Relatório Final

ALT20-03-0145-FEDER-000037

Gen-Res-Alentejo

Utilização de Genómica na Seleção de ovinos resistentes a Parasitas e Peeira no alentejo

1 de setembro de 2016 – 31 de agosto de 2020

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Resumo

O objectivo principal do projecto foi utilizar a genómica como ferramenta tecnológica para apoio à seleção de ovinos resistentes à peeira e a parasitoses por nemátodos gastrointestinais na região do Alentejo. O projecto foi liderado pela ACOS - Associação de Agricultores do Sul, em colaboração com outras entidades da Rede de Ciência e Tecnologia do Alentejo, designadamente a Universidade de Évora, o CEBAL - Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo, a DRAPAL - Direcção Regional de Agricultura e Pescas do Alentejo e o INIAV- Instituto Nacional de Investigação, Alimentação e Veterinária).

O projecto foi delineado em 5 actividades (4 actividades técnico-científicas e 1 actividade de coordenação e divulgação de resultados). Na primeira fase, no contexto da ***Actividade 1***, foi realizado um inquérito epidemiológico a 689 explorações do Alentejo para determinação dos factores de risco associados à peeira e para definição de critérios para selecção de explorações alvo para recolha de informação. Com base na avaliação dos factores de risco foram identificadas 17 explorações geograficamente dispersas por toda a região do Alentejo.No contexto da ***Actividade 2***, foram realizadas visitas às explorações para avaliação e pontuação clínica dos diversos graus da peeira, para recolha por biópsia de amostras das lesões dos animais com peeira para identificação do agente etiológico através de estudos de metagenómica e técnicas de PCR, recolha de fezes e de sangue para determinação do parasitismo causado por nemátodos gastrointestinais e colheita de amostras de sangue para extracção de ADN. Dum total de 231 animais amostrados, através de PCR foram identificados prevalências de *D. nodosus* (51%, n= 132) e *F. necrophorum* (46,4%, n=121) elevada em animais que contraíram peeira. Estes resultados foram concordantes com os obtidos através de estudos de metagenómica. Da cultura bacteriológica (dum total de 132 amostras) onde foi detectado o *D. nodosus* obtiveram-se 17 isolados caracterizados como virulentos. A maioria dos isolados pertencia as serogrupos B (40%) e C (20%) seguindo-se os serogrupos H (13%) e G (13%). A avaliação do impacto económico das doenças e das metodologias (***Actividade 3***) ainda não está finalizada. A ***Actividade 4*** consistiu na realização de estudos de associação de genómica (GWAS – Genome Wide Association Study) para os diferentes fenótipos de interesse (peeira e nemátodos gastrointestinais) identificados nas 17 explorações. Um total de aproximadamente 1500 animals foram genotipados utilizando um painel de ~50 Kb SNPs desenhado pela equipa em base a variação genética identificada em 40 genomas de merinos (Branco, Preto e cruzado). No caso dos estudos referentes à peeira os resultados obtidos indicam três SNPs significativos no cromossoma 24 e seis SNPs sugestivos nos cromossomas 2, 4, 7, 8, 9 e 15. Os três SNPs significativos localizam-se na região génica do gene SMG1.

No caso dos parasitas gastrointestinais foram encontrados 1 SNP significativo no cromossoma 16 e dois SNPs sugestivos nos cromossomas 3 e 16.

Relativamente à excução do projecto o mesmo foi alvo de 4 reprogramações (temporais e financeiras) motivadas principalmente pela Covid 19. Globalmente, a execução financeira foi de 95,1%.

Em termos de indicadores, foram submetidas 3 patentes nacionais e três patentes europeias e o número de novos investigadores (n=3) nas diversas instituições parceiras totalizou 2,4 ETI. As medidas de publicidade foram cumpridas, foi criado um site do projecto e foram efectuadas várias publicações (2 teses de doutoramento, 1 tese de mestrado, 3 artigos publicados em revistas internacionais com arbitragem científica; 1 Artigo submetido em segunda fase de revisão em revista internacional com arbitragem científica, 1 Artigo em preparação para publicação em revista internacional com arbitragem científica; 16 apresentações orais e Congressos Nacionais e Internacionais; 8 Comunicações em formato Poster em Congressos Nacionais e Internacionais; 2 Workshops temáticos e um Seminário Nacionais.

1. Identificação da operação

Acrónimo: Gen-Res-Alentejo

Título: Utilização de Genómica na Seleção de ovinos resistentes a Parasitas e Peeira no Alentejo

Referência: ALT20-03-0145-FEDER-000037

Tipologia do projeto: Projeto em co-promoção

Instituição beneficiárias:

- Associação de Agricultores do Sul (ACOS) – Coordenação

- Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL) - Coordenação

- Universidade de Évora (UEVORA)

- Instituto Nacional de Investigação Agrária e Veterinária (INIAV) – Polo de Santarém

Parceiros não executores:

- Direção Regional de Agricultura e Pescas do Alentejo (DRAPAL)

2. Enquadramento, objetivos, atividades desenvolvidas e gestão do projeto

2.1. Enquadramento e Objetivos

Esta operação tem como objetivo primordial melhorar a produtividade das explorações de ovinos no Alentejo, através da identificação por metodologias genómicas de última geração, de marcadores genéticos associados à resistência a doenças com elevada prevalência e reconhecido impacto económico, a peeira e o parasitismo gastrointestinal por nematodes. Para tal constitui-se uma parceria liderada por uma associação de produtores de ovinos, a ACOS - Associação de Agricultores do Sul, em colaboração com outras entidades da Rede de Ciência e Tecnologia do Alentejo, designadamente a Universidade de Évora, o Cebal - Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo, a DRAPAL - Direcção Regional de Agricultura e Pescas do Alentejo e o INIAV- Instituto Nacional de Investigação, Alimentação e Veterinária. Estes parceiros permitem o contacto direto com os produtores agropecuários e integram capacidade científica do ponto de vista da medicina veterinária, melhoramento genético e a utilização da genómica como ferramenta inovadora na seleção assistida por marcadores. O compromisso da União dos Agrupamentos de Defesa Sanitária do Alentejo (UADS Alentejo) garante a exequibilidade dos trabalhos de campo.

O objectivo geral do projecto foi utilizar a genómica como ferramenta tecnológica para apoio à seleção de ovinos resistentes a parasitoses e peeira na região do Alentejo. Os objetivos específicos do projeto foram:

1. Identificação dos fatores de risco associados à peeira e parasitoses por nematodes gastrointestinais em explorações ovinas do Alentejo.
2. Caracterização das doenças e de metodologias de diagnóstico para identificação de animais resistentes/suscetíveis às doenças estudadas. Utilização da metagenómica para caracterização de agente etiológico da peeira: Dichelobacter nodosus.
3. Avaliação do impacto económico da peeira e das infeções por parasitas gastrointestinais nas explorações estudadas.
4. Utilização de estudos de associação genómica para a identificação de marcadores genéticos associados à resistência a parasitoses por nematodes gastrointestinais e à peeira.
5. Avaliação o impacto económico do projeto no controlo da peeira e parasitismo por nematodes gastrointestinais e na melhoria da produtividade e da rentabilidade das explorações.

As trabalho desenvolvido neste projeto foi organizado em 4 Actividades principais e 1 Actividade de Coordenação e Divulgação do Projecto:

- **Actividade 1: Identificação de fatores de risco relevantes associados à peeira e nemátodes gastrointestinais em explorações de ovinos do Alentejo.**

  Esta actividade englobou a concepção e realização de um inquérito epidemiológico (Tarefa1) com o objectivo de selecionar explorações do Alentejo (Tarefa 2) com base na avaliação de factores de risco associados às duas enfermidades (Tarefa 3).

- **Actividade 2: Caracterização das doenças e metodologia de diagnóstico**

  Nesta actividade foram realizadas visitas às explorações para avaliação e pontuação clínica dos diversos graus da peeira (Tarefa 1), recolha por biópsia de amostras das lesões dos animais com peeira para identificação do agente etiológico através de estudos de metagenómica (Tarefa 2), recolha de fezes e de sangue para determinação do parasistimos causado por nemátodos gastrointestinais (Tarefa 3) e colheita de amostras de sangue para extracção de ADN a utilizar na Actividade 4.

- **Actividade 3: Avaliação do impacto económico da peeira e das infecções por nemátodos gastrointestinais, bem como de outras doenças na região do Alentejo**.

  O obejctivo foi identificar nas explorações amostradas os custos directos associados às doenças assim como as perdas de produtividade causadas pelas mesmas (Tarefa 1) e realizar uma análise custo-benefício do impacto económico das metodologias desenvolvidas no projecto (Tarefa 2).

- **Actividade 4: Estudos de associação genómica para a identificação de marcadores genéticos associados à peeira e aos nemátodos gastrointestinais.**

  Trata-se da actividade central do projecto que consistiu em utilizar as amostras de ADN para realizar a sequenciação com recurso à tecnologia GBS (Genotyping by Sequencing) para detecção de SNP' s (Polimorfismos de Nucleótido Simples). Esta informação será depois utilizada para estudos de associação do genoma completo (GWS – Genome Wide Association) para os diferentes fenótipos (peeira e nemátodos gastrointestinais) identificados nas explorações.

- **Actividade 5: Coordenação do Projecto e Divulgação de Resultados**

Nesta actividade tratou-se do acompanhamento geral das actividades do projecto (Tarefa 1) e da divulgação de resultados.

2.2. Atividades desenvolvidas

2.2.1. Actividade 1: Identificação de fatores de risco relevantes associados à peeira e nemátodes gastrointestinais em explorações de ovinos do Alentejo

Tarefa A1.1.- Inquérito epidemiológico

Foi delineada a estrutura do inquérito epidemiológico (Exemplar no Anexo 1) que foi aplicado em forma de entrevista aos proprietários de explorações englobadas nos diversos Agrupamentos de Defesa Sanitária da região Alentejo (11 no total) entre Dezembro de 2016 e Dezembro de 2017 Foi validado um total de 689 inquéritos, correspondendo a aproximadamente 8% das explorações de pequenos ruminantes do Alentejo. A avaliação e discussão exaustiva dos resultados do inquérito poderá ser consultada no Capítulo 3 da Tese de Doutoramento realizado no âmbito do projecto intitulada "Caracterização da Peeira ovina na região do Alentejo" realizado por Pedro Caetano (Universidade de Évora, 2020) (http://hdl.handle.net/10174/29315).

Tarefa A1.2.- Avaliação dos factores de risco associados à peeira.

Resumidamente, os resultados mais relevantes que conduziram à identificação dos factores de risco associados à peeira foram os seguintes:

- - Elevada heterogeneidade na área total das explorações (0,5 a 3000 ha; média 240,9 ha), e na dimensão dos efectivos (3 a 3446 animais; média 273 animais);
- A ocorrência de peeira observou-se em explorações de dimensão maior (média 330,1 ha), enquanto que em explorações de menor dimensão (média 130,5 ha), a doença não foi reportada.
- Aproximadamente 35% dos inquiridos reportaram casos de peeira em 2016 e os meses de maior prevalência foram janeiro, fevereiro e março.
- A prevalência da peeira foi mais elevada nas explorações do Alto Alentejo (46,4 %) e Alentejo Central (37 %) e menor Alentejo Litoral (23 %) e Baixo Alentejo (32 %).
- Explorações em que coabitavam ovinos e bovinos apresentaram uma probabilidade 1,5 vezes maior de ocorrência de peeira comparativamente às que só exploravam ovinos.
- Explorações com estabulação apresentaram uma probabilidade 1,5 superior de ocorrência de peeira do que explorações sem estabulação.
- Nos rebanhos em que se praticou a cobrição contínua ao longo do ano a probabilidade de ocorrência de peeira foi duas vezes menor comparativamente aos rebanhos sujeitos a épocas de cobrição/partos definidas no tempo.
- Ovinos explorados em áreas de montado apresentaram maior tendência para contraírem peeira comparativamente aos animais que pastoreavam em áreas desprovidas de árvores.

- Explorações com solos com fraca capacidade de drenagem apresentaram uma probabilidade de ocorrência de peeira 4 vezes superiores às de solos bem drenados.
- Relativamente a acções de prevenção e tratamento de peeira nas explorações em que a doença foi detectada, quanto maior foi a utilização de pedilúvio e a antibioterapia tópica maior foi a correlação com a percentagem de animais afectados.
- Quanto a factores climáticos, observou-se que a ocorrência está peeira está associada a condições de pluviosidade e temperaturas mais elevadas.

Tarefa A1.3.- Seleção das explorações a incluir no estudo

Com base nos factores de risco identificados procedeu-se à selecção de 17 explorações (***Tarefa A1.2***) utilizando os seguintes critérios:

- Ocorrência de casos de peeira pelo menos nos dois anos anteriores;
- Efectivo superior a 100 ovelhas;
- Animais de raças autóctones e resultantes de cruzementos;
- Ausência de tratamento contra a peeira pelo menos num período de 30 dias antes das visitas programadas às Explorações;
- Disponibilidade do proprietário de colaborar com as acctividades do projecto;
- Adequada cobertura territorial do Alentejo (Figura anexa, adaptado de Caetano, 2021)

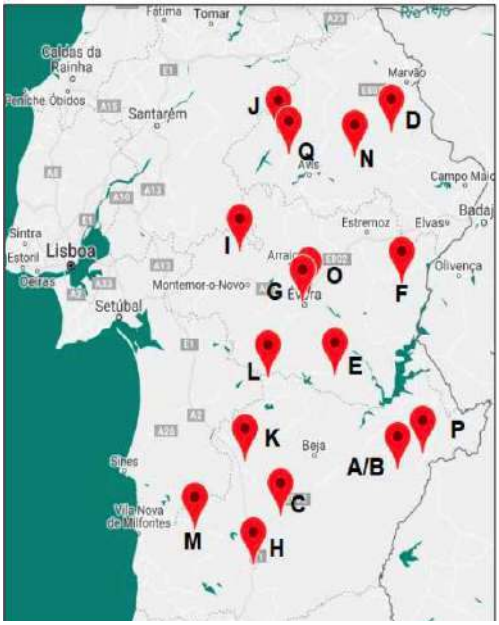


Figura 1.- Distribuição geográfica das 17 explorações seleccionadas

2.2.2. Actividade 2 - Caracterização da doença e metodologia de diagnóstico

Tarefa A2.1.- Avaliação da peeira

As visitas as explorações realizaram-se em dois anos consecutivos (dezembro 2016 a maio de 2017 e de janeiro a junho de 2018). Em cada exploração foram selecionados aleatoriamente cerca de 100 animais, para avaliação e recolha de informação e de várias amostras durante o decurso do projecto. A classificação das lesões de peeira foi realizada de acordo com a taxonomia modificada desenvolvida por Egerton and Roberts (1971) com graus de peeira compreendidos entre 0 e 5.

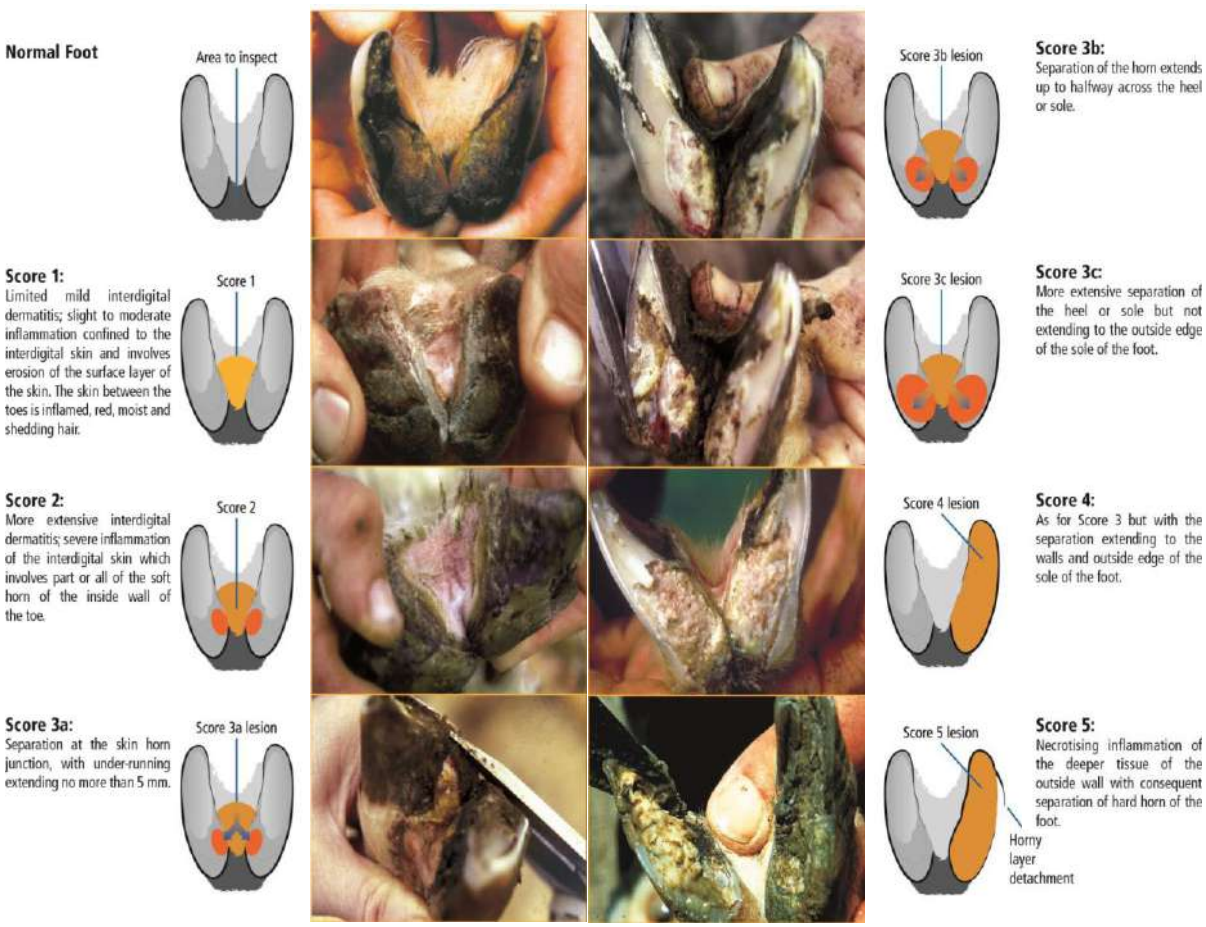


Figura 2.- Graus de afetação de peeira em base a taxonomia modificada de Egerton and Roberts (1971)

Descrição dos diferentes graus:

- **Grau 0.-** ausência de qualquer lesão, devendo ser observável o espaço interdigital com uma coloração ligeiramente rosada, apresentando-se seco e coberto por uma camada de pelo;
- **Grau 1.-** corresponde a lesão de dermatite interdigital pouco severa, podendo ser observada alopécia e a presença de eritema;
- **Grau 2.-** equivale a uma lesão de dermatite interdigital mais extensa, sendo a imagem semelhante ao grau 1, mas já se distinguem sinais de alguma necrose no espaço interdigital;

- **Grau 3.-** lesão idêntica à do grau 3, mas onde a separação da úngula já se estende até à porção anterior da parede abaxial.
- **Grau 4.-** quando a lesão deixa de estar confinada ao espaço interdigital, começando a existir sinais de destacamento da úngula na sua porção medial e na zona dos talões;
- **Grau 5.**- inflamação necrosante do tecido mais profundo da parede externa com consequente separação do casco rígido do pé.

As lesões de peeira foram classificadas por 3 médicos veterinários. Da avaliação preliminar dos dados das amostras recolhidas foi possível inferir o seguinte:

- Um total de 1910 animais observados nas duas visitas. Cerca de 15% dos animais apresentavam lesões de peeira e apena 5,81% lesões muito graves o que revela um baixo grau de incidência da doença nos dos anos em observação.
- A probabilidade de animais com idades inferiores ou iguais a 4 anos contraírem peeira foi o dobro dos animais de idades superiores.
- Machos apresentaram maior probabilidade (3,7 vezes) de contraírem lesões comparativamente às fêmeas.
- Animais da raça Merina Preta apresentaram menos predisposição para contraírem peeira (2,3%) comparativamente aos de raça Merina Branca (12,6%) ou genótipos tipo Merino (22,6%).

Foram ainda recolhidas outras amostras (Tarefas 2 e 3):

- Colheita de sangue em cada animal avaliado (determinação do hematócrito e quantificação do nível de proteínas totais do soro sanguíneo) e para extracção de ADN a utilizar na Actividade 4
- Amostras de tecido cutâneo do espaço interdigital através de biópsia com o objectivo de cacacterizar e identificar os agentes microbiológicos presentes, designadamente os agentes reconhecidos como causadores da peeira (*D. nodosus e F. necrophorum*) através de estudos de metagenómica e de detecção bacteriológica e molecular através de técnicas PCR (Polymerase Chain Reaction).
- recolha de fezes e de sangue para determinação do parasistimo causado por nemátodos gastrointeninais.

Tarefa A2.2.- Caracterização de Dichelobacter nodosus

No âmbito desta tarefa efetuaram-se duas abordagens principais para estudar e caracterizar os agentes causadores da peeira, uma via PCR e outra via a metagenômica

Caracterização via PCR

Esta primeira abordagem consistiu na utilização de técnicas de PCR para detecção de *Dichelobacter nodosus (D. nodosus) e Fusubacterium necrophorum (F. necrophorum)* em 261 amostras de DNA do tecido cutâneo do espaço interdigital de animais identificados com lesões de peeira. Detalhes sobre as metodologias usadas e os resultados desta avaliação encontram-se na Tese de Mestrado de Catarina Albuquerque (2019) realizada no âmbito deste projecto (http://hdl.handle.net/10451/41853). Em resumo, os principais resultados foram:

- Observou-se uma prevalência de *D. nodosus* (51%, n= 132) e *F. necrophorum* (46,4%, n=121) elevada em animais que contraíram peeira.
- Quanto mais severa eram as lesões maior foi a prevalência das dos dois agentes.
- Explorações com maior número de animais infectados por *D. nodosus* também apresentavam elevada prevalência de *F. necrophorum.*
- Da cultura bacteriológica (dum total de 132 amostras) onde foi detectado o *D. nodosus* obtiveram-se 17 isolados caracterizados como virulentos. Quanto o serogrupo, a maioria dos isolados pertencia as serogrupos B (40%) e C (20%) seguindo-se os serogrupos H (13%) e G (13%).

Caracterização via metagenomica

Na segunda abordagem recorreu-se à metagenómica para caracterização do *Dichelobacter nodosus.* Utilizaram-se também as 261 amostras de biópsia de tecido interdigital, com diferentes graus de classificação da doença (Tabela 1), de um total de 210 animais distribuídos em várias explorações. Destas amostras extraiu-se DNA total sendo a sua qualidade avaliada. Após a avaliação da qualidade do DNA total extraído, um total de 48 amostras foi descartado devido a uma baixa qualidade, sendo selecionadas 213 amostras para serem sequenciadas utilizando a técnica de sequenciação conhecida como "Whole Metagenome Sequencing", através da plataforma BGI-Seq 500. A sequenciação produziu um total de 13.3 biliões de reads. Os dados de sequenciação foram pré-processados, mantendo 97.2% das reads, sendo consideradas como reads de alta qualidade. Estas reads de alta qualidade foram mapeados contra o genoma de referência disponível na NCBI (GCF_002742125.1) com o intuito de identificar e retirar do conjunto de dados as reads provenientes do DNA do hospedeiro, mantendo assim só as pertencentes aos microrganismos presentes nas amostras da biopsia e supostamente associados à peeira. No fim deste processo, um total de 115,9 milhões de reads foram mantidos para as análises posteriores.

As reads finais selecionadas foram classificadas em categorias taxonómicas ao nível da espécie. Esta classificação foi a base para estimar a abundância de cada uma das espécies identificadas permitindo a realização da análise de diferenças de abundância entre espécies seguindo duas estratégias diferentes: (1) presença e ausência de afetação de peeira e (2) comparação de pares por grau de afetação. Em ambas estratégias todas as amostras com a mesma classificação de afetação foram consideradas réplicas biológicas. Cabe realçar que as amostras com classificação 5 foram excluídas desta análise devido a provocarem interferências nas análises estatísticas.

Tabela 1.- Número de amostras por grau de afetação de peeira

Grau de afetação de peeira	Nº de amostras
0	71
1	42
2	36
3	46
4	16
5	2

Primeira estratégia

As amostras com classificação 0 e 1 foram consideradas como tendo ausência de afetação, enquanto que as restantes foram classificadas como tendo presença de afetação. Após analisar as diferenças de abundância das espécies presentes em cada um dos dois grupos definidos, um total de 656 espécies foram identificadas com diferenças nas suas abundâncias (169 espécies revelaram-se mais abundantes nas amostras com ausência de afetação, e 487 nas amostras com presença de afetação).

O agente causal da doença, *D. nodosus,* e outros microrganismos associados à mesma, tais como o *F. necrophorum* e espécies de *Treponema*, foram encontrados mais abundantes nas amostras com presença de afetação.

Os principais géneros encontrados significativamente mais abundantes nas amostras com ausência de afetação foram *Streptomyces* (44 espécies), *Pseudomonas* (13 espécies), *Mycolicibacterium* (9 espécies), *Deinococcus* (7 espécies), *Brevundimonas* (7 espécies), *Micromonospora* (6 espécies), *Rhodococcus* (6 espécies), *Staphylococcus* (5 espécies), *Mycobacterium* (5 espécies) and *Gordonia* (3 espécies). Por outro lado, os principais géneros encontrados significativamente mais abundantes nas amostras com presença de afetação são *Mycoplasma* (47 espécies), *Campylobacter* (37 espécies), *Streptococcus* (35 espécies), *Clostridium* (27 espécies), *Arcobacter* (22 espécies), *Fusobacterium* (10 espécies), *Capnocytophaga* (10 espécies), *Treponema* (8 espécies), *Leptotrichia* (8 espécies) and *Vibrio* (7 espécies).

Segunda estratégia

Na comparação de pares entre diferentes graus de afetação de peeira, um total de 2.345 espécies foram encontradas significativamente mais abundantes em pelo menos uma das comparações realizadas (Tabela 2).

Tabela 2.- Sumário dos resultados das comparações por pares dos diferentes graus de peeira

A vs B	Espécies mais abundantes em A	Espécies mais abundantes em B	Total
0 vs 1	94	39	133
0 vs 2	210	325	535
0 vs 3	86	670	732
0 vs 4	845	697	1,542
1 vs 2	245	382	627
1 vs 3	104	780	884
1 vs 4	844	763	1,607
2 vs 3	44	371	316
2 vs 4	552	329	881
3 vs 4	840	122	758

A fim de identificar espécies com padrões semelhantes de abundância entre os diferentes estágios da doença, todas as espécies encontradas com diferenças significativas nas suas abundâncias nas comparações por pares foram agrupadas com base no seu perfil de abundância nos diferentes graus de afetação em 20 clusters (Tabela 3, Figura 3).

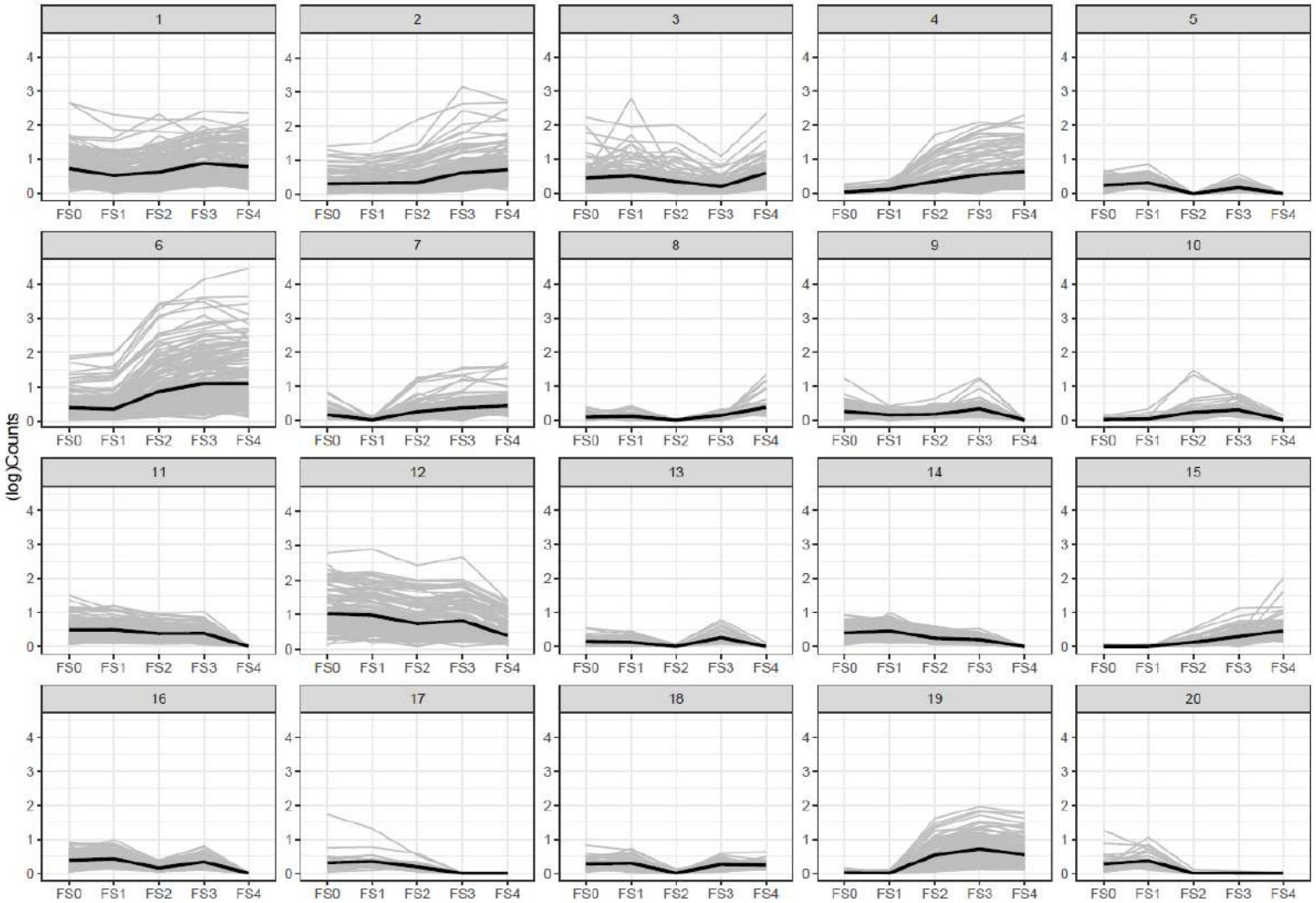
Tabela 3.- Número de espécies agrupadas em cada cluster

Cluster	#Espécies	Cluster	#Espécies	Cluster	#Espécies	Cluster	#Espécies
1	154	**6**	195	**11**	253	**16**	143
2	188	**7**	98	**12**	159	**17**	28
3	94	**8**	63	**13**	64	**18**	49
4	128	**9**	110	**14**	185	**19**	128
5	111	**10**	59	**15**	97	**20**	39

As duas principais espécies relevantes associadas à peeira, *D. nodosus* e *F. necrophorum*, foram encontradas no conjunto das 2.345 espécies com diferenças significativas na abundância. Ambas foram agrupadas no mesmo cluster, apresentando um mesmo perfil de abundância ao longo da progressão da doença (cluster 6, Figura 3). Neste cluster observa-se uma ligeira diferença nas abundâncias entre o grau 0 e grau 1, um aumento nas abundâncias entre o grau 1 e o grau 3, e um ligeiro aumento da abundância entre o grau 3 e o grau 4. Outros dois clusters (4 e 15) apresentam distribuições das abundâncias muito parecidas às descritas no cluster 6. Nestes três clusters encontram-se outros agentes patogénicos previamente identificados como importantes noutras doenças polimicrobianas, como a dermatite interdigital ou dermatite digital contagiosa ovina:

Streptococcus ssp. (n=27), *Clostridium* spp. (n=24), *Fusubacterium* spp. (n=10), *Mycoplasma* spp. (n=9), *Treponemas* spp. (n=8), *Campylobacter* spp. (n=7), *Porphyromonas* (n=4), *Veillonella* spp. (n=4) and *Gemella* genera. (n=2).

Figura 3.- Clustering do perfil de abundancias das espécies com diferenças significativas nas suas abundâncias na comparação por pares (k = 20).FS: Footrot score – Grau de afetação de peeira.



2.2.3. Actividade 3 - : Avaliação do impacto económico da peeira e das infecções por nemátodos gastrointestinais, bem como de outras doenças na região do Alentejo.

Tarefa A3.1.- Avaliação das explorações de ovinos no Alentejo.

No âmbito desta actividade e após selecção das 17 explorações foi desenvolvido um inquérito para recolha de dados económicos de modo a calcular os custos directos associados às duas enfermidades. Ainda no âmbito desta tarefa foi realizada uma análise estatística exploratória de dados produtivos e reprodutivos para avaliar o impacto das doenças e complementar assim o estudo económico previsto.

Os dados do inquérito económico foram alvo de uma avaliação preliminar ficando por adicionar a informação relativa às quebras de produtividade associadas às doenças. Esta tarefa não foi completada devido à fraca qualidade dos dados fenotípicos disponíveis. Das 17 explorações apenas 2 (Explorações A/B e P) dispunham de dados produtivos e reprodutivos que foram gentilmente facultados pela Associação Nacional de Criadores de Raça Merina. Tratou-se de duas explorações com animais das raças Merina Preta e Merina Branca inscritos nos respectivos Livros Genealógicos. Da classificação dos vários graus de peeira nestas duas explorações, observou-se uma prevalência da doença muito baixa (aproximadamente 95% não apresentavam lesões de peeira e menos de 5% apresentavam lesões classificadas como grau 1 ou 2). Além disto, o número de animais avaliados nas duas visitas foi claramente insuficiente para fornecerem a informação que se pretendia. Como resultado houve a necessidade de encontrar uma estratégia alternativa de modo a estabelecer vários cenários de baixa de produtividade causada pelas doenças, consultando especialistas em produção de ovinos.

Os resultados esperados desta tarefa ainda não foram cabalmente conseguidos estando nesta fase em preparação um novo tratamento com informação económica mais recente relativamente aos custos de produção, que, como se sabe, sofreram grandes aumentos muito significativos nos últimos tempos.

Tarefa A3.2.- Avaliação do impacto econômico do projeto.

Relativamente à análise custo-benefício do impacto económico das metodologias desenvolvidas no projecto, apesar do projecto estar concluído em 2020, apenas nesta altura estão a obter-se os resultados finais da análise genómica. Só após o conhecimento dos genes potencialmente envolvidos na resistência à peeira e às infecções por nemátodos gastrointestinais será possível avaliar que testes serão necessários e quais os respectivos custos associados. Esta tarefa será ainda alvo de avaliação.

2.2.4. Actividade 4 - Estudos de Associação Genómica para a identificação de marcadores genéticos associados à podridão dos cascos e nematoides gastrointestinais.

Como trabalho de suporte a esta actividade foi necessário criar um painel de SNPs para genotipagem.

Tarefa A4.0.- Estabelecimento de um painel de SNPs para genotipagem

Foram realizadas colheitas de sangue num total de 39 animais Merino pertencentes a várias explorações. Destas amostras extraiu-se DNA total, sendo a sua qualidade avaliada e subsequentemente sequenciado utilizando a técnica de sequenciação conhecida como "Whole Genome Sequencing", através da plataforma BGI-Seq 500. A sequenciação produziu um total de 24,338,033,027 reads. Os dados de sequenciação foram pré-processados, tendo sido mantidos 93% (22,630,205,326) das reads produzidas, sendo estas consideradas como reads de alta qualidade. Estas reads de alta qualidade foram alinhadas contra o genoma de referência disponível na NCBI (GCF_002742125.1). Apenas os alinhamentos específicos e de alta qualidade foram incluídos na identificação de variação genética. Após a identificação das diferentes variantes, mais de 110 milhões de SNPs foram identificados em todo o genoma. Foi então realizada uma filtragem para reter só SNPS de alta qualidade com base em três parâmetros: i) qualidade individual do SNP; ii) cobertura do SNP; iii) qualidade do genótipo. Após o controlo de qualidade, mais de 28 milhões de SNPs foram mantidos para a seleção do painel de genotipagem. De seguida foi realizada a anotação funcional deste conjunto de SNPs para prever o seu efeito na síntese proteica. Numa primeira fase, para o design do painel foram selecionados os SNPs com uma frequência do alelo menor (MAF) superior a 1% e um call rate superior a 90%. Foram então definidas regiões flanqueadoras (sem qualquer SNP ou INDEL) de 150 pares de bases downstream e upstream para cada SNP. Posteriormente, foram considerados os SNPs em regiões codificantes como "SNPs âncora" aos quais foram sendo adicionados SNPs de uma forma uniforme entre cromossomas. Como resultado deste processo, no total, 47,779 SNPs foram selecionados e incluídos no painel, com uma distância média entre eles de 56 Kbps. A sua distribuição por cromossoma e por região genómica está representada nas Figuras 2 e 3. O desenho das sondas e respetiva genotipagem de 1493 animais, pertencentes as 17 explorações previamente selecionadas, foi realizada pela empresa IGATech (IGA Technology Services, Udine, Itália).

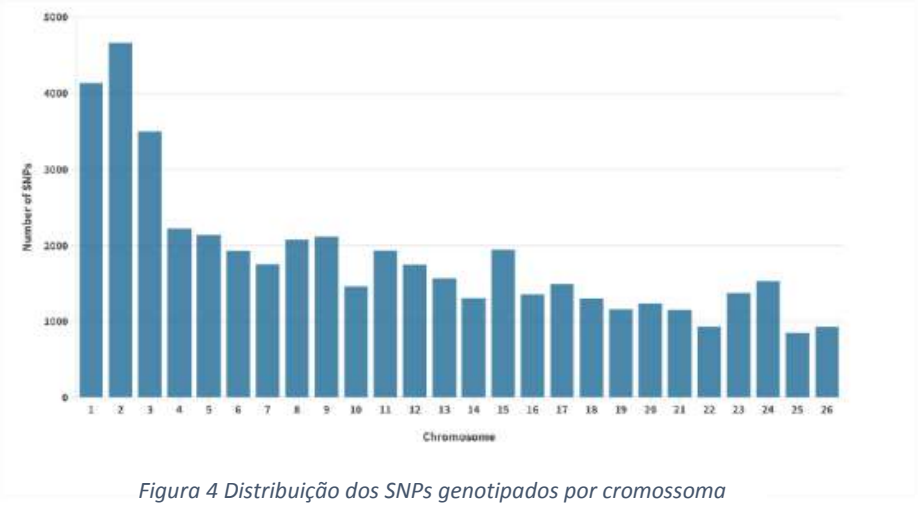


Figura 4 Distribuição dos SNPs genotipados por cromossoma

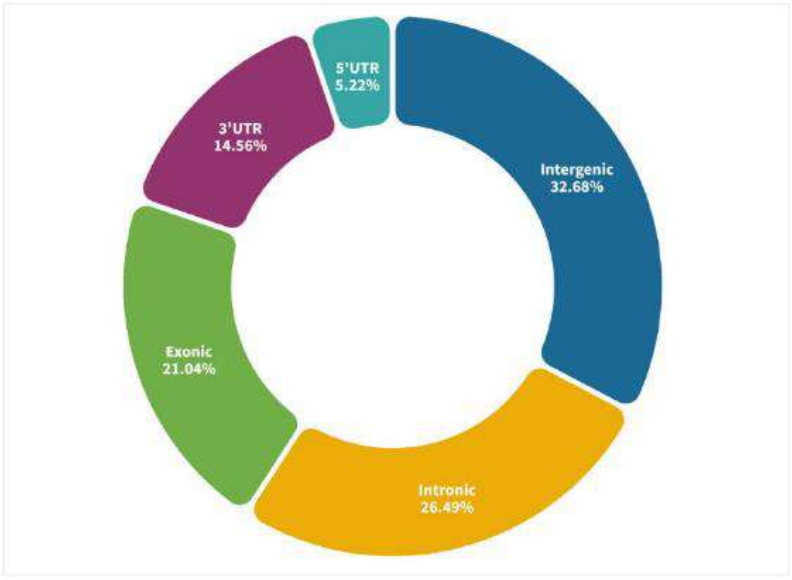


Figura 5.- Distribuição dos SNPs genotipados por região genómica

Tarefa A4.1.- Estudo de associação genómica para a resistência/susceptibilidade à peeira

Antes de iniciar o estudo de associação genómica, foi necessário verificar a qualidade dos dados genotipados. Estes foram então processados e filtrados com base nos seguintes critérios: i) MAF superior a 1%; ii) cal rate por SNP superior a 90%; iii) não foram permitidos desvios extremos ao equilíbrio de Hardy-Weinberg ($P < 10^{-6}$). Adicionalmente, animais com mais de 20% de missing data foram removidos da análise. Posto isto, um conjunto total de 29,716 SNP e 1375 animais foram mantidos para o estudo de associação. Para esse conjunto foi realizada a imputação de genótipos e a matriz de parentesco foi calculada (VanRaden kinship algorithm).

Relativamente ao fenótipo de interesse, e como descrito anteriormente, as lesões de peeira foram classificadas de forma visual nas quatro patas seguindo o método "Modified Egerton System", com valor mínimo de 0 (não afetado) e valor máximo de 5 (afetação severa). Tendo isto em consideração, foram definias três classificações para cada animal: i) score máximo (HFS), onde a cada animal foi atribuído o valor mais alto das quatro patas; ii) Score global (GFS) correspondendo ao somatório das 4 patas (valor máximo de 20); iii) Score indexado (IFS), após a aplicação de um fator de ponderação para o score individual de cada pata, para uma melhor classificação da infeção. Assim, o score 0 inicial permaneceu inalterado, e aos scores 1, 2, 3, 4 e 5, foram atribuídos os fatores 1, 2, 2.5, 3 e 3.25, respetivamente. No final, os valores iniciais foram multiplicados pelo respetivo fator de ponderação, resultando nos seguintes valores indexados por pata: 0, 1, 4, 7.5, 12 e 16.25. Esta classificação foi adotada de forma a evitar que um animal com sintomas leves de infeção tivesse um GFS superior a um animal com sintomas médios ou severos.

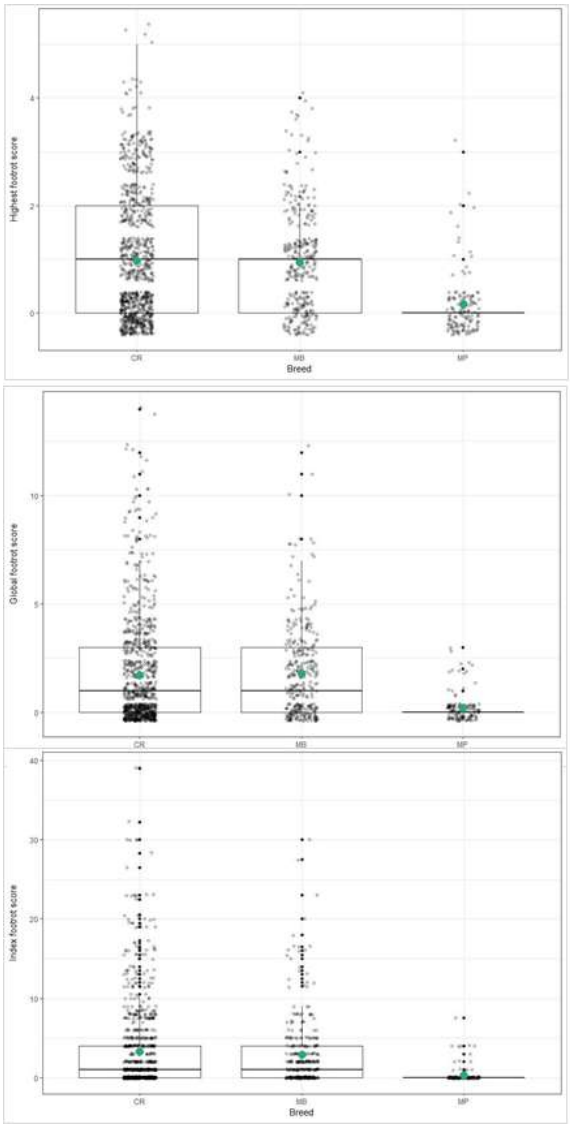


Figura 6 Distribuição dos valores fenotípicos da peeira por raça. (A) HFS. (B) GFS. (C) IFS. Os valores médios são representados por círculos verdes.

Assim, cada animal poderia ter um IFS máximo de 65. A distribuição dos valores fenótipos para cada uma destas classificações está representada na Figura 4.

Três cenários foram utilizados para fins de comparação: i) considerando o IFS como fenótipo de interesse (estratégia principal); ii) considerando o HFS como fenótipo de interesse, e iii) considerando o GFS como fenótipo de interesse. A análise de associação para cada um destes cenários foi realizada usando a função "GWAS" do software rrBLUP implementado em R, seguindo um modelo linear misto. Foram considerados como efeitos fixos a raça, idade, rebanho e o mês da visita. O cálculo da significância foi estimado usando a correção de Bonferroni, tendo sido estabelecido um threshold para a identificação de SNPs significativos (p-value = 2.06×10^{-6}). Adicionalmente, foi também considerado um threshold sugestivo (p-value = 4.13 x $x10^{-5}$).

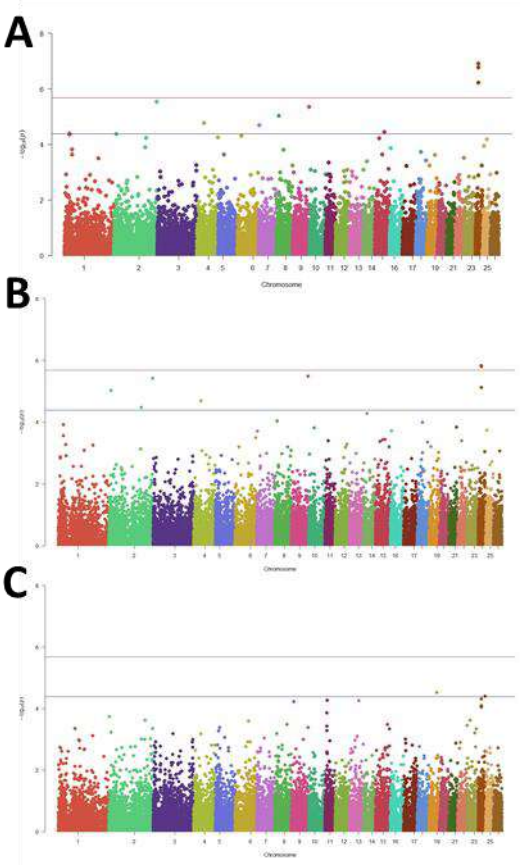


Figura 7 Resultados da análise de associação para a peeira em função do fenótipo de interesse utilizado: (A) IFS, (B) GFS e (C) HFS. O eixo vertical apresenta o valor -Log10 dos p-values, enquanto o eixo horizontal apresenta os cromossomas e a posição dos SNPs. As linhas vermelha e azul indicam o threshold de significância e sugestividade, respetivamente.

Com base nos resultados da estratégia principal, (fenótipo IFS), foram identificados três SNPs significativos no cromossoma 24 e seis SNPs sugestivos nos cromossomas 2, 4, 7, 8, 9 e 15 (Figura 5A e Tabela 4). Quando o cenário alternativo com base o fenótipo GFS foi considerado, dois SNPs significativos foram identificados no cromossoma 24 (16.664.127 pb; 16.683.234 pb), ambos comuns à estratégia principal. Além disso, seis SNPs sugestivos foram localizados nos cromossomas 2 (13.704.688 pb; 260.442.170 pb; 194.217.323 pb), 4 (42.346.212 pb), 9 (100.456.022 pb) e 24 (16.688.212 pb) (Figura 5B), dos quais, um foi identificado como significativo e três como sugestivos na estratégia principal. Quando foi considerado o fenótipo GFS para a análise, não foram identificados SNPs significativos. Apesar disso, dois SNPs sugestivos foram encontrados nos cromossomos 19 (45.832.644 pb) e 24 (38.953.050 pb) (Figura 5C).

A análise funcional dos resultados obtidos com o fenótipo IFS (estratégia principal) revelou que os três SNPs significativos estão localizados na região génica do gene SMG1, enquanto os SNPs

sugestivos estão localizados nas regiões génicas dos genes HSPG2, RALYL, CENPW, PCLO, KLHL35 e na região intergénica próxima ao gene THBS1 (Tabela 4).

Tabela 4 Sumário dos SNPs significativos e sugestivos identificados como sendo associados à resistência/susceptibilidade à peeira.

Chr	Position (bp)	A1	A2	p-value	Genomic Region	Functional effect	Gene
Genome-wide significant SNPs							
24	16,664,127	G	T	1.23E-07	UTR3	-	SMG1
24	16,683,234	G	A	1.69E-07	exon	synonymous	SMG1
24	16,688,212	T	C	6.16E-07	exon	synonymous	SMG1
Genome-wide suggestive SNPs							
2	260,442,170	C	T	2.93E-06	exon	synonymous	HSPG2
9	100,456,022	T	G	4.49E-06	intron	-	RALYL
8	13,714,405	G	A	9.38E-06	UTR5	-	CENPW
4	42,346,212	T	G	2.08E-05	intron	-	PCLO
7	3,506,174	C	G	2.06E-05	Intergenic	-	LOC106991156 (dist=553,763); THBS1 (dist=161,183)
15	57,714,827	G	A	3.66E-5	intron	-	KLHL35

As funções dos genes candidatos identificados estão relacionadas com os mecanismos de imunidade, processos de reparação de tecidos e cicatrização de feridas. Estas descobertas contribuem para uma compreensão mais aprofundada dos mecanismos subjacentes à doença de podridão dos cascos em ovelhas Merino. No entanto, a resistência/suscetibilidade à podridão dos cascos resulta da interação de muitos genes e é determinada por múltiplos fatores além da constituição genética dos animais.

Tarefa A4.2.- Estudo de associação genómica para a resistência/suscetibilidade a parasitas gastrointestinais

Com vista à identificação de marcadores genéticos associados à resistência/suscetibilidade a parasitas gastrointestinais, para além da amostragem de sangue, foi realizada colheita de fezes no conjunto de animais anteriormente descrito. Posteriormente, para cada animal foram realizadas análises sanguíneas para a determinação do micro-hematócrito, tendo sido também contabilizado o número médio de ovos parasitários por grama (OPG) de fezes (Figura 6A).

De acordo com a literatura e para uma melhor caracterização da carga parasitária, foram estabelecidas 6 categorias: (1) OPG ≤ 100, (2)100 < OPG ≤ 200, (3) 200 < OPG ≤ 500, (4) 500 < OPG ≤ 1000, (5) 1000 < OPG ≤ 2000, (6) 2000 < OPG (Figura 6B).

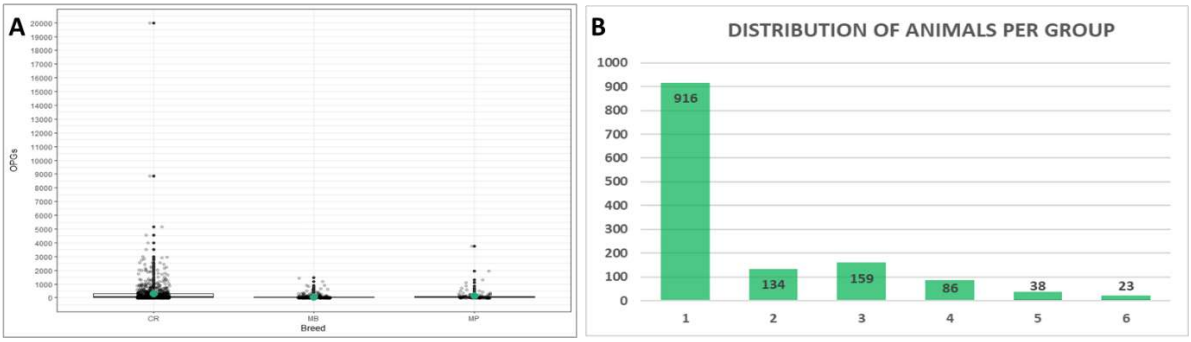


Figura 8 (A) Distribuição dos valores de OPG por raça. CR – Cruzados; MB – Merino Branco; MP – Merino Preto; (B)
Figura 6 Distribuição dos valores de OPG por raça. CR – Cruzados; MB – Merino Branco; MP – Merino Preto

Relativamente aos valores do micro-hematócrito, foram estabelecidas 3 categorias: 1 (X < 27), 2 (27 ≤ X ≤ 45), 3 (45 > X). Esta classificação permitiu agrupar os animais com valores abaixo (1), dentro (2) e acima (3) dos valores normais (Tabela 5).

Tabela 5 Distribuição dos animais por categoria de micro-hematócrito (Ht)

Grupo	Ht	Animais
1	X < 27	251
2	27 ≤X ≤ 45	1061
3	45 > X	16

Em relação aos dados genotípicos, para assegurar a sua qualidade foram aplicados os filtros descritos anteriormente para a identificação de marcadores genéticos para a peeira. Diversas abordagens foram aplicadas para efeitos de comparação, sendo duas as mais promissoras. Na primeira delas consideram-se apenas as 6 categorias de OPG como fenótipo de interesse; enquanto na segunda, para além de considerar as 6 categorias de OPG, foram considerados também as categorias definidas para os hematócritos como fenótipo de interesse. Para todas as

abordagens, a análise de associação foi realizada usando o package do R "StatgenGWAS", seguindo um modelo linear misto. Foram considerados como efeitos fixos a raça, idade, rebanho e o mês da visita. O cálculo da significância foi estimado usando a correção de Bonferroni, tendo sido estabelecido um threshold para a identificação de SNPs significativos (p-value = 2.06 × 10−6). Adicionalmente, foi também considerado um threshold sugestivo (p-value = 4.13 x x10-5).

Da análise dos resultados apresentados, verificou-se consistência entre estas duas abordagens. Em ambas foi identificado o mesmo SNP significativo no cromossoma 16 (14.439.999 pb). Sendo que, foram ainda identificados dois SNPs sugestivos em comum nos cromossomas 3 (142.391.546 pb) e 16 (19.366.442 pb). Adicionalmente, 3 SNPs sugestivos foram identificados na primeira abordagem nos cromossomas 1 (97.515.162 pb), 7 (1.050.994 pb) e 25 (8.623.199 pb) e 1 SNP sugestivo na segunda abordagem no cromossoma 12 (68.278.650) (Tabela 6).

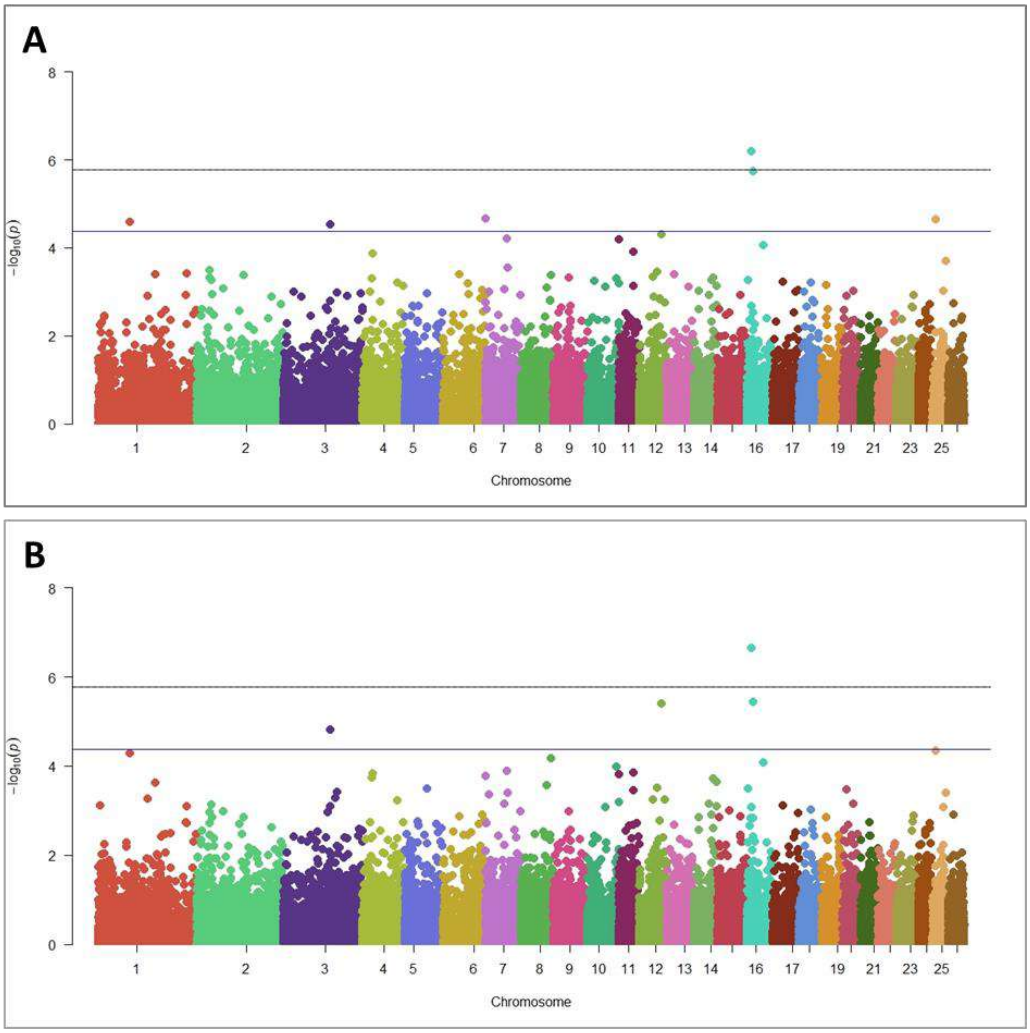


Figura 9 (A) Resultados da primeira abordagem promissora da análise de associação para os parasitas gastrointestinais. (B) Resultados da segunda abordagem promissora da análise de associação para os parasitas gastrointestinais O eixo vertical apresenta o valor -Log10 dos p-values, enquanto o eixo horizontal apresenta os cromossomas e a posição dos SNPs. As linhas vermelha e azul indicam o threshold de significância e sugestividade, respetivamente.

A análise funcional preliminar dos resultados obtidos revelou que as funções dos genes candidatos identificados estão relacionadas com mecanismos regulatórios, de sinalização de inflamações, reparação e cicatrização da mucosa do trato intestinal.

Tabela 6 Sumário dos SNPs significativos e sugestivos identificados como sendo associados à resistência/suscetibilidade a parasitas gastrointestinais nas duas abordagens.

Chr	Position (bp)	Gene
16	14,439,999	CENPK
16	19,366,442	NDUFAF2
12	68,278,650	RGS8
7	1,050,994	APC
3	142,391,546	Intergenic
25	8,623,199	ERO1B
1	97,515,162	NGF

Estes resultados preliminares contribuem para uma compreensão mais aprofundada dos mecanismos subjacentes à resistência/suscetibilidade a parasitas gastrointestinais. No entanto, a resistência/suscetibilidade a parasitas gastrointestinais resulta da interação de múltiplos genes e é determinada por múltiplos fatores além da constituição genética dos animais.

Uma análise mais aprofundada dos diferentes conjuntos de resultados gerados pelas diversas abordagens está a ser finalizada, estando já em preparação o manuscrito para submissão numa revista científica com revisão por pares.

2.3. Gestão do projeto

As atividades de gestão do projeto consistiram essencialmente na gestão de tarefas, reuniões de acompanhamento de atividades, preparação de processos de adquisição, gestão de recursos humanos, aquisição de consumíveis, orientação científica e atividades de divulgação.

Figura 10.- Fotografia do cartaz de divulgação sobre o financiamento do projeto.

Todas as instituições beneficiárias – ACOS, CEBAL, INIAV, e Universidade de Évora incluíram a divulgação do projeto nas suas páginas web oficiais:

- Gen-Res-Alentejo - INIAV
- Gen-Res_ Alentejo - Universidade de Évora
- Gen-Res-Alentejo - CEBAL
- Gen-Res-Alentejo - ACOS

Adicionalmente foi criado uma página web do projeto para uma melhor divulgação das atividades desenvolvidas e dos resultados obtidos no contexto do mesmo.

http://www.gen-res-alentejo.pt/

Figura 11.- Imagem da página inicial do site do projeto

2.4. Recursos Humanos

A operação contou com vários Recursos humanos próprios e bolseiros, cuja dedicação de tempo representou, de forma geral, o inicialmente previsto, salvaguardando as necessárias alterações exigidas pelo prolongamento excecional da execução da operação.

Recursos Humanos Próprios

Por parte da ACOS foram imputados à operação Claudino Matos, Helena Monteiro, João Santos e Miguel Madeira. Por parte do CEBAL esteve o investigador doutorado Marcos Ramos. Na Universidade de Évora foram imputados ao Projecto Elisa Bettencourt, Ludovina Padre, Sandra Branco, Pedro Henriques,Ricardo Romão. Finalmente, no INIAV esteve afeto à operação o investigador doutorado Nuno Carolino.

Todos os funcionários do projeto mantiveram ou aumentaram a dedicação de tempo inicialmente prevista e refletida nas "time-sheets" de cada membro da equipa.

Bolseiros

Por parte do CEBAL esteve imputado à operação o bolseiro **Hugo Magalhães** por um período de 33 meses.

O bolseiro esteve envolvido nos trabalhos de seleção dos animais para sequenciação assim como nas análises genómicas e bioinformáticas nas quais participou nas tarefas de identificação de SNPs nos genomas de ovelhas Merino, assim como na seleção dos mesmos para a criação do painel de SNPs para a posterior genotipagem.

Da Universidade de Évora foi imputado à operação a bolseira **Clara Dias** por um período de 18 meses.

A bolseira acompanhou as acções de campo nas explorações do Alentejo tendo sido responsável pela recolha de amostras de campo e informatização de dados do projecto.

Da ACOS, dada a natureza do projecto, foi contratado o médico veterinário **Lino Tábuas** por um período de 3 anos.

Este técnico acompanhou todos os trabalhos de recolha e processamento dos vários tipos de amostras dos animais nas várias explorações selecionadas.

3. Indicadores do Projeto

3.1. Patentes

Patentes Europeias

1. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. "Method for genetic selection of sheep with increased resistance to footrot". Ref: EP21194901

2. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. "Method for the prognosis of sheep footrot". Ref: EP21194900

3. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. "Method for sheep selection based on parasite resistant genotypes". Ref: EP21194909

Patentes Nacionais

1. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. "Method for genetic selection of sheep with increased resistance to footrot". Ref: 117397

2. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. "Method for the prognosis of sheep footrot". Ref: 117396

3. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. "Method for sheep selection based on parasite resistant genotypes". Ref: 117398

3.2. Novos Investigadores

Entidade	Pessoa	%	Início	Fim	Meses	ETIs
CEBAL	Hugo Magalhães	100	01/12/2017	31/08/2020	33	0.92
U. Évora	Clara Dias	100%	01/01/2017	30/06/2018	18	0.5
ACOS	Lino Tábuas	100%	24/10/2016	24/10/2019	36	1.0
TOTAL						**2.4**

3.3. Publicações

Artigos em revistas de circulação internacional com arbitragem científica

1. Albuquerque, C., Cavaco, S., Caetano, P., Branco, S., Monteiro, H., Ramos, M., Usié, A., Leão, C. & Botelho, A. (2022). Ovine footrot in Southern Portugal: Detection of Dichelobacter nodosus and Fusobacterium necrophorum in sheep with different lesion scores. *Veterinary Microbiology*, 266, 109339.
2. Gaspar, D., Usié, A., Leão, C., Guimarães, S., Pires, A. E., Matos, C., Ramos, A.M. & Ginja, C. (2023). Genome-wide assessment of the population structure and genetic diversity of four Portuguese native sheep breeds. *Frontiers in Genetics*, 14, 7.
3. Usié, A., Leão, C., Gaspar, D., Monteiro, H., Tábuas, L., Bettencourt, E., Caetano, P., Padre, L., Carolino, N., Ramos, A.M., de Matos, C. & Branco, S. (2023). A metagenomics approach to characterize the footrot microbiome in Merino sheep. *Veterinary Microbiology*, 281, 109745.
4. Gaspar, D., Ginja, C., Carolino, N., Leão, C., Monteiro, H., Tábuas, L., Branco, S., Padre, L., Caetano, P., Matos, C., Ramos, A.M., Bettencourt, E. & Usié, A. Genome-wide association study identifies genetic variants underlying footrot in Portuguese Merino Sheep. *Under Second revision - BMC Genomics journal*.
5. Gaspar, D., Ginja, C., Carolino, N., Leão, C., Monteiro, H., Tábuas, L., Branco, S., Padre, L., Caetano, P., Bettencourt, E., Matos, C., Ramos, A.M., & Usié, A. Genome-wide analysis of resistance to gastrointestinal parasites in Portuguese Merino sheep*. Under preparation*

Apresentações Orais em Congressos Internacionais

1. Carolino N., Monteiro M., Madeira M., Santos J., Tábuas L., Branco S., Bettencourt E., Ludovina P., Romão R., Caetano P., Damião P., Dias C., Bettencourt C., Ramos A.M. & Matos C (2019) "Parâmetros genéticos da peeira em ovinos das raças Merina Branca e Merina Preta em Portugal". XX Simpósio Iberoamericano sobre Conservação e Utilização de Recursos Zoogenéticos Corumbá, 11 a 14 novembro, Mato Grosso do Sul (Brasil).
2. Usié, A., Leão, C., Gaspar, D., Botelho, A., Cavaco, S., Monteiro, M., Madeira, M., Santos, J., Tábuas, L., Branco, S., Bettencourt, E., Padre, L., Romão, R., Caetano, P., Damião, P., Dias, C., Carolino, N., Bettencourt, C., Matos, C. & Ramos, A.M. (2020). Analyses of the sheep footrot microbiome using whole-metagenome sequencing. EAAP – 71st anual Meeting. Virtual Meeting
3. Gaspar, D., Usié, A., Leão, C., Matos, C., Padre, L., Dias, C., Ginja, C. & Ramos, A.M. (2021). Unveiling genomic regions that underlie footrot resistance in Portuguese sheep Merino. ISAG 2021 – 38th International Society for Animal Genetics Conference, Virtual Meeting.

4. Usié, A., Leão, C., Gaspar, D., Monteiro, H., Tábuas, L., Branco, S., Bettencourt, E., Caetano, P., Padre, L., Carolino, N., Ramos, A.M. & Matos, C. (2022). Characterization of the footrot microbiome in Portuguese sheep breeds through metagenomics. III Congresso Luso-Espanhol de Pecuária Extensiva e Desenvolvimento Rural: Sustentabilidade Garantida, 1-2 dezembro, Cáceres (Espanha).
5. Gaspar, D., Usié, A., Leão, C., Matos, C., Ramos, A.M. & Ginja C. (2022). Whole-genome analysis of diversity and population structure in Portuguese native sheep breeds. 73º EAAP - European Federation of Animal Science, 5-9 setembro, Porto (Portugal).
6. Gaspar D., Bruno-de-Sousa C., Dias-de-Oliveira A., Oliveira F., Guerreiro R., Pires A.E., Matos C., Usié A. & Ginja C. (2023). Estudio genómico de la raza ovina autóctona portuguesa churra algarvia. XXIV Simposio Iberoamericano CONBIAND sobre conservación y utiliación de recursos zoogenéticos, 2-6 outubro Veracruz, Ver. (México).

Apresentações Orais em Congressos e Eventos Nacionais

1. Monteiro, H. (2017) "Utilização de Metodologias Genómicas na Selecção de Ovinos Resistentes à Peeira e a Parasitas Gastrointestinais na Região do Alentejo". OviBeja, 27 abril, Beja (Portugal).

2. Equipa projeto (2017) "Utilização de Metodologias Genómicas na Selecção de Ovinos Resistentes à Peeira e a Parasitas Gastrointestinais na Região do Alentejo". Feira da Luz – Expomor, 30 agosto a 4 setembro, Montemor O Novo (Portugal).

3. Monteiro, H. (2017) "Utilização de Metodologias Genómicas na Selecção de Ovinos Resistentes à Peeira e a Nematodes Gastrointestinais na Região do Alentejo". V Jornadas Técnico-Veterinárias do Campo Branco, 17 e 18 novembro, Campo Branco (Portugal)

4. Branco, S. (2018) "GEN-RES-ALENTEJO - Utilização da Genómica na Selecção de Ovinos Resistentes a Parasitas Gastrointestinais e Peeira no Alentejo (GEN-RES-ALENTEJO - ALT20-03-0145-FEDER-000037)". VI jornadas Técnico – Veterinárias do Campo Branco, 24 novembro, Campo Branco (Portugal).

5. Caetano, P. (2019) "GEN-RES - Alentejo - Caracterização epidemiológica da peeira ovina na região". XX Jornadas da Associação Portuguesa de Buiatria, 8 e 9 novembro, Ponta Delgada (Portugal).

6. Branco, S., Padre, L., Dias, C., Albuquerque, C., Leão, C., Cavaco, S. & Botelho, A. (2019) "GEN-RES-ALENTEJO - Caracterização bacteriológica da peeira e do parasitismo gastrointestinal em ovinos na região do Alentejo". VI jornadas Técnico – Veterinárias do Campo Branco, 24 novembro, Campo Branco (Portugal).

7. Gaspar, D., Magalhães, H., Usié, A., Leão, C., Monteiro, M., Madeira, M., Santos, J., Tábuas, L., Branco, S., Bettencourts, E., Padre, L., Romão, R., Caetano, P., Damião, P., Dias, C., Carolino, N., Bettencourt, C., Ginja, C., Matos, C. & A.M. Ramos. (2020). Characterization of genomic variation in Portuguese sheep breeds using whole genome resequencing. Bioinformatics Open Days, Braga (Portugal).

8. Usié, A. (2022). Caracterização do microbioma da peeira através da utilização de novas tecnologias de sequenciação. Seminário Do diagnóstico clínico da peeira à bioinformática: desafios e oportunidades. Patrimónios do Sul, 30 setembro, Beja (Portugal).
9. Gaspar, D. (2022). Utilização de ferramentas genómicas para a identificação de marcadores moleculares associados a resistência a peeira. Seminário Do diagnóstico clínico da peeira à bioinformática: desafios e oportunidades. Patrimónios do Sul, 30 setembro, Beja (Portugal).
10. Usié, A. (2023). Utilização da genómica no estudo da peeira ovina no Alentejo. OviCapri - V Simpósio de Ovinos e Caprinos, 24 novembro, Vila Real (Portugal)

Comunicações em formato de Poster em Congressos Internacionais

1. Padre, L., Romão, R., Branco, S., Monteiro, H., Bettencourt, E., Bettencourt, C., Tábuas, L., Dias, C., Carolino, N., Henriques, P. & Matos, C. (2018) "Avaliação da resistência e do efeito do parasitismo gastro-intestinal nas raças merina branca e merina preta no Alentejo, Portugal". XI Congresso Ibérico sobre Recursos Genéticos Animales, 26 a 28 setembro, Murcia (Espanha).

2. Gaspar, D., Magalhães, H., Usié, A., Leão, C., Ginja, C., Matos, C. & Ramos, A.M. (2020). Genomic characterization of Portuguese native sheep breeds. EAAP – 71st annual Meeting. Virtual Meeting.

3. Gaspar, D., Usié, A., Leão, C., Matos, C., Padre, L., Dias, C., Ginja, C. & Ramos, A.M. (2021). Unveiling genomic regions that underlie footrot resistance in Portuguese sheep Merino. ISAG 2021 – 38th International Society for Animal Genetics Conference. Virtual Meeting.

Comunicações em formato de Poster em Congressos Nacionais

1. Costa, B. (2017) "Utilização de Metodologias Genómicas na Selecção de Ovinos Resistentes à Peeira e a Parasitas Gastrointestinais na Região do Alentejo". XIX Jornadas Da Associação Portuguesa De Buiatria, 3 a 5 novembro, Ponta Delgada (Portugal).
2. Caetano, P., Branco, S., Monteiro, H., Bettencourt, E., Dias, C., Tábuas, L., Matos, C., Henriques, P. (2017) "Identificação de fatores de risco para a ocorrência de peeira em explorações de ovinos na região Alentejo". XI Jornadas do Hospital Veterinário Muralha de Évora, 2 e 3 março, Évora (Portugal).
3. Gaspar, D., Usié, A., Magalhães, H., Leão, C., Matos, C., Ramos, A.M & Ginja, C. (2021). Genome-wide diversity and population structure analysis of four Portuguese native sheep breeds. XXII Congresso Nacional de Zootecnia, 29 e 30 Outubro. Virtual Meeting.
4. Gaspar, D., Usié, A., Magalhães, H., Leão, C., Matos, C., Ramos, A.M & Ginja, C. (2021) Unravelling the genetic diversity and population structure of four Portuguese native sheep breeds. EEDAA - VI Encontro de Estudantes de Doutoramento em Ambiente e Agricultura. Virtual Meeting.
5. Gaspar, D., Magalhães, H., Mendes, B., Leão, C., Meireles, B., Barbosa, P., Cachucho, L, Albuquerque, A., Charneca, R., Martins, J., Jerónimo, E., Matos, J., Simões, F., Genosuber Consortium, Marum, L., Branco, S., Carolino, N., Matos, C., Ginja, C., Ramos, A.M. & Usié, A. (2023). Role of different "omics" as powerful tools to face climate change and enhance breeding programs. Science Changing Policy, 2 junho, Évora (Portugal). Book of Abstracts pp 93

Organização de Eventos

1. Workshop "Peeira – Diagnostico e Prevenção, com componente prática" (2019). Universidade de Évora – Polo da Mitra, 1 março, Évora (Portugal)
2. Workshop "Estrongiloses gastrointestinais em pequenos ruminantes - Diagnóstico e controlo" (2019). OviBeja, 27 abril, Beja (Portugal).
3. Seminário "Do diagnóstico clínico da peeira a bioinformática: desafios e oportunidades" (2022). Patrimónios do Sul, 30 setembro, Beja (Portugal).

Teses mestrado

1. Albuquerque, C. (2020) “Detection and characterization of *Dichelobacter nodosus* from sheep with different clinical manifestations of Ovine footrot”. Faculdade de Ciencias, Universidade de Lisboa.

Teses doutoramento

1. Caetano, P. (2021) “Caraterização da Peeira ovina na região do Alentejo”. Universidade de Évora.
2. Gaspar, D. (expected 2024) “Genomic and bioinformatics methodologies for the identification of genetic markers in sheep”. Universidade do Porto

Patentes Europeias

4. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. “Method for genetic selection of sheep with increased resistance to footrot”. Ref: EP21194901
5. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. “Method for the prognosis of sheep footrot”. Ref: EP21194900
6. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. “Method for sheep selection based on parasite resistant genotypes”. Ref: EP21194909

Patentes Nacionais

1. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. “Method for genetic selection of sheep with increased resistance to footrot”. Ref: 117397
2. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. “Method for the prognosis of sheep footrot”. Ref: 117396
3. Matos C, Ramos AM, Usié A, Gaspar D, Monteiro MH, Leão C, Carolino RN, Branco S, Padre L, Bettencourt EM, Henriques PD. 2021. “Method for sheep selection based on parasite resistant genotypes”. Ref: 117398

3.4. Material de divulgação

Flyer e roll-up do projeto

Figura 12.- De esquerda a direita: 1) Frente flyer; 2) Verso flyer

Figura 13.- Imagem do roll-up

4. Execução Financeira

ACOS – Associação de Agricultores do Sul

Designação da Componente	Elegível Aprovado da Componente	Elegível Validado da Componente	Execução
Despesas com Pessoal	185 027,70	185 027,70	100,00%
Publicidade e Divulgação	0,00	0 €	0%
Aquisição de bens	33 152,17	32 612,89	98,37%
Deslocações e Estadas	2 500,00	0	
Estudos, pareceres e consultadoria	179 873,75	172 751,41	96,04%
Seminários, Exposições e Similares	1 000,00	0,00 €	0,00%
Equipamento básico	4 500,00	2 668,65 €	59,30%
Outras despesas	101 378,58	95 000,23	93,71%
Seguros	0	0 €	0%
Total	**507 432,20**	**488 060,88**	**96,18%**

CEBAL – Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo

Designação da Componente	Elegível Aprovado da Componente	Elegível Validado da Componente	Execução
Despesas com Pessoal	78 960,97 €	75 592,24 €	95,73%
Publicidade e Divulgação	2 000,00 €	0 €	0 %
Aquisição de bens	5 689,03 €	3 086,29 €	54,25%
Deslocações e Estadas	3 700,00 €	1 712,99 €	46,30%
Seminários, Exposições e Similares	1 500,00 €	80,00 €	5,33%
Outras despesas	23 250,00 €	19 424,83 €	83,55%
Seguros	1 150,00	0 €	0%
Total	**116 250,00 €**	**99 896,35 €**	**85,93 %**

Instituto Nacional de Investigação Agrária e Veterinária – INIAV I.P.

Designação da Componente	Elegível Aprovado da Componente	Elegível Validado da Componente	Execução
Despesas com Pessoal	17 482,98	17 482,97	100,00%
Publicidade e Divulgação			
Aquisição de bens			
Deslocações e Estadas	517,03	517,03	100,00%
Estudos, pareceres e consultadoria			
Seminários, Exposições e Similares			
Equipamento básico			
Outras despesas	4 500,00	4 500,00	100,00%
Seguros			
Total	**22 500,01**	**22 500,00**	**100,00%**

Universidade de Évora

Designação da Componente	Elegível Aprovado da Componente	Elegível Validado da Componente	Execução
Despesas com Pessoal	56 070,95	56 070,95	100,00%
Publicidade e Divulgação	672,16	672 €	100,00%
Aquisição de bens	4 578,12	4 578,12	100,00%
Deslocações e Estadas	3 469,09	3 469,09	
Estudos, pareceres e consultadoria	0,00	0,00	
Seminários, Exposições e Similares	0,00	0,00 €	
Equipamento básico	3 333,80	3 333,80 €	100,00%
Outras despesas	17 031,03	17 031,03	100,00%
Seguros	0	0 €	0%
Total	**85 155,15**	**85 155,15**	**100,00%**

Global

	Elegível Aprovado	Elegível Validado	Execução
ACOS	507 532,20	488 060,88	96,16%
CEBAL	116250,00	99896,35	85,93%
INIAV	22 500,01	22 500,00	100,00%
Universidade de Évora	85 155,15	85 115,15	99,95%
Total	**731 437,36**	**695 572,38**	**95,10%**

5. ANEXOS

Anexo I – Documentação relativa às patentes

Patentes Europeias

1. "Method for genetic selection of sheep with increased resistance to footrot". Ref: EP21194901

2. "Method for the prognosis of sheep footrot". Ref: EP21194900

3. "Method for sheep selection based on parasite resistant genotypes". Ref: EP21194909

Patentes Nacionais

1. "Method for genetic selection of sheep with increased resistance to footrot". Ref: 117397

2. "Method for the prognosis of sheep footrot". Ref: 117396

3. "Method for sheep selection based on parasite resistant genotypes". Ref: 117398

Anexo II – Publicações em revistas internacionais com arbitragem científica

Anexo III – Comunicações orais

Anexo IV – Comunicações em formato de Poster

Anexo V – Eventos/Workshops organizados

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Nº	CÓDIGO	DATA E HORA DE RECEÇÃO	MODALIDADE	PROCESSO RELACIONADO
20211000031152	0198	2021/08/13-17:01:58	PAT	117397 D

PAGAMENTO CONFIRMADO

PEDIDO DE PATENTE, MODELO DE UTILIDADE OU DE TOPOGRAFIA DE PRODUTOS SEMICONDUTORES

1 REQUERENTES

Código **Nacionalidade** PORTUGUESA
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Tipo de Representação Agente Oficial da Propriedade Industrial ou Procurador Autorizado
Nome RICARDO JORGE DINIS ABRANTES **Código** 600054
Exclusivo para este ato? NÃO

2 MODALIDADE / TIPO DE PEDIDO

Modalidade: PEDIDO PROVISÓRIO DE PATENTE
Realização de pesquisa pelo INPI: SIM

3 EPÍGRAFE OU TÍTULO

METHOD FOR GENETIC SELECTION OF SHEEP WITH INCREASED RESISTANCE TO FOOTROT

4 RESUMO

5 FIGURAS

6 INVENTORES

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7 REIVINDICAÇÃO DE PRIORIDADE

8 DOCUMENTOS ANEXOS

DOCUMENTO DO PEDIDO PROVISÓRIO DE PATENTE (20210813 Texto Depositado 2020_44679.pdf)

9 OBSERVAÇÕES

10 TAXAS

Taxa	Importância
PEDIDO PROVISÓRIO DE PATENTE	10,78 €
PESQUISA EM PEDIDO PROVISÓRIO DE PATENTE	53,87 €
Total:	64,65 €
Por Extenso:	SESSENTA E QUATRO EUROS E SESSENTA E CINCO CÊNTIMOS

11 PAGAMENTO

Tipo de Pagamento	Débito em Conta
Banco	MILLENNIUM BCP
NIB	PT50003300000000473990605
Montante	64,65 €
Débito a partir de	01-09-2021

12 ASSINATURA DO REQUERENTE OU MANDATÁRIO/REPRESENTANTE LEGAL

Assinatura/Nome Ricardo Jorge Dinis Abrantes
Nº B.I. 209863447 **Data** 2021/08/13

Atenção: Os dados relativos ao nome e morada serão publicados no Boletim da Propriedade Industrial, de acordo com o previsto no Código da Propriedade Industrial, aprovado pelo Decreto-Lei n.º 36/2003, de 5 de Março, ficando também incluídos nas bases de dados de marcas e patentes disponibilizadas neste portal.
Se desejar que a morada não seja conhecida pode optar por indicar um Apartado Postal.
Caso o requeira, poderá também aceder e retificar os seus dados. Para mais informações consulte a política de privacidade deste portal.

ABSTRACT

"METHOD FOR GENETIC SELECTION OF SHEEP WITH INCREASED RESISTANCE TO FOOTROT"

The present patent application discloses a method for the selection of sheep based on their footrot resistance profile. Said footrot resistance profile is determined based on a group of Single Nucleotide Polymorphisms (SNPs) associated with lower scores for footrot.

DESCRIPTION

"METHOD FOR GENETIC SELECTION OF SHEEP WITH INCREASED RESISTANCE TO FOOTROT"

Technical field

The present patent application relates to a method for the genetic selection of sheep based on their resistance to footrot.

Background art

Footrot is one of the main causes of lameness in sheep and represents a major animal welfare problem due to the painful nature of the lesions. This disease is caused by infection with *Dichelobacter nodosus* (*D. nodosus*), causing painful inflammation, necrotizing lesions of the interdigital skin, characteristic odor, and hoof detachment. Footrot is a multifactorial disease and its severity depends on the breed of sheep, farm management, environmental factors, the presence of co-infecting bacteria, and the virulence of the *D. nodosus* strains involved. In addition, the bacterium *Fusobacterium necrophorum* (*F. necrophorum*) has also been suggested as a secondary pathogen in the development of the disease and probably in increasing its severity. The synergistic relationship between *F. necrophorum* and *D. nodosus* is not yet fully explained, but it appears that their combined action is involved in the development and severity of wheal.

In sheep, there is variation in the individual response of each animal to the footrot. Under the same environmental conditions, for example, on the same farm, some animals are severely affected by the disease, while others show no symptoms and seem unaffected. This clearly indicates the

possibility of genetic control of footrot, in particular the severity with which each animal is affected. To date, no genetic markers for the footrot have been identified in Portuguese sheep breeds.

Summary

The present patent application relates to a method for the selection of sheep based on footrot resistance genotypes.

The method for sheep selection based on their footrot resistance genotype comprises the following steps:

Preparation of a sheep biological sample and extraction of DNA from said sample;

Genotyping the DNA sample for at least one of the following Single Nucleotide Polymorphisms (SNPs),

Chromosome	Position	Exonic alteration
NC_040254.1	14627723	-
NC_040254.1	78539086	-
NC_040254.1	237741074	synonym
NC_040263.1	62286092	-
NC_040266.1	71702732	-
NC_040269.1	21731867	synonym
NC_040276.1	24122307	-
NC_040276.1	27418667	-
NC_040259.1	13386681	-
NC_040278.1	50565062	-

Determining sheep's resistance to footrot based on the presence of at least one of said SNPs significantly associated with a lower score for footrot.

In one embodiment, the sample analyzed is preferably a blood sample.

In another embodiment, the above-mentioned Single Nucleotide Polymorphyms (SNPs) are used as biomarkers for footrot in sheep.

Detailed Description

The present patent application discloses a method for the selection of sheep based on their resistance to footrot. Said resistance to footrot is determined based on a group of Single Nucleotide Polymorphisms (SNPs) disclosed herein which are associated with lower scores of footrot in sheep.

The technology disclosed herein is based on the sequencing of genomes of animals of the breeds that will later be used in genome-wide association studies. This step of genome sequencing is essential to characterize in great detail all the type of variation present in the animals studied. The next step involves the selection of a set of SNPs to be genotyped in a larger number of animals, which in this case was 1500.All genotyped animals were also characterized from the phenotypic point of view, namely in the determination of the lameness score observed in each animal's paw, in two distinct visits made on the same farm, and spaced temporally for a period of approximately 3 months.
After all animals were genotyped, a genome-wide association study was performed, where the statistical significance of the association observed between each genotyped SNP and the phenotypic value of the wheal score was determined. This

significance was determined after a correction for multiple testing, an essential step in this type of analysis.
For each of the significant SNPs the observed mean for each of the genotypes is determined. The difference observed is the potential phenotypic gain that can be achieved if animals carrying the genotypes associated with a lower litter score are genetically selected.

The 10 SNPs identified are described in Table 1.

Chromosome	Position	P-Value	Gene ID	Genomic Localization	Exonic alteration
NC_040254.1	14627723	0.0003312	gene-DAB2IP	intron	-
NC_040254.1	78539086	0.0005151	gene-NRXN1	intron	-
NC_040254.1	237741074	0.000126	gene-CELSR1	exon	synonym
NC_040263.1	62286092	0.0005151	gene-ASTN1	intron	-
NC_040266.1	71702732	0.0005477	gene-LDLRAD3	intron	-
NC_040269.1	21731867	0.0005151	gene-ADAMTSL3	exon	synonym
NC_040276.1	24122307	0.0001633	gene-CTNNA3	intron	-
NC_040276.1	27418667	0.0003593	gene-LRRC20	UTR3	-
NC_040259.1	13386681	0.0002215		intergenic	-
NC_040278.1	50565062	0.000126		intergenic	-

For the 11 SNPs identified, the observed mean values are shown in Table 2.

NC_040254.1	NC_040254.1#14627723	GENO	A/A	A/G	G/G
NC_040254.1	NC_040254.1#14627723	COUNTS	9	121	1229
NC_040254.1	NC_040254.1#14627723	FREQ	0.006623	0.08904	0.9043
NC_040254.1	NC_040254.1#14627723	MEAN	3.961	2.444	1.491
NC_040254.1	NC_040254.1#14627723	SD	2.539	2.854	2.215

NC_040254.1	NC_040254.1#78539086	GENO	T/T	T/C	C/C
NC_040254.1	NC_040254.1#78539086	COUNTS	9	181	1247
NC_040254.1	NC_040254.1#78539086	FREQ	0.006263	0.126	0.8678
NC_040254.1	NC_040254.1#78539086	MEAN	-0.1364	0.8893	1.733
NC_040254.1	NC_040254.1#78539086	SD	0.4949	2.068	2.315
NC_040254.1	NC_040254.1#237741074	GENO	T/T	T/C	C/C
NC_040254.1	NC_040254.1#237741074	COUNTS	4	149	1268
NC_040254.1	NC_040254.1#237741074	FREQ	0.002815	0.1049	0.8923
NC_040254.1	NC_040254.1#237741074	MEAN	2.142	2.615	1.486
NC_040254.1	NC_040254.1#237741074	SD	2.307	2.919	2.174
NC_040259.1	NC_040259.1#13386681	GENO	A/A	A/G	G/G
NC_040259.1	NC_040259.1#13386681	COUNTS	5	99	881
NC_040259.1	NC_040259.1#13386681	FREQ	0.005076	0.1005	0.8944
NC_040259.1	NC_040259.1#13386681	MEAN	0.7679	2.803	1.353
NC_040259.1	NC_040259.1#13386681	SD	1.22	2.94	2.054
NC_040263.1	NC_040263.1#62286092	GENO	G/G	G/A	A/A
NC_040263.1	NC_040263.1#62286092	COUNTS	19	173	1153
NC_040263.1	NC_040263.1#62286092	FREQ	0.01413	0.1286	0.8572
NC_040263.1	NC_040263.1#62286092	MEAN	2.869	2.292	1.457
NC_040263.1	NC_040263.1#62286092	SD	3.486	2.608	2.162
NC_040266.1	NC_040266.1#71702732	GENO	C/C	C/T	T/T
NC_040266.1	NC_040266.1#71702732	COUNTS	160	631	638
NC_040266.1	NC_040266.1#71702732	FREQ	0.112	0.4416	0.4465
NC_040266.1	NC_040266.1#71702732	MEAN	1.147	1.371	1.954
NC_040266.1	NC_040266.1#71702732	SD	1.903	2.157	2.449
NC_040269.1	NC_040269.1#21731867	GENO	A/A	A/G	G/G
NC_040269.1	NC_040269.1#21731867	COUNTS	104	537	776
NC_040269.1	NC_040269.1#21731867	FREQ	0.07339	0.379	0.5476
NC_040269.1	NC_040269.1#21731867	MEAN	2.441	1.794	1.355
NC_040269.1	NC_040269.1#21731867	SD	2.877	2.391	2.077
NC_040276.1	NC_040276.1#24122307	GENO	A/A	A/C	C/C
NC_040276.1	NC_040276.1#24122307	COUNTS	28	312	1076
NC_040276.1	NC_040276.1#24122307	FREQ	0.01977	0.2203	0.7599
NC_040276.1	NC_040276.1#24122307	MEAN	2.356	2.182	1.403
NC_040276.1	NC_040276.1#24122307	SD	2.581	2.655	2.127

NC_040276.1	NC_040276.1#27418667	GENO	T/T	T/C	C/C
NC_040276.1	NC_040276.1#27418667	COUNTS	75	439	876
NC_040276.1	NC_040276.1#27418667	FREQ	0.05396	0.3158	0.6302
NC_040276.1	NC_040276.1#27418667	MEAN	2.416	1.914	1.359
NC_040276.1	NC_040276.1#27418667	SD	2.761	2.503	2.078
NC_040278.1	NC_040278.1#50565062	GENO	G/G	G/A	A/A
NC_040278.1	NC_040278.1#50565062	COUNTS	22	210	1198
NC_040278.1	NC_040278.1#50565062	FREQ	0.01538	0.1469	0.8378
NC_040278.1	NC_040278.1#50565062	MEAN	2.675	2.336	1.454
NC_040278.1	NC_040278.1#50565062	SD	2.368	2.612	2.192

The method of the present patent application involves several steps. Initially, it is necessary to collect a sample of biological material, such as a blood sample, for DNA extraction. Next, the DNA sample is genotyped for the SNPs of interest, and the genotypes obtained for each SNP are evaluated. The decision to select the animal is based on the presence of the genotype(s) significantly associated with a lower score for footrot, i.e. the goal is to select animals with higher resistance to footrot.

The method for sheep selection based on footrot resistance genotypes comprises the following steps:

Preparation of a sheep biological sample and extraction of DNA from said sample;

Genotyping the DNA sample for at least one of the following Single Nucleotide Polymorphisms (SNPs),

Chromosome	Position	Exonic alteration
NC_040254.1	14627723	-
NC_040254.1	78539086	-

NC_040254.1	237741074	synonym
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NC_040269.1	21731867	synonym
NC_040276.1	24122307	-
NC_040276.1	27418667	-
NC_040259.1	13386681	-
NC_040278.1	50565062	-

Determining sheep's resistance to footrot based on the presence of at least one of said SNPs significantly associated with a lower score for footrot.

In one embodiment, the sample analyzed is preferably a blood sample.

CLAIMS

1. Method for sheep selection based on footrot resistance genotypes comprising the following steps:

- Preparation of a sheep biological sample and extraction of DNA from said sample;
- Genotyping the DNA sample for at least one of the following Single Nucleotide Polymorphisms (SNPs),

Chromosome	Position	Exonic alteration
NC_040254.1	14627723	-
NC_040254.1	78539086	-
NC_040254.1	237741074	synonym
NC_040263.1	62286092	-
NC_040266.1	71702732	-
NC_040269.1	21731867	synonym
NC_040276.1	24122307	-
NC_040276.1	27418667	-
NC_040259.1	13386681	-
NC_040278.1	50565062	-

- Determining sheep's resistance to footrot based on the presence of the at least one of said SNPs significantly associated with a lower score for footrot.

2. Single Nucleotide Polymorphyms (SNPs) as defined in claim 1 for use as biomarkers of footrot resistance in sheep.

Europäisches Patentamt
European Patent Office
Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	10158904
Application number	EP21194901.1
File No. to be used for priority declarations	EP21194901
Date of receipt	03 September 2021
Your reference	2020/44682
Applicant	ACOS - ASSOCIAÇÃO DE AGRICULTORES DO SUL
Country	PT
Title	METHOD FOR GENETIC SELECTION OF SHEEP WITH INCREASED RESISTANCE TO FOOTROT
Documents submitted	package-data.xml application-body.xml SPECEPO-1.pdf\PPP 2021-44679 vdeposito.pdf (9 p.) f1002-1.pdf (2 p.) ep-request.xml ep-request.pdf (6 p.) 1003-1.pdf\Automatic Debit order.pdf (1 p.)
Submitted by	CN=secure.epoline.org
Method of submission	Online
Date and time receipt generated	03 September 2021, 16:30 (CEST)
Message Digest	BE:1D:E2:34:E1:0F:9D:B8:60:C7:87:C6:AF:B5:3C:37:A3:00:DA:55

/European Patent Office/

DAS access code
The access code generated for this application and used to retrieve the priority documents from WIPO's Digital Access Service (DAS) is indicated in the document appended to this acknowledgement of receipt. Please note that the appended document is non-public and will not be published.

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DAS access code

To access and retrieve the priority document from WIPO's Digital Access Service (DAS) in respect of

Application number	EP21194901.1
Applicant	ACOS - ASSOCIAÇÃO DE AGRICULTORES DO SUL

the European Patent Office has generated the following code:

DAS access code	DBE0

For further information, see OJ EPO 03/2019.

Date and time receipt generated	03 September 2021, 16:30 (CEST)

This unique access code allows the applicant to authorise participating intellectual property offices to retrieve a certified copy of the present application (as priority document) via WIPO DAS.
The code will only be valid if the requirements for according a date of filing are met (see OJ EPO 03/2019).

Europäisches Patentamt
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Request for grant of a European patent

For official use only		
1 Application number:	MKEY	
2 Date of receipt (Rule 35(2) EPC):	DREC	
3 Date of receipt at EPO (Rule 35(4) EPC):	RENA	
4 Date of filing:		

5 Grant of European patent, and examination of the application under Article 94, are hereby requested. ☒

Request for examination in an admissible non-EPO language: Solicita-se o exame do pedido segundo o artigo 94º.

5.1 The applicant waives his right to be asked whether he wishes to proceed further with the application (Rule 70(2)) ☐

Procedural language: en

Description and/or claims filed in: en

A translation will be supplied later ☐

6 Applicant's or representative's reference: 2020/44682

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14.1 The/Each applicant hereby declares that he is an entity or a natural person under Rule 6(4) EPC. ☒

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21	A general authorisation has been registered under No:	72020

Inventor(s)

23 Inventor details filed separately ☒

24 Title of invention

Title of invention: METHOD FOR GENETIC SELECTION OF SHEEP WITH INCREASED RESISTANCE TO FOOTROT

25 Declaration of priority (Rule 52)

A declaration of priority is hereby made for the following applications

	State	Filing date	Kind	Application number:	Search results under Rule 141(1) are attached
Priority 01	PT	13.08.2021	pr	117397	☐

25.2 **The EPO is requested to retrieve a certified copy of the following previous application(s) (priority document(s)) via the WIPO Digital Access Service (DAS) using the indicated access code(s):**

	Request	Application number:	Access code
Priority 01	☐	117397	

25.3 This application is a complete translation of the previous application ☐

25.4 It is not intended to file a (further) declaration of priority ☒

26 Reference to a previously filed application

27 Divisional application ☐

28 Article 61(1)(b) application ☐

29 Claims

Number of claims: 2

29.1 ☒ as attached

29.2 ☐ as in the previously filed application (see Section 26.2)

29.3 ☐ The claims will be filed later

30 Figures

It is proposed that the abstract be published together with figure No.

31 Designation of contracting states

All the contracting states party to the EPC at the time of filing of the European patent application are deemed to be designated (see Article 79(1)).

32 Different applicants for different contracting states

33 Extension/Validation

This application is deemed to be a request to extend the effects of the European patent application and the European patent granted in respect of it to all non-contracting states to the EPC with which extension or validation agreements are in force on the date on which the application is filed. However, the request is deemed withdrawn if the extension fee or the validation fee, whichever is applicable, is not paid within the prescribed time limit.

33.1 It is intended to pay the extension fee(s) for the following state(s):

33.2 It is intended to pay the validation fee(s) for the following state(s):

34 Biological material

38 Nucleotide and amino acid sequences

The European patent application contains a sequence listing as part of the description ☐

The sequence listing is attached in computer-readable format in accordance with WIPO Standard ST.25 ☐

The sequence listing is attached in PDF format ☐

Further indications

39 Additional copies of the documents cited in the European search report are requested

Number of additional sets of copies:

40 Refund of the search fee under to Article 9 of the Rules relating to Fees is requested ☐

Application or publication number of earlier search report:

42 Payment

Method of payment	Automatic debit order

The European Patent Office is hereby authorised, under the Arrangements for the automatic debiting procedure, to debit from the deposit account any fees and costs falling due.

Currency:	EUR
Deposit account number:	28140021
Account holder:	Clarke, Modet & Cº

43 Refunds

Any refunds should be made to EPO deposit account:	28140021
Account holder:	Clarke, Modet & Cº

44-A Forms

		Details:	System file name:
A-1	Request		as ep-request.pdf
A-2	1. Designation of inventor	1. Inventor	as f1002-1.pdf

44-B Technical documents

		Original file name:	System file name:
B-1	Specification	PPP 2021-44679 vdeposito.pdf Description; 2 claims; abstract	SPECEPO-1.pdf

44-C Other documents

		Original file name:	System file name:
C-1	1. Specific authorisation	Automatic Debit order.pdf	1003-1.pdf

45

General authorisation:	

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Nº	CÓDIGO	DATA E HORA DE RECEÇÃO	MODALIDADE	PROCESSO RELACIONADO
20211000031151	0198	2021/08/13-16:40:26	PAT	117396 C

PAGAMENTO CONFIRMADO

PEDIDO DE PATENTE, MODELO DE UTILIDADE OU DE TOPOGRAFIA DE PRODUTOS SEMICONDUTORES

1 REQUERENTES

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Exclusivo para este ato? NÃO

2 MODALIDADE / TIPO DE PEDIDO

Modalidade: PEDIDO PROVISÓRIO DE PATENTE
Realização de pesquisa pelo INPI: SIM

3 EPÍGRAFE OU TÍTULO

METHOD FOR THE PROGNOSIS OF SHEEP FOOTROT

4 RESUMO

5 FIGURAS

6 INVENTORES

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7 REIVINDICAÇÃO DE PRIORIDADE

8 DOCUMENTOS ANEXOS

DOCUMENTO DO PEDIDO PROVISÓRIO DE PATENTE (20210813 Texto Depositado 2020_44678.pdf)

9 OBSERVAÇÕES

10 TAXAS

Taxa	Importância
PEDIDO PROVISÓRIO DE PATENTE	10,78 €
PESQUISA EM PEDIDO PROVISÓRIO DE PATENTE	53,87 €
Total:	64,65 €
Por Extenso:	SESSENTA E QUATRO EUROS E SESSENTA E CINCO CÊNTIMOS

11 PAGAMENTO

Tipo de Pagamento	Débito em Conta
Banco	MILLENNIUM BCP
NIB	PT50003300000000473990605
Montante	64,65 €
Débito a partir de	01-09-2021

12 ASSINATURA DO REQUERENTE OU MANDATÁRIO/REPRESENTANTE LEGAL

Assinatura/Nome Ricardo Jorge Dinis Abrantes
Nº B.I. 209863447 **Data** 2021/08/13

Atenção: Os dados relativos ao nome e morada serão publicados no Boletim da Propriedade Industrial, de acordo com o previsto no Código da Propriedade Industrial, aprovado pelo Decreto-Lei n.º 36/2003, de 5 de Março, ficando também incluídos nas bases de dados de marcas e patentes disponibilizadas neste portal.
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ABSTRACT

"METHOD FOR THE PROGNOSIS OF SHEEP FOOTROT"

The present patent application discloses a method for prognosis and characterization of sheep footrot based on the use of data produced using state-of-the-art sequencing techniques to characterize the composition of the microbiome associated with the foot rot in sheep. This characterization is done qualitatively, by identifying the species of bacteria present in the microbiome of each sample collected, but also quantitatively, as the abundance of each species, in each sample analyzed, is also determined. Subsequently, in situations where the observed score for the footrot is available for each animal, it is possible to identify the most important differences between animals not affected by footrot and those more affected by this disease.

DESCRIPTION

"METHOD FOR THE PROGNOSIS OF SHEEP FOOTROT"

Technical field

This application relates to a method for the prognosis of footrot in sheep.

Background art

Sheep footrot is one of the main causes of lameness in sheep and represents a major animal welfare problem due to the painful nature of the lesions. This disease is caused by infection with *Dichelobacter nodosus* (*D. nodosus*), causing painful inflammation, necrotizing lesions of the interdigital skin, characteristic odor, and hoof detachment. Sheep footrot is a multifactorial disease and its severity depends on the breed of sheep, farm management, environmental factors, the presence of co-infecting bacteria, and the virulence of the *D. nodosus* strains involved. In addition, the bacterium *Fusobacterium necrophorum* (*F. necrophorum*) has also been suggested as a secondary pathogen in the development of the disease and probably in increasing its severity. The synergistic relationship between *F. necrophorum* and *D. nodosus* is not yet fully explained, but it appears that their combined action is involved in the development and severity of footrot.

Although the involvement of these two bacterial species is well established, the universe of bacterial species present is much more comprehensive, and no information is available on the composition and abundance of the remaining species present in this microbiome. For this reason, it is important to have available a method that can accurately decipher the bacterial species present, as well as their abundance, so

that the main differences in the microbiome between animals with contrasting phenotypes for the degree of footrot development can be evaluated.

Summary

The present patent application relates to a method for prognosis of footrot in sheep.

The method for prognosis of sheep footrot disclosed herein comprises the following steps:

- Preparation of the skin biopsy sample from an animal to be evaluated;
- Detecting the microbiome signature present in the biopsy sample comprising the genera
    - I) *Mycoplasma*, *Campylobacter*, *Streptococcus*, *Clostridium*, *Arcobacter*, *Fusobacterium (10 species)*, *Capnocytophaga*, *Treponema*, *Leptotrichia*, and *Vibrio*; and/or
    - II) *Streptomyces*, *Pseudomonas*, *Mycolicibacterium*, *Deinococcus*, *Brevundimonas*, *Micromonospora*, *Rhodococcus*, *Staphylococcus*, *Mycobacterium* and *Gordonia*;

  Concluding on footrot development probability in said animal, wherein an increase of abundance of the genera in I) over the genera in II) as detected in said sample is indicative for footrot.

Detailed Description

The present patent application discloses a method for prognosis of sheep footrot based on the use of data produced using state-of-the-art sequencing techniques to characterize the composition of the microbiome associated with the foot

rot in sheep. This characterization is done qualitatively, by identifying the species of bacteria present in the microbiome of each sample collected, but also quantitatively, as the abundance of each species, in each sample analyzed, is also determined. This method thus allows a detailed characterization of the microbiome present in each animal. Subsequently, in situations where the observed score for the footrot is available for each animal, it is possible to identify the most important differences between animals not affected by footrot and those more affected by this disease.

According to the method disclosed herein the sequencing data is first processed to remove the lowest quality sequences. A taxonomic classification is then performed to determine the number of species present, followed by an estimate of the abundance of each of these species in each of the animals analyzed. In situations where microbiomes have been collected from animals with contrasting scores for the footrot, a statistical analysis is performed to determine the number of species with differential abundance between animals affected and unaffected by the footrot.
In the data analyzed to develop this technology, 213 animals were included. In each animal a skin/tissue biopsy was collected from one of the legs, biological material from which DNA extraction was performed. On average, for each animal 545,000 sequences were used in the analyses. The results of the taxonomic classification revealed the presence of 64 phyla and 505 families of bacteria. For each footrot score (FS), at the phylum level the results obtained were similar. The most abundant phylum was *Actinobacteria* in samples with FS0 and FS1 scores (29.7% and 25.6%, respectively), *Proteobacteria* in samples with FS2 and FS3

scores (25.6% and 22.8%, respectively), *Fusobacteria* in samples with FS4 score (29.9%), and Bacteroides in samples with FS5 score (29.8%). Regarding the families identified, *Hominidae* was the most dominant in samples with FS0, FS1 and FS2 (22.6%, 25.6% and 19.7%, respectively), and the second most dominant in samples with FS3 and FS4 (10.3% and 20.80%, respectively), where the most dominant family was *Fusobacteriaceae* (21.0% and 32.2%, respectively). In the samples with FS5, the most dominant family was also *Fusobacteriaceae* (26.7%), followed by *Bacteroidaceae* (14.9%). These results are illustrated in Figure 1.

A new classification of all samples was made in order to define groups of animals considered as not affected by the footrot (scores FS0 and FS1), and another group of animals affected by the footrot(scores FS2, FS3 and FS4). Next, a differential abundance analysis was performed between the 2 groups, which revealed 656 species with significant differences in abundance between the groups (169 species were significantly more abundant in the infection-free samples and 487 in the infected samples). The top 10 genera that were significantly more abundant in footrot infection samples were Mycoplasma (47 species), Campylobacter (37 species), Streptococcus (35 species), Clostridium (27 species), Arcobacter (22 species), Fusobacterium (10 species), Capnocytophaga (10 species), Treponema (8 species), Leptotrichia (8 species), and Vibrio (7 species). Regarding the genera found significantly more abundant in the infection-free samples, the top 10 were Streptomyces (44 species), Pseudomonas (13 species), Mycolicibacterium (9 species), Deinococcus (7 species), Brevundimonas (7 species), Micromonospora (6 species), Rhodococcus (6

species), Staphylococcus (5 species), Mycobacterium (5 species) and Gordonia (3 species).

The method for prognosis of sheep footrot disclosed herein comprises the following steps:

- Preparation of the skin biopsy sample from an animal to be evaluated;
- Detecting the microbiome signature present in the biopsy sample comprising the genera
    - III) *Mycoplasma*, *Campylobacter*, *Streptococcus*, *Clostridium*, *Arcobacter*, *Fusobacterium (10 species)*, *Capnocytophaga*, *Treponema*, *Leptotrichia*, and *Vibrio*; and/or
    - IV) *Streptomyces*, *Pseudomonas*, *Mycolicibacterium*, *Deinococcus*, *Brevundimonas*, *Micromonospora*, *Rhodococcus*, *Staphylococcus*, *Mycobacterium* and *Gordonia*;
- Concluding on footrot development probability in said animal, wherein an increase of abundance of the genera in I) over the genera in II) as detected in said sample is indicative for footrot.

Brief description of drawings

For easier understanding of this application, figures are attached in the annex that represent the preferred forms of implementation which nevertheless are not intended to limit the technique disclosed herein.

Figure 1 shows the most represented phyla and families in samples with different scores for the footrot.

CLAIMS

1. Method for prognosis of sheep footrot comprising the following steps:

- Preparation of the skin biopsy sample from an animal to be evaluated;
- Detecting the microbiome signature present in the biopsy sample comprising the genera
    V) *Mycoplasma*, *Campylobacter*, *Streptococcus*, *Clostridium*, *Arcobacter*, *Fusobacterium (10 species)*, *Capnocytophaga*, *Treponema*, *Leptotrichia*, and *Vibrio*; and/or
    VI) *Streptomyces*, *Pseudomonas*, *Mycolicibacterium*, *Deinococcus*, *Brevundimonas*, *Micromonospora*, *Rhodococcus*, *Staphylococcus*, *Mycobacterium* and *Gordonia*;

  Concluding on footrot development probability in said animal, wherein an increase of abundance of the genera in I) over the genera in II) as detected in said sample is indicative for footrot.

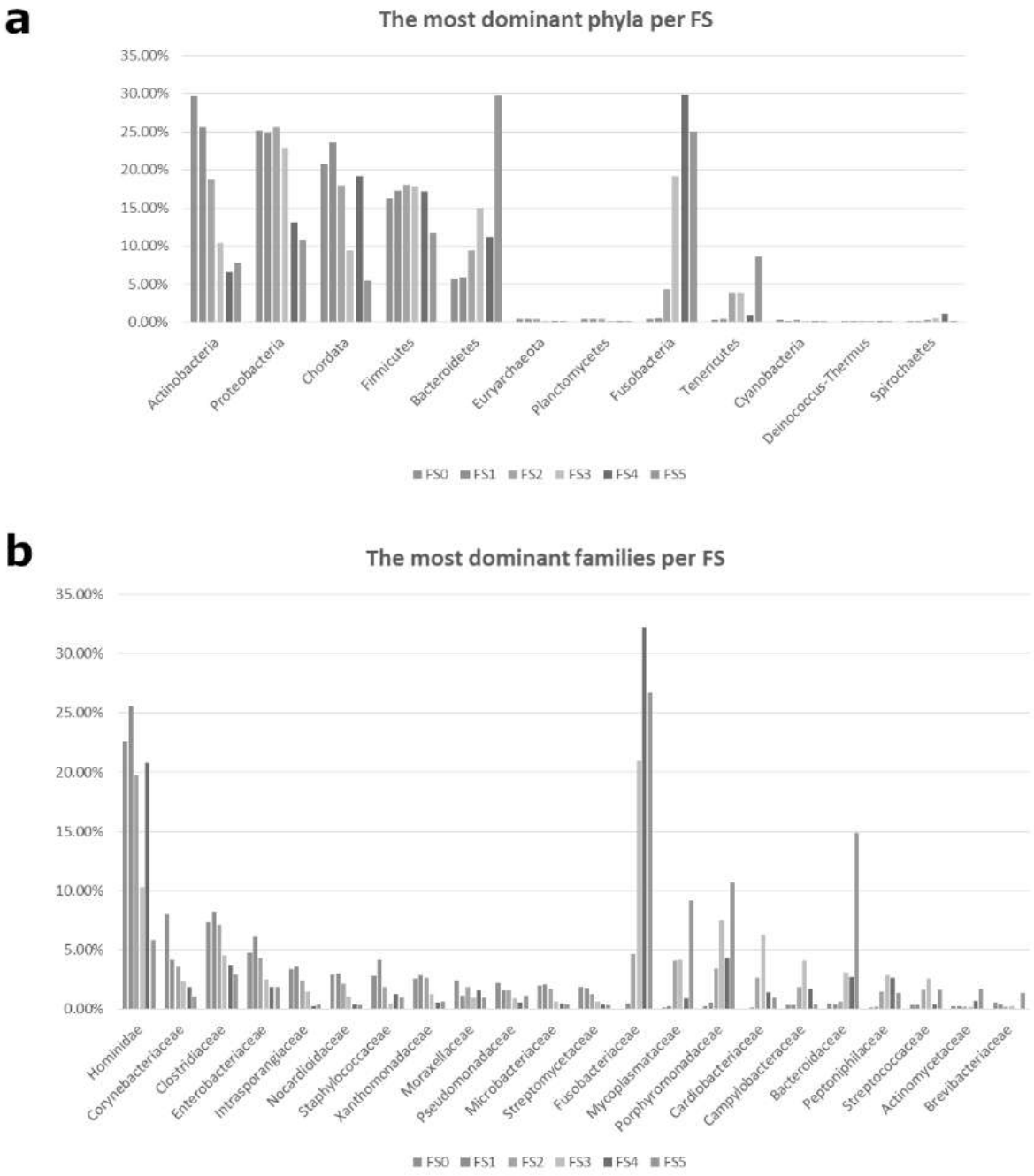
a
The most dominant phyla per FS
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
Actinobacteria
Proteobacteria
Chordata
Firmicutes
Bacteroidetes
Euryarchaeota
Planctomycetes
Fusobacteria
Tenericutes
Cyanobacteria
Deinococcus-Thermus
Spirochaetes
FS0 FS1 FS2 FS3 FS4 FS5
b
The most dominant families per FS
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
Hominidae
Corynebacteriaceae
Clostridiaceae
Enterobacteriaceae
Intrasporangiaceae
Nocardioidaceae
Staphylococcaceae
Xanthomonadaceae
Moraxellaceae
Pseudomonadaceae
Microbacteriaceae
Streptomycetaceae
Fusobacteriaceae
Mycoplasmataceae
Porphyromonadaceae
Cardiobacteriaceae
Campylobacteraceae
Bacteroidaceae
Peptoniphilaceae
Streptococcaceae
Actinomycetaceae
Brevibacteriaceae
FS0 FS1 FS2 FS3 FS4 FS5

Figure 1

Europäisches Patentamt
European Patent Office
Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	10158848
Application number	EP21194900.3
File No. to be used for priority declarations	EP21194900
Date of receipt	03 September 2021
Your reference	2020/44681
Applicant	ACOS - ASSOCIAÇÃO DE AGRICULTORES DO SUL
Country	PT
Title	METHOD FOR THE PROGNOSIS OF SHEEP FOOTROT
Documents submitted	package-data.xml application-body.xml SPECEPO-1.pdf\PPP 2021-44678 Vdeposito.pdf (8 p.) PRSR-1.pdf\Priority search report PTA117396.pdf (4 p.) ep-request.xml ep-request.pdf (6 p.) 1003-1.pdf\Ativação de Débito Automático.pdf (1 p.) f1002-1.pdf (2 p.)
Submitted by	CN=secure.epoline.org
Method of submission	Online
Date and time receipt generated	03 September 2021, 16:20 (CEST)
Message Digest	3B:94:DA:50:B7:1D:CA:9F:31:12:D7:E7:9F:82:FD:89:96:09:CE:FF

/European Patent Office/

DAS access code
The access code generated for this application and used to retrieve the priority documents from WIPO's Digital Access Service (DAS) is indicated in the document appended to this acknowledgement of receipt. Please note that the appended document is non-public and will not be published.

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To access and retrieve the priority document from WIPO's Digital Access Service (DAS) in respect of

Application number	EP21194900.3
Applicant	ACOS - ASSOCIAÇÃO DE AGRICULTORES DO SUL

the European Patent Office has generated the following code:

DAS access code	75D8

For further information, see OJ EPO 03/2019.

Date and time receipt generated	03 September 2021, 16:20 (CEST)

This unique access code allows the applicant to authorise participating intellectual property offices to retrieve a certified copy of the present application (as priority document) via WIPO DAS.
The code will only be valid if the requirements for according a date of filing are met (see OJ EPO 03/2019).

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Request for grant of a European patent

For official use only		
1 Application number:	MKEY	
2 Date of receipt (Rule 35(2) EPC):	DREC	
3 Date of receipt at EPO (Rule 35(4) EPC):	RENA	
4 Date of filing:		

5 Grant of European patent, and examination of the application under Article 94, are hereby requested. ☒

Request for examination in an admissible non-EPO language: Solicita-se o exame do pedido segundo o artigo 94º.

5.1 The applicant waives his right to be asked whether he wishes to proceed further with the application (Rule 70(2)) ☐

Procedural language: en

Description and/or claims filed in: en

A translation will be supplied later ☐

6 Applicant's or representative's reference: 2020/44681

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14.1 The/Each applicant hereby declares that he is an entity or a natural person under Rule 6(4) EPC. ☒

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21	A general authorisation has been registered under No:	72020

Inventor(s)

23 Inventor details filed separately ☒

24 Title of invention

Title of invention: METHOD FOR THE PROGNOSIS OF SHEEP FOOTROT

25 Declaration of priority (Rule 52)

A declaration of priority is hereby made for the following applications

	State	Filing date	Kind	Application number:	Search results under Rule 141(1) are attached
Priority 01	PT	13.08.2021	pr	117396	☒ PRSR-1.pdf

25.2 The EPO is requested to retrieve a certified copy of the following previous application(s) (priority document(s)) via the WIPO Digital Access Service (DAS) using the indicated access code(s):

	Request	Application number:	Access code
Priority 01	☐	117396	

25.3 This application is a complete translation of the previous application ☐

25.4 It is not intended to file a (further) declaration of priority ☒

26 Reference to a previously filed application

27 Divisional application ☐

28 Article 61(1)(b) application ☐

29 Claims

Number of claims: 1

29.1 ☒ as attached

29.2 ☐ as in the previously filed application (see Section 26.2)

29.3 ☐ The claims will be filed later

30 Figures

It is proposed that the abstract be published together with figure No.

31 Designation of contracting states

All the contracting states party to the EPC at the time of filing of the European patent application are deemed to be designated (see Article 79(1)).

32 Different applicants for different contracting states

33 Extension/Validation

This application is deemed to be a request to extend the effects of the European patent application and the European patent granted in respect of it to all non-contracting states to the EPC with which extension or validation agreements are in force on the date on which the application is filed. However, the request is deemed withdrawn if the extension fee or the validation fee, whichever is applicable, is not paid within the prescribed time limit.

33.1 It is intended to pay the extension fee(s) for the following state(s):

33.2 It is intended to pay the validation fee(s) for the following state(s):

34 Biological material

38 Nucleotide and amino acid sequences

The European patent application contains a sequence listing as part of the description ☐

The sequence listing is attached in computer-readable format in accordance with WIPO Standard ST.25 ☐

The sequence listing is attached in PDF format ☐

Further indications

39 Additional copies of the documents cited in the European search report are requested

Number of additional sets of copies:

40 Refund of the search fee under to Article 9 of the Rules relating to Fees is requested ☐

Application or publication number of earlier search report:

42 Payment

Method of payment: Automatic debit order

The European Patent Office is hereby authorised, under the Arrangements for the automatic debiting procedure, to debit from the deposit account any fees and costs falling due.

Currency: EUR

Deposit account number: 28140021

Account holder: Clarke, Modet & Cº

43 Refunds

Any refunds should be made to EPO deposit account: 28140021

Account holder: Clarke, Modet & Cº

44-A Forms		Details:	System file name:
A-1	Request		as ep-request.pdf
A-2	1. Designation of inventor	1. Inventor	as f1002-1.pdf

44-B Technical documents		Original file name:	System file name:
B-1	Specification	PPP 2021-44678 Vdeposito.pdf Description; 1 claims; 1 figure(s); abstract	SPECEPO-1.pdf

44-C Other documents		Original file name:	System file name:
C-1	1. Specific authorisation	Ativação de Débito Automático.pdf	1003-1.pdf
C-3	1. Search results under Rule 141(1) are attached	Priority search report PTA117396.pdf	PRSR-1.pdf

45

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Nº	CÓDIGO	DATA E HORA DE RECEÇÃO	MODALIDADE	PROCESSO RELACIONADO
20211000031158	0198	2021/08/13-19:08:03	PAT	117398 E

PAGAMENTO CONFIRMADO

PEDIDO DE PATENTE, MODELO DE UTILIDADE OU DE TOPOGRAFIA DE PRODUTOS SEMICONDUTORES

1 REQUERENTES

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Exclusivo para este ato? NÃO

2 MODALIDADE / TIPO DE PEDIDO

Modalidade: PEDIDO PROVISÓRIO DE PATENTE
Realização de pesquisa pelo INPI: SIM

3 EPÍGRAFE OU TÍTULO

METHOD FOR SHEEP SELECTION BASED ON PARASITE RESISTANT GENOTYPES

4 RESUMO

5 FIGURAS

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NIF 106506560

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Montante	64,65 €
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ABSTRACT

"METHOD FOR SHEEP SELECTION BASED ON PARASITE RESISTANT GENOTYPES"

The present patent application discloses a method for the selection of sheep based on their parasite resistance profile. Said parasite resistance profile is determined based on a group of Single Nucleotide Polymorphisms (SNPs) herein associated with lower scores of gastrointestinal parasite eggs (GPE) in sheep.

DESCRIPTION

"METHOD FOR SHEEP SELECTION BASED ON PARASITE RESISTANT GENOTYPES"

Technical field

The present patent application relates to a method for the selection of sheep based on parasite resistant genotypes.

Background art

In sheep, gastrointestinal parasites represent a significant problem. The eggs of these parasites are found on pasture and are then ingested by the sheep during their typical grazing behavior. These parasites are associated with a variety of symptoms in sheep that include weight loss, diarrhea, weakness, and anemia, which in turn are associated with severe production losses. Treatment for these parasites involves the use of various drugs, called anthelmintics, which are administered to infected sheep. However, these treatments represent an additional cost for producers, and there are also reports of resistance to the anthelmintic drugs used in sheep and other ruminants. Therefore, genetic selection of animals with greater resistance to gastrointestinal parasites can be a very efficient tool in combating this problem.

In sheep, there is variation in the individual response of each animal to gastrointestinal parasites. Under the same environmental conditions, for example, on the same farm, some animals are severely affected by the disease, while others show no symptoms and do not seem to be affected. This situation clearly indicates the possibility of genetic control of gastrointestinal parasites and, in particular, the severity with which each animal is affected. To date, no

genetic markers for gastrointestinal parasites have been identified in Portuguese sheep breeds.

Summary

The present patent application relates to a method for the selection of sheep based on parasite resistant genotypes.

The method for sheep selection based on parasite resistant genotype comprises the following steps:

Preparation of a sheep biological sample and extraction of DNA from said sample;

Genotyping the DNA sample for at least one of the following Single Nucleotide Polymorphisms (SNPs),

Chromosome	Position	Exonic alteration
NC_040256.1	20660687	-
NC_040263.1	45551043	synonym
NC_040264.1	80485067	-
NC_040268.1	65106946	-
NC_040272.1	26492975	-
NC_040252.1	189900946	-
NC_040258.1	21158733	-
NC_040259.1	65157854	-
NC_040267.1	61398933	-
NC_040274.1	32118726	-
NC_040278.1	102779687	-

Determining sheep's resistance to parasites based on the presence of at least one of said SNPs significantly associated with a lower score for the amount of gastrointestinal parasite eggs.

In one embodiment, the sample analyzed is preferably a blood sample.

In another embodiment, the above-mentioned Single Nucleotide Polymorphyms (SNPs) are used as biomarkers of parasite resistance in sheep.

Detailed Description

The present patent application discloses a method for the selection of sheep based on parasite resistant profile. Said parasite resistance profile is determined based on a group of Single Nucleotide Polymorphisms (SNPs) disclosed herein which are associated with lower scores of gastrointestinal parasite eggs (GPE) in sheep.

The technology disclosed herein is based on the sequencing of genomes of animals of the breeds that are subsequently used in genome-wide association studies. This step of genome sequencing is essential to characterize in great detail all the type of variation present in the animals studied. The following step involved selecting a set of SNPs to be genotyped in a larger number of animals, which in this case was 1500. All genotyped animals were also characterized from a phenotypic point of view, namely in determining the amount of gastrointestinal parasite eggs (GPE) observed in each animal.
After all animals were genotyped, a genome-wide association study was performed, where the statistical significance of the association observed between each of the genotyped SNPs and the phenotypic value of the amount of GPEs was

determined. Since the distribution of phenotypic data did not conform to the characteristics of a normal distribution, a logarithmic transformation of the data was performed before the genome-wide association analysis. The significance of each SNP was determined after a correction for multiple testing, an essential step in this type of analysis.

For each of the significant SNPs the observed mean for each of the genotypes is determined. The difference observed is the potential phenotypic gain that can be achieved if animals carrying the genotypes associated with lower OPG score are genetically selected.

The 11 SNPs identified are described in Table 1.

Chromosome	Position	P-Value	Gene ID	Genomic Localization	Exonic alteration
NC_040256.1	20660687	0.04863	gene-PDLIM4	UTR3	-
NC_040263.1	45551043	0.04016	gene-DISP3	exon	synonym
NC_040264.1	80485067	0.04016	gene-ARFGEF2	intron	-
NC_040268.1	65106946	0.04863	gene-HSPB8	intron	-
NC_040272.1	26492975	0.03608	gene-IGSF22	UTR3	-
NC_040252.1	189900946	0.04016		intergenic	-
NC_040258.1	21158733	0.03608		intergenic	-
NC_040259.1	65157854	0.04701		intergenic	-
NC_040267.1	61398933	0.04016		intergenic	-
NC_040274.1	32118726	0.04016		intergenic	-
NC_040278.1	102779687	0.04086		intergenic	-

For the 11 SNPs identified, the observed mean values are shown in Table 2.

NC_040256.1	NC_040256.1#20660687	GENO	T/T	T/C	C/C
NC_040256.1	NC_040256.1#20660687	COUNTS	171	544	661
NC_040256.1	NC_040256.1#20660687	FREQ	0.1243	0.3953	0.4804
NC_040256.1	NC_040256.1#20660687	MEAN	0.5205	0.8658	0.9259
NC_040256.1	NC_040256.1#20660687	SD	0.8768	0.9741	0.9734
NC_040263.1	NC_040263.1#45551043	GENO	C/C	C/T	T/T
NC_040263.1	NC_040263.1#45551043	COUNTS	283	589	488

NC_040263.1	NC_040263.1#45551043	FREQ	0.2081	0.4331	0.3588
NC_040263.1	NC_040263.1#45551043	MEAN	1.042	0.8812	0.7254
NC_040263.1	NC_040263.1#45551043	SD	0.9812	0.9704	0.954
NC_040264.1	NC_040264.1#80485067	GENO	T/T	T/C	C/C
NC_040264.1	NC_040264.1#80485067	COUNTS	68	481	843
NC_040264.1	NC_040264.1#80485067	FREQ	0.04885	0.3455	0.6056
NC_040264.1	NC_040264.1#80485067	MEAN	0.5441	0.7547	0.9454
NC_040264.1	NC_040264.1#80485067	SD	0.8183	0.9906	0.9695
NC_040268.1	NC_040268.1#65106946	GENO	G/G	G/A	A/A
NC_040268.1	NC_040268.1#65106946	COUNTS	133	549	703
NC_040268.1	NC_040268.1#65106946	FREQ	0.09603	0.3964	0.5076
NC_040268.1	NC_040268.1#65106946	MEAN	1.075	0.9271	0.7511
NC_040268.1	NC_040268.1#65106946	SD	1.005	0.99	0.9333
NC_040272.1	NC_040272.1#26492975	GENO	C/C	C/T	T/T
NC_040272.1	NC_040272.1#26492975	COUNTS	33	263	1094
NC_040272.1	NC_040272.1#26492975	FREQ	0.02374	0.1892	0.7871
NC_040272.1	NC_040272.1#26492975	MEAN	1.121	1.095	0.7888
NC_040272.1	NC_040272.1#26492975	SD	1.053	0.9858	0.9585
NC_040252.1	NC_040252.1#189900946	GENO	T/T	T/C	C/C
NC_040252.1	NC_040252.1#189900946	COUNTS	22	236	1145
NC_040252.1	NC_040252.1#189900946	FREQ	0.01568	0.1682	0.8161
NC_040252.1	NC_040252.1#189900946	MEAN	0.4091	0.6356	0.9048
NC_040252.1	NC_040252.1#189900946	SD	0.7964	0.9241	0.9773
NC_040258.1	NC_040258.1#21158733	GENO	C/C	C/T	T/T
NC_040258.1	NC_040258.1#21158733	COUNTS	12	244	1160
NC_040258.1	NC_040258.1#21158733	FREQ	0.008475	0.1723	0.8192
NC_040258.1	NC_040258.1#21158733	MEAN	1.417	1.094	0.7991
NC_040258.1	NC_040258.1#21158733	SD	0.793	0.9704	0.9663
NC_040259.1	NC_040259.1#65157854	GENO	C/C	C/T	T/T
NC_040259.1	NC_040259.1#65157854	COUNTS	40	400	953
NC_040259.1	NC_040259.1#65157854	FREQ	0.02872	0.2872	0.6841
NC_040259.1	NC_040259.1#65157854	MEAN	1.175	1	0.7817
NC_040259.1	NC_040259.1#65157854	SD	0.9306	1.006	0.9585
NC_040267.1	NC_040267.1#61398933	GENO	C/C	C/T	T/T
NC_040267.1	NC_040267.1#61398933	COUNTS	181	638	584

NC_040267.1	NC_040267.1#61398933	FREQ	0.129	0.4547	0.4163
NC_040267.1	NC_040267.1#61398933	MEAN	0.6409	0.7978	0.9726
NC_040267.1	NC_040267.1#61398933	SD	0.9179	0.9631	0.9867
NC_040274.1	NC_040274.1#32118726	GENO	C/C	C/T	T/T
NC_040274.1	NC_040274.1#32118726	COUNTS	100	516	746
NC_040274.1	NC_040274.1#32118726	FREQ	0.07342	0.3789	0.5477
NC_040274.1	NC_040274.1#32118726	MEAN	1.07	0.9671	0.7507
NC_040274.1	NC_040274.1#32118726	SD	0.9129	0.9679	0.976
NC_040278.1	NC_040278.1#102779687	GENO	C/C	C/G	G/G
NC_040278.1	NC_040278.1#102779687	COUNTS	12	196	1198
NC_040278.1	NC_040278.1#102779687	FREQ	0.008535	0.1394	0.8521
NC_040278.1	NC_040278.1#102779687	MEAN	1.25	1.117	0.8063
NC_040278.1	NC_040278.1#102779687	SD	0.866	0.9179	0.9764

The method of the present patent application involves several steps. Initially, it is necessary to collect a sample of biological material, such as a blood sample, for DNA extraction. Next, this DNA sample is genotyped for the SNPs of interest, and the genotypes obtained for each SNP are evaluated. The decision to select the animal is based on the presence of the genotype(s) significantly associated with a lower score for the amount of GPEs, i.e. the goal is to select animals with higher resistance to gastrointestinal parasites.

The method for sheep selection based on parasite resistant genotype comprises the following steps:

Preparation of a sheep biological sample and extraction of DNA from said sample;

Genotyping the DNA sample for at least one of the following Single Nucleotide Polymorphisms (SNPs),

Chromosome	Position	Exonic alteration
NC_040256.1	20660687	-

NC_040263.1	45551043	synonym
NC_040264.1	80485067	-
NC_040268.1	65106946	-
NC_040272.1	26492975	-
NC_040252.1	189900946	-
NC_040258.1	21158733	-
NC_040259.1	65157854	-
NC_040267.1	61398933	-
NC_040274.1	32118726	-
NC_040278.1	102779687	-

Determining sheep's resistance to parasites based on the presence of at least one of said SNPs significantly associated with a lower score for the amount of gastrointestinal parasite eggs.

In one embodiment, the sample analyzed is preferably a blood sample.

CLAIMS

1. Method for sheep selection based on parasite resistant genotypes comprises the following steps:

Preparation of a sheep biological sample and extraction of DNA from said sample;

Genotyping the DNA sample for at least one of the following Single Nucleotide Polymorphisms (SNPs),

Chromosome	Position	Exonic alteration
NC_040256.1	20660687	-
NC_040263.1	45551043	synonym
NC_040264.1	80485067	-
NC_040268.1	65106946	-
NC_040272.1	26492975	-
NC_040252.1	189900946	-
NC_040258.1	21158733	-
NC_040259.1	65157854	-
NC_040267.1	61398933	-
NC_040274.1	32118726	-
NC_040278.1	102779687	-

Determining sheep's resistance to parasites based on the presence of the at least one of said SNPs significantly associated with a lower score for the amount of gastrointestinal parasite eggs.

2. Single Nucleotide Polymorphyms (SNPs) as defined in claim 1 for use as biomarkers of parasite resistance in sheep.

Europäisches Patentamt
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Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	10158990
Application number	EP21194909.4
File No. to be used for priority declarations	EP21194909
Date of receipt	03 September 2021
Your reference	2020/44683
Applicant	ACOS - ASSOCIAÇÃO DE AGRICULTORES DO SUL
Country	PT
Title	METHOD FOR SHEEP SELECTION BASED ON PARASITE RESISTANT GENOTYPES
Documents submitted	package-data.xml application-body.xml SPECEPO-1.pdf\Text filed.pdf (9 p.) f1002-1.pdf (2 p.) ep-request.xml ep-request.pdf (6 p.) 1003-1.pdf\Automatic debit order.pdf (1 p.)
Submitted by	CN=secure.epoline.org
Method of submission	Online
Date and time receipt generated	03 September 2021, 16:43 (CEST)
Message Digest	38:74:50:E2:4A:FD:F3:5D:67:C4:72:15:58:E9:9D:B2:70:22:80:80

/European Patent Office/

DAS access code
The access code generated for this application and used to retrieve the priority documents from WIPO's Digital Access Service (DAS) is indicated in the document appended to this acknowledgement of receipt. Please note that the appended document is non-public and will not be published.

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DAS access code

To access and retrieve the priority document from WIPO's Digital Access Service (DAS) in respect of

Application number	EP21194909.4
Applicant	ACOS - ASSOCIAÇÃO DE AGRICULTORES DO SUL

the European Patent Office has generated the following code:

DAS access code	F2A8

For further information, see OJ EPO 03/2019.

Date and time receipt generated	03 September 2021, 16:43 (CEST)

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The code will only be valid if the requirements for according a date of filing are met (see OJ EPO 03/2019).

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Request for grant of a European patent

For official use only		
1 Application number:	MKEY	
2 Date of receipt (Rule 35(2) EPC):	DREC	
3 Date of receipt at EPO (Rule 35(4) EPC):	RENA	
4 Date of filing:		

5 Grant of European patent, and examination of the application under Article 94, are hereby requested. ☒

Request for examination in an admissible non-EPO language: Solicita-se o exame do pedido segundo o artigo 94º.

5.1 The applicant waives his right to be asked whether he wishes to proceed further with the application (Rule 70(2)) ☐

Procedural language: en

Description and/or claims filed in: en

A translation will be supplied later ☐

6 Applicant's or representative's reference: 2020/44683

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14.1 The/Each applicant hereby declares that he is an entity or a natural person under Rule 6(4) EPC. ☒

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21	A general authorisation has been registered under No:	72020

Inventor(s)

23 Inventor details filed separately ☒

24 Title of invention

Title of invention: METHOD FOR SHEEP SELECTION BASED ON PARASITE RESISTANT GENOTYPES

25 Declaration of priority (Rule 52)

A declaration of priority is hereby made for the following applications

	State	Filing date	Kind	Application number:	Search results under Rule 141(1) are attached
Priority 01	PT	13.08.2021	pr	117398	☐

25.2 **The EPO is requested to retrieve a certified copy of the following previous application(s) (priority document(s)) via the WIPO Digital Access Service (DAS) using the indicated access code(s):**

	Request	Application number:	Access code
Priority 01	☐	117398	

25.3 This application is a complete translation of the previous application ☐

25.4 It is not intended to file a (further) declaration of priority ☒

26 Reference to a previously filed application

27 Divisional application ☐

28 Article 61(1)(b) application ☐

29 Claims

Number of claims: 2

29.1 ☒ as attached

29.2 ☐ as in the previously filed application (see Section 26.2)

29.3 ☐ The claims will be filed later

30 Figures

It is proposed that the abstract be published together with figure No.

31 Designation of contracting states

All the contracting states party to the EPC at the time of filing of the European patent application are deemed to be designated (see Article 79(1)).

32 Different applicants for different contracting states

33 Extension/Validation

This application is deemed to be a request to extend the effects of the European patent application and the European patent granted in respect of it to all non-contracting states to the EPC with which extension or validation agreements are in force on the date on which the application is filed. However, the request is deemed withdrawn if the extension fee or the validation fee, whichever is applicable, is not paid within the prescribed time limit.

33.1 It is intended to pay the extension fee(s) for the following state(s):

33.2 It is intended to pay the validation fee(s) for the following state(s):

34 Biological material

38 Nucleotide and amino acid sequences

The European patent application contains a sequence listing as part of the description ☐

The sequence listing is attached in computer-readable format in accordance with WIPO Standard ST.25 ☐

The sequence listing is attached in PDF format ☐

Further indications

39 Additional copies of the documents cited in the European search report are requested

Number of additional sets of copies:

40 Refund of the search fee under to Article 9 of the Rules relating to Fees is requested ☐

Application or publication number of earlier search report:

42 Payment

Method of payment: Automatic debit order

The European Patent Office is hereby authorised, under the Arrangements for the automatic debiting procedure, to debit from the deposit account any fees and costs falling due.

Currency: EUR

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43 Refunds

Any refunds should be made to EPO deposit account: 28410021

Account holder: Clarke, Modet & Cº

44-A Forms		Details:	System file name:
A-1	Request		as ep-request.pdf
A-2	1. Designation of inventor	1. Inventor	as f1002-1.pdf

44-B Technical documents		Original file name:	System file name:
B-1	Specification	Text filed.pdf Description; 2 claims; abstract	SPECEPO-1.pdf

44-C Other documents		Original file name:	System file name:
C-1	1. Specific authorisation	Automatic debit order.pdf	1003-1.pdf

45

General authorisation:

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Review Article Open Access

Reviewing Footrot in Sheep

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Abstract

Ovine footrot is the main cause of lameness in sheep around the world and is responsible for extensive economic and welfare impacts. It can be an extremely contagious disease, resulting from the invasion of the interdigital tissue by a complex mixture of bacteria, in which *Dichelobacter nodosus* is a required component. Strains of *D. nodosus* can be benign or virulent, but they are not always related with the clinical expression of footrot, complicating the diagnostic process. Several efforts have been made over recent decades to control the disease, but it remains endemic in the major sheep-raising countries of the world. The use of more efficient therapeutic procedures and better farm management practices or the development of new selective breeding tools and strategic vaccination protocols are some of the key measures that may improve footrot control in the future.

Keywords: Ovine footrot; Sheep; *Dichelobacter nodosus*

Introduction

Ovine footrot is a contagious disease affecting the feet of sheep and other ruminants and presents in two different forms. Interdigital dermatitis is the disease's mildest form and involves inflammation of the interdigital skin [1]. In contrast, severe footrot is much more aggressive, presenting as the separation of the horn from the sensitive layers of the foot, known as underrunning [2]. Footrot has been known of since the early 19th century [3]. In the mid-20th century, the virulent or progressive form and the benign or non-progressive form of footrot were identified [4]. A few decades later, Stewart *et al.* identified a more complex spectrum of virulence among different strains of *Dichelobacter nodosus*, which could be associated with different clinical expressions of the disease [5]. Nowadays, ovine footrot has been reported on the vast majority sheep-farming countries around the world [6]. The disease has a prejudicial impact on animal welfare, due to pain, discomfort and weight reduction. Consequently, it leads to productivity losses by reducing the number of lambs per ewe, reducing growth rates in lambs and adult sheep and increasing the mortality rate and fertility problems [5,7]. Beyond those losses, the costs of therapeutic, control and preventive measures can be expensive [2]. The economic impact is thus extensive, estimated at an annual cost of £24-80 M in the United Kingdom and $18.4 M in Australia [8-10]. Most sheep farmers classify lameness as the condition of most concern in their sheep flocks and consider footrot as the main cause of ovine lameness [11,12]. It is estimated that approximately 5% of British sheep are lame at any one time [13]. That lameness is attributed to footrot in 80% of the cases [12]. Footrot has persisted for many decades in British flocks despite all the efforts made to control the disease, though the prevalence of lameness has halved over a period of 10 years, which means the results of recent research are being used to inform farmers about recommended management practices [13,14]. Even so, British farmers have been encouraged to keep footrot prevalence at a maximum of 2% [15].

Aetiology

Footrot results from the invasion of the epidermal tissue of the hooves by a complex mixture of bacteria, in which *Dichelobacter nodosus* is a necessary component [16]. This bacterium was formerly known as *Fusiformis nodosus* or *Bacteroides nodosus* and is a gram-negative, anaerobic bacterium [17,18]. Unlike other bacteria, *D. nodosus* is not usually found in sheep faeces or in soil [14]. It is possible to find the agent in normal healthy feet, but the load of bacteria present in affected feet is much higher [19]. Thus, disease progression from interdigital dermatitis to severe footrot is mainly attributed to *D. nodosus* [19].

The role of other bacteria in the pathogenesis of footrot is not fully understood, but Roberts and Egerton found that the presence of *Fusobacterium necrophorum*, a faecal organism, was also required for *D. nodosus* to start an infection in laboratory trials. Research

by Bennett *et al.* found evidence of a synergetic relationship between *F. necrophorum* and *D. nodosus.* More recently, Atia *et al.* proposed an opportunistic role for this pathogen, as high loads of *F. necrophorum* were only observed once severe footrot had developed. Thus, instead of attributing to *F. necrophorum* the role of precursor in footrot pathogenesis, it is now believed that this bacterium contributes to both the disease's duration and severity [1,19-22]. *F. necrophorum* promotes inflammation and damage of the *stratum corneum.* This bacterium also produces toxins that cause necrosis of the superficial layer of the interdigital skin, enabling the establishment of other bacteria, such as spirochetes [14]. *F. necrophorum* is an anaerobic gram-negative bacterium divided in two sub-species, *F. necrophorum* sub-species *necrophorum* and *F. necrophorum* sub-species *funduliforme.* The first one is commonly found in animals, while the second is usually present in humans [23]. However, Zhou *et al.* observed that the variant more frequently found in sheep and goats was different from the two sub-species previously reported, suggesting that much has yet to be learned about this issue [24]. Other unexpected bacteria such as *Fusobacterium equinum* and *Bacteroides ureolyticus* have been isolated in footrot lesions [25,26]. The presence of *B. ureolyticus*-like organisms in footrot lesions may present a problem for diagnosis as this bacterium is phenotypically similar to *D. nodosus* and it is possible that some older researches have mis-identified the agent present in footrot lesions [26]. It is possible to conclude that bacteria other than *D. nodosus* represent an important role in the pathogenesis of the disease, but there is much more to be studied to understand the exact mechanism [1]. Some decades ago it was believed that *Treponema spp*. was also involved in the disease pathogenicity, but one recent study concluded that there was no significant connection between footrot and *Treponema spp* [17,27].

Pathogenesis

Transmission of footrot starts with naturally footrot-infected sheep acting as a source of infection to the feet of healthy animals [17]. However, the infection of healthy interdigital skin of sheep with *D. nodosus* alone is not enough for the development of footrot. The activity of normal environmental microflora present on the interdigital skin, the presence of favorable environment temperatures and the water maceration of the *stratum corneum* of the hoof reflect some of the necessary prerequisites for disease development [14,17]. Disease transmission is enhanced by temperatures above 10 ºC and with consistent rainfall over several weeks, rather than a single short-lived episode of rainfall [3]. Wet weather increases vulnerability to footrot either by inducing physical changes in the hoof, making it more susceptible, or by changing the biology of the pathogens that cause footrot [1]. Therefore, transmission of footrot is higher during winter when sheep are housed in high animal densities, particularly in clay soil types [14,28].

D. nodosus can be transmitted between sheep via soil contact and is capable of surviving outside the host for long periods [1]. Myers' investigations revealed that *D.nodosus* could tolerate up to 10 days exposed to air, though more recently Muzafar has shown that it could survive longer than 30 days, despite being an anaerobic bacterium [28,29]. The duration of infectiousness of the agent outside the hoof depends on climatic conditions and Muzafar concluded that agent survival was higher at 5 ºC than at 25 ºC. *D. nodosus* feeds on collagen present in living dermis, digesting it, and this represents one of the main barriers for the eradication of footrot, since the causative agent has the capability of surviving in the inter-digital skin or in cryptic lesions within the hoof for several months [1,14,28].

Risk Factors Associated with Footrot

Expression of footrot in the field is determined by three key factors: the virulence of the bacteria involved, environmental conditions and host resistance [17]. Many environmental factors, such as temperature, rain fall or soil type, can affect the disease's progression by leading to injuries to the feet of sheep, thus increasing susceptibility to infection [22]. Some management practices are directly associated with an increased likelihood of flocks developing footrot and the probability of sheep developing footrot increases with flock size [15]. Stocking animals in high densities can create an extremely contaminated environment. The implementation of preventative measures such as quarantining or isolation of diseased animals can drastically reduce environmental contamination for the rest of the flock, decreasing disease prevalence [14]. All factors that can lead to the maceration of the interdigital skin will facilitate colonization of *D. nodosus* [17]. As such, the presence of moist ground with rough pasture in the areas where sheep graze is another risk factor [15]. Some non-genetic factors can have a significant influence on susceptibility to footrot. By sex, it has been reported that ewes are more resistant to the disease than rams, while by age, it has been concluded that yearling sheep are much less likely to have footrot than lambs [15,17,30]. Some ovine breeds, like Merino, have been shown to be more susceptible to footrot [31].

Clinical Signs

The disease is characterized by acute lameness, anorexia, reduced production, reduced wool quality and, in the worst-case scenario, can result in death [1]. In the beginning of the infection it is possible to observe an inflammatory process, characterized by erythema and diffuse superficial necrosis of the interdigital skin. If the disease evolves to a more severe phase, a break at the skin-horn junction will be seen. Underrunning, which starts at the heel and the posterior region of the sole, can progress along the sole to the toe. The separation can extend to the abaxial wall of the hoof in the most severe cases [17]. The separation process will create a cavity between the sensitive tissue of the claw and the hoof horn, which will be filled with a grey pasty scum which has a foul smell, characteristic of anaerobic bacterial activity [14]. Sheep with chronic footrot infection tend to present feet with overgrown and misshapen horns and with extensive necrotizing damage through the surrounding soft tissues [17]. Over recent decades many

scoring systems have been developed to establish criteria to differentiate the levels of progression of footrot infection. The first scoring system for footrot lesions was created by Egerton and Roberts and remains one of most widely implemented classifications to this day [32]:

Score 1: limited, mild interdigital dermatitis;
Score 2: more extensive interdigital dermatitis;
Score 3: severe interdigital dermatitis and underrunning of the horn of the heel and sole;
Score 4: similar to score 3, but with the underrunning extended to the walls of the hoof.

Diagnosis

The clinical diagnosis of footrot starts by performing a careful visual inspection of the hooves. The most frequent lesions identified visually range from a mild interdigital dermatitis to the underrunning of the sole, according to the disease's stage of developmen. The differentiation between benign and virulent forms of footrot can be a challenging process in early stages of the disease or if adverse environmental conditions are present [33]. The most significant differential diagnosis of footrot involves ovine contagious interdigital dermatis, which is a necrotizing infection caused by *F. necrophorum* in the absence of *D. nodosus* [34]. The differentiation of this disease from footrot may be difficult since the appearance of both is similar. Other diseases often mistaken with footrot include white line disease, contagious ovine digital dermatitis and foot/toe abscesses, although experienced technicians can easily distinguish these from footrot [2]. According to Kaler and Green, terminology to describe foot diseases is often used incorrectly, mainly by farmers who tend to identify any hoof horn lesion as footrot. Definitive diagnosis can only be achieved by the demonstration of *D. nodosus* in gram stain smears collected directly from suspect lesions, as some features displayed by these colonies allow for their differentiation from others [10,12,17]. These bacteria are observed as large gram-negative rods [0,6-0,8 µm wide and 3-10 µm in length] and can be straight or slightly curved, presenting a characteristic enlargement at the ends [10]. Microbiological culture of *D. nodosus* is an expensive and laborious process that requires specialized equipment and can be particularly difficult when the sample site is contaminated with other pathogens [35]. As the bacterium is an obligate anaerobe, it requires a specially enriched medium for its isolation, such as hoof agar, trypticase arginine serine agar or Eugon agar. Prior to culture, these media need to be kept in anaerobiosis jars [36]. However, as the culture takes approximately four weeks to yeld results – and thus cannot be practically used to segregate infected animals from the rest of the flock – it is rarely used as a diagnostic tool [35]. In the recent years, the use of PCR-based methods has grown because of the reliability of results [37]. A multiplex PCR is currently used for serogrouping, as it is a simple and rapid technique that may be very useful for vaccination-based footrot control [38]. Stäuble *et al.* developed a real-time PCR that detects the presence of alleles *aprV2/aprB2* directly from clinical samples, constituting a rapid and sensitive diagnostic technique to differentiate between benign and virulent footrot [39]. This technique is capable of detecting high loads of virulent strains of *D. nodosus* in clinically healthy sheep. This evidence indicates that the lesion scoring system does not always correlate with bacterial loads and the virulence of *D. nodosus* strains. Nevertheless, McPherson *et al.* have identified low rates of agreement between clinical diagnosis and PCR test results [33]. Swabs are used to collect samples directly from the interdigital skin, which can be used both for culturing and PCR analysis [27]. This technique is currently considered a better diagnostic approach than biopsy punching [39].

Classifications of Footrot

Strains of *D. nodosus* differ in virulence and in their ability to induce clinical disease, being classified as benign, intermediate or virulent strains. For descriptive purposes, these three terms are also used to describe the different clinical forms of footrot [5,10]. While benign footrot refers to the mildest state of the disease, with the lesions of dermatitis almost exclusively limited to the interdigital skin, virulent footrot is related with a much more severe disease, which frequently progresses to the separation of the soft and hard horn from the underlying hoof matrix, a phenomenon known as underrunning. The expression intermediate footrot refers to all the forms located between benign and virulent footrot [1]. Virulent strains of *D. nodosus* commonly lead to a clinical expression of virulent footrot, but it must be recognized that clinical expression of the disease is also dependent on environmental conditions and host factors [16]. Likewise, if favorable climatic conditions are present, benign strains of *D. nodosus* induce severe footrot lesions in a limited proportion of susceptible animals [33]. It is important to clearly determine which form of footrot is present in each flock, since only flocks with virulent footrot may benefit from a control or eradication program. Time and resources should not be expended on cases of intermediate and benign footrot, as they will regress without treatment if environmental conditions become less propitious for the development of footrot. Moreover, the mildest forms of footrot usually have limited economic impacts [17]. The most precise method for identifying the form of footrot requires examination of a representative number of animals from the whole flock to determine the proportion of sheep presenting score 4 lesions, rather than the presence of footrot lesions of any grade [33]. If there is any doubt as to the effect of the environment or about previous treatments, it is recommended to repeat the inspection after a minimum period of two weeks. When virulent footrot is present, it is expected that more than 10% of the animals will have severe lesions. The disease develops very fast and there is little evidence of self-curing. The presence of clinical signs, such as lameness, are associated with huge production losses. In the other hand, in case of benign footrot, less than 1% of the animals will have severe lesions, mostly confined to the interdigital skin [scores 1 or 2]. Most such lesions resolve spontaneously with improved environmental conditions [17].

In contrast to Australian researches, in the UK *D. nodosus* is not typically classified according to virulence but to the presence of key signs of clinical diagnosis, such as lameness or the severity of lesions [14].

Classifications of *D. nodosus*

There are several different criteria by which the causal agent of footrot can be classified and the presence of specific virulence factors is one relevant factor used to categorize different strains of *D. nodosus*. Some of the more important virulence factors include its proteases, fimbriae and outer-membrane proteins [1,40]. The fimbriae of *D. nodosus* play an important role in virulence expression since they are required for binding to host epithelial cells, it being generally accepted that virulence is directly proportional to the number of fimbriae present [29,41]. The outer membrane proteins of *D. nodosus* interfere with the host's immune response, rather than attacking the host [29]. The secretion of extra-cellular proteases by *D. nodosus* plays a vital role in the biology of the agent due to its inability to create any aminoacid. Thus, the bacterium obtains aminoacids by importing them from digested proteins, due to the proteolytic ability of extra-cellular proteases [29]. The thermostability of these proteases is also deeply related to the expected virulence of the microorganisms [16]. Virulent strains of *D. nodosus* produce heat stable enzymes with caseinolytic activity, while benign strains produce heat labile enzymes [17]. The gelatine gel test is used to identify heat stable proteases, but correlation between the results of this test and the clinical expression of disease can be unreliable [6,14]. Alternatively, the elastase test measures the temporal and quantitative activity of extracellular proteases, and is probably a better option for distinguishing virulent and benign strains of *D. nodosus*, since it has a good correlation with clinical diagnosis [6,33]. In the past, some segments of the *D. nodosus* genome, such as *intA or vrl*, were thought to be significant virulence factors, while the genetic segment *intD* was strongly associated with a benign phenotype of *D. nodosus* [42,43]. However, more recent research has not been able to confirm the hypothesis of the *intA* gene as a virulence factor [44]. More recently, other genetic segments have been considered important virulence factors, such as *aprV2,* which encodes the acidific protease 2 (*aprV2*), a thermostable protease. Meanwhile, the orthologous *aprB2* gene, responsible for encoding a thermolabile protease, is present in the genome of *D. nodosus* benign strains [45]. The genes *aprV2* and *aprB2* have a single nucleotidic difference (TA/CG) at position 661/662 [46]. It was believed that this difference was responsible for the definition of the elastase activity, but McPherson *et al.* have identified many strains of *D. nodosus* containing the gene *aprV2* in clinically healthy herds, proving the *aprV2* is no longer a reliable virulence marker [33,45]. Another classification of *D. nodosus* refers to serogroup, which is related to fimbrial antigenicity and which is important in the development of specific vaccines [47]. The serogroup classification is denoted by letters and there are 10 different known serogroups (A-I and M) [48]. These serogoups can further be subdivided into 21 serotypes (A1, A2, B1, B2, B3, B4, B5, B6, C1, C2, D, E1, E2, F1, F2, G1, G2, H1, H2, I and M) according to cross-absorption tests [49]. Each region and country has a specific profile of isolation frequencies for the serogroups. Serogroup B is undoubtedly the most frequent serogroup in all the countries. Other frequent *D. nodosus* serogroups are A (Australia), D (Australia, New Zealand and the United Kingdom), G (Australia) and H (Australia and the United Kingdom) [50]. The median number of *D. nodosus* serogroups found in affected hooves is one, although cases of up to seven different *D. nodosus* strains on a single hoof have been reported [51,52].

Treatment

Different treatment approaches and management methods are used worldwide according to the specific production system in question. The choice of management method depends on the size of the flock, the stocking rate, the availability of medication and other resources and the acceptance of different management and treatment policies within each market [1]. Furthermore, some farmers choose not to treat affected animals owing to a belief that treatment is expensive and may not be ultimately profitable, though Wassink *et al.* have concluded that economic losses will occur if animals are left untreated for seven days or more [7,9]. Winter and Green have produced a cost-benefit analysis of different approaches of controlling footrot in 116 sheep flocks and concluded that farms with higher prevalence of lameness had a much higher overall cost per animal per year than farms with lower prevalence rates (£6.53 versus £3.90) [9]. Some of the methods used routinely some years ago to control footrot, such as foot-trimming, are currently associated with higher levels of prevalence and incidence, even when done properly [53,54]. Foot-trimming has been used for decades and its goal was to remove diseased tissue and to promote a good hoof conformation [1]. This procedure reduces bacterial load, diminishing environment contamination, and exposes deeper tissue to oxygen, which is toxic to anaerobic bacteria. However, the use of foot-trimming needs to be done carefully, as the overuse of foot-trimming may damage the sensitive tissues and induce foot bleeding, leading to lameness [13,55]. This situation gives infectious agents an opportunity to penetrate the hooves [56]. Green *et al.* reported that the prevalence of footrot increased after routine foot-trimming sessions, which suggests that excessive use of trimming may represent a risk factor for disease transmission [53]. In addition, as foot-trimming is a time-consuming practice that requires much physical effort, its cessation could potentially save farmers significant amounts of time and money [9]. The use of foot-bathing can have a beneficial effect in lameness prevention in the initial stages of footrot, but the recommended procedures cannot be easily implemented in the vast majority of commercial farms as each sheep should remain inside the footbath for 10 minutes once a week [13,57]. Moreover, following treatment, all animals need to be moved to an area that has been free of other animals for a minimum period of two weeks [56]. Another disadvantage of footbathing is that the vast majority of chemical solutions used are toxic both to the environment and to the people applying them, with such solutions also often contain copper salts, zinc sulphate and formalin, which can be painful for animals, though it is believed there are possible alternatives to these chemical solutions [58]. Winter *et al.* performed a study based on 1260 postal questionnaires received

from British farmers and concluded that the use of footbathing in sheep presenting interdigital dermatitis has a beneficial effect preventing disease progression, as disinfectants can inactivate surface pathogens [13,17]. However, footbathing was not found to be effective against cases of severe footrot, as footbath solutions cannot penetrate deep into affected tissues. According to Winter and Green, it is extremely important to persuade both farmers and veterinarians to discontinue outdated procedures that are inefficient, physically difficult and time-consuming in favor of currently recommended methods [9]. Even though there are many farms that have altered their routine procedures, a significant number of farmers continue to regularly practice hoof-paring on their sheep, against the advice of recent research [59]. Farmers that follow the most up-to-date recommendations for reducing the prevalence of lameness in sheep have significantly lower expenses per animal than those who do not use such management methods [9]. These recommendations discourage whole-flock interventions, which are expensive and considered ineffective in reducing lameness, and focus on the early treatment of affected animals with antibiotics, which is associated with lower costs and higher treatment effectiveness in footrot [7,13]. Winter *et al.* investigated the cost-benefit of different strategies to treat ovine footrot in 116 English flocks and concluded that prompt treatment of lame ewes with parenteral antibiotics is the most efficient and cost-effective strategy [9]. However, straight extrapolations for different farming scenarios are inadvisable, as treatment effectiveness depends on flock size, footrot prevalence and the pathogenicity of *D. nodosus* strains involved. There is little evidence for antibacterial resistance in *D. nodosus*, with the microorganism showing in-vitro sensitivity to different antibiotic classes such as tetracyclines, macrolides, penicillin, cephalosporins and fluoroquinolones [54,60,61]. Oxytetracycline has been used effectively in the treatment of footrot for decades [62]. Even so, other antibiotic options have been discovered more recently. Strobel *et al*. compared the efficacy of the use of gamythromycin with oxytetracycline in the treatment of sheep presenting footrot lesions [60]. The difference between treatments was significant, with sheep injected with a single dose of gamythromycin revealing a better clinical cure rate. The use of systemic antibiotics targets anaerobic bacteria located deep within the feet, reducing inflammation. The effect of medication is rapid and lame sheep can become sound within a period of 3 to 4 days, though poor blood supply to the hoof can reduce the effectiveness of systemic therapy. Thus, the use of topical antibacterial sprays may be a useful option. Local antibiotics have another advantage as they inactivate surface *D. nodosus*, promptly reducing environmental contamination [14]. Kaler *et al.* concluded that replacing foot-paring with the use of parental antibiotics would accelerate the recovery of over a million British footrot affected lame sheep per year, with subsequent gains in production [55]. However, the extensive use of parenteral antibiotics has some limitations, since the maximum effect of antibiotics occurs when sheep are held in dry conditions for 24 hours after the injection, which may not be possible for most sheep flocks [63]. Another disadvantage is the inability to sell sheep for human consumption until after the withdrawal period, which can last several weeks for some antibiotics [64]. The overuse of antibiotics also leads to the development of drug resistance and the European commission has made limiting these medications in both human and veterinary medicine a priority [65]. Additionaly, growing numbers of organic herds, in which the use of antibiotics is prohibited, has led to demand for new environmentally friendly therapies to be found [58].

Szponder *et al.* performed a study on sheep suffering from footrot and implemented an alternative therapy consisting of ozone therapy and the application of autologous platelet-rich plasma [58]. Ozone is a strong antioxidant which promotes oxidative stress and restricts some inflammatory cell factors and is successfully used to heal many kinds of wounds [66,67]. Platelet-rich plasma has been extensively used to promote healing in lesions since it locally introduces increased concentrations of growth factors and other bioactive molecules in injured tissues [68]. The use of this therapy was successful in the totality of the animals and no side-effects were observed in treated sheep. As such, the local application of ozone and platelet-rich plasma may be considered an effective treatment for footrot as a replacement for the conventional use of antibiotics and disinfectants demands to be replaced. However, this protocol is expensive and time-consuming, which probably precludes its large-scale use [58].

Control

Control programs aim to minimize disease's adverse effects in cases where complete elimination of the pathogen is not possible [14]. The control of footrot focuses on some major goals such as limiting the spread of the disease, decreasing the severity of clinical signs in affected animals and improving resistance to disease so that sheep can better withstand environmental challenges [14]. The control involves a combination of strategies such as different treatment methods, quarantine, and vaccination, culling chronically infected sheep or selective breeding for improved genetic resistance [69]. It is expected that such measures will lead to a decrease in prevalence inside the flock and reduce the severity of clinical signs in the animals that remain infected. However, it is important to note that none of the procedures used to control the disease represent a lasting approach to disease management [17]. The development of a successful control program needs to take into consideration various factors, such as the strains of *D. nodosus* present in each region and the exact seasonality of the disease in the area where the herd is located [27]. Environmental specificities can lead to adjustments in the periods in which control measures should be implemented [17]. The non-transmission period is the most effective period to implement most control strategies, however a vaccination protocol can be successfully implemented at any time, irrespective of the flock's disease status [10,70]. Factors that increase susceptibility to footrot should be avoided. Animals should not be exposed to wet conditions or to abrasive pasture, since these conditions may damage foot integrity [14]. Footbathing is an effective strategy to limit the spread of footrot within a flock, as the disinfectant can kill bacteria present on the foot surface and reducing the environmental contamination [54]. The use of parenteral antibiotics is one of the most effective procedures to control footrot, though the costs associated with this treatment are not always justified in mild cases [17,60]. Other effective

mandatory control measures are the non-acquisition of sheep from flocks of unknown footrot status and preventing animals from grazing in the same areas as neighboring flocks [17].

Eradication

Eradication programs aim to permanently eliminate all cases of footrot in a limited region. Once eradication has been achieved, it is expected that no further cases of footrot will occur, unless it is reintroduced from another source [17]. Eradication is obviously a challenging goal, but its effects are permanent and the advantages long-lasting. As the annual cost of eradicating footrot from a flock can be over $ 10 per animal, several important factors should be taken into consideration in order to minimize financial waste before undertaking an eradication program [33]. It is important to understand the various transmission patterns occurring over the season as these determine the best period in which to implement specific preventive measures. It is also imperative that the owner of the flock is conscious that this method is costly and time-consuming. All clinical cases of footrot should be detected as soon as possible, so that operators can recognize them in the early stages of development [17]. There are several methods of eradication. The most simple and effective is whole flock disposal, though owners are often reluctant to dispose of the entire flock [70]. *Disposal of affected animals* is another effective option, but can only be applied when prevalence is low. *Identification and treatment of affected animals* is possible, but has a much lower probability of success [17]. Mills *et al.* developed a survey of 196 flocks that eradicated virulent footrot using different methods and concluded that the *whole flock disposal* was clearly the most efficient strategy [70]. Cattle are a reservoir of benign footrot strains of *D. nodosus* for sheep, but there is no evidence that virulent strains of *D. nodosus* can infect feet of cattle [17]. Goats, on the other hand, can be reservoirs of virulent footrot for sheep [71]. This indicates the improbability of eradicating footrot on those farms where sheep graze in the same area as other ruminants. All flocks to have successfully undergone an eradication program should be subjected to a regular surveillance program in following years in order to maintain footrot-free status [17]. It is important to note that an eradication programs generate flocks with animals that are highly susceptible to footrot infection if the disease is reintroduced in the herd, thus new animals should be acquired exclusively in farms free from footrot and animal contact between neighboring flocks should be avoided [14].

Vaccination

The first vaccine against footrot in sheep was developed in 1969, but only in 1974 it was possible to recognize that whole cell bacterins vaccines were not capable of protecting sheep against heterologous serogroups [72,73]. Vaccines need to induce antibody titres against fimbrial antigens from each specific serogroup of *D. nodosus,* since there is no cross-protection between them [17]. This is explained by the phenomenon of antigenic competition, in which a weak antibody production occurs against individual components if a multivalent (containing three or more antigens) vaccine is used [74]. In that way, an efficient vaccine should lead to efficient antibodies production, maintaining high antibody titres for the longest period possible [10]. Monovalent vaccines can be quite useful if used strategically in farms or regions where only one serogroup of *D. nodosus* is present [1]. The implementation of that approach was responsible for the eradication of footrot in Nepal and Bhutan [75,76]. Bivalent vaccines can also be effective, without any reduction on humoral immune response. However, multivalent vaccines are not efficient because they produce a weak and short-term antibody response [68]. While multivalent vaccines are able to protect animals for up to 10 weeks, mono or bivalent vaccines can induce an extended immunity of 16 weeks or longer [10]. Nevertheless, consecutive rounds of bivalent vaccinations, with an inter-vaccination interval of 90 days, can be successfully implemented in flocks affected by several serogroups of *D. nodosus* [77]. Vaccination against *D. nodosus* has been shown to be effective not only to prevent footrot, but to treat sheep already presenting footrot lesions [78]. The therapeutic effect can range from reduction in lameness to full healing of lesions [79]. Winter *et al.* reported an average 20% reduction in prevalence after the commencement of a vaccination program [13]. Currently, the antigenic variation is mainly attributed to fimbrae, so it is expected that specific (mono or bivalent) fimbrial vaccines will remain the best option until an antigen covering all serogroups is found [10]. Thus, future research should be focused in sequencing the *D. nodosus* genome, as it could be the key factor in developing a universal cross-protective vaccine [28].

Genetic Improvement

Some British ovine breeds such as Romney have proven to be more resistant to the development of severe footrot than the Merino breed [31]. This resistance is expressed by a quick resolution of benign lesions limited to the interdigital skin. In addition, these breeds responded more favorably to topical, parenteral and immunological therapy [10]. The natural resistance of sheep exposed to the same environmental conditions varies considerably, ranging from no clinical signs of infection to severe cases of footrot. The variation of disease resistance is expected to have an important genetic basis [80]. The estimated value of heritability of resistance to footrot could be as high as 0,31, suggesting that the use of crossbreeding with resistant animals may be very useful in producing more resilient flocks [1,80]. This will only be possible after comparing data of genetic markers with phenotypic assessment [81]. Nieuwhof *et al.* estimates that the benefits (prevalence reduction) of selecting for footrot resistance can exceed the results predicted by the existing genetic models [82]. It would be remarkable if molecular techniques could be used to identify resistant animals, which would help breeders select for footrot resistance [83]. Thanks to such techniques, it was possible to identify an association between resistance to footrot and MHC class II markers, probably because the ovine MHC class II plays an important role in modulating the response of sheep to footrot challenge [80]. This link is the basis of a commercial gene test developed in New

Zealand, which is used to select more resistant animals without the need to expose them to footrot infection [81]. However, it is expected that specific genetic tests need to be developed for each particular breed and population [1]. Mucha *et al.* attempted to identify molecular predictors of footrot resistance in Texel sheep using the genome-wide screening approach [83]. This study did not identify any potential candidate genes for footrot susceptibility, suggesting that the genetic background of footrot has a polygenic determinant. However, that study was only the first step on searching any genomic regions involved in resistance to footrot, and further researches should be employed.

Conclusion

Ovine footrot is associated with a large economic and welfare impact, remaining an important problem nowadays, despite being intensively researched for a long period. Eradication programs have been successfully implemented in limited regions, but it hasn't been possible to apply them on large-scale due to disease complexity. The diversity of *D. nodosus* strains and the environmental particularities of each region may require different control strategies. Therefore there are several areas in which researchers may gather important information that can be useful to control the disease in the future. The knowledge of the aetiology of the disease and the development of effective management practices may be key areas to control footrot. Furthermore, the production of more resilient stock can be achieved, in a short term, by implementing strategic vaccination protocols, but the protective effect will not last long if that protocols are interrupted. However, the use of genetic selection programs will be the key to produce flocks with increased resilience and resistance. In Alentejo region, Portugal, it has been developed a research in white and black Merino sheep whose goal is to identify genetic markers involved in resistance to footrot in those breeds.

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Ovine footrot in Southern Portugal: Detection of *Dichelobacter nodosus* and *Fusobacterium necrophorum* in sheep with different lesion scores

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ABSTRACT

The Mediterranean climate region of Alentejo in the Southern of Portugal is an important sheep production centre but little is known about the presence and characteristics of *Dichelobacter nodosus* in association with *Fusobacterium necrophorum* in the different footrot lesion scores. DNA from 261 interdigital biopsy samples, taken from 14 footrot affected flocks and from three non-affected flocks, were analysed for the presence of *D. nodosus* and *F. necrophorum* by real-time PCR. Both virulence and serogroup were determined for 132 and 53 *D. nodosus* positive biopsy samples, respectively. The co-infection with both bacteria was the commonest epidemiological finding associated with a greater disease severity. There was a statistically significant association ($p = 0.002$) between footrot-affected flocks and the presence of *D. nodosus*. Most *D. nodosus* positive samples were virulent (96.2 %) and belonged to serogroup B (90 %).

1. Introduction

Ovine footrot, due to *Dichelobacter nodosus* infection, is a highly contagious necrotic disease that affects hooves, with extensive economic impacts in the wool and meat industries. Disease begins as an interdigital dermatitis (ID), which may then progress to separation of the hoof horn from the underlying epidermis causing severe footrot (SFR) (Witcomb et al., 2015). The only published study in Portugal on ovine footrot was in the Alentejo region (Jiménez et al., 2003), an important Merino breed production centre, accounting for 60 % of the sheep production in Portugal (Direção-Geral de Alimentação e Veterinária - DGAV, 2019). *Fusobacterium necrophorum* has been suggested as a secondary pathogen in footrot development and, probably, in the increase of disease severity (Witcomb et al., 2014).

For the detection of these bacteria several nucleic acid based methods, more rapid and sensitive than culture, have been developed, namely PCR targeting the 16S *rRNA* gene (Frosth et al., 2012),

The thermostable AprV2 acidic protease, coded by *aprV2* gene, is found in *D. nodosus* virulent strains while the thermolabile AprB2 acidic protease, coded by the homologous gene *aprB2,* is found in benign strains (Kennan et al., 2010; Stäuble et al., 2014a).

Ten serogroups (A-I and M)) have been identified in *D. nodosus* (Dhungyel et al., 2002; Ghimire et al., 1998) based on variations in the carboxy-terminal region of the IV fimbriae subunit, and a serogroup-specific PCR assay (Dhungyel et al., 2002) targeting the *fimA* gene can discriminate nine serogroups (A-I). The identification of the predominant serogroups is the first step towards better control of the disease, through the use of both adequate prophylactic vaccines and improved biosafety measures. Additionally, prophylactic vaccines have been considered an adequate approach to footrot prevention and flock-specific vaccines has been proposed as a more efficient alternative to the multivalent commercial vaccines (Caetano et al., 2018). This work aims to detect *D. nodosus* and *F. necrophorum* in Alentejo sheep flocks, with different ovine footrot lesion scores, and to characterize *D nodosus* with respect to virulence and serogroup.

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2. Materials and methods

2.1. Sheep flocks and sampling procedure

From 13 different geographical counties in the Southern Portuguese Alentejo region (Fig. 1), 17 sheep flocks of crossbreed Merino, were selected among 689 flocks, based on epidemiological inquires to evaluate the presence of footrot in the region. The selection criteria included presence of clinical cases of footrot in the last 2 years; flocks with no less than 100 autochtone cross bread Merino sheep; no treatment for footrot within the last 30 days before sampling; availability and collaboration of the farmer. The four sheep feet were examined for footrot lesions by three DVM with large experience and training in this clinical diagnostic. Based on the Modified Egerton System (Buller and Eamens, 2014) the lesions were scored from 0 to 5, according to the extent of the lesion, as follows: score 0 - normal foot with no lesions; score 1 - limited interdigital dermatitis; score 2 - more extensive interdigital dermatitis involving part or all of the soft horn; score 3 - more extensive interdigital dermatitis with separation at the skin horn junction, extending across the heel or sole; score 4 - separation at the skin horn junction extending to the walls and outside edge of the foot; score 5 - necrotic inflammation of the deeper tissue with separation of hard horn of the foot.

Interdigital skin punch biopsies (n = 261) were collected from one foot of each animal, using disposable sterile Biopsy Punches (6 mm diameter) as described by Witcomb et al., 2015. Chlorhexidine solution (10 mg/mL) and lidocaine (Anestesin®) were applied, respectively, for local cleaning and anaesthesia. From each lesion scores the following samples were collected: score 0 n = 84, score 1 n = 48, score 2 n = 49, score 3 n = 59, score 4 n = 19 and score 5 n = 2. Samples were immediately frozen in liquid nitrogen and kept at -20 °C until being processed.

The 17 flocks, identified from A to Q, were classified in four categories (1–4) according to the criteria of Frosth et al., 2015: category 1 - majority of animals with score 0 and no animal with score above 1; category 2 - predominance of animals with score 1 and no animal with score above 1; category 3 - at least one animal with score 2; category 4 - at least one animal with score 3. Symbols representing each flock category are indicated in Fig. 1.

2.2. Reference strains

The reference strains *D. nodosus* CCUG 27824T and *F. necrophorum* subsp. *necrophorum* CCUG 9994 T (Culture Collection University of Gothenburg, Sweden) were used as controls. Nine *D. nodosus* DNA samples from serogroups A to I, used as positive controls in the serogrouping assays, were acquired to Dr. O. P. Dhungyel, University of Sydney, Australia.

2.3. Detection of D. nodosus and F. necrophorum DNA in biopsy samples by real time PCR

DNA was extracted, from 25 mg of 261 biopsy samples, using QIAamp® cador® Pathogen Mini Kit + T2 pre-treatment (Qiagen no. 50214) according to manufacturer instructions.

Detection and identification of *D. nodosus* and *F. necrophorum* was performed by real time PCR targeting, respectively, 16S *rRNA* (Frosth

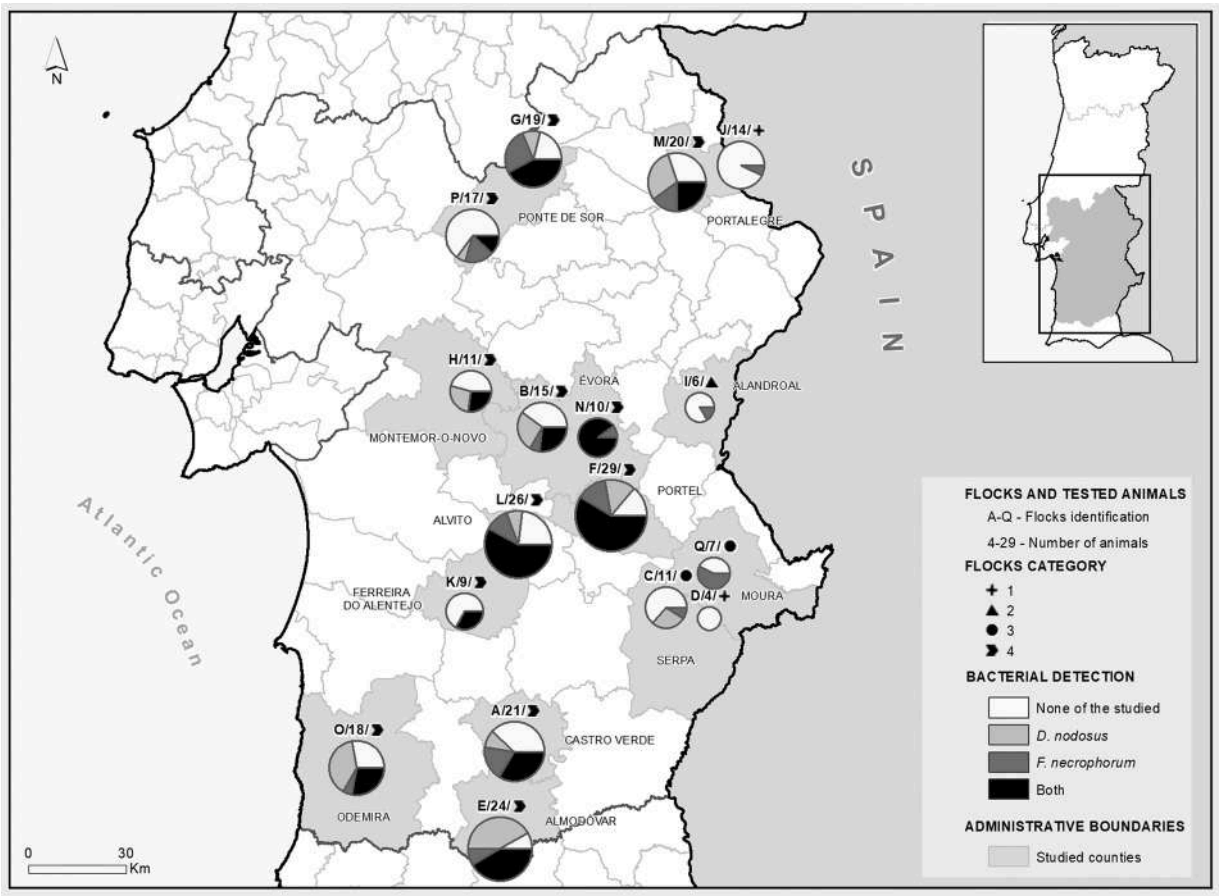

Fig. 1. Location of the 17 studied flocks in the 13 selected counties of the Alentejo region. Identification of flocks (A to Q), number of sheep tested and symbol correspondent to the flock category (1 to 4) are indicated on the top of each pie graph that marks the location of the flock. The size of this pie graph is proportional to the number of animals tested in each flock and shades of sectors corresponds to the percentage of bacterial detection (none, only *Dichelobacter nodosus*, only *Fusobacterium necrophorum* or both), according to information on the right end corner of the map.

et al., 2012) and *rpoB* (Witcomb et al., 2014) genes, according to author's description, using a Bio-Rad CFX96 system and QuantiFast Pathogen Master Mix (Qiagen no. 211354).

2.4. Determination of D. nodosus virulence

D. nodosus positive biopsy samples were analysed by competitive real-time PCR method targeting the *aprV2/B2* gene, according to Stäuble et al. (2014a), to discriminate between benign and virulent determinants. Sanger sequencing of a PCR amplified *aprV2/B2* fragment of 436 bp (Stäuble et al., 2014b) was performed (GATC, Eurofins Genomics) to confirm the real-time PCR results.

Sequences identity matching was carried out using Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nih.gov/Blast.cgi) and edited with the BioEdit Sequence Alignment Editor (Ibis Therapeutics, Carlsbad, USA) (Hall, 1999). The ClustalX2 software (Conway Institute UCD Dublin, Ireland) was used to align sequences against the reference *aprV2* nucleotide sequence from the virulent strain A198 (accession no. L38395) and the reference *aprB2* nucleotide sequence from the benign strain C305 obtained from GenBank (accession no. FN674446).

2.5. Determination of D. nodosus serogroups

To determine serogroup affinity (A-I), a multiplex PCR system targeting the *fimA* gene, described by Dhungyel et al. (2002), was performed, on positive *D. nodosus* biopsy samples, selected in each flock from sheep with lesion scores 1–5. A forward primer, common to all nine serogroups, and nine reverse primers, specific to each serogroup were used. Combinations of three serogroup specific reverse primers (ABC, DEF or GHI) were used in each multiplex PCR as suggested by Dhungyel et al. (2002). The reactions were carried out in a Biometra TOne Thermal Cycler (Analytik Jena, US) using the same conditions as described by the same authors. PCR products were visualized through 2 % (w/v) agarose gel electrophoresis at 100 V for 60 min and imaging collected using the UVP BioDoc-It® Imaging System (Analytik Jena, US).

2.6. Statistical analysis

Descriptive statistic was used to calculate proportions of different bacterial findings at flock and individual levels. These were performed using Microsoft Excel for Office 365. Fisher's Exact Test was used to analyse the connections between flock categories (1–4) and *D. nodosus* and *F. necrophorum* presence (yes/no). This test was performed using IBM SPSS Statistics for Windows, version 26.0 (Armonk, NY: IBM Corp).

2.7. Geospatial analysis

To display, explore and edit GIS datasets of the studied region, to assigned symbols and to create map layouts about the distribution and characteristics of footrot within each flock geographic region, ArcMAP software version 10.8.1 (ArcGIS) was used.

3. Results

3.1. Detection of D. nodosus and F. necrophorum DNA in biopsy samples

Considering a cycling threshold (Ct) <40 the cut-off value for the presence of *D. nodosus* or *F. necrophorum*, the Ct values of positive samples ranged between 31.28 and 39.31. Samples with a lesion score 1–5 showed always the presence of both *D.nodosus* and *F. necrophorum* (Fig. 2), with a predominance that ranged from 25 % in lesion score 1–100 % in lesion score 5, corresponding to severe footrot (SFR). *D. nodosus* alone was detected on 1.2 % of healthy feet, 25.5 % average of feet with interdigital dermatitis ID (scores 1 and 2) and 21.5 % average of feet with more severe footrot lesions (scores 3 and 4). *F necrophorum* alone was detected in 21.4 % of healthy feet, 9.2 % average of feet with ID (scores 1 and 2) and 8.86 % average of feet with more severe footrot lesions (scores 3 and 4). *F. necrophorum* was present in a lower percentage (6.9 %) than *D. nodosus* (24.1 %) in lesion score 3, but had the same percentage as *D. nodosus* in lesion score 4 (15.8 %) (Fig. 2).

Considering the flock (Fig. 1) category from 0 to 4, *D. nodosus* was detected only in severe footrot-affected flocks (categories 3 and 4) showing a significant association ($p = 0.002$) between footrot and the presence of this bacteria. On the other hand, *F. necrophorum* was detected in both footrot-affected flocks (categories 3 and 4) and in clinically healthy flocks (categories 1 and 2) and there was no significant association between flock category and detection of this bacteria ($p = 0.294$).

The flocks with higher *F. necrophorum* infection rate (Fig. 1) were flocks N (100 %), F (69 %), L (69 %), and G (68 %) and the ones with lower infection rate were flocks D (0 %), C (9 %), J (7 %) and I (17 %). *D. nodosus* was present in all but two locations (Alandroal and Moura) of the 13 studied counties (Fig. 1), with the highest presence in Almodôvar (83 %), Portel (72.4 %) and Évora (68 %).

3.2. D. nodosus virulence and serogroup

From the total of 132 *D. nodosus* positive biopsy samples, 127 (96.2 %) revealed the presence of the *aprV2* gene, coding for the thermostable AprV2 protein that is considered to confer virulence. This result was confirmed by sequencing the 436 bp amplified fragment of this gene. In five samples no amplification was obtained.

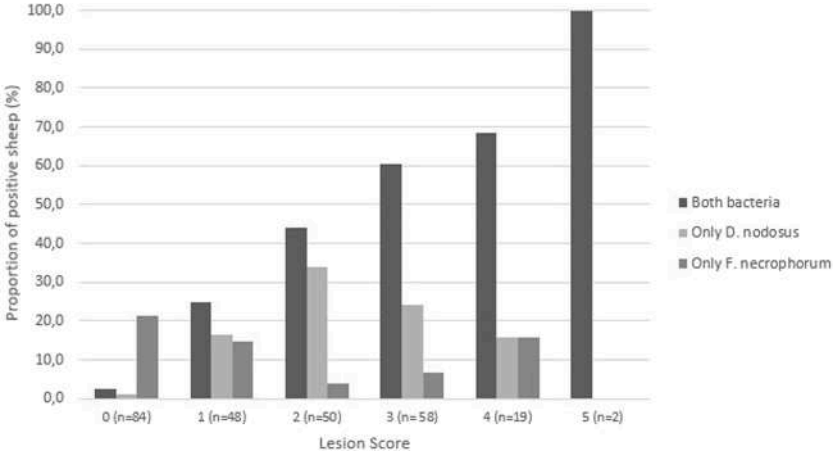

Fig. 2. Presence of *Dichelobacter nodosus*, *Fusobacterium necrophorum* or both bacteria in the different footrot lesion scores 0 to 5: score 0 – healthy foot with no lesions; score 1 - limited interdigital dermatitis; score 2 - more extensive interdigital dermatitis; score 3 - more extensive interdigital dermatitis with separation at the skin horn junction; score 4 - separation at the skin horn junction extending to the walls and outside edge of the foot; score 5 - necrotic inflammation of the deeper tissue with separation of hard horn of the foot.

Serogroups B (90 %), C (5 %) and F (5 %) were identified in 19 out of 53 *D. nodosus* positive biopsy samples.

4. Discussion

The population pattern of *D. nodosus*, along with the presence of *F. necrophorum*, were analysed with respect to the different footrot lesion scores observed in sheep from the Alentejo region. From our results the percentage of co-infections with these two bacteria increased from score 3 to 5, while in the absence of lesions (healthy feet - score 0) *F. necrophorum* predominated. An increase of *D nodosus* was observed from mild lesions (scores 1 and 2) to more severe lesions. Besides, in healthy feet (score 0) the low *D. nodosus* detection (4 %) and the high *F. necrophorum* detection (24 %) suggests that this latter bacterium may not have the ability to cause the disease, what corroborates previous observations (Roberts and Egerton, 1969; Witcomb et al., 2014).

Analysis at the flock level confirms these findings. All category 4 flocks present co-infection with *D. nodosus* and *F. necrophorum*, while in category 1, 2 and 3 flocks (flocks D/4, J/14, I/6, C/11 and Q/7) co-infection with *D. nodosus* and *F. necrophorum* was not observed. Apart from healthy flock D, category 1 or 2 flocks I, J and Q, were only infected with *F. necrophorum*. These flocks could benefit from bio sanitary measures to avoid infection with *D. nodosus* and the progression to footrot (Caetano et al., 2018). It seems that hotspots of footrot exist in the surrounding area of Évora (flocks N, F and L) since the highest percentage of *D. nodosus/F. necrophorum* co-infection was detected there. The average temperature and precipitation in the Alentejo region, when samples were collected, varied between 15.5–16.5 °C and 548–701.3 mm/m^2, respectively (Caetano, P., personal information). Serpa (flocks C and D), Moura (flock Q) and Alandroal (flock I) were the driest counties and, as expected, also the ones less infected, since wet conditions favours the development of footrot. In fact, the best environmental conditions for the development of the disease are warm and wet climate (Muzafar et al., 2016), as occurred in Évora (flock N), Portel (flock F) and Alvito (flock L) counties with an average temperature and precipitation of 15.5 °C and 701.3 mm/m^2, respectively.

Most *D. nodosus* positive biopsy samples tested were virulent (96.2 %), as expected since they were from affected sheep with footrot lesion scores 1–5, and 19 out of 53 belonged to the serogroup B. The serogroup of 34 *D. nodosus* positive biopsy samples were undetermined using the multiplex PCR, possibly because they belong to the serogroup M or to another yet unknown serogroup not targeted in this PCR. Serogroup B was also the most frequently found in New Zealand, United Kingdom and India (Caetano et al., 2018; Wani et al., 2019). However, in nearby Spain serogroups A and C were the most commonly detected by slide microagglutination technique (Hurtado et al., 1998). In Alto Alentejo region (Montemor-o-Novo, Évora, Alandroal counties and above) Jiménez et al. (2003), using the microagglutination technique, identified, predominantly, serogroups D, F and I (14.5 % each), while in this region we identified serogroups B and C. The only common serogroups identified in both works were serogroups D and F in flock F located in Portel, Baixo Alentejo. The existence of more than one serogroup in the same flock was frequent, with the exception of flocks M, N and O with single serogroups. These findings provide valuable information for the development of immunoprophylactic methods, such as herd-specific vaccines, which are made from the isolated microorganisms in the region and are more efficient than multivalent commercial vaccines (Caetano et al., 2018).

Ethical approval

This study was approved by the ethics committee for animal experimentation ORBEA-U Évora, Portugal (ID: GD/20467/2021/P1).

Declaration of Competing Interest

No competing interests to declare.

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Genome-wide assessment of the population structure and genetic diversity of four Portuguese native sheep breeds

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As the effects of global warming become increasingly complex and difficult to manage, the conservation and sustainable use of locally adapted sheep breeds are gaining ground. Portuguese native sheep breeds are important reservoirs of genetic diversity, highly adapted to harsh environments and reared in low input production systems. Genomic data that would describe the breeds in detail and accelerate the selection of more resilient animals to be able to cope with climatic challenges are still lacking. Here, we sequenced the genomes of 37 animals from four Portuguese native sheep breeds (Campaniça, Bordaleira Serra da Estrela, Merino Branco and Merino Preto) and 19 crossbred sheep to make inferences on their genomic diversity and population structure. Mean genomic diversities were very similar across these breeds ($.30 \leq H_o \leq .34$; $.30 \leq H_e \leq .35$; $1.7 \times 10^{-3} \leq \pi \leq 3.1 \times 10^{-3}$) and the levels of inbreeding were negligible ($.005 \leq F_{IS} \leq .038$). The Principal Components, Bayesian clustering and Treemix analyses split the Portuguese breeds in two main groups which are consistent with historical records: one comprising Campaniça and Serra da Estrela together with other European and transboundary dairy breeds; and another of the well-differentiated multi-purpose Merino and Merino-related breeds. Runs of homozygosity analyses yielded 1,690 ROH segments covering an average of 2.27 Gb across the genome in all individuals. The overall genome covered by ROH segments varied from 27,75 Mb in Serra da Estrela to 61,29 Mb in Campaniça. The phylogenetic analysis of sheep mitogenomes grouped the Portuguese native breeds within sub-haplogroup B1a along with two animals of the Akkaraman breed from Turkey. This result provides additional support to a direct influence of Southwest Asian sheep in local breeds from the Iberian Peninsula. Our study is a first step pertaining to the genomic characterization of Portuguese sheep breeds and the results emphasize the potential of genomic data as a valid tool to guide conservation efforts in locally adapted sheep breeds. In addition, the genomic data we generated can be used to identify markers for breed assignment and traceability of certified breed-products.

Abbreviations: CAM, Campaniça; CB, Crossbred Sheep; DNA, Deoxyribonucleic acid; HTS, High-throughput sequencing; MAF, Minor allele frequency; MB, Merino Branco; ML, maximum likelihood; MP, Merino Preto; PCA, Principal component analysis; ROH, Runs of homozygosity; SE, Bordaleira Serra da Estrela; SNP, Single-nucleotide polymorphism.

KEYWORDS

Ovis aries, high-throughput sequencing, population structure, genomic diversity, single nucleotide polymorphism, native breeds

1 Introduction

Since their domestication in the Fertile Crescent, approximately 10,500 years BP, sheep (*Ovis aries*) quickly became a valuable resource for the production of meat, milk, wool and leather products (Liu et al., 2016; Alberto et al., 2018). Nowadays, due to its physiological, morphological and behavioral characteristics, this species is well adapted to a wide range of climates and low-input agricultural environments. Local sheep are important domestic animal genetic resources for their biodiversity, role in landscape conservation and relevant contribution to the socio-economies of undeveloped and developing regions (Hassan et al., 1998; Yune and Abdela, 2017; Berihulay et al., 2019). The implementation of breeding strategies focused on environmental tolerance and specific traits of commercial interest, along with high mobility following transhumance routes, contributed to the high levels of biodiversity observed across the broad spectrum of sheep breeds worldwide (Kijas et al., 2012).

In the Iberian Peninsula, sheep are common livestock reared across the territory mainly in agrosilvopastoral systems, contributing to the environmental sustainability and heritage value in rural communities (Dinis and Simões, 2021). Selection resulted in several breeds specialized for either meat, milk or wool production or reared as dual/triple purpose animals in distinct regions. In Portugal, there are 16 native sheep breeds registered in their specific herdbook (https://www.dgav.pt/animais/conteudo/recursos-geneticos-animais/racas-autoctones/ovinos/) (Figure 1). These breeds are divided in three major groups according to their fleece characteristics, i.e., Merino (fine wool), Bordaleiro (intermediate wool), and Churra (coarse wool) (Santos-Silva et al., 2008). Among the Portuguese sheep breeds, Bordaleira Serra da Estrela (SE), Merino Branco (MB), Merino Preto (MP), and Campaniça (CAM) are some of the most abundant raised under extensive conditions (Tiberio and Diniz, 2014) (Supplementary File S1). SE is the most important Portuguese dairy breed, inhabiting the Serra da Estrela Mountain region, one of the most inhospitable areas in the country. Its milk yield can exceed .78 L per day in a lactation period of up to 248 days. The Serra da Estrela cheese is a typical high-value product deriving from this breed, which has been granted a protected designation of origin (Carolino et al., 2003). However, the commercial value of this breed is not restricted to milk products. For many years, it was the wool provided by the SE herds that supplied the industry in this mountain region (Monteiro and Santos, 2021). The MB, MP and CAM breeds are mainly distributed in the south of Portugal, throughout the Alentejo region. They have shown an extraordinary ability to adapt to arid climates, thriving under harsh conditions and with poor food resources. Their intrinsic resilience and rusticity have been explored by breeders, creating opportunities to select animals that are better suited to cope with climate changes. These breeds produce high-quality meat, dairy and wool products (Matos, 2012a; Plowman et al., 2019). The population sizes of Portuguese native breeds have declined over the last years (Tiberio and Diniz, 2014), due to agricultural land abandonment and the consequent desertification, as well as replacement by more productive (but also more demanding) transboundary commercial breeds.

In the last decade, the enormous progress in high-throughput sequencing (HTS) technologies (Van Dijk et al., 2014) provides unprecedented opportunities for understanding the genomic basis for livestock phenotypic variability, including complex production traits (Zhang et al., 2011; Ghosh et al., 2018). The availability of HTS data allows to estimate genomic diversity and investigate the impact of demographic processes across the genome bringing in new perspectives for the conservation of local breeds (Fernández et al., 2016; Eusebi et al., 2020). Likewise, genome-wide single-nucleotide polymorphisms (SNPs) have been commonly used in commercial arrays to detect genetic variability in sheep breeds and describe their population structure (Kijas et al., 2009; Grasso et al., 2014). These markers are suitable for the detection of selection signatures across the genome based on, e.g., runs of homozygosity (ROH) (Peripolli et al., 2017), to detect genetic variants associated with traits of economic interest and to obtain valuable information to manage the extent of inbreeding in livestock breeds (Purfield et al., 2017). While molecular analyses have shown that native Iberian sheep hold great maternal haplotype diversity with three haplogroups (A, B, and C) represented (Pereira et al., 2006; Pedrosa et al., 2007; Chessa et al., 2009), genomic studies in these breeds are still lacking.

Genetic diversity is a key factor underlying the adaptive capacity and resilience of livestock populations under changing conditions (Hoffmann, 2013). The purpose of this study was to estimate the genomic diversity of four Portuguese native breeds and a population of crossbred sheep using a HTS approach. We also aimed to identify and characterize genome-wide patterns of ROH in these breeds. The HTS data was used to investigate the population structure of these Iberian breeds and their relationship with worldwide sheep. This is crucial to try to disclose their evolutionary histories and understand in which ways the changes in the population dynamics, e.g., bottlenecks and admixture, impacted their genomes and their differentiation. Our results have the potential of being used for an improved management of these local genetic resources, and the implementation of breeding strategies for the long-term conservation of Portuguese native sheep.

2 Materials and methods

2.1 Ethics statement

Animal handling and blood collection were performed during routine veterinary check-ups, following the official animal healthcare program guidelines and under the consent of breeders.

2.2 Biological samples and datasets

A total of 37 blood samples of animals representative of four Portuguese sheep breeds [Campaniça ($n = 6$), Serra da Estrela ($n = 11$), Merino Branco ($n = 10$) and Merino Preto ($n = 10$)] were collected at 15 farms throughout the country. Crossbred sheep (CB) sampled at other 10 farms distributed across the Alentejo region were also included in the analysis for comparison purposes [Crossbreds ($n = 19$)]. The animals were randomly selected from each herd. Ten

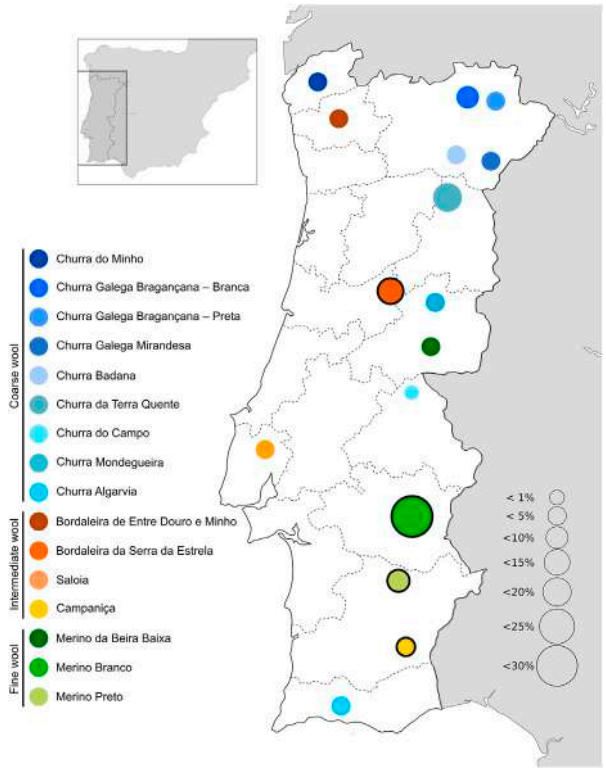

FIGURE 1
Geographical distribution and relative abundance of Portuguese native sheep breeds. The breeds sampled in this study are highlighted by a black circle.

milliliters of blood were collected from the jugular vein by vacuum puncture and stored at −20°C in collection tubes containing EDTA. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and quality were assessed with a Nanodrop spectrophotometer (Thermo Fisher Scientific™, Waltham, United States) and 500 ng of each sample were used for preparation of genomic libraries for resequencing (Protocols, 2022). Whole-genome HTS data were obtained through service acquisition (BGISEQ-500 sequencing platform, BGI, Shenzhen, China), which produced approximately 34.9 billion paired-end (2 × 100 bp) raw reads and an average depth of sequencing coverage of 21X. Details on locations, breed characteristics, sequencing statistics and accession numbers are shown in Supplementary Table S1.

We used two complementary strategies to investigate breed relationships and infer the population structure of Portuguese sheep breeds by integrating our HTS data with: 1) whole-genome data publicly available for other European, Asian, African, Australian and transboundary commercial breeds; and 2) Illumina Ovine 50 K SNP genotype data obtained for other Iberian breeds within the Sheep HapMap Consortium (International Sheep Genomics Consortium). The population structure analysis of worldwide sheep included a total of 48 animals representative of 18 breeds and the Asiatic mouflon (*Ovis orientalis*) which was used as an outgroup. Whole-genome HTS data were retrieved from the NCBI database (Bioproject ID: PRJNA624020; PRJNA160933; and PRJNA160933). Details on breeds, locations and accession numbers are shown in Supplementary Table S2. The SNP genotyping data consisted of 182 animals representing 9 Spanish breeds (Ciani et al., 2020) (see Supplementary Table S3 for details on breeds, sample sizes and locations). Furthermore, 48 mitogenomes retrieved from NCBI [Genbank accession n. NC_001941.1 (Hiendleder et al., 1998); PopSets 298110621 (Meadows et al., 2011), 583828744 (Lv et al., 2015) 158187235 (Burgstaller et al., 2007) and 528748432 (Lancioni et al., 2013)] were combined with mitochondrial consensus sequences from our shotgun data for a comprehensive phylogenetic analysis (see Supplementary Table S4).

2.3 Sequencing data pre-processing, mapping and SNP calling

The quality of paired-end raw reads was checked with the FastQC v.0.11.5 software (Andrews, 2010) and filtering was done with Trimmomatic v.0.38 (Bolger et al., 2014). Adapter sequences and low-quality bases, with less than an average quality threshold of 20 over a sliding window of 10 bp, were trimmed from the end of each read. Following, reads shorter than 80 bp were removed, resulting in ~32.5 billion high-quality reads for downstream analyses. Mapping to the sheep reference genome Oar_rambouillet_v1.0 (Bioproject ID: PRJNA414087) was performed using BWA MEM v.0.7.15-r1140 (Li and Durbin, 2009) with default settings. The alignments were indexed and sorted with SAMtools v.1.4.1 (Li et al., 2009). Non-specific matches were excluded from the analysis, considering only unique mapped reads (91.8%) for SNP calling performed with Freebayes v.1.2.0 (Garrison and Marth, 2012). A total of 115,137,724 raw SNPs uniformly distributed across all chromosomes (R^2 = .966) were filtered based on quality (minQ > 30), SNP coverage per genotype (minDP ≥ 7) and genotype quality (minGQ > 20) using VCFtools v.0.1.17 (Danecek et al., 2011). After filtering, a set of 31,320,380 high-quality autosomal SNPs was used for downstream analyses. SNPs were then annotated using ANNOVAR (downloaded 2019-10-24) (Wang et al., 2010) and categorized according to the functional effects and distribution across genomic regions that included X (65.2%), Y (33.4%) and Z (.7%) located within intergenic, intronic and exonic regions, respectively. The SNPs found in coding regions, included 120,172 (57.2%) and 80,882 (38.5%) associated with synonymous and non-synonymous effects, respectively (Supplementary Table S5).

2.4 Genomic diversity and differentiation

Estimates of genomic diversity and the levels of differentiation among Portuguese sheep breeds were determined from HTS data with VCFtools v.0.1.17 (Danecek et al., 2011), in particular: nucleotide diversity (π); observed and expected heterozygosities (H_O and H_E, respectively); genomic inbreeding coefficient (F_{IS}) and fixation index (F_{ST}). First, autosomal SNPs were filtered based on MAF (--maf .05), calling rate (--max-missing .1) and Hardy-Weinberg equilibrium (--hwe .001). SNPs that did not pass these quality criteria were excluded from the analysis. The nucleotide diversity was estimated as the average number of nucleotide differences per site within 10 Kb windows (--window-pi 10,000) across the genome. The expected and

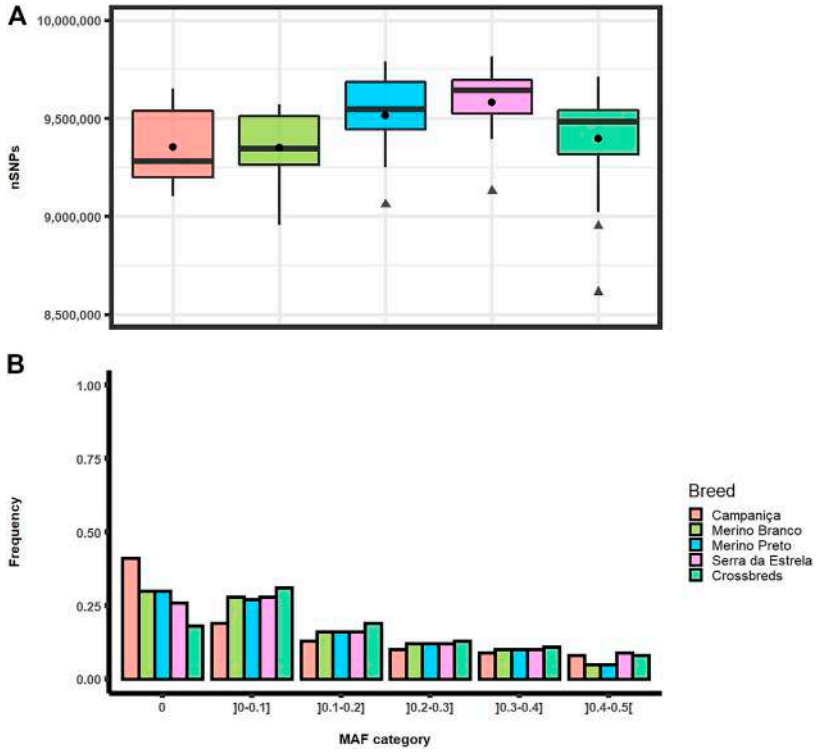

FIGURE 2
Distribution of single nucleotide polymorphisms in Portuguese sheep. **(A)** Boxplot graph of the total number of SNPs per individual observed in each population. Mean values are represented by black circles and outliers by triangles; **(B)** Frequency of minor allele frequency (MAF) by category in each breed.

observed heterozygosities, as well as the inbreeding coefficients, were estimated for each population using the functions (--hardy) and (--het), respectively. For pairwise breed comparisons, F_{ST} values were calculated following Weir and Cockerham's (Weir and Cockerham, 1984), with a sliding window of 10 Kb.

2.5 Detection and distribution of runs of homozygosity

A genome-wide detection of runs of homozygosity (ROH) was carried out in Portuguese native sheep in a sliding-window approach using PLINK software v.1.90b5.2 (Purcell et al., 2007). Briefly, ROH were defined as homozygous segments longer than 1 Mb and containing at least 50 autosomal SNPs with an average density of more than one SNP per 100 Kb. Furthermore, a segment was considered a ROH, if there was up to one heterozygous loci, no more than five missing genotypes and a maximum gap between consecutive SNPs of 250 Kb. The detected ROHs were categorized based on their length, and consensus ROH segments were estimated for each breed. Finally, the ROH-based inbreeding coefficient (F_{ROH}) was calculated either for each chromosome or genome-wide in each population as the ratio of the total length of ROH for each individual and the total length of the autosomal chromosomes. The R v.4.0.5 (Team, 2020) package detectRUNS v.0.9.6 (https://CRAN.R-project.org/package=detectRUNS) was used to obtain summary statistics and visualize the results.

2.6 Population structure of Iberian and worldwide sheep breeds

PLINK v.1.90b5.2 was used to carry out principal component analysis (PCA) and integrate the autosomal HTS data obtained for the Portuguese sheep with: 1) whole-genomes of 45 worldwide sheep and three Asian mouflon (*Ovis orientalis*) (Supplementary Table S2); and 2) SNP genotyping data available for 182 native sheep from Spain (Supplementary Table S3). To detect first-degree relationships between individuals, KING kinship coefficients (Manichaikul et al., 2010) were estimated using a cutoff of .177. The combined data sets were pruned according to the following: remove SNPs with a minor allele frequency (MAF) lower than 5%; exclude samples and markers with more than 10% missing data; account for Hardy-Weinberg equilibrium (-hwe .001) and linkage disequilibrium (--indep-pairwise 50 10 2). A total of 987,574 and 19,651 autosomal SNPs were retained for downstream analyses, respectively. Additionally, population structure was also assessed using the model-based clustering approach implemented in ADMIXTURE v.1.3.0 (Alexander and Lange, 2011). Individual ancestry proportions were calculated for *K* values ranging from 2 to 21 using the default settings. For each K value, five replicate runs with different random seeds were done. The CLUMPAK software (Kopelman et al., 2015) was used to infer the most likely K based on Evanno et al. (2005) and considering K values from 2 to 14. A graphical representation of these results was obtained using the Tidyverse collection of the R packages (Wickham et al., 2019).

TABLE 1 Genomic diversity of Portuguese sheep. Breed names and acronyms, sample sizes, observed (Ho) and expected (He) heterozygosity, nucleotide diversity (π) and the inbreeding coefficient (F_{IS}) are shown.

Breed name	Acronym	Sample size	Genomic diversity indexes			
			H_o	H_e	π	F_{IS}
Campaniça	CAM	6	.34	.35	2.016×10^{-3}	.038
Merino Branco	MB	10	.30	.30	2.006×10^{-3}	.012
Merino Preto	MP	10	.30	.30	2.032×10^{-3}	.005
Bordaleira Serra da Estrela	SE	11	.34	.35	1.956×10^{-3}	.022
Crossbreds	CB	19	.30	.31	1.981×10^{-3}	.026

TABLE 2 Pairwise-breed estimates of genomic differentiation (F_{ST}) among Portuguese sheep. Breed acronyms are shown in Table 1.

Breed	CAM	MB	MP	SE	CB
CAM	—				
MB	.037	—			
MP	.035	.021	—		
SE	.028	.029	.027	—	
CB	.035	.005	.020	.027	—

2.7 Phylogenetic analyses of autosomal and mitogenome data

The Maximum Likelihood (ML) phylogeny of sheep mitogenomes was inferred under the TN93 + R evolutionary model selected using the Akaike Information Criterion (AIC) (Posada and Buckley, 2004) in the PhyML software v.3.0 (Guindon et al., 2010) online platform, starting tree with BioNJ and branch support calculated from 100 bootstrap inferences. Briefly, after clean reads mapping to the reference sheep mitogenome, the consensus sequences were retrieved with ANGSD v.0.935 (-doFasta 3 -minQ 20 -minMapQ 30 -MinDepth 7 -doDepth 1) (Korneliussen et al., 2014) in FASTA format. Representatives of each maternal haplogroup available from public repositories were included in this analysis (Supplementary Table S4). A total of 104 mitogenomes were aligned using MUSCLE v.3 (Edgar, 2004). Finally, the phylogenetic tree was visualized and edited in FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

The Treemix software v.1.13 (Pickrell and Pritchard, 2012) was used to investigate genetic relationships (splitting and mixing) between Iberian sheep breeds using allele frequencies for 11,239 SNP positions included in the Illumina Ovine 50 K SNP array that can be unambiguously assigned to autosomal positions in the sheep reference genome Oar_rambouillet_v1.0 (Bioproject ID: PRJNA414087) following (Nicolazzi et al., 2015). Treemix was run using the default settings with a block size of 100 SNPs, 500 bootstrap replicates and Asian Mouflon as outgroup. The optimal number of migration events (m = 1–10) to add to the tree were determined using the OptM package (Fitak, 2021) in R v.4.0.5 (Team, 2020) with 10 independent replicates at each value of m. Phylogenetic networks were visualized using the Treemix R script "plotting_funcs."

3 Results

3.1 Genomic diversity

Analyses of genomic diversity based on HTS data were performed for 56 sheep of four Portuguese native breeds (Campaniça, Merino Branco, Merino Preto and Bordaleira Serra da Estrela) and the crossbred sheep population. On average, the total number of SNPs per individual ranged from 9,348,138 SNPs in Merino Branco to 9,581,638 SNPs in Serra da Estrela. Based on the Kruskal-Wallis statistical inference analysis for the p-value cutoff of .01 the average number of SNPs per individual did not differ significantly between breeds (Figure 2A).

The distributions of SNPs across MAF categories in each population are summarized in Figure 2B and are useful to evaluate the gene pool richness and genomic variability. The proportion of fixed SNPs (MAF = 0) displayed considerable differences among breeds, with CAM showing a markedly higher percentage (41.5%) than the other breeds, whereas the percentage of highly polymorphic SNPs (.4 < MAF <.5) was more uniform across breeds (overall average of 6.7%) ranging from 4.9% in MB and MP to 8.6% in SE. The overall levels of genomic variability were very similar across these sheep breeds (Table 1). On average, autosomal nucleotide diversity ranged from $\pi = 1.956 \times 10^{-3}$ (SE) to $\pi = 2.032 \times 10^{-3}$ (MP). The comparisons between breeds of expected (He) and observed (Ho) heterozygosities were not significantly different (Kruskal-Wallis, $p < .01$). On average, He varied between .3 (MB and MP) and .35 (CAM) with an overall mean of .32, and Ho ranged from .30 (MB and MP) to .34 (CAM and SE) with an overall mean of .31. The genomic inbreeding coefficient estimated from SNP data was relatively low in all breeds (MB: .012, MP: .005 and SE: .022), with the highest value observed in CAM (.038). Weir and Cockerham's mean pairwise F_{ST} was used as a measure of breed differentiation across breeds (Table 2). The pairwise-breed F_{ST} values ranged from .005 (MB and CB) to .037 (CAM and MB), showing a close genetic relationship between Portuguese native sheep. CAM and the dairy breed SE had the highest mean F_{ST} across pairwise comparisons, consistently with their relative geographic isolation, and in the case of the latter also selection for milk production.

3.2 Runs of homozygosity and inbreeding

We identified a total of 1,690 ROH comprising an average of 2.27 Gb across the genome in Portuguese sheep. The number of ROH

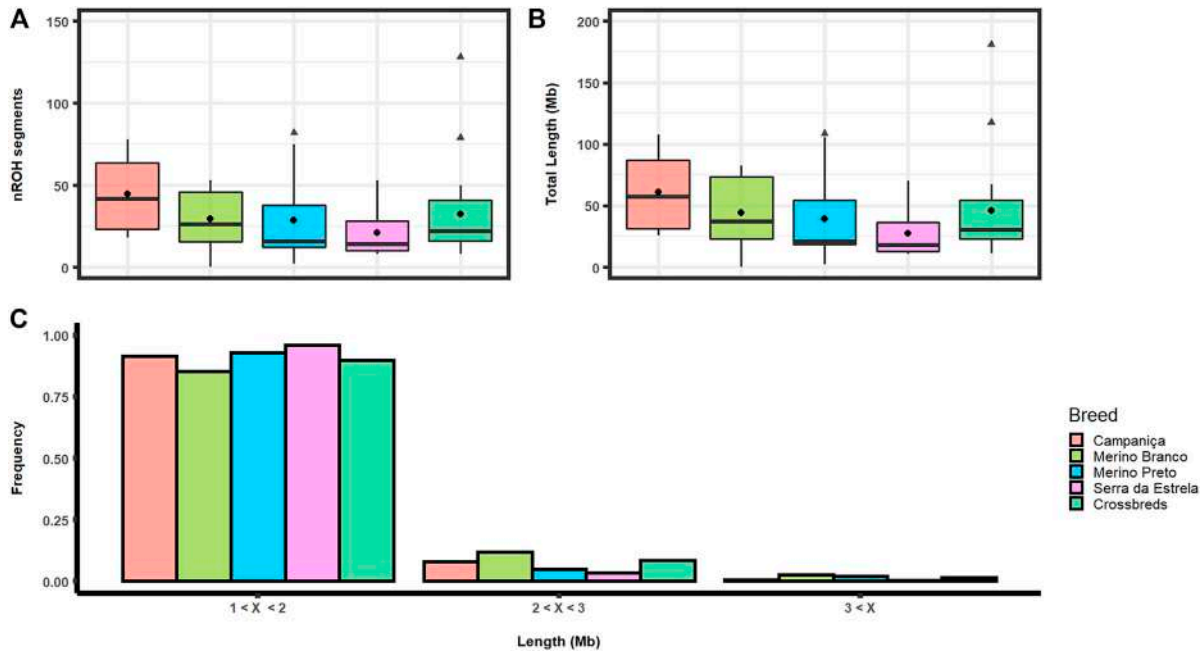

FIGURE 3
Runs of homozygosity (ROH) in Portuguese sheep. **(A)** Boxplot graph depicting the average number of ROH segments (nROH) per animal in each population. Mean values are represented by black circles and outliers by triangles; **(B)** Boxplot graph depicting average ROH length in each population. Mean values are represented by black circles and outliers by triangles; **(C)** Frequency distribution of the number of ROH by different length categories, i.e., short (1–2 Mb), medium (2–3 Mb) and large (over 3 Mb) for each population.

ranged from 11 on chromosome 24 to 213 on chromosome 3. The distribution of ROH segments was strongly correlated with the chromosome size (R^2 = .8239). CAM showed the highest average number of homozygous segments per animal (nROH = 44.5) comprising on average 61.29 Mb, whereas the lowest values were observed in the dairy breed SE (nROH = 20.9) comprising about 27.75 Mb per animal. The Merino breeds showed an intermediate number of ROH segments (MB: 29.5 and MP: 28.5) and mean lengths (MB: 44.39 Mb, and MP: 39.53 Mb) per animal (Figures 3A, B). The longest homozygous segment (~4.9 Mb harbouring 27,839 SNPs) was identified on chromosome 6 in MB. The ROH segments were grouped in three categories by length: 1) short (1 Mb–2 Mb); 2) medium (2 Mb–3 Mb); and 3) Large (>3 Mb). Most of these ROH belong to the short category (~91%), while the large category accounted for a small fraction (~1.6%) (Figure 3C).

The ROH-based inbreeding coefficient (FROH) was estimated for each population (Figure 4). On average, CAM had the highest F_{ROH} (.023) and SE the lowest (.010). F_{ROH} estimates across chromosomes varied within and between breeds (Supplementary Figure S1), which suggests that it could be associated to regions under positive selection for specific production or adaptive traits.

3.3 Population structure in Iberian and worldwide sheep

The PCA based on HTS data was conducted to assess the population structure of Portuguese native and worldwide sheep breeds and infer the proportion of the total genomic variation explained by each PC. The first two PCs, which account for the highest variation of the data set, depicted: an east-to-west cline of sheep breeds (PC1, explains 10.3% of the total genetic variation); and the differentiation between the Asiatic Mouflon, domestic sheep and the inbred Suffolk and East-Friesian breeds (PC2, 7.3%) (Figure 5). The Portuguese populations clustered into two groups, as follows: 1) the well-differentiated Merino breeds grouped together with the Merino-derived Swiss White Alpine breed indicating a close genetic relationship; 2) CAM and SE clustered in the central group along with other European and dairy sheep breeds.

For a fine resolution, the population structure of Iberian breeds was also assessed in a PCA by merging our HTS data collected for Portuguese sheep with Illumina 50 K SNP genotyping data available for nine Spanish breeds (Figure 6). The first two components accounted for over 18% of the total genomic variation. The Portuguese Merino breeds and the crossbreds clustered together with the Spanish Merino, which confirms their close genetic relationship. In addition, CAM and SE were separated from the Merino cluster by PC2 and grouped with other intermediate-fine wool Spanish breeds. The Basque breeds of coarse wool type—Latxa and Sasi Ardi, formed an isolated cluster.

ADMIXTURE analysis allowed us to infer ancestry contributions underlying the gene pool of Portuguese native sheep. The results of the model-based clustering approach are shown in Figure 7 for the most likely K value (K = 4) (for the Delta K graph see Supplementary Figure S2). Additional results were obtained for values of K ranging from K = 2 to K = 21 (Supplementary File S2). When considering two ancestral populations (K = 2), CAM and SE shared a greater proportion of the mouflon component along with other European and dairy breeds, than Merino and the SWA breeds which had little contributions. For

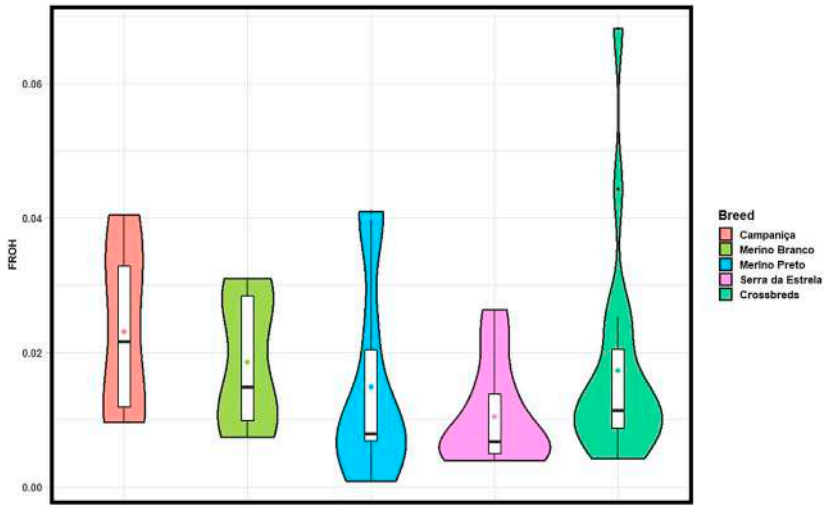

FIGURE 4
Violin plots showing ROH-based inbreeding coefficient (FROH) calculated in each population considering all ROH segments (> 1 Mb). Mean values are represented by coloured circles.

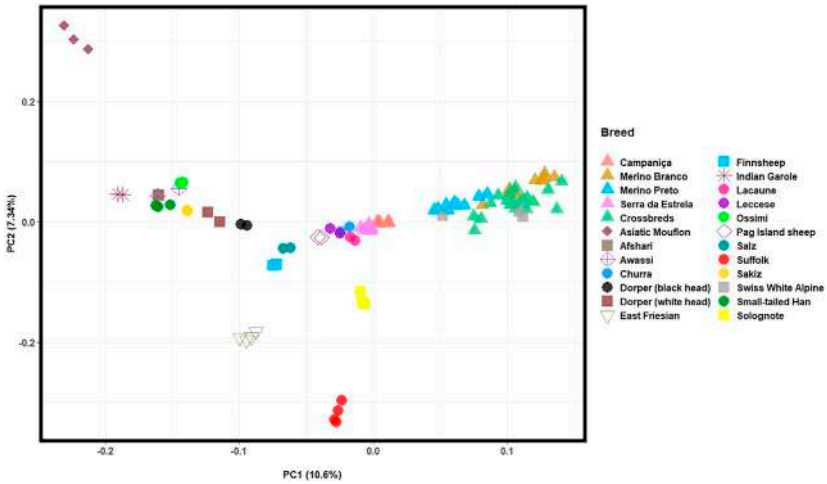

FIGURE 5
Principal component analysis (PCA) of Iberian and worldwide sheep. PC1 and PC2 account for 10.3% and 7.27% of the total genomic variation, respectively. The Portuguese sheep are represented by coloured triangles, as follows: Campaniça in salmon; Merino Branco in golden brown; Merino Preto in blue; Serra da Estrela in pink; and Crossbreds in green. See Supplementary Table S2 for details on each individual included in the analysis.

K = 3, the mouflon formed an independent cluster, with the Asian and Middle Eastern sheep showing some proportion of mouflon ancestry. For K = 4, the Merino sheep and the SWA clustered together and were more homogeneous than their Iberian counterparts CAM, SE and CHU (a coarse wool breed) which showed an admixed ancestry. Crossbreds shared MB and SWA ancestry. Also, the Asian sheep formed their own group, while VF and SFK were clearly differentiated from all other breeds. As K-values increased, CAM and SE individuals split in two clusters, with three individuals from each of these breeds showing a more heterogeneous pattern of ancestry common to other European populations (CHU, LAC, LEC, POG, and FINN). For K > 9, MP forms a separate cluster from MB, SWA and the crossbreds. These patterns of ancestry were also observed when the crossbreds were removed from the analysis (results not shown).

3.4 Phylogenetic analyses of mitogenome and autosomal data

Phylogenetic relationships inferred from the sheep mitogenomes are depicted in Figure 8 (for details see Supplementary Figure S3). Portuguese native breeds belong to haplogroup B except for three crossbred animals that were assigned to haplogroup A. We did not observe a clear

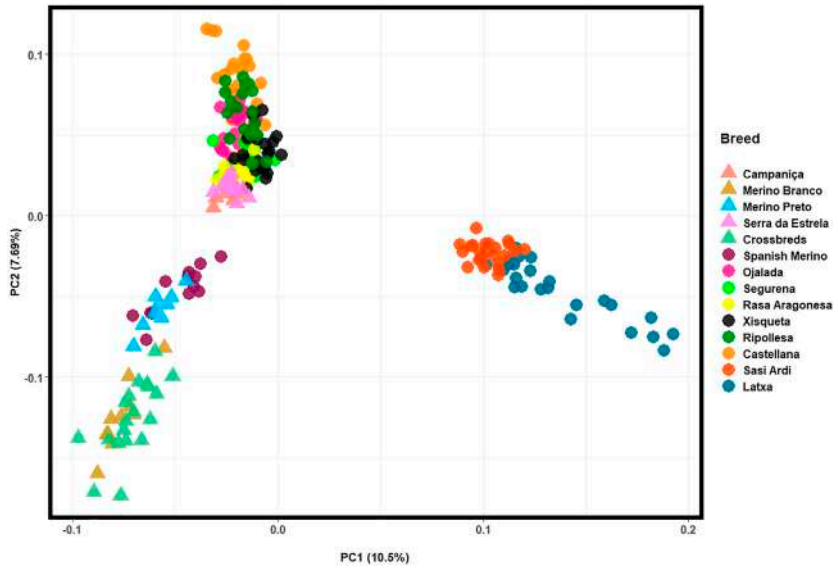

FIGURE 6
Principal component analysis (PCA) of Iberian sheep. PC1 and PC2 account for 10.5% and 7.69% of the total genomic variation, respectively. Portuguese sheep are represented by coloured triangles, as follows: Campaniça in salmon; Merino Branco in golden brown; Merino Preto in blue; Serra da Estrela in pink; and Crossbreds in green. See Supplementary Table S3 for details on each individual included in the analysis.

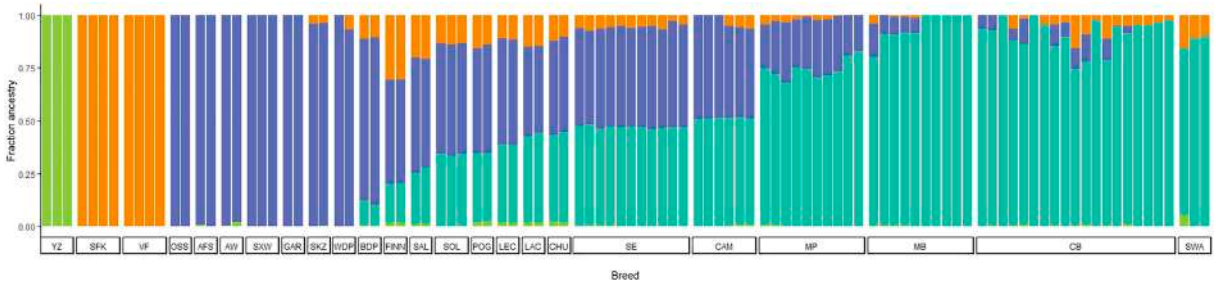

FIGURE 7
Model-based clustering analysis of Iberian and worldwide sheep. The proportions of the inferred ancestral clusters (K = 4) are depicted by the different colours with each individual represented by a bar and sorted by breed. CAM—Campaniça; MB—Merino Branco; MP—Merino Preto; SE—Serra da Estrela; and CB—Crossbreds. The results for K = 2 to K = 21 are shown in Supplementary File S2. See Supplementary Table S2 for details on each individual included in the analysis.

differentiation between the maternal lineages of the fine-wool (MB and MP) and intermediate-wool (CAM and SE) breeds. Portuguese native breeds formed a well-supported cluster within sub-haplogroup B1a along with two individuals of the Akkaraman breed from Turkey (Genbank acc. n. HM236176 and HM236177) and the reference mitogenome of a Merinolandschaf (Genbank acc. n. NC001941).

To evaluate the phylogenetic relationships and historical genetic drift events among Iberian breeds, we built a maximum likelihood (ML) tree based on the population allele frequency covariance matrix and rooted in the Asian Mouflon using TreeMix (Supplementary Figure S4). When one migration event was assumed, all domestic sheep populations clustered into one primary branch, showing the presence of gene flow between Merino Branco and Spanish Merino (Figure 9). Overall, the topology of the ML tree was consistent with the results revealed by the PCA analysis.

4 Discussion

We conducted the first whole-genome sequencing analysis of Portuguese native sheep in which four breeds and a population of crossbreds were characterized in the context of other Iberian and worldwide sheep. Improving knowledge on the genomic diversity and population structure of local sheep is especially important to disclose signatures of adaptation and improvement, but also to implement appropriate management and conservation strategies. To date, few genetic studies have been carried out on Portuguese native sheep and

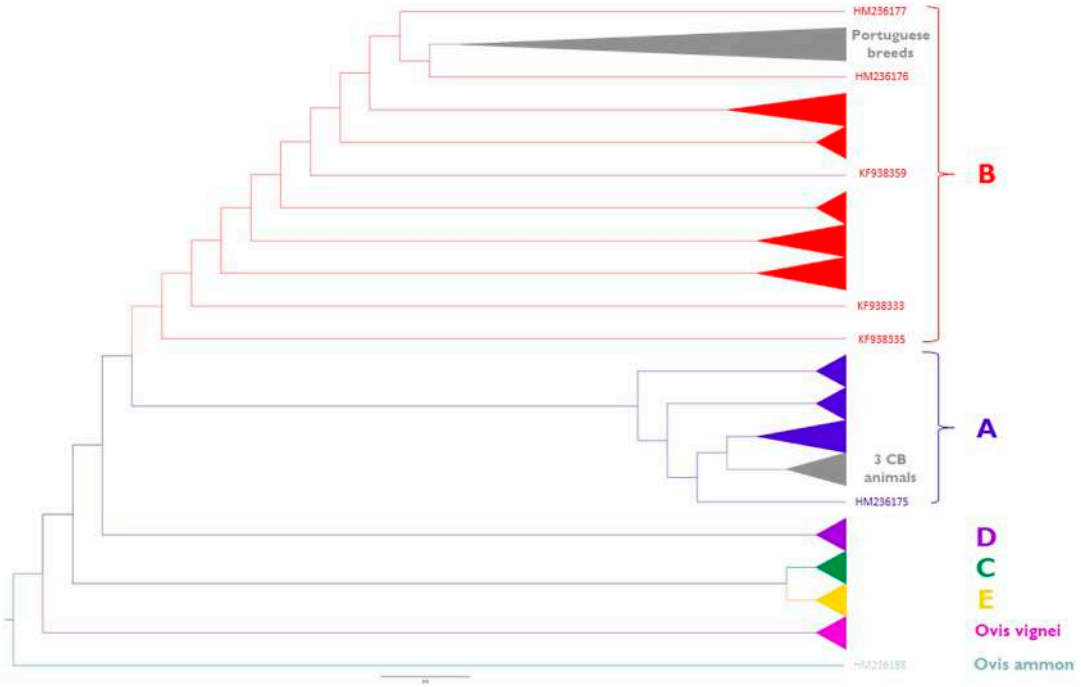

FIGURE 8
Maximum-Likelihood phylogeny of sheep mitogenomes. Portuguese native sheep (light grey) belong to haplogroup B1a and three crossbred animals clustered within haplogroup A1a. Reference sequences representing major haplogroups are colored as follows: **(A)** (dark blue); **(B)** (red); **(C)** (dark green); **(D)** (purple); **(E)** (yellow). Wild sheep mitogenomes were also included in the analysis: *Ovis musimon* (within haplogroup A); *Ovis vignei* (pink); *Ovis ammon* (petrol blue). See Supplementary Table S4 for details on each individual included in the analysis.

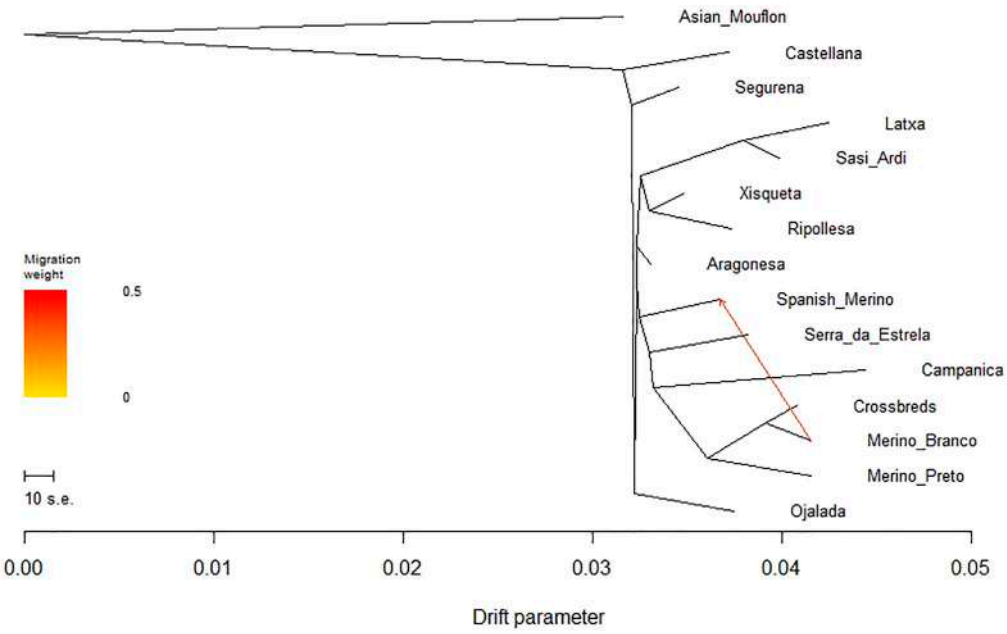

FIGURE 9
Phylogenetic network inferred by Treemix for Iberian sheep. One migration event (m = 1) among populations was allowed and is represented by an arrow indicating gene flow between Merino Branco and Spanish Merino.

were based on either mitochondrial data or a limited number of microsatellite markers (Pereira et al., 2006; Santos-Silva et al., 2008; Landi et al., 2019). We report genomic variation and ROH patterns in these breeds. Following, we used complementary population genetics and phylogenetic approaches to infer population structure, patterns of admixture and breed relationships.

Genetic variation among breeds is usually expressed in terms of allele frequencies. Our results revealed a moderate level of polymorphic SNPs (MAF >.01) with slight differences between breeds. However, a significantly high proportion of fixed SNPs (MAF = 0) was observed in CAM which can be due to a recent bottleneck from replacement by high-output transboundary breeds (Matos, 2012b), or to the fact that samples were collected in two herds. In addition, the proportion of polymorphic SNPs in Portuguese Merino breeds was lower (70% in Merino Branco and Merino Preto) than in the Merino populations analyzed by Grasso et al. (2014) (89,4%), but this might be because they used the OvineSNP50 BeadChip (Illumina) in a large number of animals. Genetic diversity is fundamental to strengthen the ability of populations to evolve to adapt to changes. Overall, our results revealed similar levels of genomic diversity across the studied breeds. The observed and expected heterozygosity values (from .30 to .34 and from .30 to .35, respectively) were slightly lower in Merino breeds. Nonetheless, they were consistent with those reported for other European breeds ($.30 \leq H_o \leq .39$; $.31 \leq H_e \leq .38$) (Luigi-Sierra et al., 2019). Nucleotide diversities were also comparable to those observed for a wide range of sheep breeds (varying from 1.7×10^{-3} to 3.1×10^{-3}) (Lv et al., 2022).

In livestock populations, high inbreeding could result in an overall decrease of their performance, which may impact economically important traits (Leroy, 2014). For instance, there is evidence that high levels of inbreeding have detrimental effects in the growth of lambs (Černá et al., 2021). Inbreeding coefficients (F_{IS}) estimated for the Portuguese native breeds were relatively low (ranging from .005 to .038). Even the highest F_{IS} value estimated for CAM is considerably lower than the observed in some European sheep breeds (between .04 and .42) (Kijas et al., 2012). As opposed to most commercial breeds, farmed under intensive conditions and subjected to intensive selection programs, Portuguese native breeds are reared by smallholder farmers in traditional agrosilvopastoral systems, where random mating predominates and admixture between flocks may occur. This might explain the low levels of inbreeding observed, as well as the somewhat low genetic differentiation between breeds (mean pairwise F_{ST} .03).

The patterns of ROH can help discriminate ancient bottlenecks (i.e., many short ROHs) from recent inbreeding and low genetic diversity (i.e., few long ROHs) (Curik et al., 2014). In our study, shorter ROH segments (1–2 Mb) were found far more frequently than longer ones (>2 Mb). The frequency of these short ROH was equal or greater than 90% in all breeds, except in MB. Our estimates are within the reported range for other local breeds (Al-Mamun et al., 2015; Deniskova et al., 2021; Liu et al., 2021), including Spanish sheep (Luigi-Sierra et al., 2019), suggesting recent autozygosity events were not frequent in the Portuguese breeds analyzed. In the absence of pedigree records, ROH has been widely used to estimate inbreeding (F_{ROH}) with a large number of SNPs (Kardos et al., 2015). The F_{ROH}, defined as the proportion of the autosomal genome covered by ROHs, was generally low in all breeds ($.010 \leq F_{ROH} \leq .023$). The F_{ROH} values obtained for Portuguese sheep were similar to those reported for the Spanish breeds Castellana, Ojalada, Ripollesa, Segurena and Xisqueta ($.008 \leq F_{ROH} \leq .025$) (Luigi-Sierra et al., 2019). Overall, F_{ROH} values agreed with the relative abundance and length of ROH, i.e., CAM had the highest number of ROH segments, the largest proportion of the genome covered by ROH and consequently the highest F_{ROH} value. The lowest F_{ROH} was observed in SE, which also displayed the lowest ROH counts and lowest length of the genome covered by ROH per individual. It is not surprising that SE animals sampled in nine herds are less related to each other than those of other breeds that derive from only 3 to 4 farms.

Population structure and breed relationships were investigated considering worldwide and Iberian sheep breeds by integrating the genomes we generated with publicly available whole-genome and SNP array data, respectively. Congruent results were obtained from complementary PCA, Admixture and phylogenetic analyses. In the PCA, Portuguese sheep split in two clusters according to breed histories, i.e., Merino populations were genetically close whereas CAM and SE belong to a distinct group of breeds. The Swiss White Alpine is a Merino-derived European breed and in agreement with previous analysis was also included in this group (Ciani et al., 2020). When only Iberian sheep were considered, the Portuguese Merino breeds grouped together in the PCA, along with their Spanish counterparts. These breeds share a common genetic background that could result to some extent from their geographic proximity (Landi et al., 2019). The CAM and SE breeds belong to a more heterogeneous group that included other Iberian breeds such as Churra from Spain, as well as other European sheep raised for milk (e.g., Lacaune originally from France), meat (e.g., Pag Island sheep from Croatia upgraded with Merino), or as dual-purpose animals (e.g., Leccese from Italy). The admixed background of some of these breeds has been interpreted as the consequence of ancient gene flow along the Mediterranean (Lv et al., 2015; Ciani et al., 2020). In the Iberian context, sheep breeds are typically classified according to the characteristics of their fleece (Pedrosa et al., 2007). Interestingly, the PCA clustering clearly depicted fine wool Merino breeds separated from intermediate and coarse-wool sheep from Portugal and Spain, including CAM and SE. Coarse wool sheep from the Basque region were highly differentiated from all other breeds probably due to their geographical isolation.

The ADMIXTURE analysis allowed us to investigate in more detail the ancestry components underlying the genetic structure of Portuguese native breeds. For low values of K, general patterns of ancestry could be inferred, such as the clear differentiation of the Asiatic mouflon and of more commercial breeds (e.g., Suffolk and East Friesian), as well as the tight clustering of the Portuguese Merino populations. For K greater than six, some sub-structure within CAM, SE, MP, and MB starts to emerge, which could be due to an effect of the herds from where the samples originate. Overall, our results are consistent with low levels of breed differentiation and a complex genetic background observed in sheep from the Iberian Peninsula (Luigi-Sierra et al., 2019). The weak differentiation observed between MB and CB suggests that this crossbred population resulted mainly from the mating of local Merino type animals with high performance individuals from transboundary commercial breeds. This result was also

observed for an admixture analysis of the Iberian dataset, which corroborated the PCA clusters shown in Figure 6, i.e., the close affinities between Portuguese Merino, Spanish Merino and the crossbreds (results not shown). In the first half of the 20th century and according to the MB Breeder's Association (https://www.merina.pt), national authorities promoted the upgrading of local Merino sheep with Merino animals from Spain, as well as Rambouillet and Merino Precóce from France. The Rambouillet is itself derived from Spanish Merino flocks (http://afs.okstate.edu/breeds/sheep/rambouillet), while the latter breed originates from Rambouillet and Île-de-France (https://www.fondazioneslowfood.com/en/ark-of-taste-slow-food/precocious-merinos-goat/). The results of our ADMIXTURE analysis show that MP and MB animals as well as crossbreds, share some ancestry with the Swiss White Alpine, a breed which derives from a cross between the Swiss White Mountain and Île-de-France (http://afs.okstate.edu/breeds/sheep/swisswhitealpine). Indeed, whole-genome 600 K SNP array data confirm the close relationship of Rambouillet and Merino breeds from Spain and France (Rochus et al., 2020), also depicting a migration event consistent with gene flow from Rambouillet to Île-de-France breed (Rochus et al., 2018). The MP that once dominated in Portugal decreased in numbers in the second half of the 20th century, as black wool became less valuable, persisting in marginal areas and maintaining its identity with minimum influences from exotic stock.

Additionally, the phylogenetic analysis of sheep mitogenomes showed that Portuguese native breeds and two animals of the Akkaraman breed from Turkey are genetically close which provides support to a direct influence of Near-Eastern stock having reached the Iberian Peninsula *via* the Mediterranean dispersion routes (Zilhã, 2001; Pereira et al., 2006; Gron et al., 2020). Our results are in agreement with a study of 501 D-loop sequences representing 19 Iberian breeds in which haplogroup B also had the highest frequency (>98%) (Pedrosa et al., 2007). Consistently, a recent study of sheep mitogenomes also showed that haplogroup B had the highest frequency in Southwest Europe (>90%) (Lv et al., 2015). The clustering of three crossbreds with Italian Merino sheep (HM236174; HM236175; KF302440; KF302445; KF302446) could reflect recent upgrading. In future studies it would be extremely interesting to extend these analyses to other local breeds from the Iberian Peninsula, in particular the coarse-wool sheep (Churra) that are raised in this region in distinct environments from north to south (see Figure 1).

5 Conclusion

We examined the patterns of genomic diversity and population structure of four Portuguese native sheep within other Iberian and worldwide breeds. Our results suggest these Portuguese breeds are not genetically compromised, showing moderate diversity and negligible inbreeding. Expanding our study to a larger number of animals and farms should allow for more comprehensive inferences on Iberian sheep biodiversity to define management and conservation plans. The population structure analyses depicted the Iberian Merino sheep as a well differentiated breed group. The Merinos are thought to have been developed in the Iberian Peninsula and their selection for wool, meat and adaptive traits for local conditions appear to have resulted in a distinct genetic make-up. The gene flow between the Portuguese and Spanish Merino breeds depicted in the phylogenetic analysis could be explained by traditional transhumance routes which increase the chance for crossbreeding. Portuguese native breeds formed a tight clade within major haplogroup B in the phylogeny of sheep mitogenomes. This is the first study of Portuguese native sheep using whole genomes and sets the ground for defining ancestry informative SNPs for breed-specific admixture analysis, i.e., a powerful tool for breed assignment and traceability of certified breed-products, but also for genome-wide association studies. In addition, the genomic data we generated will be most valuable for a combined analysis of sheep genomes retrieved from historic and archaeological specimens to investigate the origins, evolution and modes of improvement of native Iberian sheep.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material. The raw reads have been deposited in the Sequence Read Archive (SRR16085831 - SRR16085886) with the corresponding Bioproject PRJNA764662.

Ethics statement

Ethical review and approval was not required for the animal study because Animal handling and blood collection were performed during routine veterinary check-ups, following the official animal health care program guidelines and under the consent of breeders. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

CG and AR conceived and designed the study; DG, CL, CM, and AR organized sample collection and DNA extraction; DG and SG performed the analysis with the supervision of CG and AU; DG wrote the manuscript with contributions from AP and the supervision of CG and AU. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2023.1109490/full#supplementary-material

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A metagenomics approach to characterize the footrot microbiome in Merino sheep

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ABSTRACT

In the Portuguese Alentejo region, Merino sheep breed is the most common breed, reared for the production of meat, dairy, and wool. Footrot is responsible for lameness, decreased animal welfare, and higher production losses, generating a negative economic impact. The disease is caused by *Dichelobacter nodosus* that interacts with the sheep foot microbiome, to date largely uncharacterized. In fact, *Dichelobacter nodosus* is not able to induce footrot by itself being required the presence of a second pathogen known as *Fusobacterium necrophorum*. To understand and characterize the footrot microbiome dynamics of different footrot lesion scores, a whole metagenome sequencing (WMGS) approach was used. Foot tissue samples were collected from 212 animals with different degrees of footrot lesion scores, ranging from 0 to 5. Distinct bacterial communities were associated with feet with different footrot scores identifying a total of 63 phyla and 504 families. As the severity of footrot infection increases the microorganisms' diversity decreases triggering a shift in the composition of the microbiome from a dominant gram-positive in mild stages to a dominant gram-negative in the severe stages. Several species previously associated with footrot and other polymicrobial diseases affecting the epidermis and provoking inflammatory responses such as *Treponema* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Campylobacter* spp. were identified proliferating along with the lesions' severity. Although these bacteria are not able to initiate footrot, several evidences have been described supporting their association with the severity and incidence increase of footrot lesions caused by *Dichelobacter nodosus* and *Fusobacterium necrophorum*. Further investigation is required to establish the roles of particular taxa and identify which of them play a role in the disease process and which are opportunistic pathogens.

1. Introduction

Ovine footrot is a contagious disease caused primarily by *Dichelobacter nodosus (D. nodosus)*, an anaerobic gram-negative bacterium (Beveridge, 1941), being the main cause of lameness affecting sheep and other livestock animals worldwide (Zanolari et al., 2021). Footrot affects the interdigital skin and hooves, being a welfare and economic concern for the wool, milk and meat industries. Footrot disease is classified in two different clinical presentations: Interdigital Dermatitis (ID) which is characterized by the inflammation of the interdigital epidermis,

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including or not the underrunning footrot, and the severe form of the disease, denoted as Severe Footrot (SFR). In SFR, the separation of the hoof horn from the sensitive underlying tissue occurs, resulting in necrotizing lesions of the interdigital skin accompanied by a characteristic fetid odor leading to lameness (Zanolari et al., 2021).

Footrot is a multi-factorial, polymicrobial and complex disease which depends on different factors such as host susceptibility, farm management, environmental conditions, virulence of *D. nodosus* (which is known to be conferred by the presence of the aprV2 gene, coding for the thermostable AprV2 protein) and the presence of co-infecting bacteria like *Fusobacterium necrophorum* (*F. necrophorum)* (Zanolari et al., 2021). *Fusobacterium necrophorum* is another anaerobic bacterium which is known to be involved in the persistence and severity of footrot development, playing a role as an opportunistic, secondary pathogen. The synergistic relationship between *D. nodosus* and other microorganisms such as *F. necrophorum* is not clear (Zanolari et al., 2021). The bacterial community diversity observed in the sheep hooves' with footrot makes the identification of the different prevalence of taxa and its contribution to the development and expression of footrot a challenging task.

Since the mid-20th century most of the available information regarding the bacterial etiology of the ovine footrot was obtained from classical microbiological techniques, a labour-intensive cultured-based approach, which limitations are associated to a limited number of bacteria than can be cultured, and histological sections observation (Beveridge, 1941; Egerton et al., 1969). However, rapid advances in the next-generation sequencing (NGS) field as based marker-gene (16 S rDNA gene) and whole metagenome sequencing (WMGS) has enabled new insights in the research of polymicrobial diseases like ovine footrot (Calvo-Bado et al., 2011; Maboni et al., 2017; McPherson et al., 2019; Clifton et al., 2022). Despite 16 S rDNA gene sequencing is able to provide rapid information about the taxonomic composition of microbial communities, the main disadvantage of this technique is the limited amount of information produced, for instance, about metabolic pathways and functional capabilities. WMGS overcomes these limitations being able to obtain deeper insights about the functional capabilities, metabolic pathways, novel genes, host-microbiota interactions and co-evolution, offering a great specificity of identification and representation of diversity in the microbiomes (Durazzi et al., 2021). To our knowledge, the etiology of ovine footrot in the Portuguese Merino breeds and crossbreed has not yet been investigated using WMGS. However, several studies of footrot affecting different breeds of sheep have been conducted using other approaches in Australia and United Kingdom (Calvo-Bado et al., 2011; Maboni et al., 2017; McPherson et al., 2019; Clifton et al., 2022). Moreover, the role of bacterial diversity, its load and how that differs between the healthy and footrot-affected sheep feet remains unclear.

In this context, the aim of this study was to characterize the bacterial communities present on the feet of healthy and footrot-affected Merino sheep, grouped by footrot score, using WMGS. With this approach, it was intended to determine which prevalence of the different bacteria are represented in each footrot score lesions and could contribute to the development of the disease on these sheep breeds in Portugal.

2. Material and methods

a. Sample collection, DNA extraction, and sequencing

Interdigital skin punch biopsies were collected, under local anesthesia (Lidocaine, Anestesin®), from 212 sheep using disposable sterile Biopsy Punches (6 mm diameter) within seventeen flocks of White Merino and Black Merino breeds and Merino crossbreed, from different geographical locations in the Portuguese Alentejo region (Supplementary Table S1). Those flocks were randomly selected and examined between January 2017 and June 2018 for clinical diagnosis of footrot infection. Following the Modified Egerton System (scores from 0 to 5) the sheep feet lesions were scored and registered for each animal. The scores 1 and 2 correspond to ID and the scores 3, 4 and 5 to SFR. Samples were immediately frozen with liquid nitrogen and kept at − 20 ºC until being processed. Total DNA was extracted, from up to 25 mg of each 212 biopsy samples, using QIAamp® cador® Pathogen Mini Kit + T2 pretreatment (Qiagen, Hilden, Germany, Cat No. 50214) according to manufacturer instructions. DNA quality and quantity were assessed using a UV–visible spectrophotometer (Nanovue, Biochrom). DNA was sent to BGI (Shenzhen, China) for paired-end library construction and then the libraries were subjected to 2 x 100 bp sequencing using WMGS strategy on the BGISeq-500 platform.

b. Pre-processing

Prior to sequence analysis, the quality of the paired-end reads was evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then pre-processed using Trimmomatic v.0.38 (Bolger et al., 2014) in order to trim/remove low quality reads. Only the reads with a minimum quality of 12 and a minimum read length of 80 bp, screened over a sliding window of 10 bp, were kept. The pre-processed reads were then mapped against the sheep genome (NCBI: GCF_002742125.1) to remove the DNA from the host using BWA mem (v.0.7.15) with default parameters (Li and Durbin, 2009). The mapped reads were filtered out and the remaining reads were used for further analyses.

c. Taxonomy assignment and difference abundance analyses

The same set of samples used in this work was used in a previous study to identify via qPCR presence or absence of *D. nodosus* (Albuquerque et al., 2022). Hence, samples where *D. nodosus* was not identified either by qPCR or metagenomics sequencing data quantification were considered as outgroup (OG), representing the healthy control group. The samples of the remaining flocks were divided between two groups, one with ID (footrot lesion score 0 and 1) was classified as the group of no footrot infection severe signs (NFIS) and the other with the remaining samples with higher footrot lesion scores (FIS – footrot infection severe signs). Thus, the dataset was divided into three categories: i) OG samples (no footrot infection), ii) samples with NFIS and iii) samples with FIS.

The microbiome taxonomic classification of each sample was done using Kraken v.2 (Wood et al., 2019) with default parameters based on the lowest common ancestor (LCA) approach. Its partner tool Bracken (Bayesian Reestimation of Abundance with KrakEN) (Lu et al., 2017) was applied at the species level to estimate abundances. The Kraken database was built comprising the complete genomes of Refseq for the bacterial, archaeal and viral domains, along with the human genome and a collection of known core element vectors (downloaded on August 2020) while the Bracken database was built for a read length of 80 bp (the minimum read length allowed in the pre-processing step). The abundances obtained were then used to perform the difference abundance analyses with the edgeR package from Bioconductor (R v.4.2) (Robinson et al., 2009). For this analysis, as the number of samples with a footrot score of 5 was too low (only two samples), those were removed from the set to avoid noise in the statistical analyses. All samples belonging to the same footrot score were considered as biological replicates as well as all samples belonging to the outgroup (Supplementary Table S2). Taxa with low abundances were filtered out using the *filterByExp* function implemented in edgeR with default parameters. By default, this function set the minimum number of samples per condition-group, with at least 10–15 counts each, as the 70% of the smallest condition-group sample size. Hence, the larger number of biological replicates the more restrictive the filtering is. Then, a Trimmed Mean of M-values (TMM) normalization was applied. Over the normalized taxa, the test for differential expression was performed applying the GLMs method. Two different strategies were followed to

analyze the data. The first strategy was based on the comparison of (1) NFIS vs OG, (2) NFIS vs FIS and (3) OG vs and footrot infection (FI: NFIS+FIS). The second strategy was based on a pairwise comparison between all the different footrot scores (FS: 0–4). At the end, in both strategies, only species with differences in abundance with a log fold change (logFC) $\geq |2|$ and a false discovery rate (FDR) ≤ 0.05 were considered significant. Additionally, to identify species clusters with similar abundance profile within the different footrot scores, a *k*-means clustering analysis was performed (number of clusters = 10) using functions from the CummeRbund package of R (Goff et al., 2013).

Within-sample (alpha) diversity was assessed as Shannon's diversity index while the between-sample (beta) diversity was estimated based on multidimensional scaling (MDS) plot.

3. Results

a. Pre-processing

Out of 13.2 billion raw reads, 12.9 billion (97.2%) passed the Trimmomatic quality control criteria. After removing from this set those reads belonging to the host DNA (sheep) a total of 114.5 million reads remained (0.9%) which were used for downstream analyses.

b. Taxonomy assignment

Kraken2 was applied over the pre-processed reads of each sample for the taxonomy assignment resulting in 5126 species taxonomically identified. The taxonomic assignment revealed a percentage of classified reads per sample ranging from 8.5% to 66.6%, being most of them below 20%. The low percentage of classified reads obtained is expected when working with non-targeted genomics sequencing data. The taxonomic classification also revealed the presence of 63 phyla and 504 families. At the phylum level, the set of the dominant phyla was quite similar among the different footrot scores, but the percentage of reads assigned to each differed as the footrot score increase (Fig. 1A). The phylum *Actinobacteria* was the most dominant in samples from OG and FS1 (41.88% and 34.73%), the *Proteobacteria* in FS0, FS2 and FS3 samples (34.99%, 31.17% and 25.20%), *Fusobacteria* in FS4 samples (36.94%) and *Bacteroides* in FS5 samples (31.1%). At family level, the set of dominant families among the different footrot scores was different due to the change of the percentage of reads assigned to each family as the footrot score increase. Fig. 1B represents the set of dominant families obtained

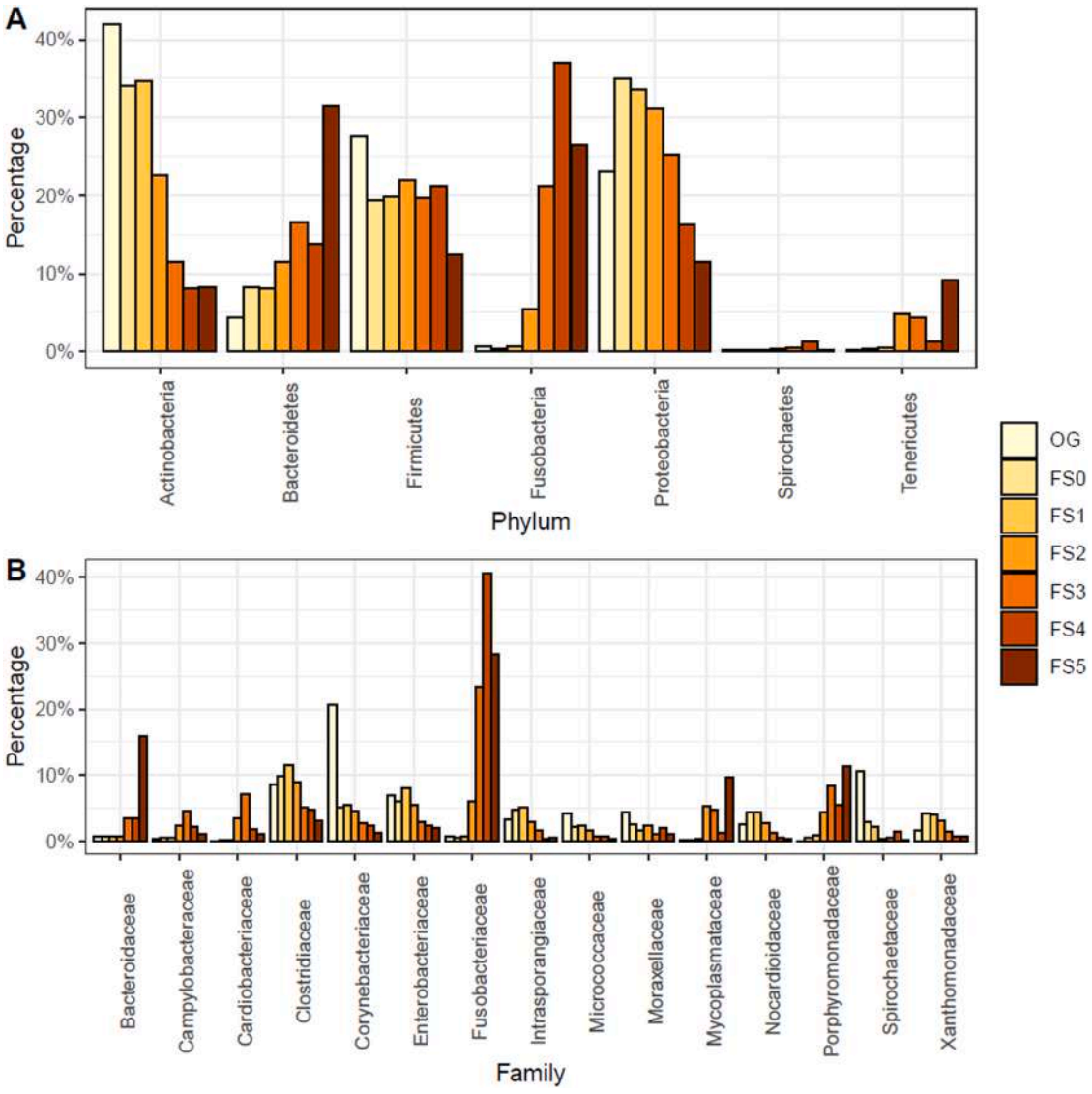

Fig. 1. Prevalence of the most dominant phyla (A) and families (B) in the footrot microbiome among the different footrot scores. All values are in percentage terms. FS: Footrot Score, OG: Out Group.

from merging the most representative families from each footrot score. The family *Corynebacteriaceae* (20.74%) was the most dominant in OG samples. In samples FS0, FS1 and FS2 the most abundant family was the *Clostridiaceae* (9.78%, 11.42% and 8.87%). Family *Fusobacteriaceae* was the second most abundant in FS2 samples (6.01%) and the most abundant in FS3, FS4 and FS5 samples (23.38%, 40.69% and 28.41%). Additionally, in FS5 samples the second most abundant family, with much lower representation in the other samples, was the family *Bacteroidaceae* (15.81%).

c. Overall difference abundance analyses

The taxonomic classification was followed by the abundance estimation of the taxa identified using Braken. As mention before, a total of 5126 species were taxonomically identified in the whole set of samples but only 869 passed the filtering of the taxa with low abundances represented in all samples of at least one group of replicates. Those taxa were then used for the differential abundance analyses, and also to assess alpha and beta diversities. The Shannon's index showed that as the severity of footrot infection increases, the microorganisms' diversity of footrot microbiota tends to decrease (Supplementary Fig. S1). Additionally, it can be observed that microorganisms' diversity of the OG and FIS samples was lower than the diversity observed in the first stages of the footrot (NFIS) infection (Supplementary Fig. S1B). However, after applying an analyses of variance (ANOVA) no significant differences were observed between diversity means between categories. Regarding beta-diversity, the MDS plot suggest that OG and NFIS samples were different than FIS samples although also no significant differences were observed (Supplementary Fig. S2).

In order to identify differences in species abundances between the three categories, pairwise comparisons between them were performed (Supplementary Fig. S3). When comparing samples with footrot infection versus OG samples (FI vs OG), 186 species with significant differences in their abundances were identified (Supplementary Table S3). From those, 146 species were found with significantly increased abundances in samples with footrot infection. These included *Mycoplasma fermentans* (logFC 11.6), *D. nodosus* (logFC 8.2), *Treponema phagedenis* (logFC 8.06), *Porphyromonas asaccharolytica* (logFC 7.6), *Treponema pedis* (logFC 4.5) and *F. necrophorum* (logFC 3.4), all species known to cause various foot diseases in sheep (Maboni et al., 2017; McPherson et al., 2019; Duncan et al., 2021; Clifton et al., 2022). Moreover, 10 species of *Planococcus* spp., nine species of *Corynebacterium* spp., eight species of *Acinetobacter* spp. and five species of *Staphylococcus* spp., among others, were identified with significantly decreased abundances in samples with footrot infection. Differences in the microbiome between the mild and severe stages of footrot infection (NFIS vs FIS) were also assessed resulting in 128 species with significant differences (Supplementary Table S4). Among the species found with significantly increased abundances in severe stages of footrot infection were *Streptococcus* spp. (n = 13), *Campylobacter* spp. (n = 13), *Fusobacterium* spp. (n = 8), *Prevotella* spp. (n = 7) and *Treponema* spp. (n = 4). The species found with significantly increased abundances in mild stages of footrot infection were *Brevundimonas* spp. (n = 5) *Streptomyces* spp. (n = 2) and *Staphylococcus* spp. (n = 2) among others. Finally, 175 species were found with significant differences between mild stages of footrot and OG samples (no NFIS vs OG) while between severe stages of footrot and OG samples (FIS vs OG) were found 219 species (Supplementary Table S5 and S6, respectively). In mild stages of footrot infection, *Psychrobacter* spp. (n = 9), *Brevundimonas* spp. (n = 8), *Marinobacter* spp. (n = 6) and *Sphingomonas* spp. (n = 5) were the main species found with significantly increased abundances while in severe stages those were *Streptococcus* spp. (n = 10), *Psychrobacter* spp. (n = 8) and *Fusobacterium* spp. (n = 8). In both comparisons, the main species in OG samples with significantly more abundances were *Planococcus* spp., *Acinetobacter* spp. and *Corynebacterium* spp. Table 1 summarizes the top 10 genera found more abundant in each sample category for all the comparisons performed.

d. Differences in the microbiome between different footrot infection stages

In the pairwise comparison between different footrot stages a total of 281 species were found significantly more abundant in at least one of the comparisons made (Supplementary Table S7, Supplementary Fig. S4). The higher number of species with significant differences in their abundances was found when comparing samples of FS0 and FS1 against the ones of FS4 (n = 218 and 185, respectively). In contrast, the lower number of species (n = 24) was found when comparing samples of FS0 against the ones of FS1, both considered as samples with NFIS. The two main relevant species associated with footrot disease, *D. nodosus and F. necrophorum* were found within all the species with significant differences in abundances. Additionally, other pathogens previously identified as important in other polymicrobial diseases such as CODD (Contagions Ovine Digital Dermatitis) and in footrot were also found with significant differences: *Campylobacter* spp. (n = 15), *Streptococcus*

Table 1
Top 10 genera more represented with significantly differences of abundances in each sample group per each pairwise comparison performed.

FI vs OG		NFIS vs FIS		NFIS vs OG		FIS vs OG	
↑FI	↑OG	↑NFIS	↑FIS	↑NFIS	↑OG	↑FIS	↑OG
Streptococcus(10 spp.)	*Planococcus*(10 spp.)	*Brevundimonas*(5 spp.)	*Streptococcus*(13 spp.)	*Psychrobacter*(9 spp.)	*Planococcus*(10 spp.)	*Streptococcus*(10 spp.)	*Planococcus*(10 spp.)
Psychrobacter(8 spp.)	*Corynebacterium*(9 spp.)	*Streptomyces*(2 spp.)	*Campylobacter*(13 spp.)	*Brevundimonas*(8 spp.)	*Acinetobacter*(10 spp.)	*Psychrobacter*(8 spp.)	*Corynebacterium*(9 spp.)
Fusobacterium(8 spp.)	*Acinetobacter*(8 spp.)	*Staphylococcus*(2 spp.)	*Fusobacterium*(8 spp.)	*Marinobacter*(6 spp.)	*Corynebacterium*(8 spp.)	*Fusobacterium*(8 spp.)	*Acinetobacter*(8 spp.)
Campylobacter(6 spp.)	*Staphylococcus*(5 spp.)	*Brevibacterium*(1 spp.)	*Prevotella*(7 spp.)	*Acidovorax*(6 spp.)	*Staphylococcus*(6 spp.)	*Campylobacter*(6 spp.)	*Staphylococcus*(5 spp.)
*Marinobacter*5 spp.)	*Tessaracoccus*(1 spp.)	*Sphingomonas*(1 spp.)	*Bacteroides*(6 spp.)	*Sphingomonas*(5 spp.)	*Campylobacter*(3 spp.)	*Marinobacter*(5 spp.)	*Tessaracoccus*(1 spp.)
Treponema(4 spp.)	*Shigella*(1 spp.)	*Sphingobium*(1 spp.)	*Treponema*(4 spp.)	*Xanthomonas*(4 spp.)	*Tessaracoccus*(1 spp.)	*Treponema*(4 spp.)	*Shigella*(1 spp.)
Porphyromonas(4 spp.)	*Pseudomonas*(1 spp.)	*Rhizobium*(1 spp)	*Porphyromonas*(4 spp.)	*Hydrogenophaga*(4 spp.)	*Shigella*(1 spp.)	*Porphyromonas*(4 spp.)	*Pseudomonas*(1 spp.)
Acidovorax(4 spp.)	*Pradoshia*(1 spp.)	*Pseudomonas*(1 spp.)	*Peptoniphilus*(3 spp.)	*Chryseobacterium*(4 spp.)	*Jeotgalibaca*(1 spp.)	*Acidovorax*(4 spp.)	*Pradoshia*(1 spp.)
Xanthomonas(3 spp.)	*Kocuria*(1 spp.)	*Plantactinospora*(1 spp.)	*Tannerella*(2 spp.)	*Campylobacter*(4 spp.)	*Dolosigranulum*(1 spp.)	*Xanthomonas*(3 spp.)	*Kocuria*(1 spp.)
Variovorax(3 spp.)	*Jeotgalibaca*(1 spp.)	*Planococcus*(1 spp.)	*Mycoplasma*(2 spp.)	*Variovorax*(3 spp.)	*Aerococcus*(1 spp.)	*Variovorax*(3 spp.)	*Jeotgalibaca*(1 spp.)

FIS: Footrot Infection severe Signs; NFIS: No Footrot Infection severe Signs; FI: Footrot Infection; OG: Outgroup.

spp. (n = 14), *Prevotella* spp. (n = 10), *Psychrobacter* spp. (n = 8), *Clostridium* spp. (n = 6), *Treponema* ssp. (n = 4), *Porphyromonas* spp. (n = 4), *Mycoplasma* spp. (n = 2) and *Gemela* spp. (n = 2). (Maboni et al., 2017; Gelasakis and Bossis, 2019).

In order to identify species with similar patterns of abundances among the different stages of footrot infection, all the species found with significant differences in their abundances in pairwise comparisons were clustered based on their abundance profile among different footrot stages into 10 clusters (Fig. 2). The number of species per cluster are 11, 61, 13, 25, 39, 42, 22, 37, 19 and 12, respectively from cluster 1–10). To get more details about which species is found in which cluster please see Supplementary Table S8.

Out of the 10 clusters obtained, five were selected for further discussion due their abundance profile (Clusters 1, 2, 7, 8 and 10, Fig. 2). The species within these clusters proliferate along the footrot infection process, with slightly differences of abundance between FS0 and FS1, and FS3 and FS4 stages. Among these species were included *D. nodosus*, *F. necrophorum* and diverse *Treponema*, *Staphylococcus*, *Streptococcus* and *Campylobacter* species.

4. Discussion

The main aim of this study was to characterize the bacterial communities present on the feet of healthy and footrot-affected Merino and Merino-related sheep and to identify the changes of the bacterial community over the different stages of footrot infection. The results of the taxonomic classification showed that footrot infection seemed to cause a shift in the composition of the microbiome as severity of the lesion (score) increases from a dominant gram-positive in mild stages of footrot infection to a dominant gram-negative in the severe stages (Fig. 3). This

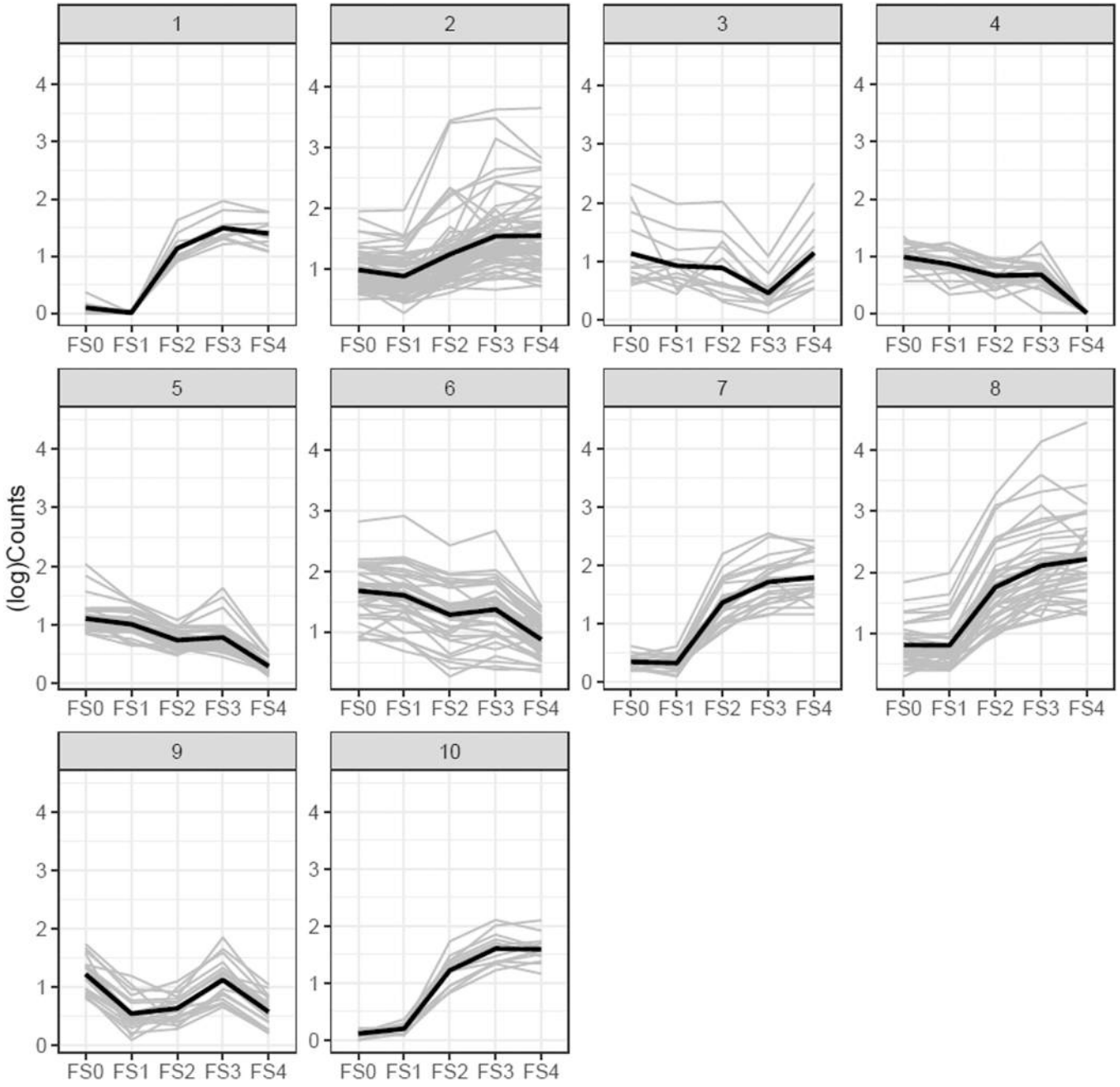

Fig. 2. k-means clustering analysis of species with significant differences in their abundance in the pairwise comparison (k = 10). The grey lines represent mean abundance profile (log10) for each species across the footrot scores. The black line represents the mean abundance profile observed in each cluster. FS: Footrot Score.

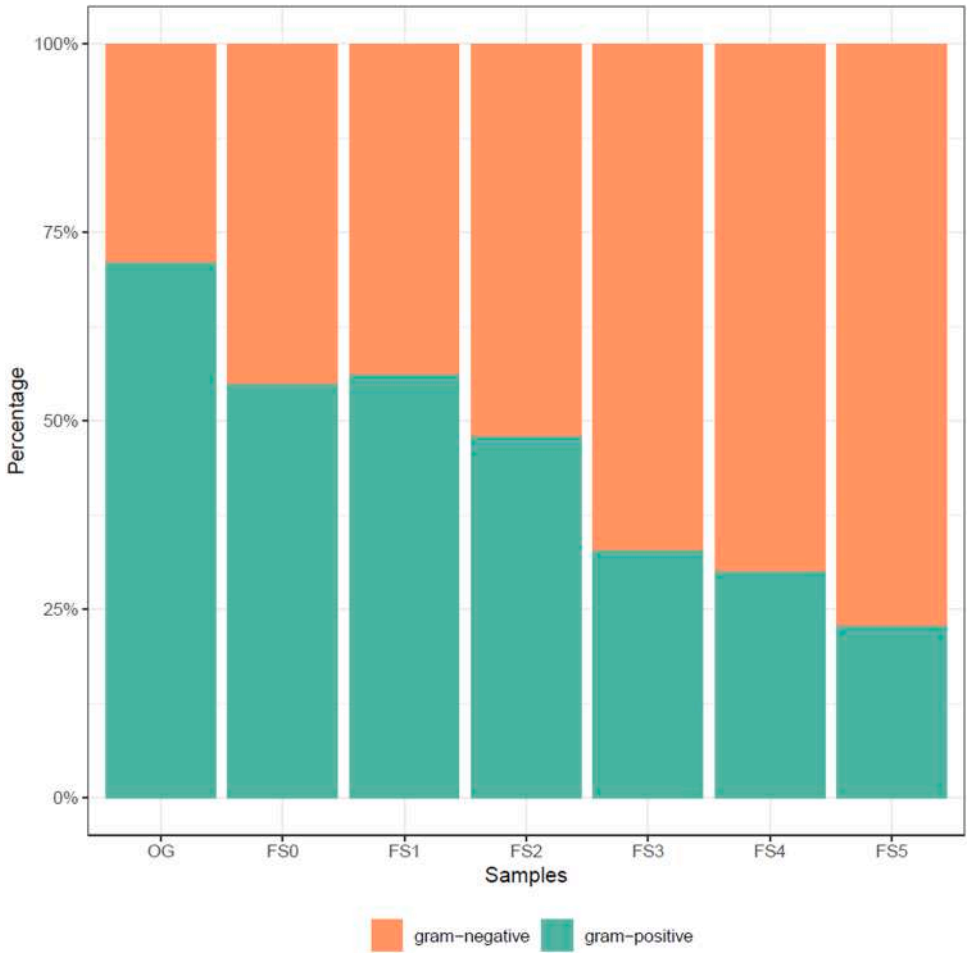

Fig. 3. The dominant phyla obtained from merging the most represented phyla (≥ 1%)) from each footrot score and with a clear definition of their gram definition were used to display the percentage of gram-positive vs gram-negative phyla as long the footrot infection disease aggravates.OG: Ourgroup; FS: Footrot Score.

shift has been also observed in previous studies of footrot as well as other polymicrobial diseases being directly associated with the evolution of the disease from the healthy to the disease stage (Zanolari et al., 2021). Additionally, the footrot microbiome showed a diminished diversity as the footrot infection aggravates (Supplementary Fig. S1) which is accompanied by the increased abundances of *D. nodosus* along with other species such as *Mycoplasma fermentans*, *F. necrophorum*, *P. asaccharolytica*, *Ezakiella massiliensis*, *Treponema* ssp. and *Staphylococcus* spp., *Streptococcus* spp. and *Campylobacter* spp. Several studies have addressed the role of *F. necrophorum*, which is known to colonize, under environmental predisposition, the epidermis facilitating other taxa proliferation along with its own due to the associated necrosis and anaerobiosis (Zanolari et al., 2021). The results obtained in this study reflects the proliferation of *F. necrophorum*, as the diseases intensifies, its abundances increase (Cluster 8, Figure2). However, other taxa following a similar proliferation profile have been identified suggesting that they may be significant to the footrot infection process.

Species from genus *Porphyromonas* have been previously associated with the pathogenesis of footrot in sheep and were related with higher levels of inflammation (Maboni et al., 2017; McPherson et al., 2019). In this study, were identified *P. asaccharolytica*, *P. cangingivalis*, *P. crevioricanis* and *P. gingivalis* with significant increased abundances along the different disease severity stages (Clusters 2 and 8, Fig. 2, Supplementary Table S8). *Porphyromonas asaccharolytica*, a synergistic wound pathogen, was also recently found associated to footrot infection (Blanchard et al., 2021). It is known that *P. gingivalis*, a keystone pathogen for periodontitis in humans, promotes the dysbiosis of the microbiome and triggers the host inflammatory response, dysbiosis that was also described to occur in footrot (Kaler et al., 2010; Maekawa et al., 2014; McPherson et al., 2019). Regarding *P. cangingivalis* and *P. crevioricanis*, both have been also associated to periodontitis and inflammatory conditions in ovine, being the latest also associated with bovine interdigital necrobacillosis (Sweeney et al., 2009; Borsanelli et al., 2017).

Different microbiological studies have associated the *Treponema* spp. with other claw diseases such as CODD and bovine digital dermatitis (BDD). Recently, it has been suggested that CODD and footrot could be different stages of the same diseases due to the similarities of the bacteriological and epidemiological features (Duncan et al., 2021). Three main *Treponema* species are known to be associated with CODD and BDD, *T. medium*, *T. phagedenis* and *T. pedis*. The latest two together with *T. denticola* and *T. putidum*, both associated to CODD and BDD lesions, were found in this work sharing a similar proliferation profile with *D. nodosus* and *F. necrophorum* (Sayers et al., 2009; Mamuad et al., 2020; Caddey, 2021). Hence, these results are highly congruent with what has previously been identified (Maboni et al., 2017; Blanchard et al., 2021; Duncan et al., 2021).

In previous studies, *Staphylococcus aureus* and *Staphylococcus epidermidis*, were significantly associated with footrot (Calvo-Bado et al., 2011; Anto et al., 2014). The *S. aureus* and *S. epidermidis*, present at the skin commensal flora in human and livestock animals, and considered as important opportunistic pathogens, are known as etiological agents of bovine, ovine and caprine mastitis leading to an inflammatory response of the mammary gland (Watts, 1988). Moreover, it has been reported that *S. aureus* can cause necrotic/staphylococcal dermatitis in sheep and also was found to be present in CODD (Duncan et al., 2014). In

accordance to this, the results obtained in this study showed that both, *S. aureus* and *S. epidermidis*, seems to take profit of the ideal environmental conditions of the disease to proliferate (Cluster 2, Fig. 2, Supplementary Table S8). However, it is important to note that, besides the competition existing between *S. aureus* and *S. epidermidis*, being the first one the most aggressive, virulent and abundant, both found a way to survive and spread based on different colonization approaches (Massey et al., 2006; Cheung et al., 2010). Further, other *Staphylococcus* spp. were found with significant differences in their abundance along the progression of the disease. In general, the proliferation of *Staphylococcus* species seems to be inhibited and diminished in the mild stages of the disease, which agrees with the interspecies competition, whereas, suddenly, in the severe stages (from FS3 to FS4) their abundance increase (Cluster 2 and 3, Fig. 2). In contrast, when we compared OG samples against NFIS, FIS and FI samples, most of these *Staphylococcus* species were always found more significantly abundant in OG samples (Supplementary Tables S3, S5 and S6). These results are in accordance with other studies (Maboni et al., 2017; McPherson et al., 2019; Blanchard et al., 2021). Hence, the comparison of the different footrot score, regarding *Staphylococcus* species, was able to clarify their abundance profile, in which abundance seems to increase at the very late stages. Another species also found associated with footrot infection was *Streptococcus pyogenes* (Calvo-Bado et al., 2011; Anto et al., 2014) which is widely known for causing diverse diseases in humans, including skin infections such as necrotizing fasciitis which destroys the tissue and has a rapid disease progression (Stevens and Bryant, 2016). *Streptococcus pyogenes* was found in the same cluster than *D. nodosus* and *F. necrophorum*. Other *Streptococcus* species were found significantly differentiated in their abundances along footrot infection, most of them with an increasing abundance profile (Clusters 1, 2, 7, 8 and 10, Fig. 2, Supplementary Table S8). Hence, the *Staphylococcus* spp. and *Streptococcus* spp. seem to be associated to the severity of the disease triggering an inflammatory response and damaging the skin of the hoof.

The species *Trueperella pyogenes* (*T. pyogenes*) was found in Cluster 8 together with *D. nododus* and *F. necrophorum* (Fig. 2, Supplementary Table S8). *Trueperella pyogenes*, which was formerly known as *Arcanobacterium pyogenes,* belongs to the commensal flora of skin and mucous membranes of animals, yet is also known as an important opportunistic pathogen, being an etiological agent of diverse animal infections, including footrot in sheep (Calvo-Bado et al., 2011; Wani et al., 2015). Our results are then in accordance with previous associations with lameness and footrot in animals such as sheep and goats (Calvo-Bado et al., 2011; Wani et al., 2015).

Little is known about the *Ezakiella* genus although it has been described as commensal flora of human and animals. It was first identified in 2015 from a human fecal sample in Peru – *Ezakiella peruensis* (Patel et al., 2015). Later in 2017 a new *Ezakiella* species, *Ezakiella massiliensis* (*E. massiliensis*), was isolated from the human vagina (Diop et al., 2017). More recently, in 2019, the species *Bacteroides coagulans*, was proposed to be classified as *Ezakilella coagulans*. (García-López et al., 2019). Hence, only three species of this genus are known. Recently, *E. massiliensis* was found with higher prevalence in samples with BDD (Caddey, 2021). Additionally, in this study, *E. massiliensis* was grouped in the same cluster with *D. nodosus* and *F. necrophorum* (Cluster 8, Fig. 2, Supplementary Table S8). Further studies are necessary in order to assess the role of this bacteria in footrot.

Regarding *Campylobacter* spp., in this study were identified 15 *Campylobacter* species with significant differences in their abundances. The clustering analysis showed that, in general, their abundances increased along with the lesions' severity, although with different abundance profiles, and therefore integrated in different clusters (Clusters 1,2,7,8 and 10, Fig. 2). This trend is in accordance with the shift, already mentioned, from dominant gram-positive species in mild stages of footrot infection to dominant gram-negative species in more severe stages, and with the results of (McPherson et al., 2019) where the genus *Campylobacter* was found more abundant in samples recovered from animals with footrot. The species of *Campylobacter* constitute a highly biologically diverse group of organisms, some of which are well-known as causative agents of clinical illness in animals and humans, whereas many other members of the genus appear to be commensals in the intestinal tract or lack clearly established associations with overt disease (Sahin et al., 2017). *Campylobacter* species are frequenctly present in the farm environment, which makes their presence in the interdigital tissues an expected result. In sheep, *Campylobacter fetus* subsp. *fetus* and *Campylobacter jejuni* subsp. *jejuni* are the major *Campylobacter* species associated with sheep abortion outbreaks (Sahin et al., 2017). The development of microscopic lesions, triggering an inflammatory response in pregnant sheep, leads to abortion. Additionally, species such as *C. jejuni* were also associated to digital dermatitis in cattle (Refaai et al., 2013). The role of *Campylobacter* species in footrot is still unknown, but the abundance profile found in this study might suggest some kind of relevance that must be taken into account in further studies.

Finally, the methodology applied, WMGS, has some inherent limitations associated with in-tissue samples such as skin biopsies where the total DNA yield contains a high level of host contamination, usually accounting for more than 99%. However, the taxa resolution achieved is higher than the one obtained with 16 S rRNA gene, allowing to perform taxonomic classifications at the species level. While WMGS screens the complete genomic DNA, 16 S rRNA gene taxonomic composition is limited and influenced by selected primers and targeted variable regions, introducing bias due to the poor taxa resolution between bacteria (genus level). Therefore, this study complements the previous studies of the ovine footrot microbiome performed using 16 S rRNA gene (Calvo-Bado et al., 2011; Maboni et al., 2017; McPherson et al., 2019; Clifton et al., 2022) providing higher resolution on the bacterial taxonomic classification. Hence, we were able to identify a variety of bacterial key species spanning multiple genera, most of which were previously identified, that are consistently associated with footrot infection.

5. Conclusions

This is the first study of the ovine footrot whole metagenome sequencing-based microbiome. The metagenomics analyses identified differences in the bacterial composition between different severity stages of footrot as well as the abundance profile of different bacteria along the disease progression. Based on improved resolution provided by this methodology *D. nodosus*, *M. fermentans*, *F. necrophorum*, *P. asaccharolytica*, *E. massiliensis*, *T. pyogenes, Treponema* ssp., *Campylobacter* spp., *Staphylococcus* spp., *Streptococcus* spp. and other species were identified as particularly abundant in the microbiome of samples from animals with footrot infection. Their abundance profile along the disease indicated that they proliferate as the diseases aggravates, being key species that differentiated mild and severe footrot lesion stages. Further analysis on the individual species of *Campylobacter* and *E. massilensis*, as well as other species with similar abundance profiles, is necessary to further understand their roles in footrot. Although these bacteria are not able to initiate footrot, several evidences had been described supporting that they are associated to the increase of the severity of footrot lesions caused by *D. nodosus* and *F. necrophorum.* Overall, our findings provided significant information to better understand disease pathogenesis and provides evidences to focus primarily on the potential footrot pathogens identified. Last but not least, the knowledge of the microbiome present in mild and severe forms of footrot, compared with healthy sheep claws, represents an important contribution for the improvement of therapeutic and prophylactic measures that are crucial for controlling the disease and improve animal welfare.

Ethical approval

This study was approved by the ethics committee for animal

experimentation ORBEA-U Évora, Portugal (ID: GD/20467/2021/P1).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The metagenomics sequencing data generated under the scope of this work has been deposited at the NCBI in the Short Read Achieve (SRA) databases under the bio project number PRJNA933156.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109745.

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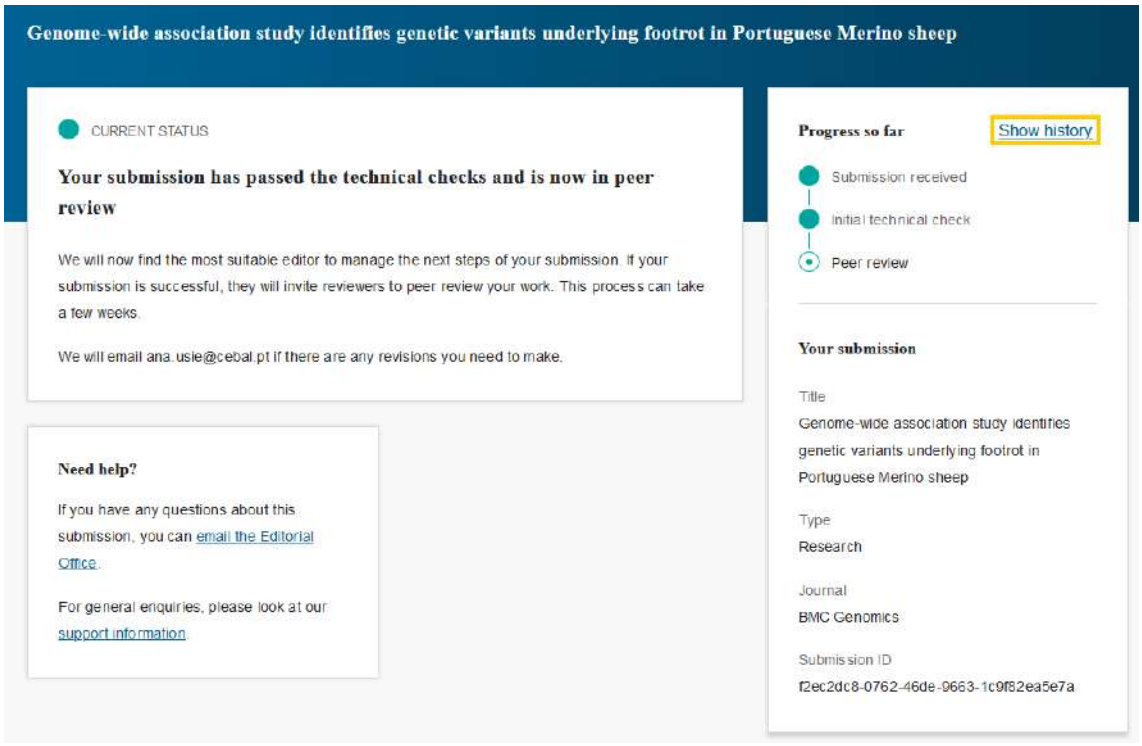
Genome-wide association study identifies genetic variants underlying footrot in Portuguese Merino sheep

Daniel Gaspar[1,2], Catarina Ginja[2], Nuno Carolino[3,4,5], Célia Leão[1,6,#], Helena Monteiro[7], Lino Tábuas[7], Sandra Branco[8,9], Ludovina Padre[8], Pedro Caetano[8], Ricardo Romão[8], Claudino Matos[7], António Marcos Ramos[1,6], Elisa Bettencourt[8], Ana Usié[1,6*]

Submitted to BMC Genomics Journal



GEN-RES-ALENTEJO

Utilização de Metodologias Genómicas na Selecção de Ovinos Resistentes à Peeira e a Parasitas Gastrointestinais na Região do Alentejo

ALT20-03-0145-FEDER-000037

Maria Helena Monteiro, ACOS
Ovibeja, 27 de Abril de 2017

Objectivos GEN-RES-ALENTEJO

1. Identificação de factores de risco em explorações de ovinos no Alentejo
2. Caracterização das doenças e desenvolvimento de metodologias de diagnóstico
3. Impacto económico das doenças
4. Identificação de marcadores genéticos
5. Impacto económico do projecto no controlo da peeira e do parasitismo GI
6. Divulgação dos resultados do projecto

Parceiros GEN- RES ALENTEJO/Financiamento

Claudino Matos (coordenador do projecto)
Helena Monteiro
Miguel Madeira
João Santos
Lino Tábuas (Bolseiro)

Elisa Bettencourt
Ludovina Padre
Pedro Henriques
Ricardo Romão
Sandra Branco
Clara Dias (Bolseira)

António Marcos Ramos

Nuno Carolino

Carlos Bettencourt (Colaborador)

Cofinanciado por:

Alentejo 2020

Portugal 2020

UE - Fundo Europeu de Desenvolvimento Regional

Parasitismo GI e Peeira

Porquê?

O parasitismo por nemátodas GI e a peeira são as doenças com maior impacto económico em explorações de ovinos

(Nieuwhof & Bishop, 2005)

Existência de diferente sensibilidade/resistência genética

Nas mesmas condições ambientais: diferentes animais apresentam diferente severidade da doença

(Davies *et al.*,2009)

Parasitismo GI

Sinais Clínicos

- Perda de Peso
- Diarreia
- Anemia
- Edema
- Letargia
- Inapetência
- Diminuição do ganho de peso

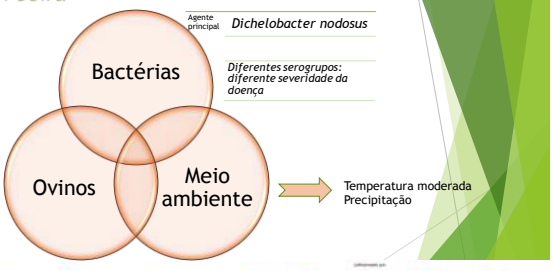
Parasitismo GI: perdas

263 milhões €/ano (2006)

99 milhões €/ano (2005)

Perdas de produção até 50%

Peeira



Peeira – sinais clínicos e perdas económicas

- Claudicação: dor
- Mortalidade
- Inapetência
- Fertilidade
- Produção de leite
- Produção de lã
- Tratamento

Peeira

Custo-perdas económicas

- Suplementação nutricional
- Diminuição do peso dos borregos ao nascimento
- Aumento da taxa de mortalidade dos borregos
- Doenças metabólicas
- Menos oportunidades de venda de animais
- Maneio mais difícil

Peeira

42-71 milhões €/ano (1991);
13 milhões €/ano (2005)

28 milhões €/ano (2005)

Controlo e profilaxia - abordagem integrada

- Controlar o hospedeiro
- Conhecer e controlar o agente etiológico
- Controlar o ambiente

Controlo parasitismo GI – factores de risco ambientais

Quarentena de animais novos na exploração	Mudar de pastagem e desparasitar	Diminuir encabeçamento
Rotação de pastagens	Pastoreio misto ou alternado (evitar ovinos + caprinos)	Alimentação nutricionalmente completa e equilibrada
Suplementação proteica no peri-parto	Suplementação mineral – Cobre (atenção dose!)	Combinar estratégias

Procurar nível óptimo de produção e não nível máximo!

Controlo parasitismo GI – controlo do agente: anti-helmínticos

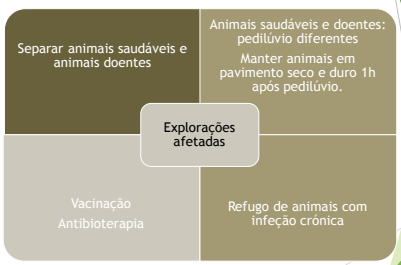
- Esquema de desparasitação selectiva - criar *refugia*
- Diminuir n.º de desparasitações anuais
- Realizar análises coprológicas
- Alternar o uso de princípios ativos de antihelmínticos (AH)
- Utilizar princípios ativos específicos para os parasitas a eliminar
- Se possível dosear para cada animal, em alternativa aplicar dose segundo animal mais pesado

Controlo parasitismo GI – novas perspectivas

Peeira prevenção – factores de risco ambientais

- Não partilhar caminhos nem pastagens com rebanhos infectados
- Adquirir animais em explorações sem peeira
- Bovinos: infeção e transporte mecânico de serogrupos benignos
- Quarentena a animais novos (4 semanas; exame de unhas e pedilúvio no início e final)
- Isolar exploração (vedações)
- Corte correctivo
- Pedilúvio a animais novos
- Vazio das pastagens (2 semanas)
- Limpeza de botas, material e transporte

Peeira – factores de risco ambientais/controlo do agente



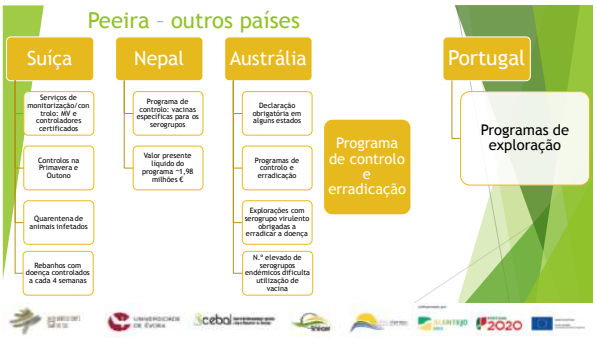
Controlo da peeira – novas perspectivas

Futuro

Vacinação contra serogrupos específicos do rebanho/região

Resistência genética

Peeira – outros países



Controlo e profilaxia - abordagem integrada

Controlar o hospedeiro

Conhecer e controlar o agente etiológico

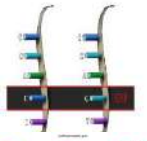
Controlar o ambiente

Selecção de animais resistentes

A) Selecção fenotípica: escolher animais sem doença

B)Identificação de marcadores genéticos: sangue/pele

Reprodução de animais identificados como geneticamente resistentes



MARCADOR GENÉTICO NÃO IDENTIFICADO NAS RAÇAS PORTUGUESAS: DIFERENÇAS ENTRE RAÇAS

Controlo e profilaxia - abordagem integrada

Controlar o hospedeiro

Conhecer e controlar o agente etiológico

Controlar o ambiente

Peeira - Controlar o agente

Como identificar o agente?

Cultura:
- **resultados podem ser falíveis**
- **Cultura longa e fastidiosa: 2-3 semanas**

METAGENÓMICA: **identificação através do ADN do agente:**
- **permite identificar serogrupos virulentos e benignos**
- **resultados em 3 dias**

Vantagem!

GEN-RES-ALENTEJO

Objectivos GEN-RES-ALENTEJO

1. Identificação de fatores de risco nas explorações de ovinos no Alentejo
2. Caracterização das doenças e desenvolvimento de metodologias de diagnóstico
3. Impacto económico das doenças
4. Identificação de marcadores genéticos
5. Impacto económico do projecto no controlo da peeira e do parasitismo
6. Divulgação dos resultados do projeto

Actividades GEN-RES-ALENTEJO

1. **Identificação de factores de risco no Alentejo:** inquéritos
2. **Caracterização das doenças e desenvolvimento de metodologias de diagnóstico:**
   1. Visitas às explorações
   2. Classificação das lesões de peeira e colheita de amostras para metagenómica
   3. Exames coprológicos
   4. Colheitas de sangue
3. **Impacto económico das doenças:**
   1. Avaliação das explorações
   2. Impacto económico do projecto
4. **Identificação de marcadores genéticos**
5. **Divulgação dos resultados do projecto**

Tarefas GEN-RES-ALENTEJO: estado actual

1. **Distribuição dos inquéritos**
2. **Visitadas 10 explorações: 2 visitas**
3. **Recolha e análise coprológica**
4. **Recolha de sangue: PT, µHct**
5. **Recolha de amostras das lesões de peeira para metagenómica**
6. **Recolha de sangue para estudo genómico do hospedeiro**

Inquérito – 5 min com vosso MV assistente!

Explorações visitadas

Até agora…


Visitas às explorações

Lesões Peeira – 1.ª Visita: 10 explorações






Grau 0	Grau 1	Grau 2	Grau 3	Grau 4	Grau 5
711 animais	277 animais	107 animais	41 animais	8 animais	3 animais

Lesões Peeira – 2.ª Visita: 9 explorações






Grau 0	Grau 1	Grau 2	Grau 3	Grau 4	Grau 5
506 animais	146 animais	72 animais	61 animais	7 animais	1 animais

Recolha de amostra para metagenómica

Agradecimentos

- União dos ADS do Alentejo
- Alunos MIMV
- Produtores:
  - DRAPAL: Herdade da Abóbada
  - Herdade das Mouras
  - Herdade da Mergulhagem
  - Herdade de S. João Batista
  - Herdade do Michão
  - Fundação Eugénio de Almeida
  - Herdade dos Montes Altos
  - Herdade de S. Lourenço
  - Herdade da Várzea


Obrigada pela vossa atenção

APORMOR Feira da Luz – Expomor 2017 (30 Ago-04 Set). II Jornadas Técnicas da Produção Animal

GEN-RES-ALENTEJO – UTILIZAÇÃO DE METODOLOGIAS GENÓMICAS NA SELECÇÃO DE OVINOS RESISTENTES À PEEIRA E PARASITAS GASTROINTESTINAIS NA REGIÃO DO ALENTEJO

Associação de Agricultores do Sul (ACOS)

Universidade de Évora (UE)

Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL)

Instituto Nacional Investigação Agrária Veterinária (INIAV)

Direção Regional de Agricultura e Pescas do Alentejo (DRAPAL)

União dos Agrupamentos de Defesa Sanitária do Alentejo (UADS Alentejo)

PROJECTO/DESAFIO:

- Identificação de factores de risco nas explorações de ovinos no Alentejo - inquéritos

- Caracterização das doenças e desenvolvimento de metodologias de diagnóstico – visitas às explorações

- Impacto económico das doenças – avaliação económica das explorações

- Identificação de marcadores genéticos

- Divulgação de resultados

Peeira: doença infecto-contagiosa

Origina claudicação severa em vários animais

Aparece sobretudo na época de chuvas mas com temperaturas moderadas

Agente bacteriano: *Dichelobacter nodosus*

Controlo:

Corte corretivo: realmente eficaz?
Pedilúvio
Antibioterapia sistémica: resistências
Isolamento dos animais doentes: mão de obra/instalações
Refugo: custos
Vazio sanitário das pastagens (15 dias): não é possível na maioria dos casos
Vacinação ???

Parasitas gastrointestinais

Não apresentam elevada mortalidade

Níveis de morbilidade elevados (diarreia, anemia, caquexia etc….)

Controlo:

Desparasitação tradicional: elevadas resistências aos anti-helmínticos nos últimos anos

O controlo de populações parasitárias com base apenas em um tratamento anti-helmíntico, é pouco sustentável e eficiente a médio prazo (Torres-Acosta & Hoste, 2005)

- Identificação de factores de risco nas explorações de ovinos no Alentejo – inquéritos √

- Caracterização das doenças e desenvolvimento de metodologias de diagnóstico – visitas às explorações √

Caracterização das doenças e desenvolvimento de metodologias de diagnóstico – visitas às explorações:

• Aproximadamente 2 explorações por ADS (critérios) – 10 explorações - duas visitas

• O que foi feito nestas visitas?

Registo da identificação (brinco)

Registo da CC

Colheita de sangue

Colheita de fezes

Lavagem e observação das 4 extremidades com registo da classificação das lesões

Colheita de material das lesões apenas na 2ª visita (terapêutica local e sistémica)

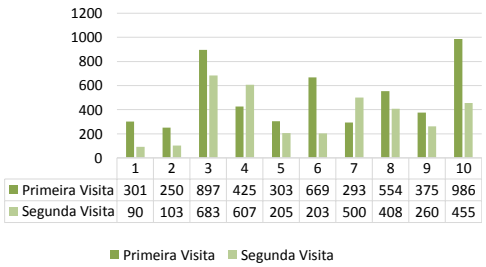
Resultados

1ª visita:
. 1147 animais observados peeira;
. 1015 amostras de fezes (OPG);

2ª visita:
. 842 animais observados peeira;
. 683 amostras fezes;

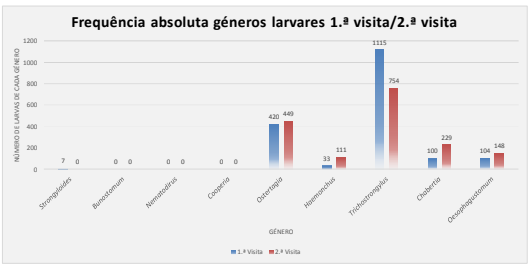
	GRAU 0	GRAU 1	GRAU 2	GRAU3	GRAU 4	GRAU 5
Primeira Visita	711	277	107	41	8	3
Segunda Visita	554	147	72	61	7	1

Média de OPG em cada visita, por exploração



	1	2	3	4	5	6	7	8	9	10
Primeira Visita	301	250	897	425	303	669	293	554	375	986
Segunda Visita	90	103	683	607	205	203	500	408	260	455

Primeira Visita Segunda Visita



Na 2ª visita colhido material das lesões para identificação do agente

Como identificar o agente?

CULTURA: longa e fastidiosa (2-3 semanas) E NÃO PERMITE DIFERENCIAÇÃO ENTRE ESTIRPES

METAGENÓMICA: identificação através do ADN do agente – PERMITE IDENTIFICAR ESTIRPES MAIS E MENOS PATOGÉNICAS

Peeira: controlar o agente

- Utilização de vacinas monovalentes para o serogrupo específico – metagenómica

- Seleção de animais mais resistentes

Parasitas gastrointestinais – controlo

- Controlo integrado

- Seleção de animais mais resistentes

MARCADOR GENÉTICO NÃO IDENTIFICADO NAS RAÇAS PORTUGUESAS: DIFERENÇAS ENTRE RAÇAS

Identificação de marcadores genéticos - SANGUE

Seleção assistida por marcadores

Reprodução de animais identificados como geneticamente resistentes

Utilização de Metodologias Genómicas na Selecção de Ovinos Resistentes à Peeira e a Nematodes Gastrointestinais na Região do Alentejo

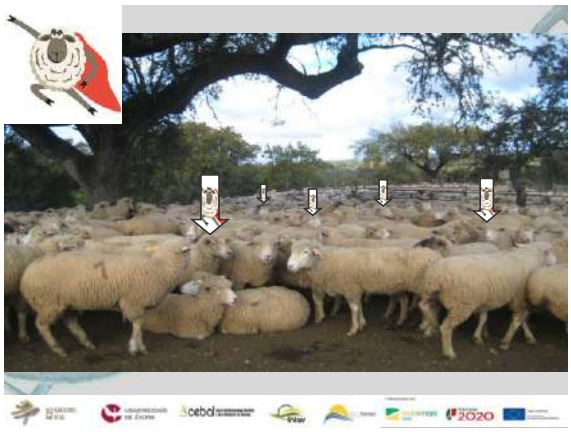
GEN-RES-ALENTEJO

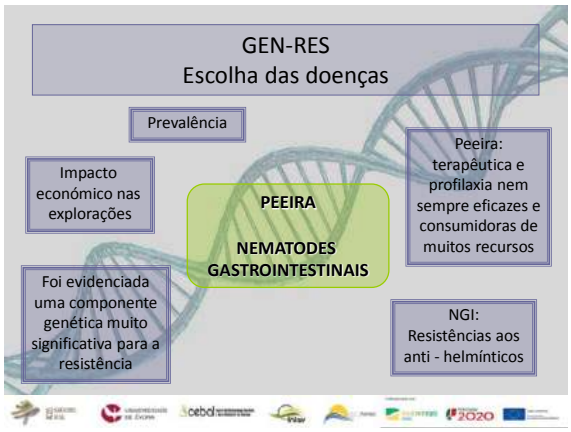
ALT20-03-0145-FEDER-000037

Resultados Preliminares

Maria Helena Monteiro

- Descrição do projecto
- Descrição das actividades desenvolvidas
- Resultados
  - Inquéritos
  - Visitas às explorações





OBJECTIVOS/TAREFAS

OBJECTIVOS	TAREFAS
1. Identificação de factores de risco em explorações de ovinos no Alentejo	*Inquéritos:* ✓ *Elaboração / Preenchimento / Avaliação* ✓ *Identificação dos factores de risco*
2. Caracterização das doenças e desenvolvimento de metodologias de diagnóstico	*1. Identificação de animais resistentes / susceptíveis nas explorações* *2. Colheita de amostras para os estudos de genómica (sangue) e metagenómica (tecidos do espaço interdigital)*

OBJECTIVOS

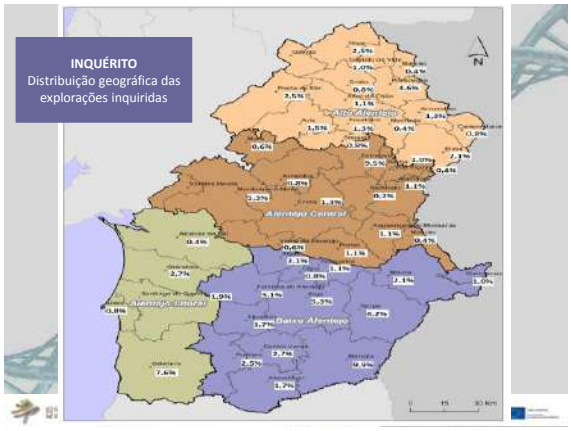
3. Impacto económico das doenças
4. Identificação de marcadores genéticos
5. Impacto económico do projecto no controlo da peeira e do parasitismo GI

Actividades desenvolvidas no 1º ano

- Elaboração do inquérito
- Preenchimento de 524 inquéritos com a colaboração dos coordenadores dos ADS do Alentejo e sua avaliação preliminar
- Duas visitas a 10 explorações
- Número de observações para Peeira: 1989
- Determinações da Condição Corporal: 1963
- Análises coprológicas: 1698
- Coproculturas: 220
- Determinações de Proteínas Totais e Microhematócrito: 1837

Inquérito

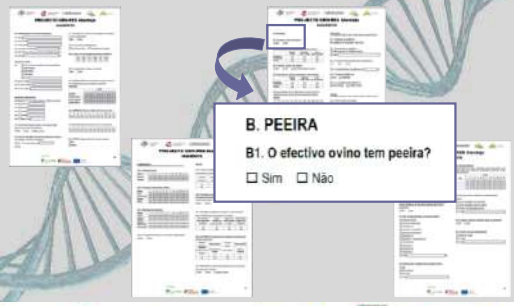
- 524 inquéritos preenchidos
- Entrevistas presenciais aos produtores, nos ADS´s
- Informação sobre os desparasitantes utilizados obtida junto do Médico Veterinário Assistente da exploração
- A informação recolhida foi registada e analisada no programa IBM SPSS Statistics (version 24).
  - Variáveis quantitativas - teste F da Anova.
  - Variáveis qualitativas ou em níveis - teste Qui-quadrado
- O preenchimento dos inquéritos ainda está a decorrer



INQUÉRITO
Distribuição geográfica das explorações inquiridas

NUTS II	Número de inquéritos	%
Alto Alentejo	110	21,0
Alentejo Central	123	23,5
Baixo Alentejo	221	42,2
Alentejo Litoral	70	13,4
Total	524	100

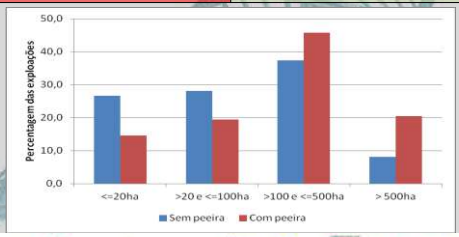
INQUÉRITO



INQUÉRITO

INQUÉRITOS
Factores de Risco Identificados para a Peeira
(***p<0,001)

Área média da exploração***	As explorações com peeira têm em média uma área maior



INQUÉRITOS
Factores de Risco Identificados para a Peeira
(***p<0,001)

Dimensão do efectivo * **	Explorações com Peeira: maior número médio de cabeças
	Explorações sem Peeira: menor número médio de cabeças
Período de cobrição e período de partos * **	Explorações com Peeira: cobrições e partos concentrados em determinadas épocas do ano
	Explorações sem Peeira: cobrições e partos ao longo de todo o ano
Montado * **	As explorações com peeira têm mais montado nos locais onde se encontram os animais

INQUÉRITOS
Factores de Risco Identificados para a Peeira
(** p<0,01 / * p<0.05)

Participação feiras / mercados**	As explorações com peeira participam em feiras e mercados (14,0%) do que as explorações sem peeira (6,5%)
Estabulação**	As explorações com peeira estabulam mais os animais (34,9%) do que as explorações sem peeira (24,3%)
Drenagem dos solos *	As explorações sem peeira referem mais (64.4%) que os locais onde os animais se encontram estão bem drenados do que as explorações com peeira (51.1%)
Textura dos solos*	As explorações com peeira têm solos predominantemente com uma textura fina ou mista, enquanto que nas explorações sem peeira também se salienta a textura grosseira
Altitude dos locais de permanência*	As explorações sem peeira têm os animais predominantemente em cerros e altos
Localização (NUTS II)*	

INQUÉRITOS
Não parecem ser factores de risco
(ns p > 0.05)

Parâmetros meterológicos (temperatura média máxima e mínima, humidade relativa e precipitação)	**Período de estabulação**
Raça dos ovinos	**Local dos partos**
Presença de espécies coabitantes	**Pastoreio directo**
Partilha de caminhos/pastagens	**Suplementação**
Origem animais de substituição	**Tipo de suplementação**
Taxa reposição do efectivo	**Período de suplementação**
	Blocos minerais
	Lesões provocadas por pedras

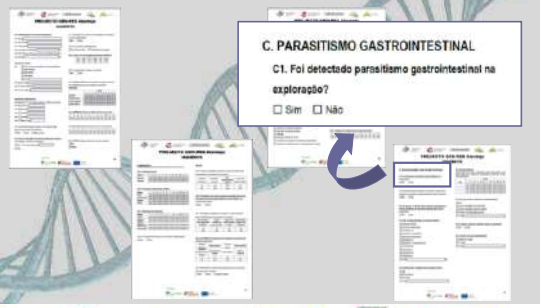
INQUÉRITO
Procedimentos utilizados para tratamento e profilaxia

Procedimento	Explorações que utilizam (%)	Explorações que não utilizam (%)
Pedilúvio	31,1	68,9
Corte de unhas	**41,6**	58,4
Tratamento tópico	33,2	66,8
Antibióticos sistémicos	23,7	76,3
Separação dos animais afectados	14,7	85,3
Vacinação	11,5	88,5
Mudança periódica das camas	3,6	96,4
Tratamento das camas	0,6	99,4
Quarentena	0,4	99,6

INQUÉRITOS
Composição dos Pediluvios

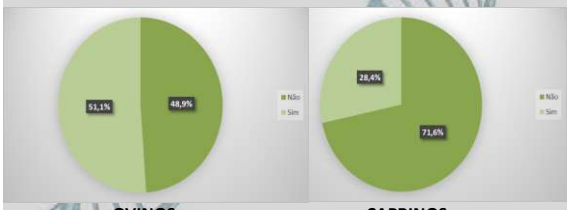
Sulfato de cobre	**Sulfato de cobre, Sulfato de Zinco e Formol**	**Sulfato de cobre, Sulfato de zinco, Cal, Formol e água oxigenada**	**Sulfato de cobre, Formol e Creolina**
Sulfato de zinco	***Podocur***	**Sulfato de cobre, Lixívia e Formol**	**Sulfato de cobre, Sal e Formol**
Oxitetraciclina	**Sulfato de zinco e Formol**	**Sulfato de cobre, Lixívia e Cal**	**Sulfato de cobre, Vinagre e Sal**
Sulfato de cobre e Oxitetraciclina em pó	**Sulfato de cobre e Sulfato de Zinco**	**Sulfato de cobre, Sulfato de zinco, Formol e Podocur**	**Sulfato de cobre, Sulfato de zinco, Enxofre e Lixívia**
Sulfato de cobre e Formol	**Sulfato de cobre, Sulfato de zinco, Lixívia e Formol**	**Sulfato de cobre, Cal viva, Água viva e Lixívia**	**Sulfato de cobre, Sal e Cal**
Sulfato de cobre e Lixívia	**Sulfato de cobre, Formol e Cal viva**	**Sulfato de zinco, Formol e Lixívia**	**Sulfato de cobre, Sal e Lixívia**

INQUÉRITO



INQUÉRITOS

Foi detectado parasitismo gastrointestinal na sua exploração?



OVINOS CAPRINOS

INQUÉRITOS

Desparasitantes mais utilizados

VISITAS ÀS EXPLORAÇÕES

Visitas às explorações

- Seleccionaram-se **10 explorações**
- A cada exploração fizeram-se **2 visitas**
  - 1ª visita: Dezembro 2016 – Janeiro de 2017
  - 2ª visita: Janeiro de 2017 – Maio de 2017
- Nas 2ªs visitas só se observaram animais que tinham sido observados na 1ª visita

Visitas às explorações

- Registo da identificação (brinco)
- Registo da CC
- Colheita de sangue (para microhematócrito e proteínas totais)
- Colheita de fezes (analise coprológica e coprocultura)
- Lavagem e observação das 4 extremidades com registo da classificação das lesões
- Colheita de material do espaço interdigital apenas na 2ª visita

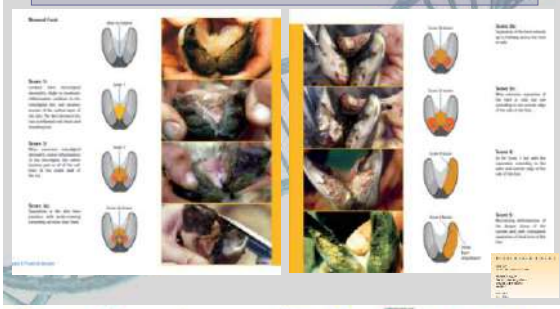
Visitas às explorações

VISITAS ÀS EXPLORAÇÕES

Lesões de Peeira

Visitas às explorações

Classificação das lesões de Peeira



Visitas às explorações

Classificação das lesões de Peeira

0

Visitas às explorações

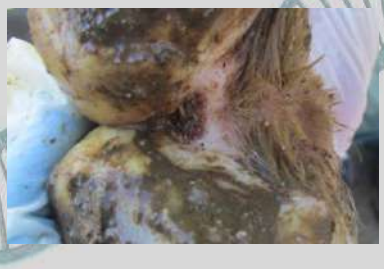
Classificação das lesões de Peeira

1

Visitas às explorações

Classificação das lesões de Peeira

2



Visitas às explorações

Classificação das lesões de Peeira

3

Visitas às explorações

Classificação das lesões de Peeira

4

Visitas às explorações

Classificação das lesões de Peeira

5

Visitas às explorações

Distribuição das lesões de Peeira encontradas

	n	GRAU 0	GRAU 1	GRAU 2	GRAU 3	GRAU 4	GRAU 5
1ª Visita	1147	711	277	107	41	8	3
		62.0%	24.1%	9.3%	3.6%	0.7%	0.3%
2ª Visita	842	554	147	72	61	7	1
		65.8%	17.5%	8.6%	7.2%	0.8%	0.1%

Visitas às explorações

Frequência de animais doentes (grau 2-5)

1 ª visita	13.9 %
2ª visita	16.7 %

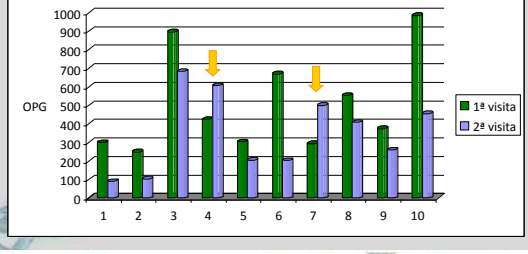
Visitas às Explorações

Analises Coprológicas

Visitas às explorações

Análises Coprológicas

Média de OPG em cada visita, por exploração



Visitas às explorações

Análises Coprológicas

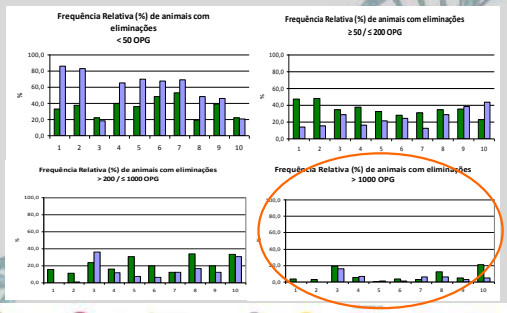
Na maioria das explorações verificou-se uma redução do número de OPG entre a 1ª e a 2ª visita

?

✓ Tempo seco

Visitas às explorações

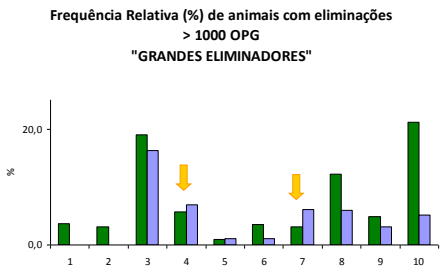
Analises Coprológicas



Visitas às explorações

Analises Coprológicas

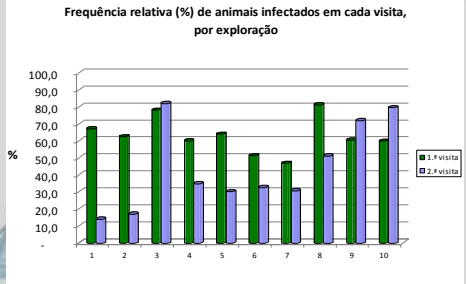
Frequência Relativa (%) de animais com eliminações
> 1000 OPG
"GRANDES ELIMINADORES"



Visitas às explorações

Análises Coprológicas

Frequência relativa (%) de animais infectados em cada visita, por exploração



Visitas às explorações
Coproculturas - Abundância Proporcional Média

Visitas às explorações
Coproculturas - Abundância Proporcional Média

Próximas actividades

- Concluir as visitas às 18 explorações previstas no projecto
- Avaliar os resultados da metagenómica
- Avaliação do impacto económico das doenças nas explorações
- Sequenciação genéticas dos animais considerados resistentes / susceptíveis
- Divulgação dos resultados do projecto

Agradecimentos

- A todos os produtores que nos permitiram fazer as visitas de campo
- A todos os alunos e estagiários que nos acompanharam e ajudaram nas visitas
- Aos coordenadores dos ADS´s que colaboraram no preenchimento dos inquéritos
- A toda a equipa do projecto

GEN-RES-ALENTEJO - Utilização da Genómica na Selecção de Ovinos Resistentes a Parasitas Gastrointestinais e Peeira no Alentejo

(GEN-RES-ALENTEJO - ALT20-03-0145-FEDER-000037)

Sandra Branco (Universidade de Évora, ICAAM)

Sumário atividades

. Inquéritos epidemiológicos: identificação de fatores de risco – 689 ✓

. Visitas explorações: 17 explorações das 18 iniciais (2 visitas a cada exploração) ✓

Sumário atividades

. Amostras de pele normal (*score* 0) e de pele com lesões (*score* 1 a 5) – bacteriologia clássica, PCR e metagenómica (260 amostras) – a decorrer

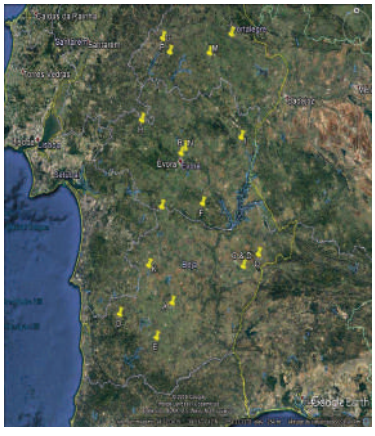
. Inquéritos económicos nas explorações visitadas para avaliação do impacto económico das duas doenças ✓

Sumário atividades

. Impacto económico que as recomendações do projeto poderão ter nas explorações ✖

. Identificação de marcadores genéticos associados à resistência às duas doenças – a decorrer

. Divulgação dos resultados finais e elaboração de Manual para MV e produtores ✖



Visitas

2017: Dezembro a Maio

2018: Janeiro a junho

Merino branco, Merino Preto e x Merino

13564 úngulas observadas - *score* das lesões √

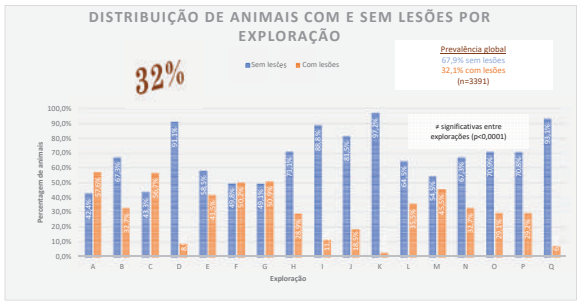
Condição corporal (CC) √

Microhematócrito (MHT) √

Proteínas totais (PT) √

Colheita de fezes (OPG e identificação de larvas) √

Prevalência Peeira nos dois anos de observações



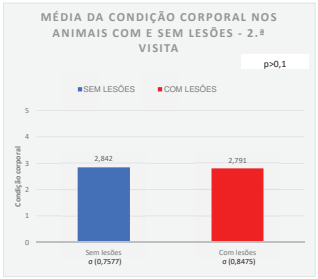
Há diferenças significativas na prevalência de Peeira nas diferentes explorações (p<0,0001)

VI JORNADAS TÉCNICO-VETERINÁRIAS DO CAMPO BRANCO
Pequenos Ruminantes

Efeito da doença – Peeira – na Condição Corporal

1ª visita: animais com lesões têm CC mais elevada (significativo)

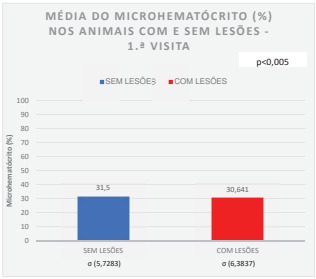
2ª visita: animais com lesões têm CC mais baixa (não significativo)



VI JORNADAS TÉCNICO-VETERINÁRIAS DO CAMPO BRANCO
Pequenos Ruminantes

Efeito da doença – Peeira – no Microhematócrito

1ª visita: animais com lesões têm MHT mais baixo (significativo)

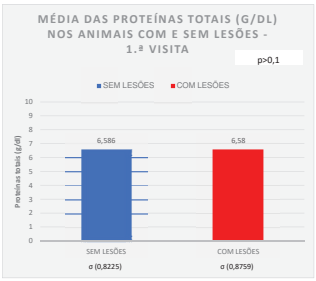


2ª visita: animais com lesões têm MHT mais baixo (significativo)

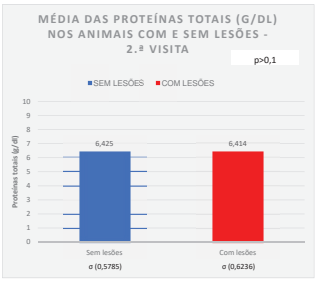
VI JORNADAS TÉCNICO-VETERINÁRIAS DO CAMPO BRANCO
Pequenos Ruminantes

Efeito da doença – Peeira – nas Proteínas Totais

1ªvisita: animais com e sem lesões não têm diferenças significativas

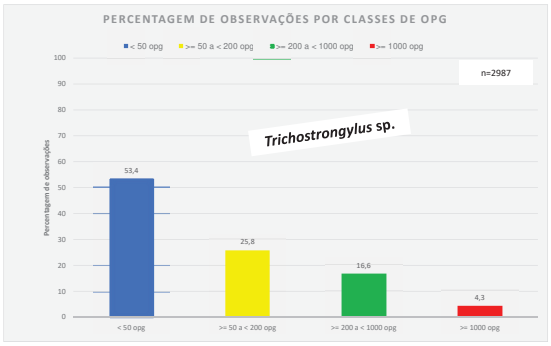


2ªvisita: animais com e sem lesões não têm diferenças significativas



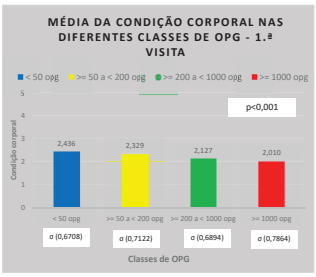
VI JORNADAS TÉCNICO-VETERINÁRIAS DO CAMPO BRANCO
Pequenos Ruminantes

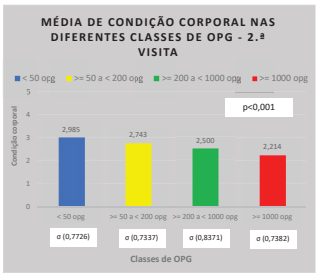
Efeito da doença – Parasitismo gastrointestinal



VI JORNADAS TÉCNICO-VETERINÁRIAS DO CAMPO BRANCO
Pequenos Ruminantes

Efeito da doença – Parasitismo gastrointestinal – na Condição Corporal

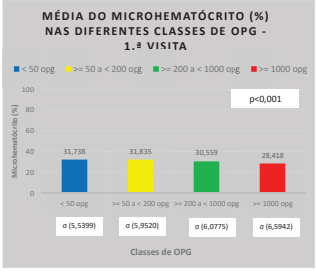


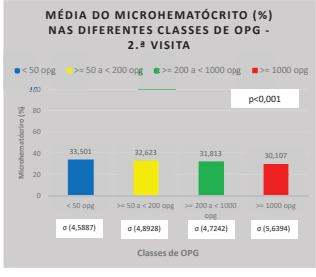


Animais com maior eliminação de OPG têm CC mais baixa e animais com menor eliminação de OPG, CC mais elevada (valores significativos em ambas as visitas)

VI JORNADAS TÉCNICO-VETERINÁRIAS DO CAMPO BRANCO
Pequenos Ruminantes

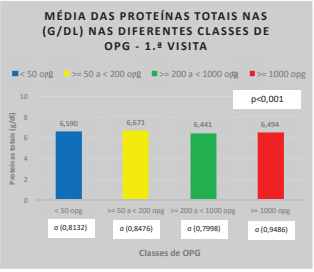
Efeito da doença – Parasitismo gastrointestinal – no Microhematócrito

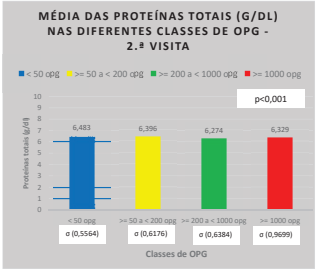




Animais com maior eliminação de OPG têm MHT mais baixo e animais com menor eliminação de OPG, MHT mais elevado (valores significativos em ambas as visitas)

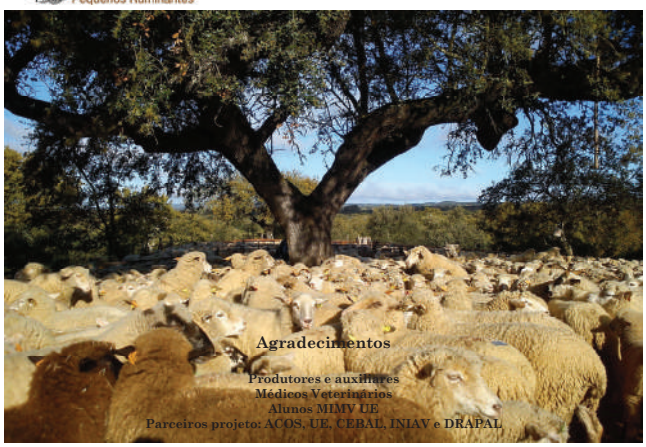
Efeito da doença – Parasitismo gastrointestinal – nas Proteínas Totais





Animais com maior eliminação de OPG têm PT mais baixa e animais com menor eliminação de OPG, PT mais elevada (valores significativos em ambas as visitas)

VI JORNADAS TÉCNICO-VETERINÁRIAS DO CAMPO BRANCO
Pequenos Ruminantes



Agradecimentos

Produtores e auxiliares
Médicos Veterinários
Alunos MIMV UE
Parceiros projeto: ACOS, UE, CEBAL, INIAV e DRAPAL

MyMail

Conheça programa e oradores do 2º dia de jornadas – Sala de Ruminantes

X Jornadas HVME - Programa e Oradores do 2º Dia - Sala Ruminantes

Exmos. Srs.

No dia 3 de Março, 2º dia das Jornadas do HVME, no auditório do Évora Hotel, promovemos a **"Saúde Animal: Boas Práticas"**. Serão apresentadas palestras sobre problemáticas emergentes na produção de bovinos, como: a **falha na transferência da imunidade dos vitelos**; a **tuberculose na interface bovinos-espécies silvestres;** e as **mastites em vacas de carne**. Relativamente à produção de pequenos ruminantes, teremos uma abordagem bastante interessante sobre **dicas práticas para o controlo de ronha**; serão também apresentados alguns **resultados do projecto GEN-RES Alentejo**, um projeto que tem como objetivo principal o desenvolvimento de ferramentas genéticas que permitam apoiar os criadores na seleção de animais resistentes a parasitoses e peeira.

A encerrar as X Jornadas do HVME, a apresentação da **nova Norma de Certificação de IBR e BVD em Portugal**.

Programa e oradores:

- **Porque falha a imunidade nos vitelos de carne? Impactos e alternativas** – Dr. Miguel Minas, *ESAE – IPPortalegre*
- **Co-habitação bovinos e fauna silveste: orientação para o controlo da tuberculose** – Dr.ª Carolina Abrantes, *UTAD-Waves*
- **Projecto Gen-Res Alentejo – Contributo para a melhoria do estado sanitário dos efectivos de ovinos** – Prof. Dr. Claudino Matos, *ACOS*
- **Dicas práticas para o controlo de ronha em pequenos ruminantes** – Dr. Miguel Figueiredo, *AMVCB*
- **Mastites em vacas de carne: realidade ou ficção?** – Prof. Dr. Luís Pinho, *SVA Expleite, ICBAS*
- **Norma de Certificação de IBR e BVD em Portugal Continental e sua aplicação** – Prof.ª Dr.ª Yolanda Vaz, *Diretora de serviços de sanidade e proteção animal da DGAV*

A inscrição do tipo A permite o acesso aos dois dias de jornadas.

As inscrições são limitadas!

Inscrições

GEN-RES-ALENTEJO

Utilização de Metodologias Genómicas na Selecção de Ovinos Resistentes à Peeira e a Nematodes Gastrointestinais na Região do Alentejo

GEN-RES-ALENTEJO

ALT20-03-0145-FEDER-000037

Claudino Matos
ACOS – Agricultores do Sul

Será Possível Obter Melhoria Genética para a Resistência aos Parasitas em Pequenos Ruminantes ?

Qual o Caracter mais apropriado para medir a resistência a parasitoses?

O Que é RESISTÊNCIA / SUSCEPTIBILIDADE a doenças ?

Ausência de sinais clínicos de doença após exposição à mesma

Caracter binomial

Qual o Caracter mais apropriado para medir a resistência a parasitoses?

Contagem ovos fecais (Fecal Egg Count)
Carga parasitária
Tamanho dos parasitas
Fecundidade

Parâmetros Genéticos

Heritabilidades

Ovinos
h_2 = (0.149) Avikalin
h_2 = (0.24) Muzafarnagri
h_2 = (0.4-0.5) Katahdin

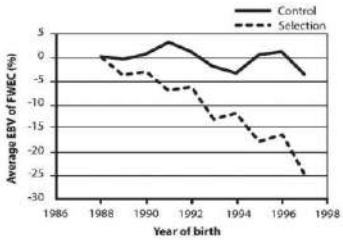
Caprinos
h_2 =0.13 (Galla and East African)
h_2 = 0.11-0.16 (Jamunapari)
h_2 = 0.37 (Creole, French West Indies)

Correlações Genéticas

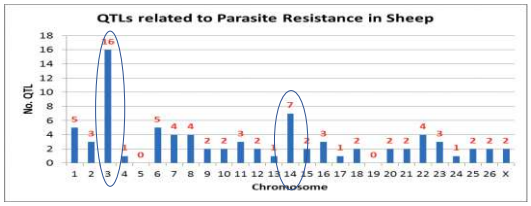
Caracteres de crescimento
(0 - 0.2)

Caracteres Lanares
(0)

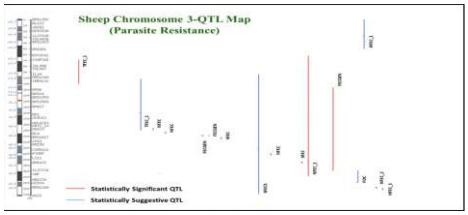
Tendência Genética na Raça Merino Rylington (Australia) de animais selecionados para resistência aos parasitas comparadas com um grupo control



81 QTLs (Quantitative Trait Loci) relacionados com a Resistência aos parasitas



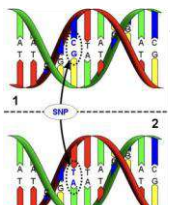
•Cromossoma 3 - 16 QTLs Cromossoma 14 - 7 QTLs



http://www.ainfo.inia.uy/digital/bitstream/item/6386/1/Kathiravan-RLA5071-Parasite-Presentation.pdf

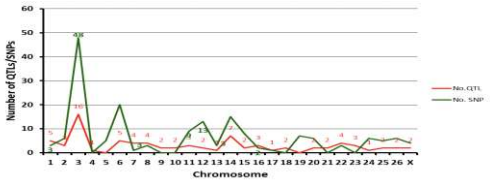
Selecção Genómica

SNP – Single Nucleotide Polymorphism
(polimorfismo de nucleotídeo simples)



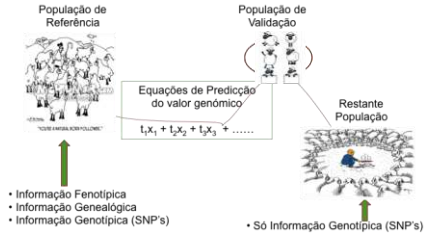
- Marcador genético de eleição
- Indivíduos podem ser genotipados para 20 a 60 mil SNP's
- SNP's explicam uma fracção importante da variação genética
- SNP's permitem estimar os valores genéticos dos animais

QTL's vs SNP's



Animal Production and Health Laboratory,IAEA Laboratories, Seibersdorf

Esquema Simplificado da Selecção Genómica



Exemplificação do cálculo do valor genómico com nucleótidos polimórficos (alelo A vs. B) com valores estimados de +8, +4, +2, e −6 para os SNP 1, 2, 3, e 4, respectivamente

Animal	SNP 1		SNP 2		SNP 3		SNP 4		Valor Genómico
1	AA	8	BB	−4	AA	2	AA	−6	0
2	AA	8	AA	4	BB	−2	AB	0	10
3	AB	0	AB	0	AB	0	BB	6	6
4	AB	0	AB	0	AB	0	AA	−6	−6

Conclusões

- **A selecção para a resistência às Parasitoses é possível**
- **O caracter a medir é a Contagem de Ovos Fecais (FEC)**
- **A selecção genómica abre grandes perspectivas ao combate às parasitoses pela via genética**.

Projeto GEN-RES ALENTEJO

PARÂMETROS GENÉTICOS DA PEEIRA EM OVINOS DAS RAÇAS MERINA BRANCA E MERINA PRETA EM PORTUGAL

Carolino N.*, Monteiro M., Madeira M., Santos J., Tábuas L., Branco S., Bettencourt E., Ludovina P., Romão R., Caetano P., Damião P., Dias C., Bettencourt C., Ramos A.M. e Matos C.

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XX Simpósio Iberoamericano sobre Conservação e Utilização de Recursos Zoogenéticos
Corumbá, Mato Grosso do Sul – Brasil, 11 a 14 de Novembro de 2019

Parâmetros genéticos da peeira em ovinos das raças Merina Branca e Merina Preta em Portugal
Carolino N., Monteiro M., Madeira M., Santos J., Tábuas L., Branco S., Bettencourt E., Ludovina P., Romão R., Caetano P., Damião P., Dias C., Bettencourt C., Ramos A.M. e Matos C.

PROJECTO GEN-RES ALENTEJO

Objetivos

- Identificação de fatores de risco relevantes associados à Peeira e aos nemátodos gastrointestinais em explorações da região do Alentejo;
- Caracterização das doenças e metodologias de diagnóstico para identificação de animais resistentes;
- Utilização de ferramentas de metagenómica para identificação dos agentes da Peeira;
- Avaliação do impacto económico do projecto no controlo da Peeira e de nemátodos gastrointestinais e melhoria da produtividade e rentabilidade das explorações de ovinos do Alentejo;
- Utilização da genómica para identificação de marcadores genéticos associados à Peeira e aos nemátodos gastrointestinais.

Resultados Esperados

- Caracterização das doenças e avaliação do seu impacto económico nas explorações de ovinos do Alentejo;
- Identificação de genes implicados na resistência à Peeira e a parasitas (nemátodos gastrointestinais) nos ovinos de várias raças do Alentejo;
- Metodologia de seleção de animais resistentes à Peeira e a nemátodos gastrointestinais, disponibilizada aos produtores de ovinos do Alentejo.

PROJECTO GEN-RES ALENTEJO

UTILIZAÇÃO DE METODOLOGIAS GENÓMICAS NA SELECÇÃO DE OVINOS RESISTENTES À PEEIRA E PARASITAS GASTROINTESTINAIS NA REGIÃO DO ALENTEJO

CANDIDATURA: ALT20-03-0145-FEDER-031674 SISTEMA DE APOIO À INVESTIGAÇÃO CIENTÍFICA E TECNOLÓGICA – PROJETOS ICDT

DURAÇÃO 36 MESES
INÍCIO: SETEMBRO 2016
FINANCIAMENTO TOTAL: 669 970,93 €

Parceiros

Apoios

IR: Claudino Matos (ACOS)

http://www.gen-res-alentejo.pt/

Merino Branco
32 Ganaderías
10042 Hembras
612 Machos

Merino Preto
57 Ganaderías
14517 Hembras
756 Machos

Razas autóctonas Portuguesas

Parâmetros genéticos da peeira em ovinos das raças Merina Branca e Merina Preta em Portugal
Carolino N., Monteiro M., Madeira M., Santos J., Tábuas L., Branco S., Bettencourt E., Ludovina P., Romão R., Caetano P., Damião P., Dias C., Bettencourt C., Ramos A.M. e Matos C.

Ação 7.8 – RECURSOS GENÉTICOS
Operação 7.8.3 CONSERVAÇÃO E MELHORAMENTO DOS RECURSOS GENÉTICOS ANIMAIS
Portaria 268/2015 (1 Set 2015)

Artigo 1.º Objeto - A presente portaria estabelece o regime de aplicação do apoio 7.8.3, «**Conservação e melhoramento de recursos genéticos animais**», integrado na ação n.º 7.8, «Recursos genéticos», da medida n.º 7, «Agricultura e recursos naturais», inserida na área n.º 3, «Ambiente, eficiência no uso dos recursos e clima», do Programa de Desenvolvimento Rural do Continente, abreviadamente designado por PDR 2020.

Objetivos de Mejoramiento

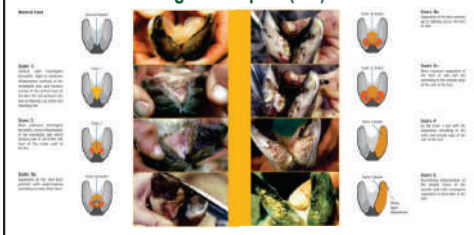
- Crecimiento
- Capacidad maternal
- Conformación

➢ Manutención de la variabilidad genética

Score Pododermatitis – "Pedero"

(John Webb, University of Melbourne, 2005)

❖ Lesiones interdigitales 4 pies (0-5)



❖ Animal "Positivo" - al menos 1 pie con lesiones Score ≥2

Objetivos – Estimar Parámetros Genéticos para el "Pedero" en las razas MB y MP

ANCORME

Información Disponible en Libro Genealógico MB y MP

MB	MP
304 185 Animales	195 530 Animales

- 745 registros de scores Pedero (positivo *vs* negativo)
- 437 hembras LG (239♀MB e 198♀MP)
- 3 Ganaderías / 2016-2018

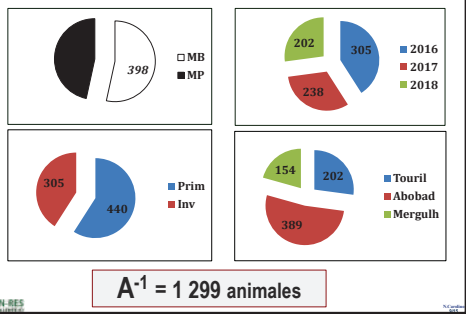
- A^{-1} = 1 229 animales (136♂ e 1093♀)

Score Pododermatitis – "Pedero"

(John Webb, University of Melbourne, 2005)

XX Simpósio Iberoamericano sobre Conservação e Utilização de Recursos Zoogenéticos
Corumbá, Mato Grosso do Sul – Brasil, 11 a 14 de Novembro de 2019

Estadísticas descriptivas - Datos Utilizados



A^{-1} = 1 299 animales

Parâmetros genéticos da peeira em ovinos das raças Merina Branca e Merina Preta em Portugal
Carolino N., Monteiro M., Madeira M., Santos J., Tábuas L., Branco S., Bettencourt E., Ludovina P., Romão R., Caetano P., Damião P., Dias C., Bettencourt C., Ramos A.M. e Matos C.

Metodología de Análisis

- BLUP – Modelo Animal
  Registros Repetidos

$$\begin{bmatrix} X'X & X'Z_1 & X'Z_2 \\ Z_1'X & Z_1'Z_1+A^{-1}\alpha & Z_1'Z_2 \\ Z_2'X & Z_2'Z_1 & Z_2'Z_2+I\gamma \end{bmatrix} \begin{bmatrix} b \\ u \\ p \end{bmatrix} = \begin{bmatrix} X'y \\ Z_1'y \\ Z_2'y \end{bmatrix}$$

- Score Pedero (0 y 1)

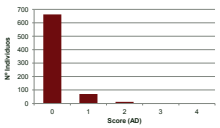
$$y = Xb + Za + Zpe + e$$

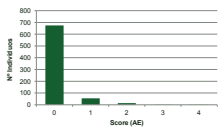
Xb: Ef. Fijos; Za + Zpe + e: Ef. Aleatórios

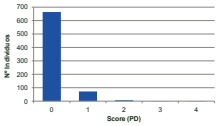
Ganadería*ano (n=5), fecha medición (Inv. y Prim),y edad a la medición (Cov linear y cuadrática)

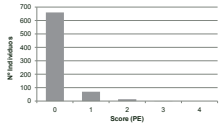
- 2 métodos análisis
  - Frecuentista - REML, Software MTDFREML
  - Bayesiana - Gibbs sampling, TM Software

Estadísticas descriptivas - SCORE









Parámetros Genéticos para el Pedero

	MTDFREML	TM (Gibbs)
Var. Genética	0.12595	0.12781
Var. Amb. Permanente	0.00000	0.00000
Var. Residual	0.86336	0.85372
Var. Fenotípica	0.98931	0.98153
Heredabilidad (h^2)	0.127± 0.089	0.130± 0.049
Ef Permanente (c^2)	0.000	0.000
Repetibilidad (re)	0.127	0.130
Desv. Est. Genética	0.355	0.358

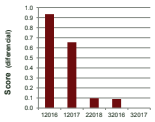
h^2 = 0.127
re = 0.127

h^2 = 0.130
re = 0.130

Parâmetros genéticos da peeira em ovinos das raças Merina Branca e Merina Preta em Portugal
Carolino N., Monteiro M., Madeira M., Santos J., Tábuas L., Branco S., Bettencourt E., Ludovina P., Romão R., Caetano P., Damião P., Dias C., Bettencourt C., Ramos A.M. e Matos C.

Efectos Fijos en el Score del Pedero

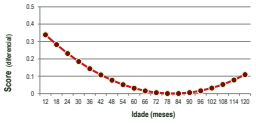
Efecto Ganadería*Año

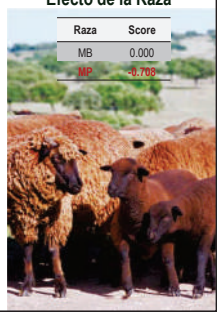


Efecto de la Raza

Raza	Score
MB	0.000
MP	-0.708

Efecto de la Edad

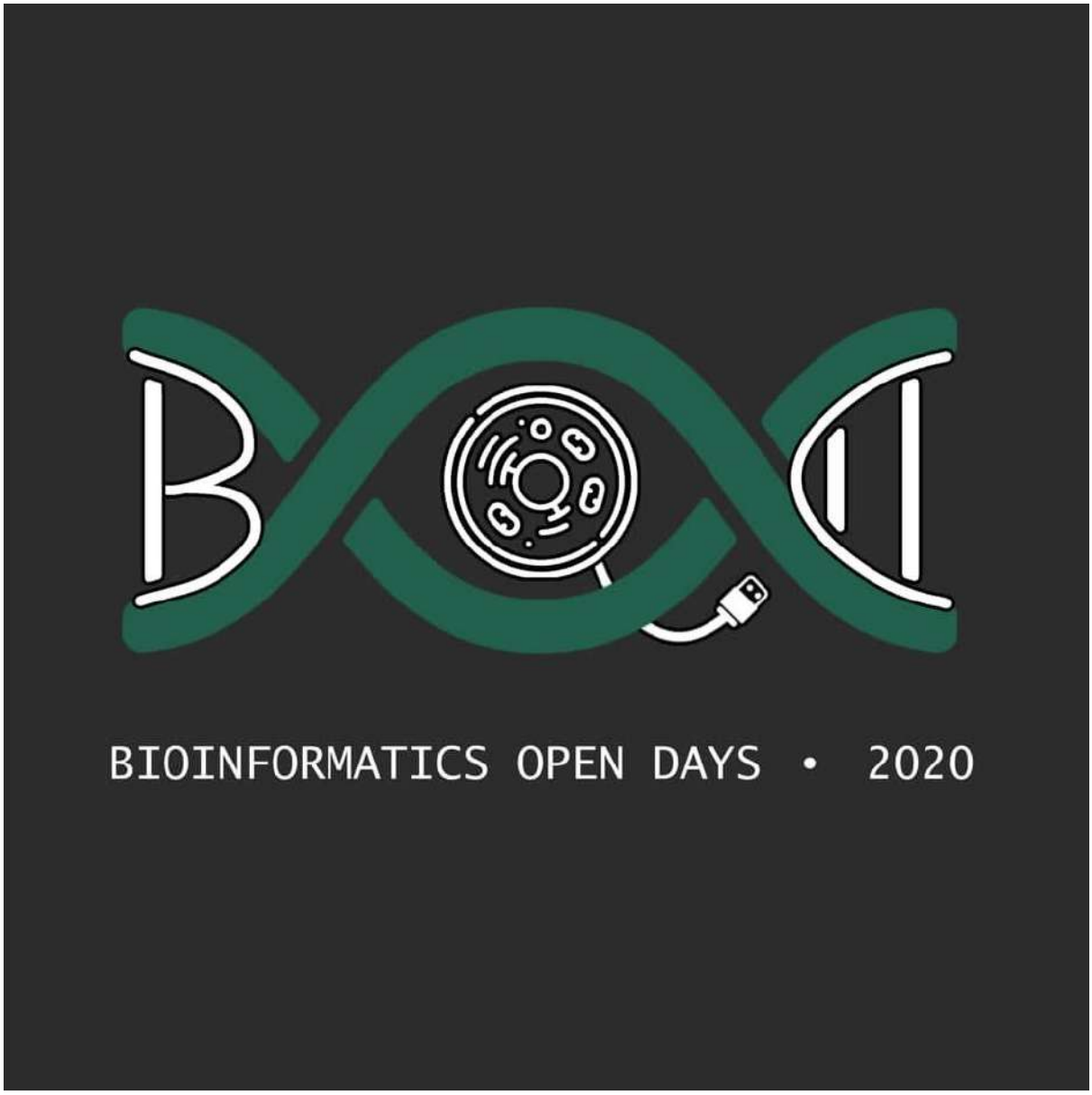




Futuro / GEN-RES

- Identificação de genes implicados na resistência à Peeira
- Metodologia de seleção de animais resistentes à Peeira

Conclusiones



19, 20 e 21 Fevereiro

Braga

Characterization of genomic variation in Portuguese sheep breeds using whole genome resequencing

D. Gaspar[1,2], H. Magalhães[1], A. Usié[1,3], C. Leão[5], M. Monteiro[4], M. Madeira[4], J. Santos[4], L. Tábuas[4], S. Branco[3], E. Bettencourt[3], L. Padre[3], R. Romão[3], P. Caetano[3], P. Damião[3], C. Dias[3], N. Carolino[5], C. Bettencourt[6], C. Ginja[2], C. Matos[4], A.M. Ramos[1,3]

[1]CEBAL - Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo, Beja, Portugal. [2]CIBIO-InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Vairão, Portugal. [3]MED-Mediterranean Institute for Agriculture, Environment and Development, Évora, Portugal. [4]ACOS – Agricultores do Sul, Beja, Portugal. [5]INIAV - Instituto Nacional de Investigação Agrária e Veterinária, Portugal. [6]Centro de Experimentação do Baixo Alentejo, Herdade da Abóbada, Vila Nova de S. Bento, Portugal

Merino, Campaniça and Serra da Estrela (SE) sheep are among the most relevant breeds reared in Portugal. Merino and Campaniça sheep are mainly distributed in the south of Portugal, in the Alentejo region, being the basis for the production of different meat, dairy and wool products. SE is the main Portuguese dairy breed, located in the Serra da Estrela region. From its milk a typical, high-value cheese is produced, awarded with the protected designation of origin mark certification. Despite their importance, the lack of significant genomic resources is a problem shared by these breeds. Thus, the purpose of the present study was to assess the variability present in the genomes of these sheep breeds, and a population of crossed Merino sheep, using whole-genome resequencing (WGRS).

A total of 34,976,564,162 paired-end raw reads were produced for 56 sheep samples, from which, 93.2% were kept for downstream analysis after quality control procedures. With a mapping rate of around 99.85%, an average of 90.01% of high-quality reads were uniquely mapped to the sheep reference genome. Variant calling was performed and an initial set of 115,137,724 raw SNPs was obtained. After SNP filtering, a final set of 31,320,381 high-quality SNPs were maintained. A total of 11,148,321 SNPs were located in genic regions, where 120,172 were annotated as synonymous and 80,882 as non-synonymous. Moreover, 20,172,060 SNPs were identified in intergenic regions. Lastly, structural variation was also characterized in all 56 sheep genomes.

The results derived from this study will be useful to develop several genomic tools for these breeds, including genome-wide association studies, genetic diversity and traceability schemes.

Preference for presentation: Oral presentation

Book of Abstracts of the 71st Annual Meeting of the European Federation of Animal Science

Invited Farmpedia: the reference toolbox for teaching animal husbandry in general education 617
A. Chouteau, C. Disenhaus, R. Baumont and G. Brunschwig

Invited Serious games to explain animal husbandry 617
A. Chouteau, E. Zanchi, R. Baumont, C. Disenhaus and G. Brunschwig

Invited Assessment and valuation of ecosystem services provided by beef farms in Alentejo, Portugal 618
M.P. Dos Santos, T.G. Morais, T. Domingos and R.F.M. Teixeira

Invited Prediction of nitrogen output in urine and faeces from beef on diets with different protein contents 618
A. Angelidis, L. Crompton, T. Misselbrook, T. Yan, C.K. Reynolds and S. Stergiadis

Invited The emissions balance of the production of Mozzarella di Bufala Campana DOP 619
R. De Vivo, L. Zicarelli and R. Napolano

Poster Session 71

Italian livestock emissions balance 619
R. De Vivo and L. Zicarelli

Session 72. Resilient sheep and goats: breeding & management strategies to overcome disease and environmental challenges

Date: Friday 4 December 2020; 13.45 – 17.30
Chair: Conington / McHugh

Theatre Session 72

Could rumen volumes measured by CT scanning help to breed sheep with lower methane emissions? 620
N.R. Lambe, A. McLaren, K. McLean, J. Gordon and J. Conington

Calibrating lamb cut weight data using computed tomography 620
A. Williams, F. Anderson and G.E. Gardner

Genetic basis of body reserves mobilisation and accretion in Manchega ewes 621
C. Diaz, I. Ureña, A. Rubio, C. Gonzalez, M.D. Perez-Guzman, M. Ramon, M. Serrano and M.J. Carabaño

Determining factors affecting feed intake in grazing sheep 621
F. McGovern, P. Creighton, N. Galvin, D. Hennessy, M. O'Donovan, B. Garry, N. McHugh and M. Beecher

The estimation of dispersion parameters for growth traits of lambs in Slovenia 622
M. Bizjak, M. Špehar, K. Potočnik, M. Štepec, B. Luštrek, G. Gorjanc and M. Simčič

Liveweight and body composition responses to differential feeding in a range of Merino genetics 622
C.J. Byrne, S. Blumer and A.N. Thompson

Weaning performance of sheep grazing on different swards under cell grazing or continuous stocking 623
M.J. Rivero, H. Fleming, O. Lawal-Adebowale, R. Pywell and J. Storkey

Differential mucin gene expression in the ovine cervix contribute to breed differences in fertility 623
L. Abril-Parreño, P. Cormican, A. Krogenæs, X. Druart, K.G. Meade and S. Fair

Analysis of the sheep footrot microbiome using whole-metagenome sequencing 624
A. Usié, C. Leão, D. Gaspar, A. Botelho, S. Cavaco, M. Monteiro, M. Madeira, J. Santos, L. Tábuas, S. Branco, E. Bettencourt, L. Padre, R. Romão, P. Caetano, P. Damião, C. Dias, N. Carolino, C. Bettencourt, C. Matos and A.M. Ramos

Analysis of the sheep footrot microbiome using whole-metagenome sequencing

A. Usié[1], C. Leão[2], D. Gaspar[1], A. Botelho[2], S. Cavaco[2], M. Monteiro[3], M. Madeira[3], J. Santos[3], L. Tábuas[3], S. Branco[4], E. Bettencourt[4], L. Padre[4], R. Romão[4], P. Caetano[4], P. Damião[4], C. Dias[4], N. Carolino[2], C. Bettencourt[5], C. Matos[3] and A.M. Ramos[1]

[1]CEBAL, Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo, Beja, 7800-072 Beja, Portugal, [2]INIAV, Instituto Nacional de Investigação Agrária e Veterinária, Portugal, Oeiras, 2780-157, Portugal, [3]ACOS, Agricultores do Sul, Beja, 7800-072, Portugal, [4]Universidade de Évora, Évora, 7000-645, Portugal, [5]Centro de Experimentação do Baixo Alentejo, Herdade da Abóbada, Vila Nova de S. Bento, 7830, Portugal; marcos.ramos@cebal.pt

In the Alentejo region Merino sheep are the most common breed, reared for the production of meat, dairy and wool. Footrot is responsible for lameness, decreased animal welfare and higher production losses, generating a negative economic impact. The disease is caused by the bacteria *Dichelobacter nodosus*, a process on which it interacts with the sheep foot microbiome, to date largely uncharacterised. To understand and characterise the footrot microbiome dynamics, a whole metagenome sequencing (WMGS) approach was used to study the microbiome of sheep with different footrot affection scores. Foot tissue samples were collected in 214 animals with different footrot degrees, ranging from 0 to 5. DNA was extracted from each sample and used in WMGS. The sequence dataset was analysed in two different ways. First, the reads were mapped directly to the *D. nodosus* and *Fusobacterium necrophorum* reference genomes. Then, a classic metagenomics approach was used, to characterise and quantify the composition of the microbial community present in each sample. The mapping results showed a general positive correlation between the number of mapped reads against the *D. nodosus* and *F. necrophorum* genomes and footrot score. The *D. nodosus* serogroups were also determined for a subset of samples using qPCR. Finally, the microbiome composition showed a higher percentage of *D. nodosus* and *F. necrophorum* in animals affected with footrot. This study showed WMGS to be useful for characterising the sheep footrot microbiome.

A survey of pathogens on lamb carcasses from Portuguese local breeds

S. Coelho-Fernandes, D. Félix-Oliveira, G. Rodrigues, V.A.P. Cadavez and U. Gonzales-Barron

Centro de Investigação de Montanha, Instituto Politécnico de Bragança, Animal Science, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; saraccoelhof@hotmail.com

This study aimed to evaluate the levels of microbial contamination on lamb carcasses from two Portuguese breeds, Bordaleira-de-Entre-Douro-e-Minho (BDM) and Churra-Galega-Bragançana (CGB). Thirty BDM and 30 CGB lambs were reared in a semi-intensive system, and slaughtered at 4 months age. On 11 visits to the abattoir, 400 cm^2 neck/loin/hind pooled areas were swabbed from dressed carcasses. Chilled *L. dorsi* sections were vacuum packed and cold stored. Swabs were analysed for mesophiles, coliforms, *Escherichia coli*, *Salmonella* spp., *Lactobacillus monocytogenes* and *E. coli* O157, while meat samples were analysed for *Salmonella* on the 3rd, 9th, and 15th day post-slaughter. Linear and logistic mixed models were adjusted to assess any effect of breed on microbial occurrence. BDM lamb carcasses presented higher counts ($P<0.05$) of mesophiles (3.52 log cfu/cm^2), coliforms (0.936 log cfu/cm^2) and *E. coli* (0.307 log cfu/cm^2) than CGB carcasses (3.03, 0.633 and 0.079 log cfu/cm^2, respectively). There was no difference between BDM and CGB in the incidences of *Salmonella* spp. (21.4% [95% CI: 10.0-40.2%] versus 16.7% [7.10-34.3%]), *L. monocytogenes* (3.50% [0.50-21.4%] versus 6.70% [1.60-23.1%]) and *E. coli* O157 (32.1% [17.6-51.1%] versus 16.7% [7.10-34.3%]). When *Salmonella* was found in a sampled batch of lamb carcasses, the odds of finding *Salmonella* in meat, at a later processing stage, increased by 8.7 times ($P=0.078$).

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ABSTRACTS

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vices. Therefore, 2 homogeneous groups of rams were identified: active (A) (7.93 ± 3.56, average mounts ± SD) and not active (NA; without any mount). Six rams of each group were killed and total RNA was extracted from HT. Sequencing was carried out generating Illumina paired-end reads of 151 bp. Gene level quantification was estimated using HTSeq, while differential expression and pathway analysis to find regulated functional groups were performed with EdgeR and Gene Set Enrichment Analysis (GSEA), respectively, using the OmicsBox package from BioBam. In the comparison A *vs* NA, 52 differentially expressed genes (DEGs) were found being 41 and 11 genes up- and downregulated, respectively. One of the most outstanding upregulated genes was the *PDYN* that has been related to sexual motivation in male European starlings. Therefore, it has been proposed as one of the neuropeptides that are involved in the control of sexual behavior at the central level. Finally, enrichment analysis including 17,003 DEGs expressed in the HT yielded 130 overrepresented pathways at FDR q-value 5%. The most interesting GO was related to behavior (GO:0007610) with 133 enriched genes including *Neuropeptide Y* (*NPY*) and *Pro-Melanin Concentrating Hormone* (*PMCH*) genes, also involved in the control of sexual behavior whereas others genes such as *CIART* and *NR1D1*, belong to circadian rhythm pathways.

Key Words: sheep, RNA-seq, ram, sexual behavior

W114 Unveiling genomic regions that underlie footrot resistance in Portuguese sheep Merino. D. Gaspar*[1,2], A. Usié[1,3], C. Leão[3,4], C. Matos[5], L. Padre[3], C. Dias[3], C. Ginja[2], and A. M. Ramos[1,3], [1]*CEBAL – Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo, Beja, Portugal,* [2]*CIBIO/InBIO – Research Centre in Biodiversity and Genetic Resources, University of Porto, Vairão, Porto, Portugal,* [3]*MED-Mediterranean Institute for Agriculture, Environment and Development, University of Évora, Évora, Portugal,* [4]*INIAV (Instituto Nacional de Investigação Agrária e Veterinária), Santarém, Portugal,* [5]*ACOS – Agricultores do Sul, Beja, Portugal.*

Footrot is an acute necrotic and highly contagious disease, caused by a co-infection of 2 g-negative anaerobic bacteria, *Dichelobacter nodosus* and *Fusobacterium necrophorum*. It affects the interdigital skin and hooves of sheep, being the main cause of lameness and a major animal welfare and economical concern for the wool, milk and meat sheep industries worldwide. Current effective strategies to control footrot are costly and rely on the use of antibiotics, which could result in the development of parasite resistance mechanisms in the long term. The development of genomic markers associated with footrot resistance can provide a more reliable strategy for classifying and selecting sheep with increased resistance, besides enhancing our understanding of the biology of this disease. We aimed to identify genomic regions and molecular mechanisms associated with resistance to footrot in Portuguese native Merino breeds. For this, a set of 50k single-nucleotide polymorphisms (SNPs) was specifically designed based on whole-genome data obtained for 39 sheep (depth of coverage >22X). A total of 1,466 Portuguese Merino sheep were genotyped using this SNP array. Genome-wide association analysis was performed using a quantitative trait approach based on the modified Egerton system (scores from 0 to 5) for foot integrity and footrot lesions. Genome-wide significance was determined using corrected p-values for multiple testing and SNPs significantly associated with footrot resistance were filtered at a genome-wise false discovery rate of 5%. Our results revealed a set of promising SNPs associated with resistance to footrot that overlaps candidate genes related to immune response and wound healing. These findings contribute to better understanding the architecture of footrot resistance in Merino sheep and to enhance the development of genomic tools to control infections. Also, the whole-genome data were used to investigate the underlying population structure of these native Iberian Merino breeds in the context of worldwide sheep, which is useful to define conservation and management programs.

Key Words: sheep, Merino, footrot, GWAS

W115 Identification of a novel loss-of-function variant in the ovine *TMCO6* gene associated with motor neuron disease of North Country Cheviot sheep. A. Letko*[1], I. M. Häfliger[1], E. Corr[2], F. Brulisauer[2], S. Scholes[2], and C. Drögemüller[1], [1]*Institute of Genetics, Bern, Switzerland,* [2]*SRUC Consulting Veterinary Services, Penicuik, Midlothian, UK.*

Motor neuron diseases (MND) occur sporadically in farm animals including sheep. The aim of our study was to characterize the phenotype and the genetic etiology of an early-onset neurodegenerative disorder observed in several lambs of purebred North Country Cheviot sheep, a native Scottish breed. Affected lambs showed progressive ataxia and subsequent histopathological analysis revealed motor neuronal degeneration including cytoplasmic vacuolation. By whole-genome sequencing of 4 affected lambs, we identified a shared homozygous loss-of-function frameshift variant in exon 6 of the ovine *TMCO6* gene on chromosome 5. Herein we present evidence for the occurrence of a familial novel form of a recessively inherited MND in sheep due to a likely pathogenic 4bp deletion that is assumed to lead to a dysfunction of a transmembrane and coiled-coil domain-containing protein 6 (TMCO6: p.Leu215PhefsTer34). The uncharacterized TMCO6 protein is proposed to interact with the ubiquilin-1 (UBQLN1) protein, which plays an important role in the regulation of different protein degradation mechanisms and pathways and is reported to be associated with sporadic forms of amyotrophic lateral sclerosis (ALS). Therefore, these findings implicate an important role of TMCO6 for proper function and survival of motor neurons and provide a novel candidate gene for human ALS or similar motor neuron disease. Furthermore, these results enable selection against the fatal disorder in sheep population.

Key Words: neurogenetic disorder, rare disease, precision medicine, whole-genome sequencing, membrane trafficking

W116 A homozygous frameshift variant in *MFSD2A* associated with congenital brain hypoplasia in a Kerry Hill sheep family. G. Lühken*[1], A. Letko[2], M. Häfliger[2], M. J. Schmidt[3], C. Herden[4], L. Herkommer[4], J. Müller[4], and C. Drögemüller[2], [1]*Institute of Animal Breeding and Genetics, Justus Liebig University, Giessen, Germany,* [2]*Institute of Genetics, Vetsuisse Faculty, University of Bern, Bern, Switzerland,* [3]*Clinic for Small Animals, Neurosurgery, Neuroradiology and Clinical Neurology, Justus Liebig University, Giessen, Germany,* [4]*Institue of Veterinary Pathology, Justus Liebig University, Giessen, Germany.*

In several consecutive years, a German breeder observed male and female purebred Kerry Hill sheep lambs with severe ataxia and in some cases with convulsions. The lambs died after some days to weeks. All affected lambs descended from the same sire or one of his sons. We hypothesized an autosomal recessive inherited brain disorder underlying the observed deaths and aimed to identify the potential causal allele. Imaging with CT and MRT of 2 affected lambs revealed a reduced skull circumference compared with age matched controls. The forebrain appeared unusually small in relation to the cerebellum and midbrain. The cerebral cortical surface pattern was simplified (pachygyria). There was moderate ventriculomegaly. The cortical and subcortical white matters were thin and the contrast between white and gray matter was diminished. Pathological examination confirmed these findings. Additionally, a multifocal mild cortical dysplasia was found. CNS infection was ruled out. Genotyping 5 affected lambs on the 50k ovine SNP array allowed us to localize the critical genome regions harboring the causative variant to 3 shared IBD segments of totally 8 Mb on different chromosomes by homozygosity mapping. By whole-genome sequencing of an affected lamb, homozygous private variants called in this single case were identified by comparison with 86 publicly available control sheep genomes. This yielded 101 private homozygous protein-changing variants affecting 73 different genes. Within the critical intervals there was only a single variant predicted to be nonsynonymous. Genotyping using Sanger sequencing showed perfect co-segregating of this variant with the observed disorder in the studied

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PROGRAMME EAAP 2022

Session 16. Integrated approach for sheep and goats Mediterranean farming systems

Room: D. Luís
Chair: Hadjipavlou / Marques
Session type: Working group session

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Whole-genome analysis of diversity and population structure in Portuguese native sheep breeds

D. Gaspar[1,2], A. Usié[1,3], C. Leão[1,3], C. Matos[4], A.M. Ramos[1,3], C. Ginja[2]

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Since their domestication, approximately 10,500 years before present, sheep accompanied humankind in all its history. In Portugal, sheep are reared nationwide mainly in agrosilvopastoral systems. Merino Branco, Merino Preto, Campaniça and Bordaleira Serra da Estrela are among the most abundant local breeds. Merino and Campaniça are raised in the Alentejo region to produce meat and wool. Serra da Estrela is the most important dairy breed in Portugal used to produce a typical high-value cheese with a protected designation of origin certification. The purpose of this study was to estimate genomic variation in these four native sheep breeds and a population of crossed Merino, as well as to describe their genetic structure in the context of worldwide sheep and other Iberian breeds. High-throughput resequencing data was generated for 56 individuals, and a total of 31,320,380 high-quality SNPs were used in subsequent analyses. The overall levels of genomic variability were very similar across Portuguese sheep breeds ($0.30 \leq Ho \leq 0.34$; $0.30 \leq He \leq 0.35$). The Principal Components and Bayesian clustering analyses separated these breeds in two clusters: one comprising Campaniça and Serra da Estrela together with transboundary dairy breeds; and another of the well-differentiated multi-purpose Portuguese Merino sheep breeds along with Spanish Merino. Runs of homozygosity analysis yielded 1,690 ROH segments comprising

an average of 2.27 Gb across the genome in all individuals. Campaniça showed the highest mean number of homozygous segments per animal (nROH = 44.5) comprising on average 61.29 Mb. The results of this study are useful to develop genomic tools for genetic improvement, management and conservation of these breeds, including the traceability of certified products.

30 de Setembro 2022

11h30 - Auditório
Parque de Feiras e Exposições

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Do diagnóstico clínico da peeira à bioinformática: desafios e oportunidades

Este seminário visa apresentar o diagnóstico e prevenção da peeira, desde o ponto de vista clínico ao uso de algumas ferramentas bioinformáticas que têm sido aplicadas pela equipa do CEBAL com o intuito de caracterizar o microbioma nos diferentes graus de lesão e associar variações genéticas à resistência à doença, mostrando ao público o potencial da sua utilização na valorização e conservação de espécies endógenas. Os trabalhos apresentados foram desenvolvidos no contexto do projeto Gen-Res-Alentejo, o qual foi liderado pela ACOS (Associação de Agricultores do Sul) em parceria com a Universidade de Évora, com o CEBAL, com o INIAV (Instituto Nacional Investigação Agrária Veterinária) e com a DRAPAL (Direção Regional de Agricultura e Pescas do Alentejo).

PROGRAMA

11h30 – Sessão de abertura

11h40 – Peeira: principais fatores de risco, diagnóstico e prevenção

Pedro Caetano (Hospital Veterinário - Universidade de Évora)

12h00 – Caracterização do microbioma da peeira através da utilização de novas tecnologias de sequenciação

Ana Usié (CEBAL, MED, CHANGE)

12h20 – Utilização de ferramentas genómicas para a identificação de marcadores moleculares associados a resistência a peeira

Daniel Gaspar (CEBAL, CIBIO)

12h40 – Discussão, notas finais e encerramento

GEN-RES ALENTEJO

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Notas biográficas

Pedro Caetano é Médico Veterinário desde 2014, ano em que concluiu o Mestrado Integrado em Medicina Veterinária na Universidade de Évora. Após um período breve em que trabalhou como médico veterinário assistente numa exploração agropecuária, iniciou funções como técnico superior no Hospital Veterinário da Universidade de Évora em 2015. Desde então e até ao início de 2022, exerce funções nas áreas clínica (médica e cirúrgica) e de assistência reprodutiva em espécies pecuárias no Hospital Veterinário e equinos no da Universidade de Évora e na Unidade Clínica de Alter do Chão. Além destas funções também acompanhou os ensinos dos cursos de Medicina Veterinária e de Ciência e Tecnologia Animal da Universidade de Évora, na componente prática relacionada com animais de produção e equídeos. A partir de 2017/2018 começou também a desempenhar funções como docente convidado dos Departamentos de Medicina Veterinária e de Zootecnia da Universidade de Évora. Em fevereiro de 2021 obteve o título de Doutor através do Programa de Doutoramento em Ciências Veterinárias da Universidade de Évora. Durante o seu doutoramento desenvolveu investigação sobre a doença infetocontagiosa peeira ovina nas áreas da clínica, epidemiologia e microbiologia. Atualmente conjuga as funções de docente auxiliar convidado com a de Médico Veterinário na Empresa Multivet – Serviços Veterinários de Equinos e Espécies Pecuárias, onde exerce nas áreas da clínica, cirurgia, medicina da produção, sanidade e assistência reprodutiva em espécies pecuárias e equinos.

Ana Usié é licenciada em Engenharia Técnica em Gestão de Computadores (2007), mestre em Engenharia Informática Sénior (2010) e em Engenharia de Software Livre (2010), pela Universidade de Lleida (Catalunha, Espanha). Obteve o doutoramento em Bioinformática através do Programa de Doutoramento em Saúde Molecular da mesma universidade em 2014. Dois meses depois mudou-se para Portugal e continuou a sua carreira no CEBAL onde integrou o grupo de Genómica Animal e Bioinformática. Ana Usié é membro integrado da unidade de investigação do Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento (MED). O seu trabalho centra-se na análise e processamento de dados de sequenciação obtidos com tecnologias de sequenciação de última geração. Tem estado envolvida em vários projetos nacionais e internacionais, trabalhando com espécies vegetais e animais. As atividades desenvolvidas nestes projetos incluíram montagem *de novo* de genomas e transcriptomas, identificação de variantes como SNPs, SVs e CNVs, metagenómica e análise de expressão diferencial, entre outros. Durante sua carreira teve a oportunidade de treinar e orientar novos membros do grupo, bem como alunos de estágio, e coorientar teses de licenciatura, mestrado e doutoramento.

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Notas biográficas

Daniel Gaspar é licenciado em Biotecnologia (2011) e mestre em Bioinformática e Biologia Computacional (2016), pela Faculdade de Ciências da Universidade de Lisboa (Lisboa, Portugal). Atualmente é aluno de doutoramento no Programa de Biodiversidade, Genética e Evolução da Universidade do Porto. O seu trabalho foca-se na análise e processamento de dados de sequenciação obtidos com tecnologias de sequenciação de última geração. Tem estado envolvido em vários projetos nacionais e internacionais em diferentes espécies vegetais e animais. As atividades desenvolvidas no contexto destes projetos incidiram principalmente em analises de dados de (re)-sequenciação total de genomas para o desenvolvimento de estudos de associação genótipo-fenótipo, diversidade genómica e caracterização de estruturas populacionais, e na análises de dados de transcriptómica para a identificação de genes candidatos associados à stresses bióticos e abióticos, entre outros.

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III

CONGRESO HISPANO-LUSO DE GANADERÍA EXTENSIVA Y DESARROLLO RURAL

2022

Cáceres, 1 y 2 de diciembre

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MESA SANIDAD

CHARACTERIZATION OF THE FOOTROT MICROBIOME IN PORTUGUESE SHEEP BREEDS THROUGH METAGENOMICS

A. Usié[a,b*], C. Leão[a,b,c‡], D. Gaspar[a,d], H. Monteiro[e], L. Tábuas[e], S. Branco[f], E. Bettencourt[f], P. Caetano[f], L. Padre[f], N. Carolino[c], A.M. Ramos[a,b], C. Matos[f*].

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Introduction

In the Alentejo region Merino sheep are the most common breed, reared for the production of meat, dairy and wool. Footrot is responsible for lameness, decreased animal welfare and higher production losses, generating a negative economic impact. The disease is caused by the bacteria *Dichelobacter nodosus* that interacts with the sheep foot microbiome, to date largely uncharacterized. To understand and characterize the footrot microbiome dynamics,of different footrot affection scores, a whole metagenome sequencing (WMGS) approach was used.

Materials and methods

Tissue samples from affected feet were collected from 212 animals with different footrot degrees of lesion severity, ranging from 0 to 5. DNA was extracted from each sample and used in WMGS. The sequence dataset was analysed with a classic metagenomics approach to characterize and quantify the composition of the microbial community present in each sample. The reads of each sample were taxonomically classified and the abundance of each species identified was estimated in order to perform a differential abundance analysis between the samples with different footrot infection degrees.

Results and discusión

Distinct bacterial communities were associated with feet with different footrot scores identifying a total of 63 phyla and 504 families. As the severity of footrot infection increases the microorganisms' diversity decreases triggering a shift in the composition of the microbiome from a dominant gram-positive in mild stages to a dominant gram-negative in the severe stages. The diminished diversity is accompanied by the increased abundances, as the disease progressed, of *D. nodosus* and *Fusobacterium necrophorum* (which plays a role as an opportunistic and is considered as a secondary pathogen), along with several species previously associated with footrot and other polymicrobial diseases affecting the epidermis and provoking inflammatory responses such as *Treponema* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Campylobacter* spp. Although these bacteria are not able to

initiate footrot, several evidences have been described supporting their association with the severity and incidence increase of footrot lesions caused by *D. nodosus* and *F. necrophorum*.

Conclusions

These results confirm the involvement of *D. nodosus* and *F. necrophorum* as two of the main species related with footrot infection. In this work were also identified a set of key-species that differentiate mild and severe footrot infection stages. Although further investigation is required to establish the roles of particular taxa, our results provided significant information to better understand diseases pathogenesis representing an important contribution for the analysis of the microbiome in animal welfare research.

ESTUDIO GENÓMICO DE LA RAZA OVINA AUTÓCTONA PORTUGUESA CHURRA ALGARVIA

D. Gaspar, **Carolina Bruno-de-Sousa**, A. Dias-de-Oliveira, F. Oliveira,
R. Guerreiro, A. E. Pires, C. Matos, A. Usié, **C. Ginja**

Programa

Sexta-feira, 24 de novembro

09h15 | Sessão de Abertura

09h30 | Sessão I – Valorização de Produtos

O equilíbrio das diferentes velocidades de degradação dos alimentos no rúmen (sincronização orgânica da fermentação das várias frações dos alimentos)
Joaquim Pais de Azevedo – De Heus

A produção de queijo: de fio a pavio
Paulo Nobre Martins – ABIASA

A valorização das raças autóctones e seus produtos – qual a estratégia?
Nuno Vieira e Brito — IUCS-CESPU

11h15 | Sessão II – Comunicação

"Era uma vez a lã", o sucesso de boa comunicação
Rosa Pomar – Retrosaria Rosa Pomar

Consultoria, a importância da comunicação de proximidade
Marlise Germer – Capril Virtual Portugal

Leilões de ovinos APORMOR: uma oportunidade e um exemplo
Joaquim Manuel Capoulas – APORMOR

12h45 | Almoço

14h30 | Sessão III – Sustentabilidade e Ambiente

Cabras Sapadoras: um método sustentável de prevenção de incêndios
Júlio Marques - Associação Vezeira

Cabra- montês: situação atual de uma espécie ameaçada
Eliana Fonseca – ICNF

Engorda com silagens de coprodutos agroindustriais
José Santos Silva – INIAV

16h15 | Sessão IV – Genética e Sanidade -

Utilização da genómica no estudo da peeira ovina no Alentejo
Ana Usié – CEBAL

Novas estratégias para o controlo de abortos infeciosos em ovinos
Deolinda Silva – HIPRA

O valor de um bom questionário epidemiológico na sanidade de pequenos ruminantes: como a mudança começa com perguntas
Ana Cláudia Coelho – UTAD

18h00 | Sessão V – Mesa redonda

Tema: *Revitalizando o setor da produção de ovinos e caprinos: estratégia e inovação para o futuro*

A utilização da genómica no estudo da peeira ovina no Alentejo

A. Usié[a,b]*, C. Leão[a,b,c‡], D. Gaspar[a,d], H. Monteiro[e], L. Tábuas[e], S. Branco[f], E. Bettencourt[f], P. Caetano[f], L. Padre[f], N. Carolino[c], A.M. Ramos[a,b], C. Matos[f]*.

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A raça Merina, predominante na região do Alentejo, é criada para a produção de carne, leite e lã em sistema agro-silvo-pastoril. A peeira (podridão dos cascos) é responsável pela claudicação, diminuição do bem-estar animal e por grandes perdas de produção, gerando um impacto económico negativo. O agente causal desta doença, *Dichelobacter nodosus*, é uma bactéria anaeróbica gram-negativa, que interage com o microbioma dos cascos das ovelhas. No entanto, o *Dichelobacter nodosus* não é capaz de induzir a peeira por si só, sendo necessária a presença de um segundo patógeno, o *Fusobacterium necrophorum*. Para compreender e caracterizar a dinâmica do microbioma da peeira nos diferentes graus de lesão, foi utilizada uma abordagem de sequenciação do metagenoma completo (WMGS). Amostras de tecido dos cascos foram obtidas de 212 animais com diferentes graus de lesão nos cascos, variando de 0 a 5. Distintas comunidades bacterianas foram associadas a cascos com diferentes graus de lesão, identificando um total de 63 filos e 504 famílias. À medida que a gravidade da infeção aumenta, a diversidade de microorganismos diminui, desencadeando uma mudança na composição do microbioma de um dominante gram-positivo em estágios leves para um dominante gram-negativo nos estágios graves. Várias espécies anteriormente associadas à peeira e a outras doenças polimicrobianas que afetam a epiderme e provocam respostas inflamatórias, como *Treponema* spp., *Staphylococcus* spp., *Streptococcus* spp. e *Campylobacter* spp., foram identificadas proliferando junto com a gravidade das lesões. Embora estas bactérias não sejam capazes de iniciar a peeira, várias evidências foram descritas apoiando a sua associação com o

aumento da gravidade e incidência das lesões causadas por *Dichelobacter nodosus* e *Fusobacterium necrophorum*. É necessária investigação adicional para poder estabelecer os papéis de taxonomias específicas e assim poder identificar quais desempenham um papel relevante no processo da doença e quais são patogénicos oportunistas.

XIX JORNADAS DA ASSOCIAÇÃO PORTUGUESA DE BUIATRIA - 3 a 5 Novembro 2017

GEN-RES-ALENTEJO

Utilização de Metodologias Genómicas na Selecção de Ovinos Resistentes à Peeira e a Parasitas Gastrointestinais na Região do Alentejo

ALT20-03-0145-FEDER-000037

Bruno Costa Estudante finalista do Mestrado Integrado em Medicina Veterinária da Universidade de Évora

I INTRODUÇÃO

A peeira e o parasitismo por nematodes gastrointestinais são as doenças com maior impacto económico na produção de ovinos na Europa (Nieuwhof & Bishop, 2005). Apesar de não haver dados referentes à prevalência e impacto económicos destas doenças em Portugal, é opinião geral dos médicos veterinários e produtores que ambas têm um impacto económico importante nas explorações de ovinos.

O parasitismo por nematodes gastrointestinais é uma doença de caracter insidioso, associada a alta morbilidade mas baixa mortalidade. É responsável pelo declínio das taxas de crescimento, diminuição do ganho médio diário, aumento do índice de conversão, perda de peso, diminuição da produção de leite e redução da fertilidade.

O agente etiológico da peeira, *Dichelobacter nodosus,* tem vários serogrupos e a imunidade é específica para cada serogrupo (Lacasta *et al*., 2015). Segundo Ware *et al* (2014) estão descritos diferentes graus de lesão (de 0 a 5) em função da sua severidade. A doença é relevante do ponto de vista económico e de bem-estar animal, na medida em que, causa claudicação, podendo, as formas mais severas, levar a anorexia, perda de condição corporal, redução do crescimento e da qualidade da lã e redução da fertilidade (Raadsma & Dhungyel, 2013).

Existem vantagens claras na identificação de marcadores genéticos associados à resistência a determinada doença, sendo que a utilização destes marcadores genéticos constitui um método imediato de selecionar animais com predisposição genética para a resistência.

II MATERIAIS E MÉTODOS

Um dos objetivos do projeto GEN-RES-ALENTEJO é a caracterização destas duas doenças em ovinos Merino Branco, Merino Preto e cruzados na região do Alentejo. O presente trabalho refere-se aos resultados preliminares obtidos, até ao momento, com base nas duas visitas realizadas a 10 explorações inscritas nos ADS/OPP do Alentejo (Figura 1). As tarefas associadas a estas visitas basearam-se na colheita de sangue para identificação de biomarcadores genéticos de resistência, colheita de fezes para cálculo de ovos por grama de fezes (OPG) e posterior identificação dos nematodes GI e colheita de fragmentos de lesão de peeira para caracterização do agente, bem como a classificação das lesões presentes em cada membro. Foram também determinados em cada animal o microhematócrito e as proteínas totais (PT).

Primeira Visita

- 10 explorações
- 1147 animais observados - peeira
- 1015 amostras de fezes – pesquisa de nematodes GI (o nº animais aos quais se colheu fezes é menor do que aquele observado para peeira, porque nem todos apresentavam fezes na âmpola rectal)
- 1106 amostras de sangue

Segunda Visita

- 10 explorações
- 842 animais observados - peeira
- 683 amostras de fezes – pesquisa de nematodes GI (o nº animais aos quais se colheu fezes é menor do que aquele observado para peeira, porque nem todos apresentavam fezes na âmpola rectal)
- 829 amostras de sangue

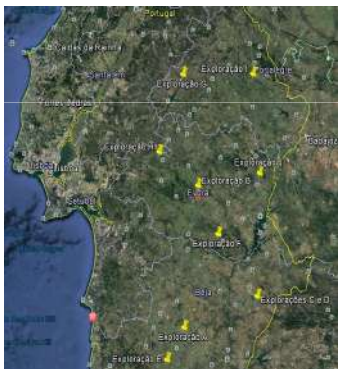


Figura 1: distribuição geográfica das 10 explorações estudadas

III PRIMEIROS RESULTADOS

Na tabela 1 apresentam-se os graus de lesão de peeira presentes nos ovinos observados em ambas visitas às 10 explorações. Foram realizadas duas visitas com o objetivo de avaliar as lesões e o parasitismo nos mesmos animais, num intervalo de tempo estabelecido, sendo que estes não foram sujeitos a tratamento para ambas as doenças. As diferenças entre o número de animais observados entre a primeira e segunda visitas devem-se à morte, venda e refugo de elementos do lote inicial.

	GRAU 0	GRAU 1	GRAU 2	GRAU3	GRAU 4	GRAU 5
Primeira Visita	711	277	107	41	8	3
Segunda Visita	554	147	72	61	7	1

Tabela 1: animais observados e respectivo grau de lesão

No gráfico 1 pode-se observar os valores de OPG por exploração, em cada visita. À excepção de duas explorações, os valores diminuíram entre a primeira e a segunda visita.

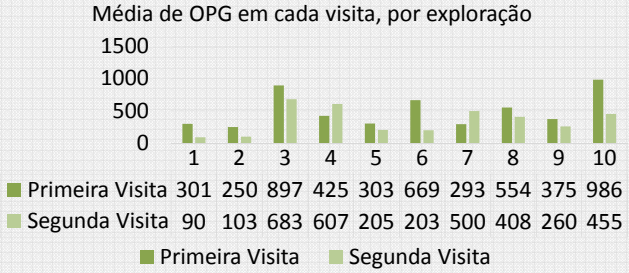
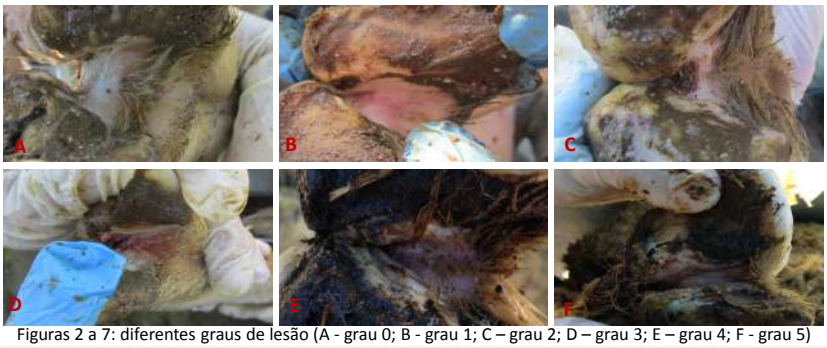


Gráfico 1: média de ovos por grama de fezes (OPG) observados em cada exploração, em ambas as visitas.



Figuras 2 a 7: diferentes graus de lesão (A - grau 0; B - grau 1; C – grau 2; D – grau 3; E – grau 4; F - grau 5)

IV CONCLUSÕES

Pela análise dos resultados obtidos pode-se concluir que o número de animais com lesões graves de peeira é reduzido. O factor clima merece destaque uma vez que no período em que decorreram as visitas, de Outubro de 2016 a Maio de 2017, a pluviosidade foi menor comparativamente a anos anteriores. Relativamente ao parasitismo gastrointestinal , as variações observadas poderão estar relacionadas com a época do ano, fase reprodutiva e fisiológica das fêmeas e diferentes condições climatéricas entre as duas visitas.

V PERSPECTIVAS E ACTIVIDADES FUTURAS

Como actividades futuras pretende-se:

- realizar visitas às restantes explorações selecionadas;
- análise dos inquéritos realizados aos produtores para o estudo de factores de risco associados a estas doenças;
- identificar os géneros dos parasitas gastrointestinais;
- identificar o agente da peeira, com recurso a metagenómica para caracterização dos diferentes serogrupos;
- identificar os biomarcadores genéticos de resistência aos parasitas e à peeira;
- realizar análise estatística completa dos resultados obtidos.

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Identificação de fatores de risco para a ocorrência de peeira em explorações de ovinos na região Alentejo

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6 - ACOS – Bolseiro do Projeto GEN-RES-ALENTEJO
7 - Departamento de Economia da Universidade de Évora – Escola de Ciências Sociais

I - INTRODUÇÃO

A peeira é uma doença altamente contagiosa que afeta a epiderme do espaço interdigital e as úngulas dos ruminantes, sendo os ovinos os mais suscetíveis (Raadsma & Egerton, 2013). Esta doença corresponde à principal causa de claudicação em ovinos (Winter *et al*., 2015), tendo por isso uma enorme relevância tanto do ponto de vista económico como de bem estar animal (Nieuwhhof & Bishop, 2005). Já foram reportados casos clínicos de peeira ovina em grande parte dos países que se dedicam à produção de ovinos (Raadsma & Dhungyel, 2013).

Existem várias bactérias que podem interferir na patogenia da doença, mas o agente etiológico corresponde à bactéria anaeróbia *Dichelobacter nodosus* (Allworth, 2014). O início da infeção carateriza-se pelo aparecimento de uma lesão de dermatite interdigital, que pode evoluir nos estadios mais severos para a separação total da úngula das estruturas sensitivas (Bennett & Hickford, 2011).

Apesar de não haver dados referentes à prevalência e aos fatores de risco de peeira ovina no Alentejo, é opinião geral dos médicos veterinários e produtores que esta tem um impacto económico bastante relevante nas explorações de ovinos. Assim, os objetivos deste estudo foram: i) estimar a prevalência de peeira em explorações de ovinos na região Alentejo; ii) identificar os fatores de risco associados à existência da doença nesta área geográfica.

II - METODOLOGIA

A metodologia utilizada baseou-se na elaboração de inquéritos epidemiológicos, que foram realizados a produtores de ovinos da região Alentejo – dividida em quatro sub-regiões. O objetivo destes inquéritos centrou-se na caraterização das explorações de ovinos que têm, e que não têm, casos clínicos de peeira. A totalidade das questões envolveu uma resposta fechada ou semifechada. A aplicação dos questionários foi feita por entrevista oral aos proprietários de explorações de ovinos, tendo as explorações sido selecionadas de forma aleatória e sendo representativas de todas as OPP's do Alentejo. A recolha de respostas decorreu entre outubro de 2016 e dezembro de 2017.

As respostas foram registadas no programa IBM SPSS Statistics (version 24), procedendo-se então à sua análise. As variáveis quantitativas foram avaliadas através do teste F da ANOVA, enquanto nas variáveis qualitativas foi utilizado o teste Qui-quadrado. Foram considerados três níveis distintos de significância (95, 99 e 99,9 %). As prevalências de peeira ovina nas explorações do Alentejo foram calculadas com base nas respostas afirmativas dos produtores à presença de peeira nas suas explorações.

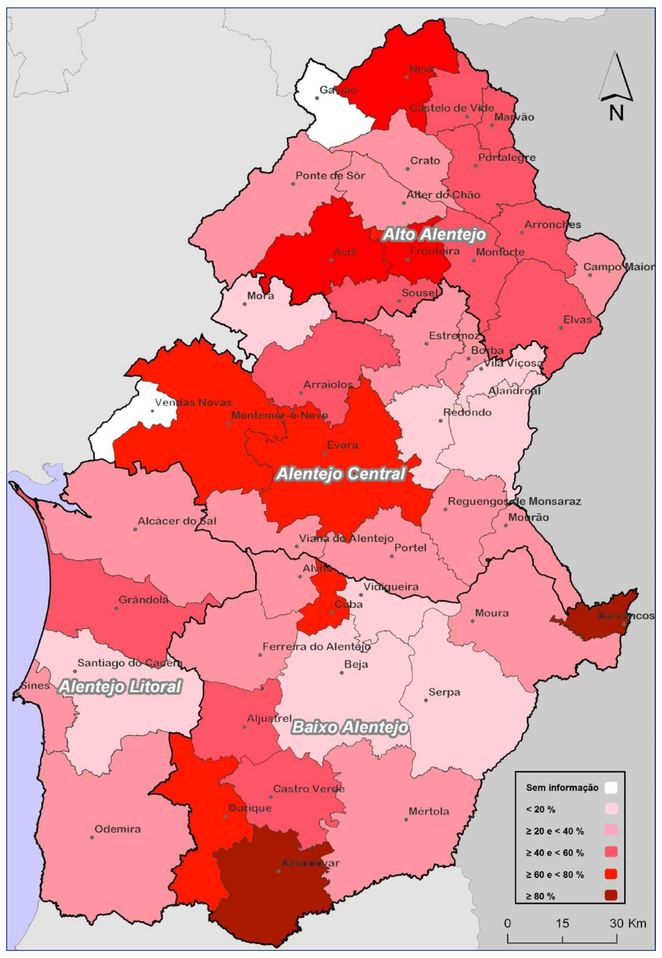

Figura 1: Prevalências de peeira ovina por concelho do Alentejo

Tabela 1: Fatores de risco para a peeira ovina em explorações do Alentejo

Variável	Amostra	p-value	OR e IC a 95%
Área (hectares)	≥ 100	<0,001	2,44
	< 100		[1,73; 3,47]
Nº ovinos	≥ 150	<0,001	3,17
	< 150		[2,23; 4,53]
Épocas de parto	Concentrada	<0,001	2,07
	Todo o ano		[1,47; 2,91]
Montado	Presente	<0,001	2,71
	Ausente		[1,71; 4,28]
Estabulação dos animais	Sim	<0,01	1,66
	Não		[1,15; 2,39]
Drenagem dos solos	Fraca	<0,01	3,75
	Boa / Média		[1,56; 8,99]
Participação em feiras	Sim	<0,05	1,86
	Não		[1,07; 3,24]

III - RESULTADOS

Foram obtidos 607 inquéritos válidos, 227 (37,4%) explorações localizam-se no Baixo Alentejo, 163 (26,9%) pertencem ao Alentejo Central, 125 (20,6%) referem-se ao Alto Alentejo e 92 (15,2%) situam-se no Alentejo Litoral. A prevalência estimada de peeira para a totalidade das explorações cifrou-se em 34,6%. O Alto Alentejo foi a sub-região que apresentou uma maior prevalência (46,4%), seguindo-se o Alentejo Central (38%), o Baixo Alentejo (29,5%) e o Alentejo Litoral (25%). Na figura 1 é possível observar a distribuição geográfica, por concelho, das prevalências estimadas de peeira. Os dois concelhos que apresentam um maior índice de prevalência são Almodôvar (88,9%) e Barrancos (80%).

Relativamente à identificação de fatores de risco, constatou-se que as seguintes caraterísticas estavam associadas a uma maior probabilidade de a exploração ter peeira:

- Maior área de exploração
- Maior dimensão do efetivo
- Concentração das épocas de cobrição / partos
- Presença de áreas com Montado
- Estabulação dos animais
- Fraca capacidade de drenagem dos solos
- Participação em feiras e mercados

A tabela 1 retrata estes fatores de risco, bem como o nível de significância e *odds-ratio* (OR) que lhes estão associados.

IV – DISCUSSÃO E CONCLUSÃO

Existem diferenças estatisticamente significativas entre as prevalências estimadas nas sub-regiões do Alentejo (p<0,01), sendo que os concelhos localizados no Alto Alentejo e Alentejo Central apresentam prevalências de peeira tendencialmente superiores. As principais exceções são os concelhos de Barrancos e Almodôvar, localizados no Baixo Alentejo e que apresentam as prevalências mais elevadas.

Os fatores de risco identificados estão de acordo com os trabalhos de Green & George (2008) e de Angell *et al*. (2018), uma vez que essas condições estão associadas a um aumento da densidade populacional, que consequentemente leva a um aumento da carga de *D. nodosus* no ambiente, favorecendo a disseminação da doença. A fraca capacidade de drenagem dos solos favorece a maceração do estrato córneo da úngula, facilitando a penetração do agente na pele (Bennett & Hickford, 2011). Os valores de OR calculados mostram que a presença de cada um dos fatores de risco determinados neste estudo, de forma isolada, está associada a um incremento na probabilidade de a exploração ter peeira entre duas e quatro vezes.

Contrariamente ao descrito por Bennett & Hickford (2011), não foi possível determinar os fatores climatéricos (pluviosidade, temperatura e humidade) como fatores de risco, possivelmente porque os dados meteorológicos usados (outubro de 2016 a maio de 2017) corresponderam a uma época de seca, pouco favorável ao desenvolvimento de peeira. Está descrita uma predisposição racial para o desenvolvimento da doença (raça merina é mais suscetível) (Emery *et al*., 1984), mas nos nossos resultados não foi possível confirmar essa evidência uma vez que grande parte das explorações têm animais de raça cruzada.

V - AGRADECIMENTOS

Este estudo engloba-se no projeto GEN-RES-ALENTEJO (ALT 20-03-0145-FEDER-000037) – "Utilização de Metodologias Genómicas na Seleção de Ovinos resistentes à Peeira e a Parasitas Gastrointestinais na região do Alentejo". Este Projeto é financiado pelo Fundo Europeu para o desenvolvimento regional e pelos programas Alentejo 2020 e Portugal 2020. Agradece-se também a todos os médicos veterinários e produtores que se disponibilizaram a divulgar e responder aos inquéritos.

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AVALIAÇÃO DA RESISTÊNCIA E DO EFEITO DO PARASITISMO GASTRO-INTESTINAL NAS RAÇAS MERINA BRANCA E MERINA PRETA NO ALENTEJO, PORTUGAL

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Introdução

A infeção por estrongilídeos gastrointestinais tem-se revelado como um dos fatores com maior impacto económico na produção de ovinos. Esse impacto manifesta-se tanto de uma forma direta (tratamento, profilaxia, morte) como de forma indireta (atraso no crescimento, quebras na produção, maior suscetibilidade a outras doenças). O controlo parasitário com base no uso exclusivo de antihelmínticos não se tem revelado uma estratégia sustentável, resultando num incremento da resistência por parte das populações parasitárias. De modo a contrariar esta tendência, tem-se dado particular atenção à relação parasita/hospedeiro, sendo a identificação de animais que revelam menor suscetibilidade à infeção parasitária um dos principais objetivos. A raça tem-se revelado como um fator importante na resistência dos estrongilídeos gastrointestinais, particularmente em raças autóctones comparativamente a raças exóticas.

Material e Métodos

Explorações amostradas	Total de animais amostrados	Animais de raça Merina Branca	Animais de raça Merina Preta
3	435	241	194

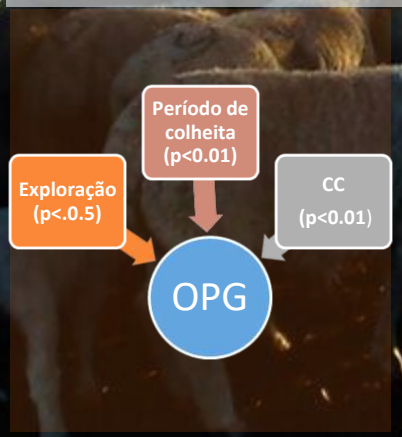
Animais avaliados em duas visitas, com um intervalo igual ou superior a 3 meses. Amostras de fezes colhidas da ampola rectal e analisadas segundo o método de McMaster modificado (Hammond & Sewell, 1978).

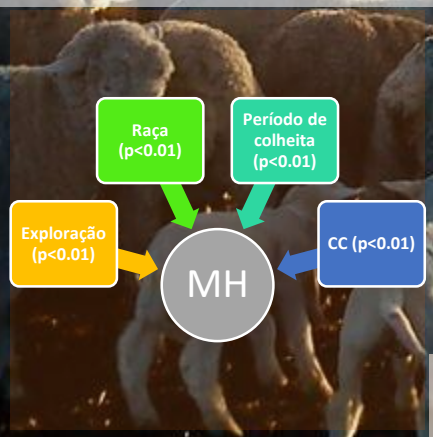
Parâmetros			
Condição corporal (CC)	Ovos por grama de fezes (OPG)	Microhematócrito (MH)	Proteínas totais (PT)

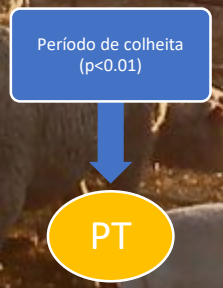
Os dados foram submetidos a análises preliminares, através do PROC MEANS e do PROC FREQ do programa do SAS (SAS Institute, 2017) e, posteriormente, analisados individualmente com um modelo misto, através do PROC MIXED do programa SAS.

Resultados e Discussão

Apesar dos ovinos de raça Merina Preta apresentarem valores médios de OPG mais elevados, o fator raça não se revelou determinante nos níveis de eliminação, mas exerce influência significativa (p<.01) nos valores obtidos para MH, com níveis mais elevados na raça Merina Branca.







Colheita de fezes

Colheita de sangue

Câmara McMaster para determinação de OPG

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The effect of rotational grazing on sheep and grassland performance

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The competitive advantage in ruminant livestock production hinges on maximising the contribution of grazed grass in the diet. Grass, our cheapest feed resource, can supply up to 95% of the energy requirements of sheep; thus, the efficient utilisation of herbage in lamb production systems is the key to profitability. Currently, there are inefficiencies in the level of herbage utilised/ha within sheep farms in Northern Ireland. The objective of this study was to examine the effect of 4 vs 8 paddock rotational grazing systems on animal and grassland performance. The study was replicated over two grazing seasons from April to November 2018 and from March to November 2019, at the Agri-Food and Bioscience Institute. The area used for the study consisted of a predominantly perennial ryegrass sward. There were two grazing treatments (4 vs 8 paddock rotational grazing system) which were balanced for ewe live weight, body condition score and lamb sire breed. Each system consisted of 1.6 ha which were rotationally grazed at a stocking rate of 14 ewes ha-1. Lambs were weighed fortnightly from 6 weeks of age using portable electronic scales and were drafted for slaughter on reaching 45 kg of live weight. Lambs were weaned on average at 14 weeks of age. Pre- and post-grazing compressed sward heights were determined on each paddock before and after grazing by taking 30 measurements across the diagonal of the paddock with a rising plate meter. Pre- and post-grazing herbage mass was determined for each paddock by taking four quadrat (0.5×0.5 m) cuts. All harvested herbage was weighed and a sub-sample was retained for DM and quality analysis. Data was analysed using linear mixed models, with ewe as a random effect and lamb sire breed, gender and deviation in lamb age from the treatment mean included as fixed effects. Lambs grazing the 4-paddock system had higher average daily gains from 10 weeks of age to weaning ($P<0.001$) compared to those grazing the 8-paddock system, which resulted in higher weaning weights ($P<0.001$) for the 4-paddock lambs. In conclusion, the 8-paddock rotational grazing system produced higher levels of herbage utilisation but resulted in lower lamb performance.

Genomic characterisation of Portuguese native sheep breeds

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Merino, Campaniça and Serra da Estrela sheep are among the most relevant native breeds reared in Portugal under extensive conditions. Merino and Campaniça are mainly distributed in the south of Portugal, in the Alentejo region, being the basis for the production of certified meat, dairy and wool products. Serra da Estrela is the main Portuguese dairy breed, producing a high-value certified cheese, and is raised in the mountain region that its name originates from. These breeds are classified as threatened genetic resources despite their importance. The purpose of this study was to assess the dynamics of population genomics in these sheep breeds using whole-genome resequencing. Blood samples were collected from 56 sheep across these breeds and a population of crossbred Merino sheep. Following DNA extraction and resequencing, the raw data were filtered by quality and used for variant calling. A total of 31,320,381 high-quality SNPs were kept for downstream analysis. Among these, 11,148,321 SNPs were located in genic regions of which 120,172 were annotated as synonymous and 80,882 as non-synonymous. The remaining 20,172,060 SNPs were identified in intergenic regions. These data were used to determine the genomic diversity and population structure of Portuguese native breeds in the context of worldwide sheep genomic variation. Structural variation was also characterised, yielding a total of 340,188 variants after filtering and 4,197 variants annotated in exonic regions. These variants were used to evaluate the patterns of copy number variation in sheep populations. The results derived from this study will be used to develop a genotyping assay specific for more diverse traditional sheep breeds, including markers useful for genome-wide association studies and traceability analysis.

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Genomic characterization of Portuguese native sheep breeds

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OVERVIEW

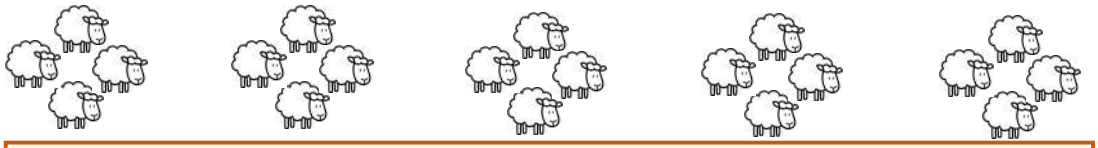
Aim: Assess the dynamics of population genomics in Portuguese sheep breeds using whole genome re-sequencing

- Merino and Campaniça are mainly distributed in Alentejo, being the basis for the production of certified products
- Serra da Estrela is the main Portuguese dairy breed, producing a high-value certified cheese
- Genetic variation of portuguese sheep breeds is needed, but has not yet been done

MATERIAL AND METHODS

Sampling and Laboratorial Procedures

Campaniça	Merino Branco	Merino Cruzado	Merino Preto	Serra da Estrela
N=6	N=10	N=19	N=10	N=11



DNA extraction

Whole Genome Re-Sequencing

BGISEQ-500 sequencing Platform (PE 2x100)

Bioinformatics analysis

Pre-processing (Trimmomatic)

Mapping (BWA-MEM)
Oar_rambouillet_v1.0 reference genome

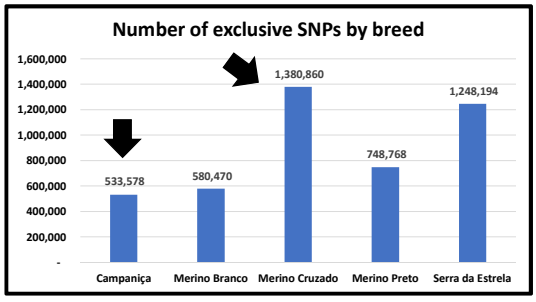
SNP calling (Freebayes)

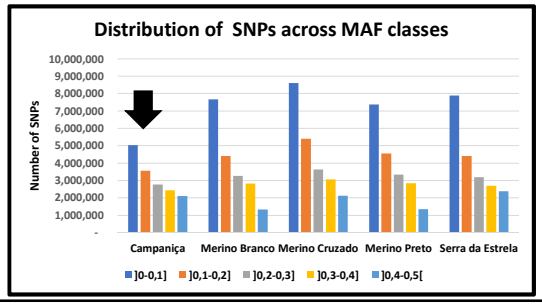
SV calling (GRIDSS)

RESULTS

SNP calling

- The highest number of SNPs was identified in Merino Cruzado (23,325,418 SNPs), of which ~5.9% were breed-specific
- The lowest number of SNPs was found in Campaniça (17,622,897 SNPs), of which ~3% were breed-specific
- High proportion of SNPs with low degree of polymorphism in all breeds (SNPs with MAF≤0.1)
- Higher degree of polymorphism in Campaniça, when comparing with the other breeds

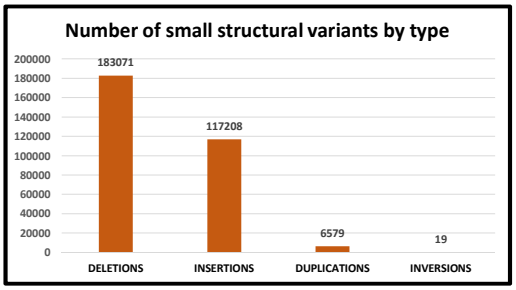


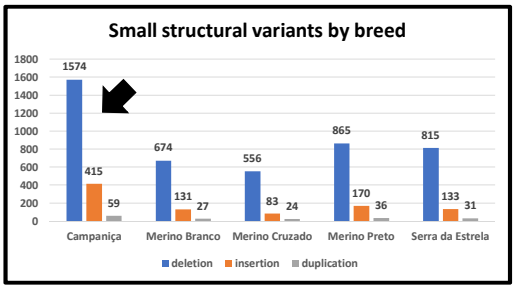


RESULTS

SV calling

- Small SVs covered a total of 3.7Mb (~0.1% of sheep reference genome)
- Significant percentage of low VAF (≤0.1) variants in all breeds, which indicates a high level of homozygosity
- The highest number of small SVs with VAF≥0.4 was identified in Campaniça (2,048)





CONCLUSIONS AND FUTURE WORK

- This study represents the first major characterization of Portuguese sheep breeds at the genome level
- A total of 31,320,380 SNPs and 306,877 small SVs were found
- Further studies are needed to identify possible phenotypic influence of the identified variants

AKNOWLEGMENTS

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ABSTRACTS

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cial Palmero cheeses were analyzed. PLINK v. 1.90 software was used to calculate MAF values and the package Adegenet for the R software and fastStructure were used for inferring population structure. The results show that this panel is useful to distinguish the cheeses prepared with a 100% of milk of Palmera, 100% of Tinerfeña and 100% Majorera with assignment coefficients of 0.9962, 0.9999 and 0.9999 respectively. However other proportions of milks are not so clearly differentiated because it is difficult to discriminate mixtures of Tinerfeña and Majorera breeds. Anyway, this panel is highly efficient for identifying variable proportions of Palmera milk in all the experimental cheeses. In conclusion, the panel of 3,385 SNPs designed is a powerful and objective tool to detect milk from other genetically related goat breeds such us Majorera and Tinerfeña. The systematic analysis of milk or cheese with this set of markers can be used by the Palmero Cheese Denomination of Origin Regulating Council to ensure the quality and the authenticity of this product. This study was funded by the RTA2014–00047–00–00 Project (INIA).

Key Words: Palmera goat, quality, Majorera

P410 Extended haplotype homozygosity analysis reveals positive selection patters in 6 Spanish goat breeds. T. E. Ziegler[1,2], A. Molina[3], G. Anaya[3], and S. Demyda-Peyrás*[2,4], [1]*IGEVET, Instituto de Genética Veterinaria, La Plata, Buenos Aires, Argentina,* [2]*FCV-UNLP, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina,* [3]*Departamento de Genética, Universidad de Córdoba, Córdoba, Spain,* [4]*CONICET, Consejo Superior de Investigaciones Científicas y Tecnológicas, La Plata, Buenos Aires, Argentina.*

Goats are a major livestock resource in Spain. Their adaptability and resilience in adverse environments and their increased productivity even in intensive schemes make them valuable livestock resources. For this reason, several local breeds were developed, focused on meat or dairy production during the last decades. In this study, we determined the presence of selection fingerprints in dairy (n = 2) and meat (n = 3) Spanish goat breeds, by estimating the integrated haplotype score (iHS) of the extended haplotype homozygosity (EHH) analysis using SNP array information. Samples from178 Spanish individuals including 46 Malagueña (MLG), 43 Florida (FLO) and 25 Murciano-Granadina (MUR) dairy goats, and 24 Bermeya (BER), 20 Mallorquina (MLL) y 20 Blanca de la Rasquera (RAS) were genotyped using the Illumina GoatSNP50 BeadChip (55,000 markers). Data were pruned by LD and MAF using PLINK and analyzed (per breed) using the *REHH* package of the statistical environment R. Finally, candidate regions (selection sweeps, SS) were selected based on the iHS P-value with a minimum length of 1Mb. Results showed a clear and distinctive iHS peak in the CHI12, despite their productive ability. This selective signature, located in a chromosome previously associated with adaptability, could be related to a genetic ability to cope with the Spanish environment, in which all these breeds were bred during the last 50 years, characterized as harsh and with low levels of forage lands. In addition, meat breeds showed an increased number of selective sweeps but more diffuses than dairy breeds (19 vs 11 on average). In particular, the less selected breeds (RAS and MLL) showed more than 20 small selective sweeps located in 15 different chromosomes, whereas the most selected breed (FLO) showed only 8 candidate regions located in 5 different chromosomes, including a clear peak in CHI6. Overall, we demonstrated that iHS could be an interesting tool to analyze differences in adaptability and selection process in Spanish goats. Further research, including functional analysis of the regions detected, is necessary to obtain more precise conclusions.

Key Words: goat and related species, population genomics, breed diversity, homozygosity

P411 Unveiling genomic regions that underlie footrot resistance in Portuguese sheep Merino. D. Gaspar*[1,2], A. Usié[1,3], C. Leão[3,4], C. Matos[5], L. Padre[3], C. Dias[3], C. Ginja[2], and A. M. Ramos[1,3], [1]*CEBAL – Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo, Beja, Portugal,* [2]*CIBIO/InBIO – Research Centre in Biodiversity and Genetic Resources, University of Porto, Vairão, Porto, Portugal,* [3]*MED-Mediterranean Institute for Agriculture, Environment and Development, University of Évora, Évora, Portugal,* [4]*INIAV – Instituto Nacional de Investigação Agrária e Veterinária, Santarém, Portugal,* [5]*ACOS – Agricultores do Sul, Beja, Portugal.*

Footrot is an acute necrotic and highly contagious disease, caused by a co-infection of 2 g-negative anaerobic bacteria, *Dichelobacter nodosus* and *Fusobacterium necrophorum*. It affects the interdigital skin and hooves of sheep, being the main cause of lameness and a major animal welfare and economical concern for the wool, milk and meat sheep industries worldwide. Current effective strategies to control footrot are costly and rely on the use of antibiotics, which could result in the development of parasite resistance mechanisms in the long term. The development of genomic markers associated with footrot resistance can provide a more reliable strategy for classifying and selecting sheep with increased resistance, besides enhancing our understanding of the biology of this disease. We aimed to identify genomic regions and molecular mechanisms associated with resistance to footrot in Portuguese native Merino breeds. For this, a set of 50k single nucleotide polymorphisms (SNPs) was specifically designed based on whole-genome data obtained for 39 sheep (depth of coverage >22X). A total of 1,466 Portuguese Merino sheep were genotyped using this SNP array. Genome-wide association analysis was performed using a quantitative trait approach based on the modified Egerton system (scores from 0 to 5) for foot integrity and footrot lesions. Genome-wide significance was determined using corrected P-values for multiple testing and SNPs significantly associated with footrot resistance were filtered at a genome-wise false discovery rate of 5%. Our results revealed a set of promising SNPs associated with resistance to footrot that overlaps candidate genes related to immune response and wound healing. These findings contribute to better understanding the architecture of footrot resistance in Merino sheep and to enhance the development of genomic tools to control infections. Also, the whole-genome data were used to investigate the underlying population structure of these native Iberian Merino breeds in the context of worldwide sheep, which is useful to define conservation and management programs.

Key Words: sheep, Merino, footrot, GWAS

P412 Comparative transcriptome analysis between suckling lambs with different levels of perirenal adipose tissue in the carcass. M. Alonso-García[1], A. Suárez-Vega[1], J. Mateo[2], H. Marina[1], R. Pelayo[1], C. Esteban-Blanco[1], J. J. Arranz[1], and B. Gutiérrez-Gil*[1], [1]*Departamento de Producción Animal, Facultad de Veterinaria, Universidad de León, León, León, Spain,* [2]*Departamento de Higiene y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad de León, León, León, Spain.*

Suckling lamb meat is very appreciated in the European-Mediterranean region. This meat is tender, juicy and shows a smooth texture. Suckling lamb carcass quality is positively related to the amount of perirenal adipose tissue, which is the predominant carcass internal fat depot. Quality traits of interest in this production show a strong influence of maternal effects as the lambs are fed exclusively of milk and slaughtered between 21 and 30 d of age. RNA sequencing (RNA-seq) has proven to help expand our understanding of the relationships between the transcriptome and the phenotype across different physiological, treatment, or disease conditions. The objective of the present study was to compare the perirrenal fat transcriptome between lambs with high and low percentages of perirenal fat in the carcass. For that, 18 male Spanish Assaf lambs born in the same flock and lambing season from primiparous ewes were initially considered. After birth the lambs had colostrum access for 4 to 8 h, and they were then fed ad libitum with reconstituted milk replacer powder. The animals were slaughtered when they reached the market live-weight (9–12 kg). At slaughter, perirenal adipose tissue samples were collected from each lamb for RNA extraction. After a phenotypic characterization of the carcass composition, RNA samples from the 4 lambs with the high-

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GENOME-WIDE DIVERSITY AND POPULATION STRUCTURE ANALYSIS OF FOUR PORTUGUESE NATIVE SHEEP BREEDS

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Introduction: Since their domestication, approximately 10,500 years before present, sheep accompanied humankind. In Portugal, native sheep are reared nationwide mainly in agrosilvopastoral systems. Merino Branco, Merino Preto, Campaniça and Bordaleira Serra da Estrela are among the most abundant local breeds. Merino and Campaniça are mainly raised in the Alentejo region to produce meat, dairy and wool. Bordaleira Serra da Estrela is the main Portuguese dairy breed, typically used to produce a high-value cheese with a Protected Designation of Origin. The lack of genomic studies is a major concern for the management of genetic diversity, thus the purposes of this study were to estimate genetic variation in these four Portuguese native sheep breeds and a population of crossed Merino, and describe their population structure in the context of worldwide sheep.

Material and Methods: Whole-genome resequencing data were obtained from DNA extracted from 56 blood samples [Campaniça (n=6), Bordaleira Serra da Estrela (n=11), Merino Branco (n=10), Merino Preto (n=10), Merino Cruzado (n=19)]. Clean reads were mapped to the sheep reference genome (Oar_rambouillet_v1.0). High-quality SNPs were filtered (SNP quality (≥30), minimum depth coverage per genotype (≥7) and genotype quality (≥20), no indels and only bi-allelic variants) and categorized according to the functional effects and distribution across genomic regions. Filtered SNPs were used to estimate genetic diversity and infer the population structure trough principal component analysis and Bayesian clustering methods.

Results and Conclusions: After filtering, 31,320,380 high-quality SNPs were obtained, of which 30,707,281 were located within intergenic (65.2%), intronic (33.4%) and exonic

(0.7%) regions. Additionally, 120,172 (57.2%) and 80,882 (38.5%) SNPs found in coding regions were associated to synonymous and nonsynonymous effects, respectively. Population structure analysis separated these breeds in two clusters: one comprising Campaniça and Serra da Estrela together with transboundary dairy breeds (e.g. Leccese and Lacaune); and another of the well-differentiated multi-purpose Portuguese Merino sheep.

Keywords: Native sheep, Whole-Genome Resequencing; Genetic diversity; Population structure

Acknowledgements: This work was co-financed by Program Alentejo 2020, through the European Fund for Regional Development under the scope "Gen-Res-Alentejo – Use of genomics methodologies to assist selection of sheep resistant to footroot and gastrointestinal nematodes in the Alentejo region" (ALT20-03-0145-FEDER-000037).

The authors also acknowledge FCT for the contract grant 2020.02754.CEECIN (CG), for the PhD fellowship SFRH/BD/140168/2018 (DG), and for UIDB/05183/2020 (AU).

Genome-wide diversity and population structure analysis of four Portuguese native sheep breeds

D. Gaspar[1,2,3], A. Usié[1,4], H. Magalhães[1], C. Leão[1], C. Matos[5], A.M. Ramos[1,4], C. Ginja[2,3]

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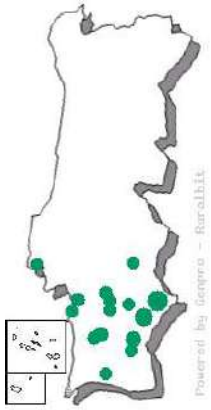
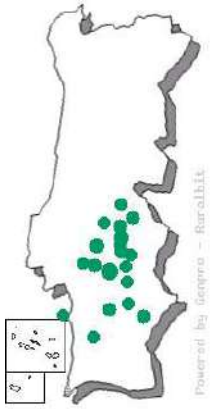
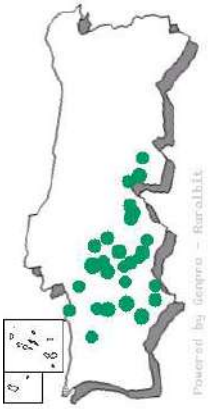
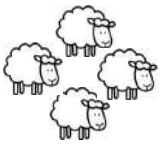
Portuguese native sheep breeds

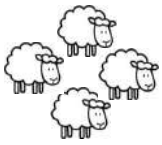
Campaniça	Bordaleira Serra da Estrela	Merino Branco	Merino Preto
			
Meat and wool	Milk	Meat and wool	Meat and wool
			

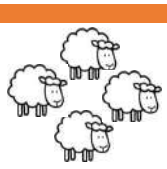
Material and Methods


Merino Branco
(N=10)


Merino Preto
(N=10)


Merino Cruzado
(N=19)


Campaniça
(N=6)


Bordaleira Serra da Estrela
(N=11)

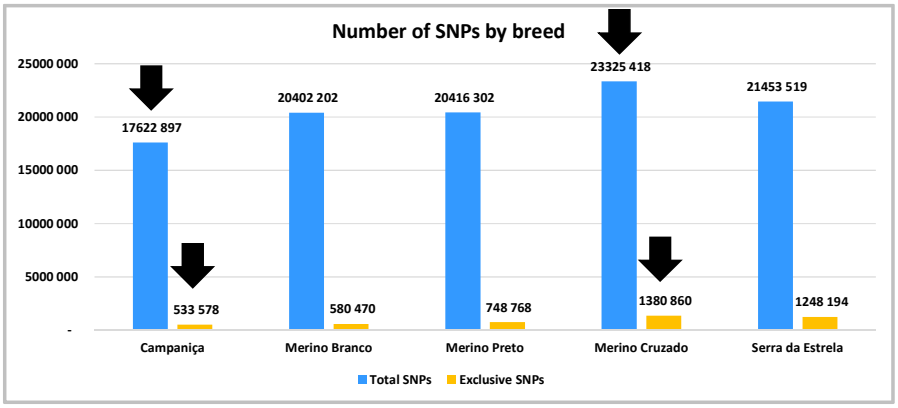
Whole Genome Re-Sequencing


BGISEQ-500 (PE 2x100)

Pre-processing (Trimmomatic) → **Mapping** (BWA-MEM) Oar_rambouillet_v1.0 → **SNP calling** (Freebayes) → **Population Genomics**

Results – Genetic diversity

SNP calling metrics



Distribution of SNPs by functional class

Region	Number of SNPs	Percentage of SNPs
Exonic	209,884	0,7
Intergenic	20,172,060	65,2
Intronic	10,325,337	33,4
UTR5	51,111	0,2
UTR3	173,004	0,6

Distribution of exonic SNPs by effect

SNP effect	Number of SNPs	Percentage of SNPs
Nonsynonymous SNV	80,882	38,5
Stopgain	880	0,4
Stoploss	111	0,1
Synonymous SNV	120,172	57,2

Results – Genetic diversity

Genetic diversity parameters

Breed	Nucleotide Diversity (π)	Expected Heterozygosity (He)	Observed Heterozygosity (Ho)	Inbreeding Coefficient (F_{IS})
CAM	0.0020	0.3521	0.3390	**0.0371**
MB	0.0020	0.3040	0.3005	0.0116
MC	0.0019	0.3112	0.3032	0.0258
MP	0.0020	0.3081	0.3065	**0.0053**
SE	0.0019	0.3484	0.3407	0.0221

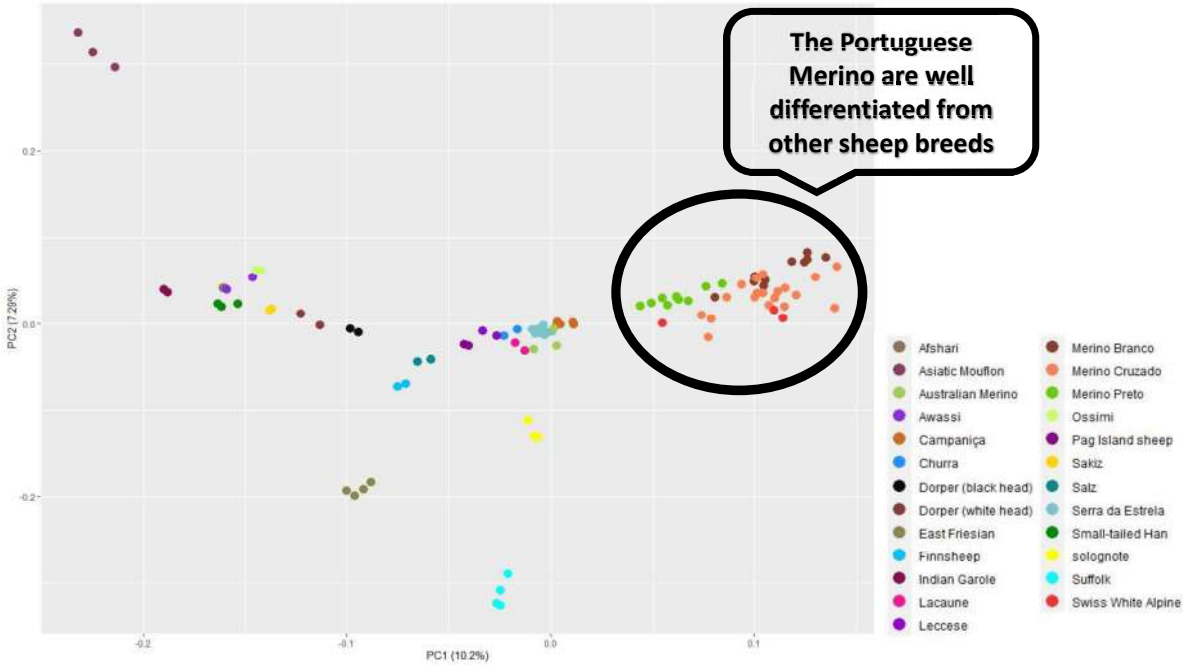
CAM – Campaniça; MB – Merino Branco; MC – Merino Cruzado; MP – Merino Preto; SE – Bordaleira Serra da Estrela

Fixation Index (Weir and Cockerham mean F_{ST} estimate)

Breed	CAM	MB	MC	MP	SE
CAM					
MB	**0.0372**				
MC	0.0347	**0.0054**			
MP	0.0355	0.0211	0.0204		
SE	0.0277	0.0288	0.0266	0.0271	

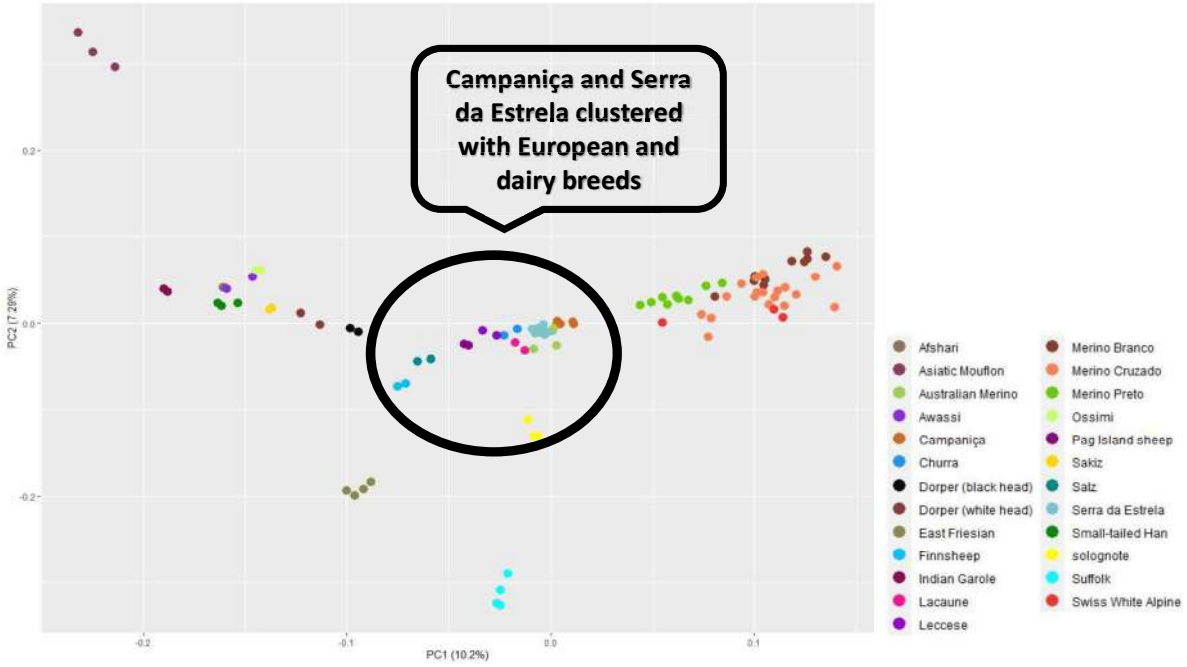
Results – Genetic structure

Principal component analysis of Portuguese Native breeds and worldwide sheep breeds



Results – Genetic structure

Principal component analysis of Portuguese Native breeds and worldwide sheep breeds



Conclusions

- **This study represents the first major characterization of Portuguese sheep breeds at the genome level**
- **Low levels of genetic diversity were identified in all breeds**
- **While Merino breeds are well differentiated from worldwide sheep breeds, Campaniça and Bordaleira Serra da Estrela clustered with European and dairy breeds**
- **Further studies are needed to identify possible phenotypic influence of the identified variants**

Aknowledgements

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Research group:




Partners:

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CIBIO Research Center in Biodiversity and Genetic Resources

InBIO Biodiversity and Evolutionary Biology

ACOS Agricultores do Sul

BIOdiv Doctoral Programme

U.PORTO FACULDADE DE CIÊNCIAS UNIVERSIDADE DO PORTO

Funding:

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Unravelling the genetic diversity and population structure of four Portuguese native sheep breeds

D. Gaspar[1,2,3], A. Usié[1,4], H. Magalhães[1], C. Leão[1], C. Matos[5], A.M. Ramos[1,4], C. Ginja[2,3]

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Since their domestication, approximately 10,500 years before present, sheep accompanied humankind. In Portugal, native sheep are reared nationwide mainly in agrosilvopastoral systems. Merino Branco, Merino Preto, Campaniça and Bordaleira Serra da Estrela are among the most abundant local breeds. Merino and Campaniça are mainly raised in the Alentejo region to produce meat, dairy and wool. Bordaleira Serra da Estrela is the main Portuguese dairy breed, typically used to produce a high-value cheese with a Protected Designation of Origin. The lack of genomic studies is a major concern for the management of genetic diversity, thus the purposes of this study were to estimate genetic variation in these four Portuguese native sheep breeds and a population of crossed Merino, and describe their population structure in the context of worldwide sheep.

Whole-genome resequencing data were obtained from DNA extracted from 56 blood samples [Campaniça (n=6), Bordaleira Serra da Estrela (n=11), Merino Branco (n=10), Merino Preto (n=10), Merino Cruzado (n=19)]. Clean reads were mapped to the sheep reference genome (Oar_rambouillet_v1.0). High-quality SNPs were filtered (SNP quality (≥30), minimum depth coverage per genotype (≥7) and genotype quality (≥20), no indels and only bi-allelic variants) and categorized according to the functional effects and distribution across genomic regions. Filtered SNPs were used to estimate genetic diversity and infer the population structure trough principal component analysis and Bayesian clustering methods.

After filtering, 31,320,380 high-quality SNPs were obtained, of which 30,707,281 were located within intergenic (65.2%), intronic (33.4%) and exonic (0.7%) regions. Additionally, 120,172 (57.2%) and 80,882 (38.5%) SNPs found in coding regions were associated to synonymous and nonsynonymous effects, respectively. Population structure analysis separated these breeds in two clusters: one comprising Campaniça and Serra da Estrela together with transboundary dairy breeds (e.g. Leccese and Lacaune); and another of the well-differentiated multi-purpose Portuguese Merino sheep. Admixture analysis revealed a wide distribution of parental lineages and corroborated the PCA clustering results.

The results derived from this study will be useful to develop several genomic tools for these breeds, including genome-wide association studies, genetic diversity and traceability schemes.

Keywords: Native sheep, Whole-Genome Resequencing; Genetic diversity; Population structure

Acknowledgements: This work was co-financed by Program Alentejo 2020, through the European Fund for Regional Development under the scope “Gen-Res-Alentejo – Use of genomics methodologies to assist selection of sheep resistant to footroot and gastrointestinal nematodes in the Alentejo region” (ALT20-03-0145-FEDER-000037). The authors also acknowledge FCT for the contract grant 2020.02754.CEECIN (CG), for the PhD fellowship SFRH/BD/140168/2018 (DG), and for UIDB/05183/2020 (AU).

Unravelling the genetic diversity and population structure of four Portuguese native sheep breeds

D. Gaspar[1,2], A. Usié[1,4], H. Magalhães[1], C. Leão[1,4], C. Matos[5], A.M. Ramos[1,4], C. Ginja[2,3]

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Introduction

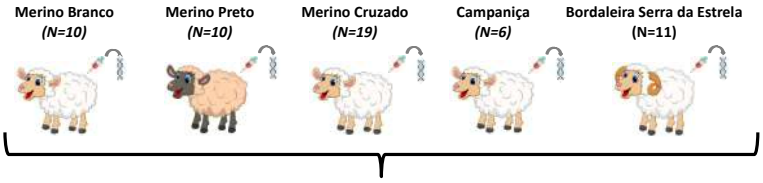
Since their domestication, approximately 10,500 years before present, sheep (*Ovies aris*) have accompanied humankind in all its history. In Portugal, native sheep are reared nationwide mainly in agro-silvopastoral systems. Merino Branco, Merino Preto, Campaniça (CAM) and Bordaleira Serra da Estrela (SE) are among the most abundant local breeds. Merino and CAM are mainly raised throughout the Alentejo region to produce meat, dairy and wool. SE is the main Portuguese dairy breed, typically used to produce a high-value cheese with a Protected Designation of Origin. Despite their role in landscape conservation and socio-economic development of rural communities, there is a lack of genomic studies for these breeds, which is a major concern for the management of genetic diversity. Thus, WGRS data was used to characterize genetic variation and investigate their population structure in the context of worldwide sheep.

OBJECTIVES

- **Estimate genetic variation and population structure of four Portuguese native sheep breeds and a crossbred Merino population**
- **Identify genome-wide ROH patterns to characterize the molecular basis underlying economically important traits**

Materials & Methods

Sampling collection



Whole Genome Re-Sequencing

BGISEQ-500 (PE 2x100)

Bioinformatics pipeline

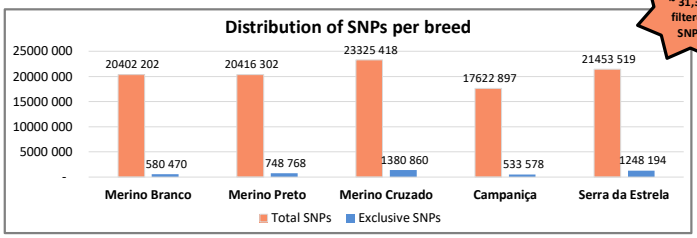
Pre-processing (Trimmomatic) → Mapping (BWA-MEM) (Oar_rambouillet_v1.0) → SNP calling (Freebayes) → Population Genomics analysis

Results & Discussion

SNP calling statistics

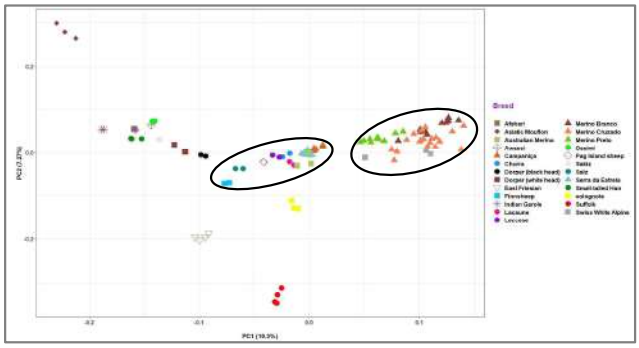
- While the highest number of SNPs (total and exclusive) were identified in Merino Cruzado, the lowest number were found in Campaniça

~ 31,3M filtered SNPs



Principal Component Analysis

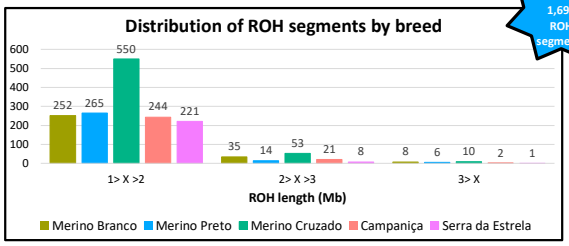
- Portuguese Merino clustered in the right-side group with Swiss White Alpine, an European Merino derived breed
- CAM and SE clustered in the central group along with European and dairy breeds



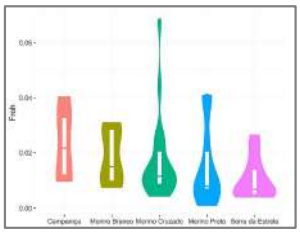
Runs of Homozygosity

- The highest number of ROH were found in **Merino Cruzado**
- High proportion of **short ROH**, which indicates **a more ancient bottleneck**

Total of 1,690 ROH segments



ROH-based inbreeding coefficient (F_{ROH}) by breed



- Largest ROH segment (**~ 4.9 Mb**) identified on **Chromosome 6**
- Highest number of ROH (**213**) identified on **Chromosome 3**
- Low levels of inbreeding in all breeds (F_{ROH}<0.02), except for Campaniça

Conclusions

- **This study represents the first major characterization of Portuguese sheep breeds at the genome level**
- **While Merino breeds are well differentiated from worldwide sheep breeds, Campaniça and Bordaleira Serra da Estrela clustered with European and dairy breeds**
- **Low levels of inbreeding derived from the proportion of ROH in genome**

Acknowledgments:
Authors gratefully acknowledge Comissão de Coordenação e Desenvolvimento Regional do Alentejo - CCDRA for the project funding Gen-Res-Alentejo – Utilização de Genómica na Seleção de ovinos resistentes a Parasitas e Peeira no Alentejo ALT20-03-0145-FEDER-000037 and Fundação para a Ciência e Tecnologia for PhD Scholarship SFRH/BD/140168/2018 (D. Gaspar) and CEEC Individual 2020.02754.CEECIND (CGinja).

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Role of different "omics" as powerful tools to face climate change and enhance breeding programs

D. Gaspar[1,2], H. Magalhães[1], B. Mendes[1], C. Leão[1,3], B. Meireles[1], P. Barbosa[1], L. Cachucho[1], A. Albuquerque[4], R. Charneca[4,5], J. Martins[4,5], E. Jerónimo[1,3], J. Matos[6], F. Simões[6], Genosuber Consortium[1,6,7,8,9], L. Marum[1,3], S. Branco[4], N. Carolino[6], C. Matos[10], C. Ginja[2], A.M. Ramos[1,3], A.Usié[1,3]

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Abstract

Climate change is the main cause of biotic and abiotic stresses affecting agriculture in different ways such as increasing temperatures, variations in rainfall, as either floods or droughts, or increasing disease incidence. In fact, it poses a major threat to species and ecosystems in Portugal and word-wide having harsh impacts on plant and animal growth, development, health and productivity. With the advent of next generation sequencing technologies, the number of sequenced genomes has increased significantly in the last decade opening new horizons in the field of genomics and transcriptomics, among other "omics". Hence, different "omics" offer new and exciting opportunities for the development of powerful strategies that can enhance the resilience of these species to climate variability accelerating their genetic characterization, a key requirement for their preservation, improvement and valorization. Understanding genetic diversity is crucial in order to identify candidate genes and genetic markers associated to agronomic traits of interest such as improved production and biotic and abiotic stress resistance. Here we present some examples, using plant and animal endogenous species, of how different "omics" approaches help to understand the molecular basis of the differences in phenotypic traits.

Role of different "omics" as powerful tools to face climate change and enhance breeding programs

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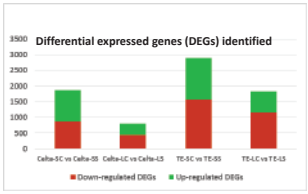
Climate change is the main cause of biotic and abiotic stresses affecting agriculture in different ways such as increasing temperatures, variations in rainfall, as either floods or droughts, or increasing disease incidence. In fact, it poses a major threat to species and ecosystems in Portugal and word-wide having harsh impacts on plant and animal growth, development, health and productivity. Different "omics" offer new and exciting opportunities for the development of powerful strategies that can enhance the resilience of these species to climate variability accelerating their genetic characterization.

Transcriptomics – RNA-Seq

Wheat, one of the world's most important crops is being threatened by climate change. Although **durum wheat** is better adapted to high temperatures and to semiarid climates, it is necessary to deeply understand how heat stress might impact wheat production and wheat biology.

Two varieties were studied: CELTA from the National Varieties Catalogue and TE 1330 from the INIAV's Cereals Genetic Breeding Program.

Leaf and stem tissues were analysed and compared:

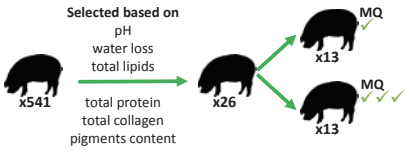


- ✓ Genes related to signaling networks and hormonal stress-specific response
- ✓ Transcription factors associated to heat stress tolerance
- ✓ Heat shock proteins
- ✓ Antioxidant defense system in response to oxidative stress

The comparative analysis between CELTA and TE 1330 transcriptome profiles suggests different molecular behaviors under heat stress

Genomics - Whole genome sequencing

A total of 541 **Alentejano pigs** were studied during the 2017 slaughter campaign. Phenotypic records for carcass and meat quality were collected and subsequently analyzed to identify the groups of animals that displayed the most contrasting phenotypes.



230 SNPs were identified with differences in allele frequency ≥ 40% between groups:

- ✓ The **SAMD4A** gene has been linked to percentage of lean meat, lipid metabolism, drip loss and feed intake. Located in QTL region associated to intramuscular fat content trait.
- ✓ The **COL23A1** gene has been reported as under selection in meat quality traits and tenderness in swine. Located in a QTL region associated to morphological body traits.
- ✓ The **COL14A1** gene has been associated with carcass, meat quality and back fat thickness.

Genomics – Genome sequencing & Genotyping

Cork oak (*Quercus suber*) trees are the main commercial source of cork, which is a renewable natural resource that has many applications due to its unique features. The availability of a **reference genome** is essential to support many of the studies needed to answer fundamental questions about cork oak biology.

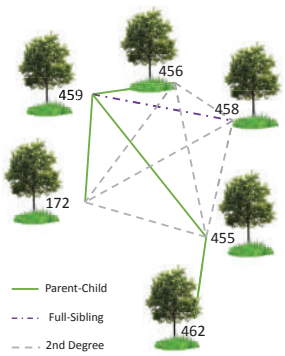
Cork oak genome size sequenced 765.7 Mb

- ✓ 2351 scaffolds
- ✓ 40,121 genes
- ✓ N50 of 1,0 Mb
- ✓ Illumina short-reads
- ✓ PacBio long reads

The measurements of the relatedness between individuals can be used to predict how a specific trait can pass through generations, producing information that can be used to improve the conservation and breeding programs of the species.

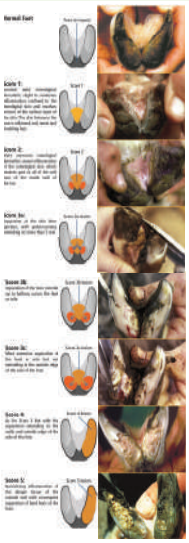
A **kinship analyses** was perform over a natural regenerating cork oak population located in the Setubal region with **494 cork oaks** sampled with **8411 SNPs genotyped**.

The **successful** identification of kinships and **establishment of some families' pedigree** indicate the potential of this approach for future studies being a **valuable tool for future management strategies of cork oak populations, including future cork oak breeding schemes**.



Genome–wide association analyses (GWAS) & Metagenomics

Ovine footrot is an acute necrotic and highly contagious disease that is caused by a gram-negative anaerobic bacterium, *Dichelobacter nodosus*. It affects the interdigital skin and hooves of sheep, being the main cause of lameness and a major animal welfare and economical concern for sheep industries worldwide.

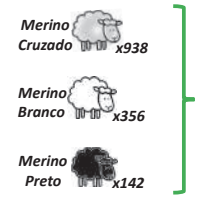


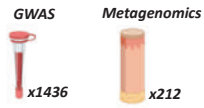
GWAS study

The identification of **genomic markers** associated with footrot resistance can provide a more reliable strategy for classifying and selecting sheep with increased resistance, besides enhancing our understanding of the biology of this disease.

Metagenomics study

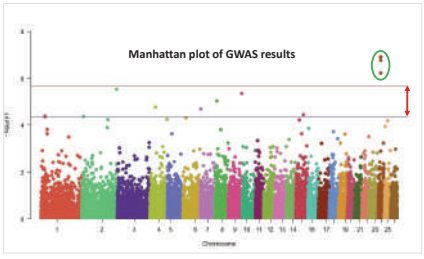
The **bacterial community** of the ovine foot is diverse being necessary to better characterize the aetiology of footrot to identify which taxa contribute to its expression.





Sampling was carried out during routine clinical diagnostics of footrot infection in 17 herds distributed throughout the Alentejo region. Phenotypes were collected as scores following the Modified Egerton System (0 to 5).

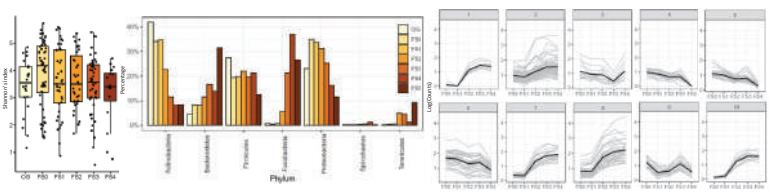
For GWAS analysis and to better adjust the impact of the injury on the animal, a weighting factor was applied to each footrot score (0,1,2,3,4 and 5) where initial scores were converted into 0, 1, 4, 7.5, 12 and 16.25 (index score),respectively



3 genome-wide **significant** SNPs found in chr 24:
- ✓ SMG1

6 genome-wide **suggestive** SNPs found in chr 2, 4, 7, 8, 9 and 15:
- ✓ CENPW
- ✓ HSPG2
- ✓ KLHL35
- ✓ PCLO
- ✓ RALYL

Most of the **SNPs identified** as genome-wide significant or suggestive were found **in genes closely related with immune response against parasite infection and wound healing.**



The **footrot microbiome** showed a **diminished diversity** as the footrot **infection aggravates** which is accompanied by the **increased abundances** of *Dichelobacter nodosus* along with other species such as *Mycoplasma fermentans*, *Fusobacterium necrophorum*, *Porphyromonas asaccharolytica*, *Ezakiella massiliensis*, *Treponema ssp.* and *Staphylococcus spp.*, *Streptococcus spp.* and *Campylobacter spp.* being **key species differentiating mild and severe footrot lesion stages**.

These works were funded through ALENT-07-0224-FEDER-001754, ALT20-03-0145-FEDER-000032, ALT20-03- 0145-FEDER-000037, ALT20–05-3559-FSE-000076, UIDB/05183/2020, LA/P/0121/2020, IF/00574/2012/CP1209/CT0001 , CEECINST/00100/2021/CP2774/CT0001, CEECINST/00131/2018, SFRH/BD/140168/2018

Workshop I

PEEIRA - DIAGNÓSTICO E PREVENÇÃO

(GEN-RES-ALENTEJO ALT20-03-0145-FEDER-000037)

1 de Março de 2019

Universidade de Évora- Pólo da Mitra
(Departamento de Medicina Veterinária)

Inscrição gratuita

COFINANCIADO

PEEIRA - DIAGNÓSTICO E PREVENÇÃO

(GEN-RES-ALENTEJO ALT20-03-0145-FEDER-000037)

PROGRAMA | 1 de março 2019

09h00 - Abertura do secretariado
09h30 - Sessão de abertura: Eng. Claudino Matos
(coordenador do projeto - ACOS)
10h00 - Diagnóstico macroscópico de lesões de Peeira
(Clara Dias, MV, bolseira GEN-RES-ALENTEJO, UE)
10h45 - Coffee break
11h15 - Factores de risco associados com a ocorrência de Peeira no Alentejo
(Pedro Caetano, MV, HVUE)
12h00 - Diagnóstico laboratorial de Peeira - Dichelobacter nodosus
(Catarina Albuquerque, bolseira Mestrado Biologia Celular INIAV)
12h45 - Almoço livre
14h30 - Avaliação prática de lesões de Peeira em ovinos
(Lino Tábuas - MV, bolseiro GEN-RES-ALENTEJO, ACOS e Pedro Caetano)
16h30 - Lanche de boas vindas

Público alvo: estudantes de MIMV, Zootecnia/CTA e Enfermagem Veterinária
Nº limite de inscrições: 20
Data limite para inscrição: 27 de Fevereiro
E-mail para inscrição: aemvue@gmail.com

Diagnóstico macroscópico de lesões de Peeira

GEN-RES-ALENTEJO ALT20-03-0145-FEDER-000037

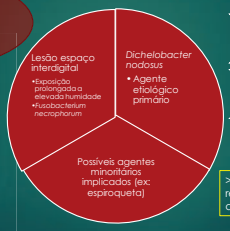
Workshop I: Peeira – Diagnóstico e Prevenção

1 de Março de 2019, Pólo da Mitra, Universidade de Évora

Clara Dias, MV, bolseira GEN-RES-ALENTEJO, UE

Etiologia

Doença infectocontagiosa que afeta a epiderme interdigital e os tecidos vivos das extremidades distais de ovinos, caprinos, entre outros.

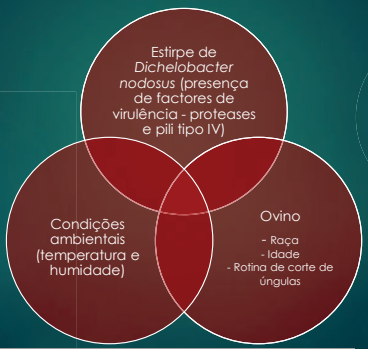


Estirpes benignas → Lesões leves

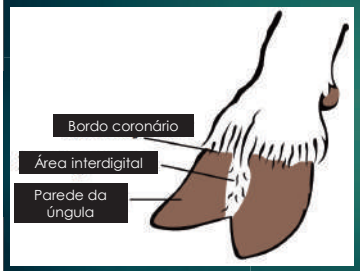
Estirpes virulentas → Lesões graves

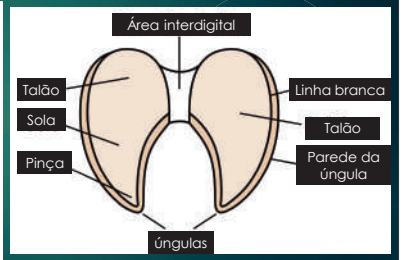
> Poder queratolítico resultante da presença de protease termoestável

Severidade das lesões



ANATOMIA DA ÚNGULA

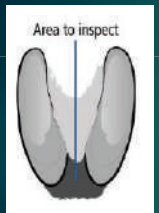




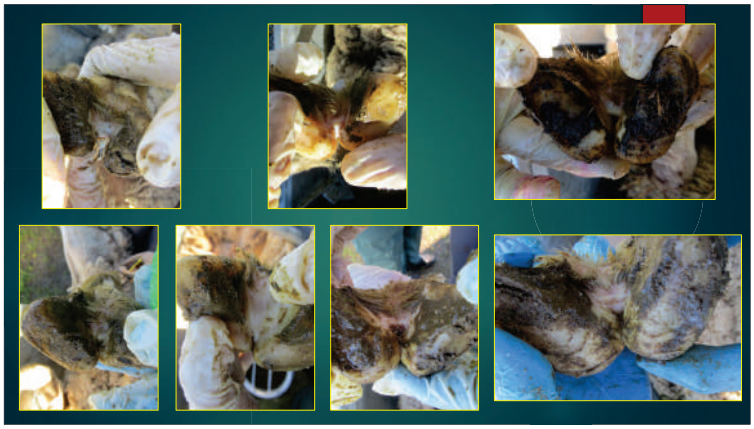
https://www.qmscotland.co.uk/sites/default/files/sheep_lameness_guide.pdf

Observar sempre os 4 membros

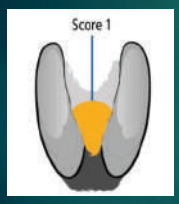
Sem lesão



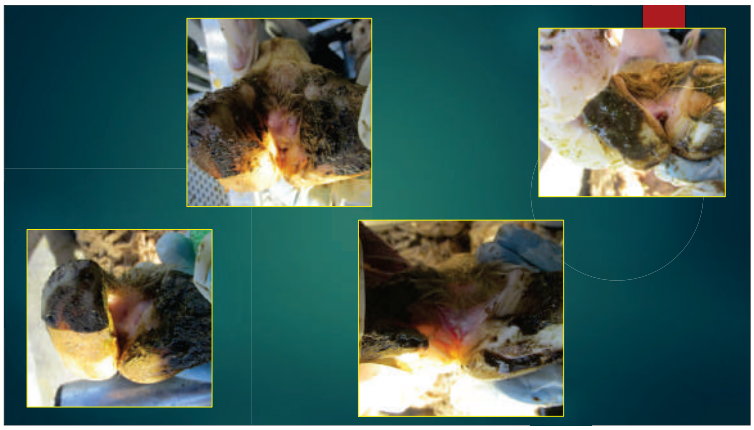
Área interdigital fisiologicamente rosada, seca e com camada de pêlo.



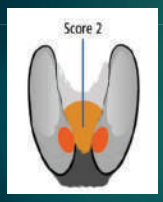
Grau I



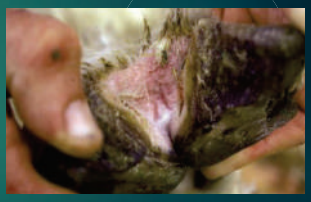
Dematite interdigital ligeira. Inflamação leve a moderada confinada à pele da área interdigital, com alopécia e erosão superficial da mesma.



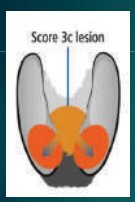
Grau II



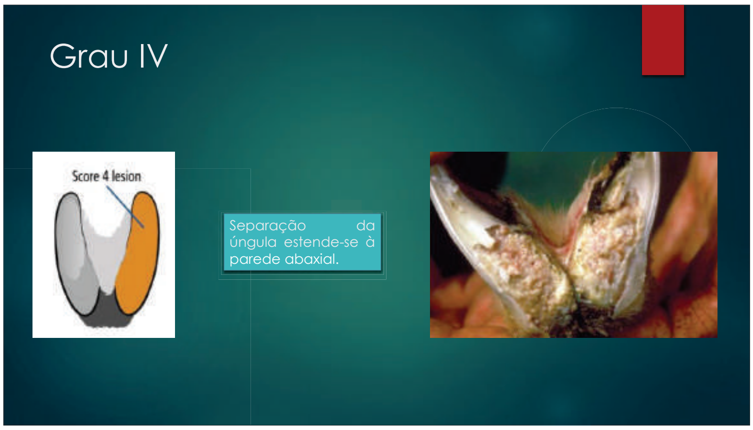
Dematite interdigital mais extensa do que no grau I. Inflamação severa da pele da área interdigital, abrangendo parte ou a totalidade do talão axial.

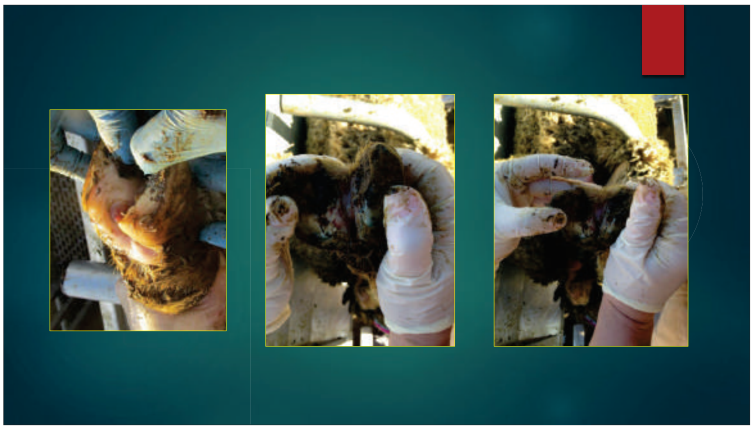


Grau III

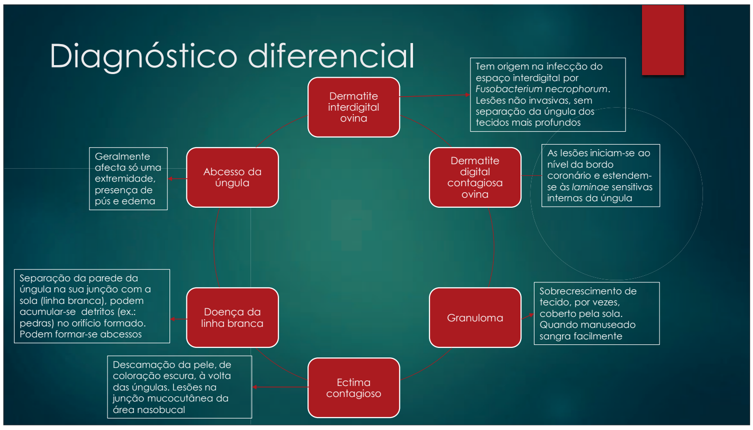


Separação da parede da úngula ao nível do bordo coronário (axial) que pode estender-se ao talão e à sola mas não à parede abaxial.


Grau IV
Score 4 lesion
Separação da úngula estende-se à parede abaxial.




Grau V
Score 5 lesion
Horny layer detachment
Necrose dos tecidos profundos da parede abaxial com consequente separação total da parede.


Diagnóstico diferencial
Dermatite interdigital ovina
Tem origem na infecção do espaço interdigital por *Fusobacterium necrophorum*. Lesões não invasivas, sem separação da úngula dos tecidos mais profundos
Dermatite digital contagiosa ovina
As lesões iniciam-se ao nível da bordo coronário e estendem-se às *laminae* sensitivas internas da úngula
Granuloma
Sobrecrescimento de tecido, por vezes, coberto pela sola. Quando manuseado sangra facilmente
Ectima contagioso
Descamação da pele, de coloração escura, à volta das úngulas. Lesões na junção mucocutânea da área nasobucal
Doença da linha branca
Separação da parede da úngula na sua junção com a sola (linha branca), podem acumular-se detritos (ex.: pedras) no orifício formado. Podem formar-se abcessos
Abcesso da úngula
Geralmente afecta só uma extremidade, presença de pús e edema


Dermatite interdigital[1]
Dermatite digital contagiosa ovina[1]
Granuloma[1]
Ectima contagiosa[2]
Doença da linha branca[1]
Abcesso da úngula[1]
1https://warwick.ac.uk/fac/sci/lifesci/research/greengroup/farmersandvets/footrotinsheep/identifycause/
2http://agriculture.vic.gov.au/agriculture/pests-diseases-and-weeds/animal-diseases/sheep/scabby-mouth-orf-a-disease-of-sheep-and-goats

BIBLIOGRAFIA

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- Mucha s., Bunger L. & Conington J., Genome-wide association study of footrot in Texel sheep, Genetics Selection Evolution (2015) 47:35, DOI 10.1186/s12711-015-0119-3
- Ware J. W. & Kluver P., Footrot Manual for Contractors, 2014

UNIVERSIDADE DE ÉVORA

Workshop
Peeira - Diagnóstico e Prevenção

Fatores de risco associados com a ocorrência de Peeira no Alentejo

Pedro Caetano

Évora, 1 de março de 2019

Introdução

- Doença bastante contagiosa, que afeta a extremidade distal dos membros dos ruminantes, sendo os ovinos os mais suscetíveis (Raadsma & Egerton, 2013)
- Descrita pela primeira vez no início do séc. XIX, no Reino Unido (Graham & Egerton, 1968)
- Enorme relevância do ponto de vista económico e de bem estar animal (Nieuwhhof & Bishop, 2005; Raadsma & Dhungyel, 2013)
- Doença já foi reportada na maioria dos países em que a produção de ovinos tem expressão na economia (Raadsma & Egerton, 2013)

Etiologia

- Doença clínica só se verifica após ocorrer colonização do espaço interdigital por determinadas bactérias (Allworth, 2014):
  - *Dichelobacter nodosus*
  - *Fusobacterium necrophorum*
  - Espiroquetas (*Treponema*)
  - Outras bactérias

Etiologia

- *D. nodosus*:
  - Verdadeiro agente etiológico da doença (Allworth, 2014)
  - Não deverá estar presente em úngulas "clinicamente saudáveis" (Atia *et al.*, 2017)
  - Bactéria *Gram* (-) e anaeróbia estrita (Raadsma & Egerton, 2013)
  - Bactéria anteriormente designada por *Fusiformis nodosus* e por *Bacteroides nodosus* (Dewhirst *et al.*, 1990; Raadsma & Egerton, 2013)
  - Tem a capacidade de se alimentar do colagénio presente nas úngulas, digerindo-as e formando lesões crípticas - Entrave à erradicação! (Green & George, 2009)

Etiologia

- *F. necrophorum*:
  - Função deste agente não está totalmente esclarecida (Bennett & Hickford, 2011)
  - *F. necrophorum* e *D. nodosus* possuem uma relação sinérgica (Bennett *et al.*, 2009)
    - Responsável por causar lesão no estrato córneo da unha, facilitando a entrada do agente primário
  - Bactéria *Gram* (-) e anaeróbia estrita (Nagajara *et al.*, 2005)
  - É um agente presente nas úngulas dos ovinos, independentemente de apresentarem ou não doença (Atia *et al.*, 2017)

Transmissão

- Ciclo inicia-se com a excreção de *D. nodosus* para o ambiente (solo), permitindo que outros animais se infetem após pisoteio de áreas infetadas (Raadsma & Egerton, 2013)
- Se determinadas condições climáticas e de pastagem estiverem reunidas, pode ocorrer transmissão do agente para animais não infetados (Abbott & Lewis, 2005)
  - Atividade da microflora presente no espaço interdigital
  - Temperatura e teor de humidade favoráveis
  - Maceração do estrato córneo das úngulas

Transmissão

- Apesar de a bactéria ser anaeróbia estrita, consegue sobreviver no meio ambiente:
  - 10 dias (Myers *et al.*, 2007)
  - 1 mês (Muzafar *et al.*, 2016)
- Pode conseguir resistir vários meses alojado em lesões cavitárias nas úngulas (Bennett & Hickford, 2011)

Expressão clínica da doença

(Green & George, 2008; Raadsma & Egerton, 2013)

Fatores de risco

- Ambiente:
  - Temperatura
    - Temperaturas ambientais baixas (< 10ºC) reduzem a transmissão da doença (Abbott & Lewis, 2005)
    - *D. nodosus* pode sobreviver no meio ambiente a 5ºC até 30 dias! (Muzafar *et al.*, 2016)
  - Pluviosidade / Humidade
    - Períodos húmidos e chuvosos (> 50 mm/m^2 mensais) maximizam a disseminação de *D. nodosus* entre os ovinos (Abbott & Lewis, 2005)
    - Chuva constante durante um período mínimo de 6 semanas é mais propícia à transmissão (Raadsma & Egerton, 2013)

Fatores de risco

- Ambiente:
  - Tipo de solo
    - Má drenagem -- Solos Argilosos
    - Pastagens alagadas / enlameadas e sujeitas a muito pisoteio ➡ Maceração do estrato córneo das úngulas
    - Solos pedregosos, fraca cobertura vegetal e pastagens com restolho grosseiro favorecem o aparecimento de feridas no espaço interdigital ➡ Porta de entrada para *D. nodosus*
    - Pastagens melhoradas ➡ Ambiente húmido

Fatores de risco

- Exploração:
  - Estabulação
    - Elevada densidade animal
    - Material utilizado para as camas (ex: palha) proporciona um micro-ambiente "quente e húmido"
    - Ambiente extremamente contaminado!!!
  - Dimensão (nº animais)
    - Maiores explorações com risco aumentado
    - Torna-se mais difícil identificar (e tratar) os casos individuais rapidamente

Fatores de risco

- Hospedeiro:
  - Raça
    - Raça Merina: maior susceptibilidade
    - "Raças Britânicas" (*Suffolk*, *Romney*...): maior resistência
  - Sexo
    - Fêmeas mais resistentes do que os machos
    - Carneiros apresentam maior percentagem de lesões e lesões mais severas

Fatores de risco

- Hospedeiro:
  - Idade
    - Animais jovens são menos suscetíveis do que os adultos
  - Afilhações
    - Ovelhas que tenham tido partos gemelares são mais vulneráveis do que aquelas que tenham tido partos simples ou que não estejam a criar nenhum borrego

Fatores de risco

- Bactéria - *D. nodosus*:
  - Virulência das estirpes
    - **Benignas**, **Intermédias** ou **Virulentas**
    - Depende da presença de certos fatores de virulência - Proteases, fímbrias, segmentos genómicos (Kennan *et al.*, 2001; Bennett & Hickford, 2011)
  - Serogrupo das estirpes
    - 10 serogrupos conhecidos – A-I e M (Chetwin *et al.*, 1991)
    - Já foram detetados até 7 diferentes no mesmo membro (Zhou & Hickford, 2000)

Tratamento / Controlo

- Diversas formas para abordar o problema ➡ escolha da melhor estratégia dependerá de:
  - Dimensão da exploração
  - Prevalência da doença
  - Taxa de reposição
  - Disponibilidade de fármacos e outros recursos
  - Legislação existente em cada país / região

(Bennett & Hickford, 2011)

- Grande parte das formas de tratamento da peeira ovina utilizadas durante a última década estão agora associadas a maiores índices de prevalência / incidência da doença !!! (Green *et al.*, 2007)

Corte corretivo de úngulas

Vantagens	Desvantagens
• Remove tecidos lesionados • Promove uma boa conformação da úngula	• Físico e demorado! •Corte excessivo causa lesão ➡ claudicação ➡ penetração bacteriana •⬆ casos de peeira após sessões de podologia • **Bastante difícil mudar a opinião de produtores e veterinários !!!**

Corte corretivo de úngulas

"*To trim or not to trim …*"

- Tema que ainda não gera consenso
- O sobrecrescimento ocorre porque existe infeção na úngula, e não o contrário!
- O importante é tratar a infeção, o que não significa que seja necessário realizar o corte corretivo

(Davies et al., 2017)

Pedilúvio

Vantagens	Desvantagens
• Impede disseminação da doença na exploração • Limita a infeção no espaço interdigital ➡ Diminui prevalência de lesões mais graves	• Permanência 10 min no pedilúvio, 1x / semana !!! • Incapaz de tratar casos peeira se utilizado de forma isolada • Produtos mais utilizados apresentam toxicidade ambiental

Antibioterapia

Vantagens	Desvantagens
• Eficaz na prevenção da disseminação da doença • Existência de poucas resistências aos AB's para as bactérias envolvidas • Taxas de cura superiores a 95% em tratamentos precoces	• Intervalos de segurança longos • Restrições ao uso de AB´s • Manter animais em ambiente seco durante 24h !!!

Opções terapêuticas

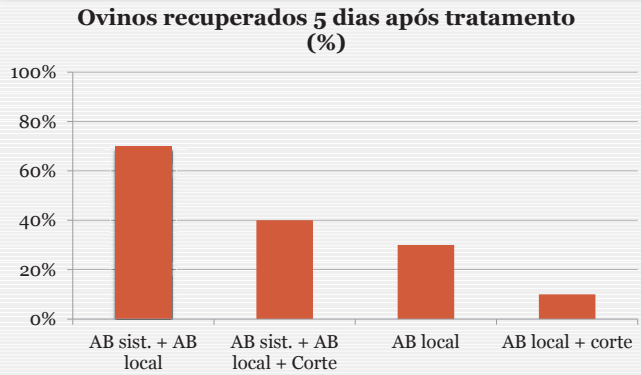


Tabela 1 - Comparação entre a eficácia de 4 protocolos de tratamento diferentes (Adaptado de Wassink & Kaler, 2010)

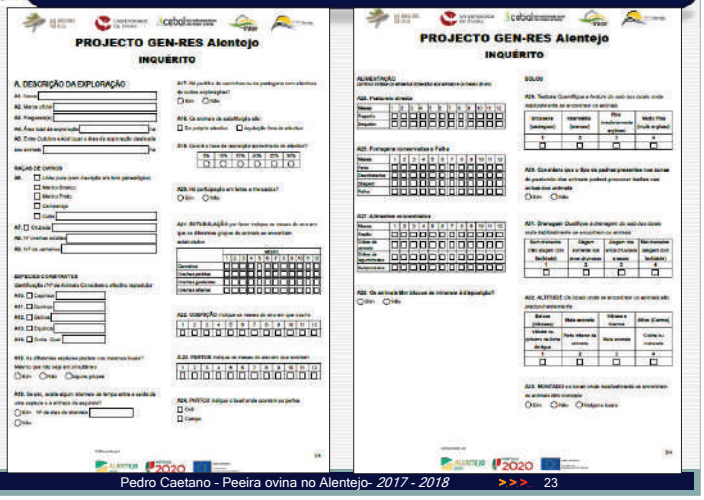
Fonte: Olifants LBA/ ACOS

Descrição do projeto

Identificação dos fatores de risco relevantes para a ocorrência de peeira em explorações de ovinos no Alentejo

- Elaboração de inquéritos para determinação de fatores de risco para a presença de peeira
- Objetivo inicial: 10% do total das explorações do Alentejo
- Respondidos pelos detentores das explorações

Inquérito epidemiológico

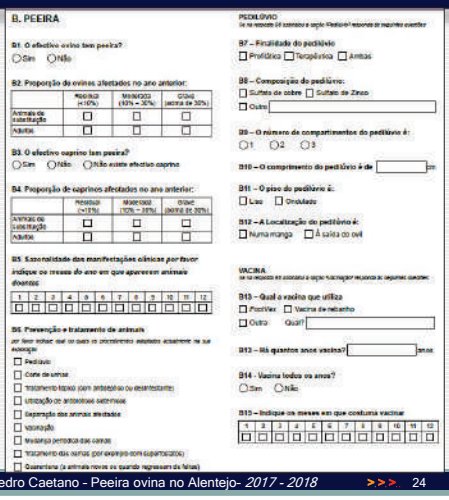


PROJECTO GEN-RES Alentejo
INQUÉRITO

A. DESCRIÇÃO DA EXPLORAÇÃO

PROJECTO GEN-RES Alentejo
INQUÉRITO

Inquérito epidemiológico



B. PEEIRA

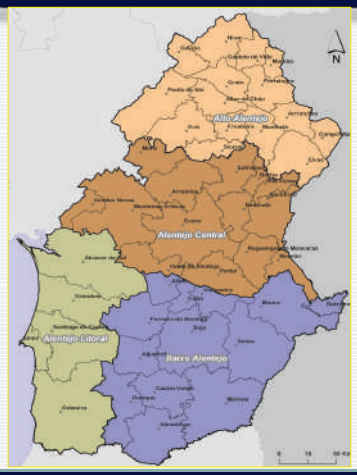
Descrição do projeto

Identificação dos fatores de risco relevantes para a ocorrência de peeira em explorações de ovinos no Alentejo

- Avaliação dos resultados obtidos nos inquéritos
  - Identificação de fatores de risco

- A informação recolhida nos inquéritos é processada e analisada com recurso ao programa IBM SPSS Statistics (*version* 24)
  - Variáveis quantitativas: teste F da ANOVA
  - Variáveis qualitativas: teste do Qui-quadrado

Área de estudo - Alentejo



Alto Alentejo
(6.230 Km²)

Alentejo Central
(7.393 Km²)

Alentejo Litoral
(5.308 Km²)

Baixo Alentejo
(8.505 Km²)

Inquéritos respondidos

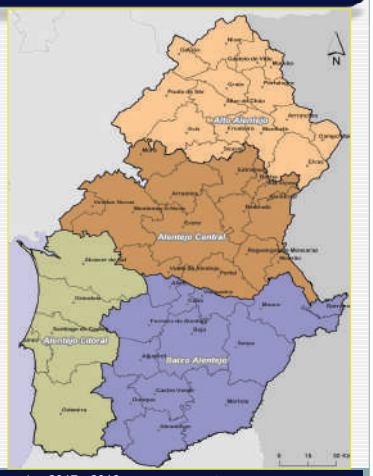
- Alto Alentejo
126 inquéritos

- Alentejo Central
171 inquéritos

- Alentejo Litoral
95 inquéritos

- Baixo Alentejo
284 inquéritos

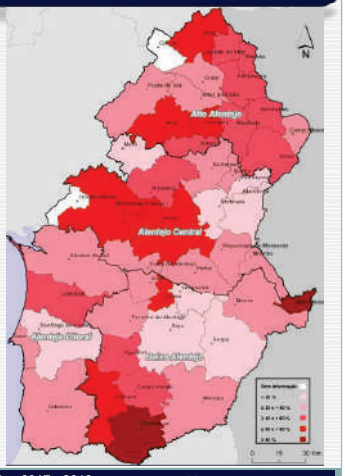
Total: 676 Inquéritos



Inquérito epidemiológico - Resultados

Região	Prevalência estimada
Alto Alentejo	46,4 %
Alentejo Central	38,0 %
Alentejo Litoral	25,0 %
Baixo Alentejo	29,5 %
Total	**34,6 %**

- Concelhos com maior prevalência estimada: **Barrancos** e **Almodôvar**



Inquérito epidemiológico - Resultados

- Maneio geral da exploração - Análise univariada:

Após analisar as respostas aos inquéritos, foi possível identificar os seguintes **fatores de risco:**

- **Maior área de exploração**
- **Maior dimensão do efetivo**
- **Concentração das épocas de cobrição / partos**
- **Presença de áreas com montado**
- **Estabulação dos animais**
- **Fraca capacidade de drenagem dos solos**
- **Participação em feiras e mercados**

Inquérito epidemiológico - Resultados

Variável	Amostra	p-value	OR e IC a 95%
Área (hectares)	≥ 100	<0,001	**2,44**
	< 100		[1,73; 3,47]
Nº ovinos	≥ 150	<0,001	**3,17**
	< 150		[2,23; 4,53]
Épocas de parto	Concentrada	<0,001	**2,07**
	Todo o ano		[1,47; 2,91]
Montado	Presente	<0,001	**2,71**
	Ausente		[1,71; 4,28]
Estabulação dos animais	Sim	<0,01	**1,66**
	Não		[1,15; 2,39]
Drenagem dos solos	Fraca	<0,05	**3,75**
	Boa / Média		[1,56; 8,99]
Participação em feiras	Sim	<0,05	**1,86**
	Não		[1,07; 3,24]

Tabela 2: Fatores de risco para a peeira ovina em explorações do Alentejo (*Odds ratio* (OR) e Intervalo de Confiança (IC))

Inquérito epidemiológico - Resultados

- Maneio geral da exploração - Análise univariada:

  Após analisar as respostas aos inquéritos, **não foi possível** identificar os seguintes **fatores de risco** (p > 0,05):

  - Raça dos animais;
  - Condições climatéricas;
    - Pluviosidade
    - Temperatura
    - Humidade
  - Local dos partos;
  - Textura dos solos.

Inquérito epidemiológico - Resultados

- Maneio geral da exploração - Análise multivariada:

  Modelo inicial com 14 variáveis:

Efetivo animal	Área da exploração
Raças "Linha Pura"	Raça "Cruzada"
Coabitantes Bovinos	Coabitantes Caprinos
Participação feiras / mercados	Partilha caminhos / pastagens
Concentração de partos	Estabulação
Pastoreio em regadio	Partos no ovil
Má drenagem do solo	Solo argiloso
Baixa altitude (várzea)	Montado

Inquérito epidemiológico - Resultados

- Maneio geral da exploração - Análise multivariada:

  Modelo final (*Logit*) com 4 variáveis:

```
                      coeficiente  erro padrão     z      valor p
  ------------------------------------------------------------------
  const                -2,01029     0,229637     -8,754  2,06e-018 ***
  Efetivo               0,00194674  0,000334421   5,821  5,84e-09  ***
  CobriAaoConcentr~     0,472504    0,176601      2,676  0,0075    ***
  MAdrenagemsolo        1,34550     0,439347      3,062  0,0022    ***
  Montado               0,638579    0,228479      2,795  0,0052    ***

Média var. dependente   0,343567   D.P. var. dependente     0,475246
R-quadrado de McFadden  0,104093   R-quadrado ajustado      0,092731
Log. da verosimilhança -394,2594   Critério de Akaike       798,5188
Critério de Schwarz     821,1586   Critério Hannan-Quinn    807,2798

Número de casos 'correctamente preditos' = 485 (70,9%)
f(beta'x) na média das variáveis independentes = 0,221
Teste de razões de verosimilhanças: Qui-quadrado(4) = 91,6159 [0,0000]

         Predito
            0     1
  Atual 0  412    37
        1  162    73
```


Inquérito epidemiológico - Resultados

- Prevenção e tratamento - Análise univariada:

  9 variáveis contempladas no inquérito:

Pedilúvio	Corte corretivo de úngulas	Tratamento tópico
Antibiótico sistémico	Separação dos animais afetados	Vacinação
Mudança das camas	Quarentena	Tratamento das camas

Inquérito epidemiológico - Resultados

- Prevenção e tratamento - Análise univariada:

  Após analisar as respostas aos inquéritos, foi possível concluir que as explorações que realizam os seguintes **procedimentos** têm **maior probabilidade de ter peeira:**

  - **Pedilúvio**
  - **Corte corretivo de úngulas**
  - **Tratamento tópico**
  - **Antibioterapia sistémica**
  - **Separação dos animais afetados**
  - **Vacinação**
  - **Mudança das camas**

Inquérito epidemiológico - Resultados

Variável	p-value	OR e IC a 95%
Pedilúvio	<0,001	**33,07** [21,13; 51,77]
Corte corretivo de úngulas	<0,001	**30,59** [19,49; 48,02]
Tratamento tópico	<0,001	**87,24** [51,53; 147,71]
Antibioterapia sistémica	<0,001	**42,46** [24,17; 74,58]
Separação dos animais doentes	<0,001	**39,49** [16,90; 92,30]
Mudança das camas	<0,001	**3,77** [2,32; 6,12]
Tratamento das camas	<0,001	**13,08** [3,83; 44,66]

Tabela 2: Fatores de risco para a peeira ovina em explorações do Alentejo (*Odds ratio* (OR) e Intervalo de Confiança (IC))

Inquérito epidemiológico - Resultados

- Prevenção e tratamento - Análise univariada:

Modelo inicial com 9 variáveis:

Pedilúvio	Corte corretivo de úngulas	Tratamento tópico
Antibiótico sistémico	Separação dos animais afetados	Vacinação
Mudança das camas	Quarentena	Tratamento das camas

Inquérito epidemiológico - Resultados

- Prevenção e tratamento - Análise multivariada:

Modelo final (*Logit*) com 6 variáveis:

	coeficiente	erro padrão	z	valor p	
const	-7,19800	0,630758	-11,41	3,66e-030	***
Pediluvio	3,33373	0,367583	9,069	1,20e-019	***
Spray	3,39483	0,418579	8,110	5,05e-016	***
ABsistemico	1,40064	0,437575	3,201	0,0014	***
SeparaAao	1,55102	0,559405	2,773	0,0056	***
MudanAacamas	2,35492	1,28611	1,831	0,0671	*
Quarentena	-7,47054	2,07347	-3,603	0,0003	***

Média var. dependente 0,344023 D.P. var. dependente 0,475395
R-quadrado de McFadden 0,699016 R-quadrado ajustado 0,683163
Log. da verosimilhança -132,9015 Critério de Akaike 279,8030
Critério de Schwarz 311,5191 Critério Hannan-Quinn 292,0746

Número de casos 'correctamente preditos' = 628 (91,5%)
f(beta'x) na média das variáveis independentes = 0,184
Teste de razões de verosimilhanças: Qui-quadrado(6) = 617,31 [0,0000]

	Predito 0	1
Atual 0	428	22
1	36	200

Inquérito epidemiológico - Resultados

- Prevenção e tratamento - Análise multivariada:

234 explorações com Peeira - Modelo Probit Ordenado

Modelo 9: Probit com ordem, usando as observações 1-687 (n = 234)
Observações omissas ou incompletas foram ignoradas: 453
Variável dependente: ClassesPeeiraSim
Erros padrão baseados na Hessiana

	coeficiente	erro padrão	z	valor p	
Pediluvio	0,837351	0,229533	3,648	0,0003	***
Podologia	-0,00891517	0,339391	-0,02627	0,9790	
Spray	0,550710	0,332129	1,658	0,0973	*
ABsistemico	-0,208665	0,189074	-1,104	0,2698	
SeparaAao	-0,338701	0,194358	-1,743	0,0814	*
Vacina	0,102954	0,212267	0,4850	0,6277	
MudanAacamas	-0,206319	0,364518	-0,5660	0,5714	
Tratamentocamas	-3,96156	1136,95	-0,003484	0,9972	
Quarentena	5,19043	1136,95	0,004565	0,9964	
cut1	2,21641	0,511285	4,335	1,46e-05	***
cut2	3,58435	0,538552	6,656	2,82e-011	***

Média var. dependente 1,354701 D.P. var. dependente 0,554187
Log. da verosimilhança -161,1092 Critério de Akaike 344,2183
Critério de Schwarz 382,2269 Critério Hannan-Quinn 359,5434

Número de casos 'correctamente preditos' = 161 (68,8%)
Teste de razões de verosimilhanças: Qui-quadrado(9) = 24,5959 [0,0035]

Inquérito epidemiológico - Resultados

- Prevenção e tratamento - Análise multivariada:

234 explorações com Peeira - Modelo Probit Ordenado

- Quanto **maior a utilização de Pedilúvio** na exploração, **maior será a probabilidade de ocorrência** de peeira;
- Quanto **maior a utilização de Tratamento Tópico** na exploração, **maior será a probabilidade de ocorrência** de peeira;
- Quanto **maior a separação de animais doentes**, **menor será a probabilidade de ocorrência** de peeira;
- Restantes variáveis não apresentam significância estatística.

Conclusão

- A prevelência estimada de peeira em explorações de ovinos não é homogénea em todo o Alentejo, tendo o Alto Alentejo e o Alentejo Central apresentado prevalências superiores.
- A maioria dos fatores de risco identificados está de acordo com o descrito na bibligrafia.
  - Aumento da densidade animal
  - Facilita a penetração do agente na pele

Conclusão

- Não foi possível determinar as variáveis climáticas como fatores de risco: Período de 2016 / 2017 correspondeu a um ano “anormalmente seco”, pouco favorável à disseminação da doença.
- Não foi possível identificar predisposição racial: Animais de “Raça Cruzada”
- Atenção à interpretação da associação da doença com as técnicas de controlo / tratamento utilizadas - Nem todos são fatores de risco: Causa ou Efeito?


OBRIGADO !!!

01/03/19

Diagnóstico Laboratorial da Peeira (*Dichelobacter nodosus*)

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Mestranda em Biologia Molecular e Genética
Faculdade de Ciências da Universidade de Lisboa (FCUL/UL) 2018/2019
Instituto Nacional de Investigação Agrária e Veterinária (INIAV)

Etiologia da Peeira

Dichelobacter nodosus → agente causador → **Peeira**

- Gravidade da doença está dependente da virulência das estirpes de *D. nodosus*.

Dermatite contagiosa

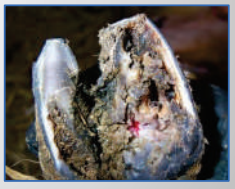
Peeira

Etiologia da Peeira

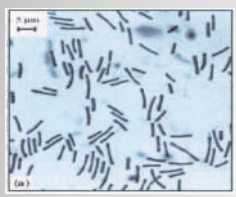
Fusobacterium necrophorum → Bactéria oportunista → **Envolvida na Peeira** → **Patologia Polimicrobiana**

Hipóteses de modo de ação

- Patogénico secundário
- **ou**
- Iniciador da doença

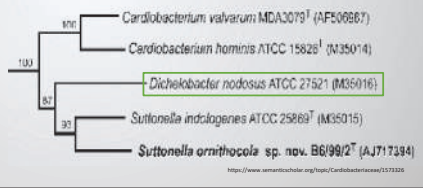


Dichelobacter nodosus



- **Bactéria gram-negativa anaeróbica**
- Genoma pequeno (1.3 Mb)
- Coloniza a epiderme interdigital de ovinos
- Forma de bastonete com 3 a 6 µm de comprimento
- 1 a 1,7 µm de diâmetro
- Fímbrias nas extremidades

Única espécie pertencente ao género *Dichelobacter* e parte da família *Cardiobacteriaceae*



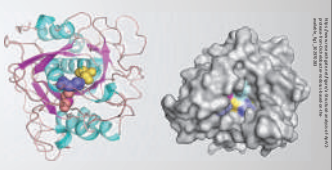
Dichelobacter nodosus: virulência

Estirpes de *D. nodosus*

- **Benignas:** versão mais moderada da doença
- **Virulentas:** versão mais agressiva da doença

Principais fatores de virulência

- Fímbrias do tipo IV
- Protéases extracelulares



- As ilhas genómicas ***vap*** e ***vrl*** estão preferencialmente associadas com isolados virulentos.

Dichelobacter nodosus: protéases extracelulares

- **AprV5/B5:** Protéase acídica isoenzima 5
- **AprV2/B2:** Protéase acídica isoenzima 2
- **BprV/B:** Protéase básica

Protéases serina extracelulares

Importante fator de virulência

Protéase acídica 2

- Gene *aprV2* – protéase termoestável (AprV2) → **Estirpes Virulentas**
- Gene *aprB2* – protéase termolábil (AprB2) → **Estirpes Benignas**

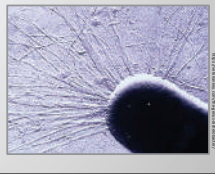
Dichelobacter nodosus: fímbrias

Fímbrias do tipo IV → Estruturas proteicas compostas por uma única proteína FimA — Codificada pelo → **Gene *fimA***

- Região amino-terminal altamente conservada
- Localização polar na superfície da célula
- Resíduo N-metilfenilalanina no N-terminal

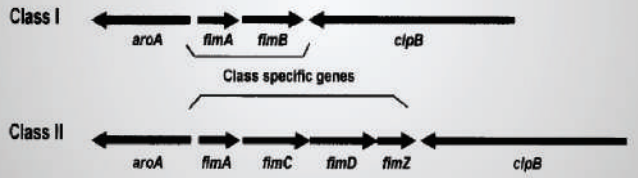
Necessárias para:

- Motilidade "twitching"
- Aderência a células epiteliais do hospedeiro
- Secreção de proteases extracelulares
- Transformação natural

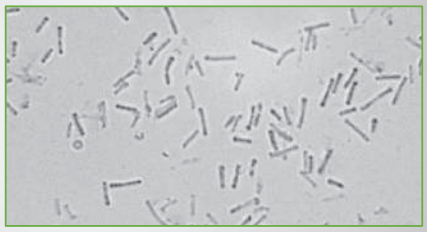


Dichelobacter nodosus: serogrupos

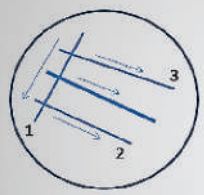
10 Serogrupos de *D. Nodosus* (A-I e M) → Baseados na → Diversidade estrutural das fímbrias → **Classe I:** A, B, C, E, F, G, I e M; **Classe II:** D e H

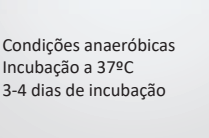


Pesquisa de *D. nodosus*



Método cultural





Condições para crescimento

- Condições anaeróbicas
- Incubação a 37ºC
- 3-4 dias de incubação

Método molecular: PCR em tempo real

Alvos de PCR em tempo real

- **Gene *16S rRNA:*** codifica para o RNA ribossómico 16S, que é um componente da subunidade pequena do ribossoma procariótico.
- **Gene *rpoD:*** codifica para o fator sigma-70 (σ 70) da RNA polimerase, uma proteína necessária para a iniciação da transcrição.

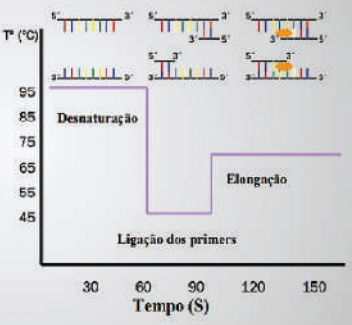
Maior sensibilidade de deteção → Três cópias do gene no genoma da bactéria

Reação de Polimerase em Cadeia (PCR)

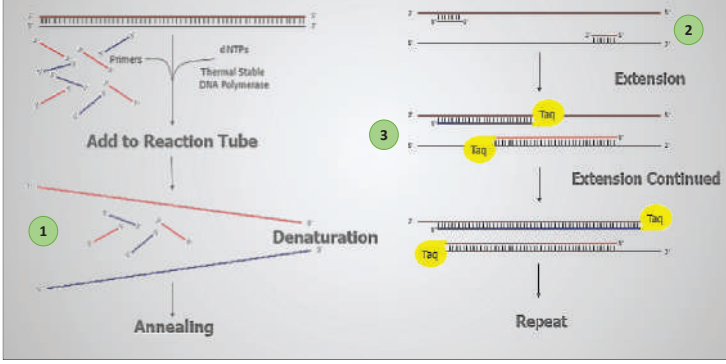
Amplificação de uma região específica de DNA de interesse

Necessário:

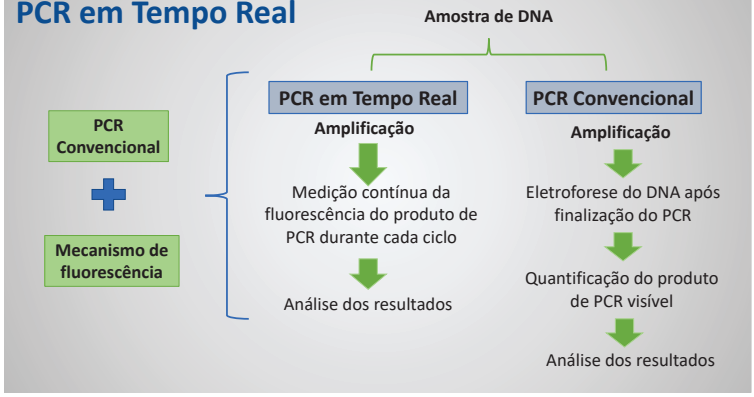
- DNA molde
- Primers específicos
- Deoxinucleótidos trifosfato ou dNTPs (dATPs, dTTPs, dCTPs e dGTPs)
- *Taq* Polimerase (DNA Polimerase termoestável)
- Iões de magnésio



Reação de Polimerase em Cadeia (PCR)



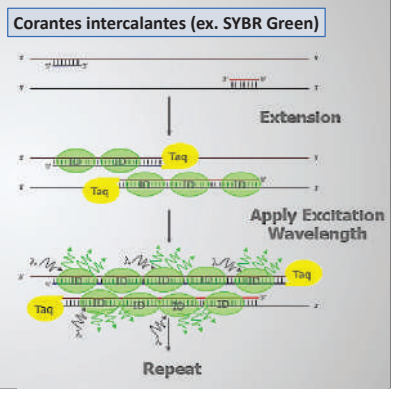
PCR em Tempo Real



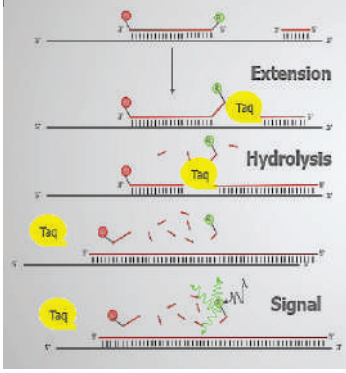
PCR em Tempo Real

Métodos de fluorescência

- Corantes intercalantes (ex. SYBR Green) → Intercalam-se em qualquer cadeia dupla de DNA (não-específico)
- Sondas de sequência específica



PCR em Tempo Real



Sondas específicas fluorescentes

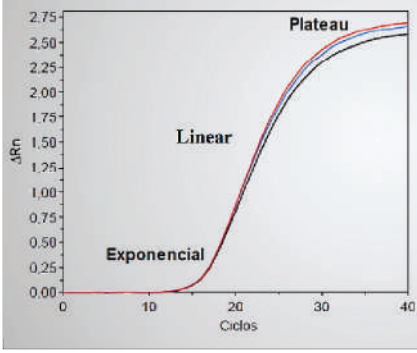
Oligonucleótidos + Fluoróforo

- **Molécula "Reporter":** emite energia
- **Molécula "Quencher":** absorve energia

Proximidade quebrada pela *Taq* Polimerase

Emissão de fluorescência

PCR em Tempo Real



Fase Exponencial

Eficiência é 100%. Reação é extremamente específica e precisa.

- A partir da Fase Linear a eficiência de reação é variável entre amostras.

PCR em tempo real

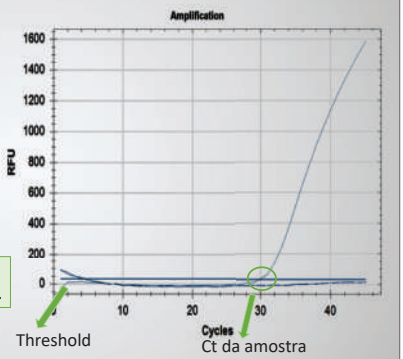
"Threshold"

Valor de sinal de fluorescência que reflete um aumento de produto significativo

Valor "Ct"

Número de ciclos necessário para o sinal de fluorescência ultrapassar o "threshold"

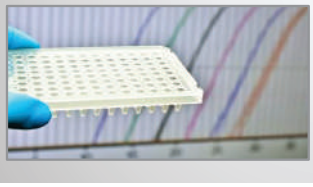
Amostras com a mesma quantidade de DNA inicial apresentam o mesmo valor Ct.



Vantagens e aplicações de PCR em Tempo Real

Vantagens de PCR em tempo real

- Recolha de dados na fase exponencial
- Aumento do sinal de fluorescência é proporcional ao número de fragmentos gerados
- Não é necessário processamento pós-PCR
- Maior sensibilidade



Aplicações de PCR em tempo real

- Quantificação de expressão de genes
- Genotipagem
- Deteção de agentes patogénicos
- Quantificação de vírus
- Entre outros

Determinação de virulência

Atividade elastase e termostabilidade

Estirpes Benignas → Protéase acídicas termolábeis AprB2 codificadas pelo gene *aprB2*

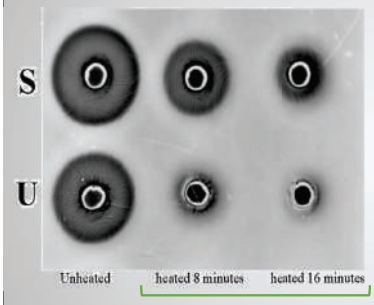
Estirpes Virulentas → Protéase acídicas termoestáveis AprV2 codificadas pelo gene *aprV2*

Diferença de um aminoácido na proteína madura

Alteração na sequência de aminoácidos vai definir a atividade elastase

Essencial para a formação de lesões no casco

Teste do "Gel de Gelatina"



Aquecimento a 67ºC

Medição da produção e termoestabilidade das protéases

Baseada no conhecimento de

Maior termoestabilidade em protéases de estirpes virulentas

- **Estirpes virulentas:** mantém atividade proteolítica após 16 minutos a 67ºC
- **Estirpes benignas:** não mantém atividade proteolítica

Teste da Elastase

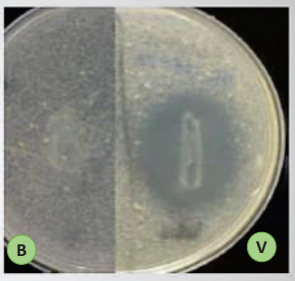
Medição temporal e quantitativa da atividade das protéases

Baseada na

Atividade elastase das protéases

Dependente de

Diferença de um aminoácido nas protéases AprV2 e AprB2



- **Estirpes virulentas:** há digestão das partículas de elastina ("zona de clearance")
- **Estirpes benignas:** não há digestão da elastina

PCR em tempo real

PCR em tempo real competitivo → **Deteção e descriminação alélica de *aprV2* e *aprB2***

- 1 par de primers com homologia para *aprV2* e *aprB2*
- 2 sondas específicas
  - **Sonda FAM:** específica para *aprV2* (estirpes virulentas)
  - **Sonda VIC:** específica para *aprB2* (estirpes benignas)

Controlos

- **Estirpe Virulenta**
- **Estirpe Benigna**
- **Água bidestilada**

Determinação de serogrupos

Determinação de serogrupos de *D. nodosus*

10 serogrupos: A-I e M → Baseados na → Diversidade antigénica do gene ***fimA***

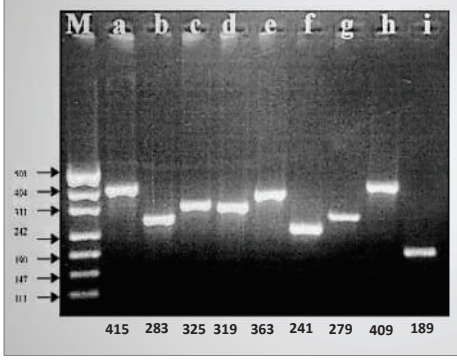
A-I detetáveis e discrimináveis

Teste de PCR multiplex → Variabilidade da região terminal-carboxilo

1 Primer Forward: comum a todos os serogrupos.

9 Primers Reverse: específicos para cada serogrupo.

Determinação de serogrupos de *D. nodosus*



Eletroforese em gel de agarose

Bandas de diferentes tamanhos

Deteção dos produtos de PCR de cada um dos 9 serogrupos

Métodos de pesquisa e caracterização de *D. nodosus*

Pesquisa de *D. nodosus*	**Métodos bacteriológicos**	• Coloração Gram • Culturas
	Métodos moleculares	• PCR em tempo real (*16S rRNA* e *rpoD)*
Determinação da virulência	**Métodos bacteriológicos**	• Teste do Gel de Gelatina • Teste da Elastase
	Métodos moleculares	• PCR em tempo real (*aprV2/B2*)
Determinação de serogrupos	**Métodos moleculares**	• PCR Multiplex (*fimA*)

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Estrongiloses gastrointestinais em pequenos ruminantes

Diagnóstico e controlo

27 Abril 2019 - Auditório da ExpoBeja

PROGRAMA

15h00 - Apresentação

1. A problemática das estrongiloses gastrointestinais em pequenos ruminantes
2. Projetos em desenvolvimento no Alentejo (Alentejo 2020):

Gen-Res-Alentejo: Utilização da Genómica na Seleção de Ovinos Resistentes a Parasitas e Peeira no Alentejo

VegMedCabras: Vegetação mediterrânica: anti-helmínticos naturais na dieta selecionada por cabras em pastoreio

CistusRumen: Utilização sustentável da Esteva (*Cistus ladanifer* L) em pequenos ruminantes - Aumento da competitividade e redução do impacto ambiental

Ludovina Neto Padre (Universidade de Évora)

15h30 – Diagnóstico

1. Importância do diagnóstico
2. Métodos de colheita, conservação e envio das amostras
3. Processamento laboratorial - técnicas de rotina
4. Interpretação dos resultados

Clara Dias (Bolseira Gen-Res-Alentejo)
Cláudia Costa (Bolseira VegMedCabras)

16h15 – Controlo: métodos e ferramentas

Controlo integrado - Sara Zúquete (UE, Projeto CistusRumen)
Gestão do pastoreio - Ana Teresa Belo (INIAV, Projeto VegMedCabras)
Seleção genética - Claudino Matos (ACOS, Projeto Gen-Res-Alentejo)

30 de Setembro 2022

11h30 - Auditório
Parque de Feiras e Exposições

Patrimónios do Sul

VINIPAX · ARTES DO SUL · BEJA EDUCA · PETISCOS · ESPETÁCULOS · SUL À MESA · CAÇA, PESCA E NATUREZA · BEJA BRAVA · AVIBEJA

Do diagnóstico clínico da peeira à bioinformática: desafios e oportunidades

Este seminário visa apresentar o diagnóstico e prevenção da peeira, desde o ponto de vista clínico ao uso de algumas ferramentas bioinformáticas que têm sido aplicadas pela equipa do CEBAL com o intuito de caracterizar o microbioma nos diferentes graus de lesão e associar variações genéticas à resistência à doença, mostrando ao público o potencial da sua utilização na valorização e conservação de espécies endógenas. Os trabalhos apresentados foram desenvolvidos no contexto do projeto Gen-Res-Alentejo, o qual foi liderado pela ACOS (Associação de Agricultores do Sul) em parceria com a Universidade de Évora, com o CEBAL, com o INIAV (Instituto Nacional Investigação Agrária Veterinária) e com a DRAPAL (Direção Regional de Agricultura e Pescas do Alentejo).

PROGRAMA

11h30 – Sessão de abertura

11h40 – Peeira: principais fatores de risco, diagnóstico e prevenção

Pedro Caetano (Hospital Veterinário - Universidade de Évora)

12h00 – Caracterização do microbioma da peeira através da utilização de novas tecnologias de sequenciação

Ana Usié (CEBAL, MED, CHANGE)

12h20 – Utilização de ferramentas genómicas para a identificação de marcadores moleculares associados a resistência a peeira

Daniel Gaspar (CEBAL, CIBIO)

12h40 – Discussão, notas finais e encerramento

GEN-RES ALENTEJO

Entidades promotoras:
cebal Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo · MED · CHANGE

Co-financiamento:
FCT Fundação para a Ciência e a Tecnologia · República Portuguesa · ALENTEJO 2020 · PORTUGAL 2020 · União Europeia Fundo Europeu de Desenvolvimento Regional Fundo Social Europeu

Notas biográficas

Pedro Caetano é Médico Veterinário desde 2014, ano em que concluiu o Mestrado Integrado em Medicina Veterinária na Universidade de Évora. Após um período breve em que trabalhou como médico veterinário assistente numa exploração agropecuária, iniciou funções como técnico superior no Hospital Veterinário da Universidade de Évora em 2015. Desde então e até ao início de 2022, exerce funções nas áreas clínica (médica e cirúrgica) e de assistência reprodutiva em espécies pecuárias no Hospital Veterinário e equinos no da Universidade de Évora e na Unidade Clínica de Alter do Chão. Além destas funções também acompanhou os ensinos dos cursos de Medicina Veterinária e de Ciência e Tecnologia Animal da Universidade de Évora, na componente prática relacionada com animais de produção e equídeos. A partir de 2017/2018 começou também a desempenhar funções como docente convidado dos Departamentos de Medicina Veterinária e de Zootecnia da Universidade de Évora. Em fevereiro de 2021 obteve o título de Doutor através do Programa de Doutoramento em Ciências Veterinárias da Universidade de Évora. Durante o seu doutoramento desenvolveu investigação sobre a doença infetocontagiosa peeira ovina nas áreas da clínica, epidemiologia e microbiologia. Atualmente conjuga as funções de docente auxiliar convidado com a de Médico Veterinário na Empresa Multivet – Serviços Veterinários de Equinos e Espécies Pecuárias, onde exerce nas áreas da clínica, cirurgia, medicina da produção, sanidade e assistência reprodutiva em espécies pecuárias e equinos.

Ana Usié é licenciada em Engenharia Técnica em Gestão de Computadores (2007), mestre em Engenharia Informática Sénior (2010) e em Engenharia de Software Livre (2010), pela Universidade de Lleida (Catalunha, Espanha). Obteve o doutoramento em Bioinformática através do Programa de Doutoramento em Saúde Molecular da mesma universidade em 2014. Dois meses depois mudou-se para Portugal e continuou a sua carreira no CEBAL onde integrou o grupo de Genómica Animal e Bioinformática. Ana Usié é membro integrado da unidade de investigação do Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento (MED). O seu trabalho centra-se na análise e processamento de dados de sequenciação obtidos com tecnologias de sequenciação de última geração. Tem estado envolvida em vários projetos nacionais e internacionais, trabalhando com espécies vegetais e animais. As atividades desenvolvidas nestes projetos incluíram montagem *de novo* de genomas e transcriptomas, identificação de variantes como SNPs, SVs e CNVs, metagenómica e análise de expressão diferencial, entre outros. Durante sua carreira teve a oportunidade de treinar e orientar novos membros do grupo, bem como alunos de estágio, e coorientar teses de licenciatura, mestrado e doutoramento.

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Notas biográficas

Daniel Gaspar é licenciado em Biotecnologia (2011) e mestre em Bioinformática e Biologia Computacional (2016), pela Faculdade de Ciências da Universidade de Lisboa (Lisboa, Portugal). Atualmente é aluno de doutoramento no Programa de Biodiversidade, Genética e Evolução da Universidade do Porto. O seu trabalho foca-se na análise e processamento de dados de sequenciação obtidos com tecnologias de sequenciação de última geração. Tem estado envolvido em vários projetos nacionais e internacionais em diferentes espécies vegetais e animais. As atividades desenvolvidas no contexto destes projetos incidiram principalmente em analises de dados de (re)-sequenciação total de genomas para o desenvolvimento de estudos de associação genótipo-fenótipo, diversidade genómica e caracterização de estruturas populacionais, e na análises de dados de transcriptómica para a identificação de genes candidatos associados à stresses bióticos e abióticos, entre outros.

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