 27, 43]. We observed higher MC and GL cells counts in the Santa Ines resistant breed, but only the GL count was significantly different between breeds. GL derive are thought to be de-granulated MC [54]. Previous study using a gut washing method has shown that GL may leave the epithelium and can be found in the gut lumen in large amounts [58]. Future studies could apply this method, which may identify an even more marked difference between resistant and susceptible animals.

Epithelial cells are the first cell type in contact with the parasite, indicating that before immune engagement there is a need for epithelial activation [59]. Epithelial chemosensory cells called tuft cells were shown to drive early signals in the small intestine to initiate type 2 immunity against helminth infection in mice [10, 60]. These cells are the dominant source of IL-25 that induces a Th2 response through the action of group 2 innate lymphoid cells (ILC2s) to produce specific type 2 cytokines [61, 62]. At present we cannot be sure that the POU2F3+ cells identified here are the equivalent of tuft cells in mice, and work is ongoing to determine this (Hildersley et al. unpublished data). Interestingly, Santa Ines sheep showed a higher percentage of POU2F3+ cells in the abomasum mucosa compared to Ile de France, indicating a possible role for these cells in resistance against *H. contortus* infection. The B cells count was similar between breeds, in agreement with other studies [6, 27], that reported no difference in B cells between resistant and susceptible breeds.

There was a marked difference between treatments groups with regard to MC, GL counts and POU2F3+ cells. The greater number of these cells in the TST groups of both breeds was possibly a result of the longer and greater GIN challenge in comparison with animals under the suppressive treatment, which showed increase in parasite egg counts in the last month of the trial and had significantly higher *H. contortus* worm burdens at post-mortem. This suggests that expansion and/or recruitment of these Th2 associated immune cells may be most sensitive to nematode continuous challenging compared to other immune responses measured in this study.

Assessing differences in the local cellular responses between breeds at early time points after infection could be informative. Particular focus would be on T cells and POU2F3+ cells (putative tuft cells), and whether earlier and/or greater activation of these putative tuft cells drive a greater type 2 response in Santa Ines breed. Determining the resistance mechanisms is important for understanding breed resistance and identifying genetic markers that may advance selective breeding as a sustainable control strategy [5].

In conclusion, Santa Ines lambs showed early and robust immune response against *H. contortus* infection, with strong local immune responses, effective in limiting the establishment and/or eliminating the worm population. This breed has potential value in improving the resistance of sheep to GIN infection, as a sustainable alternative control strategy.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13567-019-0722-3.

Additional file 1. Antibodies used in immunohistochemistry to identify positive cells on the abomasum mucosa.

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Authors' contributions
ACAA conducted the trial, all parasitological, humoral and local immune response analysis and wrote the main manuscript. CCB assisted humoral measurements and in conjunction with FAA performed parasitological analyses and managed hosts. KAH helped with immunohistochemistry methodology. TNM and CB supervised immunohistochemistry analysis. AFTA designed the study and supervised all research stages. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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CAPÍTULO 4 – Immune response to intestinal nematode infection in the Ile de France and Santa Ines sheep under different anthelmintic treatment regimens

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Immune response to intestinal nematode infection in the Ile de France and Santa Ines sheep under different anthelmintic treatment regimens

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ABSTRACT

A study was carried out to assess the differences in immune response between Santa Ines and Ile de France sheep breeds to natural infection by intestinal nematodes over five months grazing period. Nineteen Santa Ines and 19 Ile de France lambs male lambs of 3 months-old were allocated in different anthelmintic treatments regimens: suppressive treatments (SUP) or targeted selective treatments (TST). Parasitological variables, humoral immunity, local cellular immune responses were evaluated. Santa Ines lambs had significantly lower *Cooperia curticei* and *Trichuris* spp. worm burden compared to Ile de France regardless of treatment regime, but showed similar *Strongyloides papillosus* and *Trichostrongylus colubriformis* worm burden. Parasite-specific IgG levels were significantly higher and more rapidly induced in Santa Ines lambs, additionally they presented higher levels of parasite-specific IgA in intestinal mucus. Local responses were generally superior in animals under TST, which had significantly elevated number of mast cells and globule leukocytes. In general, Santa Ines presented less intestinal nematodes than Ile de France lambs, but still had an expressive *T. colubriformis* worm burden, despite showing an early and greater immune response to intestinal nematode infection.

Introduction

Gastrointestinal nematodes (GIN) are an important limiting factor to small ruminant production due to important productive and economic losses (AMARANTE et al., 2014). Their infections are responsible for 15-22% decrease of sheep meat, wool and milk production (MAVROT et al., 2015).

Sheep are usually affected by mixed infection with different parasites (MCRAE et al., 2015). Intestinal parasites such as *Trichostrongylus colubriformis*,

Cooperia curticei, *Strongyloides papillosus*, *Oesophagostomum columbianum* and *Trichuris* spp. are commonly found in sheep flocks tropical and subtropical regions (AMARANTE et al., 2004; ZAJAC, 2006).

Intestinal nematodes in heavy infections may cause severe enteritis due to tissue damage and inflammation in the intestinal mucosa (ANDRONICOS et al., 2012). The formation of tunnels by GIN in epithelium mucosa affect tissue integrity, provoke hyperplasia and crypt hypertrophy, atrophy of microvilli and epithelium erosion (CARDIA et al., 2011; DIMITRIJEVIĆ et al., 2012), compromising retention time of the digesta, nutrient digestion and absorption (CARDIA et al., 2011; SILVA et al., 2019). Furthermore, there is decrease in feed intake due to anorexia (KYRIAZAKIS et al., 1996; COOP & KYRIAZAKIS, 1999; CARDIA et al., 2011; MAVROT et al., 2015).

Gastrointestinal nematodes induces T helper 2 (Th2)-type immune response that entails innate and adaptive cells, signalled by specific cytokines such as interleukin 4 (IL-4), IL-5, and IL-13 (SOROBETEA et al., 2018; INCLAN-RICO & SIRACUSA et al., 2018). Mucosa mast cells, globule leukocyte and eosinophils are important effectors cells involved in parasite clearance herewith to IgA, IgG and IgE activity (MCCLURE et al., 1992; PERNTHANER et al., 2006; AMARANTE et al., 2007; BEASLEY et al., 2010).

Animals resistant to GIN infection develop Th2-type response earlier and more pronounced that might, impair parasite establishment and development, decreasing the worm burden (PERNTHANER et al., 2006; MCRAE et al., 2015; SOROBETEA et al., 2018). Considering the current situation of anthelmintic resistance, draw on host natural immune response is a viable alternative approach to control GIN (AMARANTE et al., 2014; ZVINOROVA et al., 2016).

Hence, as a complement of the previous study about *Haemonchus contortus* present by Albuquerque et al. (2019), here we evaluate and compare the humoral response and intestinal inflammatory cells between Santa Ines and Ile de France lambs naturally infected by intestinal nematodes and under suppressive or targeted selective treatment with anthelmintics.

Material and methods

Ethical considerations

The experiment was carried out at São Paulo State University (UNESP), Botucatu-SP, Brazil. All animal procedures were in accordance with the ethical standards and were approved by the Animal Use Ethics Committee of the FMVZ/UNESP (47/2016).

Experimental design

The experimental design was described previously (ALBUQUERQUE et al., 2017). Briefly, 38 3-month-old uncastrated male lambs were purchased from commercial farms located in São Paulo state, Brazil. Animals were distributed into groups by stratified randomization balanced as far as possible taken into consideration body weight and nematode faecal egg counts (FEC). The 19 Santa Ines (SI) and 19 Ile de France (IF) were allocated in two anthelmintic treatment groups: suppressive (SUP), lambs drenched each two weeks with monepantel (2.5 mg/kg, Zolvix®, Novartis); targeted selective treatment (TST), lambs drenched only when they showed packed cell volume (PCV) ≤ 20%, corresponding category 3 of FAMACHA method (VAN WYK et al., 1997).

Suppressive group (SUP-IF and SUP-SI) were treated with the following combination of anthelmintics seven days before being allocated to the paddocks: albendazole (10 mg/kg, Valbazen® 10% CO, Zoetis), levamisole (9.4 mg/kg, Ripercol® L 150 F, Zoetis), and monepantel (2.5 mg/kg, Zolvix®, Novartis) with no nematode eggs detected in a sequence of faecal examinations. Additional anthelmintic drenched were performed in lambs under suppressive treatment on the 7th week, with moxidectin (0.4 mg/kg, Cydectin® Zoetis), because some of them were shedding *Strongyloides* spp. eggs (mean of 42 and range of 0–200 EPG); and on the 14th week, with a combination of monepantel, levamisole (9.4 mg/kg), and albendazole (10 mg/kg), due to the shedding of strongyle eggs (379, 0–1000 EPG) and *Strongyloides* spp. (874, 0–5300 EPG). The suppressive treatment was carried out in order to keep the grazing lambs and their pastures as free of worms as possible and prevent

decrease in animal's productive performance due to parasitism. The last monepantel drench for lambs under suppressive treatment was administered on the 19th week and they were slaughtered on the 21st week.

The lambs in the TST group (TST-SI and TST-IF) were introduced into the clean pasture harbouring natural parasites infections acquired at their origin farm. They were not drenched on arrival at the experimental facilities. These lambs underwent monepantel treatment when they showed a PCV ≤ 20%, in order to prevent the occurrence of mortality and / or severe haemonchosis.

Haematology

Blood samples (5 mL) were collected weekly by jugular vein puncture into Vacutainer® tubes containing anticoagulant (EDTA). The PCV was determined by microhematocrit centrifugation at 12000 RPM for 5 minutes, and total plasma protein level (TPP) was estimated using a hand-hold refractometer (Refractometer SPR-N, Atago) (WEISER, 2012; WEISS; WARDROP, 2010). The blood in the tube was centrifuged at 2500 RPM for 15 minutes to allow plasma separation. Aliquots of plasma samples were stored at -20 ºC prior to ELISA.

Histology

Intestinal tissue samples were taken from duodenum and fixed in 4% buffered formaldehyde for 48 hours, then moved to 70% ethanol, and paraffin-embedded. Tissue sections were cut to 5 µm thick and mounted on glass slides. Eosinophils and globule leukocytes were counted on Haematoxylin and Eosin (H&E) stained sections whereas mast cells were counted on 1% toluidine blue stained sections. All cells were counted in sixty randomly selected fields of view per animal in a 0.01 mm^2 area at ×1000 magnification (adapted from BALIC et al., 2002). The counts were expressed as cells/mm^2 tissue surface.

Faecal examination

Faecal samples were collected upon animals' arrival and then weekly, directly from the rectum of each animal in polyethylene bags previously labelled and

kept refrigerated until processing. The modified McMaster technique (UENO & GONÇALVES, 1998) was used to measure faecal eggs count (FEC), in which each nematode egg counted represented 100 eggs per gram of faeces (EPG). Faecal cultures were prepared separately for each group for production of GIN infective third-stage larvae (L3) on animals' arrival and then weekly to be morphologically identified and counted (UENO & GONÇALVES, 1998). Based on the proportion of L3 identified as *T. colubriformis* in the cultures, the *Trichostrongylus* EPG of each lamb was estimated.

Intestinal worm burden

All lambs were slaughtered and the gastrointestinal tract was removed. The small intestine was opened and the contents placed in graduated buckets. Intestines were subjected to hot water bath in saline solution for 4 hours at 37ºC in 2 L graduated cylinder to recover nematodes present in the mucosa (UENO & GONÇALVES, 1998). Aliquots of 10% of the total abomasums and intestine contents were collected individually, stored in plastic flasks and preserved in -20 °C freezer. All nematodes present in the aliquots were identified and quantified, according to their developmental stage (UENO & GONÇALVES, 1998; AMARANTE, 2015; ALMEIDA et al., 2018).

The large intestine was opened and 5% aliquot of the contents was collected and preserved. Parasites attached in the mucosa of the large intestine were recovered. The results were extrapolating to the total volume of abomasums, small intestine and large intestine.

Enzyme-linked immunosorbent assay (ELISA)

Trichostrongylus colubriformis-specific IgG

Plasma samples collected at 11 time-points were used to determine IgG levels against L3-soluble extract of *T. colubriformis.* The L3 extract was prepared as previously described (AMARANTE et al., 2009). A previously described protocol was applied to determine the parasite-specific plasma IgG levels (SILVA et al., 2012), with some modifications: the plates were coated with 2 µg of antigen/mL; each wash was done three times, rotating through 180° and re-washing three

more times; the negative control (NC) sample used was from a worm free animal, as previously described (SANTOS et al., 2014); the plasma positive control (PC) sample used was from a sheep artificially infected with both *H. contortus* and *T. colubriformis* every three days for 84 days. Results were expressed as the percentage of optical density (OD) value of the PC plasma sample (KANOBANA et al., 2001).

Trichostrongylus colubriformis-specific IgA

A 5 cm tissue piece from the duodenum of each animal was sampled and stored at -20 ºC until processing for the mucus extraction. Tissues were thawed and mucus was scraped off with a glass slide and stored in a falcon tube on ice, followed by addition of PBS supplemented with protease inhibitors (Complete®, Roche) to each sample in a proportion of 4:1 (4 mL PBS + 1mL of mucus). The samples were shaken for 1 hour at 4 ºC and centrifuged for 30 minutes at 4 ºC and 3000 ×*g*. The supernatant was collected and centrifuged again for 30 minutes at 4 ºC and 15000 ×*g.*

ELISA assays for parasite-specific IgA recovered from small intestine mucous were carried out anti-*T. colubriformis* L3 and adult extract as previously described (SILVA et al., 2012) with a few differences: plates were coated with 5 µg of antigen/mL; each wash was done three times, rotating through 180° and re-washing three more times; mucus samples were diluted in PBS-GT (1:20) and rabbit anti-sheep IgA peroxidase-conjugated antibody was diluted at 1:20000. The results were expressed as OD value minus OD-blank sample (KANOBANA et al., 2001).

Statistical analysis

All data were submitted to normality test and transformed using $\log_{10}$ (x+1) when necessary. Data with single measures and data for repeated measures at several time points were analysed by ANOVA using the General Linear Model (GLM) and groups means were compared by Tukey's test using Statistical Analysis System, version 9.2 (SAS Institute, Inc., Cary, NC, USA). Values of $P<0.05$ were considered statistically significant.

Results

Parasitology

The suppressive anthelmintic treatments presented high efficacy on all intestinal nematode species infecting animals during the trial ($P < 0.05$), consequently EPG and worm burden means were always higher in lambs under TST. Animals' faecal culture data and Strongyle EPG were previously described (ALBUQUERQUE et al., 2017). *Strongyloides* EPG showed a progressive increase over time (Figure 1), of which SI presented lower EPG means than IF in all samplings, with significant breed effect on the 4th, 15th, 19th and 21st weeks.

Six species of gastrointestinal nematodes were identified in the experimental animals gastrointestinal contents: *Haemonchus contortus* in the abomasum, *T. colubriformis*, *C. curticei*, *S. papillosus* in the small intestine, and *Trichuris discolor* and *Trichuris ovis* in the large intestine.

The *H. contortus* results were previously described (ALBUQUERQUE et al., 2019). Briefly, there was breed effect on all developmental stages of *H. contortus*, with SI lambs presenting the lowest worm burden ($P<0.0001$). Lambs of both breeds under suppressive treatment presented higher means of *Haemonchus* than their TST counterparts due to emergence of anthelmintic resistance.

Conversely, because the suppressive treatment programme had a high efficacy against *T. colubriformis* (Table 1)*, C. curticei* (Table 2)*, S. papillosus* and *Trichuris* spp. (Table 3), only animals subjected to TST presented high counting. With regards to total worm burden of each of these species, there was breed effect only in numbers of *C. curticei* ($P < 0.05$, Table 2) and *Trichuris* spp. ($P < 0.05$) with lower means in the Santa Ines lambs.

However, there was significant breed effect in immature developmental stages of all intestinal worms ($P<0.05$), with IF showing higher means than SI lambs, but to adult stages similar counting was observed to both breeds.

Despite there was no breed difference in the *T. colubriformis* counting, there were large variation among animals worm burden, mainly in SI lambs. Four SI lambs showed low *T. colubriformis* burden ranging from 0 to 440, the other six presented more pronounced worm burden ranging from 4,110 to 15,670 specimens. In contrast, eight IF lambs showed *T. colubriformis* burden 4,010 to 10,090 and one presented the highest worm burden with 28,890 specimens.

A few *Trichuris* specimens were found: *T. discolor* and *T. ovis* in the IF and only *T. discolor* in the SI. The TST-IF presented higher *Trichuris* worm burden mean (8.8, range 0-22) than TST-SI lambs (0.9, range 0-7) (Table 3).

Antibody responses

IgG plasma levels against T. colubriformis L3

Significant breed effect on the mean levels of anti-*T. colubriformis* L3 IgG in plasma occurred early in the trial (on the 5^{th},7^{th} and 9^{th} weeks), with SI showing more intense *T. colubriformis* L3-specific IgG levels. In the last two samplings the means of all groups were similar (Figure 2). Treatment program had effect on IgG levels in five time-points, between on the 9^{th} and 17^{th} weeks, when animals under TST presented higher means than the suppressive treatment groups (Table 4).

Small intestine mucous IgA

There was significant breed effect ($P < 0.05$) on *T. colubriformis* L3-specific IgA levels in the intestinal mucous, with Santa Ines lambs presenting higher levels. Values of IgA against antigens of adult worms were relatively low and similar among groups (Figure 3).

Intestinal cellular immune responses

There was a significant breed x treatment interaction ($P<0.05$) on MC means and a significant effect of treatment on both MC and GL cells ($P < 0.05$), with higher MC and GL counts in lambs undergoing TST. Numbers of eosinophils

were similar among groups without any breed or treatment effect (P>0.05) (Table 5).

Discussion

Intestinal parasites are responsible for huge productive losses in sheep farming (CARDIA et al., 2011). *Trichostrongylus colubriformis* is the major parasite of the small intestine of sheep (AMARANTE, 2014). However, other species such as *C. curticei*, *O. columbianum* and *S. papillosus* are commonly found parasitizing these animals in Brazil (WILMSEN, 2014). From all species identified in our lambs, the anthelmintic treatment performed in suppressive group was effective against all intestinal nematodes species, consequently the major parasite counts were recorded in the animals under TST. Despite of high anthelmintic efficacy on intestinal parasites, suppressive treatment was not sustainable control method on account of rapid emerged of anthelmintic resistance by *Haemonchus contortus* (ALBUQUERQUE et al., 2017). In addition, the aim to used suppressive treatment was to keep animals free as possible of GIN infection, trying to simulate a non-infected control group.

Among animals subjected to TST, there were significant differences between breeds in total worm burden for *C. curticei* and *Trichuris* spp., Santa Ines lambs also presented lower *T. colubriformis* and *S. papillosus* counts than Ile de France, but breed differences were less consistent. In spite of Santa Ines being considered a resistant breed with the strong immune response against *H. contortus*, apparently shows susceptibility to *T. colubriformis* infection (AMARANTE et al., 2004). The mechanism of nematode expulsion is parasite-specific and has no effect on different worm species, which indicate that there is more than one immune way for parasites to be expelled (MCCLURE et al., 1992) or some species are more able to evade the immune system than others. Santa Ines susceptibility to *T. colubriformis* infection does not seem to be an exclusive trait of this breed, but also of some breeds known as resistant to *H. contortus* infection, such as Gulf Coast and Florida Native, which also harbour similar *Trichostrongylus* infection intensity in comparison with other susceptible breeds of sheep (AMARANTE et al., 1999; BAHIRATHAN et al., 1996). In the

other hand, all Ile de France lambs under TST needed to be drenched over the trial ranging from one to four times to prevent severe haemonchosis, in contrast to only two Santa Ines drenched one or two times (ALBUQUERQUE et al., 2017). Because the anthelmintic treatment was effective against intestinal parasites, it is possible that the treatments performed in Ile de France lambs may have influenced the worm count, masking possible differences between breeds. Furthermore, levels of anti-*T. colubriformis* IgA adult-specific was low and similar between breeds, and antibodies produced to a particular nematode stage had no effect on the other parasite stage (HARRISON et al., 2003; PERNTHANER et al., 2006), which may explain the large amount of *T. colubriformis* adults.

Furthermore, there was a large variation in the parasitological, inflammatory cells and antibodies variables among lambs. Four Santa Ines under TST, that presented high number of inflammatory cells, IgG and IgA levels, showed low worm burden of *T. colubriformis* (0-440), *C. curticei* (0-270) and *S. papillosus* (8-580), displaying more resistance than other animals in the same breed. The worm burden variation among infected animals was also observed by Cardia et al. (2011) in Santa Ines lambs artificially infected by *T. colubriformis*, where 60% of animals showed low worm burden (ranging from 13 to 1,540) and 40% presented high worm burden (ranging from 6,310–26,830).

In general type 2 response is required to protection against GIN infection where the development of immune response is adapted to specific lifecycle stages (MEEUSEN et al., 2005). The mucus is an important physical barrier to prevent nematodes establishment and its composition also carry important elements for parasite infection regulation such as mucin and nonmucin proteins as globet cells secreted products, IgG and IgA specific stage antibodies (HARRISON et al., 2003; SHARPE et al., 2018). In the present study, IgG was tested at 11 time-points in plasma and IgA in mucus at post mortem. The Santa Ines lambs were able to respond earlier and produce high levels of anti-*T. colubriformis* L3-specific IgG, as observed in other resistant sheep (PERNTHANER et al., 2006). Although Ile de France lambs have been able to reach the same IgG levels of

Santa Ines over time. Moreover, Santa Ines lambs presented significantly higher IgA levels than Ile de France regardless of the treatment performed, as expected to this breed (CARDIA et al., 2011). According to Harrison et al (2003), IgG and IgA are responsible for larvae aggregate, impairing their motility causing 80% reduction of larvae establishment, additionally the antibodies remain in mucus several days after challenge.

The inflammatory cells such as eosinophils, mast cells and globule leukocyte are related to effective immune response against GIN (DOUCH et al., 1986; MCCLURE et al., 1992; SOROBETEA et al., 2018). These cells are abundant in the intestinal tract and are associated to parasite clearance (HUNTLEY et al., 1992; SOROBETEA et al., 2018). The marked difference in MC and GL counts between treatments groups presumably occurred as a result of longer and greater GIN challenge in lambs under TST. According to Amarante et al. (1999), for MC hyperplasia is necessary continuous nematode challenge. However, in a *T. colubriformis* infection Douch et al. (1986) did not find a correlation of MC and resistance, but observed a strong association with GL and larval migration in an inhibitory assay. It is important to emphasize that GL derives from MCs (HUNTLEY et al., 1992).

Conclusion

In comparison with Ile de France sheep, Santa Ines lambs showed early and greater local immune response against intestinal parasites with ability to limiting the establishment and/or promote worm expulsion of some species like *Cooperia* and *Trichuris*. However, this breed showed lower capacity to resistant *T. colubriformis* infection, although with a considerable variation of the response among the animals.

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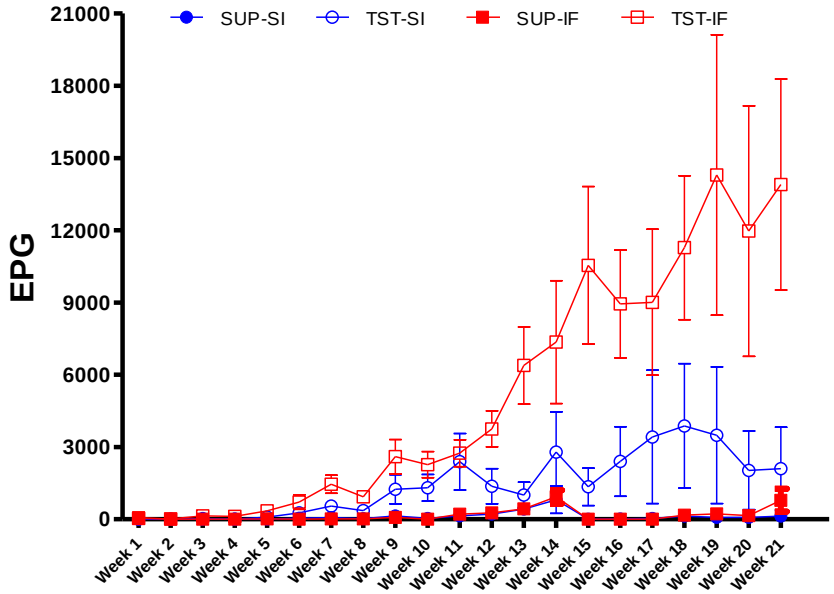

Figure 1. Means of eggs per gram of faeces (EPG) of *Strongyloides* spp. of the Santa Ines (SI) and Ile de France (IF) lambs under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Bars are standard error.

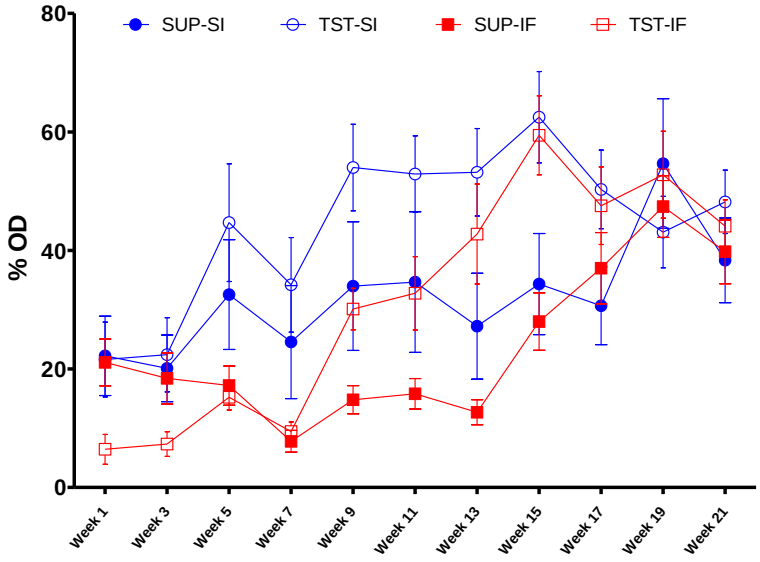

Figure 2. Mean levels of anti-*Trichostrongylus colubriformis* L3 IgG (% OD) in plasma, of the Santa Ines (SI) and Ile de France (IF) lambs under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Values represent mean ± standard error.

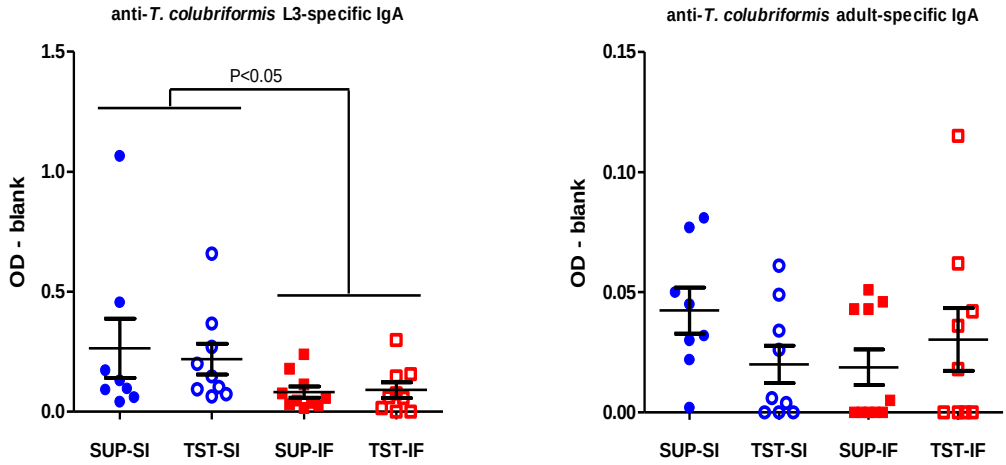

Figure 3. Mean levels of anti-*Trichostrongylus colubriformis* L3 and adult – specific IgA (OD – blank) in the intestinal mucus collected at post mortem of the Santa Ines (SI) and Ile de France (IF) lambs under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Values represent mean ± standard error.

Table 1. *Trichostrongylus colubriformis* means (minimum-maximum values) of the Santa Ines and Ile de France lambs under suppressive or targeted selective treatment (TST) programme with anthelmintics.

Development stages	Santa Ines		Ile de France		Effects (P-value)		
	Suppressive (n=9)	TST (n=10)	Suppressive (n=10)	TST (n=9)	Breed	Treatment programme	Breed x Treatment
Early L_4	0	24 (0-60)	0	156 (20-570)	0.0052	<0.0001	0.0016
	0 a *	0.83 (±0.28) b	0 a	1.97 (±0.16) c			
Female late L_4	2 (0-10)	56 (0-300)	1 (0-10)	428 (20-1060)	0.0145	<0.0001	0.0008
	0.23 (±0.15) a	1.16 (±0.29) b	0.10 (±0.10) a	2.46 (±0.16) c			
Male late L_4	1 (0-10)	90 (0-290)	4 (0-30)	487 (60-1450)	0.0095	<0.0001	0.0163
	0.12 (±0.12) a	1.44 (±0.29) b	0.25 (±0.17) a	2.56 (±0.13) c			
Female L_5	2 (0-10)	136 (0-400)	2 (0-20)	969 (440-2040)	0.0197	<0.0001	0.0012
	0.23 (±0.15) a	1.67 (±0.30) b	0.13 (±0.13) a	2.93 (±0.08) c			
Male L_5	0	166 (0-660)	1 (0-10)	1772 (640-3060)	0.0006	<0.0001	.0005
	0 a	1.55 (±0.35) b	0.10 (±0.10) a	3.19 (±0.08) c			
Adult female	1 (0-10)	2806 (0-6690)	0	4748 (1780-18030)	ns	<0.0001	0.0412
	0.26 (±0.17) a	2.61 (±0.05) b	0 a	3.55 (±0.10) b			
Adult male	1 (0-10)	2806 (0-6690)	5 (0-30)	2744 (190-14810)	ns	<0.0001	ns
	0.12 (±0.12)	2.52 (±0.49)	0.36 (±0.19)	3.07 (±0.19)			
Total worm burden	10 (0-30)	6997 (0-16190)	13 (0-50)	11253 (4670-35150)	ns	<0.0001	ns
	0.77 (±0.20)	2.93 (±0.53)	0.67 (±0.23)	3.98 (±0.08)			

* Means (± standard error) of log transformed values followed by different letter in the same line differ from each other by Tukey's test (P<0.05).
ns = not significant (P>0.05).

Table 2. *Cooperia curticei* means (minimum-maximum values) of the Santa Ines and Ile de France lambs under suppressive or targeted selective treatment (TST) programme with anthelmintics.

Development stages	Santa Ines		Ile de France		Effects (P-value)		
	Suppressive (n=9)	TST (n=10)	Suppressive (n=10)	TST (n=9)	Breed	Treatment programme	Breed x Treatment
Early L_4	16 (0-80)	296 (0-1060)	42 (0-120)	875 (240-2230)	0.0080	<0.0001	ns
	0.64 (±0.26)	1.61 (±0.39)	#1.16 (±0.27)	2.84 (±0.11)			
Female late L_4	12 (0-70)	111 (0-470)	18 (0-70)	253 (40-490)	0.0106	0.0008	ns
	0.50 (±0.25)	1.05 (±0.37)	0.84 (±0.24)	2.29 (±0.13)			
Male late L_4	3 (0-10)	23 (0-110)	4 (0-20)	149 (10-400)	0.0137	<0.0001	0.0061
	0.35 (±0.17) a*	0.87 (±0.25) a	0.34 (±0.17) a	2.01 (±0.15) b			
Female L_5	0	28 (0-120)	7 (0-50)	232 (0-490)	0.0194	<0.0001	ns
	0	0.84 (±0.29)	0.30 (±0.20)	1.90 (±0.36)			
Male L_5	0	31 (0-140)	2 (0-20)	194 (0-440)	ns	<0.0001	ns
	0	0.95 (±0.27)	0.13 (±0.13)	1.83 (±0.35)			
Adult female	1 (1-10)	517 (0-1410)	1 (0-10)	979 (0-3350)	ns	<0.0001	ns
	0.12 (±0.12)	1.92 (±0.43)	0.10 (±0.10)	2.40 (±0.38)			
Adult male	1 (1-10)	368 (0-1140)	0	660 (0-2510)	ns	<0.0001	ns
	0.12 (±0.12)	1.92 (±0.41)	0	2.20 (±0.36)			
Total worm burden	33 (0-110)	1374 (0-3360)	74 (0-190)	3339 (600-8400)	0.0292	<0.0001	ns
	0.93 (±0.31)	2.58 (±0.37)	1.58 (±0.22)	3.38 (±0.13)			

* Means (± standard error) of log transformed values followed by different letter in the same line differ from each other by Tukey's test (P<0.05).
ns = not significant (P>0.05).

Table 3. *Strongyloides papillosus* and *Trichuris* spp. means (minimum-maximum values) of the Santa Ines and Ile de France lambs under suppressive or targeted selective treatment (TST) programme with anthelmintics.

Species	Santa Ines		Ile de France		Effects (P-value)		
	Suppressive (n=10)	TST (n=10)	Suppressive (n=10)	TST (n=9)	Breed	Treatment programme'	Breed x Treatment
S. papillosus							
Early L_4	0 a	0	0	39 (0-180)	0.0259	0.0193	0.0193
	0 a *	0 a	0 a	0.74 (±0.32) b			
Female late L_4	0	15 (0-50)	10 (0-60)	62 (0-310	0.0136	0.0002	ns
	0	0.72 (±0.25)	0.44 (±0.23)	1.4 (±0.23)			
Female L_5	4 (0-20)	19 (0-80)	3 (0-20)	110 (0-790)	ns	0.0034	ns
	0.37 (±0.19)	0.77 (±0.26)	0.23 (±0.16)	1.29 (±0.30)			
Adult female	107 (10-460)	5179 (80-36370)	431 (0-2490)	20244 (760-78870)	ns	<0.0001	ns
	1.71 (±0.18)	3.01 (±0.25)	1.90 (±0.32)	4.00 (±0.20)			
Total worm burden	113 (10-470)	5244 (80-36460)	447 (0-2520)	20526 (790-79260)	ns	<0.0001	ns
	1.77 (±0.18)	3.05 (±0.25)	1.91 (±0.32)	4.00 (±0.20)			
Trichuris spp.							
Total worm burden	0	0.9 (0-7)	0	8.8 (0-22)	0.0122	0.0002	0.0071
	0 a	0.14 (±0.10) a	0 a	0.74 (±0.19) b			

* Means (± standard error) of log transformed values followed by different letter in the same line differ from each other by Tukey's test (P<0.05).
ns = not significant (P>0.05).

Table 4. Means of anti-*Trichostrongylus colubriformis* L3 IgG plasma levels (% OD) (minimum-maximum values) of the Santa Ines and Ile de France lambs under suppressive or targeted selective treatment (TST) programme with anthelmintics.

Data	Santa Ines		Ile de France		Effects (P-value)		
	Suppressive (n=9)	TST (n=10)	Suppressive (n=10)	TST (n=9)	Breed	Treatment programme	Breed x Treatment
Week 1	22.2 (0-50)	21.6 (3-58)	21.1 (6-39)	6.4 (0-23)	ns	ns	0.025
	1.11 (±0.30) a *	1.17 (±0.14) b	1.27 (±0.08) c	0.62 (±0.18) c			
Week 3	20.1 (0-44)	22.4 (2-65)	18.4 (7-43)	7.3 (1-19)	ns	ns	ns
	1.10 (±0.19)	1.21 (±0.13)	1.20 (±0.09)	0.82 (±0.10)			
Week 5	32.6 (3-82)	44.7 (8-90)	17.2 (8-36)	15.2 (7-25)	0.021	ns	ns
	1.35 (±0.15)	1.54 (±0.12)	1.20 (±0.08)	1.18 (±0.06)			
Week 7	24.6 (1-77)	34.2 (0-77)	7.8 (1-19)	9.4 (1-18)	0.045	ns	ns
	1.06 (±0.21)	1.36 (±0.17)	0.84 (±0.11)	0.96 (±0.09)			
Week 9	34.0 (6-86)	54.0 (19-87)	14.8 (6-34)	30.1 (15-53)	0.019	0.001	ns
	1.34 (±0.15)	1.70 (±0.07)	1.16 (±0.06)	1.47 (±0.04)			
Week 11	34.7 (2-108)	52.9 (17-91)	15.8 (6-29)	32.8 (11-70)	ns	0.002	ns
	1.32 (±0.17)	1.70 (±0.06)	1.18 (±0.07)	1.47 (±0.08)			
Week 13	27.2 (0-69)	53.2 (12-89)	12.7 (4-21)	42.8 (11-77)	ns	<0.001	ns
	1.16 (±0.22)	1.68 (±0.08)	1.08 (±0.08)	1.56 (±0.10)			
Week 15	34.3 (9-78)	62.5 (24-107)	28.0 (13-62)	59.4 (25-96)	ns	<0.001	ns
	1.44 (±0.11)	1.77 (±0.06)	1.42 (±0.06)	1.76 (±0.05)			
Week 17	30.7 (4-63)	50.3 (17-79)	37.0 (11-61)	47.6 (20-84)	ns	0.022	ns
	1.41 (±0.11)	1.67 (±0.07)	1.52 (±0.08)	1.65 (±0.06)			
Week 19	54.7 (20-103)	43.10 (15-62)	47.4 (15-67)	52.8 (22-85)	ns	ns	ns
	1.66 (±0.10)	1.60 (±0.07)	1.65 (±0.06)	1.69 (±0.06)			
Week 21	38.3 (17-75)	48.2 (17-71)	39.8 (16-69)	44.1 (21-69)	ns	ns	ns
	1.54 (±0.07)	1.66 (±0.06)	1.57 (±0.06)	1.63 (±0.05)			

* Means (± standard error) of log transformed values followed by different letter in the same line differ from each other by Tukey's test (P<0.05).
ns = not significant (P>0.05).

Table 5. Means (minimum-maximum values) of eosinophils, mast cells and globule leukocytes (cells/mm²) in the intestinal mucosa of the Santa Ines and Ile de France under suppressive or targeted selective treatment (TST) programme with anthelmintic.

Organ / inflammatory cells	Santa Ines		Ile de France		Effects (P-value)		
	Suppressive (n=9)	TST (n=10)	Suppressive (n=10)	TST (n=9)	Breed	Treatment programme	Breed x Treatment
Mast cells	172 (81-317) ab*	133 (20-242) ab	58 (10-102) b	155 (82-225) a	0.0264	0.0420	0.0006
Globule leukocyte	70 (8-137)	94 (10-220)	36 (10-133)	260 (10-730)	ns	0.0122	ns
Eosinophils	209 (60-388)	178 (55-470)	149 (55-293)	146 (37-381)	ns	ns	ns

*Means followed by different letter in the same line differ from each other by Tukey's test (P<0.05).
ns = not significant (P>0.05).

CAPÍTULO 5 – Influence of targeted selective treatment on productive performance of Ile de France and Santa Ines lambs naturally infected with gastrointestinal nematodes.

Nas páginas à seguir encontra-se o manuscrito que será submetido à publicação na revista Small Ruminant Research no formato “Original Research Paper”, que será finalizado e submetido.

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Influence of targeted selective treatment on productive performance of Ile de France and Santa Ines lambs naturally infected with gastrointestinal nematodes.

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Abstract

Gastrointestinal nematodes (GIN) infections are among the main sanitary problems that affect sheep farming due to substantial economic losses. The targeted selective treatment (TST) arises as an alternative approach for worm control to reduce the anthelmintic use. This research aimed to assess and compare productive performance of Santa Ines (SI) and Ile de France (IF) lambs naturally infected with GIN under TST based on packed cell volume (PCV) or suppressive anthelmintic treatments. Thirty eight lambs were allocated in four groups: suppressive treatment with monepantel every two weeks; TST when animals presented PCV≤20%, treated with the same anthelmintic. Animals were weighed weekly and faecal and blood samples were collected to determine eggs per gram of faeces (EPG), PCV and total plasma protein (TPP). After animals had been slaughtered, carcasses were weighted to perform carcass yield. The favourable environmental climate allowed the survivor and development of infective larvae on pasture. *Haemonchus contortus, Trichostrongylus colubriformis*, *Cooperia curticei*, *Strongyloides papillosus*, *Trichuris discolor* and *Trichuris ovis* were identified parasitizing the lambs, of which *H. contortus* was the major parasite, mainly in IF lambs. Animals under TST presented marked variation in haematological and parasitological variables at some experimental points, mainly in the TST-IF group. The lowest haematological means were registered to TST-IF on 25th January with 19.7% to PCV and on 23rd November with 5.4 g/dL to TPP. Substantial productive losses were observed in TST groups in comparison to their suppressive treated counterparts with reduction in body weight gain about 21.2% in the IF and 25.9% in the SI lambs. The targeted selective treatment prevents mortality, but it did not prevent productive losses, both breeds presented sharp decrease in productivity due to GIN parasitism.

Introduction

Gastrointestinal nematodes (GIN) infections are among the main sanitary problems that affect sheep farming (Chagas et al., 2011; Valcárcel et al., 2015). The economic losses caused by GIN occur due to several factors, among them: decrease in weight gain due to lower food intake and reduction in feed conversion efficiency, milk and wool productivity decrease, lower carcass quality, higher costs with veterinary products and workforce, and increase in animal death rate (Cardia et al., 2011; Brito et al., 2013; Amarante, 2014).

Commercial breeds were introduced in Brazil due to high demand for meat production. Ile de France, Texel, Suffolk and Dorper breeds have high potential of body weight gain and produce carcasses with good quality (Amarante, 2014). However, they are more susceptible to GIN infection than local breeds, which impact negatively on the productive performance of these animals (Amarante et al., 2004).

Haemonchus contortus and *Trichostrongylus colubriformis* are the most important parasites in small ruminant production in tropical regions, responsible for huge productive and economic losses (Amarante et al., 2014). *Haemonchus contortus* as blood-feeders, in heavy infection may cause progressive anaemia with continuous iron and proteins losses, inappetence and traumatic damage in the abomasal mucosa, leading to decrease in enzymatic activities and digestion disturbance (Qamar and Maqbool, 2012; Besier et al., 2016; Hoste et al., 2016). In chronic infections, *T. colubriformis* feed behaviour has negative effects, due to lesions in the small intestine mucosa, such as villous atrophy, erosion in the duodenal epithelium and mucosa thickening, resulting in decrease in nutrient digestion and absorption, besides short retention time of digesta (Cardia et al., 2011; Silva et al., 2019).

Worm control is still dependent of anthelmintic treatments, which are often used indiscriminately (Bricarello, 2015). The high frequency in anthelmintics use increases the presence of chemical residues in carcasses and in environment,

and also increase the risk of development of anthelmintic-resistant nematode populations (Brito et al., 2013; Amarante, 2014; Valcárcel et al., 2015).

There are alternative measures of GIN control, including grazing management with different species of herbivorous, selection of resistant animals, crossbreeding between resistant and commercial breeds, improvement of nutrition and targeted selective treatments (Amarante et al., 2009; Amarante, 2015; Medina-Pérez et al., 2015).

The targeted selective treatment (TST) arises as an alternative approach for worm control to reduce the anthelmintic use and decrease nematodes resistant populations (Bevilaqua et al., 2015). Several indicators could be employed in order to identify the animals in need of anthelmintic treatment, such as the evaluation of anaemia from ocular conjunctiva examination (FAMACHA©) or packed cell volume (PCV) measure; body condition; diarrhoea indicators; and count of nematode's eggs per gram of faeces (EPG) (VanWyk et al., 1997; Amarante, 2014; Medina-Pérez et al., 2015; Valcárcel et al., 2015). FAMACHA© is the TST method most used in small ruminants farms in Brazil (Maia et al., 2013). Although this method has been introduced in Brazil for more than 10 years, it has not been proved as an useful tool in preventing the development of anthelmintic resistance. Furthermore, TST did not allow any significant change in the scenario of sheep production in the country (Amarante, 2014).

The lack of anthelmintics with high efficacy against GIN was the major difficulty encountered to maintain uninfected control groups as worm-free in experiments carried out in the field. The recent launch of monepantel in Brazil gave us an opportunity to evaluate the losses caused by GIN in sheep using lambs under suppressive treatment with monepantel as a non-infected control group. It has been demonstrated that persistent challenge with *H. contortus* and *T. colubriformis* infective larvae does not affect the growth of grazing meat-breed lambs when suppressively treated with effective anthelmintics, indicating that the use of suppressively treated sheep could be a valid substitute for worm-free lambs in field experiments (Dever et al., 2015). Therefore, this research aimed to assess and compare productive performance of Ile de France and Santa Ines

lambs naturally infected with GIN under suppressive or TST anthelmintic treatments programs.

Material and methods

Ethical considerations

The experiment was carried out at São Paulo State University (UNESP), Botucatu-SP, Brazil. All animal procedures were in accordance with the ethical standards and were approved by the Animal Use Ethics Committee of the FMVZ/UNESP (47/2016).

Experimental design

The experimental design was described previously (Albuquerque et al., 2017). Briefly, 19 Ile de France and 19 Santa Ines, 3-month-old uncastrated male lambs were purchased from commercial farms, where they had been raised indoors. The animals of each breed were allocated in two anthelmintic treatment groups (suppressive or TST) by stratified randomization balanced as far as possible, taking into consideration body weight and nematode faecal egg counts (FEC).

Suppressive groups (SUP-IF and SUP-SI) were treated with the following combination of anthelmintics seven days before being allocated to the paddocks: albendazole (10 mg/kg, Valbazen® 10 Cobalto Zoetis), levamisole (9.4 mg/kg, Ripercol® L 150 F, Zoetis), and monepantel (2.5 mg/kg, Zolvix®, Novartis) with no nematode eggs detected in a sequence of faecal examinations. After the beginning of the experiment, all lambs under suppressive treatment were drenched each two weeks with monepantel. In addition, lambs received moxidectin (0.4 mg/kg, Cydectin® Zoetis) on 16th November, because some of them were shedding *Strongyloides* spp. eggs (mean of 42 and range of 0–200 EPG). On 4th January, 2017 lambs also received a combination treatment of monepantel, levamisole (9.4 mg/kg), and albendazole (10 mg/kg), because they were shedding strongyle eggs (379, 0–1000 EPG) and *Strongyloides* spp. (874, 0–5300 EPG). The suppressive treatment was carried out in order to keep the grazing lambs and their pastures

as free of worms as possible and prevent decrease in animal's productive performance due to parasitism. The last monepantel drench for lambs under suppressive treatment was administered on 8th February and they were slaughtered on 22nd February.

The lambs in the TST group (TST-SI and TST-IF) were not treated on arrival at the experimental facilities. Instead, they were introduced into the clean pasture harbouring natural parasites infections acquired at their origin farm. These lambs underwent monepantel treatment when they showed a packed cell volume (PCV) ≤20% corresponding to category 3 of FAMACHA© method (VanWyk et al., 1997), in order to prevent the occurrence of mortality and / or severe haemonchosis.

All lambs received toltrazuril (14 mg/kg, Farmacox®, Farmabase Animal Health) to prevent coccidiosis and a clostridiosis vaccine (Poli-Star®, Vallée), with a booster dose administered 2 months later.

Food and sanitary management

An area of pasture with 4,092.6 m^2 was divided into four paddocks of similar sizes. Two interleaved paddocks were designated to suppressive treatment (paddock 2 and 4) and another two to TST (paddock 1 and 3). On 7th October, 2016, the lambs were allocated and remained according to anthelmintic treatment groups in separate paddocks in a clean pasture of *Cynodon* spp. and *Urochloa decumbens*, and the paddock rotation was performed according to pasture visual availability. The gastrointestinal nematodes in the experimental area were introduced by the naturally infected experimental lambs. In the paddocks, the lambs had free access to tap water and mineral salt (Presencefós, Presence Animal Nutrition) and received a daily dietary supplement with a commercial concentrate containing 16% of crude protein (Supplementa Ovinos, Presence Animal Nutrition).

The amount of concentrate offered to each animal was adjusted according to its body weight (BW). The concentrate was offered in an amount corresponding to 2.5% of the animal's BW to lambs with less than 20 kg; 2.0% to lambs with 20-

25 kg BW; and 1.5% to lambs with more than 25 kg of BW. All animals received the same proportion of concentrate (1.5%) starting on 11st January, when all lambs were presenting more than 25 kg BW.

Production performance

In order to evaluate lambs productive performance, animals were weekly weighted to calculate daily weight gain means, and the hot carcass calculation yield was made post animals slaughtered.

Faecal examination

Faecal samples were collected weekly directly from the rectum of animals in polyethylene bags previously identified and kept refrigerated until processing time. The modified McMaster technique (Ueno and Gonçalves, 1998) was used to perform the faecal egg counting (FEC), in which each nematode egg counted represented 100 eggs per gram of faeces (EPG). Every week faecal cultures were prepared separately for each group for the production of third stage larvae (L3) of gastrointestinal nematodes, L3 obtained were identified and counted as percentage according to the descriptions by Ueno and Gonçalves (1998).

Haematology

Blood samples (5 mL) were collected weekly by jugular vein puncture into Vacutainer® tubes containing anticoagulant (EDTA). The packed cell volume (PCV) was determined by microhematocrit centrifugation at 12,000 RPM for 5 minutes, and the total plasma protein concentration (TPP) was estimated using a hand-hold refractometer (Refractometer SPR-N, Atago) (Weiser, 2012; Weiss; Wardrop, 2010).

Pasture infectivity and climate data

The extent of pasture contamination with infective nematode larvae was estimated every two weeks. Herbage was collected always in the same W track in the paddock (Taylor, 1939) by grasping it in small handfuls and cutting as close as possible to ground level. Herbage was processed and larvae were

recovered as described by (Niezen et al., 1998). The data were expressed in number of larvae per kilogram of dry material (L_3/kg DM).

Temperature means were measured at meteorological station of Faculdade de Ciências Agronômicas – UNESP located at Fazenda Lageado, Botucatu, Brasil, with 8 Km from the experimental paddock and precipitation was measured by fixed pluviometer in experimental area. The results were expressed in mean weekly of temperature (°C) and precipitation (mm).

Statistical analyses

All data were submitted to normality test and transformed using $\log_{10}$ (x+1) when it was necessary. Data with single measures and with repeated measures at several time points were analysed by ANOVA using the General Linear Model (GLM) and groups means were compared by Tukey's test using Statistical Analysis System, version 9.2 (SAS Institute, Inc., Cary, NC, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Suppressive and targeted selective treatment with anthelmintic

The details of suppressive and TST anthelmintic treatments were previously published by Albuquerque et al. (2017). Briefly, all lambs under suppressive treatment were drenched at nine time-points. These treatments presented high efficacy in Ile de France and Santa Ines lambs until 23rd November and 21st December, respectively. Then, the animals, particularly the Ile de France, showed increase in the infection levels due to emergence of *Haemonchus contortus* resistance.

The number of anthelmintic treatments in TST groups differed between breeds. All Ile de France lambs needed anthelmintic treatments during the trial, ranging from one to four drenches per animal, with the largest amount of drenches administered on 25th January. The total number of Ile de France lambs treated in each month was the following: 1 in October, 4 in November, 7 in December and 6 in January. In contrast, only two Santa Ines lambs needed treatment, due

to low PCV values (two treatments in December, one in January and one in February), although they were presenting lower EPG counts in relation to Ile de France treated. These drenches had relevant impact on parasitological and haematological variables in TST groups, mainly in Ile de France means which will be presented in the following topics.

Parasitology

The EPG and faecal culture results were previously described (Albuquerque et al., 2017). Briefly, the strongyles EPG means showed relevant changes over time, mainly to Ile de France lambs with higher EPG than Santa Ines over the experimental period. Animals subjected to suppressive treatment showed gradual increase in EPG values presenting similar means to the TST group at the end of trial due to anthelmintic resistance.

In faecal cultures of TST-SI and TST-IF, *Haemonchus* larvae were predominant with means of 50.9% and 86.8%, respectively. The TST-SI presented higher proportions of *Trichostrongylus* than the TST-IF in all samplings, of which TST-SI showed *Trichostrongylus* percentages higher than those of the *Haemonchus* in nine of the 21 time-points. *Cooperia* was also detected with maximum percentages of 11% and 14%, respectively, in the TST-IF and TST-SI groups. *Haemonchus* and *Cooperia* L3 were observed in faecal culture of suppressive groups since 16th November, and only *Haemonchus* L3 was found after 28th December.

Animals were parasitized by six species of gastrointestinal nematodes: *Haemonchus contortus* in the abomasum, *Trichostrongylus colubriformis*, *Cooperia curticei*, *Strongyloides papillosus* in the small intestine, and *Trichuris discolor* and *Trichuris ovis* in the large intestine. At the beginning of the trial lambs also presented *Eimeria* spp., that was treated with toltrazuril, and *Moniezia* spp. and *Oestrus ovis* over the trial.

Pasture contamination and climate data

In paddocks 2 and 4, grazed by groups under suppressive treatment, *Haemonchus* L3 appeared only in the last samplings with the highest numbers

recorded on paddock 4 in late January and February (respectively, 142 and 141 L3/kg DM). A few *Trichostrongylus* L3 were also detected in those paddocks (Figure 1a).

On 20th September, before the grazing, no L3 were detected on herbage. The pasture of paddocks 1 and 3 became contaminated due to the grazing by lambs under TST that were shedding eggs in faeces. In these paddocks, *Haemonchus* and *Trichostrongylus* L3 were found early in the trial, since 21st October. There was higher amount of *Haemonchus* spp. larvae than *Trichostrongylus* spp. in all samplings. The highest larvae recovery from paddock 1 occurred on 21st October and 4th November. Then, there was progressive decrease in number of infective larvae, followed by a small increase in paddock 3rd on 13th January. On 18th November and 13th January, paddocks 3 and 1, respectively, did not present larvae (Figure 1b).

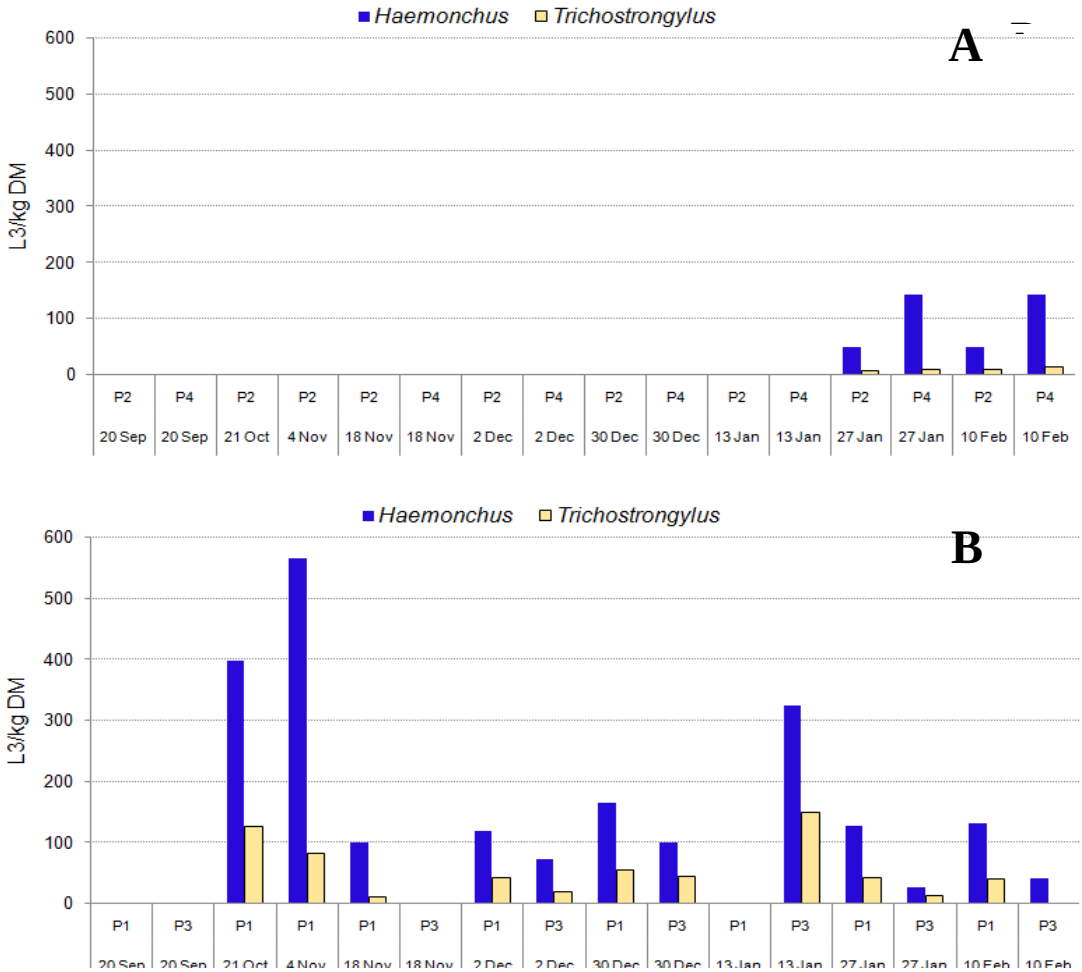

Figure 1. Number of infective larvae by kilogram of dry matter (L3/kg DM) recovered from the herbage of (A) paddocks (P) 2 and 4 grazed by Santa Ines and Ile de France lambs under suppressive treatment and (B) P1 and P3 grazed by lambs under targeted selective treatment.

The beginning of the trial (October, 2016) coincided with transition between the dry and the rainy season in our region. It presented a total of 69.8 mm of precipitation. January, 2017 was the month with the highest precipitation, with 21 days of rainfall resulting in a total of 428.5 mm. In the entire trial, the total precipitation was 868.8 mm. Temperatures means ranged from 21.6 °C in October, 2016 to 24.5 °C in February, 2017 (Figure 2).

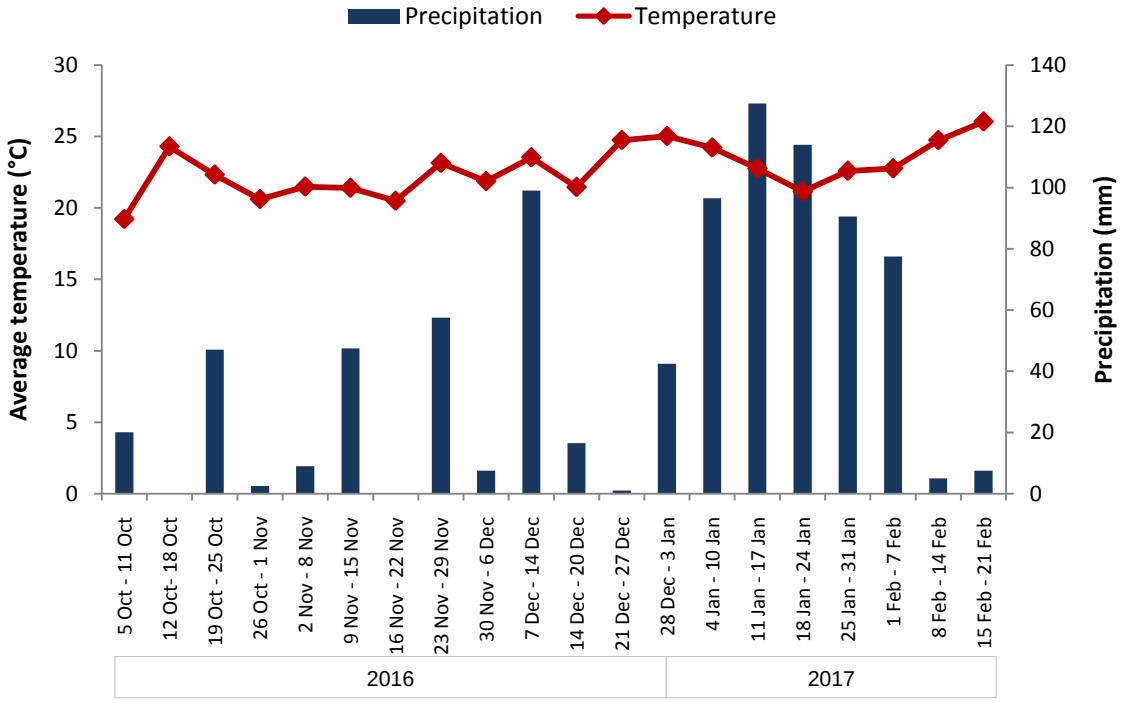

Figure 2. Total weekly precipitation (mm) and mean weekly temperature (°C) from 5th October, 2016 to 21st February, 2017, Botucatu, São Paulo state, Brazil.

Haematology

Packed cell volume (PCV)

The PCV means showed expressive changes over time, resulting in significant time x breed x treatment interaction ($P < 0.01$). At the beginning of experiment all groups showed PCV means above 34%. Then, there was a gradual decrease in all means until 23rd November. Afterwards, SI lambs retained stable values ranging between 25.9% and 31%. In the TST-IF, the progressive decrease in the PCV values continued to occur reaching the lowest mean (19.7%) on 25th January. The lowest value (13%) was recorded in an Ile de France lamb on 25th January. There was breed and treatment interaction ($P < 0.05$) at 11 time-points (Table 1), in which TST-IF showed lower PCV means in comparison with the other groups. At the end of the experiment, the differences between IF under suppressive and TST was minimal because the suppressive group was also exposed to *H. contortus* infection, which caused a decrease in their PCV values, with mean of 24.9% in the last sampling (Figure 3).

Table 1. Packed cell volume (%) means (±standard error) of the Santa Ines and Ile de France lambs under suppressive or targeted selective treatment (TST) with anthelmintics.

Data	Santa Ines		Ile de France		Effects (P-value)		
	Suppressive (n=9)	TST (n=10)	Suppressive (n=10)	TST (n=9)	Breed	Treatment programme	Breed x Treatment
5 Oct	37.2 (±1.7)	37.5 (± 2.1)	37.5 (±0.8)	36.0 (±0.9)	ns	ns	ns
12 Oct	35.1 (±1.7)	36.7 (± 1.8)	36.6 (±1.0)	34.6 (±1.7)* *	ns	ns	ns
19 Oct*	33.7 (±1.6)	35.7 (± 1.6)	35.8 (±1.0)	32.9 (±0.7)	ns	ns	ns
26 Oct	33.3 (±1.0)	35.2 (± 1.4)	35.6 (±1.2)	34.2 (±0.8)	ns	ns	ns
2 Nov*	31.4 (±0.8)	32.7 (± 1.2)	33.6 (±0.9)	32.2 (±0.5)	ns	ns	ns
9 Nov	30.2 (±1.1)	30.3 (± 1.1)	32.5 (±1.0)	27.8 (±1.7)*	ns	ns	ns
16 Nov*	28.4 (±0.7) a	28.1 (± 0.8) a	32.1 (±0.6) b	25.6 (±1.1) a**	ns	0.0002	0.0006
23 Nov	26.9 (±0.5) a	28.2 (± 0.8) ab	30.6 (±0.6) b	26.0 (±0.7) a	ns	0.0172	<0.0001
30 Nov*	28.9 (±0.9) ac	30.4 (± 0.8) bc	32.2 (±0.6) b	26.7 (±0.8) a**	ns	0.0185	0.0001
7 Dec	29.9 (±0.7) ab	28.4 (± 1.9) ab**	32.6 (±0.5) a	25.8 (±1.2) b**	ns	0.0024	0.0424
14 Dec*	28.6 (±1.6) ab	28.6 (± 1.4) ab	32.0 (±0.6) a	26.4 (±0.6) b	ns	0.0198	0.0180
21 Dec	28.4 (±1.3) ab	30.1 (± 1.2) ab	32.4 (±0.6) a	25.4 (±2.2) b**	ns	ns	0.0038
28 Dec*	28.7 (±0.5) a	29.2 (± 1.3) a	30.2 (±1.2) a	22.8 (±1.6) b**	ns	0.0086	0.0028
4 Jan*	30.3 (±0.8) a	29.9 (± 0.8) a	32.7 (±0.5) a	26.2 (±1.2) b	ns	0.0003	0.0012
11 Jan	30.7 (±0.9) a	30.3 (± 0.9) a	30.7 (±0.6) a	25.4 (±1.5) b**	0.0334	0.0095	0.0224
18 Jan*	30.4 (±0.7)	29.7 (± 1.0)	30.6 (±1.1)	26.8 (±1.2)	ns	ns	ns
25 Jan	30.8 (±0.7) a	29.3 (± 1.9) a**	29.5 (±1.4) a	19.7 (±2.2) b**	0.0038	0.0017	0.0170
1 Feb	30.1 (±0.6) a	28.9 (± 1.7) a	29.9 (±0.8) a**	21.9 (±1.6) b**	0.0138	0.0012	0.0131
8 Feb*	27.3 (±1.9)	29.5 (± 1.6)	29.1 (±0.7)	25.8 (±1.6)	ns	ns	ns
15 Feb	25.9 (±1.3)	29.5 (± 1.5.9)	24.9 (±1.1)	24.4 (±1.6)	0.0327	ns	ns
21 Feb	27.1 (±1.1)	29.4 (± 1.0)	24.9 (±1.2)	23.4 (±2.0)	0.0049	ns	ns

Means followed by different letter in the same line differ from each other by Tukey's test ($P < 0.05$).

ns = not significant ($P < 0.05$).

*Anthelmintic treatment in the suppressive groups.

**Anthelmintic treatment in the TST groups.

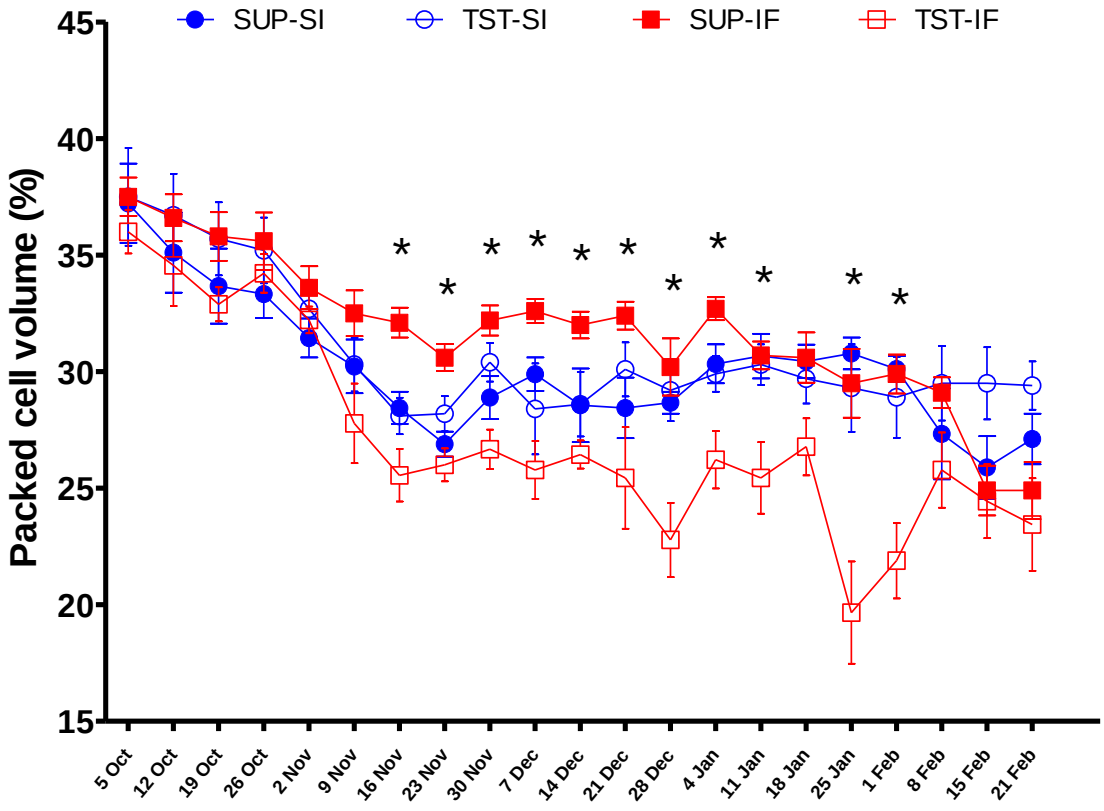

Figure 3. Packed cell volume (%) means of the Santa Ines (SI) and Ile de France (IF) lambs under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Bars are standard error. (* P<0.05 interaction between treatment x breed).

Total plasma protein (TPP)

The TPP means showed changes over time (P < 0.05), with all groups means decreasing from the beginning of experiment until 23rd November. Subsequently, the means increased and stabilized, whereby Santa Ines lambs showed higher means than Ile de France in all time-points (P < 0.05) (Figure 4). There was significant treatment differences (P < 0.05), lambs under suppressive treatment presented higher TTP means (Table 2), then their TST counterparts on 16th November, 23rd November, 7th December and 21st December.

Table 2. Total plasma protein (g/dL) means (±standard error) of the Santa Ines and Ile de France lambs under suppressive or targeted selective treatment (TST) programme with anthelmintics.

Data	Santa Ines		Ile de France		Effects (P-value)		
	Suppressive (n=9)	TST (n=10)	Suppressive (n=10)	TST (n=9)	Breed	Treatment programme	Breed x Treatment
5 Oct	6.9 (± 0.20)	7.0 (± 0.19)	6.3 (± 0.14)	6.7 (± 0.12)	0.0088	ns	ns
12 Oct	6.4 (± 0.14)	6.6 (± 0.19)	5.8 (± 0.15)	5.9 (± 0.11)	0.0002	ns	ns
19 Oct	6.7 (± 0.15)	6.9 (± 0.24)	6.0 (± 0.13)	6.4 (± 0.10)	0.0011	ns	ns
26 Oct	6.7 (± 0.14)	6.7 (± 0.27)	6.1 (± 0.13)	6.8 (± 0.22)	ns	ns	ns
2 Nov	6.6 (± 0.13)	6.4 (± 0.15)	6.1 (± 0.12)	6.4 (± 0.35)	ns	ns	ns
9 Nov	6.4 (± 0.14)	6.2 (± 0.16)	6.0 (± 0.10)	5.7 (± 0.26)	0.0260	ns	ns
16 Nov	6.5 (± 0.19)	6.1 (± 0.20)	6.1 (± 0.10)	5.6 (± 0.10)	0.0060	0.0057	ns
23 Nov	6.3 (± 0.12)	6.0 (± 0.15)	5.7 (± 0.10)	5.4 (± 0.11)	<0.0001	0.0331	ns
30 Nov	7.2 (± 0.14)	7.1 (± 0.18)	6.6 (± 0.11)	6.1 (± 0.19)	<0.0001	ns	ns
7 Dec	7.3 (± 0.20)	6.7 (± 0.11)	6.7 (± 0.07)	6.1 (± 0.20)	0.0007	0.0001	ns
14 Dec	6.8 (± 0.18)	7.1 (± 0.24)	6.2 (± 0.07)	6.2 (± 0.15)	<0.0001	ns	ns
21 Dec	7.4 (± 0.17) a*	7.0 (± 0.15) ab	6.7 (± 0.08) b	5.8 (± 0.14) c	<0.0001	<0.0001	0.0450
28 Dec	7.0 (± 0.16)	6.9 (± 0.11)	6.3 (± 0.07)	5.8 (± 0.28)	<0.0001	ns	ns
4 Jan	7.1 (± 0.13)	7.4 (± 0.18)	6.8 (± 0.11)	6.8 (± 0.19)	0.0050	ns	ns
11 Jan	7.0 (± 0.10)	6.9 (± 0.19)	6.3 (± 0.10)	6.4 (± 0.12)	0.0001	ns	ns
18 Jan	7.0 (± 0.08)	7.0 (± 0.14)	6.6 (± 0.08)	6.8 (± 0.23)	0.0272	ns	ns
25 Jan	7.0 (± 0.08)	6.8 (± 0.19)	6.8 (± 0.17)	6.5 (± 0.30)	ns	ns	ns
1 Feb	7.0 (± 0.09)	6.8 (± 0.15)	6.8 (± 0.18)	6.4 (± 0.25)	ns	ns	ns
8 Feb	7.2 (± 0.11)	7.0 (± 0.19)	6.5 (± 0.17)	6.7 (± 0.40)	0.0385	ns	ns
15 Feb	7.0 (± 0.57)	7.1 (± 0.19)	6.4 (± 0.24)	6.9 (± 0.33)	ns	ns	ns
21 Feb	7.2 (± 0.26)	6.8 (± 0.09)	6.5 (± 0.15)	6.5 (± 0.37)	ns	ns	ns

*Means followed by different letter in the same line differ from each other by Tukey's test ($P<0.05$).
ns = not significant ($P < 0.05$).

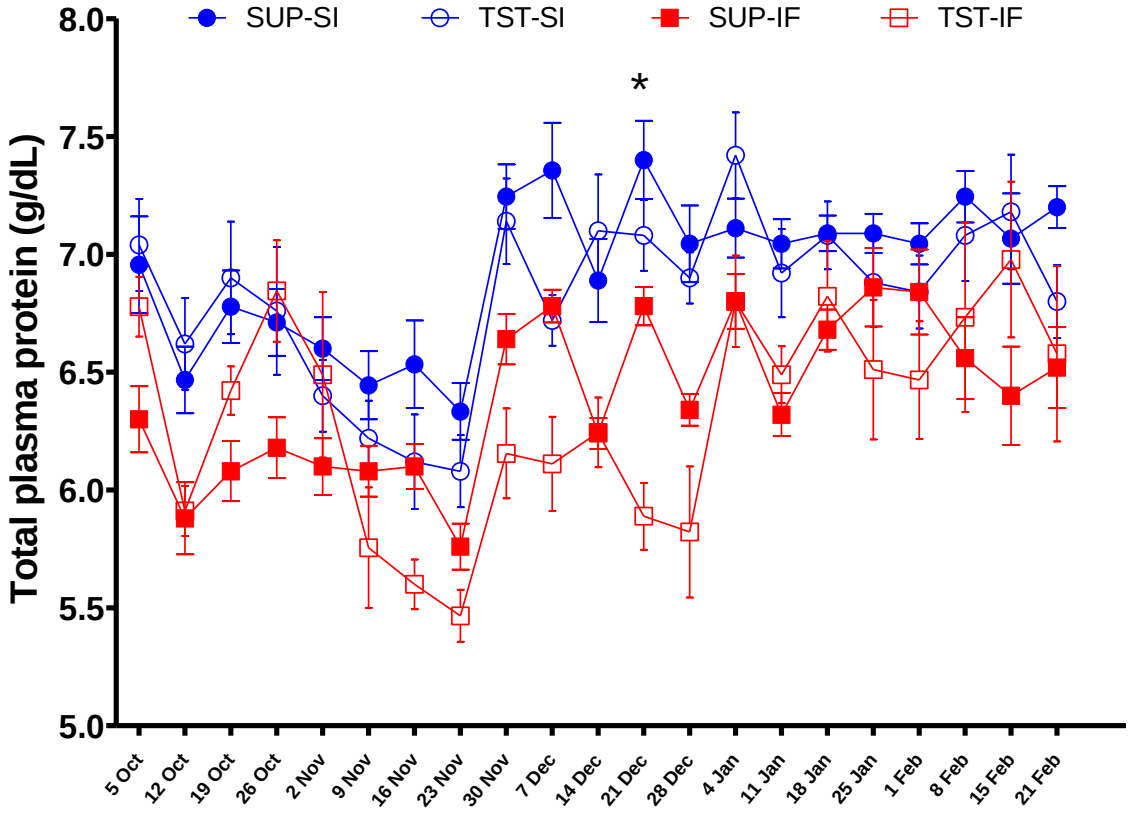

Figure 4. Total plasma protein (g/dL) means of the Santa Ines (SI) and the Ile de France (IF) lambs under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Bars are standard error. (* P<0.05 interaction between treatment x breed).

Productive performance

Body weight means increased progressively during the trial with significant time x treatment (P<0.0001) and time x breed (P<0.0009) interactions. All groups showed similar body weight means until 16th November, then, the TST groups of both breeds presented a lower body weight. There was treatment effect on body weight means in three time-points, all at the end of trial (P<0.05), whereby lambs under suppressive treatment showed the highest body weight means. The same occurred with breed effect (P<0.05), with significant difference between breeds in the last six weeks, when Ile de France lambs presented the highest body weight means (Figure 5). The lambs of the suppressive treatment showed greater final body weight gain (P≤ 0.05) compared to TST lambs. In each breed, overall the differences of body weight gain between suppressive

and TST treatments were 5.0 kg (21.6%) for the Ile de France and 4.9 kg (25.9%) for the Santa Ines lambs.

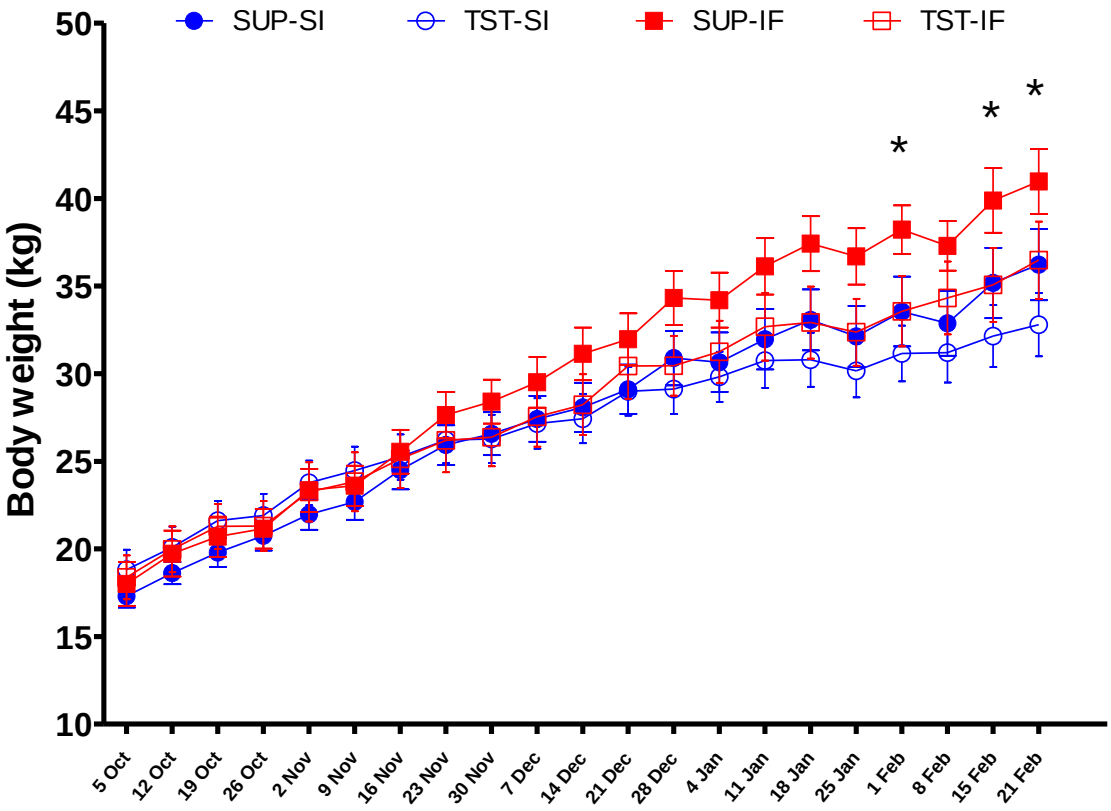

Figure 5. Body weight (kg) means of the Santa Ines (SI) and Ile de France (IF) lambs under suppressive (SUP) or targeted selective treatment (TST) with anthelmintics. Bars are standard error. (* P<0.05 between treatments).

There was significant breed and treatment programme effect on daily weight gain (DWG): suppressive treatments and Ile de France breed had the highest DWG (Table 3). The DWG means were 136 g (±0.01) to SUP-SI, 101 g (±0.01) to TST-SI, 165 g (±0.01) to SUP-IF and 130 g (±0.01) to TST-IF. The DWG difference between suppressive and TST groups in each breed were 35 g which correspond to a difference of 21.2% for the Ile de France breed and 25.7% for the Santa Ines breed.

There was significant difference between treatment programmes in carcass weight means: animals under suppressive treatment presented the highest means (Table 3). The hot carcass yield means was influenced by breed and

treatment programme (P<0.05). Overall, suppressive treatments lead to higher means than TST and interestingly, Santa Ines lambs showed the highest hot carcass yield means (Table 3).

Table 3. Means of initial body weight, final body weight, body weight gain, daily weight gain, carcass weight (kg) and hot carcass yield (%) (±standard error) of the Santa Ines and Ile de France under suppressive or targeted selective treatment (TST) programme with anthelmintic.

Productive performance	Santa Ines		Ile de France		Effects P-value	
	Suppressive (n=9)	TST (n=10)	Suppressive (n=10)	TST (n=9)	Breed	Treatment programme
Initial body weight	17.3 (±0.65)	18.8 (±1.15)	18.0 (±1.25)	18.4 (±1.26)	ns	ns
Final body weight	36.2 (±2.04)	32.8 (±1.82)	41.0 (±1.86)	36.5 (±2.21)	0.0301	0.0450
Body weigh gain	18.9 (±1.75)	14.0 (±1.51)	23.0 (±1.01)	18.1 (±1.68)	0.0064	0.0025
Daily weight gain	0.136 (±0.01)	0.101 (±0.01)	0.165 (±0.01)	0.130 (±0.01)	0.0064	0.0025
Carcass weight	16.0 (±0.98)	14.3 (±0.90)	17.7 (±1.00)	14.7 (±1.08)	ns	0.0256
Hot carcass yield	45.7 (±0.88)	44.3 (±0.50)	45.0 (±0.75)	41.9 (±0.70)	0.0498	0.0032

There was no significant breed x treatment programme interaction (P>0.05).
ns = not significant.

Discussion

Suppressive anthelmintic treatment was used in both breeds in order to keep animals as free as possible of GIN infections, trying to simulate non-infected control groups. Our approach was successful in most part of the trial, excepting the last month when *H. contortus* become resistant to monepantel treatment, as previously described (Albuquerque et al., 2017). For this reason, animals under suppressive treatment presented increase in FECs (faecal eggs count) and, at the end of the trial, presented high *H. contortus* burden compared to other groups (Albuquerque et al., 2019).

The TST emerged as sustainable parasite management to delay resistance, extending drug life (van Wyk, 2008) and reducing anthelmintic drenches as economic benefit (Molento et al., 2009). In other studies there was no significant difference in productive performance of sheep under TST using FAMACHA© compared to animals under other treatments regimens (Depner et al., 2007; Leask et al., 2013; Fernandes et al., 2019). Considering in our study all groups were subjected to similar nutritional conditions over the trial, animals submitted to TST presented marked productive losses, with reduction in body weight gain of 25.9% e 21.6% in comparison with their suppressive treated SI and IF counterparts, respectively. These losses of the animals under TST may be due to continuous infection challenge during all the trial. These results are in agreement with reports that show gastrointestinal infections being responsible for 15-23% of productive losses, affecting more the capacity to produce meat than wool and milk (Mavrot et al., 2015). By the way, GIN could cause negative consequences on metabolism with reallocation of proteins for immune response, decrease of retention time of the digesta, as consequence they cause lower efficiency in utilization of absorbed nutrients and lower nutrient availability. Moreover, the GIN are responsible for reduction in feed intake impacting on animal productive performance (Coop and Kyriazakis, 1999; Cardia et al., 2011; Mavrot et al., 2015; Silva et al., 2019).

Even though SI and IF lambs under TST were submitted to the same parasitic challenge, SI lambs presented the ability to maintain low FEC and low worm burden. Additionally, Santa Ines breed is immunologically more resistant than other breeds in different age categories (Rocha et al., 2004; Amarante et al., 2005; Rocha et al., 2005). Therefore, it is possible that the development of the immune response against GIN demanded high cost with reallocation of nutrients, resulting in lower productive performance in the Santa Ines. The nutrients reallocated to the maintenance of host vital functions and immune response during the parasitism infection may have contributed to productive losses (Coop and Kyriazakis, 1999; Vagenas et al., 2007; Cardia et al., 2011; Rocha et al., 2011). In addition, TST lambs also presented high *T. colubriformis* and *S. papillosus* burden (results shown in the previous paper), and *T. colubriformis* was proven to be responsible for substantial production losses in Santa Ines lambs, with 37% reduction in daily weight gain and inferior food conversion rate compared to uninfected lambs (Cardia et al., 2011). Despite *S. papillosus* being moderate prevalent in sheep farming (Dimitrijević et al., 2012), there is no research demonstrating the negative impact of this parasite in productive performance. However, the tissue damages caused by *S. papillosus* in the small intestine is possibly similar to those caused by *T. colubriformis* (Amarante, 2015), those pathogenic effects combined contribute to modification in tissue morphology and function. The losses caused by intestinal parasites may occur even in subclinical infections (Cardia et al., 2011), which was the case of the present trial. In general we did not observe scour, the most important clinical sign of enteritis caused by the intestinal parasites, *S. papillosus* and *T. colubriformis* in sheep (Cardia et al., 2011; Dimitrijevic et al., 2012).

The Ile de France is an European breed that present high growth rate with production with of excellent quality carcass (Moreno et al., 2010). This breed is more susceptible to GIN prevalent in Brazil (Amarante et al., 2004), requiring more frequent anthelmintic drenches due to haemonchosis (Albuquerque et al., 2017). The differences observed in FEC, PCV and TPP means between breeds under TST groups were evident and could be much more pronounced if the

animals did not receive anthelmintic treatments. In this case, we would observe a high mortality rate among these animals due to acute haemonchosis, especially in IF breed.

The IF lambs under TST were the most parasitized animals, consequently, these animals present severe anaemia and hypoproteinaemia at some points of the experiment as a result of the pathophysiological effect of GIN infection (Cardia et al., 2011; Besier et al., 2016). Even though, IF lambs showed higher productive performance than SI under the same treatment.

Both breeds under TST showed significantly lower DWG compared to suppressive groups, averaging 130 g/day and 101 g/day to IF and SI, respectively. Animals of the same breeds and age, kept indoors, free of parasitic infections, and receiving a similar diet, presented DWG of 256 g/day (IF lambs) and 223 g/day (SI lambs) (Bricarello et al., 2005). Similar to our finding, Santa Ines lambs infected by *T. colubriformis* presented DWG of 107.26 g (Cardia et al., 2011). Additionally, Dorper lambs under different nutritional regimens (basal diet – 8.1% of crude protein; supplemented diet - 18% of crude protein), showed negative impact of GIN infection (*H. contortus*, *T. colubriformis*, *C. curticei* and *S. papillosus*) on daily weight gain compared to their counterparts (control group drenched every two weeks with monepantel) of 17% to animals under supplemented diet, and this loss was more marked to animals under basal diet (26.7%) (Starling et al., 2019). This last value was similar to that recorded in the SI breed in our study (25.7%). If animals under suppressive treatment had not been infected at the end of the trial, the difference between treatment groups could have been more marked.

In the present study, IF lambs produced heavier carcasses, as expected, but conversely SI presented higher carcass yield in both treatment. Lambs of specialized breeds have higher amounts of digestive tract content at slaughter with mean of 6.5 kg for specialized breeds and 5.2 kg for Santa Ines (Furusho-Garcia et al., 2004; Endo et al., 2012). In addition to, the wool of Ile de France also may have interfered in the calculation of carcass yield. According to Louvandini et al. (2007) and Furusho-Garcia et al. (2004), Santa Ines raised in

confinement with superior nutritional intake was capable to reach satisfactory carcass yield, between 45 and 47.5%. These values were similar to those recorded for SUP-SI, however a little superior than those recorded for TST-SI in the present trial.

The *H. contortus* is blood feeding parasite capable of shed 5,000 eggs per day in contrast to 200 eggs of *T. colubriformis* (Besier et al., 2016), which explains higher numbers of *Haemonchus* spp. larvae on pasture, followed by *Trichostrongylus* spp. The detection of infective larvae (L3) on pasture grazed by suppressive groups and the highest larvae recovery from paddocks at the end of the study coincided with increase in EPG in both groups. A high amount of L3 on TST paddocks was recovered in spring season, which has favourable environmental conditions (frequent rainfalls and moderate temperature) for rapidly L3 development and survival (Amarante, 2015; Besier et al., 2016) in agreement with other studies developed in the region, that also reported higher number of *Haemonchus* and *Trichostrongylus* L3 in the spring (Silva et al., 2008; Rocha et al., 2014).

Animals parasitized by *H. contortus* and *T. colubriformis* the predominant parasites, are prone to productive losses, even when they present mild infection. Taking in consideration that resilient animals are important pasture eggs contaminants and that they are not detected by FAMACHA©, it is necessary the search for other selective methods to identify these resilient animals. Such approach associate with other alternative control methods to decrease pasture contamination, like selection of resistant animals, crossbreeding resistant breeds with commercial breeds, nutritional improvement and integrated crop-livestock system, are interesting measures to reduce environment contamination and consequently the level of animal infections.

Conclusion

The targeted selective treatment prevents mortality, however, it does not prevent productive losses in fattening Santa Ines and Ile de France lambs.

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CAPÍTULO VI

Discussão geral

O parasitismo por nematódeos gastrointestinais (NGI) é responsável por grandes perdas produtivas na ovinocultura, sendo um dos principais entraves para esse tipo de produção (AMARANTE, 2014). Em um estudo de metanálise sobre o impacto do parasitismo na produção de ovinos foi observado que os NGI são responsáveis por perdas produtivas entre 15-23%, afetando principalmente a produção de carne e lã (MAVROT et al., 2015).

No presente experimento foi possível observar o acelerado surgimento de populações de nematódeos resistentes ao monepantel, que com cinco meses de uso teve eficácia reduzida para 0% nos animais sob tratamento supressivo.

O tratamento seletivo surgiu como alternativa de controle de NGI com o propósito de desacelerar o surgimento resistência anti-helmíntica, objetivando a manutenção ou aumento da população em refugia, que é a população de nematódeos sensíveis aos anti-parasitários (MEDINA-PÉREZ et al., 2015; ROSALINSKI-MORAES; SOTOMAIOR, 2015; VALCÁRCEL et al., 2015). Esse tipo de tratamento tem sido amplamente usado no Brasil, sendo o FAMACHA© o mais difundido (MAIA et al., 2013). No entanto, no presente estudo foi observado que com cinco meses de tratamento seletivo em animais infectados naturalmente houve a redução da eficácia do monepantel para 76%. Segundo a WAAVP um anti-helmíntico é considerado altamente eficaz quando apresenta eficácia ≥ 98%, abaixo de 80% é considerado com eficácia insuficiente (WOOD et al., 1995).

No tratamento seletivo são tratados apenas os animais que apresentarem sinais clínicos aparentes, como edema submandibular, conjuntiva hipocorada, diarréia ou excessiva perda de peso (ROSALINSKI-MORAES; SOTOMAIOR, 2015). No presente estudo, os animais foram tratados quando se enquadravam na categoria 3 do FAMACHA© que compreende o intervalo de volume globular (VG) entre 18 e 22%, no qual foi

determinado o valor médio de VG ≤ 20% como valor de corte para o tratamento de animais. Para esses animais apresentarem os sinais clínicos supracitadas, é preciso que estejam com alta carga parasitária (AMARANTE, 2015) e apesar de os animais sob tratamento seletivo terem apresentado cargas parasitárias elevadas, principalmente os cordeiros Ile de France, dentre os sinais característicos de verminose foi possível observar apenas as alterações nos valores de VG, evidenciando que em tratamento seletivo que utilize como parâmetro alterações fisiológicas visíveis, os animais só seriam tratados quando ocorresse uma piora no quadro clínico.

Adicionalmente, os parasitas possuem uma distribuição agregada, no qual apenas uma parcela pequena de animais abriga boa parte dos parasitas enquanto os demais terão uma quantidade menor (BARGER, 1985). Levando em consideração que os animais tratados serão aqueles com maior quantidade de parasitas, então, a maior quantidade de parasitas do rebanho abrigados por esse animal terá contato com o anti-helmíntico, e se esse animal for suscetível, esse contato será freqüente, havendo pressão de seleção imposta pelo princípio ativo utilizado sobre esses parasitas. Fato ocorrido no presente estudo, no qual todos os Ile de France necessitaram ser tratados de uma a quatro vezes durante o experimento, e foram os animais com maiores cargas parasitárias, o que em parte explicou o aparecimento da resistência nos animais sob tratamento seletivo no presente estudo.

Estudos realizados comparando o desempenho produtivo de ovinos sob tratamento seletivo com animais submetidos a outros tipos de tratamentos anti-helmínticos evidenciaram que não houve diferença significativa entre os grupos em relação ao ganho de peso (DEPNER et al., 2007; MOLENTO et al., 2009; LEASK et al., 2013; FERNANDES et al., 2019). No presente estudo foi observado que os animais sob o tratamento seletivo tiveram perdas de 21,6 a 25,9% no ganho de peso, já no ganho em peso médio diário as perdas foram de 21,2% e 25,7% para os cordeiros Ile de France e Santa Inês, respectivamente. Resultados similares foram encontrados por Starling et al. (2019), com perdas produtivas entre 17-25,7%. Essas perdas são decorrentes da ação direta dos parasitas no organismo, causando alterações morfológicas e funcionais nos tecidos gastrointestinais, alterando a capacidade de digestão e

absorção, além de diminuição na ingestão de alimentos e realocação de nutrientes para reparo tecidual e resposta imune (COOP & KYRIAZAKIS, 1999; CARDIA et al., 2011; MAVROT et al., 2015; SILVA et al., 2019).

Os animais Ile de France foram importados com o objetivo de melhorar a produção de carne no país (MORENO et al., 2010). Contudo, não foi considerado à alta susceptibilidade aos NGI presentes no Brasil (AMARANTE, 2014). Dessa forma, apesar da alta produtividade dessa raça, os animais apresentaram produtividade inferior em relação a animais criados confinados em condições “ideais” de manejo (BRICARELLO et al., 2005). Todavia, mesmo apresentando desempenho produtivo abaixo do esperado para sua raça, os cordeiros Ile de France obtiveram maior ganho de peso e ganho em peso médio diário em relação aos animais Santa Inês.

Por outro lado, mesmos os animais Santa Inês tendo apresentado valores inferiores para boa parte dos variáveis produtivas, não apresentaram diferença no peso de carcaça em relação aos animais Ile de France e tiveram rendimento de carcaça superior em ambos os tratamentos. Este resultado pode ter ocorrido devido raças especializadas apresentarem maior quantidade de conteúdo no trato digestivo em relação a raças nativas (FURUSHO-GARCIA et al., 2004; ENDO et al., 2008). Adicionalmente, o peso da lã pode ter interferido no cálculo do rendimento da carcaça.

A resistência aos nematódeos gastrointestinais está relacionada a habilidade do hospedeiro em desenvolver resposta imunológica do tipo T helper 2 (Th2) mais rápida e robusta, limitando o estabelecimento e/ou eliminando a população de nematódeos (INCLAN-RICO & SIRACUSA, 2018; AMARANTE et al., 2009; SHAKYA et al., 2011; JACOS et al., 2016; BOWDRIDGE et al., 2015). É uma característica com herdabilidade moderada, que pode ser explorada em programa de seleção de animais para aumentar a resistência do rebanho ovino como medida de controle alternativo visando a sustentabilidade (AMARANTE, 2014; DE LA CHEVROTIÈRE et al., 2012). Os animais da raça Santa Inês foram os que apresentaram resposta imune sistêmica mais rápida e robusta com maiores contagens de eosinófilos, níveis de IgG contra L3 de *H. contortus* e *T. colubriformis*; além de resposta imune local mais intensa com maiores níveis de IgA contra L3 de *H. contortus* e *T.*

colubriformis, contagens superiores de células POU2F3+ (suposta células Tuft), células T e leucócitos globulares no tecido abomasal, e maiores contagens de mastócitos no tecido intestinal. A resposta imune local demonstrou ser a mais importante no processo de expulsão dos parasitas.

Dessa forma, é importante elucidar e entender a base imunológica da resistência aos parasitas gastrintestinais com o objetivo de desenvolver novos métodos de controle como o melhoramento genético do rebanho como estratégia de controle sustentável.

Conclusões gerais

Foi possível constatar que a utilização do tratamento supressivo é inviável para a manutenção da eficácia de princípios ativos como o monepantel, da mesma forma o tratamento seletivo não foi capaz de prevenir o surgimento de resistência anti-helmíntica em cinco meses de experimento em animais em condições naturais de infecção, também não evitou que houvesse perdas produtivas expressivas em ambas as raças. A raça Santa Inês demonstrou desenvolver resposta imune humoral precoce e robusta, e resposta imune local expressiva, principalmente no tecido abomasal. Dessa forma, essa raça possui potencial na utilização de melhoria da resistência à infecção por NGI em rebanhos de ovinos, como uma estratégia alternativa de controle sustentável.

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