

# Inmunización con una vacuna recombinante frente a *Teladorsagia circumcincta* en corderos de razas ovinas canarias



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*A mis padres,*

*“It is our choices that show what we truly are, far more than our abilities.”*

-Harry Potter and The Chamber of Secrets, J.K. Rowling

*“The separation of talent and skill is one of the greatest misunderstood concepts for people who are trying to excel, who have dreams, who want to do things. Talent you have naturally. Skill is only developed by hours and hours and hours of beating on your craft.”*

-Will Smith

*“Las preguntas que no podemos contestar son las que más nos enseñan. Nos enseñan a pensar. Si le das a alguien una respuesta, lo único que obtiene es cierta información. Pero si le das una pregunta, buscará sus propias respuestas. Así, cuanto más difícil es la pregunta, más difícil la búsqueda. Cuanto más difícil es la búsqueda, más aprendemos...”*

-El Temor de un Hombre Sabio, Patrick Rothfuss

*“Queremos conseguir cosas sin pensar que lo importante no es conseguirlas, sino saber en qué persona tenemos que convertirnos para ello.”*

-Enhamed Enhamed



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# Introducción



# *Introducción*

El incesante aumento de la población mundial implicará que el sector agropecuario necesitará incrementar su producción de cereales y carne para cumplir con las exigencias del mercado en las próximas décadas, que serán especialmente altas en los países en vías de desarrollo, y deberán hacerlo contando con una población rural cada vez más escasa y avejentada (FAO, 2009a; United Nations, 2022b). Dadas las consecuencias negativas del cambio climático sobre los rendimientos productivos y los propios efectos perjudiciales del cultivo y la producción de animales sobre el ecosistema, se necesitará incrementar los rendimientos por animal en lugar del número de cabezas de ganado (FAO, 2009b, 2013; Masson-Delmotte *et al.*, 2019; WMO, 2020). Para ello, es indispensable optimizar el control de enfermedades (FAO, 2018). En este sentido, los nematodos gastrointestinales (NGI) en rumiantes representan una de las principales preocupaciones para el sector ya que pueden llegar a reducir hasta en un 90% las producciones de carne, leche y lana, lo que supone unos 345 millones al año en pérdidas solamente en Europa (Mavrot, 2016; Mavrot *et al.*, 2015).

Entre las principales vías para el control figuran los fármacos antihelmínticos pero su vida útil se ha visto seriamente mermada por la aparición de resistencias a estos fármacos y ha impulsado el desarrollo de métodos complementarios sostenibles a largo plazo (Stear *et al.*, 2007). Entre estas alternativas destacan las vacunas y la selección de animales genéticamente resistentes a NGI de forma natural.

No obstante, el número de vacunas comerciales disponibles frente a los NGI es muy escaso debido, entre otros factores, a la complejidad estructural de los helmintos, lo que convierte en compleja la interacción parásito-hospedador, y a la dificultad de identificar antígenos protectores y producirlos *in vitro* a gran escala (Meeusen & Piedrafita, 2003; Nisbet *et al.*, 2016b). Recientemente un grupo escocés consiguió obtener alta eficacia en ensayos de vacunación con ovejas adultas y corderos mediante la vacunación con un ambicioso prototipo basado en 8 proteínas de origen recombinante frente a *Teladorsagia circumcincta* (Liu *et al.*,

2022; Nisbet *et al.*, 2013, 2019; Nisbet *et al.*, 2016a). Sin embargo, este prototipo presentó dificultades para obtener resultados protectores en corderos menores de 3 meses de edad, lo que es una problemática frecuente en el diseño de vacunas frente a nematodos dada la relativa susceptibilidad de los animales jóvenes a los parásitos y su dificultad para desarrollar respuestas inmunes protectoras frente a ellos (Kebeta *et al.*, 2022; Kooyman *et al.*, 2000; Smith *et al.*, 1982, 1985; Stear *et al.*, 1999, 2000; Vervelde *et al.*, 2001).

Por otra parte, estudios realizados en dos razas ovinas que coexisten en las Islas Canarias, las razas Canaria y Canaria de Pelo, señalaban diferencias en su susceptibilidad a los NGI, siendo la Canaria de Pelo naturalmente más resistente que la raza Canaria (González *et al.*, 2008, 2011, 2019; Guo *et al.*, 2016; Hernández *et al.*, 2016, 2017, 2020). Además, los datos sugerían que esa diferencia podría estar presente en animales jóvenes, posicionando a estas dos razas como modelos biotecnológicos en las que realizar pruebas con la vacuna recombinante frente a *T. circumcincta* en corderos menores de 3 meses de edad.

# Objetivos



# *Objetivos*

**1.** Comparar el **efecto de la vacunación** con el prototipo de vacuna recombinante frente a *Teladorsagia circumcincta* en corderos de 3 meses de edad de las razas Canaria y Canaria de Pelo tras inoculaciones seriadas con 2000 larvas del parásito, comparando los resultados obtenidos en los **animales vacunados y controles** en cada raza en relación a:

- Los resultados parasitológicos basados en el contaje de huevos en heces, el recuento de vermes en el abomaso y la medición de la longitud y del número de huevos intraútero.
- La respuesta inmune humoral a través de la medición de los niveles de IgA, IgG<sub>1</sub> e IgG<sub>2</sub> séricas frente antígenos naturales de *Teladorsagia circumcincta*.
- La respuesta inmune celular en la mucosa abomasal mediante el recuento de poblaciones celulares tales como: eosinófilos, leucocitos globulares, mastocitos, linfocitos T CD4<sup>+</sup>, T CD8<sup>+</sup>, CD45RA<sup>+</sup> y células MHC-II<sup>+</sup>.
- El fenotipo de poblaciones celulares presentes en el nódulo linfático abomasal y la producción de las citoquinas IL-4, IL-17 e IFN-γ.
- La ganancia media diaria del peso de los corderos antes y después de la infección.

**2.** Comparar la **expresión génica y el perfil transcriptómico** a nivel del nódulo linfático abomasal en los **corderos vacunados** de 3 meses de edad de las razas Canaria y Canaria de Pelo si se observan diferencias en la respuesta a la vacuna entre ambas razas.

**3.** Caracterizar las **diferencias raciales y/o individuales en la resistencia** en los **corderos controles** de 3 meses de edad de las razas Canaria y Canaria de Pelo mediante la comparación de los datos parasitológicos, la expresión de IgA de mucosas, los recuentos de células en la pared abomasal y su relación con la expresión génica y las vías metabólicas expresadas a nivel del nódulo linfático abomasal.



*Revisión*  
*bibliográfica*



# *Revisión*

## *bibliográfica*

### **1. Nuevos retos alimentarios**

#### **1.1 El mundo en el siglo XXII: Dinámica poblacional y seguridad alimentaria**

La Organización de las Naciones Unidas espera que prácticamente sobrepasemos los **once mil millones de personas** hacia finales del siglo XXI (United Nations, 2022b). Es decir, la humanidad deberá satisfacer la demanda de alimento para casi un **42% más de habitantes** en el transcurso de menos de cien años (United Nations, 2022b). Esto supondrá un reto para el sector agropecuario, que necesitará revertir la reciente tendencia a la baja de la tasa de rendimiento productivo del cultivo de **cereal**, **aumentando su producción en un 70%**, además de incrementar la obtención de **carne** un **43%** hacia mitad de siglo (FAO, 2009a). Asimismo, no se debe despreciar los novedosos requerimientos industriales de **fibra vegetal** y el **uso bio-energético** de los cultivos que también contribuirán a exacerbar la creciente demanda del producto agrícola (FAO, 2009a). Esta futura demanda agravará la **brecha en la seguridad alimentaria global** derivada por la **pandemia** del SARS-CoV-2 sumada a la incertidumbre de la **guerra en Ucrania** y la **escalada de precios del combustible**, lo que ya supone riesgos para mantener la producción agrícola y ganadera en la actualidad (FAO, 2022a, 2022b).

*La demanda mundial de cereales y carne deberá aumentar significativamente para poder alimentar a una población creciente*

*Las economías en expansión crecerán más que los países desarrollados*

La futura expansión poblacional global estará condicionada por el grado de industrialización de las regiones: mientras que los territorios más avanzados alcanzarán previsiblemente su población máxima antes del año 2040, las **economías emergentes** probablemente sigan creciendo ininterrumpidamente hasta principios del siglo XXII (FAO, 2014). De hecho, **cuarenta y siete de los países menos**

desarrollados están entre los que más rápido crecen, estimándose que el aumento de habitantes hasta 2050 se concentrará en apenas ocho de ellos, la mitad pertenecientes al continente africano (United Nations, 2022b). Estas asimetrías interregionales son ya manifiestas en otros aspectos como la disponibilidad de comida. En tanto el poder adquisitivo de los países desarrollados impulsa dietas cada vez más ricas en productos de origen animal y aumenta los índices de obesidad entre su población, casi mil trescientos millones de personas a nivel global tienen hoy en día dificultades para acceder de forma constante a alimentos que aseguren una nutrición adecuada, poniendo en riesgo su salud y bienestar (FAO, 2009a; Herrero & Thornton, 2013; Masson-Delmotte *et al.*, 2019).

Por otra parte, durante las últimas décadas se ha instaurado un patrón demográfico de **disminución de la fertilidad**. En 2021, la mitad de la población global vivía en un país o área donde el número de nacimientos por mujer es inferior a 2,1; aunque por el otro extremo, algunos países del África subsahariana alcanzaron 4,6 o en buena parte del continente Asiático se sitúa en torno al 2,4 y se prevé que estos valores disminuyan en todas las regiones en los próximos treinta años (United Nations, 2022b).

*Las zonas más empobrecidas del globo serán las que cuenten con más población en edad de trabajar*

Hacia 2050, se prevé que la esperanza de vida suba en 5 años y se espera un incremento de la proporción de ancianos, habitando en la tierra más del doble de personas por encima de 65 años que de niños menores de 5 (United Nations, 2022b). El menor número de nacimientos junto a la inclinación al envejecimiento poblacional supondrá una disminución de la proporción entre personas en edad de trabajar con respecto a los mayores de 65 años, lo que tendrá importantes repercusiones sobre el mercado laboral, el desempeño económico y los planes de protección social (United Nations, 2022b). En el contexto del desarrollo rural, estos aspectos podrán comprometer el relevo generacional e implicará que los agricultores y ganaderos contarán con escasa mano de obra para garantizar la disponibilidad de alimentos e intentar cumplir con el *reto de hambre cero* (United Nations, 2022a), forzando en su conjunto, una mayor profesionalización.

*La falta de disponibilidad de suelo cultivable amenazará con destruir zonas “vírgenes” del planeta*

Previsiblemente, las naciones darán respuesta al déficit en proteínas de origen animal al que nos enfrentaremos en el futuro mediante la intensificación de cultivos y, en menor medida, a partir de la expansión del área de tierra arable en los países en vías de desarrollo (FAO, 2009a). Pese a ello, el sector ganadero en estos países sí aumentará sensiblemente sus censos, especialmente el número de rumiantes (Thornton, 2010). Sin embargo, no se debe olvidar que la población mundial es cada vez más urbana: se estima que en 2050 el 70% vivirá en ciudades (FAO, 2009a). Esto conllevará inevitablemente transformaciones sociales y de hábitos de consumo (FAO, 2009a) que agravarán la disputa por el suelo, reduciendo aún más las posibilidades de expansión del terreno agrícola. Si bien esta opción “expansiva” todavía es viable, principalmente en algunas regiones de alto valor ecológico situadas en África subsahariana y Latinoamérica, establecer su explotación por parte del sector primario podría suponer un alto coste desde el punto de vista medioambiental dada la importancia de no perpetuar la degradación de unos recursos naturales ahora exigüos, a juzgar, entre otras cosas, por la aparente merma de nutrientes del suelo, la desertificación o la pérdida de biodiversidad (FAO, 2009a).

### **1.2. El cambio climático y el sector agropecuario: una relación circular**

Desde hace doscientos años el clima de la Tierra ha venido experimentando incrementos en la frecuencia de eventos meteorológicos adversos como la aridez, los trastornos en la disponibilidad de agua o inundaciones (FAO, 2009b, 2013; Masson-Delmotte *et al.*, 2019; WMO, 2020). Ocasionalmente, estas observaciones de variación en las características o comportamiento climático pueden tener un origen natural (IPCC, 2022) y se deben a acontecimientos de alternancia entre épocas cálidas y de glaciación (los llamados ciclos de Milankovitch) que se suceden periódicamente cada decenas de miles a cientos de miles de años (Buis, 2020). En la actualidad, las variaciones observables no son solamente atribuibles a fenómenos naturales sino que responden a

*La actividad industrial ha traído consigo un incremento en la temperatura de la Tierra*

cambios drásticos en la composición atmosférica resultado de la actividad humana, a raíz de la revolución industrial originada en Europa a mediados del S. XVIII (Buis, 2020; United Nations, 1992). El aumento exponencial en la quema de combustibles fósiles que se lleva produciendo desde hace dos siglos ha intensificado la liberación de los denominados *gases de efecto invernadero* (vapor de agua, dióxido de carbono, óxido nitroso, metano y clorofluorocarbonos) (Shaftel, 2018) que se han acumulado en nuestra atmósfera, dificultado la dinámica de liberación del calor reflejado por la superficie planetaria, dando lugar a la elevación en más de 1°C de la temperatura media de la superficie terrestre (IPCC, 2022; WMO, 2020). Este exceso de energía térmica está relacionado con la mayor frecuencia de eventos climáticos inusuales tales como temperaturas altas, fuertes lluvias e inundaciones o períodos largos de sequía (WMO, 2020).

Dichas circunstancias han dificultado la extracción de materias primas por parte de los productores, tal y como muestran la disminución del rendimiento de cultivos debido al estrés hídrico en cultivos en áreas de baja latitud o la expansión de enfermedades del ganado a zonas donde previamente no existían (FAO, 2009b, 2013; Masson-Delmotte *et al.*, 2019; WMO, 2020). En términos socioeconómicos, tan sólo la ganadería representa el principal modo de vida para

*El cambio climático afecta a la producción agropecuaria y compromete la sanidad animal y la seguridad alimentaria*

más de mil millones de personas en el mundo (FAO, 2009b; Herrero & Thornton, 2013) y, si bien las consecuencias sobre el rendimiento agrícola se manifiestan a nivel global, son las naciones próximas al cinturón ecuatorial las que se ven más perjudicadas (FAO, 2013; Parry *et al.*, 2007). Fundamentalmente, porque ahí se aglutan los estados menos industrializados con economías en expansión donde gran parte de la población

depende de actividades rurales para sustentarse (FAO, 2009a, 2014). Por otra parte, su limitada capacidad de respuesta sanitaria veterinaria ante la aparición de enfermedades emergentes acentúa su vulnerabilidad (FAO, 2009a), lo cual resulta preocupante considerando que, por ejemplo, África y Asia ostentan más del 80% del censo caprino y ovino mundial (FAO, 2019). En suma, los datos apuntan que las zonas que más se extenderán demográficamente son aquellas que menos recursos poseen para enfrentarse a esta situación (FAO, 2014) lo que

potencialmente agravaría el círculo de pobreza en estas regiones al añadir más trabas a su capacidad para garantizar la seguridad alimentaria (Parry *et al.*, 2007), generar recursos económicos y satisfacer las necesidades sociales de su población.

Por otro lado, el mundo agrícola es asimismo un gran impulsor del cambio climático. Entre 2007 y 2016, se atribuyó a la silvicultura y la agricultura la emisión del 13% del dióxido de carbono, 44% del metano y 81% del óxido nitroso a raíz de las actividades humanas (Masson-Delmotte *et al.*, 2019). Estos porcentajes también engloban una parte de las emisiones correspondientes a la producción de alimento para la ganadería, actividad que contribuye directamente al calentamiento global mediante la emisión de metano a través de la fermentación ruminal y entérica, e indirectamente debido uso de agroquímicos liberadores de óxido nitroso, así como la explotación del suelo y a otras etapas de la producción que conllevan el empleo de combustibles fósiles (FAO, 2009b, 2018; Shaftel, 2018). Adicionalmente, el sector ganadero ha sido responsable de la pérdida de biodiversidad debido al desplazamiento de especies silvestres consecuencia de la deforestación de zonas “salvajes” para generar campos destinados a la producción de plantas forrajeras o terrenos para pastoreo (Steinfeld *et al.*, 2006).

*La producción agropecuaria contribuye al calentamiento global por emisiones de gases de efecto invernadero, cerrando un círculo de retroalimentación*

Debido a que los gases de efecto invernadero perduran siglos en la atmósfera, algunos modelos señalan que incluso si las emisiones parasen bruscamente, la temperatura promedio de la superficie terrestre aumentaría otros 0,6°C durante décadas antes de empezar a percibir una desaceleración del calentamiento global y mucho más hasta notar un descenso en la incidencia de fenómenos meteorológicos extremos (Hansen *et al.*, 2007; NASA, 2007, 2020a). Por este motivo, en la actualidad se considera que la **manera de no agravar este problema a largo plazo pasa por reducir las emisiones de gases de efecto invernadero** de origen antrópico a la vez que se favorece la recaptación de carbono en sistemas terrestres como océanos o bosques, lo que permitirá ganar tiempo para atenuar las secuelas de los eventos climáticos más severos (NASA, 2020b) y adaptar nuestro modo de vida a las nuevas circunstancias. Por ello, considerando la

relación circular que existe entre el cambio climático y el mundo agropecuario, **el gran desafío** de todos los integrantes del sector será promover el desarrollo rural para generar el sustento de las personas cuyo modo de vida está ligado al mismo, mientras se asegura la disponibilidad de alimento a un precio que todos puedan pagar sin comprometer aún más los recursos naturales (FAO, 2009b; Waller, 1997b), el equilibrio ecológico y el clima del planeta.

### 1.3 Producción ganadera sostenible

Afortunadamente, aún **existen oportunidades para que esta actividad limite su huella sobre el medio ambiente**, además de ayudar a mitigar los efectos del cambio climático, a la par que cumple con las exigencias del mercado y los objetivos de desarrollo sostenible (FAO, 2013). En

*La producción agropecuaria sostenible y climáticamente inteligente según la FAO es un objetivo viable que implica combinar factores sociales, económicos y medioambientales*

2010, la FAO definió la “**agricultura climáticamente inteligente**” (CSA: *Climate-Smart Agriculture*) como una estrategia que persigue guiar a los países para desarrollar soluciones técnicas, políticas y de inversión con respecto al uso de los recursos naturales, al igual que impulsarles a adoptar métodos y tecnologías apropiados para una producción sostenible a través de la combinación de factores sociales, económicos y medioambientales (FAO, 2013). Este

enfoque requiere de asesoramiento y evaluaciones específicas in situ que identifiquen qué tecnologías y prácticas de producción agrícola resultan adecuadas en base a condiciones locales específicas (FAO, 2013). En el caso de la **agricultura**, la reducción de las emisiones por kilo de alimento producido junto con el secuestro de carbono en el suelo de cultivo son claves para alcanzar estos objetivos, que estarán basados en el uso de plantas adaptadas al medio; la implantación de tecnologías que optimicen el empleo de agua o el suelo, a la par que reduzcan la mano de obra y las pérdidas por desperdicio (FAO, 2009a, 2013). Paralelamente, los programas de selección ganadera y la conservación genética animal podrían garantizar la cría de animales mejor adaptados a un entorno particular, y por lo tanto más resilientes a condiciones cambiantes (FAO, 2018). Conjuntamente, la ganadería estará enfocada a ajustar la intensidad del pastoreo e incrementar los rendimientos por animal, extendiendo su vida productiva a través de progresos

en el manejo o la alimentación (FAO, 2018). A tal efecto, el control del desempeño productivo y el bienestar animal del ganado en las explotaciones a través de herramientas tecnológicas que permitan la monitorización continua y a tiempo real (siguiendo el concepto de *Precision Livestock Farming*) podría ayudar a detectar precozmente problemas de manejo o salud y mitigar a largo plazo el impacto sobre las emisiones generadas por cada unidad de producto animal final (Berckmans, 2014; Tullo *et al.*, 2019).

Las parasitaciones nematodos gastrointestinales en el ganado rumiante son un claro ejemplo de la repercusión que las enfermedades pueden tener sobre la huella ecológica ya que son una de las principales causas de enfermedad y pérdidas productivas entre los rumiantes domésticos, siendo los géneros incluidos dentro de la Superfamilia Trichostrongyloidea (orden Strongylida) los que tienen una mayor importancia clínica (Meana & Rojo-Vázquez, 1999) al ocasionar pérdida de peso, anemia, reducción del crecimiento y afectar a la aptitud reproductiva, la lactancia y producir la muerte en infecciones severas (Piedrafita *et al.*, 2010). Estos parásitos incrementan hasta un 33% la emisión de metano en corderos al modificar la ingesta, el aprovechamiento de nutrientes y el tiempo de retención ruminal, aunque se ha sugerido que su impacto sobre la emisión de gases de efecto invernadero podría reducirse si se garantiza un control adecuado de las parasitaciones que optimice el uso de tratamientos farmacológicos, evite la aparición de signos clínicos y el retraso del alcance del peso comercial (Fox *et al.*, 2018; Houdijk *et al.*, 2017; Kenyon *et al.*, 2013).

Aunque resulta difícil cuantificar las pérdidas que generan, una revisión y meta-análisis de 218 artículos que evaluaban el efecto de los nematodos gastrointestinales sobre uno o varias de las tres facetas de la producción ovina (carne, leche y lana), concluyó que, aproximadamente en el 60% de los ensayos, el efecto negativo de estos parásitos sobre la producción es estadísticamente significativo y éstas diferencias en términos de ganancia de peso,

***En Europa se estima que las pérdidas por nematodos gastrointestinales en corderos de carne son casi del 9% de su producción total***

***Los nematodos gastrointestinales aumentan la producción de gases de efecto invernadero al interferir en los procesos digestivos***

producción de lana y leche en relación con la producción de los animales no parasitados, son del 77, 90 y 78%, respectivamente (Mavrot, 2016). Con esta información, se estimó que la parasitación por nematodos gastrointestinales le cuesta anualmente **345 millones de euros** al sector ovino de carne (aproximadamente, 7 euros por cordero al sacrificio) lo que representa casi el 9% de su producción total al año (Mavrot, 2016). De forma similar, los productores del sector en Reino Unido y Australia ven menoscabadas sus ganancias en 93 y 267 millones de euros al año, respectivamente, por dicha causa (Lane *et al.*, 2015; Nieuwhof & Bishop, 2005). En ambos casos, tres cuartas partes del importe están ligadas a disminución del rendimiento en la cría de corderos (Lane *et al.*, 2015; Nieuwhof & Bishop, 2005). Todos estos estudios atribuyen pérdidas millonarias al sector ovino derivadas del parasitismo por nematodos gastrointestinales. A ellas, habría que sumar aspectos como el anteriormente citado impacto medioambiental, el compromiso del bienestar animal -cada vez más cuestionado socialmente- y la mayor susceptibilidad a otras enfermedades. Lo que es irrebatible es que se trata de un **factor limitante de la producción ganadera que se debe intentar minimizar**.

## 2. Nematodos gastrointestinales y la relación parásito-hospedador

### 2.1 Etiología y ciclo biológico

De entre las especies de nematodos gastrointestinales (NGI) incluidas en el Orden Strongylida, *Haemonchus contortus*, *Teladorsagia circumcincta* y *Trichostrongylus spp* son las más prevalentes y las que más afectan la rentabilidad en la cría de pequeño rumiante en todo el mundo (O'Connor *et al.*, 2006). Las dos primeras especies se encuentran en el estómago glandular, mientras que la mayoría de las incluidas en el género *Trichostrongylus* se alojan a lo largo del primer tercio del intestino delgado (Meana & Rojo-Vázquez, 1999). Los vermes adultos de estos parásitos de cuerpo filiforme miden entre menos de 1 centímetro en las especies de *T. circumcincta* y *Trichostrongylus spp*, hasta 3 centímetros en *H. contortus* (Borchert, 1975). Se caracterizan por tener un extremo anterior con una cápsula bucal poco desarrollada, en algunos casos dotada de un diente o lanceta en las especies hematófagas (Bowman *et al.*, 2004; Meana & Rojo-Vázquez, 1999). Generalmente, el extremo posterior de los vermes macho presenta una

bolsa copuladora muy aparente y en las hembras ocasionalmente destaca una “solapa” que protege la estructura vulvar (Meana & Rojo-Vázquez, 1999).

Desarrollan un **ciclo biológico directo** en el que las hembras adultas liberan huevos “de estróngilo” (Bowman et al., 2004) de forma ovoide, no embrionados, incoloros y cáscara fina que son excretados al medio junto con las heces (Meana & Rojo-Vázquez, 1999). Si las condiciones externas son favorables, la larva de primer estadio (L1) sale del huevo y sufre dos mudas a L2 y L3 (Bowman et al., 2004). Las dos primeras formas de vida libre se alimentan de bacterias presentes en el medio (Urquhart *et al.*, 1996); la larva infectante L3 mantiene la cutícula del estadio anterior, confiriéndole resistencia en el medio ambiente e impidiendo su alimentación, por lo que subsiste gracias a las reservas lipídicas que alberga en su intestino (Meana & Rojo-Vázquez, 1999; Urquhart *et al.*, 1996). Una vez en el exterior, la L3 migra de la masa fecal hacia el agua presente en el pasto circundante, donde es ingerida por el hospedador definitivo (Bowman et al., 2004). Diversos estímulos, como el gas CO<sub>2</sub> libre presente en el tracto digestivo, (Meana & Rojo-Vázquez, 1999) desencadenan la liberación de un fluido rico en una aminopeptidasa (Urquhart *et al.*, 1996) entre la cutícula y la vaina (Lee, 2002), produciendo la ruptura de ésta última. La L3 desenvainada migra hacia su nicho para desarrollar la etapa L4, donde penetrará en la mucosa fúndica (*H. contortus*), las glándulas gástricas de la zona antropilórica (*T. circumcincta*) o entre el epitelio y la membrana basal de la mucosa intestinal (*Trichostrongylus spp.*) (Meana & Rojo-Vázquez, 1999). Pocos días después ocurre otra muda a L5 y finalmente, a adulto que cerrarán el ciclo tras el apareamiento.

**La L3 envainada  
es la forma de  
resistencia e  
infectante del  
parásito**

Durante la etapa de L4 puede tener lugar un proceso llamado **hipobiosis** por el cual las larvas pueden quedar retenidas en la mucosa en un estado latente, disminuyendo su metabolismo y permitiéndoles permanecer en el interior del hospedador cuando las condiciones exteriores se vuelven desfavorables para el desarrollo de las fases de vida libre en el medio (al inicio de los meses fríos o de la estación cálida y seca) (Eysker, 1997; Meana & Rojo-Vázquez, 1999; Urquhart *et al.*, 1996). También es posible que este fenómeno esté relacionado con manifestaciones de resistencia por parte de los hospedadores (Stear *et al.*, 2004) o que sea una

estrategia para la regulación de la población de vermes adultos (Balic *et al.*, 2000). En este aspecto, las larvas inhibidas pueden salir del letargo de forma sincrónica cuando la situación del entorno vuelve a ser propicia para continuar con su desarrollo, por lo que coinciden la madurez de vermes adultos liberando gran cantidad huevos al ambiente y la presencia de corderos jóvenes en los pastos (Meana & Rojo-Vázquez, 1999; Urquhart *et al.*, 1996).

## 2.2 Patogénesis y sintomatología

La salida de las larvas de *T. circumcincta* desde las glándulas gástricas provoca su distensión y daño al tejido circundante, lo que macroscópicamente se observa como un pequeño nódulo con un orificio central en una mucosa engrosada (Meana & Rojo-Vázquez, 1999). A nivel microscópico, el daño a las células secretoras de HCl también está relacionado con la caída del pH abomasal desencadenando un aumento de la secreción de gastrina (Fox, 1997) e impidiendo la conversión del pepsinógeno (Craig, 2018), así como un aumento de la permeabilidad de la mucosa que favorece la difusión del pepsinógeno hacia la sangre y la pérdida de proteínas desde los capilares en dirección a la luz (Craig, 2018; Meana & Rojo-Vázquez, 1999). Esto da lugar a la pérdida del efecto bacteriostático del pH, el aumento el peristaltismo y malabsorción de péptidos (Craig, 2018; Meana & Rojo-Vázquez, 1999). Clínicamente, los animales muestran edema, diarrea y pérdida de apetito, que junto a las interferencias en el metabolismo proteico del propio parasitismo, explican la característica pérdida de peso (Urquhart *et al.*, 1996). De forma similar, los animales parasitados por algunas especies del género *Trichostrongylus* spp muestran diarrea y tasa de crecimiento baja debido a la atrofia de vellosidades y consecuente disminución de la superficie de absorción originada por la actividad de las fases larvarias en el espesor de la mucosa intestinal (Meana & Rojo-Vázquez, 1999; Urquhart *et al.*, 1996). Algo diferentes son las infecciones por *H. contortus*, donde la patogenia se debe principalmente a la acción hematófaga de los gusanos adultos, que producen localmente pequeñas úlceras en la mucosa (Meana & Rojo-Vázquez, 1999) y, a nivel sistémico, anemia hemorrágica aguda con pérdida de proteínas junto a descensos bruscos del hematocrito, que en los animales se observan como palidez en las membranas mucosas, edema submandibular o ascitis (Craig, 2018; Urquhart *et al.*, 1996).

### **2.3 Epidemiología y su relación con el cambio climático**

La gravedad de los síntomas está muy relacionada con la carga de vermes presentes en el hospedador, así como con la abundancia de L3 infectantes en el medio ambiente que, además, depende de **factores bióticos y abióticos**. Algunos de ellos pueden incluso condicionar la presencia de determinadas especies parásitas en zonas concretas del globo. Por ejemplo, la temperatura es uno de los factores abióticos más importantes para el desarrollo de los estadios de vida libre de los NGI, por lo que históricamente los casos autóctonos de gastritis parasitaria por *H. contortus* se describen a lo largo de regiones tropicales y subtropicales con presencia de lluvias; mientras que, las fases de vida libre de *T. circumcincta* y *Trichostrongylus* spp. cuentan con una mayor tolerancia al frío que les permite distribuirse a lo largo de la banda de clima templado (O'Connor *et al.*, 2006).

*Gran Canaria, que es descrita como un “continente en miniatura”, muestra una distribución de especies de nematodos gastrointestinales que sugiere que esta descripción es correcta, mostrando la isla como un “laboratorio natural”*

A nivel local, en la isla de Gran Canaria, algunos autores han comprobado que la orografía y la orientación del territorio dan lugar a regiones de características climáticas muy diferentes que influyen en la distribución de los géneros o especies de NGI (Hernández, 2015; Molina *et al.*, 1997). Para ello delimitaron cuatro zonas climáticas de acuerdo con la altitud sobre el nivel del mar a la que se encuentran, temperatura y precipitación media anual y la vegetación que presentan (“desierto-seco”,

“estepa-seco”, “templado-moderado” y “templado-frío”) (Molina *et al.*, 1997; Rodríguez-Ponce *et al.*, 1995). Entre 2008 y 2010, Hernández, 2015 realizó un estudio epidemiológico en dos granjas de ovino situadas en dos zonas, una al este a 380 m sobre el nivel del mar con temperatura media anual de 19 °C y precipitación media (clima “templado-moderado”) y otra localizada al sur a 147 m con media de temperatura de 22,5 °C y baja precipitación (clima “desierto-seco”), pudiendo constatar que *Trichostrongylus* spp. predominó en ambas granjas, mientras que *T. circumcincta* estaba más presente en la zona más templada y lluviosa, al igual que *H. contortus*. Molina *et al.*, 1997 describieron hallazgos similares en un estudio de prevalencia realizado en

caprino donde *T. circumcincta* y *Trichostrongylus* spp. se distribuyeron a lo largo de todas las zonas microclimáticas y *H. contortus* prefería las áreas húmedas de media altitud y temperatura cálida. Esto ejemplifica que ligeras variaciones de las características climáticas de una región pueden modular la interacción parásito-hospedador-ambiente y llegar a moldear la epidemiología de la enfermedad en la zona.

No obstante, los fenómenos ambientales derivados del cambio climático- principalmente, el aumento global de la temperatura- han repercutido sobre la prevalencia de las enfermedades parasitarias (FAO, 2009b; Mas-Coma *et al.*, 2008) al facilitar su supervivencia fuera del hospedador en las zonas templadas (Hudson *et al.*, 2006) y acortar los procesos de hipobiosis (Mas-Coma *et al.*, 2008). Se ha descrito un aumento de la incidencia de *T. circumcincta* y *Trichostrongylus* spp, al igual que una mayor uniformidad en la distribución de casos a lo largo del año (McMahon *et al.*, 2012) debido a que el menor número de días fríos resulta en una interrupción temprana del proceso hipobiótico y la reanudación simultánea del ciclo biológico cuando las condiciones ambientales vuelven a ser favorables (Kenyon *et al.*, 2009b; Sargison *et al.*, 2007). Por otra parte, los otoños cálidos y húmedos también han permitido que *H. contortus* se introduzca en el seno del rebaño durante esta época para, seguidamente, eludir las condiciones desfavorables del invierno europeo como L4 hipobiótica (Sargison *et al.*, 2007; Waller *et al.*, 2004), lo que justifica el aumento de las notificaciones de brotes de haemonchosis entre la población ovina del norte de Europa (Kenyon *et al.*, 2009b; Sargison *et al.*, 2007; Waller & Chandrawathani, 2005), tendencia que seguirá en alza en los próximos años (Rose *et al.*, 2016).

*El cambio climático  
está suponiendo  
una redistribución  
de estos parásitos y,  
por ende, de los  
parasitismos*

En cuanto al hospedador, la indulgencia climática les permite acortar su estacionalidad reproductiva, pastar hasta bien entrado el otoño y reemprender la cría antes en primavera (Hudson *et al.*, 2006; Kenyon *et al.*, 2009b). Para cuando las ovejas adultas y sus corderos recién destetados regresan a los pastos con el fin de aprovisionarse de nutrientes, éstos ya se encuentran altamente contaminados por las L3, lo que origina brotes de la enfermedad en el ganado con

gran impacto sobre la productividad de las hembras (Hudson *et al.*, 2006) y el crecimiento de los corderos (Mavrot *et al.*, 2015).

#### 2.4 Respuesta inmune

El primer mecanismo inmune que interviene tras la ingestión de las primeras larvas y su llegada al órgano diana es la **inmunidad innata**, conformada por mecanismos inespecíficos encargados de detectar la colonización por parte de los NGI. Un ejemplo es la capa de mucus que recubre el abomoso, producida por las células del cuello, que está fundamentalmente

compuesto de mucina y actúa como una barrera física a la vez que sirve de medio para sustancias bioactivas como las defensinas, lectinas y galectinas. Algunas de estas sustancias presentan actividad antimicrobiana o estimulan la inflamación (Donskow-Łysoniewska *et al.*, 2021). Por ejemplo, la galectina 11 es producida por las células epiteliales del tracto gastrointestinal y tiene la capacidad de fijarse a la superficie de las L4 y adultos produciendo inhibición del crecimiento y el desarrollo (Preston *et al.*, 2015). De forma similar, la galectina 14

es liberada al mucus por los eosinófilos y está relacionada con la expulsión de vermes (Robinson *et al.*, 2011). La contracción del músculo liso en respuesta a la parasitación también es un mecanismo de eliminación de vermes (Balic *et al.*, 2000). Por otra parte, los macrófagos y células dendríticas detectan la presencia de larvas infectantes mediante receptores de reconocimiento de patrones (PRRs) que identifican patrones moleculares asociados a patógenos (PAMPs) o bien detectan daño o estrés celular (DAMPs), lo que suele resultar en la liberación de citoquinas proinflamatorias y el reclutamiento de células como mastocitos y eosinófilos (Hansen *et al.*, 2011).

Por otro lado, estas mismas células, macrófagos y células dendríticas, están encargados de captar antígenos del parásito, procesarlos y migrar al nódulo linfático regional para presentarlos a linfocitos inactivados, iniciando y dirigiendo la **inmunidad adquirida** (o específica). Los antígenos del parásito son internalizados en la célula presentadora mediante

endocitosis y son procesados en lisosomas generando fragmentos peptídicos que se unirán a moléculas del complejo mayor de histocompatibilidad clase II (MHC-II). Los complejos péptido-molécula MHC-II son expresados posteriormente en la superficie celular para su reconocimiento por parte de linfocitos T que expresan un receptor CD4 en su superficie desencadenando su activación (Abbas *et al.*, 1999). Los linfocitos T CD4 o colaboradores (Th) se consideran clave para la regulación de la respuesta frente a helmintos (Grencis, 2015). Otros subtipos de linfocitos T son los citotóxicos (Tc), que expresan un correceptor CD8 (linfocitos T CD8) en su superficie y están relacionados con la destrucción de células infectadas con virus o patógenos intracelulares tras su activación a través presentación de antígenos extraños en moléculas MHC-I (Spellberg & Edwards, 2001). A su vez, los linfocitos Th pueden diferenciarse en subtipos Th1, Th2, Th17 o T reguladores (T reg) con funciones distintivas (Corripio-Miyar *et al.*, 2021): Los linfocitos Th1 coordinan las respuestas frente a patógenos intracelulares como bacterias, virus y parásitos; los linfocitos Th2 dirigen respuestas alérgicas y frente a patógenos extracelulares como helmintos; los linfocitos Th17 están relacionados con respuestas a bacterias extracelulares e intracelulares y hongos y los linfocitos T reg suprimen la actividad de otros linfocitos y son necesarios para mantener la tolerancia inmunológica y evitar la autoinmunidad. Las respuestas Th2, involucradas en el control de los nematodos gastrointestinales, están caracterizadas por el aumento de citoquinas Th2 (IL-4, IL-5, IL-10, IL-13), produciendo el reclutamiento de eosinófilos, mastocitos, leucocitos globulares hacia la mucosa abomasal y desencadenando la producción de inmunoglobulinas (Ig) A, G1 y E específicas por parte de los linfocitos B activados (CD45R+)(Alba-Hurtado & Muñoz-Guzmán, 2013; McRae *et al.*, 2015).

Los eosinófilos son células de núcleo bilobulado y citoplasma acidófilo dotado de gránulos que contienen sustancias citotóxicas (peroxidasa, arilsulfatasa, proteína básica mayor, proteína catiónica, entre otras) y pueden sintetizar especies reactivas del oxígeno, prostaglandinas y leucotrienos (Balic *et al.*, 2000). Estas células son reclutadas desde la medula ósea a la sangre y se desplazan hacia el tracto gastrointestinal en respuesta a la presencia de parásitos. Una vez allí, migran hacia

***Los eosinófilos, los mastocitos y leucocitos globulares son células efectoras que ayudan a controlar estos parasitismos***

las L3 adhiriéndose a su cutícula para posteriormente degranular sobre ella, produciendo daños que reducen la viabilidad de las larvas y su establecimiento en el abomaso, en presencia del complemento y de inmunoglobulinas específicas (Balic *et al.*, 2006; Rainbird *et al.*, 1998; Terefe *et al.*, 2007). Los eosinófilos juegan un papel clave en la resistencia frente a parásitos que presenta el ovino Barbados Black Belly, con mayor reclutamiento tisular de estas células, y en el ovino Canario de Pelo, donde es clave para el control, al menos, de la longitud y fecundidad de los parásitos (Hernández *et al.*, 2020).

Por su parte, los mastocitos son células mononucleares con un núcleo central y forma irregular, dotadas de gránulos citoplasmáticos que contienen potentes mediadores biológicamente activos (histamina, serotonina, prostaglandinas, leucotrienos, proteasas, entre otras) (Fong & Crane, 2022). A diferencia de los eosinófilos, los mastocitos son residentes en el tejido conectivo y las mucosas, localizándose en la lámina propia o en el epitelio. Los mastocitos intraepiteliales pueden madurar a células de mayor tamaño denominadas leucocitos globulares. Esta células, consideradas efectoras, están localizadas estratégicamente para degranular en las proximidades del parásito, lo cual es posible a través de la unión de IgE a los receptores de alta afinidad de la región Fc de esta inmunoglobulina (FcεRI) que presentan en su membrana

***La IgA secretada en la mucosa es clave para controlar la longitud y fecundidad de los vermes***

(McRae *et al.*, 2015). La liberación de estos mediadores produce contracción del músculo liso, aumento de la permeabilidad vascular, aumento de la secreción de mucus y degradación de los vasos sanguíneos y de las membranas basales de las células epiteliales (Balic *et al.*, 2000), efectos que están relacionados con mecanismos de expulsión de los parásitos (Stear *et al.*, 1995), la reducción de la fecundidad (Gruner *et al.*, 2004) y la reparación de los tejidos (Fong & Crane, 2022).

La IgA de mucosas es sintetizada por las células plasmáticas y secretada a la luz abomasal (McRae *et al.*, 2015). El reconocimiento de los estadios larvarios, en particular la L4 (Strain & Stear, 1999) y del verme adulto (Hernández *et al.*, 2016) por parte de la IgA es uno de los principales mecanismos implicados en el control de la longitud y la fecundidad de los nematodos (Stear *et al.*, 1995).

La expresión de todos estos mecanismos que intervienen en las respuestas protectoras frente a NGI es un proceso muy variable cuyo desarrollo depende de factores como la especie de nematodo parasitario y la intensidad de la exposición al mismo, el estado nutricional, el estado reproductivo (como la preñez), el desarrollo metabólico o la edad (Greer & Hamie, 2016; McRae *et al.*, 2015). En este sentido, los corderos empiezan a demostrar inmunocompetencia entre los 2 y los 3 meses a la par que se exponen regularmente a las larvas infectantes a través de la ingesta de pasto (McRae *et al.*, 2015). Generalmente, su capacidad para controlar parasitaciones por NGI se ve ampliamente sobrepasada durante este primer contacto y necesitan exposiciones antigénicas repetidas para desarrollar respuestas completas que regulen la fecundidad de los vermes, expulsen y limiten el establecimiento, lo que ocurre hacia el primer año de vida (Greer & Hamie, 2016; Smith *et al.*, 1985; Stear *et al.*, 1999, 2000). No obstante, durante este proceso de maduración inmunitaria, los NGI producen graves daños en el hospedador que resultan en el retraso del desarrollo corporal y en un aumento de la tasa de mortalidad (Charlier *et al.*, 2020). Con el fin de evitar los síntomas clínicos, reducir las consecuentes pérdidas económicas y productivas derivadas de esta interacción, se emplean tratamientos antihelmínticos para limitar la carga de vermes, pauta que se repetirá periódicamente los siguientes meses de vida de los corderos y a lo largo de toda su etapa adulta.

### 3. Control de los nematodos gastrointestinales

#### 3.1 Control químico

El control de los vermes basado en la quimioprofilaxis es el enfoque que tradicionalmente se ha seguido tras al desarrollo de los primeros antihelmínticos a principios del siglo XX. Entre la década de los 60 y los 80, el descubrimiento y la comercialización de las primeras clases de antihelmínticos de amplio espectro -benzimidazol, imidazotiazol y avermectina- fue seguida de la rápida aparición de resistencias por parte de los vermes (McKellar & Jackson, 2004), esto es, una adaptación y menor susceptibilidad de los parásitos al modo de acción de las moléculas a las que son expuestos (Kotze & Prichard, 2016).

*La quimioprofilaxis ha sido el enfoque tradicional de control, comprometida por el desarrollo de resistencias a los fármacos*

A nivel de granja, la resistencia se observa como una reducción de la eficacia del fármaco en comparación con el momento en que salió al mercado y se mide a través del test de reducción de excreción de huevos en heces (del inglés, *Fecal Egg Count Reduction Test o FECRT*), basado en comparar el número de huevos antes y después de la administración de un fármacos, considerándose sospecha de resistencia en la población de prueba si el porcentaje de reducción después del tratamiento farmacológico es inferior al 95% o el nivel de confianza del 95% es menor al 90% (Coles *et al.*, 1992; COMBAR, 2021).

Dado al escaso margen de beneficios que supone para las compañías farmacéuticas la búsqueda de nuevas moléculas efectivas para controlar los vermes gastrointestinales en el ganado, no fue hasta 2009 y 2010 que dos nuevos fármacos con mecanismos de acción novedosos se lanzaron al mercado: el monepantel (un derivado del amino-acetonitrilo) y el derquantel (de la clase de los espiroindoles), este último comercializado en combinación con abamectina (Epe & Kaminsky, 2013; Waller, 1997a). No obstante, en poco tiempo tras su lanzamiento ya empezaron a describirse los primeros casos de resistencia a estas nuevas herramientas (Sales & Love, 2016; Scott *et al.*, 2013), es por ello que era necesario implementar medidas para ralentizar el proceso.

Debido a que los mecanismos de resistencia a las distintas clases de antihelmínticos son diferentes, una de las alternativas propuestas fue la **combinación de fármacos** (Leathwick, 2012). Esto permitiría reducir el número de individuos con genotipo resistente que sobreviven al tratamiento bajo la premisa de que los portadores de múltiples alelos de resistencia son menos frecuentes que los de un único alelo (Bartram *et al.*, 2012). Algunos modelos de simulación de las condiciones de cría de ovejas en Nueva Zelanda y Australia estimaron un control adecuado de los parásitos y una mayor efectividad de la combinación para alargar la vida útil de los compuestos que el tratamiento con un único fármaco (Dobson *et al.*, 2011; Leathwick, 2012). Si bien, ambos modelos junto a estudios de campo posteriores señalaron que el éxito de esta estrategia depende del nivel preexistente de resistencias a los fármacos clásicos y, en especial, del cumplimiento de ciertas pautas de manejo en granja como evitar tratamientos innecesarios en animales que no presentan síntomas clínicos (Dobson *et al.*, 2011; Leathwick, 2012; Leathwick

*et al.*, 2015). Esto se ha señalado como un concepto fundamental para evitar seleccionar resistencias a múltiples clases de antihelmínticos mediante las combinaciones al permitir que una subpoblación de vermes dentro del rebaño queden sin exponer al tratamiento antihelmíntico y pueda transmitir a su descendencia los alelos susceptibles (población “en refugio”), diluyendo la proporción de parásitos con genotipos resistentes (Bartram *et al.*, 2012; Kenyon *et al.*, 2009a; Van Wyk, 2001).

A pesar de que las resistencias a las principales clases de estos productos disponibles en el mercado y sus combinaciones son un fenómeno ampliamente descrito en todos los continentes (Tabla 1), las desparasitaciones con antihelmínticos siguen siendo el principal recurso para el control y para frenar la expansión de los NGI a consecuencia del cambio climático, entre otros motivos. Esta dependencia de los fármacos desafía la sostenibilidad económica y medio ambiental de la ganadería en el futuro, por lo que será fundamental establecer un plan dotado de procedimientos que se complementen entre sí antes que confiar en una sola medida de control. Esto ha impulsado la búsqueda de métodos alternativos o complementarios, basados en un uso restringido del tratamiento químico y otros medidas como: manejo de pastos, suplementación nutricional, selección de razas/individuos resistentes a los vermes y desarrollo de vacunas (Bath & van Wyk, 2009; Maqbool *et al.*, 2017; Stear *et al.*, 2007; Waller, 1999).

**Tabla 1. Descripciones de resistencia a antihelmínticos en distintos países del mundo.**

<i>País</i>	<i>Género o especie implicada*</i>	<i>Clase de antihelmíntico</i>	<i>Referencia</i>
Australia	- <i>H. contortus</i>	- Derivados de amino-acetonitrilo - Espiroindoles - Lactonas macrocíclicas	Sales & Love, 2016
Brasil	- <i>Cooperia</i> spp. - <i>Haemonchus</i> spp. - <i>Oesophagostomum</i> spp. - <i>Ostertagia</i> spp. - <i>Trichostrongylus</i> spp.	- Benzimidazol - Imidazotiazol - Lactonas macrocíclicas - Salicilanilidas	Ramos <i>et al.</i> , 2016
Canadá	- <i>Haemonchus</i> spp. - <i>Teladorsagia</i> spp. - <i>Trichostrongylus</i> spp.	- Benzimidazol - Imidazotiazol - Lactonas macrocíclicas	Falzon <i>et al.</i> , 2013
Colombia	- <i>H. contortus</i> - <i>T. colubriformis</i>	- Benzimidazol - Imidazotiazol - Lactonas macrocíclicas	Chaparro <i>et al.</i> , 2017
España	- <i>Bunostonum</i> spp. - <i>H. contortus</i> - <i>T. circumcincta</i> - <i>Trichostrongylus</i> spp. - <i>Nematodirus</i> spp.	- Benzimidazol - Imidazotiazol - Lactonas macrocíclicas	Martínez-Valladares <i>et al.</i> , 2013
España	- <i>Haemonchus</i> spp. - <i>Teladorsagia</i> spp. - <i>Trichostrongylus</i> spp.	- Benzimidazol - Imidazotiazol - Lactonas macrocíclicas	Martínez-Valladares <i>et al.</i> , 2015
	- <i>O. ostertagi</i>	- Lactonas macrocíclicas	
Francia	- <i>H. contortus</i>	- Benzimidazol - Lactonas macrocíclicas	Bordes <i>et al.</i> , 2020
Francia, Grecia e Italia	- <i>Haemonchus</i> spp. - <i>Teladorsagia</i> spp. - <i>Trichostrongylus</i> spp.	- Benzimidazol - Imidazotiazol - Lactonas macrocíclicas	Geurden <i>et al.</i> , 2014
India	- <i>H. contortus</i>	- Benzimidazol	Chandra <i>et al.</i> , 2015

Nueva Zelanda	- <i>T. circumcincta</i>	- Derivados de amino-	Scott <i>et al.</i> , 2013
	- <i>T. colubriformis</i>	acetonitrilo	
	- <i>T. axei</i>	- Benzimidazol	
Reino Unido	- <i>T. colubriformis</i>	- Imidazotiazol	Waghorn <i>et al.</i> , 2014
	- <i>T. vitrinus</i>		
	- <i>H. contortus</i>	- Benzimidazol	
Sudáfrica	- <i>T. circumcincta</i>	- Imidazotiazol	Wrigley <i>et al.</i> , 2006
	- <i>T. axei</i>	- Lactonas macrocíclicas	
	- <i>C. ovina</i>		
Reino Unido	- <i>Cooperia</i> spp.		Bartley <i>et al.</i> , 2003
	- <i>H. contortus</i>		
	- <i>O. venulosum</i>	- Benzimidazol	
Sudáfrica	- <i>T. circumcincta</i>		Van Wyk <i>et al.</i> , 1999
	- <i>T. axei</i>		
	- <i>T. colubriformis</i>		
Sudáfrica	- <i>T. vitrinus</i>		
	- <i>H. contortus</i>	- Imidazotiazol	
		- Lactonas macrocíclicas	
		- Salicilanilidas	

\*Leyenda de abreviaturas:

- *Bunostomum* spp.: especies del género *Bunostomum*
- *C. ovina*: *Chabertia ovina*
- *Cooperia* spp.: especies del género *Cooperia*
- *Haemonchus* spp.: especies del género *Haemonchus*
- *H. contortus*: *Haemonchus contortus*
- *Nematodirus* spp.: especies del género *Nematodirus*
- *Oesophagostomum* spp.: especies del género *Oesophagostomum*
- *O. venulosum*: *Oesophagostomum venulosum*
- *Ostertagia* spp.: especies del género *Ostertagia*
- *O. Ostertagi*: *Ostertagia ostertagi*
- *Teladorsagia* spp.: especies del género *Teladorsagia*
- *T. circumcincta*: *Teladorsagia circumcincta*
- *Trichostrongylus* spp.: especies del género *Trichostrongylus*
- *T. axei*: *Trichostrongylus axei*
- *T. colubriformis*: *Trichostrongylus colubriformis*
- *T. vitrinus*: *Trichostrongylus vitrinus*

## 3.2 Control integrado

### 3.2.1 Tratamiento selectivo

A diferencia del sistema tradicional de tratamiento “en manta”, el tratamiento selectivo consiste en **desparasitar exclusivamente a aquellos individuos dentro del rebaño que tienen más probabilidades de estar fuertemente parasitados** y permite minimizar la presión de

*El tratamiento selectivo consiste en tratar aquellos individuos del rebaño que tienen más probabilidades de estar más fuertemente parasitados, preservando la vida útil de los fármacos*

selección sobre los vermes (Van Wyk & Bath, 2002). Para su funcionamiento es necesario definir criterios específicos que identifiquen aquellos miembros del rebaño que necesitan ser desparasitados (Kenyon *et al.*, 2009a). La metodología más utilizada es el contaje de huevos en heces (FEC) mediante el envío de muestras frescas a un laboratorio para su procesado, lo que en muchas ocasiones resulta inconveniente para los ganaderos (Vercruyse *et al.*, 2018). Además, la variabilidad

de fecundidad entre géneros de nematodos o las fluctuaciones en eliminación de huevos durante la infección son desventajas de esta técnica que deben tenerse en cuenta al interpretar los resultados (Preston *et al.*, 2014). En los últimos años se han desarrollado algunas herramientas de fácil uso para realizar un diagnóstico en granja y que ofrecen una alta sensibilidad e incluso pueden evaluarse de forma remota (Mini-FLOTAC, FECPAK<sup>G2</sup>) (Charlier *et al.*, 2018; Cringoli *et al.*, 2013; FECPAKG2, 2020).

Además del FEC, la medición del hematocrito puede indicar con precisión las pérdidas de sangre por nematodos hematófagos, si bien, la toma de muestras es invasiva y debe realizarse por personal entrenado (Preston *et al.*, 2014). Otra opción sobre el terreno útil en zonas con alta prevalencia de *H. contortus* es el *FAMACHA*®: una tarjeta-guía dotada de una escala colorimétrica basada en cinco categorías de color correlacionadas con niveles descendentes de hematocrito que se compara con el color de la conjuntiva ocular (Van Wyk & Bath, 2002). Esta herramienta está incluida en el *Five Point Check*®, un test seguro, sencillo y rápido empleado en pequeños rumiantes, basado en el examen de otras cuatro áreas del cuerpo para detectar síntomas frecuentes en animales parasitados que podrían beneficiarse del tratamiento, tales

como secreción mucopurulenta, edema submandibular, acúmulo de suciedad en el área perineal y pérdida de condición corporal (Bath & van Wyk, 2009). No obstante, el diagnóstico es sólo orientativo ya que los marcadores no son específicos de parasitaciones por un género. Por otra parte, las escalas son subjetivas y en algunos parámetros el entrenamiento del operador puede condicionar la repetibilidad de las mediciones (Bath & van Wyk, 2009).

Otros criterios para el tratamiento selectivo basados en indicadores de producción objetivos, como el peso o ganancia media diaria frecuentemente resultan más interesantes para el ganadero (Charlier *et al.*, 2014). El mayor inconveniente para implementarlos es la posible penalización en la producción en los animales sin tratar. Por ello se ha tratado de comprobar el efecto del tratamiento selectivo basado en el desempeño productivo y en los parámetros parasitológicos. Un experimento dividió animales en un grupo de “pesados” y otro de “ligeros” y trató puntualmente sólo una parte dentro de cada grupo. Los corderos que fueron privados del tratamiento antihelmíntico tuvieron FEC más altos y peores rendimientos con independencia de su peso inicial, sugiriendo que los animales del grupo “pesados” eran igual de susceptibles que los del grupo “ligeros” (Keegan *et al.*, 2018). Otras experiencias han variado el enfoque al establecer un peso objetivo para cada individuo basado en un modelo que estima la eficiencia de utilización de la energía bruta (*HappyFactor™*) (Greer *et al.*, 2009). Aquellos animales que no alcanzaban el peso objetivo estimado recibían un tratamiento antihelmíntico, y se dejaba sin tratar a aquellos que sí habían alcanzado sus metas de crecimiento (Busin *et al.*, 2013). Al compararlo con animales que se trataban rutinariamente cada 6 semanas, se consiguió reducir a la mitad los tratamientos con antihelmínticos sin afectar al rendimiento productivo. Esto sugiere que el peso como criterio para el tratamiento selectivo permitiría mantener la población refugio y presumiblemente reducir el paso del desarrollo de resistencia antihelmíntica sin poner en riesgo la producción.

*El “HappyFactor™”  
define un peso objetivo y  
se opta por desparasitar  
aquellos corderos que no  
lo alcanzan, primando  
criterios productivos en  
el modo de ralentizar las  
resistencias*

### 3.2.2 Manejo nutricional

*El aporte de proteínas en la dieta mejora tanto el desempeño productivo como la capacidad de controlar a los parásitos durante la infección*

Como se ha descrito en apartados anteriores, los nematodos gastrointestinales reducen la ingesta de alimento, producen alteraciones en la absorción de minerales y aumentan los requerimientos de proteína debido a las pérdidas por diarrea y a la demanda de componentes proteicos por parte de la respuesta inmune (Houdijk *et al.*, 2012; Ingale *et al.*, 2010; Kearney *et al.*, 2016). No obstante, el metabolismo del hospedador prioriza reparar los daños originados en el tracto digestivo, desviando parte de los nutrientes necesarios para el crecimiento de huesos y músculos o la producción de leche (Ingale *et al.*, 2010). Por ello aumentar la cantidad de **proteína** disponible en la dieta mejora el desempeño productivo durante la infección (Bisset *et al.*, 2001; Kidane *et al.*, 2009) así como la capacidad del hospedador para regular la excreción de huevos, carga y fecundidad del parásito (Bisset *et al.*, 2001; Kearney *et al.*, 2016; Strain & Stear, 2001; Valderrábano *et al.*, 2006).

Estos efectos beneficiosos se deben principalmente a un aumento de la disponibilidad de nitrógeno en forma de amoniaco para la síntesis de proteína microbiana (Knox & Steel, 1999). No es necesario que la fuente del nitrógeno sea proteína de alta calidad, dado que las bacterias ruminantes pueden producirlo a partir de fuentes no proteicas, permitiendo abaratar costes mediante la suplementación con bloques multinutricionales de **urea** y melaza (Knox & Steel, 1999; Stear *et al.*, 2007).

Por otra parte, las **partículas de óxido de cobre** se han usado tradicionalmente para paliar las deficiencias en el pasto de este mineral y, en la actualidad, para control de los NGI (Burke & Miller, 2020). Los suplementos de óxido cobre se administran por vía oral en forma de cápsulas de liberación lenta que generan un ambiente poco propicio para estos parásitos, provocan daños en la cutícula de los gusanos adultos y causan su expulsión (Burke & Miller, 2020; Kearney *et al.*, 2016). Aunque tienen gran aplicabilidad para controlar las poblaciones de *H. contortus*, sus efectos sobre otros géneros son más limitados (Kearney *et al.*, 2016). Además, deben usarse bajo

estricta vigilancia veterinaria para prevenir posibles efectos tóxicos a nivel hepático y renal (Burke & Miller, 2020).

Otra estrategia es el uso de **plantas medicinales** por su contenido en metabolitos secundarios con actividad antihelmíntica: alcaloides, compuestos fenólicos, glicósidos, lactonas, saponinas y taninos condensados son algunos ejemplos (Athanasiadou *et al.*, 2007; Burke & Miller, 2020). Aunque el uso de estas plantas es muy ventajoso, su inclusión en la dieta debe ser controlada ya que un consumo excesivo puede alterar la digestibilidad de nutrientes y la funcionabilidad ruminal y llegar a producir hemólisis o problemas neurológicos (Athanasiadou *et al.*, 2007).

Entre todos los metabolitos secundarios los taninos condensados son los más estudiados y actúan optimizando la disponibilidad de proteína ruminal además de causar daño directo sobre los vermes (Burke & Miller, 2020). Una fuente de estas sustancias es *Sericea lespedeza*, una leguminosa perenne proveniente del este de Asia y bien adaptada a las condiciones del sudeste de los Estados Unidos (Athanasiadou *et al.*, 2007; Terrill & Whitley, 2018). Puede ofrecerse al pequeño rumiante en forma de pasto, heno o pellets (Terrill & Whitley, 2018). La variedad más empleada es *AUGrazer®*, fruto de un programa de mejora genética enfocado a optimizar su tolerancia al pastoreo y su valor nutricional (Terrill & Whitley, 2018).

### 3.2.3 Manejo de pastos

Otras estrategias encaminadas al control de los NGI han considerado el manejo del pasto debido su papel en la transmisión de estos parásitos (Burke & Miller, 2020) y están ideadas para un mayor aprovechamiento de la superficie de pastoreo mientras se reduce la presencia de L3 en la misma (Stear, 2007).

Una opción es **mover al ganado** a pastos que no han sido ocupados por animales durante un tiempo suficiente que permita disminuir la carga potencial de L3, lo que dependerá del ambiente y la estación (Kearney *et al.*, 2016). Además, se puede **reducir el número de animales por unidad de terreno** ya que disminuye el número potencial de huevos excretados y condiciona la altura de la hierba, lo que afecta a la disponibilidad de L3 y a la posibilidad de sean ingeridas por un hospedador, respectivamente (Kearney *et al.*, 2016; Stear *et al.*, 2007). No obstante, estas dos medidas podrían resultar de poca rentabilidad para el ganadero o incluso inviables si no se dispone de suficiente espacio (Stear *et al.*, 2007).

*El pastoreo “en celdas” propone reducir áreas de pastoreo y tiempo de uso, reduciéndose los FEC*

Para aprovechar mejor las superficies disponibles se ha propuesto realizar **pastoreo rotacional**, esto es, dividir el terreno en secciones e ir rotando a los animales cada cierto tiempo para que cuando regresen al principio un alto porcentaje de larvas infectantes ya hayan muerto al no encontrar hospedador (Stear *et al.*, 2007). Las versiones aún más intensivas de este método proponen tiempos de pastoreo más cortos, a densidades de ganado alto en áreas más pequeñas (**pastoreo “en celdas”**), estrategias que han resultado efectivas para reducir el FEC, la presencia de *H. contortus* en el rebaño, (Colvin *et al.*, 2008; Ruiz-Huidobro *et al.*, 2019) y mejorar el hematocrito (Colvin *et al.*, 2008).

También es efectivo **combinar o alternar en el mismo terreno dos especies de ganado** aprovechando la especificidad de algunos géneros o especies de NGI por su hospedador (Burke & Miller, 2020). Por ejemplo, el pastoreo simultáneo de vacas y ovejas en zonas tropicales de Brasil redujo los recuentos de huevos de *Haemonchus* spp. en estas últimas (Brito *et al.*, 2013). Asimismo, algunas encuestas realizadas en explotaciones equinas del norte y este de Francia apuntaron que los recuentos de huevos de estróngilos en potros se reducían a la mitad en aquellas granjas donde compartían el terreno con vacas (Forteau *et al.*, 2020). Otros autores han descrito que el pastoreo mixto de vacas y ovejas puede promover incrementos en las curvas de lactación y en el crecimiento de los corderos junto a valores más altos de hematocrito (Mahieu & Aumont, 2009).

A la luz del cambio climático, resulta evidente que para un uso efectivo y sostenible de esta estrategia es necesario conocer los factores climatológicos (temperatura, humedad, lluvia) y los patrones estacionales que condicionan la disponibilidad de larvas infectantes en los pastos (Kearney *et al.*, 2016). Actualmente se están realizando estudios detallados de zonas concretas del globo para obtener datos acerca de la variabilidad de estos factores de cara a elaborar **modelos predictivos del riesgo de transmisión** de los parásitos (Rose Vineer *et al.*, 2020; Wang *et al.*, 2021).

### 3.2.4 Control biológico

Los nematodos tienen una gran variedad de organismos predadores en el medio, algunos de los cuales se han postulado como estrategias interesantes para el sector ecológico (Stear *et al.*, 2007; Szewc *et al.*, 2021). Los enemigos naturales actúan reduciendo directamente la disponibilidad de estadios de vida libre en el pasto (mediante la ingestión de huevos o larvas) o indirectamente (modificando las condiciones del suelo o por liberación de metabolitos tóxicos) (Szewc *et al.*, 2021). Entre las estrategias más prometedoras figuran los **hongos y ácaros nematófagos, los escarabajos peloteros, las lombrices de tierra y los nematodos predadores** (Szewc *et al.*, 2021). La mayoría de estos métodos no han llegado a sacarse al mercado debido a que algunos de estos organismos son susceptibles de ser predados por otros presentes en el medio o porque a su vez perjudican a nematodos no parásitos de vida libre y se temen las consecuencias ecológicas a largo plazo (Szewc *et al.*, 2021). No obstante, uno de ellos sí que se ha materializado en un producto comercial: el hongo nematófago *Duddingtonia flagrans*.

*Duddingtonia flagrans*  
es un hongo  
comercializado como  
**BioWorma® que**  
**sobrevive al tránsito**  
**digestivo y que se**  
**alimenta de los estadios**  
**larvarios en las heces**

*Duddingtonia flagrans* se vende comercialmente bajo el nombre de *BioWorma®* en forma de clamidiosporas que se suministran al ganado con el alimento (*Bioworma*, 2022). Las esporas sobreviven al paso por el tracto gastrointestinal y germinan en las heces depositadas en el medio, donde generan redes tridimensionales en el micelio para atrapar a los estadios larvarios (Ingale *et al.*, 2010). Este proceso no daña al hospedador ni a otros organismos saprofitos presentes en el

suelo (Knox *et al.*, 2002; Stear *et al.*, 2007; Szewc *et al.*, 2021). Su potencial como agente de control biológico ha sido demostrado en ganado vacuno, ovejas, cerdos y caballos (Gómez-Rincón *et al.*, 2006; Healey *et al.*, 2018; Ingale *et al.*, 2010; Szewc *et al.*, 2021). Además, no pierde efectividad cuando se combina con tratamientos antihelmínticos o con partículas de óxido de cobre para producir efectos aditivos sobre el control de parásitos (Healey *et al.*, 2018; Kearney *et al.*, 2016). El mayor inconveniente es que las esporas deben darse junto con el alimento todos los días, lo que podría soslayarse si administraran mediante dispositivos de liberación lenta o en bloques alimenticios (Ingale *et al.*, 2010; Stear *et al.*, 2007).

### 3.2.5 Selección genética

En la naturaleza, las cargas de parásitos se distribuyen de forma desigual entre los hospedadores y en muchos casos solo una pequeña parte de estos alberga el mayor número de vermes o libera mayor cantidad de huevos (Stear *et al.*, 2011; van Wyk *et al.*, 2006) debido a que algunos animales del rebaño muestran la capacidad de resistir las parasitaciones mediante la regulación el ciclo de vida de los parásitos, reduciendo carga, longitud y/o fecundidad (**resistencia**) e incluso pueden mantener niveles aceptables de salud y productividad sin mostrar signos de infección (**resiliencia**) (Bisset *et al.*, 2001), fundamentalmente debido a diferencias en la eficiencia de la respuesta inmune frente a estos parásitos (Stear *et al.*, 2011). Esta ventaja puede ser transmitida a la descendencia, formando parte de la selección natural en las poblaciones silvestres (Burke & Miller, 2020; Guo *et al.*, 2016). A la luz de la resistencia antihelmíntica, la selección en granja de animales resistentes se ha empleado para dar lugar a rebaños que contaminarían menos el pasto y necesitarían menos tratamientos químicos (Burke & Miller, 2020; Kearney *et al.*, 2016).

La resistencia y resiliencia se presentan entre y dentro de razas e incluso entre linajes (Bisset *et al.*, 2001; Woolaston & Baker, 1996). Algunos ejemplos de **razas de ovejas resistentes** a los NGI son: Barbados Black Belly (Gruner *et al.*, 2003), Churra (Martínez-Valladares *et al.*, 2005), Dorper (Mugambi *et al.*, 1997), Garole (Lalramhluna *et al.*, 2020), Gulf Coast Native (Bahirathan *et al.*, 1996), Red Massai (Mugambi *et al.*, 1997), Santa Inés (Amarante *et al.*, 2004), St. Croix (Stear & Murray, 1994) y Canaria de Pelo (González *et al.*, 2008), entre otras (Piedrafita *et al.*, 2010). A diferencia de lo que ocurre con razas comerciales que requieren un alto mantenimiento, las resistentes suelen ser razas indígenas poco caracterizadas, muy adaptadas a las condiciones locales donde sobreviven en presencia de altas cargas de parásitos y recibiendo escasos tratamientos antihelmínticos (Bahirathan *et al.*, 1996; Piedrafita *et al.*, 2010). Estos animales se han seleccionado de forma natural de acuerdo con su capacidad de desarrollar mecanismos de resistencia natural diferentes a los que desarrollan ovejas de otras razas comerciales y no necesariamente en base a caracteres productivos (González *et al.*, 2011; Hernández *et al.*, 2017, 2020; Piedrafita *et al.*, 2010). Esto las ha posicionado como una herramienta para estudiar la relación parásito-hospedador a través de modelos de estudio *in vitro*, el testaje de vacunas o la búsqueda de genes marcadores de resistencia (González *et al.*, 2019; Machín *et al.*, 2021; Piedrafita *et al.*, 2010). Aunque estas razas son muy explotadas en su contexto e incluso llegan a ser más productivas en estas zonas que las foráneas debido a su gran adaptación al medio (Woolaston & Baker, 1996), la sustitución o el cruce no siempre son bien acogidos entre los ganaderos ya que podría tener efectos negativos sobre los objetivos de selección y la rentabilidad de algunas razas (Bisset *et al.*, 2001; Woolaston & Baker, 1996).

*Las razas locales resistentes a los parásitos son un infrautilizado recurso biotecnológico para entender la respuesta inmune protectora y para mejorar prototipos vacunales, entre otros posibles usos*

comerciales que requieren un alto mantenimiento, las resistentes suelen ser razas indígenas poco caracterizadas, muy adaptadas a las condiciones locales donde sobreviven en presencia de altas cargas de parásitos y recibiendo escasos tratamientos antihelmínticos (Bahirathan *et al.*, 1996; Piedrafita *et al.*, 2010). Estos animales se han seleccionado de forma natural de acuerdo con su capacidad de desarrollar mecanismos de resistencia natural diferentes a los que desarrollan ovejas de otras razas comerciales y no necesariamente en base a caracteres productivos (González *et al.*, 2011; Hernández *et al.*, 2017, 2020; Piedrafita *et al.*, 2010). Esto las ha posicionado como una herramienta para estudiar la relación parásito-hospedador a través de modelos de estudio *in vitro*, el testaje de vacunas o la búsqueda de genes marcadores de resistencia (González *et al.*, 2019; Machín *et al.*, 2021; Piedrafita *et al.*, 2010). Aunque estas razas son muy explotadas en su contexto e incluso llegan a ser más productivas en estas zonas que las foráneas debido a su gran adaptación al medio (Woolaston & Baker, 1996), la sustitución o el cruce no siempre son bien acogidos entre los ganaderos ya que podría tener efectos negativos sobre los objetivos de selección y la rentabilidad de algunas razas (Bisset *et al.*, 2001; Woolaston & Baker, 1996).

*Actualmente hay programas de selección que incluyen la resistencia a parásitos como criterio en varios países, que recurren a los FEC como método de selección*

De esta manera, los programas de selección actuales de ovejas, carneros y corderos desarrollados en algunos países como Australia, Estados Unidos, Francia, Nueva Zelanda o Reino Unido están basados en la selección dentro de raza (Aguerre *et al.*, 2018; Bisset *et al.*, 2001; Burke & Miller, 2020; Morris *et al.*, 1995;

Vanimisetti *et al.*, 2004). Para ello es necesario definir qué carácter se quiere mejorar, cómo se va a medir y cuánto de la expresión fenotípica de dicho carácter se debe a la variación genética y no a factores ambientales (es decir, su “heredabilidad” o “ $h^2$ ”). La mayoría de los programas han incorporado el FEC como objetivo de selección ya que se trata de un carácter moderadamente heredable ( $h^2 = 0,1-0,35$ ) que puede evaluarse mediante métodos relativamente sencillos (Aguerre *et al.*, 2018; Bishop *et al.*, 1996; Woolaston & Baker, 1996). Aun así, este parámetro presenta considerables limitaciones, dado que los animales candidatos deben ser expuestos a parásitos para que la resistencia pueda manifestarse y contrastarse dentro del rebaño, lo que suscita la preocupación de que se produzcan efectos negativos en el rendimiento de los animales candidatos, especialmente en los corderos jóvenes (Dominik, 2005; Woolaston & Baker, 1996). Además, la imposibilidad de determinar el género de los nematodos, la baja representación de la carga de los nematodos menos prolíficos y la distribución desigual de los huevos en la masa fecal son también aspectos preocupantes (Hunt & Lello, 2012; Preston *et al.*, 2014). Asimismo, el pepsinógeno y el hematocrito también se han explorado para reflejar de forma indirecta la presencia de nematodos gástricos y hematófagos, respectivamente, pero son de menor utilidad en parasitaciones mixtas con vermes intestinales o histotróficos (Stear *et al.*, 2007).

Por otra parte, se han propuesto algunos **marcadores inmunitarios** basados en componentes clave en la respuesta frente a NGI. Mayores recuentos de eosinófilos en sangre periférica se han asociado con un menor FEC en corderos, pero solo a partir de 3 meses de edad y si han estado expuestos de forma continua a NGI (Stear *et al.*, 2002). Asimismo, la producción de IgA específica frente al estadio L4 está relacionada con interferencias en el crecimiento del parásito, afectando a la longitud de los vermes adultos y su fecundidad (Strain *et al.*, 2002) y, por

tanto, a la excreción de huevo en heces (Fairlie-Clarke *et al.*, 2019). Recientemente se identificó que la producción de IgA específica frente a un antígeno glucídico que se encuentra en la superficie de las L3 de tricoestrongílicos (*carbohydrate surface larval antigen*, *CarLA*) se correlaciona negativamente con el FEC y se asocia positivamente con el peso vivo (Shaw *et al.*, 2012, 2013). La IgA anti-*CarLA* es además un rasgo moderadamente heredable ( $h^2 = 0,15-0,27$ ) y repetible a partir de los 5 meses de edad que puede aislarse en hisopos de saliva, muestra biológica que está siempre disponible y puede ser recogida por parte de personal no especializado (de la Chevrotière *et al.*, 2012; Shaw *et al.*, 2012). El marcador se ha probado en rebaños de vacas, ciervos y ovejas aunque sólo ha sido comercializado en Nueva Zelanda como un kit para la selección de corderos después del destete (Mackintosh *et al.*, 2014; Merlin *et al.*, 2017; Shaw *et al.*, 2013). Si bien hay varios marcadores basados en la inmunidad, la interpretación de los mismos puede resultar complejo porque forman parte de la dinámica en la relación-parásito hospedador y a menudo varían durante el desarrollo cronológico de la respuesta inmunitaria (Bishop, 2012).

En este sentido, identificar **marcadores genéticos** responsables de la variación fenotípica permitiría seleccionar animales independientemente del estado de infección o la edad (Sweeney *et al.*, 2016). Dado que la complejidad del sistema biológico apunta a que los mecanismos que dan lugar a resistencia responden a un efecto poligénico en lugar de al de un solo gen (Dominik, 2005; Sweeney *et al.*, 2016), se han estudiado regiones del genoma asociadas a un rasgo fenotípico cuantificable (*Quantitative trait locus- QTL*) y variaciones de una sola base en la secuencia genómica (*Single-nucleotide polymorphism- SNP*) que pudieran contribuir a la variación fenotípica en la respuesta a las infecciones por NGI. De esta forma se identificaron una gran variedad de QTL con variaciones entre razas, especie de nematodo, diseño experimental, el tipo de análisis, etc. (Dominik, 2005). Esto resalta la complejidad intrínseca de estos caracteres fenotípicos y la dificultad de analizarlos (Dominik, 2005). Si bien, el estudio pormenorizado de

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genes ha destacado algunas regiones muy interesantes por su relación con el control de NGI, situadas en la vecindad de los genes del MHC-II y del interferón- $\gamma$  (IFN- $\gamma$ ) (Atlija *et al.*, 2015, 2016; Stear *et al.*, 2009). El MHC-II es una molécula relacionada con la presentación de antígeno y la activación de los linfocitos T CD4 $^{+}$ , los cuales están implicados en la respuesta protectora frente a nematodos (Abbas *et al.*, 1999; Gill *et al.*, 1993). Por su parte, el IFN- $\gamma$  es una citoquina clave para dirigir la diferenciación de los linfocitos T hacia el fenotipo Th1 – relacionado con respuestas a patógenos intracelulares- o Th2- más vinculados a la inmunidad frente a helmintos (Spellberg & Edwards, 2001; Stear *et al.*, 2009).

En los últimos años, la investigación en esta área se ha dirigido hacia la caracterización de las vías metabólicas implicadas en la respuesta al parasitismo. Esta aproximación ha venido de la mano de los avances en el ámbito de las “ómicas” (Vailati-Riboni *et al.*, 2017), concretamente, de la transcriptómica. El transcriptoma es el conjunto completo y la cantidad de transcripciones en una célula (ARN) en una etapa de desarrollo o condición fisiológica específica (Z. Wang *et al.*, 2009). Los *microarrays* y la secuenciación de ARN han permitido estudiar el transcriptoma e identificar y cuantificar los genes expresados con el fin de reconocer interacciones únicas y rasgos cuantitativos complejos como es la resistencia a parásitos (Aboshady *et al.*, 2022; Li *et al.*, 2012). Así se han realizado estudios comparativos para determinar los procesos biológicos diferencialmente expresados entre grupos de individuos resistentes y susceptibles, que generalmente suelen estar relacionados con mecanismos de la respuesta inmune o bien, con su desarrollo temporal. Por ejemplo, corderos Scottish Blackface con bajo FEC desarrollan una respuesta inmune orientada hacia helmintos más rápidamente que aquellos animales con recuentos más altos (McRae *et al.*, 2016). Esta información permitiría establecer perfiles asociados con la resistencia a NGI que se puedan aplicar en programas de selección dentro de raza (Aboshady *et al.*, 2019; Ahmed *et al.*, 2015).

### 3.2.6 Vacunas

La inmunización consiste en exponer moléculas específicas de un patógeno con capacidad infectiva reducida o nula (vacuna) al sistema inmune para generar una respuesta de memoria frente a esas moléculas cuando el organismo se vuelva a exponer al patógeno (Meeusen & Piedrafita, 2003). La vacunación persigue reducir los efectos de la infección, limitar la difusión del agente, generar protección duradera y sin dejar residuos en el organismo, lo que la ha convertido en una de las opciones atractivas para el control sostenible de enfermedades, entre

*El diseño de vacunas frente a helmintos en comparación con otros agentes se ha visto dificultado por la complejidad de éstos y de la relación con su hospedador*

ellas para el control de NGI (Matthews *et al.*, 2016; Nisbet *et al.*, 2016). No obstante, el diseño de un prototipo vacunal efectivo frente a NGI requiere un conocimiento detallado del agente y de las respuestas que origina y, precisamente, uno de los mayores obstáculos en el desarrollo de vacunas frente a estos agentes en comparación con otros microorganismos ha sido la **complejidad estructural de los helmintos y de la relación parásito-hospedador** (Meeusen & Piedrafita, 2003). Para que las vacunas frente a helmintos sean competitivas con respecto al uso de antihelmínticos éstas tendrían que poder **distribuirse por todo el mundo** y ofrecer una **protección duradera, ser eficaces en animales jóvenes y frente a más de una especie de parásitos** (Matthews *et al.*, 2016). Aunque no es probable que la efectividad de las vacunas alcance la que tienen los fármacos al salir al mercado, se estima que una **eficacia alrededor del 65%** ya ofrecería ventajas substanciales para el control de los NGI (Matthews *et al.*, 2016; Nisbet *et al.*, 2016).

El primer ejemplo de vacuna comercial frente a helmintos parásitos fue *Dictol*® (ahora comercializada como *Bovilis Huskvac*®, *MSD Animal Health*), que induce altos niveles de protección en terneros frente al verme pulmonar *Dictyocaulus viviparus* mediante inoculaciones con L3 de infectividad reducida mediante irradiación (**vacunas de larvas irradiadas**). Los intentos de aplicar esta metodología para inmunizar a animales frente a NGI produjeron algunos resultados protectores en ovejas adultas, pero no así en corderos en condiciones de campo (Smith & Jackson, 1982; Smith & Angus, 1980; Urquhart *et al.*, 1966; Nisbet *et al.*, 2016). Esta

falta de eficacia sumada a la necesidad de mantener animales infectados para producir larvas, la limitada vida útil y dificultad de almacenamiento de las vacunas de larvas irradiadas probablemente condicionaran que la vacunas de larvas irradiadas para el control de NGI no alcanzaran el mercado (Gilleard *et al.*, 2021).

Posteriormente, la estrategia se centró en la búsqueda de proteínas del parásito que fueran reconocidos por los mecanismos inmunes del hospedador durante la infección (**antígenos “naturales”**) debido a que son parte estructural del cuerpo del parásito (**antígenos somáticos**) o productos liberados por los distintos estadios (**antígenos o productos de excreción/secreción (E/S)**). También existen otros antígenos que, aunque normalmente no se expongan al sistema inmune (**antígenos “ocultos”**), pueden utilizarse como dianas estratégicas para la inmunización.

Dos ejemplos de **antígenos “ocultos”** son las glicoproteínas H-Gal-GP y H-11, localizadas en el borde en cepillo de las células intestinales de los estadios L4 y adulto, que inducen de altos niveles de protección frente a *H. contortus* (Knox *et al.*, 2005; Smith *et al.*, 2001). Los múltiples resultados prometedores con estas moléculas derivaron en la comercialización de la vacuna *Barbervax®* en el año 2014 en Australia, actualmente ya disponible en Reino Unido bajo preinscripción veterinaria y en Sudáfrica con el nombre de *Wirevax®* (<https://barbervax.com/>). Numerosos ensayos vacunales en animales adultos, ovejas al periparto e incluso corderos al destete han demostrado que los animales vacunados presentan una reducción de hasta un 80% en el FEC y tienen niveles de hematocrito significativamente más altos que los animales control, lo que contribuye a reducir la contaminación de los pastos y los síntomas clínicos de la hemoncosis (Benavides *et al.*, 2015; Kebeta *et al.*, 2020, 2021; LeJambre *et al.*, 2008; Smith, 2014; Teixeira *et al.*, 2019). La protección está basada en la generación de altos niveles de IgG circulante en el plasma del hospedador que son ingeridos por el parásito al tomar sangre, y al llegar a las células intestinales bloquean la acción proteasa de las proteínas diana, afectando a la digestión de nutrientes

*Barbervax® es la primera vacuna comercializada frente a Haemonchus contortus en ovejas, protegiendo a animales jóvenes y adultos*

(Newton & Meeusen, 2003). No obstante, para mantener niveles altos de anticuerpos circulantes durante la época de mayor incidencia de parásitos es necesario dar 3 dosis iniciales cada 3-4 semanas y 5 revacunaciones, debido a que no se produce una estimulación del mismo mediante el contacto con el parásito en el pasto al tratarse de antígenos no expuestos al sistema inmune de forma natural (Kebeta *et al.*, 2020; Nisbet *et al.*, 2016b). También se han identificado antígenos homólogos a éstos en *T. circumcincta* pero no existe protección cruzada con esta vacuna, probablemente porque este parásito histotrófico no ingiere suficientes cantidades de sangre (Smith *et al.*, 2001b). Las limitaciones de esta vacuna son similares a las detalladas anteriormente para las vacunas de larvas irradiadas dado que se necesita obtener estos antígenos de vermes adultos, dificultando las garantías de uniformidad, esterilidad, preservación y la distribución mundial de la vacuna (Claerebout & Geldhof, 2020).

Por otro lado, la proteína de superficie de L3 de *H. contortus* (*HcsL3*) es un ejemplo de **antígeno somático y natural**. La inmunización con *HcsL3* en combinación con hidróxido de aluminio como adyuvante redujo un 64% el FEC en comparación con los controles e indujo niveles altos de IgG<sub>1</sub>, IgG<sub>2</sub> e IgA aunque sin correlación con la protección, probablemente debido a que ésta se relacionaba con mecanismos inmunidad celular (Jacobs *et al.*, 1999). Estudios posteriores usando el adyuvante DEAE-Dextrano consiguieron duplicar los niveles de protección en comparación con el hidróxido de aluminio, produciendo aumentos de la IgG<sub>2</sub> y la IgE sérica (Piedrafita *et al.*, 2013). Tras realizar pruebas de hipersensibilidad retardada mediante inoculaciones intradérmicas de L3 desenvainadas se demostró una atracción masiva de eosinófilos al tejido, lo que es característico de una respuesta inmunitaria tipo 2 y evidencian el papel clave de la inmunidad celular en la respuesta a esta vacuna. Estudios recientes trataron de generar protección en corderos al destete de raza Merino mediante la combinación de *Barbervax®* y el antígeno *HcsL3* bajo la premisa de que la inmunidad natural generada por la exposición a L3 potenciaría la respuesta a *HcsL3*, reduciendo el número de revacunaciones con *Barbervax®* (Kebeta *et al.*, 2022). Sin embargo, la vacuna *HcsL3* no redujo el FEC y la administración conjunta con *Barbervax®* tampoco mejoró la eficacia ni la duración de la protección. Estos resultados resaltan en su conjunto la importancia de seleccionar el adyuvante

adecuado para dirigir y potenciar las respuestas adaptativas frente a NGI y la dificultad de obtener protección en animales jóvenes, quizás por su escasa inmunocompetencia.

***La falta de respuesta al antígeno HcsL3 y a las proteínas de E/S ejemplifican la dificultad para inducir protección vacunal en corderos jóvenes***

En este sentido, la inmunización con **productos de excreción/secreción (E/S)** de *H. contortus* con proteínas de entre 15 y 24 kDa en corderos de 9, 6 y 3 meses redujo la carga de parásitos entre un 77-83% en animales de mayor edad pero no en los de 3 meses (Kooyman *et al.*, 2000; Vervelde *et al.*, 2001). Los tres grupos etarios demostraron aumento de inmunoglobulinas (IgA, IgG1 e IgE), y de los recuentos de eosinófilos en sangre y de mastocitos en tejido abomasal. Si bien, este incremento fue mayor en corderos de 9 y 6 meses, sugiriendo que la falta de protección en los corderos más jóvenes se debía a una menor intensidad de la respuesta frente al parásito tras la vacunación.

Uno de los puntos claves para la producción a gran escala de las vacunas ha sido la **tecnología recombinante**, que permite el aislamiento y modificación de genes individuales para reintroducirlos en sistemas relativamente sencillos como virus, bacterias, levaduras o helmintos que expresen estos genes a nivel de ARN o de proteínas (Gilleard *et al.*, 2021; Khan *et al.*, 2016). Este fue el caso del antígeno somático de vermes adultos de *H. contortus* Hc23, que en su forma nativa logró reducir un 70 % y un 60% el FEC y la carga de vermes, respectivamente (Fawzi *et al.*, 2014) y recientemente se ha conseguido expresar como antígeno recombinante en *Escherichia coli* (rHc23) (González-Sánchez *et al.*, 2018). La inmunización con rHc23 redujo el 80% del FEC y el 70% de la carga de vermes, posicionando a este antígeno como un candidato interesante para el control de *H. contortus*.

En 2013, un grupo escocés publicó datos prometedores obtenidos en ensayos con ovejas adultas al periparto y corderos mediante la vacunación con un prototipo basado en un "cóctel" de proteínas recombinantes frente a *T. circumcincta* (Liu *et al.*, 2022; Nisbet *et al.*, 2013, 2019; Nisbet *et al.*, 2016a). La vacuna se desarrolló a partir de la identificación de antígenos liberados por los estadios larvarios que fueron reconocidos por la IgA de ovejas inmunes, así como

también un antígeno de superficie y proteínas con potencial papel inmunosupresor (Ellis *et al.*, 2014; Nisbet *et al.*, 2013) (Tabla 2).

**Tabla 2. Descripción de los 8 antígenos que componen el prototipo de vacuna recombinante frente a *T. circumcincta* (datos obtenidos de Nisbet *et al.*, 2013 y Nisbet *et al.*, 2019).**

	Nombre	Función	Tipo de antígeno	Estadio	Criterio de inclusión en la vacuna	Sistema de expresión	Incluida en el cóctel simplificado
Tci-APY-1	<i>Calcium-dependent apyrase-1</i>	Apirasa dependiente de la activación por calcio	Excreción/Secreción	Estadios larvarios	Molécula potencialmente inmunosupresora	<i>Escherichia coli</i>	Sí
Tci-ASP-1	<i>Activation-associated secretory protein-1</i>	Proteína secretada asociada a la activación	Excreción/Secreción	L4	Antígeno reconocido por la IgA	<i>Escherichia coli</i>	No
Tci-CF-1	<i>Cathepsin F-1</i>	Proteasa catepsina F-1	Excreción/Secreción	L4	Antígeno reconocido por la IgA	<i>Pichia pastoris</i>	No
Tci-ES20	<i>20 kDa protein</i>	Proteína de 20 kDa de función desconocida	Excreción/Secreción	Estadios larvarios	Antígeno reconocido por la IgA	<i>Pichia pastoris</i>	No
Tci-MEP-1	<i>Astacin-like metalloproteinase -1</i>	Metaloproteinasa similar a la astacina	Excreción/Secreción	Estadios larvarios	Antígeno reconocido por la IgA	<i>Escherichia coli</i>	Sí
Tci-MIF-1	<i>Macrophage migration inhibitory factor-1</i>	Factor inhibitorio de la migración de macrófagos	Excreción/Secreción	L3	Molécula potencialmente inmunosupresora	<i>Escherichia coli</i>	No
Tci-SAA-1	<i>Immunogenic homologue of a protective antigen from Ancylostoma caninum</i>	Homólogo antigénico de un antígeno protector de <i>Ancylostoma caninum</i>	Somático	L3	Homólogo de un antígeno protector	<i>Escherichia coli</i>	No
Tci-TGH-2	<i>Transforming growth protein 2-like protein</i>	Homólogo del Factor de Crecimiento Transformante Beta (TGF-β)	Excreción/Secreción	Estadios larvarios	Molécula potencialmente inmunosupresora	<i>Escherichia coli</i>	No

Los primeros ensayos se realizaron en corderos que recibieron 3 dosis separadas por 3 semanas: los animales vacunados recibieron el cóctel vacunal en combinación con el adyuvante Quil A mientras que a los controles se les administraron inyecciones de PBS y Quil A. Coincidiendo con la última vacunación se iniciaron las inoculaciones con 2000 L3 de *T. circumcincta* 3 días a la semana durante 4

semanas. Hacia la mitad de las inoculaciones experimentales se empezaron a recoger muestras de heces para el recuento de huevos. Se realizaron dos ensayos en corderos, el primero con animales de 204-206 días (6-7 meses) y el segundo con corderos 172-178 días (5-6 meses) en los que la vacuna redujo el FEC un 70% y un 58% y la carga de vermes en el abomaso un 75% y un 56% en el primer y segundo ensayo, respectivamente. Estos resultados fueron muy parecidos a los obtenidos en **ovejas al periparto**, donde se logró reducir un 45% el FEC a lo largo de la infección experimental (Nisbet *et al.*, 2016a). A partir de este punto se trabajó en la **simplificación del cóctel vacunal** para reducir los costes de producción del prototipo y mejorar sus probabilidades de comercialización. Para ello testaron un nuevo prototipo usando solamente las dos proteínas mejor reconocidas por parte del hospedador -Tci-APY-1 y Tci-MEP-1, logrando obtener porcentajes de eficacia vacunal muy similares a los que ofrecía el prototipo original (Nisbet *et al.*, 2019). Aunque los resultados de todos estos ensayos obtenidos mediante este ambicioso enfoque no tienen precedente en la bibliografía, los datos sugerían una **alta variabilidad individual** en la respuesta frente a la vacuna que era especialmente manifiesta en los animales más jóvenes.

A raíz de estos hallazgos se desarrolla el ensayo objeto de esta tesis doctoral, con la finalidad de comprobar la eficacia de esta vacuna en corderos menores de 3 meses de edad pertenecientes a las razas ovinas con diferencia en su resistencia a nematodos gastrointestinales, Canaria y Canaria de Pelo y caracterizar su respuesta inmune humoral y celular para recopilar información que ayude a optimizar este prototipo vacunal.

*El cóctel vacunal frente a *T. circumcincta* redujo la excreción de huevos y la carga de vermes pero la respuesta de los corderos tuvo una alta variabilidad individual*



# Resumen



# **Resumen**

Las infecciones por nematodos gastrointestinales son un grave inconveniente al que se atribuyen pérdidas millonarias cada año en la producción de pequeños rumiantes, siendo *Teladorsagia circumcincta* el nematodo más prevalente en las zonas templadas del globo. La expansión de la resistencia a los antihelmínticos ha catalizado el desarrollo de estrategias alternativas o complementarias al control químico. Las vacunas y la resistencia genética se han postulado como alternativas de control sostenible, ya que la adaptación de los helmintos a los mecanismos inmunitarios del hospedador se desarrolla a un ritmo mucho más lento que la resistencia a los antihelmínticos.

En el primer capítulo se realizó la vacunación con prototipo de vacuna recombinante frente a *T. circumcincta* en corderos de 3 meses de las razas Canaria y Canaria de Pelo. Los corderos de la raza Canaria no fueron protegidos por la inmunización. Por su parte, los corderos de la raza Canaria de Pelo respondieron favorablemente a la vacunación, que logró reducir los parámetros parasitológicos e inducir una fuerte respuesta humoral y celular frente al parásito. Además, la vacunación mejoró el crecimiento de los corderos durante la infección con el parásito.

En el segundo capítulo se analizó la expresión génica en el ganglio linfático abomasal de los animales vacunados de ambas razas y se pudo comprobar que los corderos de la raza Canaria de Pelo presentaron una mayor expresión de genes relacionados con respuestas inmunitarias orientadas a la protección frente a nematodos gastrointestinales en comparación con los corderos de la raza Canaria, que tuvieron una mayor expresión de genes asociadas con respuestas frente a organismos intracelulares y procesos de tolerancia inmunológica, lo que respaldaría los resultados obtenidos mediante la vacunación en cada raza.

En el tercer capítulo se estudiaron las diferencias en la susceptibilidad a la infección con *T. circumcincta* entre los corderos controles de ambas razas y no se encontraron diferencias entre

ellas, aunque sí se halló una considerable variabilidad individual independientemente de la raza, explorándose entonces la relación entre las variables parasitológicas, inmunológicas y la expresión génica del ganglio linfático abomasal. El análisis transcriptómico demostró que los animales con el menor recuento de huevos y carga de parásitos tenían una mayor activación de vías inmunitarias relacionadas con el control de nematodos gastrointestinales, sugiriendo que algunas de estas vías podrían intentar validarse como marcadores en un futuro para la selección de animales resistentes.

# Summary



# *Summary*

Gastrointestinal nematode infections are a serious problem that cause millions of euros in losses in the small ruminant sector every year, with *Teladorsagia circumcincta* being the most prevalent nematode in temperate areas of the globe. The spread of anthelmintic resistance has accelerated the development of alternative or complementary strategies to chemical control. Vaccines and genetic resistance have been postulated as sustainable control alternatives, as helminth adaptation to host immune mechanisms develops at a much slower pace than anthelmintic resistance.

In the first chapter, vaccination with a prototype recombinant vaccine against *T. circumcincta* was carried out in 3-month-old Canaria Sheep (CS) and Canaria Hair Breed (CHB) lambs. CS lambs were not protected by immunisation. On the other hand, CHB lambs responded favourably to vaccination, which reduced parasitological parameters and induced a strong humoral and cellular response against the parasite. In addition, vaccination improved the growth in CHB lambs during infection with the parasite.

In the second chapter, gene expression in the abomasal lymph node of vaccinated animals of both breeds was analysed. The CHB lambs had a higher expression of genes related to immune responses oriented to protection against gastrointestinal nematodes compared to CS lambs, which had a higher expression of genes associated with responses against intracellular organisms and immune tolerance processes, supporting the results obtained by vaccination in each breed.

In the third chapter, differences in susceptibility to *T. circumcincta* infection between control lambs of both breeds were studied and no differences were found between them, although considerable individual variability was detected in individuals across breeds. The relationship between parasitological, immunological variables and abomasal lymph node gene expression was then explored. Transcriptomic analysis showed that animals with the lowest egg

counts and worm burden had a higher activation of immune pathways involved in gastrointestinal nematode control, suggesting that some of these pathways could be candidates for resistance markers for the animal selection in the future.

# Capítulos



# *Capítulo I*

*Differences in the protection elicited by a recombinant Teladorsagia circumcincta vaccine in weaned lambs of two Canarian sheep breeds*





## Differences in the protection elicited by a recombinant *Teladorsagia circumcincta* vaccine in weaned lambs of two Canarian sheep breeds



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### ABSTRACT

Gastrointestinal nematode (GIN) infections are a serious drawback on small ruminant production. Since anthelmintic resistance has extended, optimisation of alternative non-chemical control strategies has attracted interest. Recently, a prototype recombinant vaccine protected immunologically mature sheep from Texel-cross and Canaria Sheep breeds against *Teladorsagia circumcincta*. The level of protective immunity stimulated by the vaccine varied between individuals and with age. Previous studies suggest that Canaria Hair Breed (CHB) sheep is naturally resistant to GIN infection, with some evidence suggesting that this protection is present in young lambs. Here, we sought to enhance this resistance by immunising three-month-old CHB lambs with a *T. circumcincta* prototype recombinant vaccine. Following vaccination and a larval challenge period, levels of protection against *T. circumcincta* infection were compared in CHB lambs with Canaria Sheep (CS) lambs (a breed considered less resistant to GIN). Lambs from the resistant CHB breed appeared to respond more favourably to vaccination, shedding 63% fewer eggs over the sampling period than unvaccinated CHB lambs. No protection was evident in CS vaccinated lambs. At post-mortem, CHB vaccine recipients had a 68% reduction in mean total worm burden, and female worms were significantly shorter and contained fewer eggs *in utero* compared to unvaccinated CHB lambs. A higher anti-parasite IgG<sub>2</sub> level was detected in immunised CHB lambs compared to unvaccinated control CHB animals, with data suggesting that IgA, globular leucocytes, CD45RA<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells are implicated in this protective response. The development of effective immunity in vaccinated CHB lambs did not reduce lamb growth rate as immunised CHB lambs had a significantly higher average daily weight gain after challenge than their unvaccinated counterparts. Therefore, the protection of CHB lambs was enhanced by immunisation at weaning, suggesting a synergistic effect when combining vaccination with presumed genetic resistance.

**Abbreviations:** ADG, Average daily gain; ALN, Abomasal Lymph Node; b.w, body weight; CHB, Canaria Hair Breed; CS, Canaria Sheep; ELISA, Enzyme-linked immunosorbent assay; EPG, Eggs/g of faeces; FEC, Faecal egg counts; GIN, gastrointestinal nematodes; IFN- $\gamma$ , Interferon gamma; Ig, Immunoglobulin; IL-4, Interleukin 4; IL-17A, Interleukin 17A; L3, third stage larvae; L4, fourth stage larvae; M, molar; MHC-II, Major Histocompatibility Complex II; ODI, Optical Density Index; PBS, phosphate buffered saline; SEM, standard error of the mean; SI, Stimulation Index; *T. circumcincta*, *Teladorsagia circumcincta*; Tci-APY-1, calcium-dependent apyrase-1; Tci-ASP-1, activation-associated secretory protein-1; Tci-CF-1, cathepsin F-1; Tci-ES20, 20 kDa protein of unknown function; Tci-MEP-1, astacin-like metalloproteinase-1; Tci-MIF-1, macrophage migration inhibitory factor-1; Tci-SAA-1, an homologue of a protective antigen from *Ancylostoma caninum*; Tci-TGH-2, TGF homologue.

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## 1. Introduction

Within sheep flocks, lambs are the most susceptible population to gastrointestinal nematode (GIN) infection. Generally, they are exposed to third stage larvae (L3) contaminated pasture as soon as they start grazing. However, their immune systems are still immature and therefore they are less able to control parasitic burdens. Developing a protective immune response against nematodes may require repeated contact with the parasites during the grazing season (Stear et al., 2000). Protection typically starts with a phase of hyporesponsiveness to the parasites, followed by the development of protective immunity and, finally, the expression of a mature response that prevents the development of severe disease (Greer and Hamie, 2016). Throughout the process, which has been documented as complete at around 8 months of age (Douch and Morum, 1993; Greer and Hamie, 2016), GIN can cause pathology in young individuals, resulting in economic repercussions for the sheep sector (Nieuwhof and Bishop, 2005; Lane et al., 2015; Mavrot et al., 2015). Given this, alongside the increasing emergence of anthelmintic resistance (Gilleard et al., 2021), there is a high demand for alternative methods to control GIN infections in sheep (Matthews et al., 2016).

Vaccination against GIN offers an environmentally sustainable alternative to anthelmintics but, so far, has had limited success in young lambs with reports of poor protection following immunisation in three-month-old lambs when compared to six to ten month-old animals (Kooyman et al., 2000; Vervelde et al., 2001). Indeed, it is known that younger individuals do not show evidence of immunological recognition of the parasitic antigens (McClure et al., 1998) and display weaker responses to vaccination (Winter et al., 2000; Vervelde et al., 2001), mostly ascribed to difficulties mounting secondary immune responses (McClure, 2000; Greer and Hamie, 2016; Britton et al., 2020). However, several GIN-resistant sheep breeds have shown a natural ability to control these worms at a young age (Bahirathan et al., 1996; Gruner et al., 2003; Rocha et al., 2005).

A prototype recombinant sub-unit *T. circumcincta* vaccine has been described as protective in Texel-cross lambs and in Texel-cross ewes around the periparturient period (Nisbet et al., 2013, 2016a, 2016b). It has also proven effective in 6-month-old Canaria Sheep (CS) breed, where immunisation reduced worm length and numbers of worm eggs *in utero* in vaccines (González et al., 2019). Serum IgA, IgG<sub>2</sub> and abomasal globule leucocytes and CD4<sup>+</sup> T cells may be underpinning this effect (Machín et al., 2021). However, in Canaria Hair Breed (CHB) immunisation of 6-month-old sheep did not protect vaccines, suggesting the high level of inherent resistance in CHB lambs by this age could be masking the effect of vaccination (González et al., 2019). This raises the question whether the vaccine could be protective in younger lambs of these breeds. Therefore, here we evaluated the effect of this vaccine in three-month-old CHB and CS lambs. Aspects of the immune response generated by vaccination as well as effects on lamb performance were also assessed in this study.

## 2. Materials and methods

### 2.1. Animals

The immunisation protocol was described in detail by Nisbet et al. (2013) and González et al., (2019). Three-month-old CHB ( $N = 24$ ) and CS ( $N = 24$ ) lambs were purchased from several farms located in Gran Canaria. Animals were treated on arrival with fenbendazole (Panacur 2.5%, Intervet, France) at the dosage recommended by the manufacturer (0.2 ml/kg b.w.) and were kept worm-free until the beginning of the trial. Lambs were fed with forage, feed and water ad libitum throughout the experiment. Within breed, animals were randomly assigned to vaccinated (CHB-VAC, CS-VAC) or control groups (CHB-Control, CS-Control), with each group containing 12 animals ( $N = 12$ ). During the experiment, one animal from group CHB-VAC was euthanised for

reasons not related to the experimental procedure. At the end of the trial, another animal within this group was removed due to suspected hormonal imbalance since its physical development was abnormally low (weighing 8 kg compared to the mean 26 kg of the rest of CHB lambs).

The experiment was designed according to the Spanish Legislation (RD 53/2013), approved by the Animal Welfare Ethics Committee of the Universidad de Las Palmas de Gran Canaria (OEBA\_ULPGC\_003\_2014) and subsequently ratified by the local competent authority. Local granulomas were detected in the injection site in several lambs. They were frequently examined by the designated veterinarian until they subsided without systemic consequences.

### 2.2. Experimental design

The vaccine prototype, described previously by Nisbet et al. (2013), consisted of a cocktail of 8 recombinant proteins (Tci-APY-1, Tci-ASP-1, Tci-CF-1, Tci-ES20, Tci-MEP-1, Tci-MIF-1, Tci-SAA-1 Tci-TGH-2). Tci-MEP-1 was formulated with 2 M urea in phosphate buffered saline (PBS) with 5 mg Quil A (Vax Saponin, Guinness Chemical Products Ltd) while the rest of the antigens were administered as a mixture in a single injection with 5 mg of the adjuvant in PBS. The cocktail containing 50 µg of each protein was administered three times, three weeks apart, on days 0, 21 and 42 of the experiment (Nisbet et al., 2013) in two separate sites in the anterior axilla. Concurrently, the control groups received three immunisations using the same proportions and quantities of PBS/Quil A and PBS-Urea/Quil A. Starting on day 42, coinciding with the final immunisation, sheep were trickle infected orally with 2000 L3 of *T. circumcincta* (MTCi2 strain, Weybridge, UK) three times a week, for four weeks (Nisbet et al., 2013).

### 2.3. Parasitology and performance evaluation

The protocol described in González et al. (2019) was followed. Briefly, rectal faecal samples were collected from the animals three times a week starting 14 days after the start of the L3 challenge period (i.e., day 56) until day 79. At the end of the experiment (day 82–85) animals were euthanised to perform post-mortem sampling of abomasal contents. Strongyle faecal egg counts (FEC) (expressed as eggs/g, EPG), worm burden, female worm length and its eggs *in utero* were determined using standard techniques (MAFF, 1989; González et al., 2019).

Lambs were weighed on days 8, 22, 62 and 77 of the experiment using a livestock scale (KERN EOS 150K50XL, KERN AND SOHN GmbH, Baligen, Germany). The average daily gain (ADG) was calculated for a period during the pre-challenge period (from day 8–22, ADG pre-infection) as well as for a period during the post-challenge period (from day 62–77, ADG post-infection).

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

Animals were bled from the jugular vein five days before post-mortem (day 77) (Machín et al., 2021). Antibody ELISA were carried out on serum samples (1:200) to detect serum IgA (1:8000), IgG<sub>1</sub> and IgG<sub>2</sub> (1:1000) levels specific to native somatic L3, L4 and adult *T. circumcincta* antigens (5 µg/mg) (Nisbet et al., 2010; Hernández et al., 2016; Machín et al., 2021). Further details of this protocol have been thoroughly described in Machín et al. (2021). Optical densities were transformed using the optical density index (ODI) described by Strain and Stear (2001). One point zero was added to ODI values to the prevent negative data from interfering with the statistical analysis.

### 2.5. Lymphocyte stimulation assays

Abomasal lymph nodes (ALN) were collected at post-mortem to obtain single cell suspensions. Lymphocyte stimulation assays were carried out by incubating ALN cells with L4 or adult *T. circumcincta* somatic antigen (5 µg/ml) according to the previously described method

(Machín et al., 2021). Negative (ALN+PBS) and positive (ALN+Concavalin A) controls were run in parallel to the samples. Proliferation was measured by the incorporation of methyl-<sup>3</sup>H thymidine during the final 18 h of culture and expressed as Stimulation Index (SI) by dividing the proliferation of samples incubated with *T. circumcincta* antigen by that from PBS controls.

## 2.6. Cytokine ELISA

Stimulated ALN were examined through capture ELISAs to evaluate the antigen specific secretion of interferon (IFN)- $\gamma$ , interleukin (IL)-4 and IL-17A as seen in Machín et al. (2021). To quantify the cytokines of interest, samples were diluted 1:2 for IFN- $\gamma$  or undiluted for IL-4 and IL-17A. All values were blank corrected, and concentrations determined from the standard curves included in all plates (Machín et al., 2021).

## 2.7. Phenotyping of ALN cells by flow cytometry

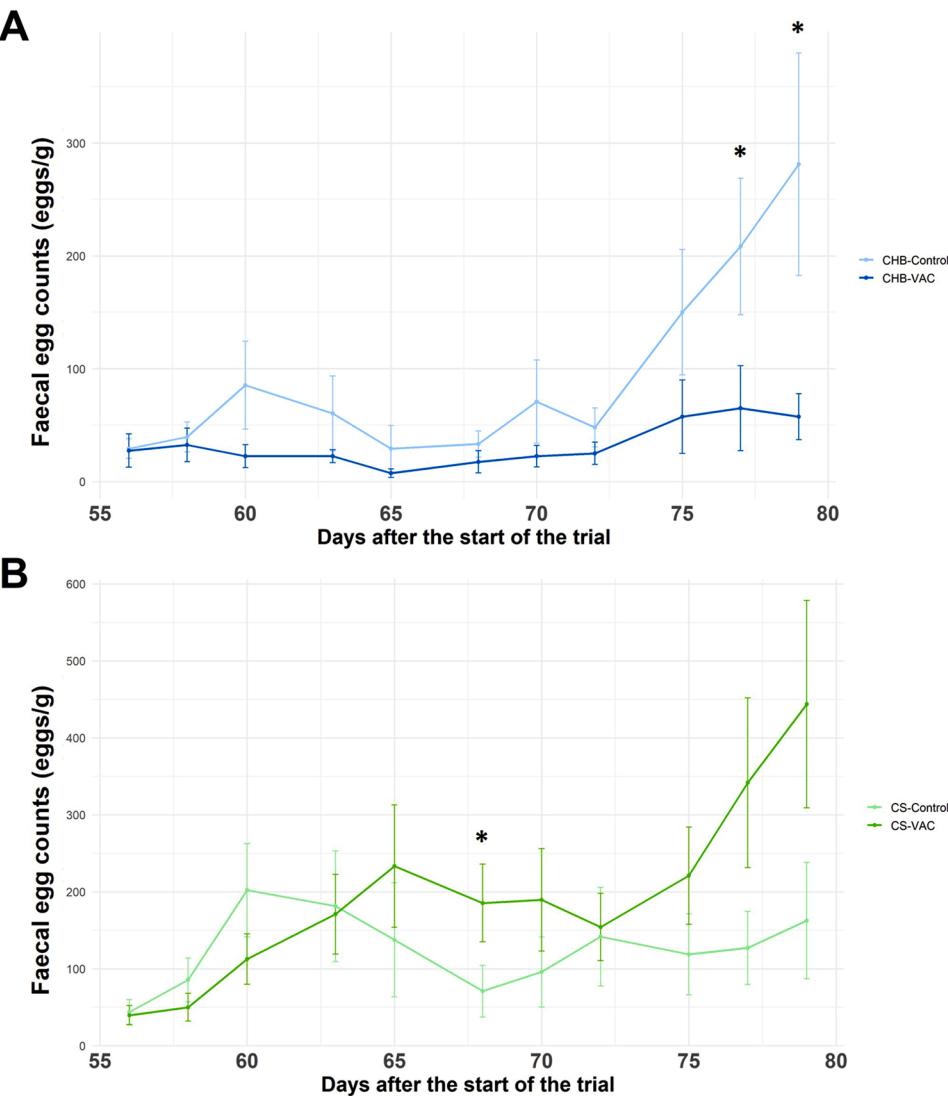
Single colour flow cytometry was carried out in resuscitated ALN cells using the monoclonal antibodies at pre-optimised concentrations using a MACSQuant® Analyzer 10 (Miltenyi Biotech, Germany) and were analysed using FlowJo vX for Windows 7 (Machín et al., 2021).

## 2.8. Histology and immunohistochemistry

Two abomasal tissue samples of the anthropiloric region were sampled to perform histological and immunohistochemical staining following previous protocols (Balic et al., 2000a; González et al., 2011; Machín et al., 2021). For each individual histological sample, cells were counted in 30 fields of 0.049 mm<sup>2</sup> at 400x magnification (40x) (Motic BA310E) and expressed as cells/mm<sup>2</sup>. Eosinophils and mast cells were counted in fields adjacent to the lamina propria while globule leucocytes were counted in the luminal margin of the mucosa. In the immunohistochemistry-stained samples, positively stained cells were counted in 20 fields of 0.06 mm<sup>2</sup> (CD4<sup>+</sup> using Olympus CX31) or 0.049 mm<sup>2</sup> (CD8<sup>+</sup>,  $\gamma\delta$ <sup>+</sup>, CD45RA<sup>+</sup> and MHC-II<sup>+</sup> using Motic BA310E) located next to the lamina propria and luminal margin of the mucosa at 400x magnification (40x).

## 2.9. Statistical analysis

Statistical analysis was performed with the IBM SPSS Statistics 24.0 programme and the R version 4.0.2 (R Core Team, 2021) and figures were also produced using the R software. FEC, cumulative FEC, worm burden, length and eggs *in utero*, immunoglobulins, mean abomasal cell counts, proliferation, cytokine data and flow cytometry data were transformed, analysed and correlated as specified in Machín et al.



**Fig. 1.** Faecal egg counts after parasite challenge in two Canarian sheep breeds vaccinated against *Teladorsagia circumcincta*. Mean FEC  $\pm$  SEM are shown for Canaria Hair Breed (CHB) (Panel A) and Canaria Sheep (CS) (Panel B) trickle infected with *T. circumcincta* L3 after the final vaccination. Vaccinated ("VAC") groups are represented by dark lines; adjuvant-recipient only ("Control") groups are represented by lighter lines. Asterisks indicate statistically significant differences ( $p < 0.05$ ) between groups for a specific time-point.

(2021). Weight data were tested for normality with the Shapiro and Wilk test and were compared through general linear model. Pre- and post-infection ADG values were analysed using the non-parametric Mann-Whitney U test. Least Significance Difference test was used as reference. Probabilities with  $p$  value  $< 0.05$  were considered statistically significant.

### 3. Results

#### 3.1. Parasitological and performance data

Lambs from all groups began to shed *T. circumcincta* eggs by day 14 after challenge (day 56 after the first vaccination) (Fig. 1A-B). Within the CHB lamb group, FEC was lower in vaccines than in controls throughout the experiment, reaching statistical significance on days 77 and 79 of the experiment ( $p < 0.05$ ) (Fig. 1 A). Both vaccinated and unvaccinated CS lambs showed similar levels of egg shedding after challenge, except on day 68 when vaccines excreted significantly more eggs than controls ( $p < 0.05$ ) (Fig. 1B). Mean  $\pm$  standard error of the mean (SEM) cumulative FECs in CHB were 711 ( $\pm 254$ ) EPG in vaccines and 1953 ( $\pm 632$ ) EPG in the control group, representing an overall 63% reduction in egg excretion in vaccinated CHB lambs ( $p < 0.05$ ). In CS, mean cumulative FECs were 4278 ( $\pm 1037$ ) EPG in vaccinated and 2905 ( $\pm 1088$ ) EPG in the control group. When comparing between breeds, CHB vaccines shed significantly fewer eggs than CS vaccines and controls ( $p < 0.05$ ).

At post-mortem, CHB-VAC lambs harboured mean worm counts of 1147 ( $\pm 374$ ) compared with 3587 ( $\pm 817$ ) in CHB-Control lambs ( $p < 0.05$ ). The adult female worms from CHB-VAC lambs were shorter than those from CHB-Control lambs ( $7.75 \pm 0.1$  mm and  $8.31 \pm 0.08$  mm, respectively) ( $p < 0.01$ ) and less prolific ( $11 \pm 0.48$  eggs in *utero* in CHB-VAC as opposed to  $17 \pm 0.1$  in CHB-Control lambs) ( $p < 0.01$ ). This represents a 62%, 70% and 36% reduction in worm burden, length and eggs in *utero*, respectively (Figs. 2A, 2B, 2C). Worm burden levels at post-mortem between CS vaccines ( $4613 \pm 652$ ) and controls ( $2900 \pm 644$ ) were not statistically different ( $p = 0.163$ ), and neither were female length ( $7.95 \pm 0.07$  vs  $7.99 \pm 0.07$ ) ( $p = 0.190$ ) or numbers of eggs in *utero* ( $15 \pm 0.6$  vs  $14 \pm 0.6$ ) ( $p = 0.506$ ) (Figs. 2A, 2B, 2C). When comparing vaccinated groups, significantly fewer worms were recovered from CHB vaccines than CS vaccines ( $p < 0.01$ ) and the levels of stunting and reduction in eggs in *utero* were also significantly different ( $p < 0.01$ ) between these groups. However, between control CHB and CS groups, statistical analysis did not find differences in worm burden ( $p = 0.509$ ), worm length ( $p = 0.530$ ) or eggs in *utero* ( $p = 0.217$ ).

When comparing lamb growth during pre- and post-infection periods within breed, ADG was significantly higher in vaccinated CHB lambs than in CHB-Controls after challenge had begun ( $p < 0.05$ ) (Fig. 3A). CS groups showed comparable ADG during both pre- and post-challenge periods (Fig. 3B).

#### 3.2. Humoral immune response

Immunoglobulin G<sub>2</sub> isotype levels against L3 antigen were significantly higher in CS control groups than in CHB control groups ( $p < 0.05$ ) (Table 1). Similarly, the same isotype against L4 *T. circumcincta* antigen was statistically elevated in vaccinated CHB lambs when comparing with the other groups ( $p < 0.05$ ) (Table 2). IgA levels against the L4 antigen were significantly higher in control CHB than in vaccinated and control CS groups ( $p < 0.05$ ) (Table 2). Immunoglobulin production against the adult antigen was not statistically significantly different between groups for any of the isotypes studied (Table 3).

A negative correlation between serum levels of IgA against L3 antigen and worm length was observed only in CHB vaccinated group, while the association was positive with cumulative FEC (Table 1). In addition, in sera from CS vaccinated lambs, a negative association between

specific L4 serum IgG<sub>1</sub> against cumulative FEC was observed (Table 2). A positive correlation between IgG<sub>1</sub> against L4 antigen and eggs in *utero* was shown in CHB vaccinated group. Only IgG<sub>2</sub> against L4 *T. circumcincta* antigen was negatively and significantly associated with eggs in *utero* in CHB control group (Table 1). No statistically significant correlations were detected between immunoglobulin levels against the adult antigen and parasitological parameters (Table 3).

#### 3.3. Cellular immune response

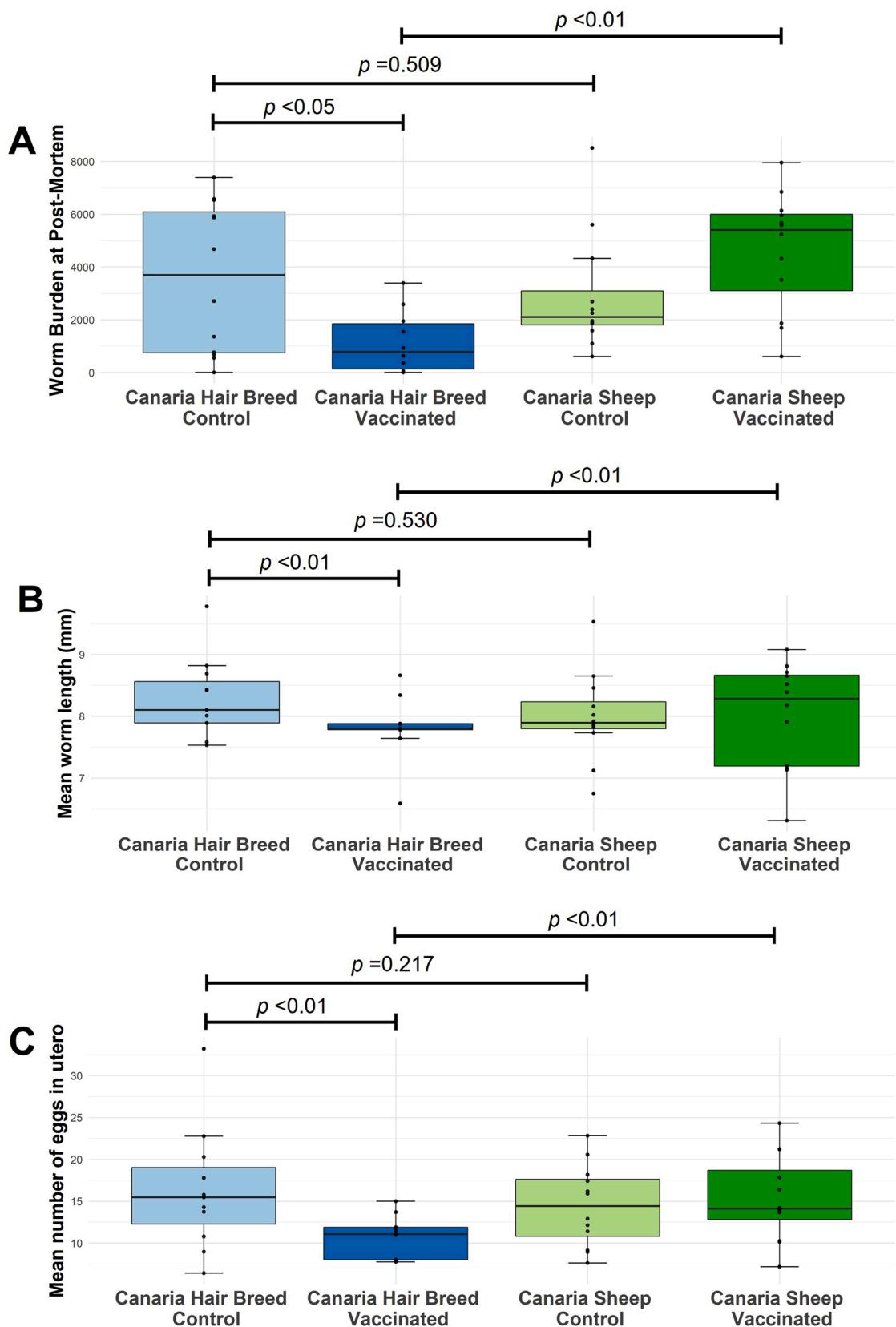
Proliferation of ALN cells collected at post-mortem was higher following stimulation with somatic antigens from adult when compared to L4 stages of *T. circumcincta* (L4 relative to adult estimate =  $-1.27 \pm 0.25\text{SE}$ ,  $F = 25.71$ ,  $p < 0.001$ ). Meanwhile, there were no effects of breed, vaccination, or any of the interactions between the three variables. Supernatants from stimulated cultures were collected to analyse IL-4 and IFN- $\gamma$  secretion. Generally low levels of IFN- $\gamma$  were detected in all groups, but there was a statistically supported effect of vaccination status, with vaccinated animals showing lower IFN- $\gamma$  secretion (estimate =  $-0.81 \pm 0.26\text{SE}$ ,  $F = 10.02$ ,  $p = 0.002$ ). There was, however, no support for effects of breed or antigen, or any interactions between the variables. IL-4 secretion was influenced by both vaccine and antigen treatments, with lower IL-4 production in vaccinated animals (estimate =  $-1.14 \pm 0.49\text{SE}$ ,  $F = 5.46$ ,  $p = 0.021$ ) and in cells treated with media compared to adult and L4 antigens (estimate for media versus adult antigen =  $-2.23 \pm 0.60\text{SE}$ ; overall effect of antigen  $F = 7.71$ ,  $p < 0.001$ ). There were, however, no effects of breed, or any of the interactions. In general, there were no significant differences between breeds or vaccine treatment in the cell markers studied, except for a lower percentage of WC1 cells in the CS breed ( $F = 6.31$ ,  $p = 0.016$ ; Fig. 4).

Several cell populations were identified and enumerated in samples from the abomasal wall of the lambs (Table 4). Eosinophil counts were significantly higher in vaccinated CHB lambs than in vaccinated CS lambs and CD4<sup>+</sup> cell counts were higher in vaccinated CHB than in CHB controls ( $p < 0.05$ ). CD45RA<sup>+</sup> cell counts were significantly higher in CHB-Control lambs in comparison with the other three experimental groups ( $p < 0.05$ ). The other cell populations studied did not show significant differences in their counts between the four experimental groups.

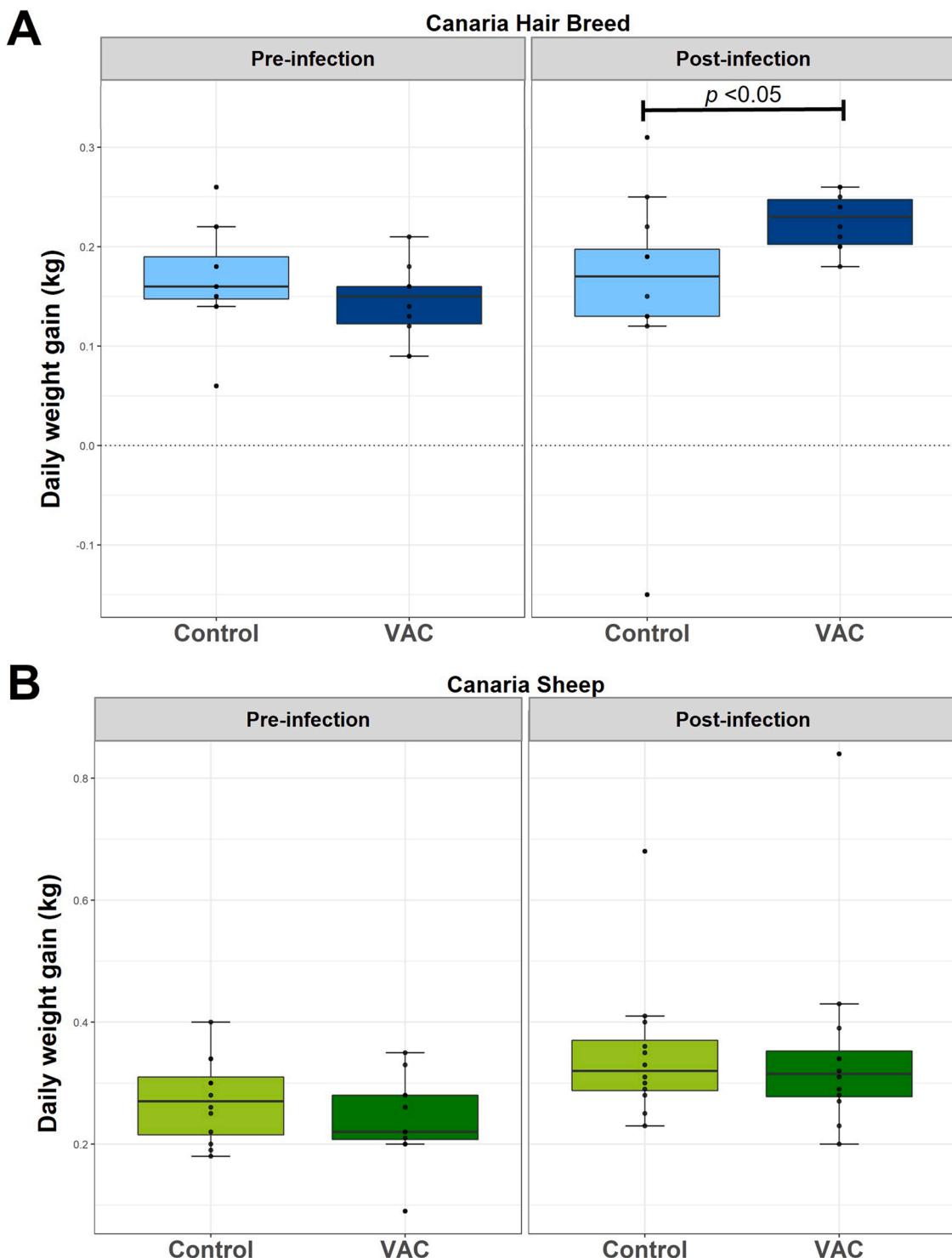
Correlation studies between abomasal cell populations and parasitological parameters showed negative associations between the numbers of some of these cells and several parasitological parameters (Table 4). Eosinophil counts were negatively correlated with cumulative FEC, worm burden and fecundity in the CHB-Control lambs. Globule leucocyte counts were negatively associated with numbers of eggs in *utero* in both CHB groups and in vaccinated CS lambs. Mast cell numbers were negatively associated with parasitological variables in the CS groups only. CD4<sup>+</sup> cell numbers were significantly, and negatively, correlated with adult worm burden in vaccinated CHB lambs. Similarly, CD8<sup>+</sup> cell numbers were negatively associated with adult worm burden and cumulative FEC in vaccinated CHB lambs only. CD45RA<sup>+</sup> cell numbers were also negatively correlated with parasitological variables in both vaccinated groups. MHC-II<sup>+</sup> stained cell numbers were negatively associated with egg excretion and fecundity in both CHB groups but not in CS groups (Fig. 5).

### 4. Discussion

This study demonstrated that CHB lambs were protected against *T. circumcincta* through immunisation with a prototype recombinant subunit vaccine. Vaccinated CHB lambs were able to control egg excretion, worm burden, length of female worms and egg levels in *utero*. Anti-parasite IgA and IgG<sub>2</sub> levels and the presence of globular leucocytes, CD45RA<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells in abomasal tissue appears to be associated with this response. In contrast, no evidence of protection



**Fig. 2.** Effects of immunisation against *Teladorsagia circumcincta* in two Canarian sheep breeds on worm establishment, length and fecundity. Mean worm burden (Panel A), worm length (Panel B) and eggs in utero (Panel C)  $\pm$  SEM are shown for sheep trickle infected with *T. circumcincta* L3 after the final vaccination. Vaccinated ("VAC") groups are represented by dark boxplots; adjuvant-recipient only ("Control") groups are represented by lighter boxplots.



**Fig. 3.** Effects of immunisation against *Teladorsagia circumcincta* in two Canarian sheep breeds on production performance. Daily weight gain  $\pm$  SEM for Canaria Hair Breed (CHB) (Panel A) and Canaria Sheep (CS) (Panel B) are shown before and after trickle infection with *T. circumcincta* L3 after the final vaccination. Vaccinated ("VAC") groups are represented by dark boxplots; adjuvant-recipient only ("Control") groups are represented by lighter boxplots.

was determined in CS vaccinated lambs.

Within the flock, young lambs are more likely to suffer severe GIN infections, partly because at the time they start grazing and contacting with parasites their immune system may be relatively immature and therefore less able to control the nematode burden (Manton et al., 1962; Smith et al., 1985; Barger, 1988; Colditz et al., 1996; McClure et al., 1998; Vervelde et al., 2001). Consequently, by vaccinating lambs against GIN at an early age, young animals could be better protected

against larval challenge and overcome the production effect of parasitism, preserving animal health and welfare, all of which are the priority goals of immunisation. However, a lack of response to immunisation has been recurrently observed with different vaccine prototypes against several GIN in young lambs (Bitakaramire, 1966; Urquhart et al., 1966; Smith and Angus, 1980; Smith et al., 1985; Kooyman et al., 2000; Vervelde et al., 2001; Nisbet et al., 2016b). An exception to this paradigm is when hidden antigens, such as H11 and

**Table 1**

Levels of serum immunoglobulins against L3 *Teladorsagia circumcincta* antigen in Canaria Hair Breed (CHB) and Canaria Sheep (CS) after challenge and vaccination against *T. circumcincta* and correlation with parasitological variables. IgA, IgG<sub>1</sub> and IgG<sub>2</sub> levels against L3 antigen are shown as mean of Optical Density Index (ODI) ± SEM.

L3	Isotype	Group	Mean ODI ± SEM	Correlation			
				Cumulative FEC	Worm burden	Worm length	Eggs in utero
IgA	CHB-VAC	1.459 ± 0.126 <sup>a</sup>	0.659*	0.491	-0.667*	-0.517	
		1.546 ± 0.189 <sup>a</sup>	0.119	-0.140	-0.100	-0.318	
	CS-VAC	1.867 ± 0.184 <sup>a</sup>	-0.196	-0.308	0.014	-0.084	
		1.899 ± 0.159 <sup>a</sup>	0.071	-0.056	-0.105	-0.371	
IgG <sub>1</sub>	CHB-VAC	1.200 ± 0.101 <sup>a</sup>	0.207	0.200	-0.05	0.15	
		1.078 ± 0.043 <sup>a</sup>	-0.274	-0.539	-0.405	-0.597	
	CS-VAC	1.175 ± 0.089 <sup>a</sup>	-0.284	-0.175	0.147	-0.046	
		1.265 ± 0.012 <sup>a</sup>	-0.418	-0.434	-0.189	-0.301	
IgG <sub>2</sub>	CHB-VAC	1.212 ± 0.079 <sup>ab</sup>	0.507	0.432	-0.126	-0.017	
		1.097 ± 0.042 <sup>a</sup>	0.042	0.091	-0.023	-0.191	
	CS-VAC	1.278 ± 0.120 <sup>ab</sup>	-0.305	-0.392	0.231	-0.056	
		1.413 ± 0.138 <sup>b</sup>	-0.426	-0.315	-0.224	-0.203	

Statistically significantly differences ( $p < 0.05$ ) between groups for a specific isotype and antigen are represented with different letters. Significant correlations are represented with “\*” at  $p < 0.05$ .

**Table 2**

Levels of serum immunoglobulins against L4 *Teladorsagia circumcincta* antigen in Canaria Hair Breed (CHB) and Canaria Sheep (CS) after challenge and vaccination against *T. circumcincta* and correlation with parasitological variables. IgA, IgG<sub>1</sub> and IgG<sub>2</sub> levels against L4 antigen are shown as mean of Optical Density Index (ODI) ± SEM.

L4	Isotype	Group	Mean ODI ± SEM	Correlation			
				Cumulative FEC	Worm burden	Worm length	Eggs in utero
IgA	CHB-VAC	1.393 ± 0.106 <sup>ab</sup>	0.056	-0.285	0.050	-0.200	
		1.639 ± 0.205 <sup>a</sup>	0.221	0.126	-0.027	-0.391	
	CS-VAC	1.239 ± 0.063 <sup>b</sup>	-0.100	0.361	0.168	0.312	
		1.162 ± 0.071 <sup>b</sup>	-0.262	-0.392	0.140	-0.147	
IgG <sub>1</sub>	CHB-VAC	1.322 ± 0.082 <sup>a</sup>	-0.270	-0.018	0.500	0.767*	
		1.158 ± 0.023 <sup>a</sup>	-0.544	-0.501	-0.068	-0.314	
	CS-VAC	1.324 ± 0.091 <sup>a</sup>	-0.725**	-0.531	-0.126	-0.406	
		1.206 ± 0.089 <sup>a</sup>	0.121	0.140	0.21	-0.140	
IgG <sub>2</sub>	CHB-VAC	1.37 ± 0.085 <sup>a</sup>	0.145	0.036	0.117	0.109	
		1.042 ± 0.026 <sup>b</sup>	-0.357	-0.406	-0.573	-0.682*	
	CS-VAC	1.053 ± 0.081 <sup>b</sup>	-0.557	-0.483	-0.049	-0.329	
		0.980 ± 0.112 <sup>b</sup>	0.504	0.615	0.566	0.238	

Statistically significantly differences ( $p < 0.05$ ) between groups for a specific isotype and antigen are represented with different letters. Significant correlations are represented with “\*” at  $p < 0.05$ .

**Table 3**

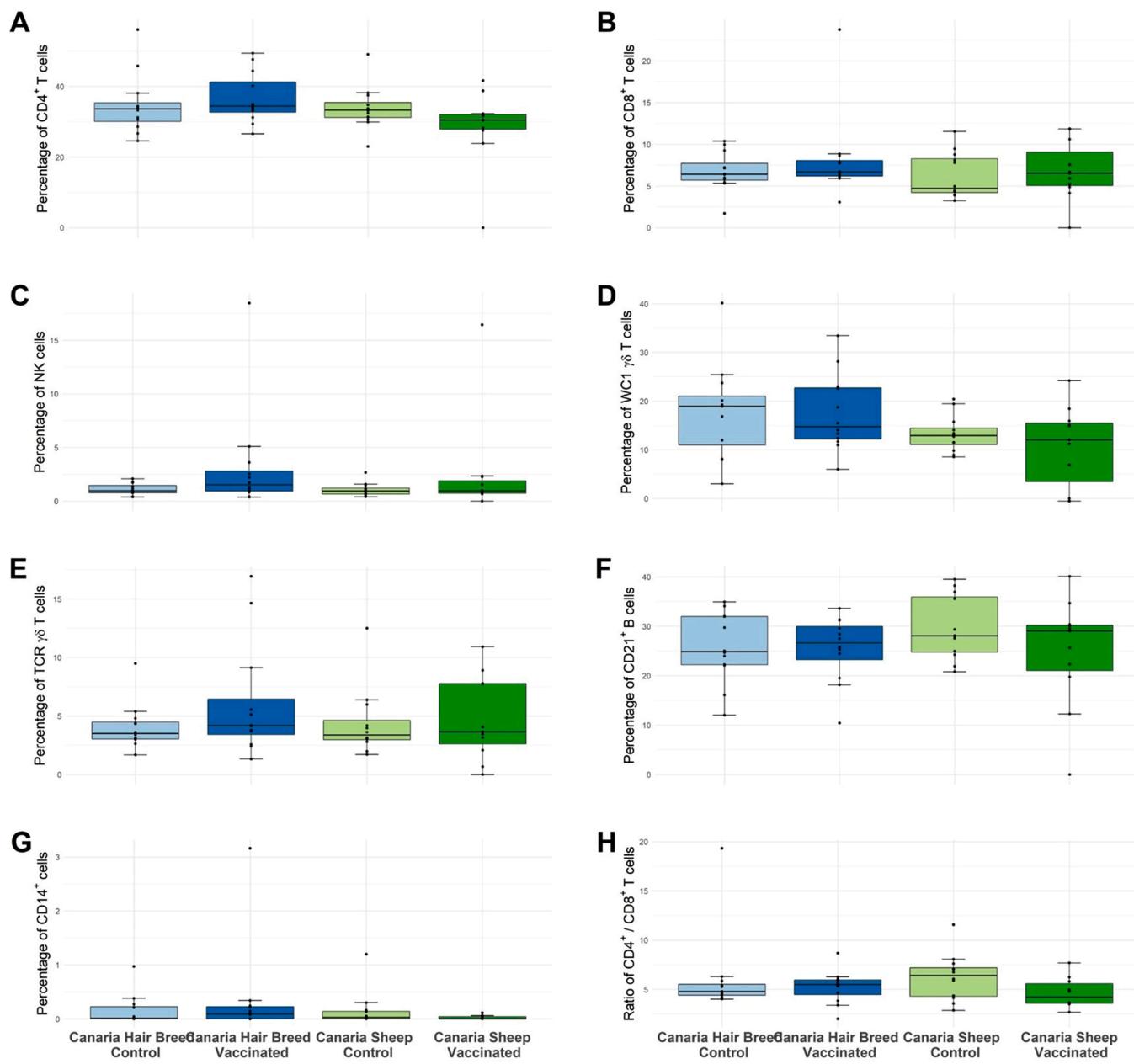
Levels of serum immunoglobulins against adult *Teladorsagia circumcincta* antigen in Canaria Hair Breed (CHB) and Canaria Sheep (CS) after challenge and vaccination against *T. circumcincta* and correlation with parasitological variables. IgA, IgG<sub>1</sub> and IgG<sub>2</sub> levels against adult antigen are shown as mean of Optical Density Index (ODI) ± SEM.

AD	Isotype	Group	Mean ODI ± SEM	Correlation			
				Cumulative FEC	Worm burden	Worm length	Eggs in utero
IgA	CHB-VAC	1.252 ± 0.059 <sup>a</sup>	0.496	0.455	-0.217	-0.050	
		1.284 ± 0.130 <sup>a</sup>	0.123	-0.133	-0.451	-0.556	
	CS-VAC	1.330 ± 0.081 <sup>a</sup>	-0.381	-0.217	-0.200	-0.214	
		1.307 ± 0.057 <sup>a</sup>	-0.366	-0.347	-0.098	-0.060	
IgG <sub>1</sub>	CHB-VAC	1.078 ± 0.040 <sup>a</sup>	0.370	0.539	-0.083	0.350	
		1.050 ± 0.031 <sup>a</sup>	-0.118	-0.462	-0.159	-0.369	
	CS-VAC	1.091 ± 0.038 <sup>a</sup>	-0.473	-0.490	-0.091	-0.357	
		1.068 ± 0.042 <sup>a</sup>	-0.007	0.056	0.049	-0.385	
IgG <sub>2</sub>	CHB-VAC	1.029 ± 0.014 <sup>a</sup>	0.527	0.576	-0.317	-0.300	
		1.018 ± 0.011 <sup>a</sup>	0.119	-0.133	-0.255	-0.045	
	CS-VAC	1.020 ± 0.018 <sup>a</sup>	-0.423	-0.119	-0.249	-0.322	
		1.038 ± 0.035 <sup>a</sup>	-0.203	-0.056	-0.200	-0.396	

Statistically significantly differences ( $p < 0.05$ ) between groups for a specific isotype and antigen are represented with different letters. Significant correlations are represented with “\*” at  $p < 0.05$ .

H-gal-GP from *H. contortus* have been used in vaccines, inducing high levels of circulating IgG in a range of ages of lambs (Broomfield et al., 2020; Kebeta et al., 2021). However, as this type of antigens are normally not exposed to the immune system, a natural boost from exposure

to the parasites in the field does not occur (Kooyman et al., 2000). Hence, lambs need around 3 priming doses and booster vaccinations every 6 weeks to maintain acceptable levels of protection (Kebeta et al., 2020).



**Fig. 4.** Phenotypic profile of abomasal lymph node cells in two Canarian sheep breeds after challenge and vaccination against *Teladorsagia circumcincta*. The expression of CD4<sup>+</sup>, CD8<sup>+</sup> and γδ<sup>+</sup> T cells, NK, CD21<sup>+</sup> B cells and CD14<sup>+</sup> (monocytes and macrophages) cells is represented. Results are shown as boxplots with IQR ± SEM. Vaccinated ("VAC") groups are represented by dark boxplots; adjuvant-recipient only ("Control") groups are represented by lighter boxplots.

The age of lambs can be key when it comes to vaccine efficacy, as low responses observed in young lambs to some immunogens can be due to the animals not being fully immunocompetent. In which case, vaccination can be impacted by the inability of an immature immune system to translate vaccination into effective immunity. Young lambs develop weak adaptive immune responses (Manton et al., 1962; Smith et al., 1985; Barger, 1988; Colditz et al., 1996; McClure et al., 1998; Vervelde et al., 2001) with low CD4<sup>+</sup> and CD8<sup>+</sup> counts and immunoglobulin levels (Watson et al., 1994; Colditz et al., 1996), although populations of γδ T cells and B cells in peripheral blood are similar to mature sheep (Colditz et al., 1996). This suggests that while there is an adequate pool of B cells in young lambs, a relative deficiency in T cell help may result in less efficient induction of adaptive immune responses and, consequently, protection would be negatively impacted. This would be consistent with our recent study of the abomasal transcriptome of Texel-cross lambs immunised with the same *T. circumcincta* vaccine used

in this study, in which protective immune pathways associated with T cell activation and polarisation were present in 6-month but not 3-month-old lambs (Liu et al., 2021). Likewise, great individual and inter-breed variation in immunity, which may be more related with metabolic than chronological lamb age, has been observed (Greer and Hamie, 2016).

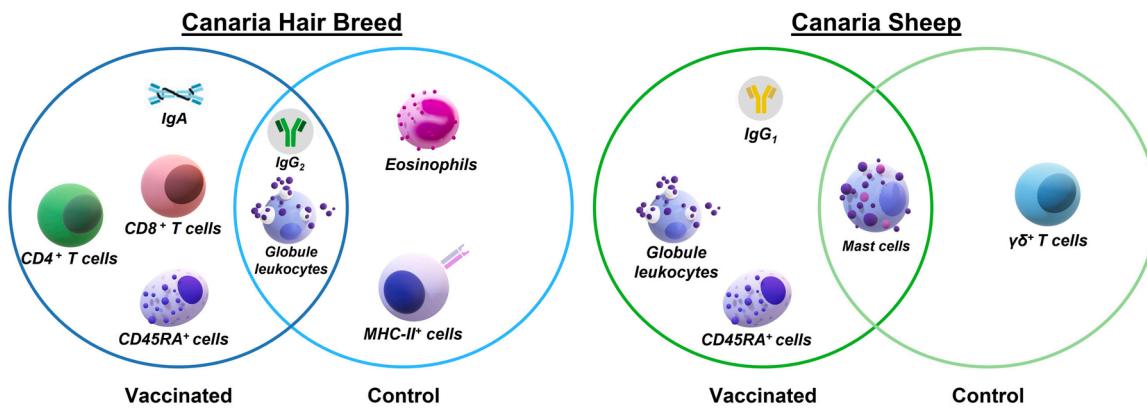
Not all sheep breeds are equally susceptible to GIN (Piedrafita et al., 2010). Several breeds have shown ability to control these worms at a relatively young age (Bahirathan et al., 1996; Gruner et al., 2003; Rocha et al., 2005) and it is possible that some breeds may respond earlier to vaccines (Piedrafita et al., 2010). The results of our study are in agreement with these reports, as protection from the impacts of GIN infection in CHB lambs, a breed previously described as having some levels of resistance to *H. contortus* (González et al., 2008) and *T. circumcincta* (González et al., 2019), was enhanced through vaccination at weaning, indicating synergy between genetic and vaccine-induced protection.

**Table 4**

Immune cell counts in the abomasal wall in Canaria Hair Breed (CHB) and Canaria Sheep (CS) after challenge and vaccination against *Teladorsagia circumcincta* and correlation with parasitological variables. Mean (cells/mm<sup>2</sup>) ± SEM in abomasal tissue are shown.

Cell population	Group	Mean (cells/mm <sup>2</sup> ) ± SEM	Correlation			
			Cumulative FEC	Worm burden	Worm length	Eggs in utero
Eosinophils	CHB-VAC	119.18 ± 27.89 <sup>a</sup>	-0.075	-0.079	0.267	0.067
	CHB-Control	91.21 ± 11.73 <sup>ab</sup>	-0.669*	-0.580*	-0.027	-0.655*
	CS-VAC	60.15 ± 19.41 <sup>b</sup>	-0.161	-0.469	-0.042	-0.301
	CS-Control	81.92 ± 18.44 <sup>ab</sup>	-0.369	-0.252	0.028	-0.189
Globule Leucocytes	CHB-VAC	243.4 ± 57.08 <sup>a</sup>	0.458	0.079	-0.233	-0.767*
	CHB-Control	213.15 ± 73.68 <sup>a</sup>	-0.347	-0.217	-0.236	-0.727*
	CS-VAC	164.11 ± 58.99 <sup>a</sup>	-0.592*	-0.559	-0.385	-0.580*
	CS-Control	132.25 ± 36.68 <sup>a</sup>	-0.489	-0.238	-0.462	0.091
Mast cells	CHB-VAC	30.06 ± 8.73 <sup>a</sup>	0.634*	0.515	-0.333	-0.650
	CHB-Control	40.98 ± 12.54 <sup>a</sup>	0.151	0.273	-0.173	-0.218
	CS-VAC	40.81 ± 15.06 <sup>a</sup>	-0.744**	-0.444	-0.465	-0.479
	CS-Control	18.87 ± 4.47 <sup>a</sup>	-0.707*	-0.609*	-0.266	-0.298
CD4 <sup>+</sup>	CHB-VAC	69.25 ± 22.38 <sup>a</sup>	-0.376	-0.673*	0.583	0.283
	CHB-Control	26.33 ± 3.74 <sup>b</sup>	0.178	0.091	0.297	-0.006
	CS-VAC	60.87 ± 13.75 <sup>ab</sup>	-0.525	-0.308	-0.322	-0.217
	CS-Control	54.48 ± 12.91 <sup>ab</sup>	-0.170	-0.182	-0.343	0.189
CD8 <sup>+</sup>	CHB-VAC	109.18 ± 22.37 <sup>a</sup>	-0.857**	-0.867**	0.567	0.500
	CHB-Control	79.76 ± 31.72 <sup>a</sup>	0.039	-0.350	-0.036	0.027
	CS-VAC	68.79 ± 14.87 <sup>a</sup>	0.361	0.119	-0.063	0.098
	CS-Control	68.67 ± 11.77 <sup>a</sup>	-0.213	-0.315	-0.035	0.217
γδ <sup>+</sup>	CHB-VAC	28.16 ± 6.91 <sup>a</sup>	-0.462	-0.620	0.251	0.059
	CHB-Control	13.95 ± 3.83 <sup>a</sup>	0.174	0.165	0.278	0.155
	CS-VAC	27.42 ± 9.71 <sup>a</sup>	-0.039	0.175	0.014	0.280
	CS-Control	22.15 ± 4.26 <sup>a</sup>	-0.675*	-0.480	-0.529	-0.497
CD45RA <sup>+</sup>	CHB-VAC	10.45 ± 2.96 <sup>b</sup>	-0.032	-0.141	-0.770*	-0.728*
	CHB-Control	23.08 ± 5.44 <sup>a</sup>	0.186	0.301	0.282	0.045
	CS-VAC	7.31 ± 2.67 <sup>b</sup>	-0.552	-0.593*	-0.470	-0.474
	CS-Control	7.90 ± 1.88 <sup>b</sup>	-0.170	-0.056	-0.399	0.182
MHC-II <sup>+</sup>	CHB-VAC	40.82 ± 21.88 <sup>a</sup>	-0.584	-0.477	0.623	0.563
	CHB-Control	21.01 ± 9.88 <sup>a</sup>	-0.758**	-0.547	-0.205	-0.683*
	CS-VAC	64.46 ± 22.59 <sup>a</sup>	-0.060	-0.203	0.357	-0.007
	CS-Control	44.39 ± 13.09 <sup>a</sup>	-0.277	-0.070	-0.413	0.238

Statistically significantly differences ( $p < 0.05$ ) between groups for a specific cell population are represented with different letters. Significant correlations are represented with “\*” at  $p < 0.05$  and “\*\*” at  $p < 0.01$ .



**Fig. 5.** Graphic representation of the possible immune mechanisms implicated in the response for Canaria Hair Breed and Canaria Sheep trickle infected with *Teladorsagia circumcincta* L3 after the final vaccination. Humoral and cellular elements associated with protection were represented within a circle for each group (dark lines for vaccines and lighter lines for controls). Elements included in the overlapped surface between vaccines and controls indicate it is shared by both groups.

The vaccine cocktail used had a high impact in this experimental *T. circumcincta* study in 3-month-old CHB lambs. Worm establishment, growth and fecundity were impaired in vaccinated CHB lambs. Previous vaccination trials using this vaccine in older Texel-crossbred have also shown reductions in worm number and FEC (Nisbet et al., 2013) and use of this vaccine in 6-month-old CS lambs impacted worm length and fecundity but not on worm burden (González et al., 2019). Although vaccinated 3-month-old Texel-crossbred lambs were protected in comparison to their non-vaccinated counterparts, FEC and worm burdens were higher than in older (6 month old) Texel-cross vaccinated lambs

(Liu et al., 2021). Interestingly, parasitological data (FEC levels, worm counts) in 3-month-old vaccinated CHB lambs was very similar to that recorded in 6-month-old vaccinated CHB lambs and substantially lower than in vaccinated six-month-old CS lambs, when animals were exposed to an identical experimental infection (González et al., 2019).

Lambs of 3–4 months are able to develop similar immune response mechanisms to older sheep, although quantitatively, these are lower than 10–12 months lambs, at which point immunity is considered mature (Smith et al., 1985; McRae et al., 2015). Sheep immune responses against *T. circumcincta* initially impact worm length and, later,

worm burden (McRae et al., 2015). However, in the work described here, both worm length and worm burden were impacted by day 40 post-infection in vaccinated CHB lambs, suggesting a strong and effective immune response to immunisation.

Interestingly, in 6-month-old CHB lambs, vaccination did not add to the already substantial impact of natural breed resistance to *T. circumcincta*, (González et al., 2019) (Fig. 6). However, the data presented here demonstrate that, at weaning, vaccination of CHB lambs can significantly boost natural breed resistance to control worm numbers and enhance weight gain in this breed. It is notable, however, that abomasal lymph node lymphocyte responses to parasite antigens in all lamb groups were low, with some animals not responding at all, and no differences in cell population counts were observed across groups. This still suggests certain level of immaturity in the immune response of this age of lamb, in contrast to lambs of 6 months (Machín et al., 2021).

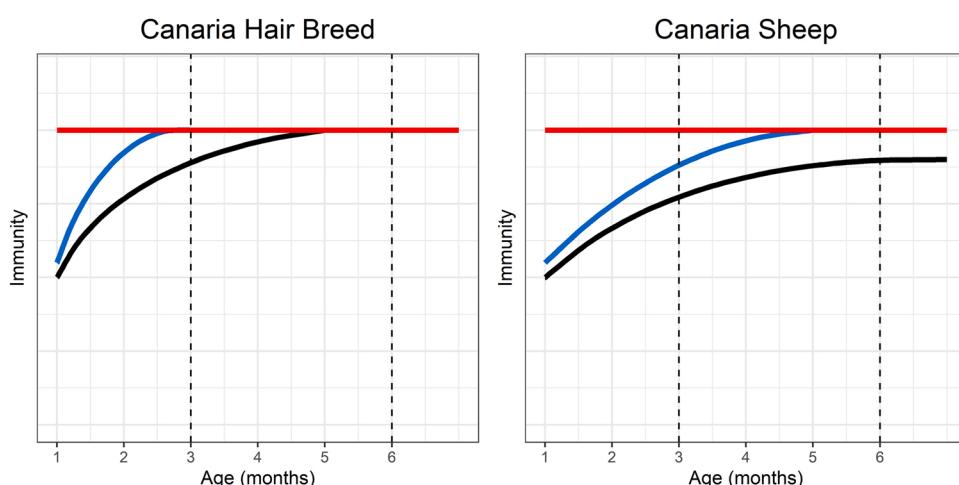
Although lymphocytes did not proliferate in vitro, all groups produced IL-4 when they were cultured with parasitic antigens. IL-4 is a key cytokine in GIN control (Venturina et al., 2013) in addition to GL activation (McRae et al., 2015). A similar response was observed in a previous vaccination trial with CHB/CS lambs of six months of age. Local lymph node lymphocytes cultured in the presence of parasite antigens produced IL-4 independently of vaccination status or breed (Machín et al., 2021). Interestingly, lymphocytes from older lambs also produced IFN- $\gamma$ , particularly in CHB (Machín et al., 2021), while only the control group of three-month-old CHB lambs produced this cytokine in the present work. Lower production of IFN- $\gamma$  by blood lymphocytes has also been previously described in young lambs (Watson et al., 1994; Colditz et al., 1996).

Similarly to a previous vaccination trial with 6-month-old CS lambs, no major differences in cell populations in the abomasum between vaccinees and controls were observed, which is in contrast with trials in other breeds using vaccines for related worms such as *H. contortus* (Kooyman et al., 2000). The only exceptions were the elevated eosinophil and CD4 $^{+}$  counts in vaccinated CHB lambs compared to vaccinated CS lambs and control CHB, respectively. Globular leucocytes have been associated with the control of *T. circumcincta* in sheep with acquired resistance (Gruner et al., 2004; McRae et al., 2015; Albuquerque et al., 2019), manifested as rapid larval rejection (Balic et al., 2000b). This cell type was negatively associated with parasitological data in the work here, independently of vaccination status or breed and a similar response was observed in older animals (Machín et al., 2021), suggesting that this response is independent of age in these breeds.

The levels of some other cell types were also negatively associated with parasitological parameters and some of these potential immune responses might be associated with breed resistance. For instance, MHC-II $^{+}$  cells in the abomasum were only negatively associated with

parasitology in CHB controls, suggesting recruitment of antigen presenting cells is important for protection in this breed. Also, CD4 $^{+}$  and B cells (CD45RA) were associated with protection here; other authors have described increases in these cell types with another prototype vaccine against *H. contortus* in goats (Zhao et al., 2012). The vaccine may have induced similar response in both breeds, but it was more effective in the CHB lambs and, perhaps, this difference could be unravelled with a more detailed study of the response. CD8 $^{+}$  could be a critical cell type in protection because it was only negatively associated with worm burden and cumulative FEC in the vaccinated CHB group. CD8 $^{+}$  cells were not associated with protection in six-month-old vaccinated CS lambs in a previous study but they were present at elevated levels in six-month-old CHB lambs (Machín et al., 2021). CD8 $^{+}$  cells are involved in type-1 responses (Spellberg and Edwards, 2001) and, in terms of natural immunity, have been traditionally linked with susceptibility to GIN (Gill et al., 2000; Pernthaner et al., 2005; Craig et al., 2014; McRae et al., 2015), with type 2 responses associated with protection. However, in this study, CD8 $^{+}$  cells may play a role in successful vaccination as early type 1 responses have been shown to be predictive of vaccine-induced protection in older Texel cross-bred lambs vaccinated with this *T. circumcincta* vaccine (Liu et al., 2021). In fact, recent studies have found upregulation of transcripts encoding proteins associated with type 1 response in small ruminants resistant to GIN at 35 days after challenge (Aboshady et al., 2020) and it is possible that a mixed, or sequential, type1/type 2 response could be optimal instead of the type-2-biased response, traditionally accepted as being protective. In addition, a successful vaccine incorporating E/S antigens of GIN developed both type 1 and type 2 responses (Vervelde et al., 2001).

A negative association between *T. circumcincta*-specific IgA and worm length was observed in vaccinated CHB lambs and *T. circumcincta*-specific IgG<sub>2</sub> may be also involved in protection because higher levels of this isotype were detected in vaccinated CHB than in the other groups. Both immunoglobulins showed similar patterns in successfully vaccinated six-month-old CS lambs (Machín et al., 2021). Besides, vaccinated cross-bred Texel lambs produced higher total IgG against *T. circumcincta* L4 ES antigens than control animals (Nisbet et al., 2013). These immunoglobulins have been considered relevant in protection of sheep against GIN infections through natural immunity (Stear et al., 1995; Pernthaner et al., 2005; McRae et al., 2015). Worm-specific IgG<sub>2</sub> has also been associated with protection in sheep with a vaccine against *H. contortus* (Knox et al., 2005). Using different immunogens against *H. contortus*, IgG<sub>1</sub> and IgA were associated with protection, and lower production was observed in younger lambs that responded less well to the vaccine (Vervelde et al., 2001). Generally, lower levels of immunoglobulins can be detected in younger lambs than in mature lambs (Smith et al., 1985; Watson and Gill, 1991; Watson et al., 1994). It is



**Fig. 6.** Schematic representation of immunity dynamics in 3 and 6-month-old Canaria Hair Breed (CHB)(left panel) and Canaria Sheep lambs (CS)(right panel) in two vaccination trials with a prototype recombinant sub-unit *Teladorsagia circumcincta* vaccine and after challenge with *T. circumcincta*. 3-month-old CHB lambs demonstrated detectable levels of protective immunity (horizontal red line) when vaccinated (light blue line) in comparison with controls (dark blue line) whereas at 6 months the effect of immunisation was not evident. In contrast, in CS lambs, 3-month-old vaccinees (light blue line) were not protected by the vaccine when compared to controls (dark blue line) whilst vaccination proved protective by 6 months of age.

possible the CHB lambs were able to produce enough immunoglobulins at this younger age.

Finally, daily weight gain was higher in vaccinated CHB lambs than in control CHB lambs in this trial. Protection against *H. contortus* with the commercial vaccine Barbervax® -composed of two native integral membrane proteins isolated from the intestinal brush border of adult worms- has not been associated with weight gain in lambs in other trials (Kebeta et al., 2020, 2021) in contrast with what is described here. This is particularly intriguing because, even though the vaccine induced a protective immune response, it did not have (in the context of this relatively short time frame) a negative production cost. There has been concern about the “metabolic cost” of the development of a protective response against worms in young lambs due to the energy and protein required to develop an effective response (Greer, 2008; Greer and Hamie, 2016). Both negative -favourable- and positive -unfavourable- associations between lamb weight and worm burden have been observed in different trials. However, lamb growth is generally stunted while developing an immune response (Kimambo et al., 1988; Greer and Hamie, 2016). In the work presented here, the vaccine did not impair growth in the CHB lambs.

In conclusion, the combination of genetic resistance and an effective vaccine, could be a good example of integrated control strategy for *T. circumcincta*. Several immunoglobulins and cells may be involved in the effective response of 3-month-old CHB lambs. In future, a more detailed study of the response to the vaccine in lambs could unravel the most relevant mechanisms in protection to improve this vaccine prototype.

#### CRediT authorship contribution statement

**Tara Pérez-Hernández:** Formal analysis, Investigation, Writing – original draft, Visualization. **Yolanda Corriño-Miyar:** Formal analysis, Investigation, Writing – original draft, Visualization. **Julia N. Hernández:** Investigation, Writing – original draft, Supervision. **Cynthia Machín:** Investigation. **Yania Paz-Sánchez:** Investigation. **Adam D. Hayward:** Formal analysis, Writing – review & editing. **Harry W. Wright:** Investigation. **Daniel R.G. Price:** Investigation. **Jacqueline B. Matthews:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition. **Tom N. McNeilly:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Alasdair J. Nisbet:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. **Jorge F. González:** Conceptualization, Methodology, Writing – original draft, Supervision, Project administration.

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#### Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jacqueline Matthews has patent #EP2812023A1 SHEEP NEMATODE VACCINE pending to Moredun Research Institute. Alasdair Nisbet has patent #EP2812023A1 SHEEP NEMATODE VACCINE pending to Moredun Research Institute.

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## *Capítulo II*

*Exploring the transcriptomic changes underlying recombinant vaccine efficacy against Teladorsagia circumcincta in 3-month-old lambs*



# Exploring the transcriptomic changes underlying recombinant vaccine efficacy against *Teladorsagia circumcincta* in 3-month-old lambs

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## Abstract

*Teladorsagia circumcincta* is an abomasal parasitic nematode that can cause serious issues in small ruminant production, which are aggravated by drug resistance. Vaccines have been suggested as a feasible, long-lasting alternative for control since adaptation to the host's immune mechanisms by helminths develops at a much slower pace than anthelmintic resistance. In a recent trial, a *T. circumcincta* recombinant subunit vaccine yielded over a 60% reduction in egg excretion and worm burden and induced strong humoral and cellular anti-helminth responses in vaccinated 3-month-old Canaria Hair Breed (CHB) lambs, but Canaria Sheep (CS) of a similar age were not protected by the vaccine. In the present study, we compared the transcriptomic profiles in the abomasal lymph nodes of 3-month-old CHB and CS vaccines after vaccination and infection with *T. circumcincta* in order to understand differences in responsiveness at the molecular level. In the CS, differentially expressed genes (DEG) identified were related to innate immunity processes such as antigen presentation or antimicrobial proteins and down-regulation of inflammation and immune response through regulatory T cell-associated genes. However, DEG in CHB vaccines were associated with type-2 oriented immune responses, i.e., development of immunoglobulins; activation of eosinophils and other effector cells, as well as tissue structure and wound repair-related genes and protein metabolism pathways such as DNA and RNA processing. These results highlight potentially more optimal timing and orientation of immune responses in CHB sheep compared to CS after vaccination, which are associated with vaccine-induced protection. The data obtained in this study thus deepens our understanding of variations in responsiveness to vaccination in young lamb and provides insights for vaccine refinement strategies.

## Keywords

Gastrointestinal nematode, *Teladorsagia circumcincta*, lambs, transcriptome, recombinant vaccine

### 1. Introduction

Climate change-driven expansion of diseases such as gastrointestinal nematode (GIN) infections threatens the profitability of ruminant livestock farming (Mavrot *et al.*, 2015), contributing to food insecurity in an increasing human population scenario (Fitzpatrick, 2013). The pathogenic effects of GIN and their ability to induce economic loss is particularly concerning in lamb production (Nieuwhof & Bishop, 2005). At the same time, we have historically relied on anthelmintics as the main control strategy, but their efficacy has been steadily decreasing due an increase in drug resistance in nematode populations across the globe (Gilleard *et al.*, 2021).

Vaccines are considered a sustainable alternative because of their long-lasting protective effect in the host without residues in the final product. However, GIN vaccine development has been slow since the 1970s, yielding one commercially available vaccine based on native *Haemonchus contortus* antigens: Barbervax® (Britton *et al.*, 2020). The main constraints to vaccine development have been attributed to: 1)

difficulty in expressing complex nematode antigens as recombinant proteins, limiting product standardization and safe, large-scale production; and 2) variability in host protective immune responses, which may be influenced by physiological status, nutrition, sex, and age (Nisbet *et al.*, 2016b). Young lambs have a limited ability to mount full anti-nematode responses during their first grazing season when their immune system is still maturing (Greer & Hamie, 2016; Stear *et al.*, 2000). Aligned with this, is the observation that 3 to 6-month-old vaccinated lambs of certain breeds develop incomplete immune responses that may not control worm infection (Kooyman *et al.*, 2000; Nisbet *et al.*, 2013), which can lead to clinical symptoms and subsequent economic loss. A recent study demonstrated that a recombinant subunit *T. circumcincta* vaccine induced a protective immune response to helminth infection in 3-month-old Canaria Hair Breed (CHB) lambs, while stabilizing lamb growth during infection (Pérez-Hernández *et al.*, 2022). In contrast, age-matched Canaria Sheep (CS) counterparts were not protected by vaccination. Pinpointing the mechanisms behind protective responses to vaccination in young sheep is thus critical to improve vaccination strategies in young sheep of different breeds. In this sense, transcriptomic analysis has been successfully used to explore and

compare changes in gene expression during GIN infection between different sheep lineages or breeds with marked phenotypic differences in their susceptibility to GIN (Aboshady *et al.*, 2020; McRae *et al.*, 2016). In this study, we explored the differences in the transcriptomic signatures of CHB and CS vaccines after infection with *T. circumcincta* by comparing their gene expression profiles in draining abomasal lymph node tissue samples.

## 2. Materials and methods

### 2.1 Experimental design

The vaccination and challenge protocol followed in this study has previously been published in detail (Pérez-Hernández *et al.*, 2022). Briefly, 3-month-old CHB (N=24) and CS (N=24) were purchased, dewormed on arrival, and kept in conditions that prevented strongyle infection. The study was designed as a 2 (breed) x 2 (treatment) factorial arrangement, assigning animals randomly to vaccinated (CHB-VAC, CS-VAC) or control (CHB-Control, CS-Control) groups (N=12). During the study, two animals in group CHB-VAC were excluded from the experiment because of reasons not related to the procedure. For this study, only the data obtained from CHB (N=10) and CS (N=12) vaccinated (VAC) groups were used.

The prototype recombinant vaccine, described previously by Nisbet *et al.* (2013), consisted of a cocktail of 8 recombinant proteins. The protein mixture (400 µg) was administered with Quil A and PBS on days 0, 21 and 42 of the experiment. Simultaneously, control groups received PBS/Quil A injections. From day 42 until day 68, sheep were infected orally with *T. circumcincta* 2,000 L3 three times a week (Nisbet *et al.*, 2013). On days 82-85, animals were euthanised to collect abomasal lymph node samples, which were stored in E.Z.N.A. RNA Lock Reagent (Omega Bio-tek) at -80°C prior to RNA extraction.

The experimental protocol was designed following the Spanish Legislation (RD 53/2013) and was subsequently approved by the Animal Welfare Ethics Committee of the Universidad de Las Palmas de Gran Canaria (OEBA\_ULPGC\_003\_2014) and by the local competent authority.

### 2.2 RNA extraction and sequencing

The extraction protocol has been described in Machín, 2022. Briefly, total RNA was extracted from lymph node samples using RNeasy mini-isolation kits (Qiagen Ltd, UK) following the manufacturers' protocol. RNA quantity and integrity were assessed prior to sequencing on an Illumina HiSeq 4000 at The Centre for Genome

Research (CGR) at the University of Liverpool, UK, generating 2x150 bp strand-specific, paired-end reads.

### 2.3 RNA-Seq Quality Control and alignment

R version 3.4.4 was used for the RNAseq data quality control (QC) based on samples obtained from CHB and CS vaccines with the array QualityMetrics package. Sequences were processed, then aligned to the *Ovis aries* genome assembly Oar\_v3.1 (GCA\_000298735.1) using the STAR aligner and the numbers of mapped read pairs counted based on the *Ovis aries* genome annotation (Ensembl release 91), also within STAR (Dobin *et al.*, 2013). Following data QC, a subset of 18 RNAseq samples (CHB: N=9; CS: N=9) was considered for further analyses.

### 2.4 Statistical analysis

For the RNAseq analysis, count data for the samples were normalized using TMM (Trimmed Mean of M-value) and transformed with VOOM (Law *et al.*, 2014) to log2-counts per million with associated precision weights. A comparison between CHB vaccines *versus* CS vaccines in abomasal lymph node tissue was performed using linear modelling. Subsequently, empirical Bayesian analysis was applied including adjustment for multiple testing, which controls for false discovery rate (FDR) based on an FDR p-

value cut-off of <0.01 and a 1.2-fold change in gene expression. The null hypothesis was that there was no difference between the groups being compared. The Bioconductor package, Limma (Ritchie *et al.*, 2015), was used to identify differentially expressed genes and gene lists were then annotated based on the *Ovis aries* genome, with further manual curation of genes to the gene symbol level.

Gene network analysis annotations of the differentially expressed genes (DEG) identified in the CHB *versus* CS vaccines in abomasal lymph node tissue were manually obtained using different tools. DAVID v6.8 (*DAVID: Database for Annotation, Visualization and Integrated Discovery*, 2021) was used to annotate some gene symbols of *Ovis aries* species. The remaining gene symbols were studied through Ensembl Oar v3.1 (Ensemble, 2022), Uniprot (*UniProt: Universal Protein Knowledgebase*, 2021) and BLAST (*BLAST Tool: Basic Local Alignment*, 2021), whilst biological process, cellular component and molecular function were investigated using GeneCards webpage (*GeneCards: The Human Gene Database*, 2021).

### 2.5 Pathway analysis

To explore potential functions of the DEG in the vaccinated CHB and the CS breeds, a cluster annotation analysis was performed using the CTD bioinformatics tool (*CTD: The Comparative*

*Toxicogenomics Database*, 2022), classifying these genes into pathways using KEGG (*KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway Database*, 2022) and the REACT (*Reactome Pathway Database*, 2022) directories.

### 3. Results

#### 3.1 Parasitology and immunology

The results of the effect of the vaccine on parasitological and immunological parameters have been described in detail previously (Pérez-Hernández, 2022). In brief, the *T. circumcincta* recombinant sub-unit vaccine elicited protection in CHB lambs but not in CS lambs. Vaccinated CHB had significant lower egg excretion in faeces than vaccinated CS (CHB:  $711 \pm 254$  EPG; CS:  $4278 \pm 1037$  EPG) ( $p < 0.05$ ) and lower abomasal worm counts (CHB:  $1147 \pm 374$ ; CS:  $4613 \pm 652$ ) ( $p < 0.05$ ) than vaccinated CS. Worms were also significantly shorter (CHB:  $7.75 \pm 0.1$  mm; CS:  $7.95 \pm 0.07$  mm) ( $p < 0.01$ ) and less prolific (CHB:  $11 \pm 0.48$  eggs in utero; CS:  $15 \pm 0.6$  eggs in utero) ( $p < 0.01$ ) in the CHB vaccines. The expression of antigen-specific IgA and IgG2, combined with the presence of CD4<sup>+</sup>, CD8<sup>+</sup>, CD45RA<sup>+</sup> cells and globule leukocytes in the abomasal wall, were associated with this protective effect of the vaccine in the CHB lambs. On the other hand, in CS lambs, antigen-specific IgG1 levels and CD45RA<sup>+</sup> cells, globule leukocytes, mast cells were negatively

associated with parasitological parameters (Pérez-Hernández, 2022).

#### 3.2 Transcriptomic analysis

A total of 239 significantly DEG were identified between vaccinated CHB and CS lamb lymph node tissue after establishing an FDR-adjusted p-value threshold of  $<0.01$  and a FC cut-off of  $>1.2$ . When comparing breeds, 91 and 148 DEG were upregulated in the vaccinated CHB lambs (Annex- Table 1) and CS lambs (Annex- Table 2), respectively.

In the CHB group, a considerable number of DEG were immune-related. For instance, several genes identified are known to be involved in the regulation of the innate immune system, such as antigen presentation through Class I Major Histocompatibility Complex (MHCI) molecules (RNF14, HECW2, ASB2, BTNL2) and activation of type I interferon responses (TMEM173). Similarly, other DEG identified had a role in direct killing of infectious and parasitic agents (for example, SC5, LYZ, HMGN2, SCG2 genes). Genes regulating immunity (HGSNAT) were also differentially expressed in the CHB lambs, with one set of genes involved in up-regulation of inflammatory processes (GBP1, GBP2, MTHFD2, TCAF2, SLCO3A1, HS1BP3, MGB1, IL-36 $\beta$ ), whereas others had anti-inflammatory functions (BTNL2, OSGIN1, RGS22, NLRP12, RPL13A).

Genes encoding lymphocyte (LY6L) antigens, T cell receptor (TCR) (TCRB- T cell beta chain, TCRG- T cell Gamma chain) and immunoglobulins (JCHAIN, FCGR2A- Fc Gamma Receptor IIa) were highly expressed in this breed. A further set of identified DEG was related to effector cells (MBP2, GNLY), or had tissue structure and wound repair-related functions, such as keratinocyte differentiation (ETV4), protection of the epithelial barrier (SERPINB12), angiogenesis (ECM1) and fibrinolysis (PLAU).

In the CS breed, a considerable proportion of the identified DEG were involved in innate immune processes such as antigen presentation (RNF144B, ABCC4, HLA-B- Class I MHC antigen B alpha chain, SLA-DQA- Class II MHC antigen DQ alpha 1 chain) and damage to infectious and parasitic agents (CST9L, DUOXA2, PRSS2, DEFB104A). Also identified as DEG, were up-regulated genes participating in inflammation and immune regulation and signaling (GPNMB, PTEN), with some genes involved in pro-inflammatory (FANCC, RHEBL1, PELI3, GBP1, MSMP, USP29, MAPK10, GBP4) and anti-inflammatory processes (LIPH, CSMD1, RNF144B, RASAL3, PARG, ALOX15B, LCTL, MYADML, CST9L). Also, some genes encoding  $\alpha\beta$  (TCRA, TRAV24, TCRB),  $\gamma\delta$  (TCRD, WC1,

CD163L1) and T reg (ART1) population markers were differentially expressed. Also, NK cell (B3GAT1) markers, B-cells (TLE3, DLK1) and immunoglobulin components (IGKV2D-26) were found to be highly expressed in this group. A further subset of genes involved in maintaining tissue architecture were also identified as DEG in the CS lambs (MYADML & AFDN).

In the cluster annotation analysis, a total of 35 and 21 pathways were obtained from the DEG in CHB and CS lambs, respectively. In CHB lambs, the clusters with the largest numbers of genes was associated with the immune system, neutrophil degranulation, the innate immune system, metabolism of proteins and metabolism (Annex-Table 3). Similarly, in the CS lambs, a high proportion of DEG were clustered in the immune system, the innate immune system, metabolism and signal transduction (Annex- Table 4).

#### 4. Discussion

Developing stable, long-lasting protection against *T. circumcincta* for lambs to reduce the economic impact of this disease and ensure animal welfare has been a long-term driver for vaccine development against this parasitic nematode. However, attempts to protect young lambs by vaccination against challenge have yielded inconsistent results due to difficulties in inducing appropriate protective, immune responses after

vaccination in various breeds. The recombinant prototype vaccine against *T. circumcincta* has been found to induce significant protection against challenge periparturient Texel crossbred ewes (Nisbet *et al.*, 2016a) and in lambs (Nisbet *et al.*, 2013), albeit with considerable age-dependent variability in vaccine efficacy (Liu *et al.*, 2022). An exception to this was the favourable outcome obtained previously in 3-month-old CHB lambs, a GIN-resistant breed (González *et al.*, 2008), where immunization impacted egg excretion, abomasal worm establishment and yielded shorter and less prolific worms (Pérez-Hernández *et al.*, 2022). Vaccination did not lead to reductions in these parasitological measurements in 3-month-old CS lambs, a more GIN-susceptible breed. After analyzing the immune responses at the infection site, these differences seem to be due to a more rapid immune response in vaccinated CHB group, in which CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes are likely to play a role in coordinating a protective response, along with globular leukocytes, B lymphocytes, and *T. circumcincta* specific IgA and IgG2 (Pérez-Hernández *et al.*, 2022). However, there is still a lack of knowledge about the dynamics of the immune and non-immune related pathways that could predict a protective response in young individuals. In this study, we sought to obtain more knowledge of response variability after vaccination and challenge with *T.*

*circumcincta* in young lambs by exploring breed differences in the transcriptome profile in the abomasal lymph node in coordinating host response at the site of infection.

The transcriptomic analysis showed substantial numbers of DEG between breeds (239), possibly reflecting the considerable differences in parasite viability following vaccination. The number of genes that were more highly expressed between breeds was higher in the CS lambs (148) than in the CHB lambs (91). In both breeds, a major proportion of DEG were involved in the immune system process, which may be a consequence of the responses induced by the vaccine, subsequent exposure to the parasite and the intrinsic nature of the sampled tissue.

Some genes involved in innate immunity were identified as DEG in both breeds, such as those involved in antigen presentation through MHC molecules. Additionally, in the CS breed, some genes were implicated in dendritic cell migration (ABCC4) (van de Ven *et al.*, 2009) and Toll-Like receptor activation (PELI3, USP29). Toll-like receptors are an innate pathogen recognition system with an important role in the induction of cytokines, and other trigger signals, for the adaptive immune response (McRae *et al.*, 2015). Similarly, dendritic cells are able to sample foreign antigens, migrate to local lymph nodes and

present antigens to naïve T cells through an MHC molecule, initiating the adaptive immune response (Abbas *et al.*, 1999). These results could represent the effect of pathogen infection on activation of immune mechanisms.

Similarly, some genes encoding proteins with antibiotic, antifungal and, in some cases, antiprotozoal activity in mucus were significantly expressed in both breeds after vaccination. Some examples in the CHB lambs include secretogranin II (SCG2) (Shooshtarizadeh *et al.*, 2010), antibacterial peptide SMAP-29 (SC5) (Giacometti *et al.*, 2003; Skerlavaj *et al.*, 1999) and lysozyme (LYZ), all of which are characterized as defensins in the mucosal barrier. In CS vaccinated lambs, DEG identified include cystatin 9 (CST9L), which has demonstrated antimicrobial activity (Eaves-Pyles *et al.*, 2013), and DUOXA 2, which is reportedly expressed in respiratory tract epithelium and in gastrointestinal mucosa, where it plays a role in antimicrobial defense by participating in generation of H<sub>2</sub>O<sub>2</sub> (Carré *et al.*, 2015). There is growing interest in the potential role of these proteins in parasitic nematode infections due to their ability to mediate in host-parasite interactions. For example, local expression of galectins and interlectins is characteristic of GIN infections and is known to influence nematode survival, probably by

modifying mucus properties (Artis, 2006; Donskow-Łysoniewska *et al.*, 2021).

A high number of DEG with immunomodulatory properties were identified in both breeds. In the CHB lambs, some of these genes encode proteins that activate cell signaling, chemotaxis and cell proliferation (MGB1, MTHFD2, TCAF2) (Acharya *et al.*, 2021; Sugiura *et al.*, 2022) or control differentiation and proliferation through cell death (OSGIN1), whereas other molecules suppress immune responses (NLRP12) and T cell activity (BTNL2) (Abeler-Dörner *et al.*, 2012). Interestingly, in vaccinated CS lambs, regulatory genes were numerous. Some of these are known to increase regulatory T cells such as ART1 (Cortés-Garcia *et al.*, 2016) and LIPH, which reduces CD8<sup>+</sup> populations (Zhuang *et al.*, 2022). Others, such as GPNMB, reduce T cell functionality (Saade *et al.*, 2021), and B3GAT1, which is a marker of NK and T cells with reduced functionality and inability to proliferate (Kared *et al.*, 2016). Several of the identified DEG in the vaccinated CS lambs (PARG, ALOX15B, MYADML, CST9L) are known to participate in anti-inflammatory responses (Aranda *et al.*, 2013; Eaves-Pyles *et al.*, 2013; Snodgrass & Brüne, 2019; Wang *et al.*, 2019). Other DEG, such as CSMD1, may inhibit complement (Blom, 2017), and RASAL3, which

negatively regulates neutrophils and NK cells (Saito *et al.*, 2021).

Several genes (GBP1, GBP2, GPB4, FANCC) that participate in interferon- $\gamma$  (INF- $\gamma$ ) activation were significantly differentially expressed in both breeds. IFN- $\gamma$  is a molecule traditionally associated with responses against intracellular pathogens (Type 1 responses) and not with protection against GINs (Type 2 responses) (Gill *et al.*, 2000; Spellberg & Edwards, 2001). However, there is increasing evidence to support that GIN-infected animals display a concomitant activation of type 1 and 2 pathways (Aboshady *et al.*, 2020). This could be due to the ability of some animals to balance immune responses as observed in mixed infections with intracellular and extracellular pathogens in wild ruminants (Corripi-Miyar *et al.*, 2021). Furthermore, experimental assays have highlighted the importance of an early and wide activation of these routes in parasitic nematode rejection (Aboshady *et al.*, 2022; Liu *et al.*, 2022).

In the CHB lambs, several identified DEG were T lymphocyte associated. Some encoded parts of T-cell receptor (TCR) structure ( $\beta$  and  $\gamma$  chains), whilst others were T cell- activating proteins such as TCAF2 and MTHFD2. TCAF2 is a surface  $\text{Ca}^{+2}$  channel in T cells, recently described as an important regulator of TCR activation and

differentiation to effector cells (Acharya *et al.*, 2021), while MTHFD2 is an enzyme belonging to the *de novo* purine synthesis pathway that participates in signaling in activated T cells to promote proliferation and inflammatory cytokine production (Sugiura *et al.*, 2022). In addition, interleukin-36 $\beta$  is involved in promoting dendritic cell maturation and inducing type 1 responses (Dong *et al.*, 2022), but also induces type 2 responses and downregulates T cell (Treg) responses in mice and humans with inflammatory bowel disease (Zhu *et al.*, 2022). In the CS lambs, putative immune mechanisms involved in responses to vaccination and challenge involving T cells were more limited. In fact, sequences encoding TCR  $\alpha$  (TCRA) and TRAV24),  $\beta$  (TCRB) and  $\delta$  chains (TCRD), as well as WC1 antigen, a  $\gamma\delta$  T cell subpopulation marker, which is a cell associated with innate responses, were identified as DEG in this breed. Moreover, DLK1, a gene instrumental for B cell development (Raghunandan *et al.*, 2008), and MSMP, a peripheral blood monocyte- and lymphocyte-attracting chemokine (Pei *et al.*, 2014) were more highly expressed in this breed.

These results highlight an important component of T cell presence, activation, differentiation and cytokine production, which has long been recognized as a main control

mechanism against parasitic nematode establishment, fecundity and egg output through B-cell activation and IgA and IgE production that stimulate eosinophil and mast cell degranulation (Balic *et al.*, 2000). The predominant role of T cells, described here, is in keeping with the previous histological analysis, especially in CHB lambs, where CD4<sup>+</sup> T cells were involved in stimulating a strong adaptive type 2 response (Pérez-Hernández *et al.*, 2022). Regarding the marked differences between breeds in T cell involvement, it is possible that the balance of genes stimulating T-cell proliferation and activation may be different between breeds at this age, resulting in differences in vaccine efficacy. This interpretation is based on the results obtained in 6-month-old CS lambs, where vaccines had higher IgA and IgG2 levels and a higher CD4/CD8 ratio than control CS lambs (Quil A-only recipients) (Machín *et al.*, 2021). Also, subsequent transcriptomic analysis demonstrated TCR alpha variable (TRAV41) and delta locus (TRD) were more highly expressed in CS vaccines than in controls (Machín, 2021), suggesting the limited participation of T cells at 3 months may be age-related.

Certainly, the effects of breed differences after vaccination were most evident in effector responses against the parasite. In CHB vaccines,

increased expression of MBP2 compared to CS, associated with activation of eosinophil granules, was identified. This protein has an important role in synthesis of histamine, which is present in mast cells granules. Eosinophils, mast cells and globule leukocytes are cells associated with worm growth and fecundity impairment and burden regulation in *T. circumcincta* (Stear *et al.*, 1995) and *H. contortus* (Nisbet *et al.*, 2016b) infection. Here, DEG were observed that are linked with IgG receptor expression (FCGR2A) and a J-chain protein (JCHAIN), key in the formation of IgA dimers and IgM pentamers. As alluded to above, IgA has a main role in protection against *T. circumcincta* (Stear *et al.*, 2004), especially against L4, which is the parasitic stage against which most of the proteins in this vaccine cocktail were developed (Ellis *et al.*, 2014). Interestingly, in the more susceptible breed, only one gene (IGKV2D-26), which encodes a region of light immunoglobulin chains and participates in antigen recognition, was upregulated.

Epithelial integrity appears to be important in the GIN-resistant breed (CHB), represented by the increased expression of SERPINB12, which is a highly expressed epithelial protein that has been attributed to the ability to protect cells from the damaging action of proteases and maintaining barrier function

(Niehaus *et al.*, 2015). Moreover, production of extracellular matrix proteins, angiogenesis (ECM1) and keratinocyte migration (ETV4), typically occur during classical stages of wound repair and regeneration (Gurtner *et al.*, 2008). Previous studies have suggested heightened cell proliferation and tissue repair contributing to host resistance to GIN infection in this breed (Guo *et al.*, 2016).

Pathway analysis demonstrated enriched immune responses, innate immunity and cell signaling pathways were upregulated in both breeds. In CS lambs, innate immunity, defensins and TLR routes were particularly numerous. In the CHB lambs, enriched routes included haemostasis and protein metabolism processes such as ribosome metabolism, RNA transcription and DNA replication.

The study of abomasal lymph nodes transcripts here suggests that young vaccinated CHB lambs are able to coordinate T cells more efficiently than age-matched vaccinated CS lambs, triggering a wider range of humoral and cellular responses, based on participation of immunoglobulins and effector cell populations as identified in the DEG analysis. Interestingly, CHB lambs responded with enhanced innate responses to intracellular agents, which may be a suitable formula for balancing the protective response in

the context of an increased type 2 response and may be important in maintaining balance with gut microbiota. In CS sheep, vaccination generated differences in expression of innate protective responses and was predominantly oriented towards intracellular pathogens such as viruses or bacteria, which is presumably not an adequate response to protect against GIN such as *T. circumcincta*. The upregulation of immunoregulatory cells (Treg) and components in this breed is noteworthy. Perhaps, including adjuvants that control this type of immunoregulation could be useful to reverse a lack of response in young lambs of more nematode-susceptible breeds.

Other studies on resistant breeds have highlighted that timing of the anti-parasitic response could be key in resistance. Since this study evaluated transcriptome dynamics after 40 days of infection, this could explain why both breeds showed an array of immune mechanisms, but the protective effect on parasitological parameters was only evident in CHB lambs. It is possible that susceptible CS lambs have a higher stimulation threshold, taking them longer to develop a protective response. Another aspect to consider is that this study was performed on lymph node tissue and may not accurately represent all effector mechanisms participating at

the site of infection in the abomasal wall. Future studies should focus on following development of immune responses using sequential biopsies and investigating the effect of the immunogens in pathway activation in each breed. This could elucidate differences between responder and non-responder individuals and provide new approaches to improve protection elicited by the vaccine in young lambs.

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# *Anexo*

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## *Capítulo II*

*Exploring the transcriptomic changes underlying recombinant vaccine efficacy against Teladorsagia circumcincta in 3-month-old lambs*



**Table 1.** Differentially expressed genes (DEG) at *p* value 0.05 and 1.2-fold in lymph node tissue in CHB lambs vaccinated with a recombinant subunit vaccine against *Teladorsagia circumcincta* and subsequently challenged with the parasite.

Gene ID	Symbol	Gene description	Fold change (FC)	<i>p</i> value	<i>p</i> value (adjusted)
ENSOARG00000016936	MPAN	Protein C19orf12 homolog	-17.201	1.016E <sup>-3</sup>	4.644E <sup>-1</sup>
ENSOARG00000001588	GBP2	Guanylate-binding protein 2-like	-12.935	1.946E <sup>-5</sup>	1.051E <sup>-1</sup>
ENSOARG00000013171	-	Uncharacterized protein C21orf58 homolog isoform X2	-6.049	5.908E <sup>-3</sup>	7.498E <sup>-1</sup>
ENSOARG00000015226	GBP1	Guanylate-binding protein 1-like	-5.713	5.473E <sup>-4</sup>	3.873E <sup>-1</sup>
ENSOARG00000002372	GBP2	Guanylate-binding protein 2	-5.047	3.156E <sup>-4</sup>	2.730E <sup>-1</sup>
ENSOARG00000005233	SNTG2	Syntrophin gamma 2	-5.002	5.397E <sup>-3</sup>	7.301E <sup>-1</sup>
ENSOARG00000006441	SERPINB12	Serpin family B member 12	-4.971	8.073E <sup>-3</sup>	8.023E <sup>-1</sup>
ENSOARG000000017541	BTNL2	Butyrophilin-like protein 1	-4.744	6.677E <sup>-3</sup>	7.763E <sup>-1</sup>
ENSOARG00000002154	SC5	Cathelin-related peptide SC5 precursor	-4.568	7.856E <sup>-3</sup>	7.977E <sup>-1</sup>
ENSOARG00000002301	MBP2	Pro eosinophil major basic protein 2	-4.381	7.980E <sup>-3</sup>	8.023E <sup>-1</sup>
ENSOARG00000002036	GNLY	Granulysin	-3.646	5.360E <sup>-3</sup>	7.301E <sup>-1</sup>
ENSOARG00000005930	FIGN	Fidgetin, microtubule severing factor	-3.606	3.145E <sup>-4</sup>	2.730E <sup>-1</sup>
ENSOARG00000001348	LY6L	Lymphocyte antigen 6 family member L	-3.539	1.383E <sup>-3</sup>	4.918E <sup>-1</sup>
ENSOARG00000014805	MGB1	Mammaglobin-A	-3.396	5.965E <sup>-3</sup>	7.511E <sup>-1</sup>
ENSOARG00000019783	SCG2	Secretogranin II	-3.390	1.680E <sup>-3</sup>	5.302E <sup>-1</sup>
ENSOARG00000015335	CBLN4	Cerebellin 4 precursor	-3.237	4.558E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000016275	TCRB	Hypothetical protein M91_00608	-3.228	4.984E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000015650	DPYS	Dihydropyrimidinase	-3.202	1.046E <sup>-3</sup>	4.644E <sup>-1</sup>
ENSOARG00000016474	CLNS1A	Chloride Nucleotide-Sensitive Channel 1A	-3.195	4.961E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000020912	RNF14	E3 Ubiquitin-Protein Ligase RNF14	-3.142	8.560E <sup>-3</sup>	8.030E <sup>-1</sup>

ENSOARG00000010509	FAM174B	Family with sequence similarity 174 member B	-3.104	2.871E <sup>-3</sup>	6.510E <sup>-1</sup>
ENSOARG0000001692	RGS22	Regulator of G protein signaling 22	-3.048	8.552E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG0000000492	NLRP12	NLR family pyrin domain containing 12	-3.018	5.997E <sup>-3</sup>	7.511E <sup>-1</sup>
ENSOARG00000014538	SLC25A45	Solute carrier family 25 member 45	-2.985	9.952E <sup>-4</sup>	4.644E <sup>-1</sup>
ENSOARG00000020263	IQCGL	IQ motif containing G	-2.960	2.342E <sup>-3</sup>	5.974E <sup>-1</sup>
ENSOARG00000007582	CAVIN4	Caveolae associated protein 4	-2.921	1.197E <sup>-3</sup>	4.918E <sup>-1</sup>
ENSOARG0000000327	LAMA1	Laminin subunit alpha 1	-2.915	2.676E <sup>-5</sup>	1.051E <sup>-1</sup>
ENSOARG00000020476	LYZ	Lysozyme	-2.891	3.118E <sup>-3</sup>	6.806E <sup>-1</sup>
ENSOARG00000008405	CASQ1	Calsequestrin 1	-2.848	2.783E <sup>-3</sup>	6.460E <sup>-1</sup>
ENSOARG00000010648	GNG8	G protein subunit gamma 8	-2.838	9.774E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000004418	RPL7	60S ribosomal protein L7	-2.779	6.071E <sup>-3</sup>	7.511E <sup>-1</sup>
ENSOARG00000004153	NKX2-5	NK2 homeobox 5	-2.710	4.914E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000012744	NRAP	Nebulin related anchoring protein	-2.693	1.942E <sup>-4</sup>	2.221E <sup>-1</sup>
ENSOARG00000007103	PRSS44P	Serine protease 44-like	-2.633	9.961E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000003549	CNIH2	Cornichon family AMPA receptor auxiliary protein 2	-2.598	9.741E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000001797	TRG	T-cell receptor gamma locus	-2.504	2.354E <sup>-3</sup>	5.974E <sup>-1</sup>
ENSOARG00000015845	TRB	T-cell receptor beta chain	-2.499	3.305E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000016157	MTHFD2	Methenyltetrahydrofolate Cyclohydrolase	-2.485	4.994E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000004381	TMEM26	Transmembrane protein 26	-2.458	7.114E <sup>-3</sup>	7.840E <sup>-1</sup>
ENSOARG00000017008	GNPDA	Glucosamine-6-phosphate isomerase 1	-2.432	2.006E <sup>-3</sup>	5.511E <sup>-1</sup>
ENSOARG00000020874	TMEM54	Transmembrane protein 54	-2.313	2.111E <sup>-3</sup>	5.667E <sup>-1</sup>
ENSOARG00000017714	CCDC184	Coiled-coil domain containing 184	-2.298	9.571E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000016475	ANKRD26	Ankyrin repeat domain-containing protein 26-like	-2.245	9.630E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000010840	RSPH1	Radial spoke head 1 homolog	-2.237	1.944E <sup>-3</sup>	5.508E <sup>-1</sup>

ENSOARG00000019998	SLC2A13	Solute carrier family 2 member 13	-2.178	9.963E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000020843	IL36B	Interleukin 36 beta	-2.094	2.827E <sup>-3</sup>	6.473E <sup>-1</sup>
ENSOARG00000017884	NXPH4	Neurexophilin 4	-2.055	9.017E <sup>-3</sup>	8.069E <sup>-1</sup>
ENSOARG00000010051	FCGR2A	Fc Gamma Receptor IIa	-2.013	7.041E <sup>-3</sup>	7.830E <sup>-1</sup>
ENSOARG00000018199	TCAF2	TRPM8 channel associated factor 2	-2.006	4.630E <sup>-4</sup>	3.379E <sup>-1</sup>
ENSOARG00000016321	KAZALD1	Kazal Type Serine Peptidase Inhibitor Domain 1	-1.925	3.891E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000020383	BDH1	3-hydroxybutyrate dehydrogenase 1	-1.868	1.289E <sup>-4</sup>	2.201E <sup>-1</sup>
ENSOARG00000005011	TTC21B	Tetratricopeptide repeat domain 21B	-1.868	9.154E <sup>-4</sup>	4.644E <sup>-1</sup>
ENSOARG00000012863	SRSF12	Serine and arginine-rich splicing factor 12	-1.856	9.147E <sup>-3</sup>	8.101E <sup>-1</sup>
ENSOARG00000000262	TRAM1L1	Translocation associated membrane protein 1-like 1	-1.836	8.942E <sup>-3</sup>	8.063E <sup>-1</sup>
ENSOARG00000005637	ETV4	ETS variant 4	-1.757	8.054E <sup>-3</sup>	8.023E <sup>-1</sup>
ENSOARG00000004915	TM4SF18	Transmembrane 4 L six family member 18	-1.756	7.318E <sup>-3</sup>	7.861E <sup>-1</sup>
ENSOARG00000009792	OSGIN1	Oxidative stress induced growth inhibitor 1	-1.717	4.617E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000008610	CYP2J2	Cytochrome P450 2J2-like	-1.702	6.787E <sup>-3</sup>	7.763E <sup>-1</sup>
ENSOARG00000020159	RPL13A	60S ribosomal protein L13a	-1.662	3.750E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000000998	DHX35	DEAH-box helicase 35	-1.652	1.919E <sup>-3</sup>	5.508E <sup>-1</sup>
ENSOARG00000015882	PPFIBP2	PPFIA binding protein 2	-1.641	8.696E <sup>-4</sup>	4.644E <sup>-1</sup>
ENSOARG00000013467	HMGN2	High Mobility Group Nucleosomal Binding Domain 2	-1.640	8.229E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG00000002377	UGGT2	UDP-glucose glycoprotein glucosyltransferase 2	-1.639	1.463E <sup>-3</sup>	4.944E <sup>-1</sup>
ENSOARG00000012675	TKFC	Triokinase and FMN cyclase	-1.635	1.997E <sup>-4</sup>	2.221E <sup>-1</sup>
ENSOARG00000007112	VMO1	Vitelline membrane outer layer 1 homolog	-1.616	6.083E <sup>-4</sup>	4.125E <sup>-1</sup>
ENSOARG00000014836	HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	-1.607	3.957E <sup>-3</sup>	7.001E <sup>-1</sup>

ENSOARG00000010528	SLCO3A1	Solute carrier organic anion transporter family member 3A1	-1.598	1.779E <sup>-3</sup>	5.432E <sup>-1</sup>
ENSOARG00000008327	TBC1D32	TBC1 domain family member 32	-1.587	8.868E <sup>-3</sup>	8.063E <sup>-1</sup>
ENSOARG00000019510	RPS26	Ribosomal protein S26	-1.584	8.650E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG00000011599	JCHAIN	Joining chain of multimeric IgA and IgM	-1.560	4.501E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000014168	ASB2	Ankyrin repeat and SOCS box containing 2	-1.541	4.918E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000006046	ICA1	Islet cell autoantigen 1	-1.531	2.930E <sup>-3</sup>	6.579E <sup>-1</sup>
ENSOARG00000015106	WIPI1	WD repeat domain phosphoinositide-interacting protein 1	-1.522	8.062E <sup>-4</sup>	4.626E <sup>-1</sup>
ENSOARG00000017265	TMEM173	Transmembrane protein 173	-1.478	3.312E <sup>-4</sup>	2.762E <sup>-1</sup>
ENSOARG00000015506	STX2	syntaxin 2	-1.455	1.890E <sup>-3</sup>	5.508E <sup>-1</sup>
ENSOARG00000006295	RIDA	Reactive Intermediate Imine Deaminase A Homolog	-1.441	1.374E <sup>-3</sup>	4.918E <sup>-1</sup>
ENSOARG00000002176	DNAJC3	DnaJ heat shock protein family (Hsp40) member C3	-1.390	2.388E <sup>-3</sup>	5.997E <sup>-1</sup>
ENSOARG00000016355	CCDC112	Coiled-coil domain containing 112	-1.385	4.981E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000010339	YJU2	YJU2 splicing factor homolog	-1.385	2.621E <sup>-3</sup>	6.311E <sup>-1</sup>
ENSOARG00000020820	ECM1	Extracellular matrix protein 1	-1.378	3.602E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000018095	ABCA3	ATP binding cassette subfamily A member 3	-1.373	2.794E <sup>-3</sup>	6.460E <sup>-1</sup>
ENSOARG00000019897	APLF	Aprataxin and PNKP like factor	-1.363	3.871E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG0000001796	JDP2	Jun dimerization protein 2	-1.341	3.527E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000014838	NAP1L1	Nucleosome assembly protein 1 like 1	-1.320	7.949E <sup>-3</sup>	8.023E <sup>-1</sup>
ENSOARG00000017302	HS1BP3	HCLS1 binding protein 3	-1.308	4.435E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000005918	SARS2	Seryl-tRNA synthetase 2, mitochondrial	-1.300	2.684E <sup>-3</sup>	6.397E <sup>-1</sup>

ENSOARG00000012342	KNOP1	Lysine rich nucleolar protein 1	-1.297	9.837E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000005846	TMC8	Transmembrane channel like 8	-1.290	7.778E <sup>-3</sup>	7.977E <sup>-1</sup>
ENSOARG00000003777	HGSNAT	Heparan-alpha- glucosaminide N- acetyltransferase	-1.288	4.479E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000008473	PLAU	Urokinase-type plasminogen activator precursor	-1.286	1.652E <sup>-3</sup>	5.284E <sup>-1</sup>
ENSOARG00000013449	AK3	Adenylate kinase 3	-1.250	6.111E <sup>-3</sup>	7.511E <sup>-1</sup>



**Table 2.** Differentially expressed genes (DEG) at *p* value 0.05 and 1.2-fold in lymph node tissue in CS lambs vaccinated with a recombinant subunit vaccine against *Teladorsagia circumcincta* and subsequently challenged with the parasite.

Gene ID	Symbol	Gene description	Fold change (FC)	<i>p</i> value	<i>p</i> value (adjusted)
ENSOARG000000011671	CADPS	Calcium dependent secretion activator	19,356	3.165E <sup>-5</sup>	1.051E <sup>-1</sup>
ENSOARG00000001535	GBP4	Guanylate-binding protein 4-like	15,671	3.921E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000002250	ABCC4	Multidrug resistance-associated protein 4-like	11,392	2.187E <sup>-4</sup>	2.321E <sup>-1</sup>
ENSOARG00000001169	-	Hypothetical protein M91_13651	10,613	7.825E <sup>-3</sup>	7.977E <sup>-1</sup>
ENSOARG000000020807	IGKV2D-26	Immunoglobulin kappa variable 2D-26	8,776	1.388E <sup>-3</sup>	4.918E <sup>-1</sup>
ENSOARG00000000222	ABCC4	Multidrug resistance-associated protein 4-like	7,992	1.741E <sup>-5</sup>	1.051E <sup>-1</sup>
ENSOARG00000002078	ABCC4	Multidrug resistance-associated protein 4-like	5,915	4.031E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG000000013397	MRAP2	Melanocortin 2 receptor accessory protein 2	4,993	3.003E <sup>-5</sup>	1.051E <sup>-1</sup>
ENSOARG00000001232	ABCC4	Multidrug resistance-associated protein 4-like	4,858	1.408E <sup>-4</sup>	2.201E <sup>-1</sup>
ENSOARG00000001895	ABCC4	Multidrug resistance-associated protein 4-like	4,704	1.342E <sup>-3</sup>	4.918E <sup>-1</sup>
ENSOARG00000001594	ABCC4	Multidrug resistance-associated protein 4-like	4,673	1.158E <sup>-4</sup>	2.201E <sup>-1</sup>
ENSOARG00000001404	ABCC4	Multidrug resistance-associated protein 4-like	4,499	4.727E <sup>-7</sup>	1.104E <sup>-2</sup>
ENSOARG000000016054	MYO7B	Myosin VIIB	4,480	1.092E <sup>-5</sup>	1.051E <sup>-1</sup>
ENSOARG00000003887	MAPK10	Mitogen-activated protein kinase 10	4,465	6.220E <sup>-4</sup>	4.125E <sup>-1</sup>
ENSOARG00000003367	SMIM17	Small integral membrane protein 17	4,330	1.503E <sup>-4</sup>	2.201E <sup>-1</sup>
ENSOARG00000006141	CST9L	Cystatin-9-like	4,257	3.989E <sup>-4</sup>	3.105E <sup>-1</sup>
ENSOARG00000003855	KLHDC8A	Kelch domain containing 8A	4,137	1.294E <sup>-4</sup>	2.201E <sup>-1</sup>
ENSOARG00000001459	-	Antigen WC1.1-like	4,054	3.453E <sup>-4</sup>	2.781E <sup>-1</sup>
ENSOARG000000016990	RPL22L1	60S ribosomal protein L22-like 1	4,041	4.884E <sup>-3</sup>	7.026E <sup>-1</sup>

ENSOARG00000020726	NLGN1	Neuroligin 1	3,905	1.725E <sup>-3</sup>	5.372E <sup>-1</sup>
ENSOARG0000000833	TPO	Thyroid peroxidase-like	3,803	4.954E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000003432	USP29	Ubiquitin Specific Peptidase 29	3,760	1.054E <sup>-3</sup>	4.644E <sup>-1</sup>
ENSOARG00000016555	PRSS2	Anionic trypsin	3,705	1.434E <sup>-3</sup>	4.944E <sup>-1</sup>
ENSOARG00000002307	DLK1	Delta like non-canonical Notch ligand 1	3,702	8.733E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG00000014376	B3GAT1	Beta-1,3-glucuronidyltransferase 1	3,672	8.121E <sup>-4</sup>	4.626E <sup>-1</sup>
ENSOARG00000002582	ART3	GPI-linked NAD(P)(+)-arginine ADP-Ribosyltransferase 1-like	3,570	9.816E <sup>-4</sup>	4.644E <sup>-1</sup>
ENSOARG00000001067	ABCC4	Multidrug resistance-associated protein 4-like	3,555	1.814E <sup>-3</sup>	5.432E <sup>-1</sup>
ENSOARG00000019553	TRAV24	T-cell receptor alpha variable 24	3,512	2.325E <sup>-3</sup>	5.974E <sup>-1</sup>
ENSOARG00000017240	HECW1	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1	3,498	1.482E <sup>-3</sup>	4.944E <sup>-1</sup>
ENSOARG00000016365	DEPTOR	DEP Domain Containing MTOR Interacting Protein	3,484	1.791E <sup>-3</sup>	5.432E <sup>-1</sup>
ENSOARG00000005250	TPO	Thyroid peroxidase-like	3,450	8.416E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG00000016417	-	-	3,443	7.882E <sup>-4</sup>	4.626E <sup>-1</sup>
ENSOARG00000012071	DERA	Deoxyribose-phosphate aldolase	3,399	6.776E <sup>-3</sup>	7.763E <sup>-1</sup>
ENSOARG00000011784	MSMP	Microseminoprotein, prostate associated	3,396	6.359E <sup>-4</sup>	4.125E <sup>-1</sup>
ENSOARG00000005756	BRINP1	BMP/Retinoic acid inducible neural specific 1	3,302	8.939E <sup>-3</sup>	8.063E <sup>-1</sup>
ENSOARG00000006582	KCNQ3	Potassium voltage-gated channel subfamily Q member 3	3,278	8.428E <sup>-4</sup>	4.644E <sup>-1</sup>
ENSOARG00000010193	PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	3,104	1.451E <sup>-4</sup>	2.201E <sup>-1</sup>
ENSOARG0000000913	NAXD	ATP-dependent (S)-NAD(P)H-hydrate dehydratase-like(LOC105606082)	3,069	7.568E <sup>-3</sup>	7.977E <sup>-1</sup>
ENSOARG00000001690	-	-	3,007	3.683E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000015756	AHR	Aryl Hydrocarbon Receptor	3,006	1.193E <sup>-3</sup>	4.918E <sup>-1</sup>

ENSOARG00000014160	SAXO1	Stabilizer of axonemal microtubules 1	2,992	5.647E <sup>-3</sup>	7.319E <sup>-1</sup>
ENSOARG00000002561	CFAP61	Cilia and flagella associated protein 61	2,983	2.596E <sup>-3</sup>	6.311E <sup>-1</sup>
ENSOARG00000019694	TPPP2	Tubulin polymerization promoting protein family member 2	2,973	3.654E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000002076	ART1	GPI-linked NAD(P)(+)-arginine ADP-ribosyltransferase 1-like	2,957	4.983E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000001772	FOXP2	Forkhead box P2	2,956	1.026E <sup>-3</sup>	4.644E <sup>-1</sup>
ENSOARG00000007477	GBP1	Guanine nucleotide-binding protein subunit beta-like protein 1	2,938	3.048E <sup>-3</sup>	6.738E <sup>-1</sup>
ENSOARG00000002772	TDRD10	Tudor domain containing 10	2,915	1.780E <sup>-4</sup>	2.221E <sup>-1</sup>
ENSOARG000000015485	SLA-DQA	SLA class II histocompatibility antigen, DQ haplotype D alpha chain precursor	2,885	1.369E <sup>-3</sup>	4.918E <sup>-1</sup>
ENSOARG00000014208	-	Transmembrane protein 202-like	2,873	8.347E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG00000000812	ABCC4	Multidrug resistance-associated protein 4-like	2,853	3.419E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000005572	DLX4	Distal-less homeobox 4	2,853	7.348E <sup>-3</sup>	7.861E <sup>-1</sup>
ENSOARG00000010572	HLA-B	HLA class I histocompatibility antigen, B-40 alpha chain-like	2,802	3.406E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000004213	MYO3A	Myosin IIIA	2,794	3.704E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000011015	MYADML	Myeloid-associated differentiation marker-like	2,777	1.976E <sup>-3</sup>	5.508E <sup>-1</sup>
ENSOARG00000007062	CYP2F	Cytochrome P450 2F2-like	2,750	5.621E <sup>-3</sup>	7.319E <sup>-1</sup>
ENSOARG00000001163	ABCC4	Multidrug resistance-associated protein 4-like	2,746	5.382E <sup>-3</sup>	7.301E <sup>-1</sup>
ENSOARG00000001727	DISP3	Dispatched RND transporter family member 3	2,679	4.419E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000001210	CHRM2	Cholinergic receptor muscarinic 2	2,657	4.770E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG000000017470	SLC11A2	Natural resistance-associated macrophage protein 2-like	2,645	1.208E <sup>-3</sup>	4.918E <sup>-1</sup>

ENSOARG00000001543	ABCC4	Multidrug resistance-associated protein 4-like	2,629	1.390E <sup>-3</sup>	4.918E <sup>-1</sup>
ENSOARG00000019498	TRD	Uncharacterized protein LOC102390654	2,625	3.291E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000005901	SERP2	Stress Associated Endoplasmic Reticulum Protein Family Member 2	2,595	5.585E <sup>-3</sup>	7.319E <sup>-1</sup>
ENSOARG00000001522	TAF7L	TATA-box binding protein associated factor 7 like	2,584	4.047E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000000608	CD163L1	Antigen WC1.1	2,549	4.949E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000001860	BEAN1	Brain expressed associated with NEDD4 1	2,537	7.253E <sup>-3</sup>	7.861E <sup>-1</sup>
ENSOARG000000017391	CRH	Corticotropin releasing hormone	2,519	7.702E <sup>-3</sup>	7.977E <sup>-1</sup>
ENSOARG00000001553	ABCC4	Multidrug resistance-associated protein 4-like	2,488	1.568E <sup>-3</sup>	5.159E <sup>-1</sup>
ENSOARG00000001940	MLPH	Melanophilin	2,448	6.423E <sup>-3</sup>	7.737E <sup>-1</sup>
ENSOARG00000005351	PELI3	Pellino E3 ubiquitin protein ligase family member 3	2,447	7.322E <sup>-4</sup>	4.621E <sup>-1</sup>
ENSOARG00000001808	ABCC4	Multidrug resistance-associated protein 4-like	2,429	7.683E <sup>-4</sup>	4.626E <sup>-1</sup>
ENSOARG00000004851	HSD17B13	Hydroxysteroid 17-beta dehydrogenase 13	2,418	6.661E <sup>-3</sup>	7.763E <sup>-1</sup>
ENSOARG00000001058	LCN12	Lipocalin 12	2,394	2.615E <sup>-3</sup>	6.311E <sup>-1</sup>
ENSOARG00000003388	KLHL41	Kelch like family member 41	2,387	4.362E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000007898	PRR19	Proline rich 19	2,377	5.539E <sup>-3</sup>	7.319E <sup>-1</sup>
ENSOARG000000015543	ARHGAP44	Rho GTPase activating protein 44	2,374	3.449E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000001948	ERVK-9	Uncharacterized protein LOC105613207	2,351	4.596E <sup>-4</sup>	3.379E <sup>-1</sup>
ENSOARG000000012404	WDR66	WD repeat-containing protein 66	2,345	8.321E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG000000010570	TMEM213	Transmembrane protein 213	2,342	3.820E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG000000012904	WC1	Antigen WC1.1	2,326	9.512E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG000000021055	DUOXA2	Dual oxidase maturation factor 2	2,320	3.370E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG000000015991	TRB	T cell receptor beta chain	2,293	8.601E <sup>-3</sup>	8.030E <sup>-1</sup>

ENSOARG00000019509	TRDC	T cell receptor delta chain precursor	2,289	7.801E <sup>-3</sup>	7.977E <sup>-1</sup>
ENSOARG00000018240	LCTL	Lactase like	2,278	9.987E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000012510	UCP1	Mitochondrial brown fat uncoupling protein 1	2,272	9.917E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000017895	-	-	2,262	6.081E <sup>-3</sup>	7.511E <sup>-1</sup>
ENSOARG00000000873	PRKCG	Protein kinase C gamma	2,256	4.343E <sup>-5</sup>	1.127E <sup>-1</sup>
ENSOARG000000020199	SPEG	Striated muscle preferentially expressed protein kinase-like	2,242	1.448E <sup>-3</sup>	4.944E <sup>-1</sup>
ENSOARG00000005968	CSMD1	CUB and Sushi multiple domains 1	2,227	8.914E <sup>-3</sup>	8.063E <sup>-1</sup>
ENSOARG00000018725	ADGRF3	Adhesion G protein-coupled receptor F3	2,206	1.577E <sup>-4</sup>	2.201E <sup>-1</sup>
ENSOARG00000003000	Tnfrsf26	Tumor necrosis factor receptor superfamily member 26-like	2,188	8.991E <sup>-3</sup>	8.069E <sup>-1</sup>
ENSOARG00000019496	TRAC	Hypothetical protein M91_16822	2,180	9.695E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG0000000972	PLCXD1	Phosphatidylinositol specific phospholipase C X domain containing 1	2,120	1.890E <sup>-4</sup>	2.221E <sup>-1</sup>
ENSOARG00000009805	-	Uncharacterized protein LOC105613868	2,083	4.213E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000010505	CDHR1	Cadherin related family member 1	2,081	3.827E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000018062	GUCY2F	Guanylate cyclase 2F, retinal	2,026	4.917E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000005941	TNC	Tenascin C	1,974	1.207E <sup>-3</sup>	4.918E <sup>-1</sup>
ENSOARG00000004079	DEFB104A	Beta-defensin 104A-like	1,974	7.722E <sup>-3</sup>	7.977E <sup>-1</sup>
ENSOARG00000004801	CBLN2	Cerebellin 2 precursor	1,948	6.023E <sup>-3</sup>	7.511E <sup>-1</sup>
ENSOARG00000020481	TPRG1	Tumor protein p63 regulated 1	1,920	4.446E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG0000000403	FAM217A	Family with sequence similarity 217 member A	1,919	3.731E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000003360	ESRP2	Epithelial splicing regulatory protein 2	1,903	1.281E <sup>-3</sup>	4.918E <sup>-1</sup>
ENSOARG00000008870	GPR179	G protein-coupled receptor 179	1,890	8.687E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG00000007590	CFAP70	Cilia and flagella associated protein 70	1,862	4.461E <sup>-3</sup>	7.026E <sup>-1</sup>

ENSOARG00000011062	ANKS6	Ankyrin repeat and sterile alpha motif domain containing 6	1,860	6.198E <sup>-3</sup>	7.578E <sup>-1</sup>
ENSOARG00000017570	VXN	Uncharacterized protein C8orf46 homolog	1,850	2.557E <sup>-3</sup>	6.311E <sup>-1</sup>
ENSOARG00000019296	ZFHX2	Zinc finger homeobox 2	1,844	2.804E <sup>-4</sup>	2.730E <sup>-1</sup>
ENSOARG00000020562	LIPH	Lipase H	1,831	8.265E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG00000018613	ALOX15B	Arachidonate 15-lipoxygenase, type B	1,804	4.742E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000015261	ANAPC10	Anaphase-promoting complex subunit 10	1,801	9.660E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000014172	KCNN1	Potassium calcium-activated channel subfamily N member 1	1,799	1.053E <sup>-3</sup>	4.644E <sup>-1</sup>
ENSOARG00000007008	ABHD15	Abhydrolase domain containing 15	1,766	7.309E <sup>-3</sup>	7.861E <sup>-1</sup>
ENSOARG00000015062	SEC31B	SEC31 homolog B, COPII coat complex component	1,765	1.602E <sup>-4</sup>	2.201E <sup>-1</sup>
ENSOARG00000019317	KRTCAP3	Keratinocyte associated protein 3	1,745	7.743E <sup>-3</sup>	7.977E <sup>-1</sup>
ENSOARG00000012703	ZNF791	Zinc finger protein 791-like	1,739	6.969E <sup>-3</sup>	7.787E <sup>-1</sup>
ENSOARG00000002319	PARG	Poly(ADP-ribose) glycohydrolase	1,736	3.975E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000016363	HOXC13	Homeobox C13	1,710	9.160E <sup>-3</sup>	8.101E <sup>-1</sup>
ENSOARG00000008039	NKAIN2	Sodium/Potassium Transporting ATPase Interacting 2	1,708	6.790E <sup>-3</sup>	7.763E <sup>-1</sup>
ENSOARG00000008821	SLC25A34	Solute carrier family 25 member 34	1,700	9.640E <sup>-3</sup>	8.251E <sup>-1</sup>
ENSOARG00000009579	PLPPR3	Phospholipid phosphatase related 3	1,673	6.248E <sup>-3</sup>	7.600E <sup>-1</sup>
ENSOARG00000015260	ZFYVE28	Lateral signaling target protein 2 homolog	1,653	3.708E <sup>-3</sup>	7.001E <sup>-1</sup>
ENSOARG00000014159	-	Hypothetical protein JEQ12_005599	1,641	6.467E <sup>-3</sup>	7.737E <sup>-1</sup>
ENSOARG00000018635	DZANK1	Double zinc ribbon and ankyrin repeat domains 1	1,639	1.638E <sup>-3</sup>	5.284E <sup>-1</sup>
ENSOARG00000018016	PASK	PAS domain containing serine/threonine kinase	1,614	6.905E <sup>-3</sup>	7.763E <sup>-1</sup>

ENSOARG00000002686	TRA	T-cell receptor alpha chain V region 2B4	1,610	7.372E <sup>-3</sup>	7.861E <sup>-1</sup>
ENSOARG00000017597	VN1R1	Vomeronasal type-1 receptor 1- like	1,595	4.293E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000010836	DNM1	Dynamin 1	1,577	4.750E <sup>-3</sup>	7.026E <sup>-1</sup>
ENSOARG00000003583	WNK4	WNK lysine deficient protein kinase 4	1,568	5.142E <sup>-3</sup>	7.123E <sup>-1</sup>
ENSOARG00000011867	RFLNB	Refilin B	1,524	6.914E <sup>-3</sup>	7.763E <sup>-1</sup>
ENSOARG00000018925	RHEBL1	RHEB like 1	1,516	5.409E <sup>-3</sup>	7.301E <sup>-1</sup>
ENSOARG00000012748	GPNMB	Glycoprotein Nmb	1,515	7.559E <sup>-3</sup>	7.977E <sup>-1</sup>
ENSOARG00000017527	ADHFE1	Alcohol dehydrogenase, iron containing 1	1,504	6.718E <sup>-3</sup>	7.763E <sup>-1</sup>
ENSOARG00000003099	RASAL3	RAS protein activator like 3	1,490	3.601E <sup>-5</sup>	1.051E <sup>-1</sup>
ENSOARG00000008608	FANCC	Fanconi anemia complementation group C	1,448	2.351E <sup>-3</sup>	5.974E <sup>-1</sup>
ENSOARG00000004988	AFDN	Afadin, adherens junction formation factor	1,433	6.860E <sup>-3</sup>	7.763E <sup>-1</sup>
ENSOARG00000008719	NDE1	NudE neurodevelopment protein 1	1,384	8.480E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG00000015698	MNT	MAX network transcriptional repressor	1,375	8.069E <sup>-3</sup>	8.023E <sup>-1</sup>
ENSOARG00000017110	PIGG	Phosphatidylinositol glycan anchor biosynthesis class G	1,363	5.517E <sup>-3</sup>	7.319E <sup>-1</sup>
ENSOARG0000000630	IQSEC1	IQ motif and SEC7 domain- containing protein 1	1,329	8.597E <sup>-3</sup>	8.030E <sup>-1</sup>
ENSOARG00000013554	CFAP298	Cilia and flagella associated protein 298	1,311	6.614E <sup>-3</sup>	7.763E <sup>-1</sup>
ENSOARG00000004838	NIPSNAP1	Nipsnap homolog 1	1,299	5.041E <sup>-3</sup>	7.049E <sup>-1</sup>
ENSOARG00000007835	PCMTD2	Protein-L-isoaspartate (D- aspartate) O-methyltransferase domain containing 2	1,298	1.981E <sup>-3</sup>	5.508E <sup>-1</sup>
ENSOARG00000018695	TLE3	Transducin like enhancer of split 3	1,294	2.770E <sup>-3</sup>	6.460E <sup>-1</sup>
ENSOARG00000010343	CHD2	Chromodomain helicase DNA binding protein 2	1,267	5.781E <sup>-3</sup>	7.378E <sup>-1</sup>
ENSOARG00000021113	KIAA0586	KIAA0586	1,255	4.917E <sup>-3</sup>	7.026E <sup>-1</sup>

ENSOARG00000013780	CNNM4	Cyclin and CBS domain divalent metal cation transport mediator	1,230	8.400E <sup>-3</sup>	8.030E <sup>-1</sup>
		4			
ENSOARG00000010155	RNF144B	Ring finger protein 144B	1,224	9.372E <sup>-3</sup>	8.228E <sup>-1</sup>
ENSOARG00000005741	FAM117A	Family with sequence similarity 117 member A	1,215	5.687E <sup>-3</sup>	7.319E <sup>-1</sup>
ENSOARG00000016661	ZNF740	Zinc finger protein 740	1,206	4.862E <sup>-3</sup>	7.026E <sup>-1</sup>

**Table 3.** Enriched pathways identified from differentially expressed genes (DEG) in CHB lambs vaccinated with a recombinant subunit vaccine against *Teladorsagia circumcincta* and subsequently challenged with the parasite.

Pathway	Pathway ID	Annotated Genes Quantity	Annotated Genes
Immune System	REACT:R-HSA-168256	17	ASB2, BTNL2, DNAJC3, FCGR2A, GBP1, GBP2, GNLY, HECW2, HGSNAT, IL36B, LYZ, PLAU, PRG3, RNF14, SERPINB12, STING1, TKFC
Neutrophil degranulation	REACT:R-HSA-6798695	8	DNAJC3, FCGR2A, HGSNAT, LYZ, PLAU, PRG3, SERPINB12, STING1
Viral mRNA Translation	REACT:R-HSA-192823	4	DNAJC3, RPL13A, RPL7, RPS26
Influenza Viral RNA Transcription and Replication	REACT:R-HSA-168273	4	DNAJC3, RPL13A, RPL7, RPS26
Influenza Life Cycle	REACT:R-HSA-168255	4	DNAJC3, RPL13A, RPL7, RPS26
Innate Immune System	REACT:R-HSA-168249	10	DNAJC3, FCGR2A, GNLY, HGSNAT, LYZ, PLAU, PRG3, SERPINB12, STING1, TKFC
Influenza Infection	REACT:R-HSA-168254	4	DNAJC3, RPL13A, RPL7, RPS26
NOD-like receptor signaling pathway	KEGG:hsa04621	4	GBP1, GBP2, NLRP12, STING1
Ribosome, eukaryotes	KEGG:hsa_M00177	3	RPL13A, RPL7, RPS26
Peptide chain elongation	REACT:R-HSA-156902	3	RPL13A, RPL7, RPS26
Selenocysteine synthesis	REACT:R-HSA-2408557	3	RPL13A, RPL7, RPS26
Eukaryotic Translation Termination	REACT:R-HSA-72764	3	RPL13A, RPL7, RPS26
Eukaryotic Translation Elongation	REACT:R-HSA-156842	3	RPL13A, RPL7, RPS26
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	REACT:R-HSA-975956	3	RPL13A, RPL7, RPS26
Formation of a pool of free 40S subunits	REACT:R-HSA-72689	3	RPL13A, RPL7, RPS26
Metabolism of proteins	REACT:R-HSA-392499	10	ABCA3, CNIH2, DNAJC3, GNG8, LYZ, RPL13A, RPL7, RPS26, UGGT2, WIPI1
SRP-dependent cotranslational protein targeting to membrane	REACT:R-HSA-1799339	3	RPL13A, RPL7, RPS26

GTP hydrolysis and joining of the 60S ribosomal subunit	REACT:R-HSA-72706	3	RPL13A, RPL7, RPS26
L13a-mediated translational silencing of Ceruloplasmin expression	REACT:R-HSA-156827	3	RPL13A, RPL7, RPS26
Nonsense-Mediated Decay (NMD)	REACT:R-HSA-927802	3	RPL13A, RPL7, RPS26
Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC)	REACT:R-HSA-975957	3	RPL13A, RPL7, RPS26
Selenoamino acid metabolism	REACT:R-HSA-2408522	3	RPL13A, RPL7, RPS26
Cap-dependent Translation Initiation	REACT:R-HSA-72737	3	RPL13A, RPL7, RPS26
Eukaryotic Translation Initiation	REACT:R-HSA-72613	3	RPL13A, RPL7, RPS26
Ribosome	KEGG:hsa03010	3	RPL13A, RPL7, RPS26
Hemostasis	REACT:R-HSA-109582	5	AK3, ECM1, GNG8, JCHAIN, PLA2U
Infectious disease	REACT:R-HSA-5663205	4	DNAJC3, RPL13A, RPL7, RPS26
IRE1alpha activates chaperones	REACT:R-HSA-381070	2	DNAJC3, WIPI1
Major pathway of rRNA processing in the nucleolus and cytosol	REACT:R-HSA-6791226	3	RPL13A, RPL7, RPS26
Metabolism	REACT:R-HSA-1430728	10	BDH1, CYP2J2, DPYS, GNG8, GNPDA1, MTHFD2, RPL13A, RPL7, RPS26, TKFC
RIG-I-like receptor signaling pathway	KEGG:hsa04622	2	STING1, TKFC
rRNA processing	REACT:R-HSA-72312	3	RPL13A, RPL7, RPS26
rRNA processing in the nucleus and cytosol	REACT:R-HSA-8868773	3	RPL13A, RPL7, RPS26
Translation	REACT:R-HSA-72766	3	RPL13A, RPL7, RPS26
XBP1(S) activates chaperone genes	REACT:R-HSA-381038	2	DNAJC3, WIPI1

**Table 4.** Enriched pathways identified from differentially expressed genes (DEG) in CS lambs vaccinated with a recombinant subunit vaccine against *Teladorsagia circumcincta* and subsequently challenged with the parasite.

Pathway	Pathway ID	Annotated Genes Quantity	Annotated Genes
Alpha-defensins	REACT:R-HSA-1462054	2	ART1, PRSS2
Immune System	REACT:R-HSA-168256	15	ANAPC10, ART1, DERA, DNM1, GBP1, GBP4, HLA-B, KLHL41, MAPK10, PELI3, PRKCG, PRSS2, PTEN, RASAL3, RNF144B
Thyroid hormone synthesis	KEGG:hsa04918	3	DUOXA2, PRKCG, TPO
Autoimmune thyroid disease	KEGG:hsa05320	2	HLA-B, TPO
Autophagy - animal	KEGG:hsa04140	3	DEPTOR, MAPK10, PTEN
cAMP signaling pathway	KEGG:hsa04024	4	ABCC4, AFDN, CHRM2, MAPK10
Cholinergic synapse	KEGG:hsa04725	3	CHRM2, KCNQ3, PRKCG
Defensins	REACT:R-HSA-1461973	2	ART1, PRSS2
Endocrine and other factor-regulated calcium reabsorption	KEGG:hsa04961	2	DNM1, PRKCG
Focal adhesion	KEGG:hsa04510	4	MAPK10, PRKCG, PTEN, TNC
Hepatitis B	KEGG:hsa05161	3	MAPK10, PRKCG, PTEN
Innate Immune System	REACT:R-HSA-168249	10	ART1, DERA, DNM1, HLA-B, MAPK10, PELI3, PRKCG, PRSS2, PTEN, RASAL3
Interferon gamma signaling	REACT:R-HSA-877300	3	GBP1, GBP4, HLA-B
Metabolism	REACT:R-HSA-1430728	13	ADHFE1, AHR, ALOX15B, B3GAT1, CYP2F1, DERA, HSD17B13, LIPH, NAXD, PTEN, RPL22L1, TPO, UCP1
mTOR signaling pathway	KEGG:hsa04150	3	DEPTOR, PRKCG, PTEN
Ras signaling pathway	KEGG:hsa04014	4	AFDN, MAPK10, PRKCG, RASAL3
Signal Transduction	REACT:R-HSA-162582	15	ARHGAP44, CHRM2, CRH, DLK1, DNM1, ESRP2, GPNMB, GUCY2F, HECW1, NDE1, PLPPR3, PRKCG, PTEN, RASAL3, TLE3
Sphingolipid signaling pathway	KEGG:hsa04071	3	MAPK10, PRKCG, PTEN
Toll Like Receptor 4 (TLR4) Cascade	REACT:R-HSA-166016	3	DNM1, MAPK10, PELI3

Toll-Like Receptors Cascades	REACT:R-HSA-168898	3	DNM1, MAPK10, PELI3
Xenobiotics	REACT:R-HSA-211981	2	AHR, CYP2F1

## Capítulo III

*Characterisation of resistance against Teladorsagia circumcincta in lambs of two Canarian sheep breeds*



# Characterisation of resistance against *Teladorsagia circumcincta* in lambs of two Canarian sheep breeds

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## Abstract

Increasing resistance to anthelmintics has necessitated the exploration of alternative control strategies of gastrointestinal nematode (GIN) infections. A sustainable option is genetic selection based on differences in susceptibility to GIN infection between and within breeds of sheep. Here, 3-month-old Canaria Hair breed (GIN-resistant) and Canaria Sheep breed (GIN-susceptible) showed no significant between-breed differences after trickle infection with *Teladorsagia circumcincta*, whereas considerable individual variability was found in both breeds. Next, data from lambs of both breeds were used to explore relationships between parasitological variables and *T. circumcincta*-specific IgA levels, local immune cell populations and abomasal lymph node gene expression to understand the possible mechanisms underlying resistance. Mucosal IgA levels as well as numbers of globular leukocytes and MHC-II<sup>+</sup> cells were associated with protection. Analysis of lymph node gene expression revealed associations between lower parasite numbers and cumulative faecal egg counts and several immune pathways, such as leukocyte cell adhesion, activation and differentiation of T cells, in particular CD4<sup>+</sup> and IL-4 production. The data obtained here may inform on the relationship between phenotypic resistance variability and protective responses at the humoral, cellular and transcriptomic levels, thus contributing to identifying immune responses in young lambs that could be used as markers for selection.

## Keywords

Gastrointestinal nematode, genetic resistance, sheep, immunology, transcriptomic analysis.

### 1. Introduction

Gastrointestinal nematodes (GIN) represent an increasing concern for the domesticated ruminant farming sector. Recent estimates of direct and indirect losses attributable to GIN in ruminants amount to approximately 686 million euros per year in Europe alone (Charlier *et al.*, 2020). Despite resistance against the main anthelmintic classes being reported worldwide (Bordes *et al.*, 2020; Chandra *et al.*, 2015; Geurden *et al.*, 2014; Ramos *et al.*, 2016; Sales & Love, 2016), the farming sector still relies largely on chemotherapeutic intervention to control ovine GIN. This, alongside the climate-driven expansion of GIN prevalence across the globe, poses a serious threat to the economic and environmental sustainability of the sector (Rose *et al.*, 2015). Consequently, the quest for alternative approaches to management based on more restricted use of anthelmintics, such as pasture management, nutritional supplementation, biological control, vaccine development or genetic selection has increased in importance (Burke & Miller, 2020).

Genetic selection relies on purposely breeding individuals with the ability to prevent worm establishment and/or to expel parasitic nematodes based on the timely development of an effective immune response (Stear *et al.*, 2001). This usually manifests as reductions in egg excretion, worm burden and fecundity after the development of a type 2 immune response, characterised by CD4 activation, increases in Th2 (IL-4, IL-13 and IL-5) cytokine levels, IgE and IgA production and recruitment of mast cells, globule leukocytes and eosinophils in parasitised tissues (Venturina *et al.*, 2013). However, there is a lack of comprehensive data on how these immune response mechanisms operate in young lambs. It has been suggested that lambs generally need repeated exposure during their first year to fully acquire protective immunity, even though some individuals demonstrate repeatable low GIN egg excretion from 3 months of age and demonstrate some features of an effective immune response (Smith *et al.*, 1985; Stear *et al.*, 2000). Also, some sheep breeds, such as the Canaria Hair Breed (CHB), display a natural resistance against GIN, with evidence that such breeds could manifest resistance mechanisms at a younger age than other breeds (Amarante *et al.*, 2004; Bahirathan *et al.*, 1996; González *et al.*, 2008, 2019; Gruner *et al.*, 2003; Lins *et al.*, 2022). In this regard, an in-depth study of

the phenotypic variation in lambs within and between breeds, and the genetic basis of this variation, could improve understanding of the immunological dynamics that participate in resistance against nematodes in young lambs. This is important as many of the key impacts of ovine GIN infection on production occur in early life. To this end, global transcriptomic analyses have been used to explore changes in gene expression in host tissue throughout infection and to characterise the immune-related pathways associated with the resistance across breeds in an unbiased manner (Aboshady *et al.*, 2020; Ahmed *et al.*, 2015; Sweeney *et al.*, 2016).

The present study aimed to determine whether there are breed and/or individual differences in resistance in 3-month-old lambs of either worm-resistant (CHB) or susceptible (Canaria breed, CS) breed lambs after repeated infection with the GIN, *Teladorsagia circumcincta*. After comparing the parasitological data, histological techniques, immunohistochemistry and ELISA studies were used to investigate the immune response at the mucosal level and transcriptomic analysis of the draining lymph node response to explore the local adaptive immune response.

## 2. Materials and methods

### 2.1 Animals and parasitology

The experimental design was previously described in detail (Pérez-Hernández *et al.*, 2022). Briefly, 3-month-old CHB (n=12) and CS (n=12) lambs were dewormed with fenbendazole. Coprological examination was performed two weeks after drenching to confirm the effectiveness of the anthelmintic treatment and animals were kept in conditions that excluded further parasite transmission until the start of the trial. All animals were challenged with 2,000 *T. circumcincta* L3 three times a week for a total of four weeks (from day 0 to 26).

Starting on day 14, faeces were directly collected from the rectum of the lambs three times per week until day 37 after the start of the infection regime. Faecal egg counts (FEC, measured as eggs per gram, EPG) were determined using the modified McMaster technique (sensitivity 50 EPG). At the end of the experiment (days 40-43), animals were euthanised to recover juvenile and adult parasites from aliquots of the abomasal contents. Estimations of worm burden, female worm length and numbers of eggs *in utero* were performed following previously described protocols (González *et al.*, 2019; MAFF, 1989).

### 2.2 Enzyme-linked immunological assay (ELISA)

The ELISA protocol was previously described elsewhere (Pérez-Hernández *et al.*, 2022). Mucus samples were obtained at post-mortem (days 40-43) from the abomasal surface after washing and preserved at -80°C until use. ELISA was used to assess mucosal IgA levels against native *T. circumcincta* antigens (somatic L3, L4 and adult, 5 µg/ml plate coating concentration). Each sample was assayed in duplicate, and all test plates contained positive and negative control samples to account for plate-to-plate variation. The optical densities were transformed into an optical density index (ODI) as previously described (Strain & Stear, 2001). These values were then increased by a value of 1.0 to avoid negative values and to prevent statistical errors (Machín *et al.*, 2021; Strain & Stear, 2001).

### 2.3 Histology and immunohistochemistry

Full details of this protocol have been published (Pérez-Hernández *et al.*, 2022). Briefly, at post-mortem, two abomasal tissue samples of the antropyloric region were taken. One sample was processed and embedded in paraffin-wax and stained with hematoxylin and eosin to count eosinophil and globule leukocyte numbers, whilst toluidine blue staining was used to determine mast cell numbers. Cells were counted adjacent to the

lamina propria (eosinophils and mast cells) or in the luminal margin of the mucosa (globule leukocytes) and expressed as cells/mm<sup>2</sup>.

The other tissue sample was embedded in OCT™ solution (Optimal Cutting Temperature, Tissue Tek, Sakura Finetek, Europe B.V. Zoeterwoude, The Netherlands), preserved at -80°C and sectioned with a cryostat. Immunohistochemical staining was performed as described previously by González *et al.* (González *et al.*, 2011). Primary antibodies against CD4, CD8, γδ, MHC-II and Galectin 14 sheep markers were used to stain the tissue sections. Positively stained cells were counted in the upper and lower half of the mucosa (Pérez-Hernández *et al.*, 2022).

### 2.4 RNA Extraction and sequencing

Abomasal lymph node samples were collected at post-mortem and kept in E.Z.N.A. RNA Lock Reagent (Omega Bio-tek) at -80°C for RNA extraction. Total RNA was extracted from the lymph node samples by first cutting the primary sample (20 mg) into smaller pieces with a sterile scalpel and then further homogenising each sample in 1ml RLT buffer (Qiagen Ltd, UK) using a Precellys bead basher (Bertin Instruments, UK) within a Precellys CK28 bead tube (Stretton Scientific, UK) for 3 x 23-second cycles at 5,800 rpm with 2 minutes between

cycles, on ice. Samples were cleared by centrifugation at 14,000 g at 4°C for 10 minutes and supernatants processed using a RNeasy mini-isolation kit (Qiagen Ltd, UK) according to the manufacturer's protocol and including an on-column DNase I digestion for 15 mins at room temperature. RNA quantity and integrity were assessed using a Nanodrop spectrophotometer (Thermo Fisher, UK) and a Bioanalyser RNA Nanochip (Agilent Technologies Ltd, UK). The mean RNA integrity number (RIN) value across all samples was 8.1, indicating the successful extraction of high-quality RNA from the abomasal lymph nodes. Prior to sequencing library preparation, the yield of total RNA was determined on a Qubit Fluorometer (ThermoFisher, UK) using the Broad Range RNA kit (Thermo Fisher, UK). The resulting RNA samples were sequenced on an Illumina HiSeq 4000 by The Centre for Genome Research (CGR) at the University of Liverpool, UK, generating 2 x 150 bp strand-specific, paired-end reads with a minimum of 30 million reads per sample.

### 2.5 RNA-seq quality control and alignment

R version 3.4.4 (2018-03-15) was used for the RNAseq data QC based on all 21 samples with the array QualityMetrics package. Base calls were made using the Illumina CASAVA 1.8 pipeline and

cutadapt (v1.11) was used for adapter trimming. Processed sequences were then aligned to the *Ovis aries* genome assembly Oar\_v3.1 (GCA\_000298735.1) using the STAR aligner and the numbers of mapped read pairs were counted based on the *O. aries* genome annotation (Ensembl release 91) also within STAR (Dobin *et al.*, 2013).

### 2.6 Statistical analysis

Statistical analysis and graph production were conducted using the IBM SPSS Statistics 24.0 programme. Cumulative FEC (cFEC) values were estimated using the trapezoidal method for calculation of area under the curve (González *et al.*, 2019). Parasitological data were initially compared between breeds and evaluated using a Generalised Linear Model (Machín *et al.*, 2021). Thereafter, animals from both breeds were considered together as a single group. Associations between parasitological parameters, immunoglobulin level data and mucosal cellular counts were evaluated through Spearman's correlation coefficients. Probabilities with *p*-value <0.05 were considered statistically significant. Two linear regression models were performed examining the relationships between levels of gene expression and cFEC or worm burden. Functional enrichment analysis was performed to identify gene ontology (GO) terms

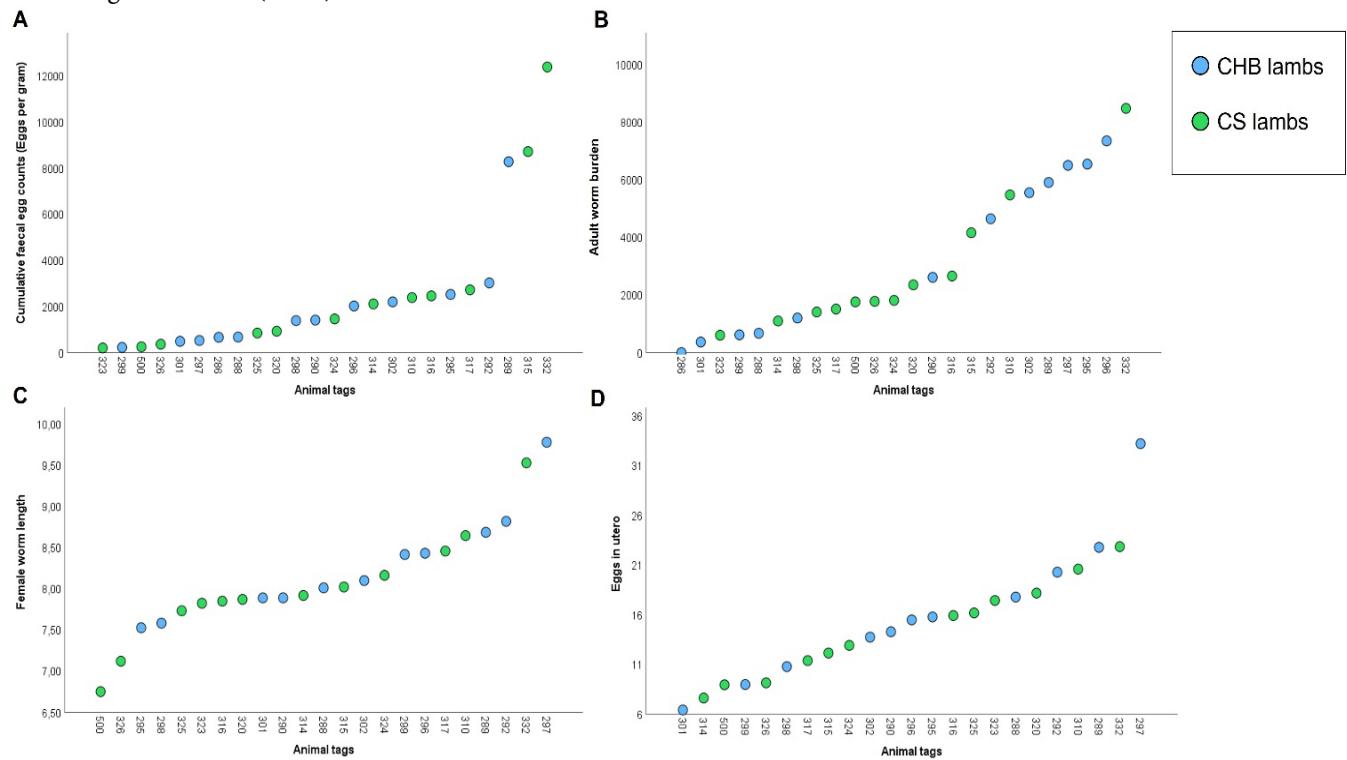
that were enriched in genes with statistically significant associations with parasitological parameters using GO annotations obtained from Ensembl. Significantly associated genes (raw  $p$ -value <0.05, up to a maximum of 1,000 genes ranked by  $p$ -value) from each comparison were analysed for enrichment of GO terms across all three GO categories using a hypergeometric test; this was then corrected for tests over multiple terms using a Benjamini-Hochberg correction (FDR correction) to yield an adjusted  $p$ -value ( $p < 0.01$ ).

### 3. Results

#### 3.1 Parasitology

At the end of the trial, differences in cFEC, worm burden, female worm length and eggs *in utero* were not statistically significant between breeds (Figure 1). Therefore, all animals were studied within the same group. Mean cFEC, adult worm burden, female worm length and eggs *in utero* values were 2429( $\pm 623$ ), 3128( $\pm 518$ ), 8.13( $\pm 0.14$ ) and 15( $\pm 1$ ), respectively. Also, values showed high variability between individuals (Figure 1A-D). Nevertheless, several parasitological values were positively correlated, implying that those lambs with lower egg excretion also harboured fewer, shorter and less prolific parasites (Table 1).

**Figure 1.** Parasitological variables in three-month-old sheep after trickle infection with *Teladorsagia circumcincta* infective larvae. Total cumulative faecal egg counts (A), worm burden at post-mortem (B), length of female worms (C) and eggs *in utero* (D) are shown as individual means. Animal tags in the X-axis are arranged from left to right according to ascending mean value ( $n=24$ ).



**Table 1.** Parasitological correlations in three-month-old sheep after trickle infection with *Teladorsagia circumcincta* L3.

	Adult worm burden	Female worm length	Eggs in utero
Cumulative FEC	0.654 **	0.476 *	0.332
Adult worm burden	-	0.464 *	0.578 **
Female worm length	-	-	0.501 *

Significant correlations between variables are indicated by \* $p < 0.05$  and \*\* $p < 0.01$  ( $N=24$ ).

### 3.2 Humoral and cellular immune response

Levels of somatic L3, L4 and adult *T. circumcincta* antigen-specific IgA within the abomasal mucus, and several immune cell populations within the abomasal mucosa were quantified at post-mortem (Table 2). Correlation studies between antigen-specific IgA levels or abomasal cell populations with parasitological variables showed significant negative associations between several of these parameters (Table 2). Mucosal IgA targeting *T. circumcincta* L3, L4 and adult antigens was significantly negatively correlated with the number of eggs *in utero*. Additionally, mucosal IgA levels against the adult stage were significantly negatively associated with worm burden. Numbers of MCH-II<sup>+</sup> cells were significantly negatively associated with cFEC and worm burden. Lastly, globule leukocyte numbers were significantly negatively correlated with cFEC.

### 3.3 Transcriptomic analysis

The relationships between cFEC and worm burden with gene expression in the abomasal lymph node tissue were evaluated using linear regression models. When comparing the top 50 enriched terms for genes negatively associated across the cFEC and worm burden contrasts, 48% of terms overlapped and the majority were immunity and cell adhesion-

related. For cFEC correlation analysis, expression of transcripts involved in IL-4 production, membrane and signalling terms were enriched (Figure 2A). Similarly, for the observation of decreased worm burdens, transcripts involved in cell cycle and immune-related terms were enriched (Figure 2B). Thirty-eight and 148 significantly upregulated genes were involved in the top 50 pathways negatively correlated with cFEC and worm burden comparisons, respectively. Full details regarding individual significant genes included in each GO term for cFEC and worm burden contrasts can be found in Annex- Supplementary file 1 & 2, respectively.

## 4. Discussion

In contrast to previous experimental GIN infection studies that included older (approximately 6 month-old) animals (González *et al.*, 2019; Machín *et al.*, 2021), we found no evidence that CHB lambs were more resistant to *T. circumcincta* than CS lambs at three months of age. However, great individual variability in resistance regarding parasitological parameters was observed within both breeds. On this basis, the analysis here focused on characterising the humoral and cellular immune response among individuals in relation to their *T. circumcincta* burdens and faecal egg excretion

profiles. The analysis indicated that MHC-II<sup>+</sup> cells, globule leukocytes and *T. circumcincta*-specific IgA appeared to have a role in controlling nematode burden, egg production and excretion. By comparing transcriptional data from the abomasal lymph node with cFEC, worm burden and gene expression, negative correlations were identified between these parasitological parameters and multiple enriched immune-related GO terms and key immunological genes.

Gastrointestinal nematode infections in lambs represent one of the main concerns for the sheep industry; these parasites have a major negative impact on animal welfare and considerably contribute to poor production performance and economic loss. This is partly due to the relative immaturity of the lamb immune system during the first months of life (Greer & Hamie, 2016; Mavrot *et al.*, 2015), in which adaptive immune responses appear to be poor (Liu *et al.*, 2022). In addition, lambs generally require repeated contact with the parasites whilst grazing to develop a protective response against these nematodes (Stear *et al.*,

2000). A high individual variation in FEC excretion has been demonstrated in 3-month-old lambs, implying that some animals can control GINs better than others at this age and prior data suggest that these differences are sustained over time (Stear *et al.*, 2000). This is similar to the unequal distribution of GIN within a normal population of adult sheep, where a small proportion of susceptible animals harbour most of the parasitic burden (Stear *et al.*, 2011; van Wyk *et al.*, 2006). Hence, exploring the differences in immune response and dynamics in young animals could unravel the basis for resistance to these parasites from an early stage.

In this regard, a recent comparative study showed that suckling Santa Ines lambs were more resistant than Ile de France lambs due to a strong abomasal cellular immune response when infected with *Haemonchus contortus*, with significant intra-breed variability (Lins *et al.*, 2022). The CHB and the CS breeds have been used previously as models in several studies because of differences in their resistance to *H. contortus* and *T. circumcincta* in lambs older than six months of age (González *et al.*, 2019; Machín *et al.*, 2021).

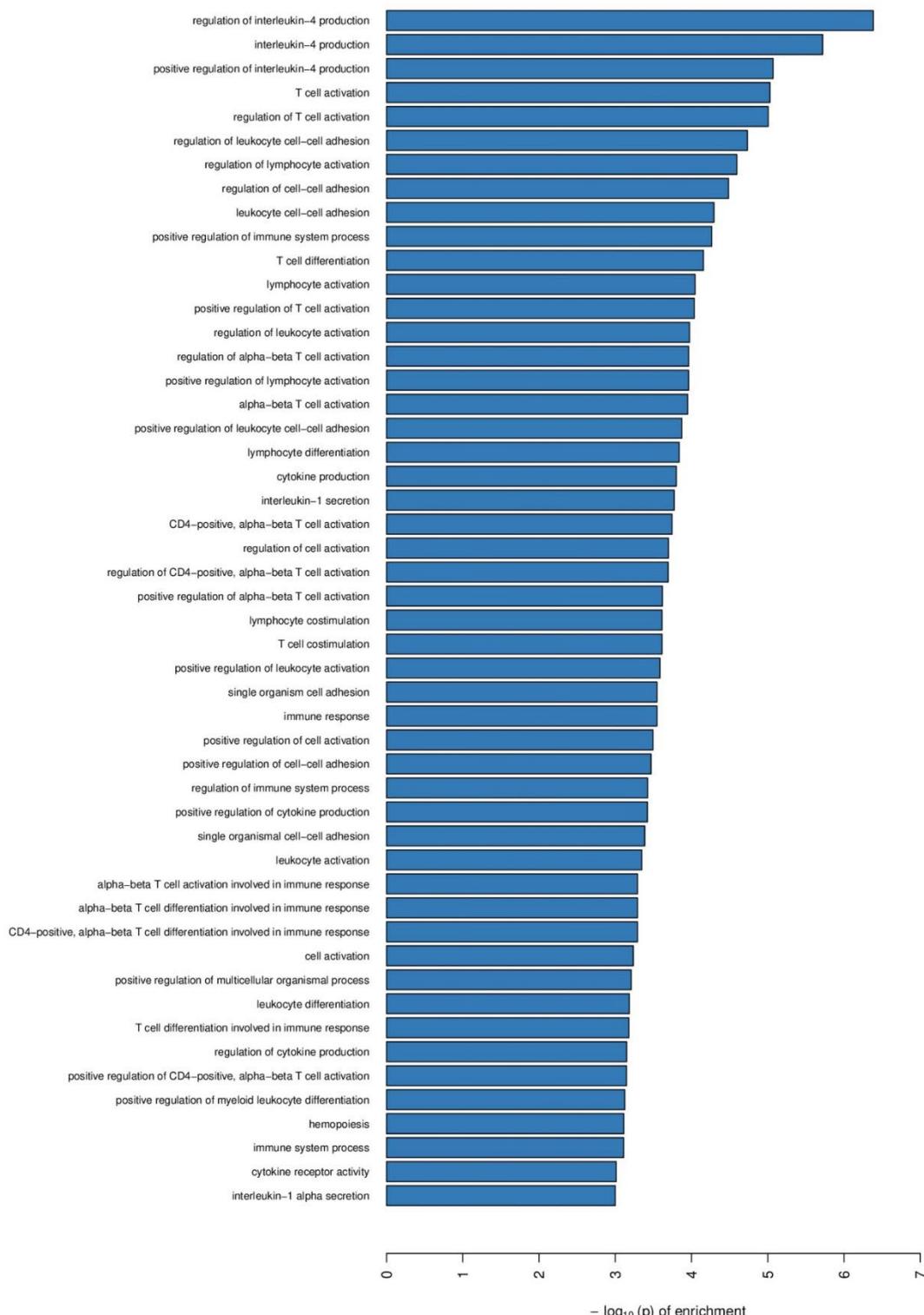
**Table 2. Immunological means and correlations in three-month-old sheep after trickle infection with *Teladorsagia circumcincta* L3.** Values are shown as mean Optical Density Index (ODI) for IgA expression or cells/mm<sup>2</sup> for cell recruitment (N=24).

Variable	Mean (ODI or cells/mm <sup>2</sup> ) ± SEM	Correlation			
		Cumulative FEC	Adult worm burden	Female worm length	Eggs in utero
Mucus IgA-L3	1.10±0.07	-0.069	-0.241	-0.331	-0.669**
Mucus IgA-L4	1.15±0.04	-0.189	-0.294	-0.377	-0.668**
Mucus IgA-AD	1.01±0.02	-0.141	-0.543**	-0.175	-0.419*
CD4 <sup>+</sup>	41.01±7.45	-0.147	0.042	-0.209	0.035
CD8 <sup>+</sup>	74.21±16.59	-0.001	-0.198	0.045	0.082
γδ <sup>+</sup>	18.05±2.93	-0.122	-0.065	-0.214	-0.126
MHC-II <sup>+</sup>	33.21±8.50	-0.476*	-0.476*	-0.030	-0.269
Gal 14 <sup>+</sup>	4.00±0.88	-0.207	-0.104	-0.179	-0.180
Eosinophils	86.56±10.73	-0.336	-0.375	0.126	-0.314
Globule leukocytes	172.70±41.13	-0.443*	-0.350	-0.287	-0.323
Mast cells	29.93±6.91	-0.129	-0.137	-0.159	-0.251

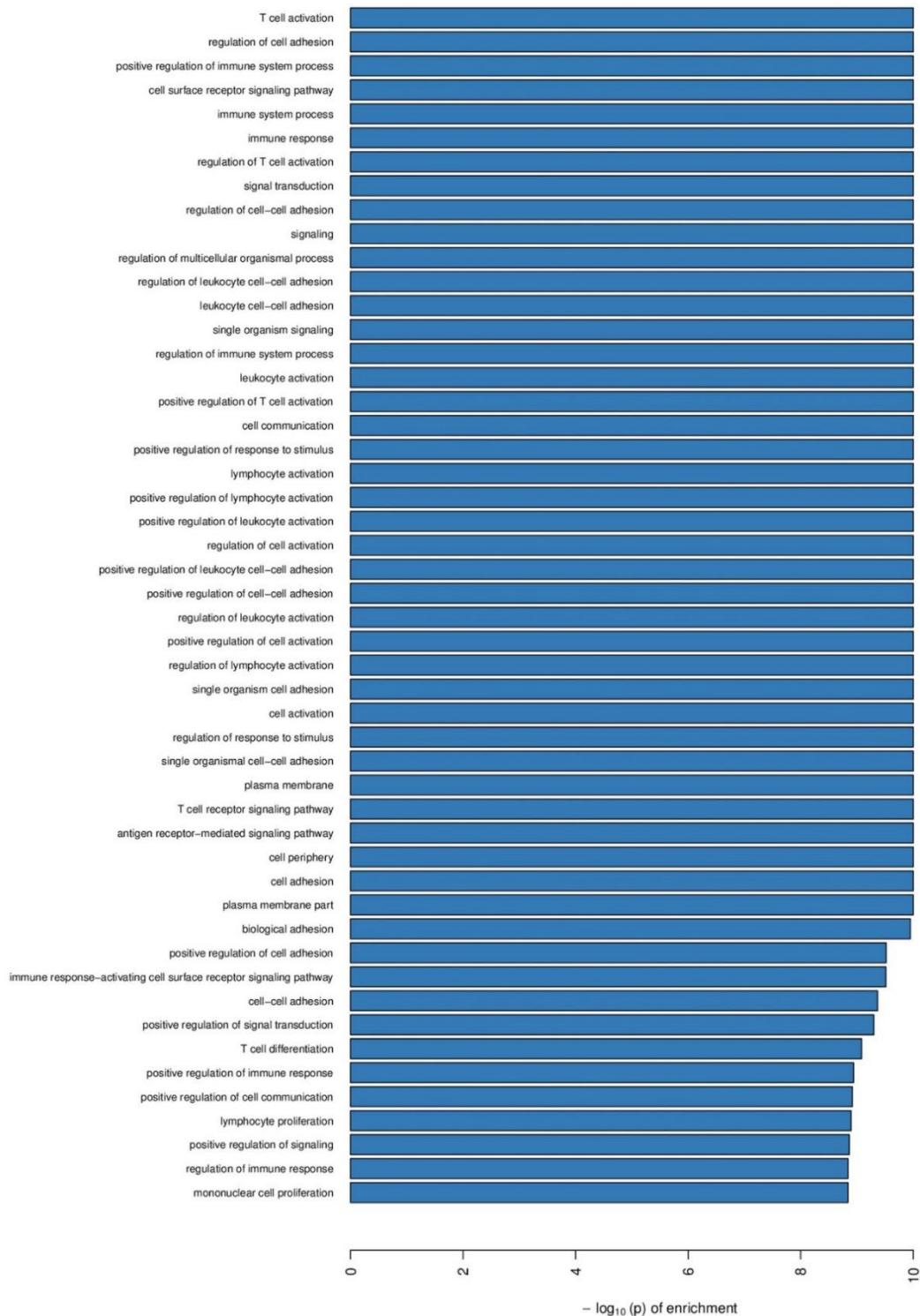
Significant correlations between variables are indicated by \**p* <0.05 and \*\**p* <0.01.

**Figure 2.** The top 50 enriched terms identified in a transcriptomic analysis of abomasal lymph nodes from three-month-old sheep after trickle infection with *Teladorsagia circumcincta* infective larvae. Panel A depicts those transcripts negatively correlated with cumulative faecal egg counts and Panel B depicts those transcripts negatively correlated with worm burden. Enrichment analyses with enrichment score are represented on the X axis and GO terms are displayed along the Y axis ( $p < 0.01$ ).

### A Enriched terms, negatively correlated with cumulative FEC



## B Enriched terms, negatively correlated with adult worm burden



Moreover, a recent study demonstrated that 3-month-old CHB lambs responded to vaccination with recombinant *T. circumcincta* proteins, with reductions in parasitological variables and involvement of well-characterised anti-GIN immune mechanisms described in adult animals, whereas such differences were not observed in 3-month-old CS lambs (Pérez-Hernández *et al.*, 2022). In contrast, here, significant differences in susceptibility to *T. circumcincta* in unvaccinated lambs of these two breeds at three months of age were not identified. Interestingly, as alluded to above, great variability in individual response to infection was detected. Hence, data from both breeds was used in this study to examine the relationship between parasitological variables with the abomasal immune cell recruitment, local IgA production and the analysis of the regional lymph node dynamics to explore possible resistance mechanisms. While this would not uncover breed-specific mechanisms of resistance to *T. circumcincta*, it allowed the investigation of resistance mechanisms in operation at a young age which are shared between the two breeds.

In general, immune response correlated well with parasitological differences. Animals with lower worm and egg burdens had a higher expression of

pathways related to inflammation cascades, such as cell migration and adhesion, hematopoiesis, mononuclear cell proliferation, response to stimulus, cell activation and signalling (see Figure 2A-2B). This is consistent with recent research on gene expression profiles in selected resistant and susceptible Yichang goats in which 31 genes related to immune processes and cell adhesion molecules pathways were more highly expressed in a more resistant group (Bhuiyan *et al.*, 2017).

Also, several pathways which were negatively associated with cFEC and worm burden contained genes involved in dendritic cell activation (CCR7, FLT3) and antigen presentation through MHC-I (B2M, LMO7) and MHC-II (ARL14) molecules. Counts of abomasal MHC-II<sup>+</sup> cells were negatively associated with egg excretion and the abomasal worm population. These results are in line with the characteristics of anti-nematode adaptive immune responses, which usually start with macrophages or dendritic cells presenting parasitic antigens to naïve CD4<sup>+</sup> T helper cells through MHC-II receptors leading to their activation and differentiation towards a protective type 2 phenotype (Gill *et al.*, 1993; McRae *et al.*, 2015; Oliphant *et al.*, 2014; Peña *et al.*, 2006). Similarly, a comparative study between GIN-resistant and

susceptible Scottish Blackface lamb strains infected with *T. circumcincta* showed that resistant animals had a higher expression of pathways involved in inflammatory responses and attraction and binding of T-lymphocytes early in infection, implying they had quicker cell migration responses after challenge than susceptible lambs (McRae *et al.*, 2016). Early expression of immune processes and intermittent activation of type 2-related pathways involving CD4<sup>+</sup> T cells and IL-4 expression in abomasal mucosa have also been reported in resistant Creole goat kids infected with *H. contortus* for 35 days (Aboshady *et al.*, 2020). Amongst the top 50 pathways negatively associated with cFEC and worm burden were regulation and differentiation of leukocytes, lymphocytes, T cells, gd T cells and CD4<sup>+</sup> T cells and IL-4 cytokine production. These pathways reflect a classical type 2 response, likely leading to increases in IL-4, IL-13 and IL-5 production, prompting IgE and IgA antigen-specific production by B cells, along with recruitment of mast cells, globule leukocytes and eosinophils (McRae *et al.*, 2016; Oliphant *et al.*, 2014). In this study, some genes were implicated in B cell activation and immunoglobulin production, related to the regulation of the IgE receptor (DOCK10). Globule leukocytes, thought to be degranulated mast cells, have been linked with expulsion of

worms from their niche through contraction of abomasal smooth muscle and stimulation of mucus secretion (Huntley *et al.*, 1992; Venturina *et al.*, 2013). Interestingly, globule leukocytes were negatively correlated with egg excretion and the abomasal worm population here. Also, mast cells and globule leukocyte degranulation is associated with IgE production (Sayers *et al.*, 2008). Several genes related to mast cell activation and degranulation (EDN1, PTGDR, RASGRP1) were present in several pathways negatively associated with parasitological parameters. Moreover, levels of antigen-specific IgA were correlated with reduced egg production *in utero* and worm establishment here, in line with literature that associates the presence of this immunoglobulin isotype with impaired nematode development and detrimental effects on worm length and fecundity (Strain *et al.*, 2002).

In conclusion, the most resistant lambs here were able to generate protective type-2 immune responses, similar to those developed by adult animals (Machín *et al.*, 2021). Future study designs will confirm the data presented; for example, by creating resistant and susceptible lineages and exploring the dynamics of the immune response with changes in gene expression throughout

infection. This strategy will be key to identifying immune responses associated with protection, thus

pinpointing resistance genetic markers for the selection of animals for breeding.

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# Anexo

## Capítulo III

*Characterisation of resistance against Teladorsagia circumcincta in lambs of two Canarian sheep breeds*



**Supplementary file 1A. Pathways negatively correlated with cumulative FEC at  $p < 0.01$**

Ontology	Name of the pathway	Significant genes	S	N	S/N (%)	OR	Z-score	p-value	p-value (adjusted)
BP	Regulation of interleukin-4 production	CD3E, CD40LG, LEF1, PRKCQ, PRKCZ	5	13	38.46	49.26	-1,387	4.15E-07	5.65E-04
BP	Interleukin-4 production	CD3E, CD40LG, LEF1, PRKCQ, PRKCZ	5	17	29.41	32.83	-1,213	1.91E-06	1.30E-03
BP	Positive regulation of interleukin-4 production	CD3E, CD40LG, PRKCQ, PRKCZ	4	11	36.36	44.64	-1,206	8.53E-06	2.69E-03
BP	T cell activation	B2M, CD5, IFNG, CD3E, CD4, CD40LG, LEF1, CCR7, LAT, PRKCQ, PRKCZ, IL27	12	197	6.09	5.35	-0.855	9.36E-06	2.69E-03
BP	Regulation of T cell activation	CD5, IFNG, CD3E, CD4, CD40LG, CCR7, LAT, PRKCQ, PRKCZ, IL27	10	135	7.41	6.51	-0.861	9.87E-06	2.69E-03
BP	Regulation of leukocyte cell-cell adhesion	CD5, IFNG, CD3E, CD4, CD40LG, CCR7, LAT, PRKCQ, PRKCZ, IL27	10	145	6.90	6.03	-0.830	1.85E-05	4.21E-03
BP	Regulation of lymphocyte activation	CD5, IFNG, CD3E, CD4, CD40LG, NOD2, CCR7, LAT, PRKCQ, PRKCZ, IL27	11	183	6.01	5.23	-0.813	2.55E-05	4.96E-03
BP	Regulation of cell-cell adhesion	CD5, IFNG, CD3E, CD4, CD40LG, LEF1, CCR7, LAT, PRKCQ, PRKCZ, IL27	11	188	5.85	5.08	-0.802	3.27E-05	5.57E-03
BP	Leukocyte cell-cell adhesion	CD5, IFNG, CD3E, CD4, CD40LG, CCR7, LAT, PRKCQ, PRKCZ, IL27	10	163	6.13	5.31	-0.783	5.08E-05	7.41E-03
BP	Positive regulation of immune system process	B2M, CD5, IFNG, CD3E, CD4, CD40LG, NOD2, LEF1, CACNB3, CCR7, CRTAM, IL34, PRKCQ, GPLD1, PLA2G7, PRKCZ	16	397	4.03	3.52	-0.803	5.44E-05	7.41E-03

BP	T cell differentiation	B2M, IFNG, CD3E, CD4, LEF1, CCR7, PRKCZ, IL27	8	106	7.55	6.54	-0.777	6.96E-05	8.61E-03
BP	Lymphocyte activation	B2M, CD5, IFNG, CD3E, CD4, CD40LG, NOD2, LEF1, CCR7, LAT, PRKCQ, PRKCZ, IL27	13	287	4.53	3.91	-0.767	8.98E-05	9.00E-03
BP	Positive regulation of T cell activation	CD5, IFNG, CD3E, CD40LG, CCR7, PRKCQ, PRKCZ	7	82	8.54	7.43	-0.773	9.21E-05	9.00E-03
BP	Regulation of leukocyte activation	CD5, IFNG, CD3E, CD4, CD40LG, NOD2, CCR7, LAT, PRKCQ, PRKCZ, IL27	11	214	5.14	4.41	-0.752	1.06E-04	9.00E-03
BP	Regulation of alpha-beta T cell activation	IFNG, CD3E, PRKCQ, PRKCZ, IL27	5	37	13.51	12.28	-0.822	1.09E-04	9.00E-03
BP	Positive regulation of lymphocyte activation	CD5, IFNG, CD3E, CD40LG, NOD2, CCR7, PRKCQ, PRKCZ	8	113	7.08	6.10	-0.753	1.09E-04	9.00E-03
BP	Alpha-beta T cell activation	IFNG, CD3E, LEF1, PRKCQ, PRKCZ, IL27	6	59	10.17	8.96	-0.781	1.12E-04	9.00E-03
BP	Positive regulation of leukocyte cell-cell adhesion	CD5, IFNG, CD3E, CD40LG, CCR7, PRKCQ, PRKCZ	7	87	8.05	6.97	-0.750	1.34E-04	1.02E-02
BP	Lymphocyte differentiation	B2M, IFNG, CD3E, CD4, CD40LG, LEF1, CCR7, PRKCZ, IL27	9	150	6.00	5.14	-0.735	1.45E-04	1.04E-02
BP	Cytokine production	B2M, IFNG, CD3E, CD40LG, NOD2, LEF1, ADAMTS3, IL1R2, CCR7, S100A13, CRTAM, PRKCQ, PRKCZ, IL27	14	346	4.05	3.48	-0.753	1.58E-04	1.08E-02
BP	Interleukin-1 secretion	NOD2, IL1R2, CCR7, S100A13	4	22	18.18	17.34	-0.853	1.69E-04	1.10E-02
BP	CD4-positive, alpha-beta T cell activation	IFNG, LEF1, PRKCQ, PRKCZ, IL27	5	41	12.20	10.91	-0.781	1.80E-04	1.12E-02

BP	Regulation of cell activation	CD5, IFNG, CD3E, CD4, CD40LG, NOD2, CCR7, LAT, PRKCQ, PRKCZ, IL27	11	230	4.78	4.08	-0.725	2.01E-04	1.15E-02
BP	Regulation of CD4-positive, alpha-beta T cell activation	IFNG, PRKCQ, PRKCZ, IL27	4	23	17.39	16.42	-0.834	2.03E-04	1.15E-02
BP	Positive regulation of alpha-beta T cell activation	IFNG, CD3E, PRKCQ, PRKCZ	4	24	16.67	15.60	-0.816	2.41E-04	1.23E-02
BP	Lymphocyte costimulation	CD5, CD3E, CCR7	3	10	30.00	33.19	-0.949	2.44E-04	1.23E-02
BP	T cell costimulation	CD5, CD3E, CCR7	3	10	30.00	33.19	-0.949	2.44E-04	1.23E-02
BP	Positive regulation of leukocyte activation	CD5, IFNG, CD3E, CD40LG, NOD2, CCR7, PRKCQ, PRKCZ	8	128	6.25	5.33	-0.707	2.60E-04	1.26E-02
BP	Single organism cell adhesion	CD5, IFNG, CD3E, CD4, CD40LG, LEF1, CCR7, LAT, PRKCQ, OLFM4, PRKCZ, IL27	12	280	4.29	3.65	-0.717	2.84E-04	1.29E-02
BP	Immune response	B2M, IFNG, CD3E, CD4, CD40LG, NOD2, LEF1, LOC101102230, CACNB3, FYB1, CCR7, LAT, CRTAM, PRKCQ, IKBKE, GPLD1, PRKCZ, IL27	18	554	3.25	2.82	-0.765	2.84E-04	1.29E-02
BP	Positive regulation of cell activation	CD5, IFNG, CD3E, CD40LG, NOD2, CCR7, PRKCQ, PRKCZ	8	132	6.06	5.16	-0.696	3.21E-04	1.41E-02
BP	Positive regulation of cell-cell adhesion	CD5, IFNG, CD3E, CD40LG, CCR7, PRKCQ, PRKCZ	7	101	6.93	5.92	-0.697	3.40E-04	1.45E-02
BP	Regulation of immune system process	B2M, CD5, IFNG, CD3E, CD4, CD40LG, NOD2, LEF1, CACNB3, CCR7, LAT, CRTAM, IL34, PRKCQ, GPLD1, PLA2G7, PRKCZ, IL27	18	567	3.17	2.75	-0.756	3.77E-04	1.52E-02

BP	Positive regulation of cytokine production	B2M, IFNG, CD3E, CD40LG, NOD2, CCR7, CRTAM, PRKCQ, PRKCZ, IL27	10	208	4.81	4.08	-0.693	3.79E-04	1.52E-02
BP	Single organismal cell-cell adhesion	CD5, IFNG, CD3E, CD4, CD40LG, LEF1, CCR7, LAT, PRKCQ, PRKCZ, IL27	11	250	4.40	3.73	-0.696	4.11E-04	1.60E-02
BP	Leukocyte activation	B2M, CD5, IFNG, CD3E, CD4, CD40LG, NOD2, LEF1, CCR7, LAT, PRKCQ, PRKCZ, IL27	13	338	3.85	3.27	-0.707	4.49E-04	1.70E-02
BP	CD4-positive, alpha-beta T cell differentiation involved in immune response	IFNG, LEF1, PRKCZ, IL27	4	29	13.79	12.47	-0.743	5.12E-04	1.79E-02
BP	Alpha-beta T cell differentiation involved in immune response	IFNG, LEF1, PRKCZ, IL27	4	29	13.79	12.47	-0.743	5.12E-04	1.79E-02
BP	Alpha-beta T cell activation involved in immune response	IFNG, LEF1, PRKCZ, IL27	4	29	13.79	12.47	-0.743	5.12E-04	1.79E-02
BP	Cell activation	B2M, CD5, IFNG, CD3E, CD4, CD40LG, NOD2, LEF1, CCR7, LAT, PRKCQ, PRKCZ, IL27, ZP2	14	393	3.56	3.03	-0.706	5.81E-04	1.98E-02
BP	Positive regulation of multicellular organismal processes	FGF7, B2M, IFNG, CD3E, CD4, CD40LG, NOD2, LEF1, BMPR2, F12, RND2, CCR7, CRTAM, IL34, PRKCQ, VASH2, CPNE6, GPLD1, PRKCZ, IL27	20	695	2.88	2.49	-0.759	6.19E-04	2.06E-02
BP	Leukocyte differentiation	B2M, IFNG, CD3E, CD4, CD40LG, LEF1, CCR7, IL34, PRKCZ, IL27	10	223	4.48	3.78	-0.670	6.55E-04	2.11E-02
BP	T cell differentiation involved in immune response	IFNG, LEF1, PRKCZ, IL27	4	31	12.90	11.55	-0.718	6.64E-04	2.11E-02
BP	Regulation of cytokine production	B2M, IFNG, CD3E, CD40LG, NOD2, LEF1, IL1R2, CCR7, CRTAM, PRKCQ, PRKCZ, IL27	12	310	3.87	3.28	-0.682	7.10E-04	2.16E-02

BP	Positive regulation of CD4-positive, alpha-beta T cells	IFNG, PRKCQ, PRKCZ	3	14	21.43	21.11	-0.802	7.13E-04	2.16E-02
BP	Positive regulation of myeloid leukocyte differentiation	IFNG, CD4, LEF1, IL34	4	32	12.50	11.13	-0.707	7.52E-04	2.20E-02
BP	Hemopoiesis	B2M, IFNG, CD3E, CD4, CD40LG, LEF1, CCR7, FLT3, IL34, KIRREL3, PRKCZ, IL27, SERPINB12	13	358	3.63	3.08	-0.687	7.72E-04	2.20E-02
BP	Immune system process	B2M, CD5, IFNG, CD3E, CD4, CD40LG, NOD2, LEF1, LOC100422799, LOC101102230, CACNB3, FYB1, CCR7, LAT, CRTAM, FLT3, IL34, PRKCQ, KIRREL3, IKBKE, GPLD1, PLA2G7, PRKCZ, IL27, SERPINB12	25	984	2.54	2.23	-0.797	7.74E-04	2.20E-02
MF	Cytokine receptor activity	CD4, IL1R2, CCR7, CSF2RA, CCRL2	5	56	8.93	7.33	-0.668	9.70E-04	2.70E-02
BP	Interleukin-1 alpha secretion	IL1R2, S100A13	2	4	50.00	76.80	-1,000	9.99E-04	2.72E-02

### Key

**BP:** Biological process

**MF:** Molecular function

**S:** Significantly differentially expressed genes

**N:** Total number of genes in term

**OR:** Odds ratio

**Z-score:** (up regulated genes-down regulated genes) / (term size)<sup>2</sup>

**p value:** Raw (unadjusted) p-value

**p-value (adjusted):** FDR adjusted p-value (Benjamini-Hochberg procedure)

**Supplementary file 1B. Genes present in the pathways negatively correlated with cumulative FEC at  $p < 0.01$ .**

ADAMTS3	IKBKE
B2M	KIRREL3
BMPR2	LEF1
CD3E	LAT
CD40LG	LOC101102230
CD5	LOC100422799
CD4	NOD2
CCR7	OLFM4
CACNB3	PRKCQ
CRTAM	PRKCZ
CPNE6	PLA2G7
CSF2RA	RND2
CCRL2	S100A13
FYB1	SERPINB12
FGF7	VASH2
F12	ZP2
FLT3	
GPLD1	
IFNG	
IL27	
IL34	
IL1R2	

**Supplementary file 2A. Pathways negatively correlated with worm burden at  $p < 0.01$**

Ontology	Name	Significant genes	S	N	S/N (%)	OR	Z-score	p-value	p-value (adjusted)
BP	T cell activation	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, LEF1, FOXP3, STAT3, RASGRP1, FYN, CCR7, LAT, IL7R, ZAP70, ITK, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, THEMIS, CD6, SLA2, STAT5B, ANXA1	28	197	14.21	8.60	-1.995	1.14E-15	2.63E-12
BP	Regulation of cell adhesion	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, LEF1, FN1, FOXP3, LMO7, SEMA4D, RASGRP1, CCR7, LAT, IL7R, UBASH3B, ZAP70, PRKCQ, CYLD, CYTIP, ITGA6, SLK, CD27, PELI1, CD274, PDCD1LG2, LAMA2, UTRN, PDE3B, OLFM4, CD6, STAT5B, ANXA1	34	328	10.37	6.14	-1.877	1.28E-14	1.48E-11
BP	Positive regulation of immune system process	CTLA4, CD5, IL6, CD3E, CD4, TLR3, CD40LG, NOD2, LEF1, FOXP3, STAT3, TXK, CD84, CACNB3, LCP2, RASGRP1, PRKCH, FYN, CCR7, IL7R, RPS6KA3, CRTAM, CD226, ZAP70, ITK, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, THEMIS, TESPA1, CD6, STAT5B, ANXA1, EVI2B	37	397	9.32	5.52	-1.857	2.22E-14	1.71E-11
BP	Cell surface receptor signaling pathway	CASP3, CTLA4, ARNTL, IL6, STAT5A, CD3E, EDN1, CD4, TLR3, LEF1, NRP2, FASLG, FOXP3, STAT3, SPRY1, LOC101102230, TXK, TGFBR3, CHRND, MLLT3, ADAMTS3, SEMA4D, CACNB3, LCP2, PRKCH, SPART, STK3, ST18, BEND6, FYN, CCR7, DIXDC1, TIPARP, LOC101110429, LAT, IL7R, SGPL1, UBASH3B, FLT3, CD226, ZAP70, ITK, ZMYND11, PRKCQ, CYLD, ITGA6, DAPK1, TNFSF10, CD27, PELI3, PELI1, ZRANB1, CD274, CSF2RA, GPR161, PDK4, THEMIS, ABCA1, AGT, PDCD4, TESPA1, CD6, STAT5B	63	1082	5.82	3.67	-1.915	2.98E-14	1.72E-11
BP	Immune system process	CASP3, CTLA4, CD5, IL6, CD3E, CD4, TLR3, CD40LG, NOD2, LEF1, FASLG, FOXP3, STAT3, LOC101102230, TXK, TGFBR3, CD84, CACNB3, LCP2, FYB1, RASGRP1, PRKCH, STK3, GPR15, FYN, CCR7, TIPARP, LAT, IL7R, SGPL1, RPS6KA3, UBASH3B, CRTAM, FLT3, CD226, ZAP70, ITK, ZMYND11, PRKCQ, CYLD, ITGA6, DAPK1, TNFSF10, CD27, TRIM21, KIRREL3, PELI1, CD274, PDCD1LG2, IKBKE, THEMIS, GPR171, TESPA1, CD6, SLA2, STAT5B, ANXA1, EVI2B, SAMHD1	59	984	6.00	3.72	-1.881	7.52E-14	3.01E-11
BP	Immune response	CTLA4, IL6, CD3E, CD4, TLR3, CD40LG, NOD2, LEF1, FASLG, FOXP3, STAT3, LOC101102230, TXK, TGFBR3, CD84, CACNB3, LCP2, FYB1, RASGRP1, PRKCH, FYN, CCR7, LAT, IL7R, RPS6KA3, CRTAM, CD226, ZAP70, ITK,	43	554	7.76	4.61	-1.827	8.34E-14	3.01E-11

PRKCQ, CYLD, DAPK1, TNFSF10, TRIM21, PELI1, CD274, IKBKE, THEMIS,  
TESPA1, CD6, STAT5B, ANXA1, SAMHD1

BP	Regulation of T cell activation	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, FOXP3, RASGRP1, CCR7, LAT, IL7R, ZAP70, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	22	135	16.30	9.81	-1.893	9.12E-14	3.01E-11
BP	Signal transduction	CASP3, CTLA4, ARNTL, CD5, IL6, STAT5A, CD3E, EDN1, CD4, TLR3, CD40LG, NOD2, LEF1, NRP2, FASLG, ADRA1D, FN1, FOXP3, STAT3, PPP2R5C, E2F7, SPRY1, LOC101102230, TXK, TGFBR3, CHRND, MLLT3, PIK3CG, ADAMTS3, TIAM1, PITPNM1, CHN2, ARHGEF3, SEMA4D, CACNB3, LOC101105165, LCP2, ARL14, RASGRP1, IQGAP2, PRKCH, ADCY6, SPART, STK3, ST18, BEND6, GPR15, LOC101106767, FYN, CCR7, DIXDC1, TIPARP, LOC101110429, LAT, CHRM5, IL7R, SGPL1, RPS6KA3, RAPGEF2, UBASH3B, PAQR3, FLT3, CD226, ZAP70, ITK, ZMYND11, DOCK4, PRKCQ, CYLD, ITGA6, DAPK1, TNFSF10, CD27, PELI3, PELI1, ZRANB1, CD274, TNIK, CSF2RA, ARHGAP22, RCAN3, IKBKE, GPR161, GRK3, PDK4, THEMIS, GPR171, AKT3, ABCA1, AGT, PDCD4, TESPA1, RASSF4, PDE3B, PTGDR, CD6, LOC101122356, SLA2, STAT5B, DOCK10, ANXA1, SLA, LMO3	103	2532	4.07	2.98	-2.047	1.41E-13	4.08E-11
BP	Regulation of cell-cell adhesion	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, LEF1, FOXP3, RASGRP1, CCR7, LAT, IL7R, UBASH3B, ZAP70, PRKCQ, CYLD, ITGA6, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	25	188	13.30	7.82	-1.823	2.09E-13	5.34E-11
BP	Signaling	CASP3, CTLA4, ARNTL, CD5, IL6, STAT5A, CD3E, SLC16A1, EDN1, CD4, TLR3, CD40LG, NOD2, LEF1, NRP2, FASLG, ADRA1D, FN1, FOXP3, STAT3, PPP2R5C, E2F7, SPRY1, LOC101102230, TXK, TGFBR3, CHRND, LMO7, MLLT3, PIK3CG, ADAMTS3, TIAM1, PITPNM1, CHN2, ARHGEF3, SEMA4D, CACNB3, LOC101105165, LCP2, ARL14, RASGRP1, IQGAP2, PRKCH, ADCY6, SPART, STK3, ST18, BEND6, GPR15, LOC101106767, FYN, CCR7, DIXDC1, TIPARP, LOC101110429, LAT, CHRM5, IL7R, SGPL1, RPS6KA3, RAPGEF2, UBASH3B, PAQR3, FLT3, CD226, ZAP70, ITK, ZMYND11, DOCK4, PRKCQ, CYLD, ITGA6, DAPK1, TNFSF10, CD27, PELI3, PELI1, ZRANB1, CD274, TNIK, CSF2RA, ARHGAP22, RCAN3, IKBKE, GPR161, GRK3, LAMA2, PDK4, THEMIS, GPR171, AKT3, ABCA1, AGT, PDCD4, TESPA1, RASSF4, PDE3B, PTGDR, CD6, LOC101122356, SLA2, STAT5B, DOCK10, ANXA1, SLA, LMO3	106	2678	3.96	2.93	-2.048	2.82E-13	6.49E-11

BP	Regulation of multicellular organismal process	CTLA4, ARNTL, IL6, STAT5A, CD3E, EDN1, CD4, TLR3, CD40LG, NOD2, LEF1, FASLG, FN1, FOXP3, STAT3, SPRY1, TXK, CD84, MLLT3, TIAM1, SYTL2, SEMA4D, ADTRP, RASGRP1, PRKCH, SPART, STK3, CNMD, AMIGO1, BEND6, F12, KIF13B, FYN, CCR7, DIXDC1, IL7R, SGPL1, RAPGEF2, UBASH3B, CRTAM, PAQR3, ZAP70, DISP3, DOCK4, PRKCQ, CYLD, VASH2, CD27, PELI1, CD274, TNK, PDCD1LG2, GPR161, LAMA2, PDK4, PLAC8, GPR171, CPNE6, AGT, PDCD4, PDE3B, STXBP5, CD6, STAT5B, ANXA1, EVI2B	66	1239	5.33	3.35	-1.875	4.14E-13	7.99E-11
BP	Regulation of leukocyte cell-cell adhesion	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, FOXP3, RASGRP1, CCR7, LAT, IL7R, ZAP70, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	22	145	15.17	9.00	-1.827	4.16E-13	7.99E-11
BP	Leukocyte cell-cell adhesion	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, FOXP3, SEMA4D, RASGRP1, CCR7, LAT, IL7R, ZAP70, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	23	163	14.11	8.30	-1.801	5.73E-13	1.02E-10
BP	Single organism signaling	CASP3, CTLA4, ARNTL, CD5, IL6, STAT5A, CD3E, SLC16A1, EDN1, CD4, TLR3, CD40LG, NOD2, LEF1, NRP2, FASLG, ADRA1D, FN1, FOXP3, STAT3, PPP2R5C, E2F7, SPRY1, LOC101102230, TXK, TGFBR3, CHRND, MLLT3, PIK3CG, ADAMTS3, TIAM1, PITPNM1, CHN2, ARHGEF3, SEMA4D, CACNB3, LOC101105165, LCP2, ARL14, RASGRP1, IQGAP2, PRKCH, ADCY6, SPART, STK3, ST18, BEND6, GPR15, LOC101106767, FYN, CCR7, DIXDC1, TIPARP, LOC101110429, LAT, CHRM5, IL7R, SGPL1, RPS6KA3, RAPGEF2, UBASH3B, PAQR3, FLT3, CD226, ZAP70, ITK, ZMYND11, DOCK4, PRKCQ, CYLD, ITGA6, DAPK1, TNFSF10, CD27, PELI3, PELI1, ZRANB1, CD274, TNK, CSF2RA, ARHGAP22, RCAN3, IKBKE, GPR161, GRK3, LAMA2, PDK4, THEMIS, GPR171, AKT3, ABCA1, AGT, PDCD4, TESPA1, RASSF4, PDE3B, PTGDR, CD6, LOC101122356, SLA2, STAT5B, DOCK10, ANXA1, SLA, LMO3	105	2673	3.93	2.87	-2.031	7.22E-13	1.19E-10
BP	Regulation of immune system process	CASP3, CTLA4, CD5, IL6, CD3E, CD4, TLR3, CD40LG, NOD2, LEF1, FOXP3, STAT3, TXK, CD84, CACNB3, LCP2, RASGRP1, PRKCH, FYN, CCR7, LAT, IL7R, RPS6KA3, UBASH3B, CRTAM, CD226, ZAP70, ITK, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, THEMIS, GPR171, TESPA1, CD6, STAT5B, ANXA1, EVI2B, SAMHD1	42	567	7.41	4.35	-1.764	8.41E-13	1.29E-10
BP	Leukocyte activation	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, NOD2, LEF1, FOXP3, STAT3, CD84, LCP2, RASGRP1, FYN, CCR7, LAT, IL7R, CD226, ZAP70, ITK, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, THEMIS, CD6, SLA2, STAT5B, ANXA1	32	338	9.47	5.47	-1.741	1.06E-12	1.52E-10

BP	Positive regulation of T cell activation	CD5, IL6, CD3E, CD40LG, FOXP3, RASGRP1, CCR7, IL7R, ZAP70, PRKCQ, CYLD, CD27, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	17	82	20.73	12.88	-1.877	1.16E-12	1.58E-10
BP	Cell communication	CASP3, CTLA4, ARNTL, CD5, IL6, STAT5A, CD3E, SLC16A1, EDN1, CD4, TLR3, CD40LG, NOD2, LEF1, NRP2, FASLG, ADRA1D, FN1, FOXP3, STAT3, PPP2R5C, E2F7, SPRY1, LOC101102230, TXK, TGFB3, CHRND, MLLT3, PIK3CG, ADAMTS3, TIAM1, PITPNM1, CHN2, ARHGEF3, SEMA4D, CACNB3, LOC101105165, LCP2, ARL14, RASGRP1, IQGAP2, PRKCH, ADCY6, SPART, STK3, ST18, BEND6, GPR15, LOC101106767, FYN, CCR7, DIXDC1, TIPARP, LOC101110429, LAT, CHRM5, IL7R, SGPL1, RPS6KA3, RAPGEF2, UBASH3B, PAQR3, FLT3, CD226, ZAP70, ITK, ZMYND11, DOCK4, PRKCQ, CYLD, ITGA6, DAPK1, TNFSF10, CD27, PELI3, PELI1, ZRANB1, CD274, TNK, CSF2RA, ARHGAP22, RCAN3, IKBKE, GPR161, GRK3, LAMA2, PDK4, THEMIS, GPR171, AKT3, ABCA1, AGT, PDCD4, TESPA1, RASSF4, PDE3B, PTGDR, CD6, LOC101122356, SLA2, STAT5B, DOCK10, ANXA1, SLA, LMO3	105	2706	3.88	2.82	-2.018	1.70E-12	2.18E-10
BP	Positive regulation of response to stimulus	CTLA4, ARNTL, IL6, CD3E, EDN1, CD4, TLR3, CD40LG, NOD2, FASLG, FOXP3, STAT3, TXK, MLLT3, PIK3CG, ADAMTS3, SEMA4D, CACNB3, LCP2, RASGRP1, PRKCH, STK3, ST18, F12, FYN, CCR7, DIXDC1, LAT, IL7R, RPS6KA3, RAPGEF2, CRTAM, CD226, ZAP70, ITK, PRKCQ, CYLD, TNFSF10, CD27, PELI1, ZRANB1, TNK, IKBKE, THEMIS, AKT3, AGT, PDCD4, TESPA1, CD6, SLA2, STAT5B, ANXA1, SLA, LMO3	54	915	5.90	3.55	-1.785	2.38E-12	2.89E-10
BP	Lymphocyte activation	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, NOD2, LEF1, FOXP3, STAT3, RASGRP1, FYN, CCR7, LAT, IL7R, ZAP70, ITK, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, THEMIS, CD6, SLA2, STAT5B, ANXA1	29	287	10.10	5.81	-1.712	2.71E-12	3.01E-10
BP	Positive regulation of lymphocyte activation	CD5, IL6, CD3E, CD40LG, NOD2, FOXP3, RASGRP1, CCR7, IL7R, ZAP70, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	19	113	16.81	10.03	-1.787	2.74E-12	3.01E-10
BP	Positive regulation of leukocyte activation	CD5, IL6, CD3E, CD40LG, NOD2, FOXP3, RASGRP1, CCR7, IL7R, CD226, ZAP70, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	20	128	15.62	9.23	-1.768	2.94E-12	3.08E-10
BP	Regulation of cell activation	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, NOD2, FOXP3, CD84, RASGRP1, CCR7, LAT, IL7R, UBASH3B, CD226, ZAP70, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	26	230	11.30	6.51	-1.714	3.12E-12	3.11E-10

BP	Positive regulation of leukocyte cell-cell adhesion	CD5, IL6, CD3E, CD40LG, FOXP3, RASGRP1, CCR7, IL7R, ZAP70, PRKCQ, CYLD, CD27, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	17	87	19.54	11.95	-1.823	3.23E-12	3.11E-10
BP	Positive regulation of cell-cell adhesion	CD5, IL6, CD3E, CD40LG, FOXP3, RASGRP1, CCR7, IL7R, ZAP70, PRKCQ, CYLD, ITGA6, CD27, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	18	101	17.82	10.72	-1.791	3.78E-12	3.49E-10
BP	Regulation of leukocyte activation	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, NOD2, FOXP3, CD84, RASGRP1, CCR7, LAT, IL7R, CD226, ZAP70, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	25	214	11.68	6.73	-1.709	4.05E-12	3.59E-10
BP	Positive regulation of cell activation	CD5, IL6, CD3E, CD40LG, NOD2, FOXP3, RASGRP1, CCR7, IL7R, CD226, ZAP70, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	20	132	15.15	8.90	-1.741	5.30E-12	4.53E-10
BP	Regulation of lymphocyte activation	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, NOD2, FOXP3, RASGRP1, CCR7, LAT, IL7R, ZAP70, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	23	183	12.57	7.25	-1.700	6.81E-12	5.61E-10
BP	Single organism cell adhesion	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, LEF1, FN1, FOXP3, SEMA4D, RASGRP1, CCR7, LAT, IL7R, UBASH3B, ZAP70, PRKCQ, CYLD, ITGA6, CD27, PELI1, CD274, PDCD1LG2, OLFM4, CD6, STAT5B, ANXA1	28	280	10.00	5.71	-1.673	8.65E-12	6.88E-10
BP	Cell activation	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, NOD2, LEF1, FOXP3, STAT3, CD84, LCP2, RASGRP1, FYN, CCR7, LAT, IL7R, UBASH3B, CD226, ZAP70, ITK, PRKCQ, CYLD, CD27, PELI1, CD274, PDCD1LG2, THEMIS, CD6, SLA2, STAT5B, ANXA1	33	393	8.40	4.80	-1.665	1.25E-11	9.59E-10
BP	Regulation of response to stimulus	CTLA4, ARNTL, IL6, CD3E, EDN1, CD4, TLR3, CD40LG, NOD2, LEF1, FASLG, FN1, FOXP3, STAT3, SPRY1, TXK, CD84, MLLT3, PIK3CG, ADAMTS3, TIAM1, CHN2, ARHGEF3, SEMA4D, CACNB3, ADTRP, LOC101105165, LCP2, RASGRP1, PRKCH, SPART, STK3, ST18, BEND6, F12, FYN, CCR7, DIXDC1, LAT, IL7R, RPS6KA3, RAPGEF2, UBASH3B, CRTAM, PAQR3, CD226, ZAP70, ITK, ZMYND11, PRKCQ, CYLD, ITGA6, DAPK1, TNFSF10, CD27, PELI3, PELI1, ZRANB1, TNIK, IKBKE, GPR161, THEMIS, AKT3, ABCA1, AGT, PDCD4, TESPA1, STXBP5, CD6, LOC101122356, SLA2, STAT5B, ANXA1, SLA, SAMHD1, LMO3	76	1684	4.51	2.88	-1.852	1.67E-11	1.24E-09

BP	Single organismal cell-cell adhesion	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, LEF1, FOXP3, SEMA4D, RASGRP1, CCR7, LAT, IL7R, UBASH3B, ZAP70, PRKCQ, CYLD, ITGA6, CD27, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	26	250	10.40	5.91	-1.644	2.13E-11	1.53E-09
CC	Plasma membrane	CTLA4, CD5, IL6, CD3E, SLC16A1, CD4, CD40LG, NOD2, FASLG, ADRA1D, CYP24A1, FN1, STAT3, TGFBR3, CHRND, PIK3CG, TIAM1, ABCG1, SYTL2, ATP6V0A4, SEMA4D, CACNB3, ADTRP, LCP2, FYB1, RASGRP1, PRKCH, ADCY6, SPART, AMIGO1, GPR15, SYNE2, DYSF, FYN, CCR7, LOC101110429, LAT, CHRM5, IL7R, RAPGEF2, CRTAM, SLC3A1, SEMA6D, KCNN3, ZAP70, DOCK4, CDHR1, PRKCQ, CYLD, ICOS, ITGA6, MYO7A, DAPK1, CD27, KIRREL3, CD274, TNIK, MGST2, EHBP1, LAMA2, PTPRE, CDH20, SLC7A6, CPNE6, ESYT2, ABCA1, MPP7, UTRN, SLC26A11, OLFM4, PTGDR, CD6, SLA2, ANXA1, SAMHD1	75	1808	4.15	2.84	-1.764	3.36E-11	2.31E-09
BP	T cell receptor signaling pathway	CD3E, FOXP3, CACNB3, LCP2, FYN, CCR7, CD226, ZAP70, ITK, CYLD, THEMIS, TESPA1	12	41	29.27	19.89	-1.874	3.41E-11	2.31E-09
BP	Antigen receptor-mediated signaling pathway	CTLA4, CD3E, FOXP3, CACNB3, LCP2, PRKCH, FYN, CCR7, CD226, ZAP70, ITK, CYLD, THEMIS, TESPA1	14	63	22.22	13.86	-1.764	4.54E-11	2.99E-09
CC	Cell periphery	CTLA4, CD5, IL6, CD3E, SLC16A1, CD4, CD40LG, NOD2, FASLG, ADRA1D, CYP24A1, FN1, STAT3, TGFBR3, CHRND, PIK3CG, TIAM1, ABCG1, SYTL2, ATP6V0A4, SEMA4D, CACNB3, ADTRP, LCP2, FYB1, RASGRP1, PRKCH, ADCY6, SPART, AMIGO1, GPR15, SYNE2, DYSF, FYN, CCR7, LOC101110429, LAT, CHRM5, IL7R, RAPGEF2, CRTAM, SLC3A1, SEMA6D, KCNN3, ZAP70, DOCK4, CDHR1, PRKCQ, CYLD, CYTIP, ICOS, ITGA6, MYO7A, DAPK1, CD27, KIRREL3, CD274, TNIK, MGST2, EHBP1, LAMA2, PTPRE, CDH20, SLC7A6, CPNE6, ESYT2, ABCA1, MPP7, UTRN, SLC26A11, OLFM4, PTGDR, CD6, SLA2, ANXA1, SAMHD1	76	1868	4.07	2.79	-1.758	6.01E-11	3.85E-09
BP	Cell adhesion	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, LEF1, FN1, FOXP3, LMO7, TIAM1, SEMA4D, RASGRP1, AMIGO1, CCR7, LAT, IL7R, UBASH3B, ZAP70, CDHR1, PRKCQ, CYLD, CYTIP, ITGA6, SLK, CD27, KIRREL3, PELI1, CD274, PDCD1LG2, LAMA2, CDH20, UTRN, PDE3B, OLFM4, CD6, STAT5B, ANXA1	39	572	6.82	3.90	-1.631	8.20E-11	5.11E-09
CC	Plasma membrane part	CTLA4, CD5, IL6, CD3E, SLC16A1, CD4, CD40LG, NOD2, FASLG, FN1, TGFBR3, CHRND, TIAM1, ABCG1, SYTL2, ATP6V0A4, SEMA4D, CACNB3, ADTRP, LCP2, AMIGO1, SYNE2, DYSF, CCR7, LOC101110429, LAT, CHRM5,	46	828	5.56	3.48	-1.599	9.12E-11	5.53E-09

IL7R, RAPGEF2, SLC3A1, ZAP70, CDHR1, PRKCQ, CYLD, ICOS, ITGA6, MYO7A, CD27, CD274, TNIK, ESYT2, ABCA1, MPP7, UTRN, CD6, ANXA1

BP	Biological adhesion	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, LEF1, FN1, FOXP3, LMO7, TIAM1, SEMA4D, RASGRP1, AMIGO1, CCR7, LAT, IL7R, UBASH3B, ZAP70, CDHR1, PRKCQ, CYLD, CYTIP, ITGA6, SLK, CD27, KIRREL3, PELI1, CD274, PDCD1LG2, LAMA2, CDH20, UTRN, PDE3B, OLFM4, CD6, STAT5B, ANXA1	39	578	6.75	3.85	-1.622	1.12E-10	6.63E-09
BP	Positive regulation of cell adhesion	CD5, IL6, CD3E, CD40LG, FN1, FOXP3, RASGRP1, CCR7, IL7R, ZAP70, PRKCQ, CYLD, ITGA6, CD27, CD274, PDCD1LG2, UTRN, OLFM4, CD6, STAT5B, ANXA1	21	182	11.54	6.50	-1.557	3.03E-10	1.72E-08
BP	Immune response-activating cell surface receptor signaling pathway	CTLA4, CD3E, FOXP3, CACNB3, LCP2, PRKCH, FYN, CCR7, CD226, ZAP70, ITK, CYLD, THEMIS, TESPA1	14	72	19.44	11.69	-1.650	3.06E-10	1.72E-08
BP	Cell-cell adhesion	CASP3, CTLA4, CD5, IL6, CD3E, CD4, CD40LG, LEF1, FOXP3, SEMA4D, RASGRP1, CCR7, LAT, IL7R, UBASH3B, ZAP70, CDHR1, PRKCQ, CYLD, ITGA6, CD27, KIRREL3, PELI1, CD274, PDCD1LG2, CDH20, CD6, STAT5B, ANXA1	29	353	8.22	4.59	-1.544	4.31E-10	2.36E-08
BP	Positive regulation of signal transduction	ARNTL, IL6, CD3E, EDN1, CD4, TLR3, CD40LG, NOD2, FASLG, STAT3, TXK, MLLT3, PIK3CG, ADAMTS3, SEMA4D, RASGRP1, PRKCH, STK3, ST18, FYN, CCR7, DIXDC1, LAT, IL7R, RAPGEF2, CD226, ZAP70, CYLD, TNFSF10, CD27, PELI1, ZRANB1, TNIK, IKBKE, AKT3, AGT, PDCD4, TESPA1, SLA2, SLA, LMO3	41	663	6.18	3.52	-1.592	5.02E-10	2.69E-08
BP	T cell differentiation	CTLA4, CD3E, CD4, LEF1, FOXP3, STAT3, RASGRP1, CCR7, IL7R, ZAP70, ITK, CYLD, CD27, THEMIS, STAT5B, ANXA1	16	106	15.09	8.68	-1.554	8.28E-10	4.34E-08
BP	Positive regulation of immune response	CTLA4, CD3E, TLR3, NOD2, FOXP3, TXK, CACNB3, LCP2, RASGRP1, PRKCH, FYN, CCR7, RPS6KA3, CRTAM, CD226, ZAP70, ITK, PRKCQ, CYLD, PELI1, THEMIS, TESPA1, STAT5B, ANXA1	24	256	9.38	5.20	-1.500	1.15E-09	5.88E-08
BP	Positive regulation of cell communication	ARNTL, IL6, CD3E, EDN1, CD4, TLR3, CD40LG, NOD2, FASLG, STAT3, TXK, MLLT3, PIK3CG, ADAMTS3, SEMA4D, RASGRP1, PRKCH, STK3, ST18, FYN, CCR7, DIXDC1, LAT, IL7R, RAPGEF2, CD226, ZAP70, CYLD, TNFSF10, CD27, PELI1, ZRANB1, TNIK, IKBKE, LAMA2, AKT3, AGT, PDCD4, TESPA1, SLA2, SLA, LMO3	42	711	5.91	3.35	-1.575	1.20E-09	6.04E-08

BP	Lymphocyte proliferation	CASP3, CTLA4, IL6, CD3E, CD40LG, LEF1, FOXP3, FYN, IL7R, ZAP70, PRKCQ, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	17	125	13.60	7.71	-1.521	1.27E-09	6.24E-08
BP	Positive regulation of signaling	ARNTL, IL6, CD3E, EDN1, CD4, TLR3, CD40LG, NOD2, FASLG, STAT3, TXK, MLLT3, PIK3CG, ADAMTS3, SEMA4D, RASGRP1, PRKCH, STK3, ST18, FYN, CCR7, DIXDC1, LAT, IL7R, RAPGEF2, CD226, ZAP70, CYLD, TNFSF10, CD27, PELI1, ZRANB1, TNK, IKBKE, LAMA2, AKT3, AGT, PDCD4, TESPA1, SLA2, SLA, LMO3	42	714	5.88	3.34	-1.572	1.37E-09	6.58E-08
BP	Regulation of immune response	CTLA4, CD3E, TLR3, NOD2, FOXP3, TXK, CD84, CACNB3, LCP2, RASGRP1, PRKCH, FYN, CCR7, IL7R, RPS6KA3, CRTAM, CD226, ZAP70, ITK, PRKCQ, CYLD, PELI1, THEMIS, TESPA1, STAT5B, ANXA1, SAMHD1	27	325	8.31	4.60	-1.498	1.44E-09	6.66E-08
BP	Mononuclear cell proliferation	CASP3, CTLA4, IL6, CD3E, CD40LG, LEF1, FOXP3, FYN, IL7R, ZAP70, PRKCQ, PELI1, CD274, PDCD1LG2, CD6, STAT5B, ANXA1	17	126	13.49	7.64	-1.514	1.44E-09	6.66E-08

**Key**

**BP:** Biological process

**CC:** Cellular component

**S:** Significantly differentially expressed genes

**N:** Total number of genes in term

**OR:** Odds ratio

**Z-score:** (up regulated genes-down regulated genes) / (term size)<sup>2</sup>

**p value:** Raw (unadjusted) p-value

**p-value (adjusted):** FDR adjusted p-value (Benjamini-Hochberg procedure)

**Supplementary file 2B. Genes present in the pathways negatively correlated with worm burden at  $p < 0.01$ .**

ANXA1	CD84	FLT3	MLLT3	SLA2	TNIK
ARNTL	CACNB3	FYB1	MYO7A	STAT5B	TRIM21
ADAMTS3	CD226	F12	MGST2	SEMA4D	UBASH3B
ABCA1	CHRND	GPR161	MPP7	SLK	UTRN
AGT	CSF2RA	GPR15	NOD2	STAT5A	VASH2
ADRA1D	CHN2	GRK3	NRP2	SPRY1	ZAP70
ARHGEF3	CHRM5	GPR171	OLFM4	SPART	ZMYND11
ARL14	CYP24A1	IL6	PRKCQ	STK3	ZRANB1
ADCY6	CDHR1	IL7R	PELI1	ST18	
ARHGAP22	CDH20	ITK	PDCD1LG2	SLC16A1	
AKT3	CPNE6	ITGA6	PDE3B	SGPL1	
ABCG1	CNMD	IQGAP2	PRKCH	SLA	
ATP6V0A4	CRTAM	IKBKE	PELI3	SYTL2	
ADTRP	DIXDC1	ICOS	PDK4	SYNE2	
AMIGO1	DAPK1	LOC101102230	PDCD4	SLC3A1	
BEND6	DOCK4	LOC101110429	PPP2R5C	SEMA6D	
CASP3	DOCK10	LOC101105165	PIK3CG	SLC7A6	
CTLA4	DYSF	LOC101106767	PITPNM1	SLC26A11	
CD5	DISP3	LOC101122356	PAQR3	SAMHD1	
CD3E	EVI2B	LMO3	PTGDR	STXBP5	
CD4	EDN1	KCNN3	PTPRE	THEMIS	
CD40LG	E2F7	KIRREL3	PLAC8	TLR3	
CCR7	EHBP1	KIF13B	RASGRP1	TXK	
CYLD	ESYT2	LEF1	RPS6KA3	TESPA1	
CD27	FOXP3	LAT	RAPGEF2	TGFBR3	
CD274	FYN	LMO7	RCAN3	TIPARP	
CD6	FN1	LAMA2	RASSF4	TNFSF10	



# Discusión

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## -general



# *Discusión*

## *general*

El trabajo plasmado en esta tesis forma parte de un único ensayo enmarcado en el proyecto “*PARAGONE: Vaccines for animal parasites (635408)*”, financiado por la Comisión Europea dentro del Programa Marco Horizonte 2020, cuyos objetivos coinciden con los contemplados en esta tesis. Dada la dimensión del trabajo, los resultados se han dividido en tres artículos para facilitar su análisis y presentación.

La protección frente a *Teladorsagia circumcincta* obtenida mediante la vacunación en los corderos de la raza Canaria de Pelo de 3 meses de edad y la falta de ésta en sus homólogos de la raza Canaria forma parte de los resultados que se esperaba obtener al probar la vacuna en estas dos razas, dadas las diferencias en la susceptibilidad de estas razas debido a sus distintos orígenes ancestrales. La vacuna redujo todas las variables parasitológicas estudiadas en los corderos de la raza Canaria de Pelo al inducir una fuerte respuesta humoral y celular frente a *T. circumcincta* de características similares a las que se describen en animales adultos, lo que resultó sorprendente considerando la inherente susceptibilidad a los parásitos de los animales más jóvenes y las dificultades que señala la bibliografía para generar respuestas frente a ellos a una corta edad. Además, la vacunación también estabilizó el crecimiento de los corderos de esta raza. Estos resultados refuerzan la hipótesis de que la combinación de medidas alternativas de control frente a nematodos gastrointestinales puede tener un efecto sinérgico, lo que podría ser un valor añadido para los sectores implicados de cara a la comercialización de la vacuna.

Debido a las marcadas diferencias entre razas encontradas en el ensayo vacunal, se decidió ahondar en el origen de éstas mediante la comparación de la expresión génica a nivel del nódulo linfático regional como posible reflejo de los procesos metabólicos que ocurren en el abomaso. Los resultados obtenidos fueron consistentes con los del ensayo de vacunación, al poner de relieve una mejor orientación y desarrollo de las respuestas inmunitarias inducida por

la vacunación en la oveja Canaria de Pelo –con mayor expresión de procesos de inmunidad frente a helmintos y reparación tisular– en comparación con la Canaria –más orientada a organismos intracelulares y a procesos de inmunotolerancia. Estos datos podrían ofrecer nuevas pistas acerca de cómo mejorar el prototipo vacunal para revertir la falta de respuesta en corderos jóvenes de razas más susceptibles a los nematodos, por ejemplo, mediante la inclusión de adyuvantes que controlen la inmunorregulación.

La hipótesis de partida sugería que los animales de la raza Canaria de Pelo serían más resistentes que los de la raza Canaria a los 3 meses de edad, como está descrito en ovejas adultas. Sin embargo, no se observaron diferencias entre los corderos controles de ambas razas a esta edad pero sí se detectaron grandes variaciones en la susceptibilidad individual. Entonces se decidió considerar a todos los corderos controles en un solo grupo poblacional, para estudiar la relación entre las variables parasitológicas con la respuesta inmune humoral y celular y la expresión génica a nivel del nódulo linfático abomasal a fin de explorar los posibles mecanismos de resistencia en funcionamiento en una edad temprana que comparten las dos razas, y que por ende podrían ser mecanismos universales de protección. Los resultados señalaron varios elementos de la inmunidad relacionados con el control del parásito. Por su parte, el estudio de regresión de las variables parasitológicas con la expresión de vías biológicas en el nódulo linfático regional destacó que los animales con menores recuentos parasitológicos tenían una mayor activación de vías relacionadas con procesos inmunitarios en el control de helmintos. Los resultados obtenidos en este trabajo podrían contribuir a la identificación de respuestas inmunitarias protectoras en corderos jóvenes que, de ser validadas en futuros estudios, podrían utilizarse como marcadores para la selección de animales resistentes desde edades tempranas.

# Conclusiones



# **Conclusiones**

- 1.** La vacunación con el prototipo de vacuna recombinante frente a *Teladorsagia circumcincta* en corderos de 3 meses de edad consiguió proteger a los animales de la raza Canaria de Pelo, a juzgar por la reducción de la excreción de huevos en heces, el menor recuento de vermes en el abomaso y la reducción de la longitud y del número de huevos intraútero hallados en los animales vacunados en comparación con los controles.
- 2.** La expresión de IgA e IgG<sub>2</sub> específica frente al parásito y la presencia de leucocitos globulares, linfocitos T CD4<sup>+</sup>, T CD8<sup>+</sup> y CD45RA<sup>+</sup> en el tejido abomasal parecen estar asociados con la protección conferida por el prototipo vacunal en la raza Canaria de Pelo.
- 3.** La vacunación no produjo diferencias en los fenotipos celulares presentes en el nódulo linfático abomasal y redujo la producción de IFN-γ y de IL-4.
- 4.** La vacunación aumentó la ganancia de peso media diaria durante la infección en la raza Canaria de Pelo.
- 5.** La mayor expresión de genes asociados a respuestas inmunitaria tipo 2, es decir, la producción de inmunoglobulinas, la activación de eosinófilos y otras células efectoras, así como a la estructura de los tejidos y genes relacionados con la reparación de heridas y a las vías del metabolismo de las proteínas en los corderos vacunados de 3 meses de edad de la raza Canaria de Pelo pueden estar asociados a la protección generada por la vacuna.
- 6.** No hubo evidencia de protección mediante la inmunización con el prototipo de vacuna recombinante frente a *T. circumcincta* en corderos de 3 meses de edad de la raza Canaria.

**7.** En los corderos vacunados de 3 meses de edad de la raza Canaria, los genes significativamente más expresados se relacionaron con procesos de inmunidad innata como la presentación de antígenos o las proteínas antimicrobianas y la regulación a la baja de la inflamación y la respuesta inmunitaria a través de los genes asociados a las Treg. Todos estos procesos están orientados hacia respuestas frente a patógenos intracelulares, lo que podría explicar la falta de protección frente a *T. circumcincta* en esta raza.

**8.** Los corderos controles de 3 meses de edad de la razas Canaria y Canaria de Pelo no mostraron diferencias significativas entre razas en la resistencia a la infección con *T. circumcincta*, aunque se encontró una considerable variabilidad individual en ambas razas.

**9.** La expresión de IgA en la mucosa abomasal, el número de leucocitos globulares y células MHC-II<sup>+</sup> parecieron tener un papel en el control de la carga de nematodos, la producción y excreción de huevos. El análisis de la expresión génica del ganglio linfático abomasal reveló que una menor carga de parásitos y acumulado de recuentos de huevos en heces estaban asociados con la expresión de vías relacionadas con la inmunidad e inflamación tales como la hematopoyesis, señalización y transducción de señales, respuesta a estímulos, migración y adhesión celular de los leucocitos, la activación y diferenciación de linfocitos T  $\gamma\delta^+$ , T CD4<sup>+</sup> y la producción de IL-4.

# Conclusions



# **Conclusions**

- 1.** Vaccination with the prototype recombinant vaccine against *Teladorsagia circumcincta* in 3-month-old lambs was successful in protecting the Canaria Hair Breed lambs, considering the reduction in faecal egg excretion, lower worm counts in the abomasum and reduction in the length and number of eggs *in utero* found in vaccinated animals compared to controls.
- 2.** The expression of specific IgA and IgG<sub>2</sub> against the parasite and the presence of globular leukocytes, CD4<sup>+</sup>, CD8<sup>+</sup> and CD45RA<sup>+</sup> lymphocytes in the abomasal tissue seem to be associated with the protection conferred by the vaccine in the Canaria Hair Breed.
- 3.** Vaccination did not produce differences in the cell phenotypes present in the abomasal lymph node and reduced IFN- $\gamma$  and IL-4 production.
- 4.** Vaccination increased mean daily weight gain during infection in the Canaria Hair Breed.
- 5.** Increased expression of genes associated with type 2 immune responses, i.e. immunoglobulin production, activation of eosinophils and other effector cells, as well as tissue structure and genes related to wound repair and protein metabolism pathways in vaccinated 3-month-old Canaria Hair Breed lambs may be associated with vaccine-induced protection.
- 6.** There was no evidence of protection by immunisation with the recombinant prototype vaccine against *T. circumcincta* in 3-month-old Canaria Sheep lambs.

**7.** In vaccinated 3-month-old Canaria Sheep lambs, the significantly more expressed genes were related to innate immunity processes such as antigen presentation or antimicrobial proteins and down-regulation of inflammation and immune response through Treg-associated genes. All these processes are oriented towards responses against intracellular pathogens, which could explain the lack of protection against *T. circumcincta* in this breed.

**8.** The control 3-month-old Canaria Sheep and Canaria Hair Breed lambs showed no significant differences between breeds in resistance to *T. circumcincta* infection, although considerable individual variability was found in both breeds.

**9.** IgA expression in the abomasal mucosa, the number of globular leukocytes and MHC-II<sup>+</sup> cells appeared to play a role in the control of nematode burden, egg production and excretion. Gene expression analysis of the abomasal lymph node revealed that lower worm burden and accumulated faecal egg counts were associated with the expression of immune and inflammation-related pathways such as haematopoiesis, signaling and signal transduction, response to stimuli, leukocyte migration and cell adhesion, activation and differentiation of  $\gamma\delta^+$  and CD4<sup>+</sup> T lymphocytes and IL-4 production.

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# *Entidades*

## *financiadoras*

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